POLISH ACADEMY OF SCIENCES
NENCKI INSTITUTE OF EXPERIMENTAL BIOLOGY

ACTA BIOLOGIAE EXPERIMENTALIS

FUNDATOR

KAZIMIERZ BIAŁASZEWICZ

VOL XVIII

PAŃSTWOWE WYDAWNICTWO NAUKOWE
WARSZAWA 1958

Papers submitted for publications should be sent to the Editor, Prof. Dr. M. Bogucki. Nencki Institute of Experimental Biology, 3 Pasteur Str. Warszawa

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Ośrodek Rozpowszechniania Wdawnictw PAN
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Wydanie pierwsze. Nakład 850+150 egz. Ark. wyd. 18,25. Ark. druk. 18,5.
Papier druk. sat. V kl., 70 g, 70×100. Oddano do skład. w lutym 1958 r.
Druk ukończono w listopadzie 1958. Zam. 254/58 A-69 Cena zł 57.—

Drukarnia im. Rewolucji Październikowej, Warszawa

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258	Table IV column 4th item 3		1	—
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275		11	Magary	Macary

CONTENTS

Acta Biologiae Experimentalis, Vol. XVIII, 1958

EXPERIMENTAL INVESTIGATIONS ON THE BEHAVIOUR AND ACTIVITY OF PIGEONS (*COLUMBA LIVIA LIVIA* L.) DURING THE PARTIAL SOLAR ECLIPSE ON 30th JUNE 1954

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(Received for publication 16 February 1957)

INTRODUCTION

At the time of the solar eclipse there exist on earth special physical conditions which do not occur at any other time. First of all, there are marked changes as regards the quantity and quality of electro-magnetic radiation. The differences which occur in the visible part of the spectrum can be perceived, at least partially, by the sense of sight; the disturbances in the electromagnetic waves of the remaining lengths, and changes of other kind in the world of physical phenomena are registered by suitable measuring apparatus.

The above mentioned deviations take place during all degrees of the solar eclipse; however, the greater part of the disk of the sun is obscured the higher is the intensity of the changes.

All animals, species as well as individuals, have adapted themselves essentially to the established physical conditions of the environment they live in. The solar eclipse introduces a serious disturbance into this established order.

Hence the questions arise whether animals react in some perceptible way on these exceptional changes in the physical world, and in case the answer is affirmative, at what percent of totality of the solar eclipse their reactions begin; lastly, which of the

external factors mentioned above in a general way are responsible for the corresponding behaviour.

Since the year 1544 (Coolidge 1935) there appear scanty and more or less extensive accounts regarding this problem (Booth 1927, Bayard, Marden, Mousley 1933; Wheeler, MacCloy, Griscom, Coolidge 1935).

According to the statement of the authors of the work mentioned last "the first comprehensive and scientifically conducted study of animal life during a total eclipse" is submitted in their elaboration of observations concerning the solar eclipse in the year 1932 and sent in by "the general public including anyone who was sufficiently interested to send in a record of what he or she saw; by the game wardens of various States; by the naturalist who occupied definite places in the zone of totality, especially adapted for the observations of some group of animal or insect life in which they were particularly interested" (p. 34).

According to the authors mentioned above no fundamental changes were noticed during the solar eclipse in the reaction of the particular species. As regards birds, these observations are in brief the following (Griscom 1935):

A. The behaviour of birds in the zone of total solar eclipse.

a. There is no ground to suppose that the birds feel the approaching eclipse in the way they anticipate heavy storms or an earthquake.

b. During the time of the eclipse they did not show any exceptional signs of unusual panic or fear.

c. Most individuals of the same species showed a certain excitement or some kind of unusual behaviour in that it was not typical for the time of the day e.g. the birds went to rest although it was early afternoon.

d. Some individuals of the same species did not react at all to the changes occurring in their surrounding.

e. A detailed analysis of the causes of differences in the reaction of the individuals of the same species is impossible; inborn differences of the individuals may prove responsible for these variations.

B. The behaviour of birds in the zone of partial solar eclipse. "The evidence is absolutely conclusive that birds of all kinds showed practically no unusual or abnormal behaviour in regions where totality was 98 % or less" (p. 54).

C. The factors responsible for a changed behaviour of birds at the time of solar eclipse.

No such problem has been specially taken into consideration yet. It is accepted as obvious that the cause of such a reaction of birds was the fear of the coming night. In cases in which this interpretation meets with difficulties the problem is left open. Such point of view is visible from the attitude towards the information sent in by Mrs. E. B. Holdt at Denmark, Maine: "here a flock of turkeys started to return to the barn from the pastures at three p.m. when the sun was half obscured only, so that it was no darker than if there has been a light cloud. Mrs Holdt makes the very interesting suggestion that as it was so light when the flock began making for the barn, they were possibly influenced more by the sudden drop in temperature than by the very slight failure of daylight (p. 56).

All the above mentioned conclusions are founded on information of doubtful value. They are based on more or less accurate single observations made in uncontrolled conditions. In consequence many contradictions arise in the records sent in, which puzzle the scientific workers mentioned above: "In fact the amazing contradictions in the testimony of the observers is the outstanding feature of inquiries of this kind and is at first very puzzling" (p. 54).

It seems that observations made in unusual conditions, which besides were short and unrepeteable, are especially liable to be subjective and generalizing. In such cases it is very difficult, if not impossible, to perform control investigations.

In the investigations concerning the behaviour of animals during the solar eclipse methods should be used besides accurate observations which might grasp the course of the phenomena quantitatively. An approach of this kind is possible, when the activity of the animals observed is recorded. Besides, to reveal eventual reactions which do not appear in special disturbances of activity, other physiological methods as measurements of the speed of breathing or of pulse etc. might also be applied.

The explanation of evident differences in the reaction of animals to the solar eclipse by factors not subject to objective analysis seems to be premature, considering the evidential material as mentioned above.

While making their ultimate conclusions, the authors of the works of the year 1935 did not take into account either the physiological state of single individuals, or the external conditions in which their reactions on the solar eclipse took place. Animals when sick or hungry may react differently than healthy and well-fed ones; those kept together with other individuals and liable to stimuli caused by companionship may react differently than those living in separation. These circumstances ought to be taken into consideration when the investigations concern a partial solar eclipse; the less is the degree of obscuration of the solar disk, the more detailed should be the consideration of the circumstances.

The behaviour of animals during the solar eclipse may also depend on meteorological factors taking place accidentally with a greater intensity at the time of the eclipse, which however, does not interfere with the phenomenon of the eclipse. They may modify the behaviour of the animals deforming additionally the real picture of the reactions essential for the eclipse. Simultaneously with the observations concerning the behaviour of animals, local meteorological measurements should be taken and considered while drawing ultimate conclusion. Such proceeding has certain advantages. As the observations made at the time of the solar eclipse cannot be repeated when desired, and as the material acquired is often not sufficient to draw well-grounded conclusion, the results of such incomplete investigations may serve as material for analysis in subsequent solar eclipses. Meteorological data may render them comparable.

At the time of the solar eclipse observed in Poland on 30th June 1954 it was decided to undertake investigations on the behaviour of pigeons, and to take into account the above mentioned considerations.

From the descriptions of the behaviour of pigeons during former total solar eclipse it is known that these birds take heed of this sort of phenomena. According to the information of Dr S o m m e s of Portugal (1900) "Pigeons being fed were much alarmed and disturbed stretching their necks upwards to the sky as if apprehensive of some bird of prey. They recommenced eating when the sun reappeared" (G r i s c o m 1935, p. 61).

At the time of a total solar eclipse in 1932 pigeons behaved slightly different: "All reports show that pigeons went to roost.

There is only report of an individual pigeon remaining outside the dovecot when its companions retired" (Griscom 1935, p. 56).

As far as it is known from the accessible literature there are no papers concerning the activity of pigeons.

While applying methods recording activity in conjunction with direct observations it was the aim of the present paper to ascertain, how pigeons behave in the zone of a partial solar eclipse of less than 98 %. The investigations were performed in the zone of a partial solar eclipse amounting to 89 % in Cracow. Another problem concerned the factors responsible for eventual characteristics of ecliptical reactions. As the changes in the intensity and quality of the visible light are considered to be such causes, it was decided to eliminate this factor from the environment of the animals examined. Therefore a part of the investigations were performed in conditions of constant light. If any additional factors were active at the time of the solar eclipse, besides the changes of light, which would modify the behaviour of pigeons apart from the sense of sight, they would have been manifested.

MATERIAL AND METHODS

For these investigations pigeons (*Columba livia livia* L.) living in wild state in the market place of Cracow were employed. The birds were accustomed to the noise of the busy street and were not much afraid of people. All specimens were of mature age. There were males as well as females among them. Judging by their appearance and their general behaviour they were normal and healthy.

They were caught a long time before the beginning of the investigations and immediately placed in of experiment conditions. Each individual was put into a separate large cage 80 cm wide, 75 cm long and 55 cm high. These cages were covered with large-mesh wire net.

From the very beginning they were given water and food in abundance. Their food consisted of maize, corn and wheat. The food was supplied early in the morning at an established hour. Beside this necessary visit the birds were left in peace.

For registration of activity kymographs were used. The bottoms of the cage hung on delicate springs. The movements of the birds caused deformations of the springs, which in turn shut the link of the electric current, put in motion the electromagnets and pulled the recording levers provided with pens.

Investigations were performed outside the town in almost rural conditions.

The birds were divided in two groups; some cages were located in open places with a wide view towards the south, others in an attic of the

laboratory with shield windows and a free access of fresh air. Besides, care was taken to assure equal conditions for these two separated groups. It was not possible, however, to avoid certain differences in temperature on some control days, when investigations were carried on simultaneously on both these groups. On cloudy days was a little warmer ($\pm 1°C$) in the laboratory, on sunny days was contrarily.

The cages in the open air as well as those in the laboratory were placed in one row but at certain distances and (besides) the single cages were divided by non-transparent curtains; in this way the birds could not see one another during the investigations.

In the dark room electrical light was used with a power of 300 W. It was 2 metres above cage. Light was switched on regularly at 5 o'clock and put off at 20. Thus the length of the light phase corresponded more or less to the daytime in June. As the current ran unevenly it was not possible to avoid certain transitory and sudden oscillations in the intensity of the light used.

Kymographs were placed at certain distances from the cages with the birds, both in the open air and in the laboratory, and behind the partitions so that neither their operation nor service could be seen or heard by the birds under investigations. During the investigations the birds were observed from hidden places.

On the last days of investigations a certain kind of artificial eclipse was applied for the purpose of making comparisons concerning the pigeons in the laboratory. By reducing the power of the current in the electric net, conditions of lighting were imitated as they were on the day of the total eclipse, i.e. on 30th June 1954 at Suwałki, the place of totality in Poland. The measurements of the intensity of light made there at the hours eclipse had been recorded by the research group of the Institute of Psychology and Ethology of Animals at the Jagellonian University of Cracow. Only the last minutes before and after the maximum of the eclipse were practically taken into account; at the initial power of light in the laboratory amounting to 300 W it was practically impossible to get a relatively conspicuous decrease in the intensity of light distributed over a long period of time as it was at Suwałki: the light of the sun is by far more intensive than artificial light in the laboratory. The aim of our experiments was to test whether a decrease in the light at the same rate as during eclipse would have any characteristic effect on the behaviour of the pigeons examined.

While working on the results of experiments meteorological data recorded by the State Hydro-Meteorological Institute in Cracow and the Astronomical Observatory of the Jagellonian University were used. The author is grateful to both these Institutions for allowing him to make use of their observations.

For the manifold help offered in my research work by my best friend Mr. St. Bąk I wish to express him in this place my sincere thanks. Best thanks are also due to Madam K. Wojtusiak, Eng. W. Węglarski, and Messrs. A. Miodoński and W. Łodziński for their help in the preparation and performance of the investigations.

THE COURSE OF THE INVESTIGATIONS

I. Behaviour of pigeons in the zone of 89 % solar eclipse

A. Day of eclipse: 30th June 1954

1. The day was cloudy from the very morning and the percentage of cloudiness persisted to be very high reaching on the average 8.7. According to the indications of the heliometer the sun appeared for several minutes about 10 o'clock a.m., at 12.45 and next it was shining for a longer period since 15.35 (Table I).

Although it was full summer the day was rather cold. The minimum temperature was 13.9°C, maximum 22.9°C.

Table I

Records* of the heliometer (Cracow)

Date \ h	10 — 11	11 — 12	12 — 13	13 — 14	14 — 15	15 — 16
30.VI	0.1	0.0	0.1	0.0	0.0	0.3
1.VII	0.7	1.0	0.4	0.0	0.0	0.0
2.VII	0.0	0.0	0.0	0.0	0.1	0.2
3.VII	0.0	0.0	0.0	0.0	0.0	0.0
4.VII	1.0	1.0	1.0	1.0	1.0	1.0
5.VII	1.0	0.8	1.0	1.0	0.8	0.2
6.VII	0.9	1.0	1.0	1.0	1.0	1.0
7.VII	0.3	0.3	0.1	0.4	0.0	0.4

* 0.1 = 6 minutes.

The air pressure was on almost the same level throughout the day, on the average 746.56 mm. Relative humidity reached 77 % on the average.

2. The eclipse began at 12.47 and lasted till 15.14. From 12.45 on detailed meteorological notes were made every 15 minutes. The beginning of the eclipse started at a cloudiness amounting to 7. As the eclipse of the solar disk proceeded the accidental cloudiness increased reaching the value 8 at 13.15 and 9 during the maximum of the eclipse. In spite of the considerable cloudiness, the disk of the sun was visible through the clouds almost all the time the phenomenon lasted.

The temperature of the air was subject to insignificant oscillations. From 12.47 (20.9° C) it was falling irregularly reaching its minimum of 19.4° C a few minutes after the maximal obscurity of the disk of the sun i.e. at 14.08 and from that time on it went back slowly almost to its starting point, i.e. at 15.15 it amounted to 20.3° C.

The relative humidity of the air showed tendency towards increase which reached its maximum (78) several minutes after the eclipse. At the end of the eclipse its irregular fall was recorded.

Detailed data concerning the meteorological phenomena are shown in Table II.

Table II

Meteorological observations at the time of the solar eclipse on 30th June 1954 (Cracow)

h	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
12.45	996.7	20.9	16.5	15.3	62	9.4	48	21.3	13.1	N	1	7	ScCuAc	4.9	15
13.00	996.6	20.5	16.0	14.6	61	9.5	48	21.3	13.1	ENE	1	7	ScCuAc	5.0	15
13.15	996.6	20.7	16.4	15.2	62	9.2	49	21.3	13.1		0	8	ScCuAc	5.1	15
13.30	996.6	20.5	16.4	15.4	64	8.7	51	21.3	13.1		0	8	ScCuAc	5.1	15
13.45	996.6	20.1	16.3	15.5	66	8.0	53	21.3	13.1		0	8	ScCuAc	5.2	15
14.00	996.7	19.9	16.8	16.7	72	6.5	58	21.3	13.1		0	9	ScCu	5.3	15
14.15	996.7	19.6	16.7	16.7	73	6.1	61	21.3	13.1		0	9	Sc	5.3	15
14.30	996.6	19.6	17.2	17.7	78	5.1	62	21.3	13.1		0	9	ScAc	5.3	10
14.45	996.5	20.0	16.9	16.8	72	6.6	59	21.3	13.1		0	9	ScAc	5.3	8
15.00	996.4	19.9	16.5	16.0	68	7.4	57	21.3	13.1		0	9	ScAc	5.4	6
15.15	996.2	20.3	16.9	16.6	69	7.2	55	21.3	13.1		0	8	ScCuFeAc	5.4	8
15.30	996.2	20.5	16.8	16.2	67	7.9	56	21.3	13.1		0	7	ScCuAc	5.5	8

Explantations: 1 — the atmospheric pressure in mm 700+, 2 — temperature in °C, bulb thermometer, 3 — wet-bulb thermometer, 4 — vapor pressure, 5 — relative humidity, 6 — saturation deficit, 7 — hair hygrometer, 8 — maximum temperature, 9 — minimum temperature, 10 — wind direction, 11 — wind velocity, 12 — cloudiness, 13 — clouds, 14 — Piche's evaporometer, 15 — visibility in km.

The intesity of the visible electromagnetic radiation during the eclipse was subject to evident changes. The solarimeter being damaged measurements of the intensity of light were performed by means of a photoelectric cell. From the very moment of the eclipse (357 lux) the intensity of light diminished gradually reaching its minimum (137 lux) at the time of the greatest eclipse, and then

it returned slowly almost to its initial value reaching 299 lux at the end.

The changing cloudiness caused the irregularity in the decrease and increase of the intensity of radiation (Fig. 1).

3. Observations on the behaviour of pigeons together with setting in motion the apparatus for registration of their activity were started 2 hours before the eclipse i.e. at 10.45.

Time of observation: 10.45 to 12.47. Birds behaved freely in their typical way, each individual in a slightly different manner (Heinroth 1948). Periods of activity alternated with mo-

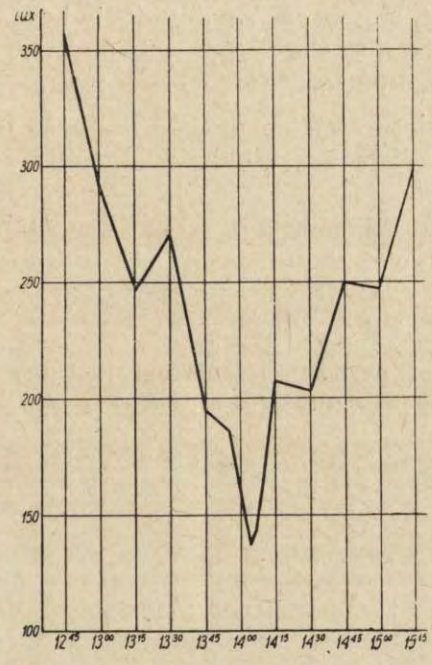

Fig. 1. The intensity of the solar radiation at the time of the solar eclipse in Cracow on 30th June 1954

ments of rest. Their activity was shown by walking or running along the cage, trying to get out of the cage, picking up food or drinking water, preening their feathers, scattering corn on the floor, and in other similar movements. Their rest passed in a position of sitting or standing in some place of the cage. Several times it was observed that all the specimens were resting at the

same time, although in the posture acquired, as well as the places chosen the birds were quite at ease. The same concerns the activity occurring sometimes in all cages simultaneously.

Time of observations: 12.47 to 13.50. From the first moments of the eclipse (12.47) up to 13.50 no essential changes were noticed in the reactions of the birds, their behaviour being similar as before. From 13.35 the birds began to take their positions in corners at the back walls of the cages, where they used to settle down at dusk for the coming night; their activity abated.

Time of observations: 13.50 to 14.10. At 13.50 all specimens were at rest, sitting or standing, their heads turned at different angles in relation to the sun. In this position, uniform for them all, they persevered up to 14.10.

Time of observations: 14.10 to 15.14. From this moment (14.10) on their usual individual activity returned slowly, slight at the beginning and increasing more and more. Not all specimens interrupted their rest at the same time, neither did all of them become quiet simultaneously before the maximum of the eclipse. Later on the behaviour of the pigeons was similar to that of the beginning of the eclipse.

4. Diagrams of activity. The diagrams enable a quantitative analysis of the behaviour of individual specimens. The time of

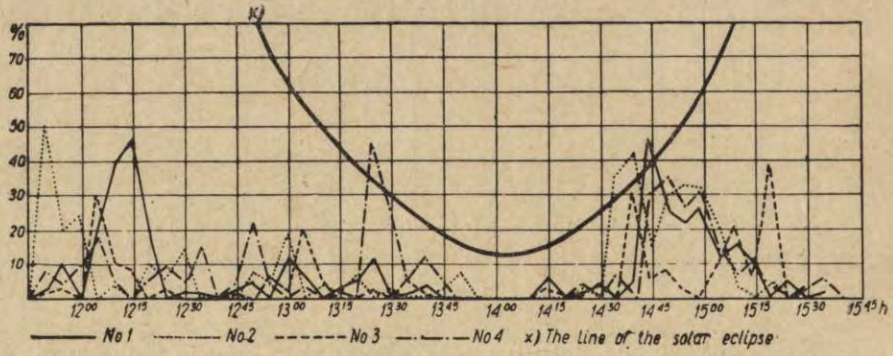

Fig. 2. The activity of pigeons at daylight on 30th June 1954

observations was divided into five-minutes intervals, and the respective duration of activity of individual specimens corresponding to the intervals has been expressed in percentage (Fig. 2).

While comparing the course of activity of all specimens in the diagrams at about 14 o'clock there strikes that at the same time all the birds show a complete quietness. In all specimens it lasted for at least 20 minutes i.e. from 13.50 to 14.10. Immediately before this period and closely after it a weak activity was manifested. It was only from 14.30 that all specimens showed increased activity. The state of general rest was contemporaneous with the period of maximum occultation of the sun's disk. From the observations mentioned above it is known that at that time birds behaved in a manner characteristic for them at evening time.

Table III

Average percentage values for the periods of activity of the pigeons kept out of doors (Explanations in the text)

Pigeon	No 1			No 2			No 3			No 4			Averages		
Period	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
30.VI	3.5	0	12.8	4.0	0.	16.6	3.6	0	7.4	9.7	0	13.2	5.2	0	12.5
1.VII	14.8	15.7	9.5	9.0	25.5	21.0	12.6	8.2	10.8	5.5	9.0	2.4	10.5	14.6	10.7
2.VII	12.3	6.0	19.7	9.6	0.2	5.7	12.2	24.0	10.1	4.8	12.5	3.6	9.7	10.7	9.8
3.VII	12.9	21.0	5.8	3.0	19.0	17.1	13.0	18.5	22.5	6.3	2.2	4.7	8.8	15.1	12.5
4.VII	19.7	24.5	26.1	9.7	36.7	12.0	6.5	0	14.6	4.5	4.2	6.3	10.1	16.3	14.7

The simultaneous quietness of all the birds investigated which took place at the maximum occultation of the sun's disk divides the activity of the pigeons during the eclipse into 3 unequal periods: I-lasts from the beginning of the eclipse up to 13.50; II-period of rest-comprises the time from 13.50 to 14.10; III-lasts from 14.10 to the end of eclipse. Each of the periods mentioned may be characterised quantitatively by calculating the mean percentage values for the average sector of the 5-minutes intervals of the given period. These calculations are given in Table III.

B. Control research

The behaviour of pigeons at the time of the total eclipse did not bear such exceptional characteristics as to ascribe them exclusively to this phenomenon. It was necessary therefore to undertake similar investigations on the following days to make sure whether

the behaviour of pigeons observed during the eclipse is not their usual behaviour at this time of the day.

Investigations were carried out on the same specimens, in the same place, at the same hours, and in the same way as on the day of the eclipse. From a series of experiments which, as far as their

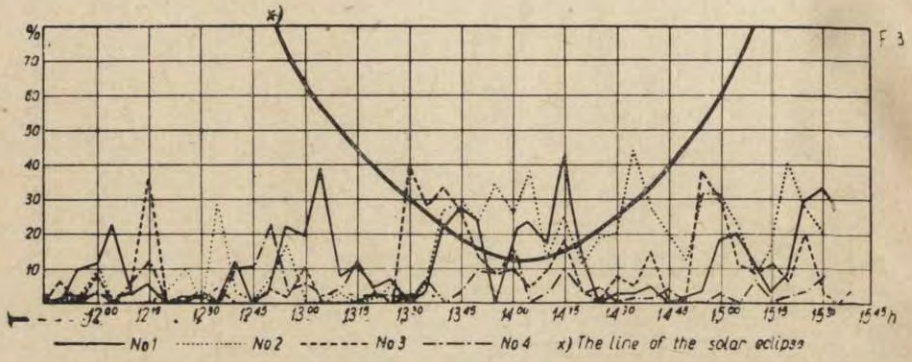

Fig. 3. The activity of pigeons at daylight on 1st July 1954

results are concerned, do not differ essentially among themselves, some typical ones will be cited, performed on the days closely following the day of the eclipse (Fig. 3).

1st July 1954. The day was sunny at the beginning; about midday, however, the sky became heavily overcast and at 14 o'clock cloudiness reached value 10. The period of the investigations proper was completely sunless. It was slightly warmer than on 30th June. The temperature amounted to 25.6° C at 14 o'clock. The air pressure showed tendency to decrease in comparison with the morning. Relative humidity fell from 82 % in the morning to 63 % at midday. Detailed data are shown on Table IV.

All birds except No 4 showed greater activity than on 30th June. Their mobility was distributed more or less evenly over the whole period under consideration. There was lack of the characteristic Phase II of rest which occurred on the day of the eclipse. About 14.05 o'clock only No 4 was quiet although by its posture and by place chosen for rest it did not remind the behaviour of the birds at the time of the maximum of eclipse.

2nd July 1954. The day was sunless at the beginning, at midday the cloudiness was very great (10). At the end of the obser-

vations, about 15 o'clock the sun appeared for a short time (Table I). It was colder than on 30th June. At 14 o'clock the temperature was 20.1° C. The air pressure did not show any major changes during the day: the average of the day was 731.31 mm. The relative humidity of the air was greater than at that time on the day of the eclipse (at 14 o'clock it attained 87 %). Detailed meteorological data are represented on Table IV.

Table IV

Meteorological observations at the time of the control days (h 14.00), Cracow

Date	1	2	3	4	5	6	7
1.VII	38.74	25.6	15.1	63	10	E 13	AsCuSc
2.VII	30.80	20.1	15.2	87	10	SSE 3	StScCu
3.VII	37.84	14.2	10.2	85	10	WNW 13	StScCu
4.VII	38.42	22.8	9.7	47	4	W 10	CuAc
5.VII	37.79	26.4	12.8	50	6	ESE 6	CuAc
6.VII	41.67	21.8	13.0	67	3	WNW 4	CuAc
7.VII	41.04	22.6	14.8	72	10	ENE 4	AsCuSc

Explanations: 1 — the atmospheric pressure in mm 700+, 2— temperature in °C, 3 — vapor pressure in mm reduced to of wind velocity, 4 — relative humidity in percentage reduced to of wind velocity, 5 — cloudiness, 6 — wind direction and velocity in km/h, 7 — clouds.

The degree and distribution of the mobility of pigeons (Table III) were much like on the previous day. About 14 o'clock there was lack of the characteristic Phase of rest (II); No 2 was slightly mobile at that time, but its behaviour did not show characteristics of the period of eclipse.

3rd July 1954. Heavy clouds (10) throughout the day. The sun did not appear at all (Table I). It was cold. Air temperature at 14 o'clock attained 14.2°C. Air pressure 737.84 mm. Relative humidity 85 %. Meteorological data are shown on Table IV.

The behaviour of the birds was similar as on the previous days. Lack of the phase of rest (II) common to all about 14 o'clock. No 1 was very active at that time (Table III).

4th July 1954. The whole day was sunny (Table I) and warm. Temperature about 14 o'clock was 22.8° C. Air pressure was almost on the same level (on the average 738.66 mm). Relative humidity

of the air at 14 o'clock was 47 %. Detailed meteorological data are shown on Table IV.

Some of the birds were more lively than on the previous days. Nos 1 and 2 very lively in Phase II, No 3 was at rest about 14 o'clock though it did not show any features characteristic of the day of 30th June. All birds were more active in Phase III than in Phase I (Table III).

Discussion of the results

The experiments carried out on the days after the eclipse showed that the activity of birds between 12.45—15.14 o'clock was not subject to a stable and uniform suspension in determined periods of time, especially between 13.50 and 14.10. It was distributed irregularly with alternating periods of rest. With the birds observed during the control days at the times mentioned above the behaviour was not characteristic of that observed before night fall and on the day of eclipse at about 14 o'clock i.e. when the birds were in complete rest.

In spite of the fact that on some control days the weather was different than on days of experiments no marked differences were noticed as regards distribution of activity as well as the behaviour of the birds.

On the background of the control investigations it became the more conspicuous how different was the behaviour of the same specimens of pigeons of the day of the eclipse at the time of the maximum obscurity of the disk of the sun. While the average activity for 5 minutes on the days of control experiment in phase II, with the exception of one case, amounted always to a certain value, sometimes quite a considerable one, it showed 0 with the same specimens during the day of eclipse (Table III). Besides, as a feature connected exclusively with the period of the maximum obscurity of the disk of the sun characteristic of behaviour were recorded which are peculiar for the hours of dusk.

As a result of these investigations it may be stated that pigeons reacted to the maximum phase of a partial eclipse of the sun in quite an evident and quantitatively measurable way. The behaviour of pigeons ascertained does not differ from that observed during a total eclipse in the year 1932. Hence, the statement that pigeons react to the ecliptical phenomena only when 98 % of the disk

of the sun is obscured, is not justified. This limit of reaction ought to be transferred at least to 89 % if not below, taking into account that the birds began to react in the way described above already at 14 minutes before the maximum of the eclipse.

The observations made in 1932 by E. B. Hold, who stated the return of the turkeys at the moment when the sun was scarcely half obscured and it was not darker outside than it is at the time of a slight cloudiness are in conformity with conclusions arrived at in the present paper. The cause of this fact, if it was not accidental, however, have been the progressing dusk, and not the fall of temperature, as Mrs. E. B. Hold suggests which must have been minimal, as results from her description and from the present observations.

Negative results obtained in the year 1932 in the zone of eclipse amounting to less than 98 % should be ascribed to imperfect methods of observation and lack of a suitable preparation of the animals. Such methods of observation of the influence of the eclipse of the sun on the behaviour of animals as were used in the work of 1935 mentioned above may serve only as a basis for suggestions and very vague conclusions.

During the eclipse of the sun described in the present work, a minute after the maximum of obscuration of the disk of the sun there appeared a strange pigeon descending on the neighbouring field in search of food. Very probably the physiological state of hunger was the cause of a behaviour so much different from the behaviour of the birds in optimal conditions. It is also by physiological conditions that we should explain the different behaviour of pigeons in the year 1900 (Dr Sommes) mentioned at the beginning. The eclipse of the sun that year took place soon after daybreak, the birds were hungry and just awake, hence they did not go to their resting places and only outside they showed adequate reactions.

II. Factors responsible for the behaviour of animals

The characteristic behaviour of pigeons at the time of the solar eclipse was caused, as control investigations permit to suppose, by external factors acting only on such occasions. From among the meteorological factors only the changes in quality and intensity of

sunlight are taken into account; all other weather constituents were subject only to slight, common changes (Table II). If it were only the changes of light at the time of the eclipse which would be exclusively responsible for the behaviour of birds in open spaces, then, pigeons which at that time were kept in conditions of permanent lighting, ought have behaved in a quite normal way. If, however, in both cases identical behaviour were manifested, one ought to presume that some other physical factors, and not the changes in the intensity of light were responsible for similar reaction in pigeons.

A. Day of solar eclipse: 30th June 1954

Observations and recording of the reactions of pigeons were started 2 hours before the beginning of the solar eclipse. The essential meteorological conditions such as: air pressure, humidity and temperature were the same as in the open space outside, except for slight deviations (Table II).

Time of observations: 10.45 to 12.47. The behaviour of pigeons was quite normal and reminded of that described in both

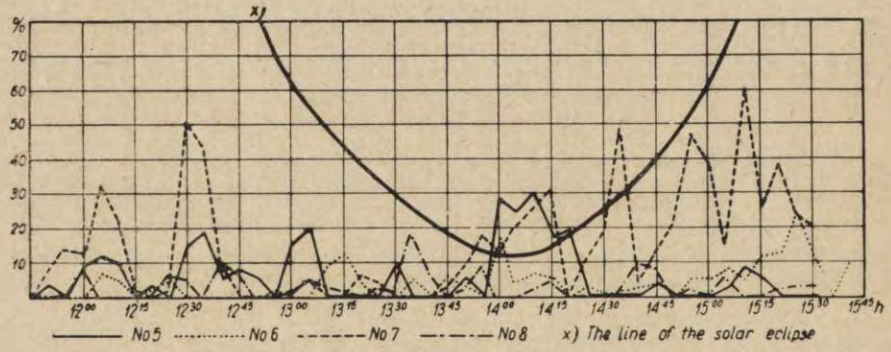

Fig. 4. The activity of pigeons in electric lighting on 30th June 1954

previous series i.e. during the solar eclipse on 30th June and during the control researches in July.

Time of observations: 12.47 to 15.14. No essential changes were observed in the behaviour of the pigeons. About 14.04 o'clock (maximum of the eclipse) all birds except No 8 moved about;

neither in their posture nor in the kind of their activity anything worth mentioning was observed. After 14.15 the pigeons except No 7 were less lively.

Table V

Average percentage values of activity of the pigeons kept in the laboratory (Explanations in the text)

Pigeon	No 5			No 6			No 7			No 8			Averages		
Period	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
30.VI	4.3	20.2	4.6	3.2	7.0	4.0	2.2	18.2	25.5	3.0	2.5	7.2	3.1	11.9	10.3
3.VII	9.8	4.5	9.0	4.3	5.0	3.6	23.3	2.5	28.0	5.4	18.5	7.7	10.7	7.6	12.0
4.VII	2.5	10.7	21.1	4.7	4.5	4.0	21.3	47.0	29.0	8.0	3.0	5.5	9.1	16.3	14.9
5.VII	3.0	4.7	3.1	3.8	0.7	8.3	38.0	13.7	31.1	4.3	3.5	7.7	12.2	5.6	12.5
6.VII	18.8	0.0	16.4	2.7	15.0	7.6	21.7	3.5	37.3	4.2	0.0	6.0	11.8	4.6	16.8

Diagrams of activity. As the birds were in their normal activity at the time of the eclipse there is no division of the diagrams into three phases (Fig. 4). The average activities calculated for the period of time between 13.50 and 14.10 show throughout the percentage value above 0 (Table V).

B. Control investigations

The behaviour of the birds kept in artificial light on 30th June, although not in accordance with the behaviour of those in the open space might, however, possess some elements connected with occurring astronomical phenomenon. Hence, in connection with this possibility it was necessary to perform additional investigations on the non-ecliptical days with the same birds and in the same conditions. From the numerous investigations performed only some records of certain days are quoted, as there are no essential discordances (Fig. 5).

3rd July 1954. Meteorological data for that day were given in the description of the previous series and in Table IV.

The birds were on the whole more lively than on the day of the eclipse, especially No 7. About 14 o'clock all birds were active (Table V). The behaviour of the pigeons throughout the whole time under observation reminded on the whole on that of the day of the eclipse.

4th July 1954. Meteorological data of that day are given at the description of the previous series of investigations and in Table IV.

The degree of activity and kind of motions performed were similar to those recorded on the preceding day.

The activity does not show any greater differences between the day of the eclipse and the one under consideration at the time

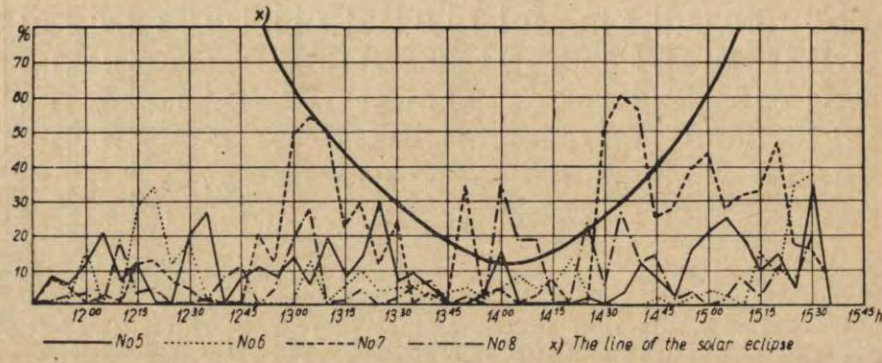

Fig. 5. The activity of pigeons in electric lighting on 3rd July 1954

of observation about 14 o'clock. The mean values of activity for the specified intervals of the time are given on Table V.

5th July 1954. The air temperature at 14 o'clock was 26.4° C. Relative humidity 50 %, air pressure 737.79 mm (Table IV).

The birds with the exception of No 7 behaved in the same way as on the day of the eclipse. No 7 was less lively about 14 o'clock. The mean values of activity for the particular intervals of time are given on Table V.

6th July 1954. On that day the temperature of the air about 14 o'clock was similar to that on the day of the eclipse. The air pressure was not subject to any greater variations (741.67 mm at 14 o'clock); the relative humidity was at that time 67 % (Table IV).

The behaviour of birds was similar as on the day of the eclipse. About 14 o'clock (Phase II) Nos 5 and 8 at rest, but with no character of that at twilight. The mean values of activity for the chosen period of time of observation are given on Table V.

Discussion of the results

The observations performed on the days after the eclipse showed that the activity of pigeons kept in conditions of permanent lighting did not show any exceptional characteristic features; the activity of the individual specimens alternated with periods of rest, long or short, throughout the whole time under consideration. During the observations no uniform suspension of activity has been established, neither was the behaviour of the birds typical of that at twilight.

The behaviour of the pigeons kept in laboratory conditions at the time of the eclipse as well as on the control days was similar to the behaviour of the birds of the same species observed on the control days in natural conditions. Thus all those series taken together represent control material in comparison with the fundamental investigations in the open air on 30th June.

The behaviour of pigeons on the day of the eclipse in this series of investigations was typical for that time of the day, because, with the exception of slight differences in the duration and degree of activity, it did not differ fundamentally from the results obtained on the control days. Thus the behaviour of the birds at the time of reduction of light in the laboratory did not possess any special characteristics connected exclusively with this phenomenon.

From the factors subject to considerable oscillations at the time of the solar eclipse the most important was the intensity of the visible electromagnetic radiation. This factor underwent gradual diminution reaching at time maximum of obscurity of the solar disk its lowest value. In the environment of the birds kept in artificial light no changes in the intensity of light took place (all other meteorological factors and their oscillations, such as temperature, air pressure and humidity were common to both groups of birds). It might seem that the factor responsible for the twilight behaviour of birds immediately exposed to the changes of the solar eclipse were the oscillations in the intensity of solar light and eventually some additional factors the influence of which, however, did not reach the birds in the laboratory room.

III. Behaviour of pigeons in "artificial" solar eclipse

If oscillations in the intensity of solar radiation were the factor responsible for the behaviour of birds kept in the open air during the solar eclipse then an experimental diminution of the intensity of light at the rate ot the solar eclipse should cause similar results in the birds observed.

A series of control investigations was carried out with a group of pigeons kept in laboratory conditions. The results obtained were in all cases similar and uniform. As an example the record of the day 7th July 1954 may be quoted.

The day was on the whole cloudy, the temperature at 14 o'clock was 22.6° C, the air pressure 741.04 mm, relative humidity 72 %. Further details are given in Table IV.

Observations on the behaviour of pigeons and the registration of the activity were started at 10.45. The intensity of light in the

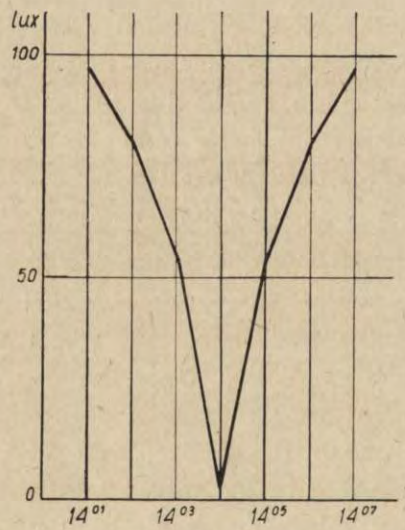

Fig. 6. The intensity of electric light at the time of the experimental eclipse on 7th July 1954

accomodation for birds was measured with a photo-electrical cell and was 97 lux. From 14 o'clock the intensity of the electrical current was gradually reduced at the same rate at which intensity

of the electromagnetic radiation diminished in the zone of the solar eclipse at Suwałki on 30th June 1954. The minimum of intensity of light at 14.04 was 2 lux (Fig. 6).

Up to the moment of the diminution of the electric current (14 o'clock) the behaviour of the birds was similar as of those of the previous series. Just before 14 o'clock No 5 stood still, No 6 cleaned its feathers. Nos 7 and 8 were active (No 8 drank water).

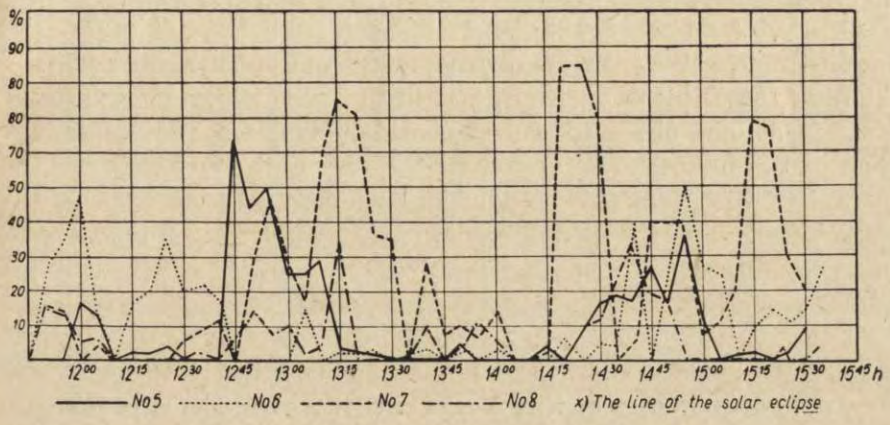

Fig. 7. The activity of pigeons at electric lighting at the time of the experimental eclipse on 7th July 1954

At the intensity of light 80 lux the same state lasted. At 54 lux Nos 6 and 7 sat down, the others stood still. This state persisted up to 14.08, although already at 14.07 the intensity of light returned to its initial value. After 14.08 the behaviour of the birds was the same as on the previous days. In the period of immobility the birds took their places in the middle of their cages (Fig. 7).

Discussion of the results

The pigeons subjected to an artificial diminution of the intensity of light in conditions which resembled those of the solar eclipse showed reactions similar to those recorded for the birds kept in the open spaces during the solar eclipse. At the time of the greatest reduction of the intensity of radiation all specimens showed quietness manifested in their postures of rest, or by giving up all movements.

A gradual fall of darkness takes place every day at evening time, and reverse changes in the growing intensity of the light occur at dawn.

With most diurnal animals, and especially distinctly with birds, there occurs (at certain periods of time, i.e.) at the morning and evening time a characteristic growth of general activity (Wagner 1930). With certain species of birds this greater activity before twilight may easily pass unobserved because it is limited to very short period of time (Haartmann 1940) e.g. with the bullfinch (Aschoff 1953).

In the investigations of pigeons at the time of the solar eclipse, both on those in the open air, and in the present series, there was applied a light phase shortened considerably by a gradual reduction of the intensity of light from the centre at 14.04 o'clock. The period of the "twilight" and of the "night" was very short. No wonder that in these conditions the morning and the evening maxima might have not appeared even if they occur with the pigeons.

The investigations of this series, although fragmentary, seem to corroborate the rightness of the conclusions arrived at in the first and second part of this work, that the pigeons reacted to the partial solar eclipse on 30th June 1954 displaying a period of twilight rest, and that the factor immediately responsible for such behaviour was the decrease in the intensity of the visible electromagnetic radiation which the birds noticed with their sight.

SUMMARY

Investigations on the behaviour and activity of pigeons (*Columba livia livia* L.) in controlled conditions were undertaken in Cracow at the time of a partial solar eclipse (89 %).

The observations and experiments were carried out on birds, some left in the open, others kept in conditions of artificial unchangeable light at the same time of the day.

The pigeons in the open reacted to the solar eclipse by a period of a total twilight rest: they showed no special increase of activity, neither immediately before nor after the maximum of the eclipse. The birds kept at that time in the laboratory room in artificial unchangeable light behaved quite normally, they was active. Thus the factor responsible for the reactions of birds kept out of

doors were visual impressions of the decrease in the intensity of visible radiation; no other factors exerting extraoptical influence have been perceived.

The conclusions obtained were tested in control investigations during the following days, and a kind of artificial eclipse was applied in laboratory conditions as an additional control. On those control days the pigeons kept both in the open and in laboratory did not show at the time of the solar eclipse on 30th June any distinct fall of activity or a behaviour peculiar for the twilight. However, in conditions of an artificially caused fall of the intensity of light, resembling a natural eclipse of the sun at the period of the lowest intensity of light (2 lux) they manifested a behaviour and a fall of activity similar as during the solar eclipse in nature. Control investigations corroborate the conclusions concerning the susceptibility of pigeons to 89 % solar eclipse (as the maximal phase), and the importance of the sun.

The investigations have also proved the inaccuracy of some former statements, that in zones of eclipse under 98 % the birds do not react to this phenomenon; they have also pointed to the cause of the contradictions in the accounts concerning the behaviour of bird at the time of the solar eclipse. The limit of the solar eclipse able to modify the behaviour of birds ought to be shifted from 98 % to at least 89 %. In the investigations the physiological state of the animals (the optimal conditions) should be taken into account.

The investigations have proved the suppositions existing so far that visual impressions are the factors responsible for the behaviour of birds at the time of the solar eclipse.

REFERENCES

ASCHOFF J. 1953 — Aktivitätsperiodik bei Gimpeln unter natürlichen und künstlichen Belichtungsverhältnissen. Z. Vergl. Physiol. *35*, p. 159—166.

BAYARD H. Ch., MARDEN A., MOUSLEY H. 1933 — Birds Action during the Total Eclipse of the Sun, August 31, 1932. Auk *1*, p. 125—126.

BOOTH H. B. 1927 — Birds and the Solar Eclipse. Naturalist. London, p. 228.

HAARTMANN L. von. 1940 — Über den Tagesrythmus des Mauerseglers, Apus a. Apus. Orn. Fenn. *17*, p. 7—11.

HEINROTH O. und K. 1948 — Verhaltensweisen der Felsentaube (Haustaube) *Columba livia livia* L. Zeitschr. f. Tierpsychol. *6*, p. 153—201.

WAGNER H. O. 1930 — Über Jahres- und Tagesrhythmus bei Zugvögeln. Z. vergl. Physiol. *12*, p. 704—724.

WHEELER W. M., MACCOY C. V., GRISCOM L., ALLEN G. M., COOLIDGE H. J. Ir. 1935 — Observations on the Behaviour of Animals during the Total Solar Eclipse of August 31, 1932. Proc. Amer. Acad. Arts a. Sc. *LXX*, 2, p. 34—70.

WHITMAN Ch. O. 1919 — The Behaviour of Pigeons. Carnegie Inst. Wash. Publ. *257*, 3, p. 1—161.

Acta Biologiae Experimentalis, Vol. XVIII, 1958

SOME REMARKS ON HERTER'S THERMOPREFERENDUM APPARATUS

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(Received for publication 29 April 1957)

The essential method of the investigations on the thermopreferendum consists in placing the animal concerned in apparatuses creating a gradient of temperature. They were constructed on various principles, and the most popular and simple ones were those designed by Herter in 1924 and modified by him in 1934 and 1939. They are known as the "temperature organs".

The most important part of this apparatus is a metal plate, generally of aluminium, one end of which is cooled by running water or ice, while the other is heated by a burner. Thus it is possible to get a gradient from nearly 0° C to about 60° C, which is quite sufficient for any experiment. The animal is placed in a glass cage the bottom of which is the metal plate mentioned above. The temperatures are measured with a row of thermometers which in the first model of 1924 stand on the upper surface of the metal plate, and in the modified model of 1934 are embedded in special holes bored in it. A scale on the surface of the plate facilitates a precise interpolation of the temperature of any given point.

This apparatus was used for many investigations (see: Deal 1941, Herter 1939). Nevertheless we see in its structure two deficiencies greatly affecting the results of any investigation. These deficiencies are to be discussed in this paper. They are as follows:

1) the thermometres are embedded i n the metal plate, while the animal observed stands o n its surface, 2) the animal observed is kept immediately on the metal plate, which obviously is an artificial factor never found in nature.

Both these problems will be analysed successively with the help of our experiments.

TEMPERATURE MEASUREMENTS BY MEANS OF EMBEDDED AND STANDING THERMOMETER

Let us begin with the first problem, i.e. that of the position of the thermometers. This problem arose in 1934 when Herter modified his first model of the "temperature organs" arguing as follows:

"Einer der grössten Mängel dieser Konstruktion ist, meiner Ansicht nach, dass bei ihr die Quecksilberkugeln der Thermometer a u f dem Metalboden des Versuchskäfigs stehen. Dadurch misst man weder die B o d e n temperatur noch die L u f t temperatur einer bestimmten Schicht über dem Boden, sondern eine Temperatur die zwischen den beiden liegt. Wie gross der Anteil de reinen oder der anderen Komponente... ist, lässt sich nicht ermitteln. Das Verhältniss beider zueinander ist abhängig von der Form der Quecksilberkugeln der Thermometer..."

"Um diesem Mangel abzuhelfen, habe ich vor einigen Jahren (1934) eine verbesserte Temperaturorgel beschrieben, bei der nur die Bodentemperaturen gemessen werden... Mancher wird es vielleicht als Mangel empfinden, dass man mit dieser Temperaturorgel nur die Bodentemperaturen ermitteln kann, da bei einigen Insecten auch die Lufttemperaturen in gewissen bodennahen Schichten für die Orientierung von Wichtigkeit sein können (N i e l s e n 1938). Nach meinen Erfahrungen ist aber für die meisten Insecten die B o d e n temperatur einer der wichtigsten thermotaktischen Orientierungsfaktoren. Der grösste Vorteil der neuen Anordnung ist jedoch, dass die gemessenen Temperaturen von den Formen der Quecksilberreservoire der verwendeten Thermometer unabhängig sind, so dass sich die Ergebnisse, die man mit verschiedenen Apparaten dieser Konstruktion erhält, auch zahlenmässig miteinander vergleichen lassen".

In his argumentation H e r t e r is right to a certain degree, nevertheless he obviously ignores the fact, that the thermometers embedded in the metal plate are located in quite a different place

than the animal observed which is influenced during the experiment by the temperature of the surrounding air and by that of the surface of the metal plate, while the thermometers show the temperature of the interior of the plate. This temperature certainly is different not only from that of the air but also from that of the surface of the plate. The primary position of the thermometers in the first model of 1924 seems therefore much more justified even taking into account the difficulties which Herter wished to avoid.

In order to base our thesis not on words only we have carried out two series of measurements of the preferred temperature on five white mice and on eight adult meal worms (*Tenebrio molitor*) measuring in each case the temperature preferred with the embedded and the standing thermometers simultaneously. In each experiment we put the animal observed into the cage of the apparatus, and after nearly 15 minutes, when it became accustomed enough to the new conditions we recorded the temperature of the place it was standing on measured every three minutes with both thermometers. If the animal was tramping about we did not take any records. The results of our observations are represented by the curves on Fig. 1 and the data in Table I. Moreover, we wish to emphasize here the difference of gradients: those measured by the standing thermometers ranged from 12°C to 45°C, while those measured by the embedded ones varied from 2°C to 65°C on the average.

Already at a first glance at the table we see the very great difference between the results obtained from the two series of thermometers. The curve for the mice illustrating the results from the standing thermometers (primary model 1924) begins at 13°C and ascends steeply to the temperature of 21°C, being at the same time the mean and the modal value. Then the curve falls equally steeply to the temperature of 31°C. The curve for the meal worms (*Tenebrio molitor*) is very similar beginning at 11°C, culminating at 17°C and falling to 25°C. The same observations measured with the embedded thermometers (model 1934) are represented by a curve running rather zig-zaglike. It begins at 7°C for the mice, and at 1°C for the meal worms and reaches 39°C in both cases, culminating rather indistinctly at 25°C for the mice and twice: at 17°C and 27°C for the meal worms.

The curves show clearly that the temperatures indicated by the embedded thermometers are much higher in the warm and much lower in the cold end of the apparatus than those measured by the standing thermometers. It should be emphasized that there is no method for comparing the data from the embedded and the standing thermometers.

The difference analysed is also very striking in regard to the statistical values. While the arithmetic mean (calculated strictly after Herter 1924) for the standing thermometers is 21.8°C for the mice, the standard deviation being $\sigma = \pm 2.58$°C and for the meal worms 18.6°C, with a standard deviation $\sigma = \pm 1.70$°C the same data for the embedded thermometers are: for the mice the arithmetic mean 25.1°C, the standard deviation being $\sigma = \pm 7.34$°C. (!), for the meal worms arithmetic mean 22.4°C. with the standard deviation $\sigma = \pm 4.16$°C. These numbers deserve more than a passing glance, especially as they originate from the same experiment and we shall consider them as well as the curves cited as a very serious argument in favour of the standing thermometers of the model 1924.

A few words should be devoted to Herter's opinion that for such animals, as the small rodents as well as the insects, it is the temperature of the ground, and not that of the air, which is the most important. We cannot agree with this in examining the curves of Fig. 1. Both curves for the standing thermometers indicating the air temperature with the ground temperature are much closer than those showing only the ground temperature. Besides, we made a special experiment with the mice. We put them in the cage standing on the table and in one end we placed a glass with water of 33°C which is the preferred temperature of the white mouse, according to Herter (1936) by the use of this modified model. If the ground temperature were important for the animal the mice would press themselves close to the glass, which they did not for a long time although they sniffed at the glass many times. The mice stayed in various places of the cage with absolutely no regard to this "preferred temperature".

KIND OF FLOOR IN HERTER'S APPARATUS

Another problem concerns the ground on which the animal examined stands during the experiment. In Herter's original

apparatuses it is simply the surface of the aluminium plate which is their main part. We consider this to be a great deficiency, as the animal in nature never stands on any metal plate, and we know very well from our own experience how disagreable the contact with any cold or hot metal object appears to us, while wood, wool or even stone of the same temperature appears rather indifferent. And the animal cannot avoid the contact with the metal in the apparatus!

For a thermopreferendum apparatus a floor similar to that the animal lives on would be most suitable. Unfortunately, this is often practically difficult to provide and to use. Therefore we propose a wooden floor made of narrow ledges of ply-wood fastened at a distance of several millimetres from one another on a longitudinal list. Thus, the animal as well as the thermometers stand on such a floor about 1—2 cm from the metal plate; this prevents their immediate contact with the hot or cold metal, and at the same time it does not eliminate the air being heated by it. For very small animals e.g. insects the same floor may be covered with gauze.

For the sake of testing this modification we carried out another two series of observations on the thermopreferendum with the same white mice and the meal worms as above. The first series was the same as the one described above, the animals being placed immediately on the metal plate. In the second series the wooden floor was used. All the experiments were carried out in the same manner as described above, and the animals were observed in turns on the wooden floor and on the metal plate during the same experiment.

The results of both these series of observations are illustrated by the curves of Fig. 2 and by the data of Table II. They differ for the mice and for the meal worms. For the mice the difference is obvious: the thermopreferendum measured on the wooden floor is by 2.3°C higher than that measured immediately on metal plate. The contact with the hot metal is obviously disagreable for the mice and drives them towards the cool end. Both the curves and the standard deviations are rather similar: they are steep and based on a rather narrow span of temperature. This is narrower for the wooden floor. The mice easily find their preferred temperature although they visit much higher temperatures for an instant — but never stay in them, even for a short time. We observed many short visits at 35°C on the wooden floor, while on the metal plate

Table I

Percent of visits at different temperatures measured with standing and embedded thermometers. Statistical data: M — mean value, σ — standard deviation, m — mean error of probability, n — total number of observations

	Temperature in °C	0	+2	+4	+6	+8	+10	+12	+14	+16	+18	+20	+22	+24	+26	+28	+30	+32	+34	36	+38	+40	n	M °C	σ °C	±3m °C	M+3m °	M−3m °C
White mice	Standing thermometers	—	—	—	—	—	—	0.2	3.2	4.4	19.4	35.2	22.2	11.6	2.8	0.8	0.2	—	—	—	—		500	21.78	±2.58	±0.34	22.12	21.44
	Embedded thermometers	—	—	—	0.6	2.0	0.6	5.4	4 2	7.4	5.8	8.2	5.8	15 2	10.4	6.4	7.2	8.2	5.4	4.8	2.4		500	25.08	±7.34	±0.98	26.06	24.10
Meal worms	Standing thermometers	—	—	—	—	—	1.1	2.6	8.4	50.1	27.0	5.7	2.6	2 4	—	—	—	—	—	—	—		800	18.56	±1.70	±0.18	18.74	18.38
	Embedded thermometers	1.1	1.6	0.6	0.8	2.5	1.2	2.0	3.0	30.3	19.9	3.9	5.7	9.6	12.2	3.—	0.1	0.5	0.1	1.4	0.6		800	22.39	±4.16	±0.44	22.83	21.95

Table II

Percent of visits at different temperatures observed on the metal plate and on the openwork wooden floor. Statistical data: M — mean value, σ — standard deviation, m — mean error of probability, n — total number of observations

	Temperature in °C	+10	+12	+14	+16	+18	+20	+22	+24	+26	+28	+30	+32	n	M °C	σ °C	±3 m °C	M+3 m °C	M−3 m °C
White mice	On metal plate	—	0.2	3.2	4.4	19.4	35.2	22.2	11.6	2.8	0.8	0.2		500	21.78	±2.58	±0.34	22.12	21.44
	On wooden floor	—	—	—	—	0.4	11.8	28.6	34.8	18.4	5.6	0.4		500	24.09	±2.04	±0.27	24.36	23.82
Meal worms	On metal plate	1.1	2.6	8.4	50.1	27.1	5.7	2.6	2.4	—	—	—		800	18.56	±1.70	±0.18	18.74	18.38
	On wooden floor	—	10.6	17.3	28.5	23.9	7.2	6.6	4.6	1.3	—	—		800	18.78	±2.82	±0.30	19.08	18.48

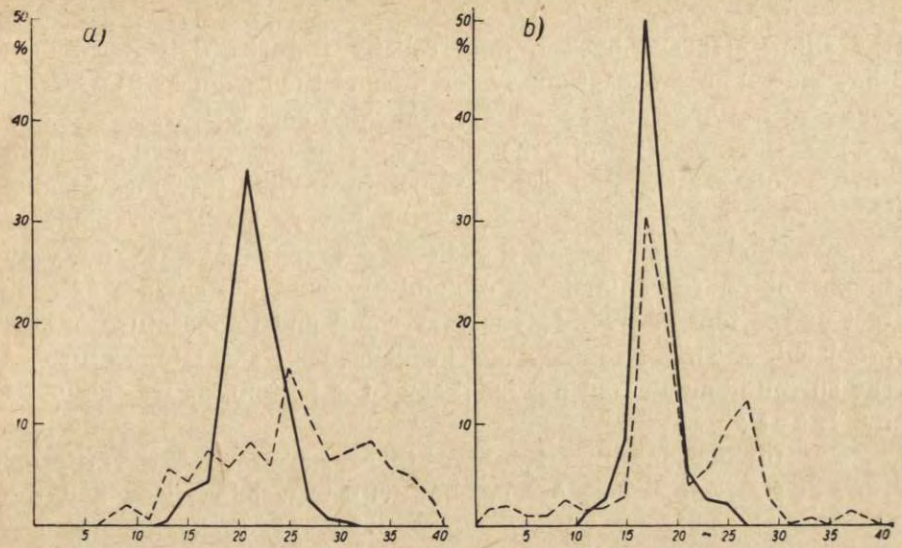

Fig. 1. A comparison of thermotactic behaviour of white mice and meal worms measured during the same experiment in Herter's thermopreferendum apparatus with standing and embedded thermometers. Solid curve: standing thermometers, broken curve: embedded thermometers

a — white mice, b — meal worms

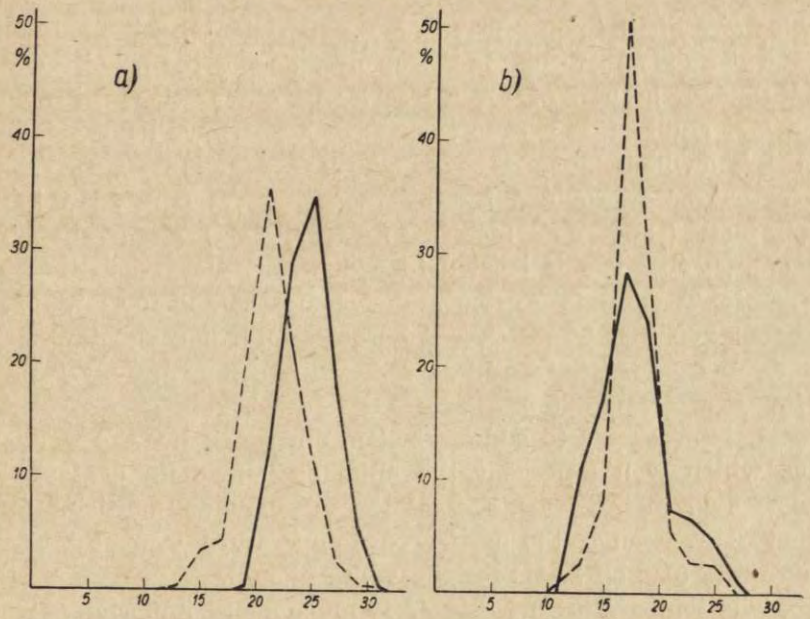

Fig. 2. A comparison of thermotactic behavior of animals kept on metal plate (broken curve) in Herter's apparatus and on openwork wooden floor (solid curve)

a — white mice, b — meal worms

the animals turned back rapidly usually from 27°C. Nevertheless they stayed only exceptionally for a short time even at 31°C. This was also the highest stay observed on the wooden floor.

For the meal worms the difference is not so distinct. Both curves culminate at the same temperature 17°C (the mean value being 18.6°C and 18.8°C) and base on a very similar gradient of temperature. The kind of the floor does not seem to be very important for the animals — probably because the surface of contact of the tops of their feet is very small and temperature organs are lacking. Therefore they are influenced by the temperature of the surrounding air and not by that of the bottom, as was assumed by Herter.

For further investigations we therefore propose the use of Herter's temperature organs, but with a wooden floor and the thermometers standing on it. We consider this to be an arrangement immitating best the natural conditions which were not provided by the original apparatus of 1934.

SUMMARY

The aim of this paper was to test experimentally the suitability of Herter's apparatus for the examination of the preferred temperature. On ground of several series of observations with white mice and adult meal worms (*Tenebrio molitor*) we have established the following:

1. The data provided by the thermometers embedded in the metal plate (Herter's modified model 1934) are not reliable, as the animal observed is located in a different place than the thermometers. These data are too high for the hot and too low for the cold temperatures. There should be used only thermometers standing on the floor of the aparatus, as it was in the first model of 1924.

2. It is necessary to add a wooden, openwork floor to the apparatus which would prevent the animal's immediate contact with hot or cold metal. This contact is obviously an artificial factor which may change the preferred temperature even by several centigrades (e.g. by 2.3°C for the white mouse).

3. The temperature of the air is much more important for the choice of the preferred temperature than the temperature of the ground.

REFERENCES

BESKROVNYJ M. A. 1953 — Praktikum po ekołogii żyvotnych. Charkov.

DEAL J. 1941 — The temperature preferendum of certain insects. Journ. Anim. Ecol. *10*, p. 323.

GRABER V. 1887 — Thermische Experimente an der Küchenschabe. Pfl. Arch. *41*, p. 240.

HERTER K. 1924 — Untersuchungen über den Temperatursinn einiger Insecten. Z. Vergl. Phys., *I*, p. 221.

HERTER K. 1934 — Eine verbesserte Temperaturorgel und ihre Anwendung auf Insecten und Säugetiere. Biol. Zbl. *54*, p. 487.

HERTER K. 1936 — Das thermotaktische Optimum bei Nagetieren ein mendelndes Art- und Rassenmerkmal. Z. Vergl. Phys. *23*, p. 605.

HERTER K. 1939 — Über den Temperatursinn der Insecten. Verh. VII Intern. Kongr. Entom., p. 740.

KRÜGER F. 1952 — Zwei neue Temperaturorgeln. Zool. Anz. *16*, Supl., p. 263.

Acta Biologiae Experimentalis, Vol. XVIII, 1958

THE INFLUENCE OF THE TEMPERATURE ON THE PULSE RATE OF TADPOLES AND METAMORPHOSED INDIVIDUALS OF *XENOPUS LAEVIS* D., *RANA TEMPORARIA* L. AND *RANA ESCULENTA* L.

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(Received for publication 7 July 1957)

PROBLEM AND MATERIAL

The hearts of poikilothermic animals react by increased or diminished number of contractions to the changes of temperature. All the investigators agree that in conformity with van't Hoff's rule the heart rate increases steadily with the rise of temperature. Barcroft's and Izquierdo's (1931) investigations confirmed by Taylor (1931) point to the fact that the isolated hearts of frogs *Rana temporaria* L. pulsate in a regular manner in temperatures ranging from +4° to +22°. Lower or higher temperatures are noxious for them. Taylor also states that the hearts of thermophil species *Xenopus dactylethra* pulsate in higher temperatures ranging from +2° to +35° and their pulse rate increases steadily up to +28.5° or +31.5°.

The purpose of our work is to confront our own results with those of the above mentioned authors. The problem is also extended by the investigation of the behaviour of tadpoles' hearts.

In the first stage of our work we examined the thermophil frogs *Xenopus laevis* D. and we made experiments with isolated hearts of tadpoles and of frogs and with hearts in tadpoles of the same species. Next we examined two Polish species: *Rana tempo-*

raria L. and *Rana esculenta* L., which spawn at different periods, namely *Rana temporaria* L. in March and in the first half of April and *Rana esculenta* L. in May. We supposed that this fact might cause differences in their pulse rate. It was too difficult to observe the hearts in very strongly pigmented tadpoles therefore only the isolated hearts were examined.

The experimental material was taken out of the following sources: *Xenopus laevis* D. tadpoles and frogs out of the cultures of the Department of Evolution J.U., *Rana temporaria* L. tadpoles out of the pond near Tenczynek and frogs out of Koło Tynieckie, *Rana esculenta* L. tadpoles and frogs out of the pond behind Skały Twardowskiego.

According to the stage of development the whole material may be divided into the following groups: 1) the young tadpoles already without external gills, 10—15 mm long (*Rana temporaria* L. and *Rana esculenta* L.), and 5—20 mm long (*Xenopus laevis* D.); 2) metamorphosing tadpoles with two pairs of legs already (*Rana temporaria* L.) or with the fully developed hind legs and the beginning of the front legs (*Rana esculenta* L.) and 3) young, completely metamorphosed frogs (*Xenopus laevis* D., *Rana temporaria* L. and *Rana esculenta* L.).

METHOD

A tadpole killed by the destroying of spinal cord is placed in a small shallow dish in Tyrode's solution. The jaw and the adjoining part of the trunk containing the heart are cut with the scissors. The heart is excised with ophtalmological scalpes but some adherent tissues, specially a part of peritoneum are left. It is just here that the sinus pacemaker which may be damaged easily, is placed. The heart is removed from the pericardium. This procedure is an easy one in the case of almost transparent *Xenopus laevis* D. tadpoles, more difficult in the case of strongly pigmented and opaque *Rana temporaria* L. and *Rana esculenta* L. tadpoles. The whole operation is carried out under a binocular dissecting microscope at 24 X magnification and it lasts 5—10 minutes if it is made by a skilled operator. A well excised heart pulsates regularly immediately the operation is over. Relatively many hearts are damaged during the procedure by streching, incision or crushing. The results of damages often revealed themselves later on in course of further observations as weakening, disturbances or complete stopping of pulsation. Such hearts were not taken into account in discussing the results.

The excised heart with a drop of Tyrode's solution are placed on a coverklip where a paraffin ring with 5—6 compartments has been prepared.

One heart is placed in each compartment. The coverslip with the hearts is glued to the slide by means of a vaseline ring. The space between the edges of a coverslip and of the slide is filled with paraffin or wax. So the preparation is in an air chamber completely cut off from its environment. The slide with the hearts is placed in a metal — glass chamber (Grodziński 1955), where the water of desired temperature may be let through. The

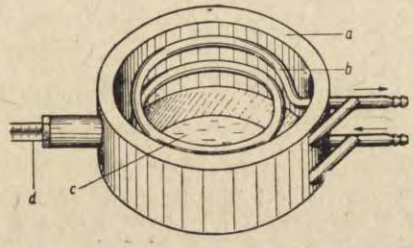

Fig. 1. Experimental chamber
a — double walls of chamber, b — spiral tube, c — glass bottom of chamber, d — thermometer, direction of water current

water is heated and driven to the chamber by Höpler's ultrathermostate type B. A thermometer placed in the chamber in the neighbourhood of preparations controls the temperature. The hearts are watched through the glass cover by means of a binocular dissecting microscope at 96 X magnification. The same heart was always observed in all the temperatures. The number of contractions per minute for each heart and each temperature was counted. Each minute was rung by an interval timer. Changes in pulse rate are controlled every 5°. It took about 10 minutes to obtain the rise in temperature of 5°.

Xenopus laevis D. tadpoles whose contractions rate is to be watched are placed in a shallow glass dish 5—6 in number. Each tadpole is shut up in a celluloid chamber where it can move in a small space. The dish is filled with clean water to the height of 6 mm. The dish with the tadpoles is submerged in a round double — walled glass — metal chamber. (Fig. 1). A water current diven by Höpler's ultrathermostate circulates between its walls and maintains the desired temperature in the chamber. Under the binocular dissecting microscope the heart is well visible through the cover of tadpoles body.

The hearts of metamorphosed frogs were excised after the removal of skin and of the breast bone; they were excised together with adhering tissues and brought into a shallow glass dish filled with Tyrode's solution. Here the heart was cleaned of adhering tissues with scalpels and excised out of pericardium. Frog's hearts are too big to be watched in a chamber on the slide. So the Petri dish 6 cm in diameter is divided into six partitions with

mica and a heart is placed in each partition filled with Tyrode's solution. The dish with hearts is placed into water in a round glass — metal chamber like that with *Xenopus laevis* D. tadpoles.

Xenopus laevis D.

Two series of experiments both with tadpoles' and frogs' hearts were carried out; they differed in the initial temperature. The first series was started by heating from the temperature of +2°, the other from +10°. The procedure was based on the fact that these frogs are thermophil animals and their optimal temperature of water ranges from +18° to +25°. This suggested that the temperature of +2° would be lethal for them.

Table I

The pulsation rate of the isolated hearts of *Xenopus laevis* D. tadpoles and frogs

	Total number of examined hearts	Rejected	Taken into consideration	Temperature								
				2°	5°	10°	15°	20°	25°	30°	35°	40°
Tadpoles	60	50	10	4	10	23	44	67	90	114	40	43
	25	15	10	—	—	38	69	109	135	161	153	99
Frogs	26	16	10	9	12	24	37	54	78	95	115	74
	18	8	10	—	—	23	36	48	65	81	106	75

The hearts not pulsating satisfactorily in experimental conditions that is the hearts which did not react distinctly by the number of contractions on the changes of temperature, the hearts which pulsated irregularly in higher temperatures and stopped contracting, as well as the hearts destroyed in the last stage of experiment on account of faults in technic were rejected and not taken into consideration. The ten best working hearts of each group were chosen to be discussed here.

The results of observation are represented in the Table I and the Fig. 2. The contractions of the isolated heart of tadpole at the temperature +2° are very slow, but distinct. It happens that some hearts do not pulsate at this temperature but when they are heated to higher temperatures (+3°, +4°, +5°) they begin to contract. In accordance with van't Hoff's rule the pulse rate increases with the rise of temperature attaining 114 contractions per minute at the temperature +30°. This is the optimal temperature for heart

contractions. Above +30° the number of contractions diminishes quickly. At +40° only 5 hearts were pulsating: 3 rhythmically, 2 vibrated irregularly. When the temperature was lowered quickly from +40° to +20° two of the examined hearts continued to pulsate but the number of contractions was lower than that at the beginning of the experiment in the same temperature. The tadpoles hearts cooled to +10° only attain a much higher pulse rate. The temperature of +30° is as well optimal for them as for the preceding

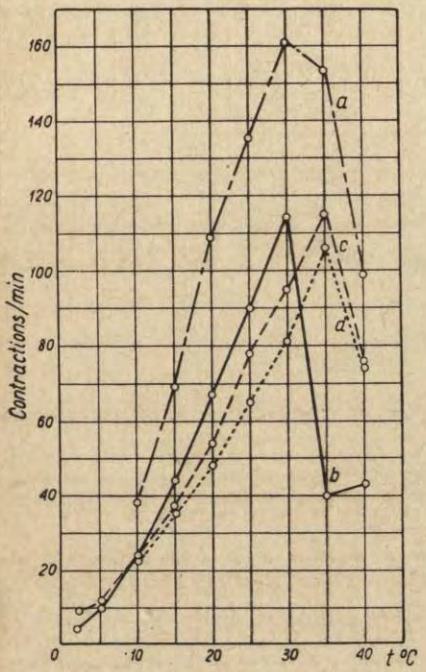

Fig. 2. The pulsation rate of the isolated hearts of tadpoles and frogs of *Xenopus laevis* D.
a — from 10° tadpoles, b — from 2° tadpoles, c — from 2° frogs, d — from 10° frogs

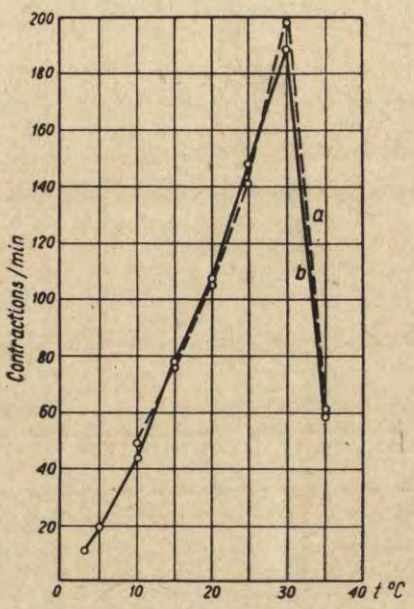

Fig. 3. The heart rate of tadpoles of *Xenopus laevis* D.
a — from 10°, b — from 2°

ones, they attain 161 contractions per minute (that is 47 contractions more than the hearts cooled to +2°). At the temperature of +35° the pulsation rate drops to 153 contractions per minute. All the hearts beat then similarly as at the temperature of +40°, though their pulse rate continues to drop to 99 contractions per minute. At the temperature of +40° they contract either very slowly and regularly, or oscillate quickly and irregularly.

The temperature of +2° is not lethal for the isolated hearts of metamorphosed animals. In both series of experiments with initial temperatures +2° and +10° the results are similar. In both series the maximal number

of contractions is attained at the temperature of +35°. Above it the contractions become irregular, indistinct, or they pass into fibrillation within the sinus. Above +40° the hearts die.

Table II

The heart rate of *Xenopus laevis* D. tadpoles

Total number of examined hearts	Rejected	Taken into consideration	Temperature								
			3°	5°	10°	15°	20°	25°	30°	35°	40°
40	30	10	11	19	44	78	114	155	189	58	—
62	52	10	—	—	49	76	112	149	198	61	—

The pulsation rate of the heart in the body of *Xenopus laevis* D. tadpoles was examined too, in order to find if there were any differences in the pulse rate of isolated and not isolated hearts. The results are presented in the Table II and Fig. 3. The tadpoles cooled to +3° have the highest pulse rate at the temperature of +30°, that is 189 contractions per minute. Above +30° the number of contractions diminishes. Only 6 tadpoles survived in the temperature of +35°. The pulsation rate of tadpoles cooled to +10° behaves in the very similar way. Only 6 of them survived the temperature of +35°. The hearts in the tadpoles' bodies pulsate more intensively than the isolated ones, but they completely stop at the temperature of +40°.

Rana temporaria L.

The division of experiments into two series differing in the initial temperature is groundless in case of *Rana temporaria* L. hearts because their development takes place in low temperatures too, so all the hearts could be watched from the temperature +2°. The whole experimental material was

Table III

The pulsation rate of the isolated hearts *Rana temporaria* L.
I — young tadpoles, II — metamorphosing tadpoles, III — young frogs

Stage	Total number of examined hearts	Rejected	Taken into consideration	Temperature								
				2°	5°	10°	15°	20°	25°	30°	35°	40°
I	41	31	10	7	12	23	43	70	99	123	138	41
II	14	4	10	5	11	23	41	61	81	94	104	38
III	89	79	10	12	15	28	43	63	76	85	75	64

divided into three developmental stages: 1) young tadpoles 10—15 mm long, 2) metamorphosing tadpoles and 3) young frogs. The ten best working hearts of each group were chosen for further consideration. The results are presented in the Table III and Fig. 4.

The hearts of young *Rana temporaria* L. tadpoles react on the rise of temperature in the range from +5° to +35° by increase of the number of contractions almost in a steady way and consistently with van't Hoff's rule. The increase of the number of contractions between +2° to +5° is a little lower. Its maximum at +35° is 138 contractions per minute. The hearts heated above +35° contract more and more slowly. When they are kept for 5—6 minutes in the temperature +40° they stop contracting. Being kept not too long in the temperature of +40° they begin to pulsate again after cooling to the normal temperature.

The hearts of metamorphosing tadpoles behave in similar way. At the temperatures ranging from +2° to +35° they react consistently with van't

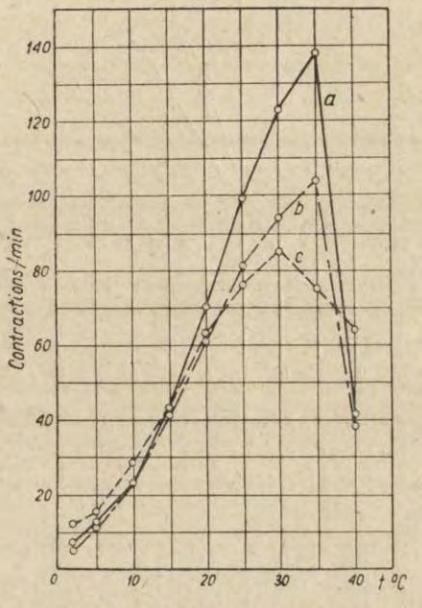

Fig. 4. The pulsation rate of the isolated hearts of *Rana temporaria* L.
a — young tadpoles, b — metamorphosing tadpoles, c — young frogs

Hoff's rule, higher temperatures are lethal, the highest number of contractions at +35° is 103 contractions per minute.

The highest pulsation rate for the young frogs is 85 contractions per minute at the temperature +30°. Above +30° it drops.

Confronting all the obtained data for all the three stages we state that the number of contractions at the similar temperature drops with the age of an animal.

Rana esculenta L.

All the hearts of *Rana esculenta* L. were watched like those of *Rana temporaria* L. at the initial temperature +2°. 140 hearts were examined, namely 39 hearts of young tadpoles, 45 hearts of metamorphosing tadpoles with hind legs developed and the beginning of front legs, and 56 hearts of

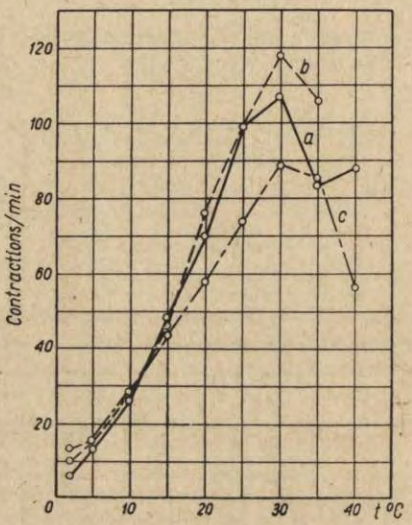

Fig. 5. The pulsation rate of the isolated hearts of *Rana esculenta* L.
a — young tadpoles, b — metamorphosing tadpoles, c — young frogs

young frogs. The ten best working hearts were chosen from each group. The results observation are presented in the Table IV and Fig. 5.

The hearts of young *Rana esculenta* L. tadpoles react on the rise of temperature in the range from +2° to +25° by the increase of the number of contractions in an almost steady way consistently with van't Hoff's rule. In the temperature above +25° the number of contractions increases more slowly than it would be expected from van't Hoff's rule. The highest pulse rate at +30° is 107 contractions per minute. The hearts kept in the temperature above +30° pulsate irregularly, more and more slowly. Any longer stay at the temperature +40° is lethal for them.

The hearts of metamorphosing tadpoles increase their contraction rate in the range of temperature from +2° to +30°, attaining in this last temperature 118 contractions per minute. Above +30° their pulse rate drops. The temperature +35°proved lethal for all the examined hearts of this stage.

Table IV

The pulsation rate of the isolated hearts *Rana esculenta* L.
I — young tadpoles, II — metamorphosing tadpoles, III — young frogs

Stage	Total number of examined hearts	Rejected	Taken into consideration	Temperature								
				2°	5°	10°	15°	20°	25°	30°	35°	40°
I	39	29	10	6	13	26	48	70	99	107	84	88
II	45	35	10	10	15	28	46	76	99	118	106	—
III	56	46	10	13	15	28	44	58	74	89	85	56

The hearts of young frogs behave in similar way with the exception that they survive up to +40°. They contract a little more slowly in the temperatures ranging from +2° to +5°, as it would result from van't Hoff's rule.

DISCUSSION

Our experiments have proved that the range of temperatures, in which the hearts of the tadpoles and of the frogs of the three species examined by us pulsate, is much wider than that reported by Barcroft, Izquierdo and Taylor in their papers (1931). The hearts of all the three species pulsate in the range of temperatures from +2° to +40° and their pulse rate is disturbed only when the temperatures rises above +30°. There are specially large divergences between our results and those of the above mentioned authors concerning the species: *Rana temporaria* L. and *Rana esculenta* L. One of the reasons explaining them is the fact that discussing our results we rejected a great percentage of examined hearts namely those with disturbances of pulsation rate in the temperature above +20°. The remainder considerably exceeds in number the material examined by Barcroft and Izquierdo. Other reasons which may have contributed to the divergency of the results are: the use of different medium (Barcroft, Izquierdo — Ringer's solution: we — Tyrode's solution), as well as the time of

the duration of experiment, which was longer than that of the authors, because they counted the contraction rate during cooling and heating. We counted it only while heating and we shortened as much as possible the time of keeping the hearts at the disadvantageous high temperatures.

At any rate it is certain that the hearts under the conditions of our experiments survived up to the temperature +40° and even lived in this temperature for 6—8 minutes, that is the time necessary for counting the number of contractions of 4—6 hearts. After a quick coming back to normal temperature they pulsated again a little slower than before. In a few cases the hearts pulsated still some moments in the temperature +41.5°.

Table V

The comparison of the pulsation rate of isolated tadpoles hearts

Species	Temperature								
	2°	5°	10°	15°	20°	25°	30°	35°	40°
Rana temporaria L.	7	12	23	43	70	99	123	138	41
Rana esculenta L.	6	13	26	48	70	99	107	84	88
Xenopus laevis D.	4	10	23	44	67	90	114	40	43
	—	—	38	69	107	137	161	153	99

The hearts of all the three species of amphibians behave in the similar way. The temperature +2° proved noxious only for the hearts of *Xenopus laevis* D. tadpoles; hearts cooled to +2° pulsated all the time more slowly than those cooled to +10°. Some hearts did not pulsate in this temperature at all, and began to pulsate at +3°, +4°, +5°. This temperature was not a noxious one for all other hearts, only the rise of the pulsation in the range +2° to +5° was a little lower than it would be expected of van't Hoff's rule.

The heart rate of young tadpoles of *Rana temporaria* L. and *Rana esculenta* L. is almost conformable to each other. The heart rate of young *Xenopus laevis* D. tadpoles cooled to +2° is lower

during all the time and in all temperatures than the pulsation of above mentioned species. The heart rate of *Xenopus laevis* D. tadpoles cooled to +10° is in all temperatures higher (Table V and Fig. 6).

The heart rate of the metamorphosing *Rana esculenta* L. tadpoles is a little higher in all temperatures than that of *Rana temporaria* L.

The heart rate of *Xenopus laevis* D. young frogs is a little lower in the temperatures ranging from +2° to +20° than that of *Rana*

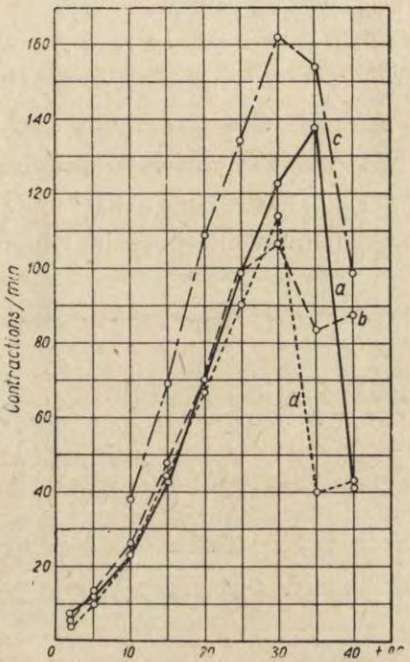

Fig. 6. The comparison of the pulsation rate of the isolated tadpoles hearts

a — *Rana temporaria* L., b — *Rana esculenta* L., c — *Xenopus laevis* D. from 10°, d — *Xenopus laevis* D. from 2°

temporaria L. and *Rana esculenta* L., only above +20° it becomes a little higher and attains its maximum at +35° namely 115 contractions per minute. The frogs' hearts of the other two species attain their maximum at +30°, their pulsation rate is: 85 — *Rana temporaria* L. and 89 — *Rana esculenta* L.

The Tables V and VI present the heart rates of isolated hearts of species we have examined: Table V — tadpoles' hearts and Table VI — the hearts of already metamorphosed individuals.

Table VI

The comparison of the pulsation rate of isolated frogs' hearts

Species	Temperature								
	2°	5°	10°	15°	20°	25°	30°	35°	40°
Rana temporaria L.	12	15	28	43	63	76	85	75	67
Rana esculenta L.	13	15	28	44	58	74	89	85	56
Xenopus laevis D.	9	12	24	37	54	78	95	115	74

Special attention was paid to the behaviour of the frogs' hearts in high temperatures, namely in those in which the heart begins

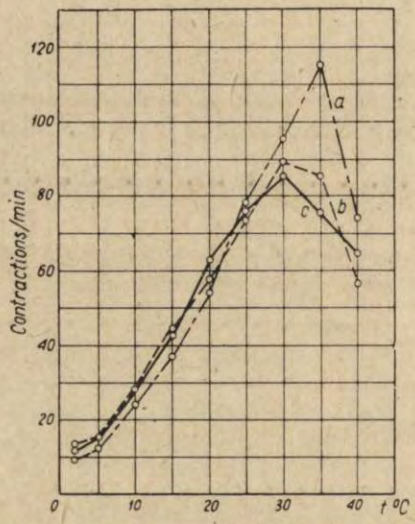

Fig. 7. The comparison of the pulsation rate of the isolated frogs' hearts

a — *Xenopus laevis* D., b — *Rana esculenta* L., c — *Rana temporaria* L.

to contract irregularly. Already Pickford in 1851 and Schelske in 1860 described the phenomenon that the ventricle already

stopped contracting, while the auricle and the sinus venosus worked on. This phenomenon is the result of the impairment of conductivity between the ventricle and the auricle. The next investigators as Cyon (1866), Aristow (1879) and Stewert (1892) established that the ventricular block occurs in the range of temperature from +30° to +40° and is reversible by the lowering of temperature (Unger 1912).

We observed this phenomenon too. The ventricular block was usually preceded by inconsistency of the pulsation rate between the ventricle and the auricle, the ventricle worked on with its own rhythm controlled by auriculo-ventricular pacemaker. This phenomenon usually occurred in the temperature above +30° and with some hearts already in above +25°. Other hearts beat conformably to auricle up to +40° and almost simultanously the auricle and the ventricle stopped contracting.

It was stated that the heart rate diminishes with the age in all three species. It is contrary to what is observed in fish in which, the heart rate increases with the age of the embryo. (Grodziński 1955).

The comparative experiments with isolated and not isolated hearts of *Xenopus laevis* D. tadpoles have pointed to the fact that pulse rate of hearts in tadpoles is much higher than that of isolated hearts. It can be partly explained by the fact that the heart in tadpole is not subjected to mechanical injuries during operations. The hearts in tadpoles die quickly in the temperatures above +30° on account of smaller endurance of skeletal muscles for higher temperatures. The higher temperature paralyses them. A tadpole is most animated in the temperatures ranging from +18° to +23°, the respiratory movements are distinctly visible, and as the temperature rises they become vibratory. In the temperature +25° tadpoles begin to turn upside down, and to lie motionlessly with their mouths open. Their respiratory movements stop causing lack of oxygen in the heart muscle and bringing about its death. On the other hand the isolated hearts are supplied with oxygen directly from the medium and therefore they survive easier in the higher temperatures than the hearts within tadpoles.

At the end of our discussion we want to confront the behaviour of the examined frog's hearts with that of hearts of other amphibians and fish.

Not isolated hearts of *Hylobius lichenatus* larvae pulsate like the frogs' hearts in certain temperatures the pulse rate is higher. The hearts of these larvae pulsate still at +35° (Inukai 1922).

The investigations of the dependency of the sea-trout's (*Salmo trutta* L.) embryos' heart rate on the temperature were carried out in the experimental conditions the most similar to ours (Grodziński 1955). The range of temperatures in which these hearts pulsate is narrower than that of the examined frogs' hearts — the sea-trouts' hearts contract in the temperatures ranging from 0° to +34°. The temperatures above +34° are detrimental for them. The sea-trouts' heart rate increases with the age of the embryo, a phenomenon contrary to that observed in the amphibians. Their number of contractions per minute exceeds the number of contractions of heart of amphibians in every temperature.

The same phenomenon was observed by Markowsky — Senta (1933) who examined the hearts of adult fish (the eel and the tench). In low temperatures the hearts of these fish pulsate irregularly. In the temperatures ranging from +5° to +25° the hearts work rhythmically and their pulsation rate increases with temperature. Above +30° the functional dependence between the auricle and the ventricle is broken. The ventricle contracts more slowly with its own rhythm and next stops. It can begin to contract again after lowering of temperature.

SUMMARY

The pulse rate of isolated hearts of *Xenopus laevis* D., *Rana temporaria* L. and *Rana esculenta* L. tadpoles and metamorphosed animals was observed in temperatures ranging from +2° to +40°.

The heart rate increases with temperature in accordance with van't Hoff's rule.

In the temperatures ranging from +30° to +35° the heart rate is disturbed, the conductivity between the ventricle and the auricle is impaired and the ventricle block comes. The auricle pulsates longer and sinus venosus the longest.

Any stay in a temperature of about +40° longer than 6—8 minutes is lethal for the heart; a shorter stay sometimes stops heart action. After lowering the temperature the heart contracts again but at a slower rate.

The number of heart contractions in the same temperature lessens with the age of an animal.

The heart rate in *Xenopus laevis* D. tadpoles was also observed. In temperatures ranging from +2° to +30° it is much higher than that of tadpole's isolated heart. The heated hearts in tadpoles stopped contracting in lower temperature than isolated hearts.

No substantial differences were found in the rate of isolated hearts of examined animals. Only hearts of *Xenopus laevis* D. tadpoles cooled to +2° pulsate more slowly than those of other species of the same developmental stage and those of *Xenopus laevis* D. tadpoles cooled only to +10°.

REFERENCES

BARCROFT J., IZQUIERDO J. I. 1931 — The relation of temperature to the pulse rate of the frog. J. Physiol. *71*, p. 145.

CZUBALSKI F. 1956 — Fizjologia układu krążenia. Warszawa PZWL.

GRODZIŃSKI Z. 1954 — Initiation of contractions in the heart of sea-trout *Salmo trutta* L. embryo. Bull Acad Sc. Cl. II, *II*, p. 3.

GRODZIŃSKI Z. 1955 — Tętno odcinków izolowanego serca troci *Salmo trutta* L. Folia Biol. *1*, p. 65.

GRODZIŃSKI Z. 1955 — Reactions of the isolated heart of the sea-trout *Salmo trutta* L. embryos to temperature. Zool. Pol. *6*, 3, p. 187.

INUKAI T. 1922 — Über den Einflus der Temperatur auf die Pulsationzahl bei Amphibienlarven und Vogelembrionen. Japan J. Zool. *1*, p. 67.

IZQUIERDO J. I. 1929—30 — The influence of excision and treatment with saline on the pacemaker of the frog's heart. J. Physiol. *68*, p. 363.

KOLLER G. 1933 — Versuche an nervenfreien embrionalen Amphibienherzen. Zeitschr. Vergl. Physiol. *18*, p. 186.

MARKOWSKY — SENTA 1933 — Über die Beeinflussung der Herztätigkeit durch die Temperatur. Biologia Generalis *9*, p. 231.

SMITH C. L. 1951 — The temperature — pulse rate curve of the isolated frog's heart. J. Exper. Biol. *28*, p. 141.

TAYLOR N. B. 1931 — The relation of the temperature to the heart rate of South African frog (*Xenopus dactylethra*). J. Physiol. *71*, p. 156.

WEIGMANN R. 1929 — Die Wirkung starker Abkühlung auf Amphibien und Reptilien. Zeitschr. Wissensch. Zool. *134*, p. 641.

Acta Biologiae Experimentalis, Vol. XVIII, 1958

PARTITION OF FORAGING GROUNDS AND MODES OF CONVEYINGS INFORMATION AMONG ANTS

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(Received for publication 5 June 1957)

It is a known fact that animals having a permanent place of nesting usually have also a defined area of feeding or preying. The existence of permanent feeding grounds with ants is of special interest owing to the social mode of life of these animals. The problem is not limited to the simple question, whether a given nest has a defined area of feeding; it is also necessary to explain how this area is taken possession of by a social unit such as a given nest of ants, that is how a social foraging takes place.

One the possible ways of penetration of the territory by an ants nest would consist in a secondary partition of the foraging grounds by single individuals, or groups of individuals. Such phenomenon will be called the partition of foraging grounds.

Conveying of information is understood as a certain narrow part of a more general problem of intercourse in ants. We shall deal here only with information exchanged by workers concerning the findings of food.

The purpose of this study is to analyse the way or ways in which social foraging takes place outside the nest.

METHODS

In all experiments described in this study we applied a mass or individual marking of workers. The marking was carried out when the ants were moving. The catching of animals was avoided. The choice of a suitable paint was one of the most difficult methodical problems. The best results were obtained with paints for leather. This difficulty greatly hinders prolonged observations, as the paint used must not only be permanent and harmless, but also quickly drying. It has not been so far possible to find a paint

which would hold for longer than two months. Only two workers Buckingham (1909) and Ehrhardt (1931) — used paints which kept for years, unfortunately however they do not mention the kind of paints used. On the other hand other authors, both previous and recent, complain of the lack of permanent paints. So for example, Carthy (1951) in his studies used a paint which kept only for a period of several weeks.

The problem of permanent paint is very important in studies concerned with the division of work performed by ants. In the experiments described in the present paper, the paints used were sufficiently permanent.

One of the methods employed here consisted in mass marking of individuals moving along a given ant trail. A similar method was used previously by Ökland (1931) nad Kiil (1934).

In these experiments, in which the tracks to aphides were investigated, the marking was carried out on the trunks of trees with aphids. This was followed by observation of this trail over a longer period of time. As a control other trails surrounding the nest were also surveyed.

During the observations the marked and the unmarked individuals, passing in one direction only were counted during a limited period of time.

It is technically impossible to mark all the ants moving along a given road. For this reason additional workers were marked at each session and so the total number of marked individuals was increased each day. The marking was of course always carried out after the observation, and the ants marked lastly were not taken into consideration until the following day. The computations were made of the number of observed marked individuals in per cent of the total number of workers observed on the tree during a given day.

In one of the experiments the marking was done separately in the morning and separately in the evening with various colours. The observations were then carried out at various times of the day.

The next method used consisted of marking single individuals found outside the trails. A plot which was rarely frequented by the workers, was selected within the foraging territory of a given nest, at a certain distance from the track; a certain number of individuals passing over this plot was marked, and then the plot was observed over a number of days at various periods of the day.

In contrast to previous investigations concerning the partition of foraging grounds, this comparative study was carried out on several species:

Formicinae subfamily — *Formica rufa* L., *F. pratensis* De Geer, *F. truncicola* Nyl., F. *sanguinea* Latr., *Lasius niger* L.,

Myrmicinae subfamily — *Myrmica scabrinodis* Nyl., *Tetramorium caespitum* L., *Leptothorax acervorum* Mayr.

In the experiments concerning the conveying of information about a source of food, the method of Eidmann (1925, 1927) was applied. It consisted of placing the food near the nest, marking the first worker which found it, and observing its behaviour. Sometimes pupae were used instead of food.

These experiments were also conducted for comparative purposes on species belonging to various subfamilies. The same species were used, which are enumerated above, with the exception of *F. sanguinea.*

RESULTS

I. Partition of foraging grounds

Two pine-trees with aphides, A and B, standing some 5 meters from one another, were selected. A track leading to these trees forked near the first tree A. The distance from a large nest of *Formica rufa* to the trees was about 40 meters (see Fig. 1 a).

Ants on the tree A were marked with a white paint, on the tree B with a green one. Some 500 individuals were marked on each tree. Observation lasting 15 minutes each, were then carried out every day over a period of 11 days on each tree. During this period 25 ants marked white and 5 marked green were noted on tree A, and 34 marked green on the tree B.

This experiment was made on a large ant-nest about 1 m high. It is particularly significant that even with such big nest the marked workers were systematically found on corresponding tracks after marking of 1000 individuals only. Their number however was so large that it was impossible to survey the other tracks. Therefore the experiment was repeated on a smaller nest.

Moreover it was necessary to account for the presence of green ants observed several times on the tree A. In view of the fact that

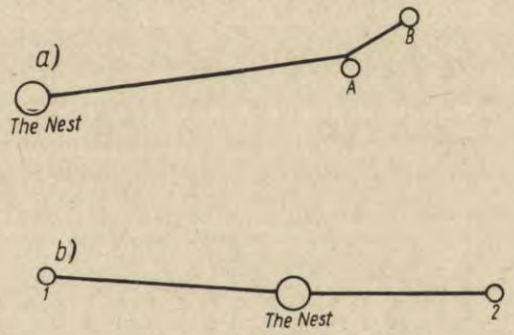

Fig. 1. Situation of nest

white ants were not found on the tree B, it could be assumed that the location (Fig. 1a) of the trees on the road was here the deciding factor.

For this reason a small nest was selected for a further experiment, as well as trees situated in different directions from the nest, one at a distance of 12 meters, another at a distance of 9—10 meters

(see Fig. 1b). 660 individuals were marked on the first track, and 250 (with a different colour) on the second, as the latter track was less frequented. After ten days of observations (a total of 10 hours of observation per tree), not a single case of confusion of tracks by the workers was noted. The results of observations on the first tree are presented in Table I.

Table I

Frequency of appearance of marked individuals on the tree A

1	Marking	Observation		
	2	3	4	5
Day of observation	Number of marked individuals	Total number of individuals per tree per hour	Number of marked individuals per hour	Percentage of marked individuals (in relation to total observed)
1	55	2400	18	0.75
2	55	2280	15	0.66
3	100	1980	22	1.1
4	125	1050	18	1.7
5	225	1200	13	1.1
6	300	720	24	3.3
7	410	1080	37	3.4
8	550	1320	91	6.8
9	550	1500	82	5.5
10	660	780	48	6.1

Comparison of column 5 and column 2 shows that the percentage of marked individuals increases parallely with the increase of the total number of marked ants (column 2). This fact proves that the results are not fortuitous and that the same ants always move to a definite object along a given track.

It should be noted that marking was always carried out on the tree trunk, and it can therefore be assumed that the majority of workers moved for only one purpose — to the aphides.

Similar experiments in which the tracks lead to other objects, not the aphides, were performed three times with *Formica rufa* and *Formica pratensis*, and similar results were obtained.

The experiments so far described were made mostly at the same hour. Thus, the constant appearance of the marked individuals on the tracks could be connected with the time of the day. In order to elucidate this question, another experiment was performed in

which a group of workers was marked in the morning an another group in the evening with different colours. The observations were then carried out at various times of the day. The results did not show any differences, both colours occurred in the same proportion at each time of the day.

The next method that of marking single ant found beyond the tracks, was — as already mentioned above — applied for the first time.

The course of the experiment was as follows:

At a distance of 1.5 meters from a very large track of *F. rufa* (described in the first experiment with trees A and B), and at 25 meters from the large nest 10 individuals passing through a selected plot of 20 × 20 cm were marked during a period of 35 minutes. During the following 5 days the plot was observed 7 times every day at various times. Not a single case was noted in which there was not at least one marked ant on the plot (the marking was not individual, and hence it is not known whether different individuals were present each time). The results are shown in Table II.

Table II

Frequency of singly marked individuals on the selected plot (10 individuals were marked on the first day)

Day of observation	Time of the day	Number of observed individuals
2	12.05	1
	16.17	2
3	6.07	1
	17.23	1
4	15.10	2
	23.50	1
5	0.55	1

This type of experiment was repeated 14 times with *F. rufa*, *F. pratensis* and *F. truncicola*. In general the results obtained were always similar. In only one experiment the marked individuals were seen twice during the seven days of observation. The results presented in the Table II for the remaining 10 experiments are typical.

All the results presented above relate to *Formica rufa*, *F. pratensis* and *F. truncicola*. As concerns *Formica sanguinea* the results are totally different.

Marking of individuals on one area repeated many times did not give results similar to those obtained with other species of *Formica*, nor was it possible to observe the marked individuals in the same spot or on the same track; this was due to the fact, that *F. sanguinea* do not use any permanent trails, they are formed for short periods, usually for one day, are less frequented than in the *F. rufa* and *F. pratensis*, and finally the tracks disappear only to reappear close by in a new spot.

There is one exception to this rule. When the *F. sanguinea* go to the aphides they use constant tracks. During the experiments it was observed several times that workers of *F. sanguinea* systematically went to aphides. Incidentally, these observations invalidate the opinion of Forel (1921—1923), who thought that *F. sanguinea* never go to the aphides and that this is done for them by the slaves. It is interesting that these ants are no less persevering in this task than the other species of *Formica*. Only for this function do the *F. sanguinea* establish a normal permanent track with a stable team of individuals.

The *Lasius niger* also has no permanent tracks. The results of all experiments, with the exception of those concerning the collection of the excreta from aphides, were negative, as in *F. sanguinea*. However taking into consideration, that the workers of this species are better at removing the marks by rubbing them of, the negative results obtained cannot be considered as certain.

As concerns the species *Tetramorium caespitum*, certain facts indicate that this species does not have any partition of foraging grounds. Experiments show that even in the nearest neighbourhood of the nest the individuals pass at random, and in general they do not reappear at the same spot. This species has no defined tracks. It may be therefore concluded, that *Tetramorium caespitum* have no partition of foraging grounds. Similar relations are observed with *Myrmica scabrinodis* and *Leptothorax acervorum*. It is therefore necessary to elucidate how the social foraging takes place in these species.

II. Conveying of information about the findings of food

The experiments described here are not yet completed. Preliminary results are however presented, as they supplement the data previously obtained relating to the partition of the foraging grounds in various species.

With all *Myrmicinae*, as well as with *Lasius niger*, similar results were obtained: if the amount of food is large, the foraging worker goes to the nest, and returns after some time again to the food followed by a string of several workers. The experiments of this type are especially demonstrative with *Tetramorium caespitum*.

Similar results were obtained by Eidmann (1925, 1927) with *Myrmica rubra*.

As concerns *Lasius niger*, it was observed that if the source of food is large, the first worker is followed by such a large number of helpers that a track is rapidly formed between the food and the nest. This track disappears immediately when the source of food is exhausted.

Numerous experiments with species of the *Formica* genus (*F. rufa*, *F. pratensis*, *F. truncicola*) did not give any positive results. It was not possible to observe any indication that workers of these species communicate with the nest when food is found. It can be frequently observed that a worker loaded with food, going towards the nest meets another worker: if the latter is hungry, it feeds from the loaded worker, and then moves along its way, irrespective of the direction of the former. *Myrmicinae* in similar cases change their direction, and follow a scent-trail to the source of food.

According to our observations, *Formica* species reacts differently to food that is found; within a short period almost all the ants passing near the food crowd around it, so that almost all of the ants foraging on a given ground are assembled at the food. Each worker stops near the source of food, and after carrying a part of it to the nest, returns again. Thus, as long as the source of food is not exhausted, there is always a small crowd of ants around it.

In order to prevent the worker which found the food from carrying it to the nest, we presented, as a bait, an insect pinned to the ground by a small rod. In such conditions the workers of *Tetramorium caespitum*, *Myrmica scabrinodis*, *Leptothorax acervorum* and *Lasius niger*, after a few unsuccessful attempts to re-

move the prey, returned to the nest to get helpers. The bait was then, sooner or later, torn to pieces and transported to the nest. The workers of genus *Formica* even under such circumstances never looked for help. Just the few individuals that happened to be there, tried to get the bait and sometimes when they could not manage it, abandoned the prey.

When the pupae were used as a bait, the effect was with every species the same as with food.

The experiments described above point to the necessity of carrying out further investigations on the ways of conveying information in various species and genera of ants.

DISCUSSION

The composition of individuals traversing trails in the species *Formica rufa* was the subject of previous investigations by Adlerz (1886), Natzmer (1915), Stäger (1925). Most extensive studies in this respect were carried out by Ökland (1931) and Kiil (1934). All these authors found that the composition of individuals on tracks is stable in this species. Moreover Eidmann (1927) also investigated the composition of individuals in *Lasius niger* on trails leading to aphides. He also found this composition to be stable.

Ökland and Kiil were of the opinion that practically it is not possible to mark workers in a territory individually. These authors assumed that no definite partition of the foraging grounds exists, apart from the above mentioned definite tracks. Our observations however showed that in *F. rufa*, *F. pratensis* and *F. truncicola* the social partition of the foraging grounds is more thoroughgoing. A stable distribution of individuals exists throughout the foraging grounds of a given nest; this area is divided into plots frequented constantly by definite individuals. Every forager has also a particular, individual or team foraging area, and remain on the assigned plot all the day long, only rarely returning to the nest. Other experiments concerning the division of labor showed, that during hot summer periods the workers of *F. rufa* and *F. pratensis*, also spend the whole day with the aphides, and return to the nest rarely, for several minutes only.

The recurrence of the same individuals in definite points of the territory was already observed previously by Reichle (1943) and Dobrzański (1956).

The results of observations reported here confirm previous studies on the constant composition of individuals on given tracks. If specific workers are assigned to a permanent foraging ground, then the trails must also have a permanent composition of individuals as it is along the trails that the workers invade the territory.

Very interesting is the fact, that the *Formicidae* family shows marked ethological differences between species in respect to the partition of foraging grounds. This confirms once more that the investigations conducted on a limited number of species cannot lead to generalizations including all ants. The family of *Formicidae* does not constitute a uniform group, but shows such a large biological divergences, that some authors (Wheeler 1937, Gösswald and Bier 1953) admit a polyphyletic origin of this family.

Thus our investigations have shown that although there is no doubt as to the strict partition of the foraging grounds in some species of the *Formica* genus, in the species *Lasius niger*, belonging also to the sub-family of *Formicinae*, such partition is no longer so distinct. In the *Myrmicinae* sub-family however many experiments did not permit to discover any partition of the territory. Although our results do not allow a definite conclusion on this point — it is always difficult to draw conclusion from negative results — there is no doubt, that the ethology of the species of *Myrmicinae* studied here, is totally different from the ethology of *Formicinae*, and that both from a quantitative as well as a qualitative point of view.

Special attention should be paid to results of investigations on the species of *Formica sanguinea*, as they differ from those obtained with other species of the same genus. This species is slave-making and one of its important life functions consists on finding new nests of ants of slave-providing species and of stealing their pupae. With such mode of life stable foraging grounds, such as were found to exist in other species of the *Formica* genus, would constitute a harmful factor, as the *Formica sanguinea* is bound to invade constantly new areas to find the foreign nests. Thus the absence of permanent trails and of any permanent division of the foraging grounds seems to constitute a regular feature of behaviour of this species.

It is all the more interesting to note that in the breeding of aphides the *F. sanguinea* behaves similarly to other *Formica* species, i.e. it shows then the same stability of individuals assigned to the

task. This is a very vivid example of the plasticity of *F. sanguinea*. This species is universally recognized as presenting the highest level of social life. Comparison with another slave-making species, which shows an exceptional lack of plasticity, namely with *Polyergus rufescens* — is of special interest. Apparently similar modes of life are manifested differently in both these species. It seems that this is connected with various degrees of plasticity. *Polyergus rufescens*, as it is well known, is strongly adapted to slave-making modes of life and depends totally upon slaves, losing thereby all normal instincts of workers. On the other hand, the *Formica sanguinea* maintains all its social instincts, supplementing them by the instinct of slave-raiding; hence slave-making for this species is not an absolute necessity, and does not limit its possibilities of adaptation; it supplements and expands them.

Concerning the problem of conveying information about the discovered prey, it should be noted that Eidmann (1925) made a special study of this problem. His results are similar to those presented here for various species of *Myrmicinae*. Certain reservations must be made, however, concerning his conclusion, in view of the fact that the behaviour of various species differs considerably, and the mentioned author conducted his investigations mostly on *Myrmica rubra* (without mentioning other species), and generalized his findings to ants generally.

Eidmanns' generalizations is proved incorrect by the results of Stäger (1931), who found no direct conveying of information in the genus *Formica*. He postulates that crowding of some number of workers near the food is caused by transmission of the founders excitation, which is purely visually, to the neighbouring individuals. The author calls it kinopsis; in consequence, the search becomes more intensive.

I failed to observe Stäger's kinopsis in Formica. I rather suppose that the scanty crowding near the food is involved by the territory partition: other individuals, feeding on the same foraging ground, constantly circulate on it and find this food accidentally in the same manner as the first finder.

It is evident, that in the problem of conveying of information, as well as in that of the partition of the foraging grounds, the results obtained are characteristic for various species. It is interesting to note that conveying of informations can be easily observed in species which do not show division of the foraging grounds (*Myr-*

mica scabrinodis, Tetramorium caespitum, Leptothorax acervorum and probably *Lasius niger*), whereas in species where this division does exist, it was not possible to reveal the conveying of information concerning the discovered prey (*F. rufa, F. pratensis, F. truncicola*).

These relation are summarized in the following scheme:

	Species where the conveying of information could not be detected	Species conveying information
Species presenting partition of foraging grounds	*F. rufa* *F. pratensis* *F. truncicola*	—
Species in which the partition of foraging grounds could not be detected	—	*L. acervorum* *T. caespitum* *M. scabrinodis* *L. niger* (?)

Thus it seems, that the partition of the foraging grounds and the conveying of information about the findings of food are two forms of social foraging. A definite partition of foraging grounds in some species may be considered as an adaptation replacing the conveying of information about the pray in other species. In cases, in which each plot of ground is surveyed in detail by the individuals constantly present on this spot, there is always a sufficient number of workers arround to carry the discovered food to the nest. In the species without partition of foraging grounds, a passing worker, finding food accidentally, informs the community about its finding. In these species, as with bees, the unemployed workers are probably always in the nest, where they form a reserve of foraging workers.

These two modes of social foraging were probably elaborated adaptively in the course of evolution.

In order to test the hypothesis, that the two modes of social foraging replace one another it is necessary to investigate experimentally, whether in those species where the partition of the foraging grounds exists, the conveying of information is always absent, and reciprocally, where the conveying of information exists, there is lack of partition of the foraging grounds. It is also possible that

such investigations might disclose other methods of social foraging differing from the two described here.

SUMMARY

1. The stable composition of individuals on ant tracks was discovered in *Formica pratensis* and *F. truncicola* and was confirmed in *F. rufa*. The constant composition of individuals is observed also in *F. sanguinea* and *Lasius niger* on tracks leading to aphides.

2. The social partition of the foraging grounds in *F. rufa*, *F. pratensis* and *F. truncicola* is very marked. Not only the tracks, but also the foraging grounds are allotted to a constant group of individuals.

3. Numerous experiments did not reveal any division of foraging grounds in the following species: *Formica sanguinea* and probably *Lasius niger* (apart from the function of collection of the excreta from the aphides), *Myrmica scabrinodis*, *Tetramorium caespitum*, *Leptothorax acervorum*.

Although it is not possible to draw final conclusions, in the case of negative results, it can be nevertheless definitely stated that partition of the foraging ground in these species has a totally different character than in the species *F. rufa*, *F. pratensis* and *F. truncicola*.

4. It is especially interesting that *F. sanguinea* behaves differently than other *Formiica species*. This is probably connected with slave-making practised by this species, and with the high plasticity of its behaviour.

5. Experiments concerning conveying of information about the findings of food show that in the species, showing a definite division of the foraging grounds, it was not possible to disclose any conveying of information concerning the discovered prey (*F. rufa*, *F. pratensis*, *F. truncicola*); and reciprocally, the species without partition of the foraging grounds possess a precise system of information (*M. scabrinodis*, *Tetramorium caespitum*, *Leptothorax acervorum*).

6. On this basis the existence of at least two forms of adaptation to social foraging may be assumed. In some species this adaptation consists of a definite partition of the foraging grounds, in other species, which have no permanent partition of the territory, the individual, which discovered prey, notifies it to other workers.

REFERENCES

ADLERZ G. 1886 — Myrmecologiska studier. II. Svenska myror och deras letnadsforhallanden. Bihang Kgl. Svenska Vet. Akad. Handlingar, *11*.

BUCKINGHAM E. 1911 — Division of labor among Ants. Proc. Am. Acad. of Arts a. Sci *46*, p. 425—507.

CARTHY J. D. 1951 — The orientation of two allied species of British ant. Behaviour. *3*, p. 275—303.

DOBRZAŃSKI J. 1956 — Badania nad zmysłem czasu u mrówek. Folia Biologica. *4*, p. 385—397.

EHRHARDT S. 1931 — Über Arbeitsteilung bei Myrmica und Messor-Arten. Zeitschr. Morphol. Ökol. Tiere *20*, p. 755—812.

EIDMANN H. 1925 — Mitteilungsvermögen bei Ameisen. Naturwis. *13*, p. 126—128.

EIDMANN H. 1927 — Ameisen und Blattläuse. Biol. Zentralblt. *47*, p. 537—555.

EIDMANN H. 1927 — Jazyk murawiew. Rus Zool. Żurnal. *71*, p. 39—47.

FOREL A. 1921—23 — Le monde social des fourmis du globe. T. I—V. Généve, Libraire Kundig.

GÖSSWALD K., BIER R. H. 1953 — Untersuchungen zur Kastendetermination in der Gattung Formica. Naturwiss. *40*, p. 38—39.

KIIL V. 1934 — Untersuchungen über Arbeitsteilung bei Ameisen. Biolog. Centralblatt *54*, p. 114—146.

NATZMER G. 1915 — Das biogenetische Grundgesetz im Leben der Insektenstaaten. Biol. Cbl. *35*, p. 30—36.

ÖKLAND Fr. 1931 — Studien über die Arbeitsteilung und die Teilung der Arbeitsgebietes bei der roten Waldameise (*F. rufa* L.). Zeitschr. Morph. Ökol. Tiere. *20*, p. 63—131.

REICHLE Fr. 1943 — Untersuchungen über Frequenzrythmen bei Ameisen. Zeitschr. f. vergl. Physiol. *30*, p. 227—251.

STÄGER R. 1924—25 — Über die näheren Umständen beim Heimschaffen der Beute durch die Waldameisen. Entom. Z., Internat. Ent.-Ver. 1925, pp. 58—60, 66—67, 72—73, 78, 80—81, 84—85, 87—88, 91—92.

WHEELER W. M. 1926 — Les sociétés d'insectes. Paris, Gaston Doin.

Acta Biologiae Experimentalis, Vol. XVIII, 1958

EXPERIMENTAL STUDIES ON THE CONDUCTIVE ROLE OF ECTOPLASM AND THE SILVERLINE SYSTEM IN CILIATES

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(Received for publication 23 October 1957)

In accordance with the classical views of many authors (Sharp 1914, Rees 1922, Klein 1942, Gelei 1936), coordination of the motion of cilia, and conduction of impulses in ciliates takes place by means of neuromotor apparatuses and silverline systems, although opinions of the mentioned authors differ considerably. All of these theories were frequently criticised, and the problem remains open.

In recent times a number of studies have appeared relating to electronic microscopy, and giving new data concerning the fine structure of silverline systems. The majority of authors in hypothesis concerning the role of fibril formations base their assumptions on morphological data; experimental studies were few. From amongst such studies the following authors should in the first place be mentioned: Taylor (1920), Worley (1934), Milicer (1935), Parducz (1954), (1955) and Okijama (1954). The method applied in this work was that of removing and damaging definite protoplasmic elements, and observing disruptions in the coordination of motion, and reactions to stimuli in ciliates. Efforts were thus made to solve the problem of localization of such functions, and the directions of conductivity. Attempts were made to determine: 1) which elements must be present for various forms of coordinated motion and reaction to take place, 2) to what degree

does the character of coordinated motion change parallel with damaging of the structure and especially parallel with incisions in various directions.

MATERIAL AND METHODS

The experiments were conducted with *Paramecium caudatum* Ehrb., as a representative of ciliates showing a zonal silverline system and as a classical object of experimental investigations. Our knowledge of the morphology of fibrillar systems in this species is the greatest, and notwithstanding many obscurities, it can be assumed that the general picture of these systems and of their connection with motion organella is known. Experiments were conducted both with pure lines, as also with mixed ones — the principle was accepted that more representative results can be obtained with mixed cultures while in pure lines results might have a specific character.

However no actual differences were found in the reaction of individuals of various origin. The individuals used for breeding were from the neighborhood of Warsaw. A hay infusion was used for propagation. The method of selecting individuals for investigations experiments will be described in discussing particular, at any rate each individual was checked for normal activity.

Observations on the motion of cilia were conducted in a dark field by means of a Lumipan microscope at medium and low enlargements (objectives 10×, 20×, 40× and immersive objective 60×). A dark field is much more suitable for observing cilia than a phase contrast. A phase microscope was used for controlling operations; phase contrast frequntly gave better results than the dark field in distinguishing protoplasmic structure. A Zeiss phase contrast was used.

The majority of the microphotographs were made with a Rathenow Microphote, and with an FF Isopan film. A binocular Spencer A. O. magnifying glass or a Zeiss Cytoplast were used for small enlargements.

Operations were made by means of two methods: by hand and by means of a Goldacre glass micromanipulator. Usually scalpels made of minutia were used for the operations by hand. Initial material consisted of silver minutia 0.02 mm in diameter, which were hammered on a small anvil, and sharpened on unpolished porcelain under a binocular. Steel minutia were also used, which were heat treated and tempered. These blades were next set in needle holders or fused into glass tubing.

The Goldcare micromanipulator was constructed in the Nencki Institute of Experimental Biology according to description by this author. Its great simplicity, cheapness and ease of operation constitute its merits, although precision obtained is not as great as that of the Zeiss or de Fonbrune micromanipulators. During operations this micromanipulator was connected with the microscope stage by means of a vice. The microneedle made according to the method described above was mounted in a thin glass rod, constituting a part of the micromanipulator.

Glass needles were drawn by means of an electrically heated wire spiral and a weight attached to the glass rod from which the needle was to be

made. Advantage was taken of equipment made in the Neurophysiological Department of the Nencki Institute of Experimental Biology on the basis of a prescription given by Weal, and somewhat improved. Microneedles were made of Jena glass.

OBSERVATIONS ON NORMAL MOTION OF CILIA

For proper interpretation of experiments, it was found necessary to accurately investigate the normal motion of cilia. Notwithstanding numerous studies conducted on this subject, even the general character of ciliary motion had not been hitherto definitely explained in Paramecium. This is especially true of the problem of so called ciliary waves, as concerns their occurrence and biological significance, and even their direction — in all of these questions, the opinions of different authors differ considerably. This is due in the first place to methodical difficulty of observing the motion of cilia in normal conditions because of its seeming rapidity under microscopic enlargement, as also due to the lack of data as to what extent various methods of artificial retardation of this motion change its normal picture. The author carried out a number of observations in this respect. Observations conducted in normal conditions in a dark field point to the distinctly metachronic character of ciliary motion during normal forward movement of ciliates i.e. left-turn spiral, sometimes during forward movement without rotation. During Jennings avoiding reaction, the character of the motion seems to be synchronic, at least during certain stages of reaction.

These observations prove facts known previously. The wave character of metachronic motion can be observed only in certain cases under favorable optic conditions. More precise observations can also be made if we have to do with operated individuals or individuals pressed by a cover glass, however even in such cases it is not always possible to observe the motion of cilia on the whole surface simultaneously. From amongst methods used for retarding the motion of cilia for observing normal movement, low temperature was applied according to Parducz. Temperature in the room during the experiments was +2°C; the microscope and cultures were also previously cooled.

In such conditions individuals possess a somewhat changed form; they are distinctly thicker, especially in the rear part of the body,

move forward very slowly, rotary motion is considerably retarded or totally lacking. During the lack of rotary motion individuals move forward along a zigzag path, bending left and right. Forward movement makes the impression of being broken. These changes of motion and rate are however very slight, and therefore noticeable only with difficulty.

Ciliary waves can be seen on all of the individuals. These waves are highly labile, frequently changing the line along which they run, however during progressive motion the wave crests always run under an acute angle to the longitudinal axis of the body, i.e. waves run obliquely. Waves are variously distinct, and a number of transitions more or less standing out can be distinguished. The most distinct waves can be observed in the peristomal depression (see Plate II, Fig. 5), in the sector from the cytopharynx to the front end of the body, although these waves are also visible on the whole body.

The most important result of the observations described was the fact that ciliary waves move very rapidly from the rear to the front. This observation confirms the view of Parducz, but is not in agreement with the opinion of other authors who always assumed motion of waves from the front to the rear.

Motions of the membrana quadripartita are distinctly visible within the peristome. The general picture of the run of waves possesses the same character as the picture obtained by means of the method of rapid fixation.

RAPID FIXATION DURING GALVANOTROPIC MOTION

One of the important sources of our knowledge relating to ciliary motion of ciliates is based on the method of rapid fixation. The main difficulty consists in stating the connection between the various forms of the ciliary motion fixed in the preparation and the behaviour of the individual as a whole. The fixed picture shows a number of individuals with various arrangement of cilia. Which of these, if such exist, correspond to the motion of ciliates taking place before fixation? In normal conditions ciliates move in drop of water in various ways and directions before fixation; not knowing the initial state, nothing can be said of disturbances caused by the fixative. It therefore became necessary to force

a uniform and undirectional motion upon all of the ciliates before fixation. Galvanotropism was taken advantage of for this purpose. A contrivance was used for evoking electric shocks in ciliates, where by it was possible to regulate the interval between electrodes, and the tension and voltage of the current. A large drop of water containing clean ciliates was placed on a glass previously deprived of all fatty substances and placed in the field of vision of a binocular. The water was spread to take a shape as shown on the Table II, Fig. 7, electrodes were placed at both ends of the drop, and the current connected. Lowest voltage was so regulated as to evoke active galvanotropic motion in all of the individuals. Thus for example at a distance of 3.3 cm between the electrodes, a voltage of 3 V and a current of 30 milliamperes was applied.

These values furthermore depend upon many other factors, as for instance the cross-section of the water connection between electrodes. After changing the direction of the current several times, and obtaining one direction of movement of ciliates between the electrodes, the Parducz fixative was injected in the center of the water connection by means of a specially drawn pipette rendering possible simultaneous injection over a given surface. There was 3—4 times more fixative in the spot of fixation than water with ciliates. After fixation the ciliates were removed only from the spot of fixation thereby assuring that fixation took place rapidly and that the ciliates were not in the immediate neighborhood of the electrodes. This was followed by staining the ciliates according to the P a r d u c z method, however in view of the relatively small number of individuals in each experiment, the liquids were changed in the glass receptacles instead of centrifuging.

As a result of this procedure i twas found that undulative motion of cilia is preserved on a majority (about 84%) of the ciliates.

It was furthermore found that in cases in which fixation took place rapidly, a higher percentage of individuals with undulating cilia was obtained.

In general the data obtained prove that undulative motion of cilia takes place with progressive movement, and that the general picture obtained with the rapid fixation method is true. The fact however, that not all of the ciliates have a uniform character of motion in the samples fixed, cannot be passed over.

OBSERVATIONS OF CILIARY MOTION ON FRAGMENTS OF CILIATES

The author repeated experiments conducted by W. Milicer. using a somewhat different method; instead of using carragan for retarding motion, an infusion of flax-seed was used, and observations also carried out without retarding motion. Cutting was carried out by means of the previously described needles from silver minutia. Results were basically in agreement with results obtained by the mentioned author, although ciliary waves were more frequently observed on fragments.

In order to obtain more definite data, a new method of rapid Parducz fixation was used for fragments. The purpose of the described method consisted of obtaining a large number of operated individuals, which on the one hand increased credibility of results, on the other made it possible to apply a fairly simple technique. The ciliates were taken from a geotropic ring of a well developed hay culture, washed, and placed in a test tube containing water, and then lightly centrifuged by means of a hand centrifuge. It was not possible to carry out the trials in a culture medium, containing large amounts of impurities. A large drop of water with ciliates was then placed on a black glass slide attached by means of a small vice in the field of vision on the binocular. Side lighting made observations possible. Experimentation was begun after passing of the eventual influence of centrifuging, and return of normal motion.

The drop of water containing ciliates was chopped by means of a normal safety razor blade without paying attention to single individuals. Effects of this chopping were checked under a binocular. Chopping was stopped after the number of fragments was around one fourth the number of individuals, this usually being after around five minutes. Material so prepared was immediately, or after a certain period of time depending upon the principles of the experiment, fixed by means of the Parducz fixative either by placing a drop containing ciliates in a test tube with fixative or by injecting an excess of the fixative into drop with ciliates. Further procedure was in accordance with the Parducz method of staining. Microphotographs and drawings by means of a camera lucida were made. (Plate I, Figs. 2, 3, 4, 5. Plate II, Figs. 2, 3, 4). Undamaged individuals present on the same slides served for control and comparative purposes.

On the basis of this material, the following conclusions were reached:

1. The general character of ciliary motion on a given slide is similar in fragments and in control individuals.

2. The most distinct ciliary undulations occur around the peristome.

3. Undulations appear more frequently on fore fragments than on rear ones.

4. Undulations on fore fragments in general show a longitudinal direction; on the ventral side around the cytostome-circular.

Reaction of cut individuals

The reaction of reverse ciliary motion was selected as the reaction to be investigated. Experiments and observations on the motion Paramecium fragments are extensively described in literature (Jennings and Jamieson 1903, Worley 1934, Koehler 1936, Milicer 1934 and others), notwithstanding the above, this problem requires further experimentation.

The experiments were made by means of a knife from silver minutia. For comparison of results with results obtained by W. Milicer (1934), cuttings were made at the same levels as

	Front fragment	Hind fragment
1	OO	+++++
2	O–OO–	+++++
3	+––+O	+++++
4	++––+	+++++
5	+++++	++

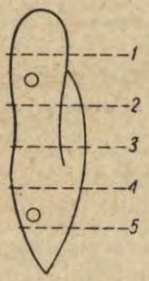

Fig. 1. Reaction of Paramecium caudatum fragments to chemical factor o lack of forward movement, — further forward movement (lack of reversion), + backward movement (reversion). Numbers denote levels at which incisions were made. Explanations in the text

those made by the mentioned author i.e. in five variants: before the fore pulsating vacuole, past the fore vacuole, before the cytopharynx, after the cystome and before the rear vacuole, and past

the rear vacuole. The cut fragments were carried in a minimum amount of water to 25 mM of potassium bromide solution, which causes reversion of motion in normal individuals (D r y l 1952) and backward movement lasting around two minutes. Results of this series of investigations are presented in the Fig. 1. Obtaining of the most forward and rear fragments was fairly difficult, as they easily disintegrate. As can be seen from the Fig. 1, the rear fragments submitted to reversion most easily.

It should be furthermore noted that rear fragments moved backwards along a straighter line than fore fragments.

It is obvious that the fore parts show a greater sensitiveness, however the majority of data indicate that each part of Paramecium can show reversion with the exception of the most forward sections.

A similar experiment was also carried out by means of a somewhat different method, namely ciliates after cutting were placed with a larger drop of water in a solution of potassium bromide, and observed at the line of contact between the two liquids. This line of contact could be defined by observing several control individuals simultaneously let into the same drop. Reversion was observed along the line of contact in individuals without a rear end, however not in each case. General results are similar to those described above.

Observations were also carried out on fragments of ciliates in paraffined square shaped dishes by applying a drawing apparatus; however distinct results were not obtained here. Also in this case the fore part shows slower movement, and a more zigzag course. Fore fragments perish within a short period of time. Larger rear fragments show a certain constancy in angles of reflection, similarly as normal individuals, although not in every case.

Enucleation

In order to prove, whether the presence of the nucleus is a necessary condition for maintaining normal motion of cilia, a number of tests were made after its removal. A broad cut was made by means of a scalpel from minutia in the pellicula of Paramecium which was then lightly pressed around the nucleus. Emergence of the macronucleus was observed together with a small amount of endoplasm (Plate III, Fig. 1). The nucleus observed in a dark field

had a steel blue shine, and without doubt a harder consistency than the surrounding plasma. After removal the macronucleus maintained its initial shape.

The micronucleus was not observed. Only 8 successful operations were performed in which emergence of the macronucleus together with a small amount of cytoplasm was observed, and the whole individual did not disintegrate that it was possible to observe motion. In all of the cases distinct coordination of motion could be observed, this being proved both directly, as well as by observing the movement of particles around the ciliate. Motion of cilia in general had a metachronic character.

This experiment shows that the presence of the nucleus is not an indispensable condition for maintaining coordination of ciliary motion.

Pressing with a coverglass

Further investigations were aimed at separating other constitutional elements of ciliates, such as endoplasm and the subpellicular silverline system.

Pressing with a cover-glass was applied, by gradual removal of water and ciliary motion observed in a dark field. Two types of vesicles were formed, outflows of hyaloplasm in the form of large drops which did not separate from the individual, and protrusions on the surface of which cilia and pellicula are found. An accurate description of these formations is given by the author elsewhere (Doroszewski 1953).

These experiments do not give uniform results; as an example description of two experiments are given (Plate III, Figs. 2 and 3).

A tuft of constantly moving cilia can be seen on the surface of the vesicle. At times distinct coordination of motion was observed. The course of undulation on the tuft was independent of the remaining ciliary motion. At times cilia separate into 2 or 3 tufts moving independently of each other, but within each tuft cilia can be seen bending in one direction. During short moments, no coordination can be observed. This observation lasted for around 30 minutes at a very good visibility of the cilia.

In another experiment an infusion of linseed (Plate III, Fig. 2) was used for slowing motion. Vesicles were formed by pressing the cover glass followed by making two incisions in the vesicles. In this way slices of pellicula together with the subpellicular silverline

system were obtained. Vesicles were formed from the pressed and cut pellicula, from which ectoplasm slowly oozed. The pellicula and the silverline system were very distinctly visible. On the pellicula of the vesicles, ciliary undulation is much more distinct than in normal individuals. Undulations moved from the rear to the front similarly as in a low temperature. To the rear of the cutting, motion was coordinated, but undulations were observed once only, and that not at the same time as in fore part of the body.

In other experiments no undulative motion was observed on slices of the pellicula; synchronic or non-coordinated motion sometimes occurred. It is difficult to state if ectoplasm was present directly under the surface layers of vesicles or whether they touched with hyaloplasm, as hyaloplasm and ectoplasm are optically uniform. At any rate the fact that undulative movement can take place in pressed slices of the pellicula with subpellicular elements proves, that the presence of endoplasm is not a necessary condition for its occurrence.

OBSERVATIONS ON INCISED CILIATES

After proving that the outer parts of the body of ciliates are the only necessary element for metachronic motion to take place, efforts were made to explain the problem of direction of motor impulses, and eventually the interrelation between this direction and the silverline system, so as to supply material for explaining the mutual role of ectoplasm and this system.

For this purpose the following series of experiments was carried out: single longitudinal and transverse incisions, double parallel longitudinal and transverse incisions.

Longitudinal incisions

This type of incisions are much more difficult to make than transverse; the pellicula is more resistant in a lengthwise direction, and in attempting this type of incisions, ciliates rapidly fall apart. Thus obtaining a large number of experimental material is difficult. Operations were made by means of scalpels from silver minutia.

Disruptions in the motions of ciliates were relatively small; only small groups of several cilia in the immediate neighborhood of the

injury moving irregularly were noticed. Non-coordinated motion was not noticeable on both sides of the cut. If reversion occurs, it involves the whole individual.

Transverse incisions

Efforts were made to make relatively shallow incisions. Observations were carried out after retarding motion by means of a flax seed infusion, as also without retarding ciliary motion. A phase contrast microscope was used for controlling the incision, and a dark field for observing ciliary motion. Incisions were made in the middle of the body, usually on the dorsal side. Immediatelly along both sides of the incision, tufts of cilia were visible irregularly moving (called "flames" by W. Milicer). Frequently non-coordinated undulations were observed before and past the incision (Plate III, Figs. 4, 5, 6, 7, 8). Very distinct, rapid undulations were observed before the incision in the case of transverse incisions made at the level of the fore vacuole. In this case metachronic motion past the incision was fairly slow. Sudden accelerated motion of cilia from the fore part of the body did not reflect upon the rapidity of ciliary motion below the incision. In some cases the reverse could be observed, i.e. movement past the incision is more rapid. It was also noted that in some cases motion at one side of the incision is less regular than at the other side. Sometimes non-coordinated undulation can be observed at both sides of the incision at various angles — up to 180°, as also non-coordinated motion on one of the sides.

In the case of deep and broad incisions resulting in strong damaging the ciliate, specific effects of incising cannot be noted on the background of such far reaching disruptions. If the reversion of ciliary motion takes place during observations, it involves the whole individual irrespective of the incision, thereby proving Worley's theory regarding the independence of reversion mechanisms and metachronic motion. However according to this author the only type of disruptions with transversal incisions constitute non-coordinated or synchronized motion near the incision; the present observations show that also disruptions in metachronic motion can occur as sequence of transversal incision.

Two parallel longitudinal incisions

These experiments were conducted as controls for experiments on isolating the ciliary? field by two transverse incision, which will be discussed later. Due to methodical difficulties with longitudinal incisions, only several trials of this type were carried out, and that with deep incisions. Longitudinal incisions were made on an individual flattened by a surface water film in such a way that an incision was cut through the edge of the individual thereby giving two parallel incisions (Plate III, Figs. 9, 10). If the region of "flames" did not occupy the whole field between incisions, then undulations passed through this part without breaking down or changing their rapidity.

Two parallel transverse incisions

The methods of experimentation were similar to those applied with single transverse incisions, i.e. operations were made with and without retarded motion. Motion was observed in a dark field and by means of phase contrast. The purpose of these experiments was to obtain an isolated ciliary field between two parallel transverse surface incisions. Elements of the subpellicular silverline system are longitudinally directed in the central part of the body on the dorsal and lateral side, hence efforts were made to observe the motion of cilia after severing the argentophile fibres from both sides.

In the case of two transverse incisions, observations were also conducted on the direct effects of the injury as much, i.e. on tufts of cilia irregularly moving on both sides of the injury. The major purpose of the observation was to compare the motion of cilia between the incisions, and on the remaining part of the body. As a result of several experiment it was proved that ciliary undulations in the isolated field can differ considerably in respect to their rapidity, and hence in respect to their phase of motion and distinctness, from metachronic motion on the remaining part of the body. A region of more rapid or slower undulation can occur between incisions irrespective of motion on the remaining part of the body (Plate III, Figs. 4, 5, 6, 7, 8). Total conformance between metachronic motion and motion on the remaining surface was not noted even once. If however reversion of motion takes place, it also includes the isolated field. In case non-coordinated or slower

PLATE I

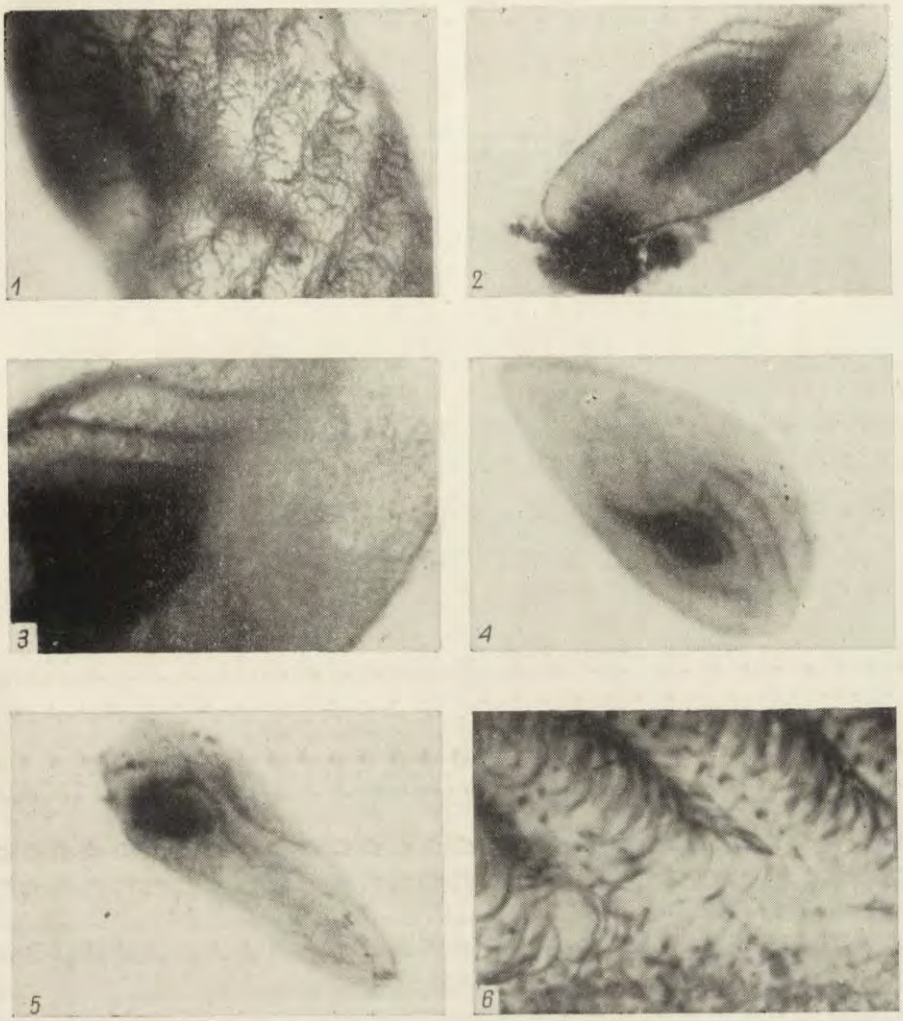

Fig. 1. Ciliary undulations on the surface of P. caudatum. Parducz method. Enlarged around 570 times

Fig. 2. Ciliary undulations around the peristome on a fragment of P. caudatum. Fore part of ciliate cut off. Modified Parducz method. Enlarged around 194 times

Fig. 3. Fragment of Fig. 2 enlarged 570 times

Fig. 4. Ciliary undulations of the rear fragment of P. caudatum, incision before peristome, enlarged around 240 times

Fig. 5. Fragment of P. caudatum, ciliary undulations, enlarged around 240 times

Fig. 6. Ciliary undulations on surface of P. caudatum, enlarged around 1500 times

undulative motion takes place betwen incisions, the individuals begin to revolve in the direction of the injury.

Undulations do not always move constantly along the isolated part, a description of one of the experiments is given as follows.

Immediately after the operation cilia usually remain immobile; undulations move along them every several seconds. During this time movement of cilia along the rest of the body is normal. After five minutes cilia of the isolated field are in constant metachronic motion, which is slower than the motion on the remaining part of the body. At the beginning of observations the individual is bent somewhat in the direction of the injury, and circles in this direction, gradually movement of the whole ciliate becomes more and more normal.

GENERAL REMARKS

Studies on the course of ciliary undulations, which confirm Parducz's opinion, lead to certain general conclusion in agreement with this author. Let us assume that during the movement of ciliary undulations from the rear to the front, the ciliate is moving forward. If some chemical or mechanical obstacle is met with and reaction takes place in the form of a change of direction of ciliary motion, then the direction of impulses transmitting the changing of this motion must differ by around 180° from the normal motor impulse. It is obvious that we can also have here to do with a classical avoiding reaction and synchronic reversion, however this problem has also been recently explained by Parducz. Without going into details as to the theoretical consequence of opposite directions of two impulses, it can be stated that the phenomena of transmission in ciliates are differentiated and specialized to a much greater extent than can be imagined.

Experiments on rapid fixation of ciliates can constitute a certain contribution to the problem of relative sensitivity of various parts of the Paramecium body. No specific stimuli were here applied, nevertheless the region in which undulations were most frequently excited was the peristomal sector. In accordance with other authors this region can be therefore treated as the most sensitive in respect to the formation of undulation.

Results of experiments on the reaction of fragments of ciliates to chemical factors are not fully in agreement with results obtain-

ed by Koechler (1934) or by Alverdes (1922). The lack of reaction in the fore fragments can be explained either by greater sensitivity of these parts to injury according to Child's gradient sensitivity theory, or by the role in this reaction of the elements placed farther to the rear.

It is also possible that sensitivity differentiation occurs in a certain longitudinal sector in accordance with that stated above.

The mentioned experiments are further proof of the fact that the presence of nuclear material and endoplasm do not constitute a condition necessary for coordinated ciliary motion. Localization of centers of transmission should be sought for in the peripheral layers of the protoplasm.

The results of experiments with surface incisions and application of an isolated field, indicate conformance in the direction of transmitting metachronic stimuli with the general direction of the silverline system. These experiments also confirm Worley's (1934) opinion as to the separate localization of centers governing

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PLATE II

Fig. 1. Elements of the subpellicular silverline system in individuals of P. caudatum fixed by applying high temperature and pressing with cover-glass
a — argentophile fibre and basal bodies, b — pellicula
c — protoplasm, d, e — hyaloplasm extrusions.
Observations in dark field, immersive objective 60 times. Sketch

Fig 2. Rear fragment of P. caudatum fixed after cutting. Modified Parducz method
a — place of cutting, b — ciliary undulations. Enlarged around 300 times

Fig. 3. Fore fragment of P. caudatum
a — place of cutting, b — ciliary undulations. Enlarged around 300 times

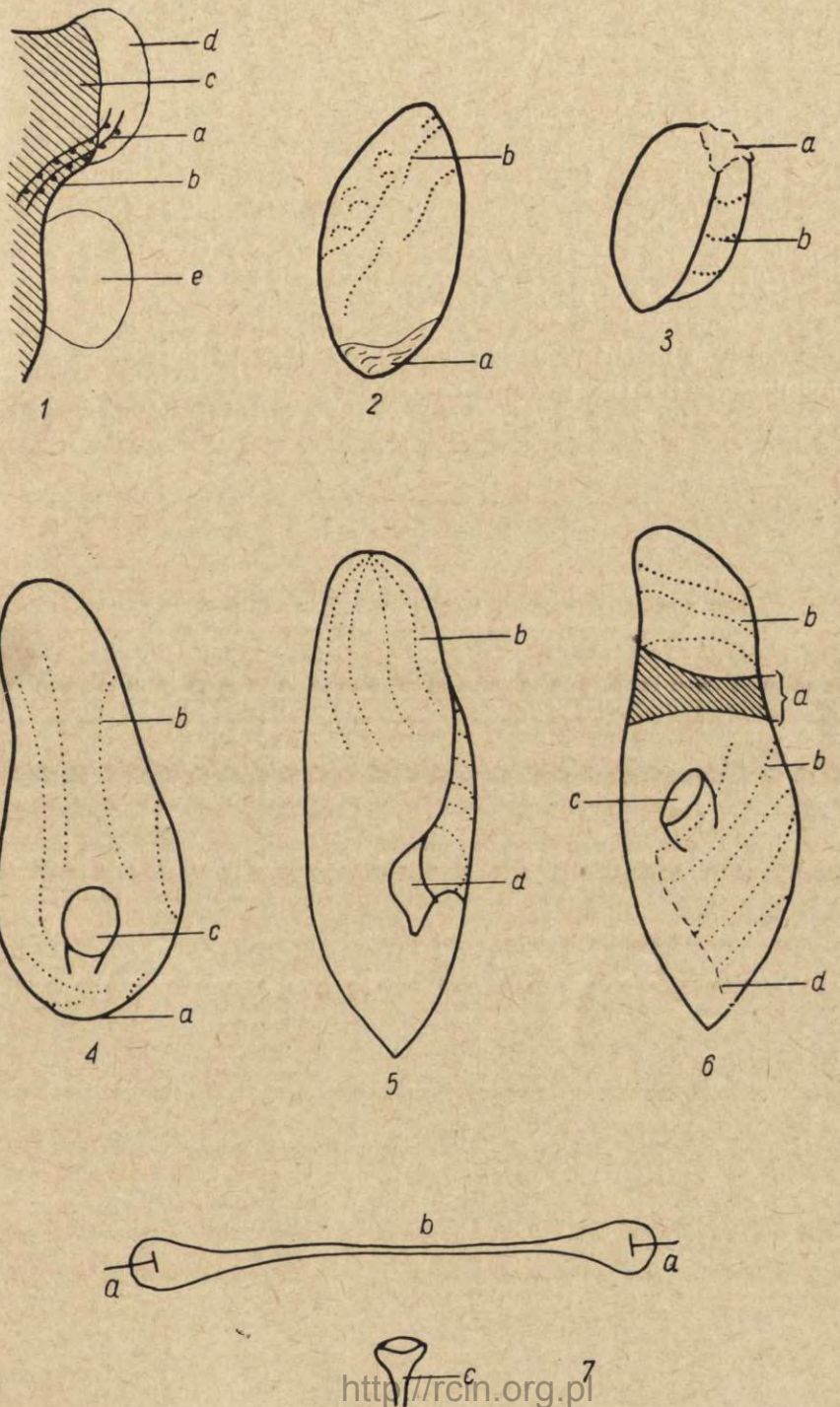
Fig. 4. Fore fragment of ciliate fixed after cutting
Enlarged around 300 times
a — place after cutting, b — ciliary undulations, c — cytopharynx

Fig. 5. Course of ciliary waves on living individual, motion retarded by means of low temperature
a — cytopharynx, b — ciliary undulations. Sketch without drawing equipment

Fig. 6. Fixed individual with transverse surface incisions
a — surface of incision, b — ciliary undulations, c — cytopharynx, d — silverline suture. Enlarged 300 times

Fig. 7. Experimental schema, fixation of ciliates during galvanotropic movement. Explanations in the text
a — electrodes, b — drop of water, c — pipette tip with fixative

PLATE II



reversion, which probably move through surface spreading. They also show that in certain conditions there is a possibility of maintaining metachronic motion outside of the incision with the exception of local effects of injury. By applying a different method, Worley found only synchronic motion.

No influence of Rees motorium on metachronic cilliary motion was observed, on the contrary ciliary undulations remained on fixed fore fragments, especially in the peristomal sector.

By comparing results obtained by Worley (1934), Parducz (1954), Okijama (1954) and herein, it can be assumed that there are two types of mechanisms in Paramecium for coordinating ciliary motion. The mechanism of reversion, change in pattern of ciliary undulation and the general character of motion could be identified in the light of studies by Parducz (1954) and Okijama

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PLATE III

Fig. 1. Enucleation of P. caudatum. Explanations in the text
a — macronucleus, b — endoplasm. Enlargement around 200 times

Fig. 2. P. caudatum pressed with the cover glass. Explanations in the text
a — convexity on the surface of the ciliate, b — the observed cilia, c — the hyaloplasm extrusions. Enlargement around 200 times

Fig. 3. P. caudatum after being pressed. Explanations in the text
a — the pellicular lobes obtained by the longitudinal incision of the superficial convexities, b — the direction of the ciliary propagation. Enlargement around 200 times

Fig. 4. Two transverse incisions
a, b — the fields of constantly disturbed ciliary movement and immobilized cilia (flames), c — the field of isolated ciliary waves. Enlargement around 200 times

Fig. 5. Two transverse incisions
a — the anterior incision, b — the wide posterior incision, c — the field of isolated ciliary waves. Enlargement around 200 times

Fig. 6. Two transverse incisions
a, b — the field of constant disturbances, c — the field of independent undulation. Enlargement around 200 times

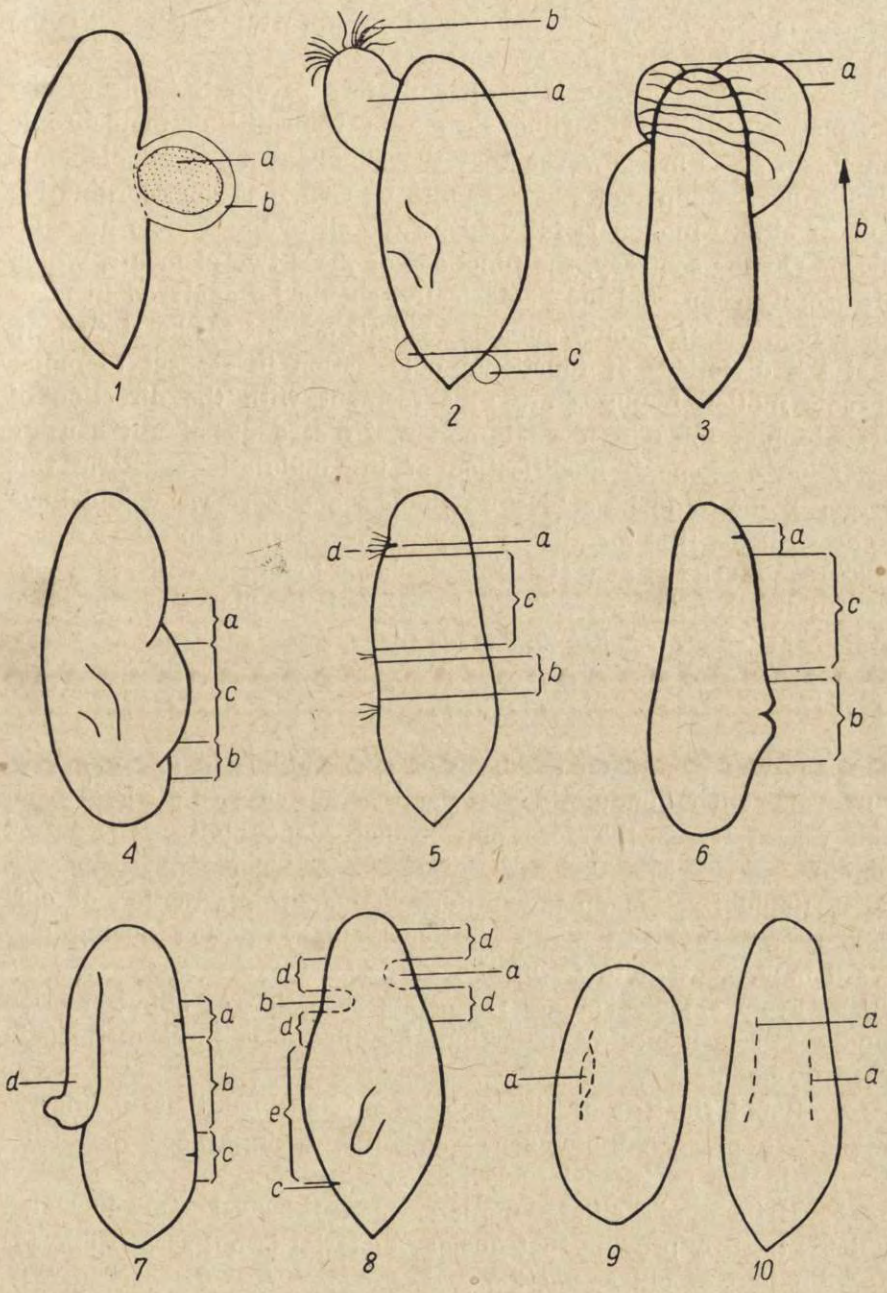
Fig. 7. Two transverse incisions
a, c — the field of constant disturbances, b — the field of independent undulation. d — the pressed out peristome. Enlargement around 200 times

Fig. 8
a, b — wide incisions (area of torn cilia), c — scarred incision, d — the field of constant disruptions, e — area of independent undulation. Enlarged around 200 times

Fig. 9. Longitudinal incision running through individual Ciliate flattend by surface water film

Fig. 10. The same individual after adding water. Two cuts visible (a, b) formed on surface. Areas of separate undulations not observed. Enlarged 200 times

PLATE III



(1954). This type of impulses could disperse in the ectoplasm in various directions. On the other hand we would have to do with ciliary motion mechanism connected in a certain way with the direction of the silverline system.

As mentioned above, it is difficult to separate the role of the ectoplasm and the silverline system in transmitting metachronic impulses. It seems however that the functions of these elements should not be opposed, and it cannot be said that the coordinative role of ciliary motion is fulfilled by the silverline system or ectoplasm. On the contrary, the functions of these two elements, which are closely connected morphologically, should be analyzed in common. It is therefore only possible to limit oneself to the statement, that the directions of functional polarization in respect to metachronic motion impulses are in agreement with the direction of argentophile lines in normal motion. In case of stimuli causing reversion or general modification of the undulative pattern, this arrangement could be subject to change, and here we would have to do with the first mentioned mechanism described by P a r d u c z (1954) and O k i j a m a (1954). Ectoplasm together with the subpellicular silverline system can be treated in ciliates as the basic system in which transmission takes place.

SUMMARY

1. It was proved on the basis of observations in low temperatures that ciliary undulations in Paramecium as a rule travel from the rear towards the fore end in accordance with P a r d u c z opinion.

2. Rapid fixation during uniform galvanotropic motion of ciliates shows, that this method reflects ciliary motion during forward movement quite faithfully.

3. From amongst ciliate fragments reversion of ciliary motion under the influence of potassium bromide was most distinct in rear fragments.

4. Rapid fixation of ciliate fragments showed that undulative motion is preserved on such fragments, taking place most distinctly in the peristomal sector.

5. Metachronic ciliary motion was preserved on enucleated ciliates thereby proving that nuclear material is not responsible for coordination of ciliary motion.

6. Ciliary motion was maintained on separated from endoplasm pieces of pellicula containing the silverline system, thereby proving that the endoplasm does not exert any direct influence on ciliary motion.

7. Isolated field of ciliary motion was formed by applying two transverse incisions. Ciliary motion of this field was independent, in its phase and rapidity, of motion on the remaining parts of the body. Besides this local effects of injuries were observed in the form of immobilized cilia and non-coordinated motion.

Two parallel longitudinal incisions in control experiments gave only local effects in respect to ciliary motion. This would seem to prove conformance between the course of stimuli and that of the silverline system. However, if reversion of ciliary motion took place, then the isolated field was also included.

8. The author sees a connection between the subpellicular silverline system and metachronic motion impulses; however reversion takes place independently of this system in accordance with Worley's viewpoint.

9. Ectoplasm together with the supbellicular silverline system should be treated as the basic system in which transmitting of impulses takes place in ciliates.

The author expresses his thanks to Dr Bela Parducz and Dr Joseph Gellert for their personal help and for numerous discussions from which he greatly benefited.

REFERENCES

ALVERDES F. 1922 — Zur Lokalisation des chemischen und thermischen Sinnes bei Paramecium und Stentor. Zool. Anz. *55*, p. 19—21.

DOROSZEWSKI M. 1952 — Z badań nad działaniem fitoncydów cebuli na *Paramecium caudatum* Ehrb. Annal. Univ. M. Curie-Skłod. Sectio C. *8*, 3, p. 67—87.

DRYL. S. 1952 — The dependence of chemotropism in *Paramecium caudatum* on the chemical changes in the medium. Acta Biol. Exp. *16*, 2, p. 23—53.

GELEI J. 1936 — Das Erregungsleitende System der Ciliaten. C. A. Int. Congr. Zool. XII. Lisbon, p. 174—209.

JENNINGS H. S. and JAMIESON C. 1902 — Studies on reactions to stimuli. The movements and actions of pieces of ciliate infusoria. Biol. Bull. *3*, p. 229—234.

KLEIN B. M. 1942 — Das Silberlinien oder neuroformative System der Ciliaten Ann. Not. Mus. Wien. *53*, 1, p. 156—327.

KOEHLER C. 1934 — Beiträge zum Verhalten von Paramecium Teilstückchen. Zool. Ann. 7. Suppl.

LUND E. E. 1933 — A correlation of the silver-line and neuromotor systems of Paramecium. Univ. Calif. Publ. Zool. *38*, p. 35—57.

MILICER W. 1935 — Badania doświadczalne nad systemem neuromotorycznym *Paramecium caudatum*. Acta Biol. Exp. *9*, 10, p. 174—194.

OKIJAMA A. 1954 — Studies on the metachronal wave in Opalina II. The regulatory mechanism of ciliary metachronism and of ciliary reversal. Annot. Zool. Japon. *27*. 1, p. 40—45.

PARDUCZ B. 1954 — Reizphysiologische Untersuchungen an Ziliaten. II. Neuere Beiträge zum Bewegungs — und Koordinationsmechanismus der Ziliaten. Acta Biol. Ac. Sc. Hung *5*, p. 196—212.

PARDUCZ B. 1953 — Reizphysiologische Untersuchungen an Ziliaten. IV. Über Empfindungs bzw. Reaktionsvermögen von Paramecium Acta Biol. Ac. Sc. Hung. *6*, 3—4, p. 289—316.

REES C. W. 1922 — The neuromotor Apparatus of Paramecium. Univ. Calif Publ. Zool. *20*, p. 333—36.

SHARP H. G. 1914 — *Diplodinium ecaudatum* with an account of its neuromotor apparatus, Univ. Calif. Publ. Zool. *13*, p. 42—121.

WORLEY L. G. 1934 — Ciliary metachronism and reversal in Paramecium, Spirostomum and Stentor. J. Cell. Comp. Physiol. *5*, p. 53—72.

Acta Biologiae Experimentalis, Vol. XVIII, 1958

THE MANIFESTATION OF THE DEFENSIVE REACTIONS IN NEUROTIC STATES

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(Received for publication 29 April 1957)

INTRODUCTION

It is well known that in neurotic states an animal manifests certain types of behaviour which may be considered as special forms of defensive reactions. W. H. Gantt in his monograph "Experimental basis for neurotic behavior" (1944) enumerates, among the neurotic symptoms the defensive reactions, which he divides into two groups: "direct active defense responses aimed at escaping from the situation and passive defense, leading to immobility and various motor disturbances (catalepsy etc.)". Also Masserman 1943, Arnold 1944, Dworkin 1936, Petrova 1945, Wolpe 1952, 1954 and others, when describing the neurotic disturbances specially drew attention to the appearance of phobic of fear reactions, which are of the same nature as the defensive ones. Many authors (Fonberg 1953, 1956, Gantt 1944, Jakovleva 1938, Karn 1938 a, b, Masserman 1943, Podkopaev 1926, Petrova 1937, Rikman 1932, Szenger-Krestovnikova 1921 and others) observed the appearance in the neurotic state of such reactions as scratching or tearing off of the salivary capsula and other recording apparatus, destroying laboratory furniture, biting, hiding in a corner or under the table etc. These were considered by Pavlov and his school as subcortical defensive reactions.

It is therefore interesting to examine whether the instrumental conditioned defensive reflexes, which are undoubtedly of cortical origin, could also appear in the neurotic states. If this really was the case, it would follow that there exists a more general principle, by which the centers of all the defensive reactions, both subcortical and cortical, would be excited in the neurotic states. The aim of this work is to test this supposition.

METHOD

The experiments were carried out by the method of conditioned reflexes in a reflex-chamber usually used for such purposes. The experimenter was sitting in the ante-chamber, separated from the reflex-chamber by a wall with a small observation window, through which the behaviour of the dog could be observed. During the experiments the dog remained standing in the reflex-chamber, tied up on a stand, and before him was placed a food-tray about 40 cm high with mechanically moved bowls. Fine bread crumbs or bread cubes soaked in broth were used as alimentary reinforcement. For defensive reinforcement we used either air-puffs into the ear from a small tube fastened to the inner edge of the pinna, or electric stimulation of the skin by single electric shocks delivered from a condenser of 1/2 MF capacity, previously loaded from an anodic battery to the tension of 60—120 V, or else we sometimes introduced into the dog's mouth an 0.06 % solution of acetic acid, about 6 cub.cm, by means of a tube fastened to the corner of the mouth and bent inwards. The limb movements were registered on a cylinder after the unpublished method of Kozak (1947) described in a previous paper (Fonberg 1953 b). Bands for movement registration were placed on all the four limbs.

During the series of conflicting experiments, the apparatus for producing noxious stimuli (electrodes, tubes for introducing acid, tubes for air-puffs etc.) were not fastened.

GENERAL COURSE OF EXPERIMENTS

The experiments were carried out on 4 male dogs, mongrels, weighing from 9 to 19 kgs. The course of the experiments went as follows:

1. First instrumental conditioned alimentary reflexes (of the II type) to various visual and auditory stimuli were established. In the first dog (Neron) the instrumental reaction consisted in the lifting of the right hind leg; in the next three dogs (Azor, Dog, Filut) it consisted in the lifting of the right foreleg and putting it on the food-tray.

2. Then in the same reflex-chamber, defensive instrumental conditioned reflexes were elaborated. The metod by which they were developed was as follows (Konorski and Miller me-

thod 1933, 1936). To an entirely new stimulus, a classical defensive conditioned reflex was elaborated. As a negative reinforcement we used with Neron an electric shock, applied to the right foreleg, and in Dog, Azor and Filut — a strong air-puff into the ear. When the conditioned defensive reflex was fully established, the dog was trained to perform a certain movement in response to the conditioned stimulus. In some trials the execution of this movement was followed by the immediate cessation of the stimulus without application of the reinforcing noxious stimulus. After a number of such trials the dog learned to perform this movement to the conditioned stimulus and thus to avoid the negative reinforcement. The motor reactions used in avoiding training were always different from the previously established alimentary movements.

Thus in Neron the effect of the defensive reflex was the movement of lifting the right foreleg, in Azor — the lifting of the left foreleg, in Dog — barking, and in Filut — the lifting of the right hind leg. During the training of the defensive reflexes, the alimentary reflexes were not trained.

3. After the defensive reflexes were firmly established the experiments with alimentary reflexes were resumed. In all our dogs we introduced a new stimulus — a tone of 50 cycles, the intensity of which could be changed by a graduated rheostat. To the strongest tone (T_0) the excitatory instrumental conditioned reflex was established (the motor effect being the same as in the first alimentary series), while to the weekest one (T_{16}) the inhibitory reflex was formed.

4. The differentiation between T_0 and T_{16} being established, we now proceeded to bring gradually the two stimuli nearer and nearer to each other. This was done by means of progressive changes in the intensity of the tones, so that in every successive series of experiments the differentiation between them became gradually more and more difficult, until in the end it became completely impossible. This gradual intensification of the difficulties presented to the dog was achieved either by the simultaneous strengthening of the inhibitory and weekening of the excitatory tone, 1 grade on the rheostat at a time (Neron, Dog, Filut), or else by changing only the inhibitory tone, so bringing it nearer to the excitatory one, which remained fixed (Azor). Each series of experiments lasted on an average several weeks. Usually a new series was started only

when the differentiation in the preceding one was mastered and all disturbances in the behaviour of the animal had disappeared.

5. In three dogs (Azor, Dog, Filut) some additional conflicting tasks were introduced. They are described in detail in a later section.

6. Finally we tested in all dogs the preservation of the particular conditioned instrumental defensive reflexes to the respective conditioned defensive stimuli. Also tested was the generalisation of the instrumental defensive reflexes to stimuli reinforced by other kinds of noxius reinforcements.

During the training of conditioned alimentary and defensive reflexes, and also during the conflicting experiments detailed observations were made on all dogs, regarding their general behaviour and conditioned reflex activity.

RESULTS

Dog No 1 — "Neron"

"Neron" is a male, mongrel dog, weighing about 12 kgs; very quiet, rather timid, and very gluttonous. We started to use him for experiments when he was about 1 year old.

First the positive instrumental alimentary conditioned reflexes, consisting in lifting of the right hind leg to the metronome and then the differentiation between two tones of different intensity were established. Next the defensive conditioned reflex was elaborated. It consisted in the dog lifting right foreleg to the lamp and so avoiding the application of an electric shock to that leg.

After the defensive training, which lasted about 4 months, was completed, we returned again to the alimentary reflexes. Despite the long break in the training, these reflexes were fully preserved. To the positive conditioned stimulus the dog lifted his right hind leg ("alimentary") several times and turned towards the bowl containing food. Whereas to the inhibitory stimulus he stood quietly without moving. The defensive movements as a rule did not appear. After 2 weeks of such normal functioning, the dog was set more difficult tasks in differentiation.

The introduction of the first of such tasks (the differentiation between T_1 and T_{14}) did not cause any change in the behaviour of the dog. Likewise the next differentiation (2nd series) did not

at first produce any visible disturbances. However in the next experiment a disinhibition of the differentiation was observed. Towards the end of the experiment the motor alimentary reflex to the excitatory stimulus disappeared, the dog refused to finish his food and during the interval between the stimuli the defensive movements appeared several times. During the experiments which followed, the disturbances subsided and the defensive movements were absent. Also in the next series (3rd) no deviation from normal was noted in the dogs behaviour.

However in the 4th series a number of various disturbances again appeared. The dog was restless and fidgety on the stand, frequently looked around, the conditioned alimentary reflexes were disinhibited during the intervals and also to the application of the negative stimuli. Sometimes he also tore off the elastic attached to the band on the right foreleg.

These disturbances gradually became more pronounced, the dog whined, the conditioned alimentary reflexes to the positive stimulus became irregular or were even completely absent; sometimes the dog would refuse to eat, although normally he is very gluttonous and greedily devours his food. The movements of the defensive limb appeared almost in all the experiments. They were mostly single, but sometimes several were observed during the course of an experiment, which lasted about 15 minutes. All the disturbances described did not as a rule appear together at the same time; they alternated and appeared with varying strength.

Towards the end of the 4th series the disturbances diminished, the defensive reactions ceased and the differentiation became complete.

In the 5th series the course of the experiments followed much the same pattern. The disturbances, slight at first, gradually gained in strength and at the same time the defensive movements began to appear. Usually they appeared singly, except in one experiment, when we recorded 14 defensive movements of high amplitude (not counting over 30 smaller ones) during the 15 minutes experimental session. Then the disturbances gradually began to subside, and the defensive reactions also disappeared.

The most pronounced disturbances took place during the 6th series of experiments (differentiation between tones — T_6 and T_9). At first the dog, although unable to cope with the differentiation,

stood quietly in the stand waiting for food. The positive conditioner reflexes were high and fairly regular. After several days, however, they diminished, became more irregular and sometimes even completely dissapeared. The dog began to whine softly during the intervals, looked around warily, hung down his tail. Gradually the power of differentiation began to reappear but was still incomplete. This state lasted for more than 3 weeks. Then the disturbances became more pronounced and by degrees a definite neurotic state developed. During the experiments the dog stood with legs bent, tail under; the motor conditioned alimentary reflexes disappeared completely, so that the lifting of the leg had to be retrained by passive movements. After several days of such training the motor conditioned alimentary reflexes returned, although irregular. Other disturbances, however, still persisted. The dog usually stood motionless, with his back arched, "frightened", often turned away from the food-tray, hiding his head between the food-tray and the wall. Sometimes he would jump about and tear off the lines. Most often he tore off the elastic connected to the right foreleg (the "defensive" one).

Simultaneously with the onset of the neurotic state, the intensive movements of the "defensive" limb began to appear. They appeared in almost every experiment; at first singly, then more and more frequent, until finally they numbered over 20 during one 15 minutes session (Fig. 1).

The state just described lasted for about 2 weeks and then gradually slightly improved.

In order to see whether the changes of alimentary excitability have any influence upon the appearance of the defensive movements, we performed other experiments (series 6a), in which the dog was either completely satiated (5 experiments) or more hungry than usually (5 experiments). It turned out than neither of these changes had any effect on the appearance of the defensive movements.

In the next series of experiments (7th series) disturbances of the conditioned reflexes and in the dog's behaviour generally were again insignificant, also the defensive movements did not appear. An irregular reinforcement of one conditioned stimulus, the middle one between the inhibitory and excitatory tones in the 8th last series, causes an increase in observed disturbances. The dog whined,

tore elastic connecting the band on the right foreleg, the alimentary reflexes became irregular, single defensive movements also appeared.

With this the experiments on the gradual approximation of differentiated stimuli finished.

Table I shows the course of the successive series of experiments.

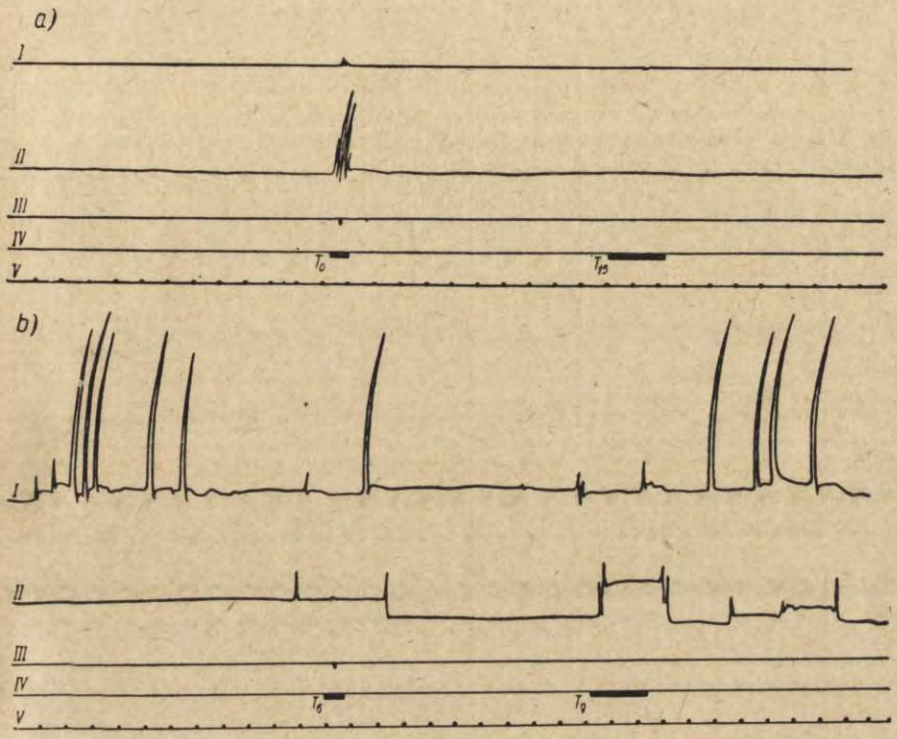

Fig. 1. Appearance of defensive movements in neurotic state in dog No 1 ("Neron")

a — kymographic record of the experiment carried out prior to the gradual approximation of the differentiated tones. Alimentary conditioned reflexes (line II) are strong and regular, defensive movements do not appear (line I)

b — kymographic record of the experiment carried out at the time when express neurotic state appeared (6 series). Alimentary conditioned reflexes are diminished, disinhibition appears in the intervals of the action of the conditioned stimuli (line II); strong defensive movement are seen (line I)

I — movements of the right foreleg (instrumental defensive reaction), II — movements of the right hind leg (instrumental alimentary reaction), III — alimentary reinforcement. IV — conditioned stimuli, V — time in sec (each five sec)

After a break in the experiments lasting about 7 months, the preservation was tested of the defensive instrumental reaction to the conditioned stimulus for which it was previously elaborated. The defensive conditioned stimulus (the light of a lamp) was applied eight times, but the conditioned instrumental defensive

Table I

"Neron"

No of series	Number of experiments in a series	Number of experiments in which instrumental defensive reactions appeared	Number of instrumental defensive reactions Total	The extent of neurotic disturbances (estimated approximately)	Type of disturbances
1	8	—	—	—	—
2	4	1	4	+	conditioned reflexes somewhat diminished
3	7	—	—	—	—
4	15	11	31	++++	bites the apparatuses, turns away from the bowl, refuses to take food, alimentary conditioned reflexes diminished, absent or disinhibited in the intervals
5	29	6	19	++++	turns away from the bowl, does not eat or leaves some food untouched, alimentary conditioned reflexes irregular, disinhibited in the intervals
6	36	20	112	+++++	whines, bites the apparatuses, strong neurotic state described in the text
6a	10	1	1	+	alimentary conditioned reflexes disinhibited
7	9	—	—	—	whines softly
8	12	6	12	++	bites apparatuses, whines

reaction — the lifting of the right foreleg — did not appear. It had to be elaborated anew. It should be stressed here that all through the difficult tasks the dog performed this defensive movement, although no conditioned or unconditioned defensive stimulus was applied during the experiments and even though 9 months had elapsed since the defensive training.

When this reflex was again renewed and well established, a series of experiments was performed, in which a number of conditioned stimuli, reinforced by noxious stimuli other than the ones used when elaborating the defensive reflexes, were applied. The instrumental defensive movement developed as a "defence" against the stimulation of the skin by electric shock did not appear when the conditioned stimuli were reinforced by an air-puff into the ear, or by the introduction of acid into the mouth.

Dog No 2 "Azor"

He is a mongrel dog, weighing about 19 kgs, lively, gay, easily stimulated by food.

He is in the laboratory from 1947 and several series of experiments were previously performed on him.

The instrumental conditioned alimentary reflexes to a number of visual and auditory stimuli, the effect of which was the placing of the right foreleg on the food-tray, had already been elaborated in him a long time before. Now we established the instrumental defensive reflex to a rhythmically swinging rattle (the visual-auditory stimulus). Apart from the instrumental reaction of lifting up the left foreleg, which movement protected the dog against the air-puff into the ear, the dog performed sometimes to the conditioned defensive stimulus the movement of "shaking off".

After the defensive reflexes were established, the experiments were interrupted for 10 months. It was found that after this interval the instrumental conditioned defensive reflex was fully retained. Then we returned to the conditioned alimentary reflexes. The differentiation of 2 tones was established, and then, gradually, in successive series of experiments the intensity of the inhibitory tone was changed, so that it became more and more similar to the positive tone, which remained unchanged. At first this task did not cause any visible disturbances. Only during the 10th series of experiments did the behaviour of the dog begin to change. The dog became listless, whined softly during intervals between the stimuli,

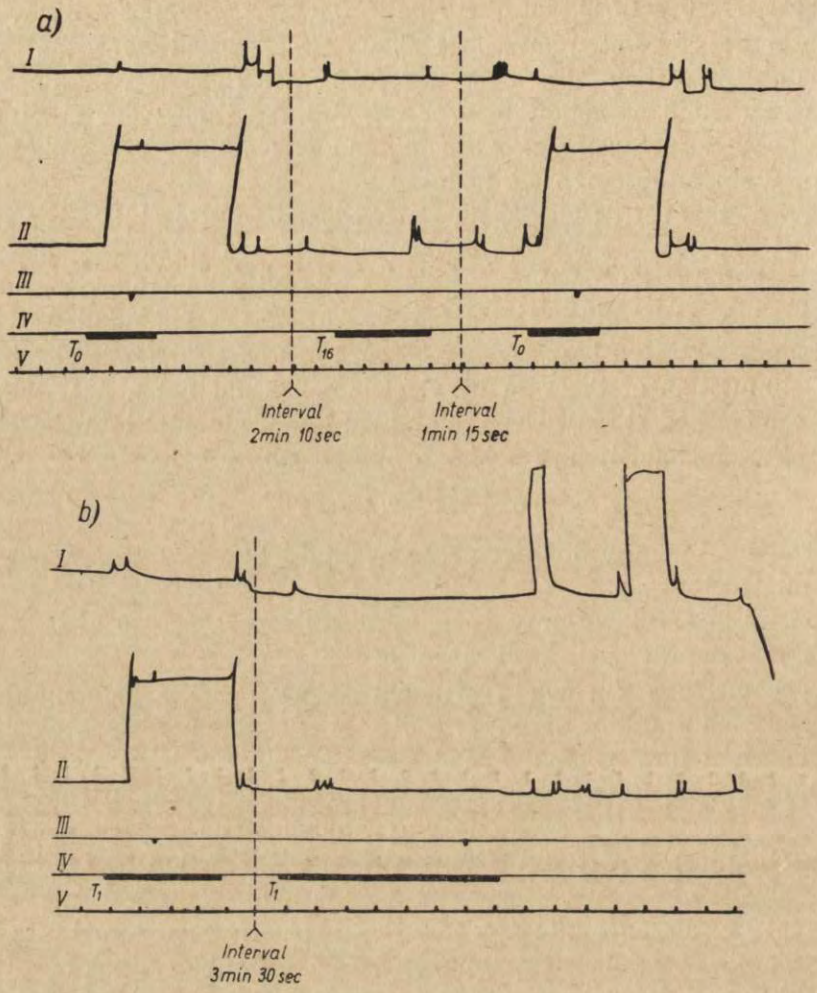

Fig. 2. Apperance of defensive movements in neurotic state in dog No 2 ("Azor")

a — kymographic record of the experiment carried out prior to the gradual approximation of the differentiated tones. Alimentary conditioned reflexes (line II) are regular, defensive movements do not appear (line I)

b — kymographic record of the experiment carried out during 10 series of gradual approximation of the differentiated tones. Alimentary conditioned reflexes (lineII) irregular (absent in the second trial); notice the movement of the "defensive" leg (line I)

I — movements of the right foreleg (instrumental defensive reaction), II — movements af the right foreleg (instrumental alimentary reaction), III — alimentary reinforcement, IV — conditioned stimuli, V — time in sec (each five sec)

kept turning towards the door and sometimes even refused to take food. In 3 experiments definite movements of the "defensive" leg appeared. In the series which followed, the disturbances were still not very pronounced and the defensive movements appeared sporadically as the disturbances gained in strength.

In the 16th series of experiments the inhibitory tone approached so close to the positive one that they became almost one. Then the disturbances in the behaviour of the dog became very pronounced. The dog whined, howled, tried to escape towards the door, or else he became dull and would remain motionless, with glassy gaze focused on one point; sometimes he would leave the food unfinished, or even completely refuse to eat (in the normal state it never happened with this gluttonous dog). In this series the defensive reactions at first did not appear in their typical form. It was however observed that on the way to the reflex-chamber, the dog limped on the left foreleg (the "defensive" one), and kept iti raised before the experiment. The dog did not allow this leg to be touched, just as if he had a sore wound on it. However if his attention was diverted and his "sore" leg cought unexpectedly, even strong pressure did not cause the reaction of pain. It was also discovered that although the dog limped from the moment of entry into the antichamber outside the laboratory building he ran around quite correctly. The possibility of an organic ailment was therefore excluded.

After 4 days this distrubance disappeared, but the other neurotic signs became more marked. During the experiments the dog was turned away from the food-tray straining towards the door, his breath spasmodic, whining. After a certain time some new disturbances were added, such as the "shaking off" reaction, the tearing off of the salivary capsula, and movements of the "defensive" leg. Towards the end of the experiment the dog barked. When the disturbances, just described, diminished, we performed yet one more series of experiments with this dog. This time the difficult task of purely motor differentiation between two whistles was introduced. This consisted in that to a positive alimentary conditioned stimulus the dog received food only when he placed his rigiht foreleg on the food-tray, whereas to the second stimulus, on the contrary, he received food only when he did not place his leg there. This differentiation was very difficult for the dog and caused various disturbances: the dog whined, tried to rush to the door, often refused food, sometimes remained motionless with

half closed eyes, and at the same time the movements of the "defensive" leg appeared — (Fig. 2). Table II shows the course of successive series of experiments.

Table II

"Azor"

No of series	Number of experiments in a series	Number of experiments in which instrumental defensive reactions appeared	The extent of neurotic disturbances (estimated approximately)	Type of disturbances
1	6	—	—	—
2	1	—	—	—
3	5	—	—	—
4	12	—	—	—
5	6	—	—	—
6	6	—	—	—
7	2	—	—	—
8	3	1	—	—
9	4	—	—	—
10	11	3	+++	dull, refuses to eat, whines
11	9	1	+	alimentary reflexes disinhibited during the intervals
12	5	—	+	whines slightly
13	8	2	++	alimentary reflexes disinhibited during the intervals, slight erection
14	9	—	—	—
15	10	—	+	dull
16	14	4 (atypical)	++++	dull, whines, refuses to eat, erection, shaking off movements, panting
II (motor differentiation)	22	6	++++	whines, very restless, or dull, refuses to eat, shaking-off movements, panting

Then, as with dog No 1, the reaction to the conditioned defensive stimulus for which it was elaborated was tested. To this purpose the defensive stimulus was applied between the alimentary stimuli. The instrumental defensive reaction did not appear although several days before the same reaction appeared during the conflicting experiments, which did not include any defensive elements.

After a 3 months' break in the experiments the reaction to the defensive stimulus was once more tested. This time the defensive stimulus was applied at the beginning of the experimental session and not as a supplementary one, as was the case in the alimentary experiment. But also this time the defensive movement did not appear. Only in the next experiment, after two reinforcements of the conditioned stimulus by air-puff into the ear, did the defensive instrumental reaction appear, but it was so unstable that both in this experiment and in the succeeding ones it had to be reinforced several times by air-puffs into the ear to restore it to its normal strength.

The application of a new conditioned stimulus reinforced by weak electric shock produced an instrumental defensive reaction after 36 trials. This movement, however, was not stable. The reinforcement of yet another stimulus by acid produced this reaction after 10 trials, and yet another conditioned stimulus reinforced by a strong electric current produced the instrumental defensive reflex after only two reinforcements.

Dog No 3 "Dog"

He is a male mongrel dog, weighing about 9 kgs. very lively, gay, gluttonous. Came to the laboratory when he was one year old. Neurotic disturbances were developed in this dog three times and it must be stressed here, as it is important for further discussion, that while in these neurotic states, this dog never barked during the experiments.

At first alimentary conditioned reflexes were established in him to several visual auditory stimuli, and next the instrumental conditioned defensive reflexes were elaborated. The effect of the defensive reflex was the movement of barking "protecting" against the noxious reinforcement of the air-puff into the ear.

After we elaborated and established the defensive reflexes, experiments on the alimentary reflexes were resumed and, as with

Table III

"Dog"

No of series	Number of experiments in a series	Number of experiments in which the defensive reaction of barking appeared	The extent of neurotic disturbances	Type of disturbances
1	7	—	—	
2	5	1	+	once refuses to eat, softly whines
3	5	—	+	ditto
4	8	2	++	alimentary reflexes disinhibited in the interval, licks his right foreleg, waves it in the direction of door, twice refuses to eat
5	4	—	+	twice refuses food
10 months break in the experiments				
1a	7	—	—	
2a	11	1	+++	alimentary reflexes disinhibited during the intervals, restless, 4 times refuses food
3a	17	—	+++	restlessness, erection, does not eat in 6 experiments
4a	6	2	+++	restlessness, alimentary reflexes disinhibited in the intervals
5a	11	1	+++	restlessness, whines
6a	3	—	—	—
7a	15	3	++++	marked restlessness, tries to break loose, whines, disinhibition of alimentary reflexes during the intervals, refuses to eat in 5 experiments
8a	6	1	++++	restlessness marked, disinhibition of alimentary reflexes during the intervals, whines, shaking off movements
9a	17	—	++++	very restless, whines, alimentary reflexes disinhibited in the intervals, sometimes refuses to eat, shaking off movements

Table III continued

No of series	Number of experiments in a series	Number of experiments in which the defensive reaction of barking appeared	The extent of neurotic disturbances	Type of disturbances
10a	5	—	+	slight restlessness, shaking off movements
11a	6	—	+	quiet, once refuses to eat shaking off movements
12a	28	8	++++	marked restlessness, whines, howls, tries to escape to the door, alimentary reflexes disinhibited in the intervals, 3 times refuses to eat, shaking off movements
II (motor differentiation)	20	7	+++++	very marked restlessness, motor disturbances, alimentary reflexes disinhibited in the intervals erection, return to previous motor neurosis, shaking off movements
III (motor dtfferentiaton and new feeding procedure)	13	9	+++++	same as above but to a greater extent, very often refuses to eat, strong shaking off movements

dog No 1, successive series of more and more difficult differentiations were applied, during which both the positive and the inhibitory tones were gradually brought closer to each other. These successive series of gradual approximation of the differentiation tones caused, as it was also the case with dogs Nos 1 and 2, the neurotic disturbances. And again like in the provious dogs, during the strengthening of the neurotic disturbances, defensive reactions appeared in "Dog" in the form of barking. Sometimes also the reaction of "shaking off" was observed. Beginning from 11th series of experiments the "shaking off" reactions appeared invariably during the experiments and were very intensive.

The most pronounced neurotic disturbances appeared during the next conflicting task, which was the purely motor differentiation of two positive conditioned alimentary stimuli. In order to obtain food the dog had to perform an antagonistic reaction to both of the conditioned alimentary stimuli (see p. 11). For a dog who had elaborated as primary the motor alimentary reflexes and had them well established which means that between the alimentary and motor centres there existed a very strong connection, (Wyrwicka 1952 a, b), this was a very difficult task. It was made even more difficult by the additional introduction of a delay in the reinforcement from 1 to 10 seconds after the beginning of conditioned stimulus. Therefore the neurotic disturbances in this series of experiments were very violent. The dog became very restless, tried to rush towards the door, put both legs on the food-tray, whined, sometimes refused food, vigorous "shake off" reactions occurred. In many experiments intensive barking (defensive) also appeared. The old motor neurosis consisting in "ambivalent" movements of the right foreleg was also renewed in the same form as it appeared in him four years before (see Fonberg 1953 a).

As the motor differentiation began to be established, the neurotic disturbances became less noticeable. Also the defensive movements ceased to appear. Then an extra difficulty was introduced. While not withdrawing from the experiments the difficult motor differentiation, which was not yet fully elaborated, a new method of giving food was introduced.

The combination of these two difficult tasks produced a very noticeable increase in the neurotic disturbances, also the defensive movements appeared in a hitherto unprecedented strength. They appeared now in all experiments. There were no more single, isolated barkings; the dog barked now almost non-stop, it was a virtual "avalanche of barking". Besides, while barking previously appeared only in the intervals between the stimuli, now it appeared also during the action of the conditioned stimuli.

Gradually, as the dog got used to the new way of receiving food he became more quiet, the neurotic disturbances slowly disappeared and the defensive movements became less and less marked. Finally they disappeared altogether during the experiments and the dog's behaviour returned to normal. Here the conflicting experiments ended. The course of the successive series of experiments is shown in Table III.

To finish, just as with dogs No 1 and 2, the preservation and generalization of the defensive reflexes were tested. The results however were slightly different from those in the previous cases, because the barking appeared at the very first experiment, in which defensive stimuli were applied and reappeared afterwards in all experiments, where any defensive stimuli, either conditioned or unconditioned, were used. The barking took place not so much to the conditioned stimuli themselves as during the intervals, gaining in intensity as the experiment proceeded. On this the experiments were finished.

Dog No 4 "Filut"

Male, mongrel dog, weighing about 12 kgs, quiet with the inhibitory process predominating. Acquired by the laboratory in 1947, he was used for several experiments on the disturbances of the higher nervous activity. Motor conditioned alimentary reflexes to a number of stimuli were established in him a long time ago, the effect of which was the movement of placing the right leg on the food-tray.

Now we elaborated in him the following motor defensive conditioned reflexes: to the conditioned stimuli (the noise of running water splash 1 and a small soundless propeller placed in front of him) the dog had to raise his right hind leg, thus avoiding the negative reinforcement of an air-puff into the ear. The reaction of the dog to the latter kind of reinforcement was the "shaking off" movement. After a certain time this "shaking off" movement began occasionally to appear also to the conditioned stimulus itself, preceding the movement of the hind leg. Apart from the positive conditioned reflexes we established also the inhibitory defense reflex to the noise of running water rhythmically interrupted — Splash 2.

The elaborating and establishing of the defensive reflexes lasted three and a half months. After a break in the experiments, which lasted 10 months the defensive reflexes were preserved so well, that not once was it found necessary to apply a negative reinforcement.

Next we returned to the experiments with the conditioned alimentary reflexes and as with the dogs already described the differentiation of 2 tones was elaborated in him. After three months

of regular functioning we began the gradual approximation of the differentiation tones. Seven experimental series were performed, in each series, both the positive and the inhibitory tones were approximated — 1 grade on the scale each. In the 8th series, only one tone was used (T_8 — the middle one between the positive and the inhibitory tones of the previous series) reinforced irregularly.

The first disturbances, namely the diminishing of the conditioned alimentary reflexes and disinhibition in the intervals, already appeared in the 2nd series of approximation of the differentiation tones. At the same time the "shaking off" movement also appeared. However in the next series, the disturbances did not become more pronounced. Comparatively the biggest disturbances took place during the middle series (5th, 5th A, and 6th). The dog whined softly, from time to time looking round towards the door, the conditioned alimentary reflexes were diminished and irregular, sometimes disinhibited during the intervals. At other times the dog either left the food untouched or did not finish it (this however very often happened with this dog even in the normal state). Very vigorous "shaking off" movements appeared. In one experiment the movement of the right hind (defensive) leg was noted. Further series of experiments showed the disturbances gradually disappearing. The "shaking off" movement appeared sporadically, every two or three experiments.

After concluding the experiments on the gradual approximation of the differentiated tones yet some more experiments were performed on "Filut". He was given other difficult tasks to perform such as the purely motor differentiation between two positive alimentary stimuli and elaboration of conditioned inhibition with growing interval between the conditioned inhibitor and the conditioned stimulus. However we did not succeed in producing any pronounced disturbances of the higher nervous activities; on the contrary, the dog functioned better and better. Also the instrumental defensive reflexes did not appear at all.

The testing of the preservation of the conditioned reflexes showed that the instrumental reflex did not appear to the stimulus for which it had been previously elaborated. Only after 4 experiments, during which it was reinforced 5 times by air-puffs into the ear did the movement appear but it was irregular and had to be again re-established. When new stimuli were reinforced

by an electric shock delivered to the paw, the instrumental defensive reaction appeared after several trials. On the other hand this reaction as a rule did not appear at all to other stimuli reinforced by acid.

DISCUSSION

The experiments described in this report show that in the neurotic states along with other disturbances, also appear the defensive reactions which are effects of previously elaborated instrumental defensive reflexes (2nd type). These defensive reactions did not appear in experiments where the dog was confronted with tasks which he had previously mastered or which were not very difficult to perform. On the other hand they appeared in the series where the differentiation of two gradual approximated tones was trained, during the difficult purely motor differentiations and also in other difficult tasks.

In no dog used for the experiments was a severe and long lasting neurosis provoked, however distinctive neurotic disturbances appeared in all dogs. These disturbances consisted in the unwillingness of the dogs to take part in the experiments or enter the reflex-chamber. They refused to eat although hungry, were very restless, climbed the food-tray, tried to break loose, bit the lines and demolished any laboratory furniture within reach. Sometimes the opposite took place, they remained motionless listless, and indifferent, with half closed eyes. The conditioned alimentary reflexes were diminished, became irregular and disinhibited during the intervals. In three dogs typical instrumental defensive reactions previously elaborated as a "defence" against a particular negative reinforcement appeared during the neurotic disturbances just mentioned. In two of them, in which air-puff into ear was the unconditioned stimulus used during the elaboration of the defensive reflexes, the reaction of "shaking off" also appeared. In the 4th dog only the "shaking off" reactions appeared. The absence of the instrumental defensive reactions in this dog can either be explained by the fact, that his neurotic disturbances were very mild or else, because he had in addition to the positive instrumental defensive reactions, also the defensive differentiation elaborated previously. He was, therefore, taught to react by a definite movement only to a strictly defined stimulus and not to execute this movement to any other stimulus.

The appearance of the defensive reactions in the neurotic state was observed by many workers (see p. 1). They were mainly the reactions of escape, immobility or aggressive reactions such as the demolishing of furniture, biting etc. The appearance of such reactions was considered either as the expression of an emotional state (Masserman, Gantt and others) or else as a result of the inhibition of the cortex followed by the "release" of the subcortical centres (Pavlov and his school). Both these groups considered therefore the defensive reactions appearing in the neurotic states as subcortical reactions of rather unconditioned character.

So evidence showing that the instrumental defensive reflexes, the cortical character of which seems obvious, have also a tendency to appear in the neurotic states, seems to be novel.

Many authors (Tinbergen 1940, Armstrong 1950, Lorentz 1939 and others) stated that in conflicting situations, or in such situations where the normal reactions of the animal were inhibited, the animal manifests various inadequate, accidental reactions. This was called the displacement activity. Therefore we might assume, that the appearance of the defensive reactions in the neurotic state is also accidental, i.e. that the animal, not being able to react adequately, performs movements which were elaborated in him proviously, irrespective of their character.

Yet our experimental dogs had in their repertory many different motor reactions, such as the sitting down and getting up movements, jumping, standing on two legs, defecation movements, copulation movements etc., but they did not show any tendency to perform them in the neurotic states. Even if some accidental movement did occasionally appear, it did not have any tendency to repetition. On the other hand the defensive reactions, both instrumental and other, appeared constantly and regularly.

Neither was the appearance of the instrumental defensive reactions in our case a regression to the reactions primarily elaborated in a given situation (Sanders 1937, Tolma and Krechevsky 1933 and others) because in that particular situation it was the instrumental conditioned alimentary reflexes which were first elaborated and applied over a long period. Therefore the fact seems to be beyond doubt, that in the neurotic state it is all kinds of defensive reactions that are in the first place released.

In our experiments we also observed that the instrumental defensive reactions appeared in neurosis even in those dogs where these reactions ceased to be evoked by the conditioned defensive stimuli for which they were elaborated. This then testifies to the fact that the tendency for defensive reactions to appear in neurosis is so great that it can even exceed the capacity of their "mother" conditioned stimuli to evoke them.

Now we proceed to discuss the mechanism of this phenomenon. In the previous report (Fonberg 1956) it was interpreted in this way, that the difficult tasks which cause the neurotic disorders, constitute a certain kind of noxious stimulus, against which the dog "defends himself" by means of the same movement, which previously proved to be an effective defence against definite unconditioned reinforcement. For, as follows from the works of Konorski and Miller 1936, Kellog and Walker 1938 and Brodgen 1940, the conditioned defensive instrumental reaction can appear not only in response to the stimulus for which it was elaborated but also to other conditioned stimuli, signaling some other kind of negative reinforcement. It could therefore be assumed, that if an animal has in his repertory a certain instrumental defensive reflex, he will use this movement always, whenever confronted with any noxious stimulus. And this being so, then including the conflicting stimuli, which undoubtedly are of a negative (unpleasant) character, in the group of negative stimuli, we may rightly expect that the animal when confronted with them, will also make the same movement with which he always "defended himself" against danger.

The present experiments have shown that although the generalization of instrumental defensive reflexes does in fact exist, it occurs only in some conditions. The animal does not perform a specific defensive movement to the signals of any negative reinforcement but only in response to those which are in some way similar to the original noxious stimulus, or those which evoke defensive excitation of a very high degree.

On the other hand we know, that the majority of the visceral and humoral reactions appearing in neurosis (Gantt 1942, 1943, 1944, Gantt and Dykman 1952, Reese, Doss and Gantt 1953, Liddell 1934 and others) are identical with the visceral and humoral reactions appearing to the defensive stimuli (Solo-

mon 1953, 1954, Dykman and Gantt 1954, 1956, Bersh et al., 1956, Wolpe 1952, 1954 and others). The appearance of these reactions in the defensive situation suggests that the above mentioned visceral-vascular reactions form the basis of the anxiety state (Mowrer 1944, Solomon 1954, Farber 1948). The fact that the pharmacological or surgical exclusion of the autonomic system (Wynne and Solomon 1955) weakens or even completely disperses the state of anxiety in the animal, handicaps the formation of defensive reactions and causes their extinction, seems to favour this hipothesis.

Lissac (1955) and Delgado (1956) have shown in their experiments that the instrumental defensive reflexes may by evoked by means of stimulation of the subcortical centres, such as hypothalamus and nucleus amygdale. Also Brady (1954) and Schreiner and Kling (1953) proved that the injury of the nucleus amygdale hampers the formation of defensive reflexes of the avoidance type which are typical cortical reactions.

All these facts suggest that there may exist a main centre (not in a strictly anatomical sense) which controls and coordinates all the defensive reactions, both the lowest and the most specialized ones. This governing centre is probably connected with all the centres of the particular defensive reactions and is alerted only if a definite degree of defensive excitation in the specialized centres is reached and superpassed. When the threshold of alertness is reached, the excitation of the main centre may irradiate into the various specific defensive centres and provoque their activities. As far as the neurotic state is concerned it may be assumed that it produces a more or less strong excitation of this very centre. This assumption can explain why in neurotic states a great variety of defensive reactions takes place, namely various autonomic reactions, general defensive motor reactions (such as reactions of escape), and specific instrumental defensive reactions.

Thus the signals of the noxious stimuli — "imminent danger" signals — evoke a certain state in organism, called the anxiety, state, which consists mainly in a number of visceral and humoral reactions. The neurotic state which is a state of "emergency", heralding damage to the functionning of such an important organ as is the central nervous system, would also cause a state of anxiety similar to the state of expectancy for impending danger.

The performance of an instrumental movement, a movement which always before removed the threatening danger, would reduce the anxiety state (Mowrer 1950, Miller 1948, Solomon 1953, 1954 and others), thus providing a positive reinforcement. Therefore the appearance of the anxiety state would always produce a tendency to its reduction, that is a tendency to perform a movement associated with the reduction or abolition of this state.

The performance of a particular instrumental movement in a state of neurosis would also reduce the anxiety state caused by the conflict and thus probably preventing the occurrence of more serious disturbances. Godson and Marx

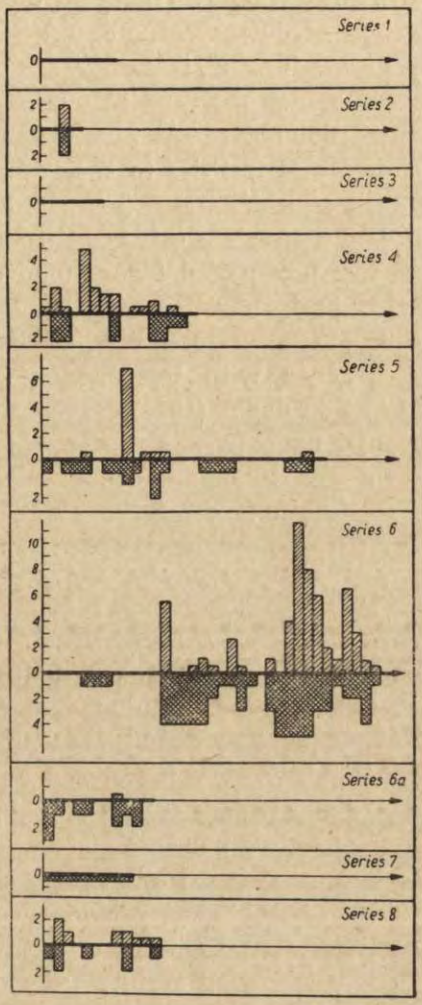

Fig. 3. The successive series of gradual increase in difficulties of differentiation in dog No 1. The rectangles above the line indicate the number of defensive movements performed by the dog during one experiment. The rectangles below the line show the extent of the neurotic disturbances (estimated approximately). As we can see the intensity of the defensive reactions runs parallel the increase in the neurotic disturbances

(1953) have shown that opportunity to make a well learned instrumental avoidance response would increase resistance to audiogenic seizures. Bersh et al. (1956) showed that the cardiac conditioned reflex (related to fear) diminished after elaboration of the avoidance instrumental reaction. And from the paper of Arnold (1944) it follows, that not only the instrumental, but also a certain number

of other reactions, called by this author "fear reactions", such as trembling, whining, hiding, defecation and urination may diminish the state of neurosis, judged by the proneness to audiogenic seizures. Also Wolpe (1954) proved in human subjects, that the elaboration of defensive notor reaction to shock (escape reaction) could reduce neurotic states.

The fact observed in our experiments that the neurotic distubances did not progressively gain in intensity but held a wavering course also testifies to the "buffer" role of the defensive reactions in the neurotic states (see Fig. 3). In no dog was a long-lasting and severe neurosis provoked. It seems, therefore, that the performance of instrumental defensive movements reduces the anxiety state, what in turn relieves the neurotic state and thus prevents more severe and permanent disturbances from developing. This is in accordance with the suggestion of Liddell (1934, 1935) and Masserman, (1943) who considered that the main cause of experimental neurosis was the restriction of the animal's freedom, which deprived it, among other things, of the possibility to manifest freely natural defensive reactions, such as for instance, escaping from the experimental situation.

SUMMARY

The aim of the paper was to test whether the instrumental defensive reactions would appear in a neurotic state caused by the conflict of two conditioned alimentary stimuli. For this purpose experiments were performed on 4 dogs, in which both alimentary and defensive reflexes were elaborated. Then in several series of experiments a neurotic state was evoked by difficult differentiation of two conditioned alimentary stimuli, and observations were made as to whether the previously elaborated instrumental defensive reactions would emerge under these conditions.

The following results were obtained.

In three dogs the special instrumental defensive reactions which had been elaborated as a "defence" against some particular noxious stimulus, appeared in the neurotic state. In two of them, where air-puff into the ear was used as noxious stimulus for the elaboration of defensive reflexes, the "shaking off" reaction also appeared. In the 4th dog, where the neurotic disturbances were the weakest, only the "shaking off" reactions were present.

Apart from that, in all our dogs some ordinary reactions appeared, such as trying to escape, to bite, to demolish the apparatuses etc.

The defensive instrumental reactions appeared in the neurotic state even in those dogs where due to a long break in its application they had already ceased to appear to the conditioned stimulus for which they were elaborated.

The appearance of defensive reactions in the neurotic state was explained by the theory of anxiety reduction.

I wish to extend my sincere thanks to Prof. Jerzy Konorski for his valuable advice and criticism. I wish also to thank Mrs J. Dunin who translated this paper into English.

REFERENCES

ARMSTRONG E. A. 1950 — The Nature and Function of Displacement Activities. Symp. Soc. Exper. Biol. No 4, p. 361.

ARNOLD M. B. 1944 — Emotional Factors in Experimental Neuroses. J. Exper. Psych. Vol. *34*. No 4, p. 257.

BERSH P. J., NOTTERMAN J. M. and SCHOENFELD W. N. 1956 — Extinction of a Human Cardiac-Response during Avoidance-Conditioning, Amer. J. Psychol. Vol. *LXIX*. No 2, p. 244.

BRADY J. V., SCHREINER L., GELLER I., and KLING A. 1954 — Subcortical Mechanisms in Emotional Behavior. J. Comp. Physiol. Psychol. Vol. *47*. No 3, p. 179.

BRODGEN W. J. 1940 — Conditioned Flexion Responses in Dogs Re-established and Maintened with Change of Locus in the Application of the Unconditioned Stimulus. J. Exper. Psych. Vol. *27*. No 6, p. 583.

DELGADO J. M. R., ROSVOLT H. E. and LOONEY E. 1956 — Evoking Conditioned Fear by Electrical Stimulation of Subcortical Structures in the Monkey Brain. J. Comp. Physiol. Psychol. Vol. *49*. No 4, p. 373.

DWORKIN S., BOURNE W., and RAGINSKY B., 1936 — Conditioning Neurosis Treated by Sedatives. Amer. J. Physiol. Vol. *116*. No 1, p. 40.

DYKMAN R. A. and GANTT W. H. 1954 — Blood Pressure Conditioned to Pain. Fed. Proceed. Vol. *13*. No 1, p. 127.

DYKMAN R. A. and GANTT W. H. 1956 — Relation of Experimental Tachycardia to Amplitude of Motor Activity and Intensity of the Motivating Stimulus. Amer. J. Physiol. Vol. *185*. No 3, p. 495.

FARBER J. E. 1948 — Fixation Response Under Anxiety and Non Anxiety Conditions. J. Exper. Psychol. Vol. *38*. No 2, p. 111.

FONBERG E. 1953a — Zaburzenia wyższych czynności nerwowych wywołane za pomocą nieregularnego wzmacniania jednego bodźca warunkowego (Disorders in the higher nervous function caused by the irregular reinforcement of the conditioned stimulus). Neurol. Neuroch. Psychiatr. Pol. No *2*, p. 117.

FONBERG E. 1953b — Przewlekła nerwica doświadczalna u psa z dominującymi zaburzeniami ruchowymi (Chronic experimental neurosis with dominating motor symptoms in dog). Soc. Sc. Lodziensis, Wydz. III. Sec. III. No 27, p. 1.

FONBERG E. 1956 — On the manifestation of conditioned defensive reactions in stress. Bull. Soc. et Lettres de Łódź. Cl. III. Vol. *VII*, p. 1.

FONBERG E. 1957 — Przyczynek do mechanizmu występowania reakcji obronnych w stanach nerwicowych (Concerning the mechanism of conditioned defensive reactions, occurring in experimental neuroses). Acta Physiol. Pol. Vol. *VIII*. p. 321.

GANTT W. H. 1942 — The Origin and Development of Nervous Disturbances Experimentally Produced. Amer J. Psychiatr. Vol. *98*, No 4, p. 475.

GANTT W. H. 1953 — Measures of Susceptibility to Nervous Breakdown. Amer. J. Psychiatr. Vol. 99. No 6, p. 839.

GANTT W. H. 1944 — Experimental Basis for Neurotic Behavior, Psychosom. Med. Monogr. Vol. *III*. Nos III and IV, p. 1.

GANTT W. H. and DYKMAN R.A. 1952 — Experimental Psychogenic Tachycardia. Amer. J. Physiol. Vol. *171*. No 3.

GOODSON F. E. and MARX M. H. 1953 — Increased Resistance to Audiogenic Seizure in Rats Trained on an Instrumental Wheel-turning Response, J. Comp. and Physiol. Psychol. Vol. *46*. No 3, p. 225.

JAKOVLEVA V. V. 1940 — Fiziologiczeskij mechanism obrazovanija trudnoj differencirovki. Trudy Fiziol. Labor. Akad. I. P. Pavlova. T. *IX*, p. 130.

JAKOVLEVA V. V. 1938 — Rezultaty dlitelnego primenenija trudnych zadanij dlja sobaki wozbudimogo tipa. Trudy Fizjol. Labor. Akad. I. P. Pavlova. T. *VIII*, p. 244.

KARN H. W. 1938a — A Case of Experimentally Induced Neurosis in the Cat. J. Exper. Psych. Vol. *22*. No 6, p. 589.

KARN H. W. 1938b — The Behavior of Cats on the Double Alternation Problem. J. Comp. Psych. Vol. *26*. No 1, p. 201.

KELLOG W. N. and WALKER E. L. 1938 — "Ambiguos Conditioning" a Phenomenon of Bilateral Transfer. J. Comp. Psychol. Vol. *26*. No 1, p. 63.

KONORSKI J. i MILLER S. 1933 — Podstawy fizjologicznej teorii ruchów nabytych. Ruchowe odruchy warunkowe. Warszawa.

KONORSKI J. i MILLER S. 1936 — Uslovnyje refleksy dvigatelnogo analizatora. Trudy Fiziol. Labor. Akad. I. P. Pavlova. T. *VI*, vyp. 1.

KOZAK W. 1947 — unpublished.

LIDDELL H. S., JAMES W. T. and ANDERSON O. D. 1934 — The Comparative Physiology of the Conditioned Motor Reflex. Comp. Psych. Monogr. Vol *11*. No 1, p. 1.

LIDDELL S. H., ANDERSON O. D., KOTYUKA E., HARTMAN F. A. 1935 — The Effect of Cortin upon the Experimental Neurosis in Sheep. Amer. J. Physiol. Vol. *113*. No 1, p. 87.

LIDDELL H. S., SUTHERLAND G. F., PARMENTER R. and BAYNE T. L. 1935 — A Study of the Conditioned Reflex Method for Producing Experimental Neurosis. Amer. J. Physiol. Vol. *113*. No 1, p. 95.

LISSAK K. 1955 — Nowyje eksperimentalnyje aspekty w issledovanii diencefaliceskich mechanizmov i procesov wysszej nervnoj dejatelnosti. Zurn. Vys. Nerv. Dejat. T. *V*, wyp. 5.

LORENTZ K. 1939 — Vergleichende Verhaltenforschung. Zool. Anz. Suppl. Bd. *12*, p. 69.

MASSERMAN J. H. 1943 — Behavior and Neurosis. Univ. Chicago Press.

MILLER N. E. 1948 — Studies on Fear as Acquirable Drive. J. Exper. Psychol. Vol. *38*. No 1, p. 89.

MOWRER O. H. 1950 — Learning Theory and Personality Dynamics. Ronald Press. Comp.

PAVLOV I. P. 1938 — Dvadcatiletnij opyt objektivnogo issledovanija wysszej nervnoj dejatelnosti (povedenija) životnych. Biomedgiz., izd. 6-e.

PODKOPAEV N. A. 1926 — Osobennyj słuczaj dvigatelnoj reakcji sobaki w swjazi s razvitiem tormoženija w kore bolszych poluszarij. Trudy Fizjol. Labor. Akad. I. P. Pavlova, T. *I*, zeszyt 2—3.

PETROVA M. K. 1937 — Trudy Fiziol. Labor. Akad. I. P. Pavlova. T. *VII* (Total).

PETROVA M. K. 1945 — Ob eksperimentalnych fobijach. Trudy Fiziol. Labor. Akad. I. P. Pavlova. T. *XII*, wyp. 1, p. 5.

REESE W. G., DOSS R. and GANTT W.H. 1953 — Autonomic Responses in Differential Diagnosis of Organic and Psychogenic Psychoses A. M. A. Arch. Neurol. Psychiatr. Vol. *70*, p. 778.

RIKMAN V. V. 1954 — Obnaruženije davnych sledov razdraženija centrov oboronitelnoj reakcji kak analog travmaticzeskogo nevroza, Trudy Fiziol. Labor. Akad. I. P. Pavlova. T. IV, izd. 2e. p. 90.

SANDERS M. J. 1937 — An Experimental Demonstration of Regression in the Rat. J. Exper. Psychol. Vol. *21*. No 5, p. 493.

SCHOENFELD W. N., BERSH P. J. and NOTTERMAN M. J. 1954 — Interaction of Instrumental and Autonomic Responses in Avoidance Conditioning. Science *120*, p. 788.

SCHREINER L. and KLING A. 1953 — Behavioral Changes Following Rhinen-Cephalic Injury in the Cat. J. Neurophysiol. Vol. *16*, p. 643. (Cit. BRADY J. V. 1956, Transac. N. Y. Acad. Sc. Ser. II. Vol. *18*. No 7).

SOLOMON R. L. and WYNNE L. C. 1953 — Traumatic Avoidance Learning. Psych. Monographs. Vol. *67*. No 4, p. 1.

SOLOMON R. L. and WYNNE L. C. 1954 — Traumatic Avoidance Learning. Psych. Rev. Vol. *61*. No 6, p. 533.

SZENGER-KRESTOVNIKOVA N. R. 1921 — K voprosu o diferencirovanii zritelnych razdražení. Izw. Petrograd. Inst. Lesgafta. T. *III*.

TINBERGEN N. 1940 — Die Übersprungbewegung. Z. Tierpsychol. *4*, p. 1.

TOLMAN E. C. and KRECHEVSKY J. 1933 — Means-end-readiness and Hypothesis. Psych. Rev. Vol. *40*, p. 60.

WOLPE J. 1952 — Experimental Neuroses as Learned Behaviour, Brit. J. Psychol. Vol. *XLIII*. Part. 4, p. 243.

WOLPE J. 1954 — Reciprocal Inhibition as the Main Basis of Psychotherapeutic Effects, A. M. A. Arch. Neurol. Psychiatr. Vol. *72*, p. 205.

WYNNE L. C. and SOLOMON R. L. 1955 — Traumatic Avoidance Learning:

Acquisition and Extinction in Dogs Deprived of Normal Peripheral Autonomic Function. Gen. Psych. Monographs. *52*, p. 241.

WYRWICKA W. 1952a — Zagadnienie mechanizmu ruchowej reakcji warunkowej. Acta Physiol. Pol. Vol. *III*. Fasc. 1.

WYRWICKA W. 1952b — On the Mechanism of the Motor Conditioned Reaction. Acta Biol. Exper. *16*, p. 131.

Acta Biologiae Experimentalis, Vol. XVIII, 1958

SHORT INTERNODES "INTERCALATED" IN NERVE FIBRES

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(Received for publication 3 September 1957)

In examining teased fibres from peripheral nerves or nerve roots one sometimes comes across a thin short internode, or several in succession, intercalated between thicker and much longer normal internodes of the fibre. Such short internodes have been described by Rénaut (1881) in peripheral nerves of healthy horses and asses as "segments courts intercalaires". Rénaut interpreted the existence of these segments as a proof that the nerve fibre grows not only from the central end but also by intussusception of new internodes. An occasional occurrence of internodes much shorter than other of the same fibre was sometimes mentioned by later workers (for exp. Vizoso and Young, 1948).

At about the same time as Rénaut published his description of "segments courts intercalaires" there appeared a fundamental work of Gombault (1881) on morphological lesions in peripheral nerve fibres of guinea-pigs after lead poisoning. In the nerve fibres of his experimental animals some individual internodes were profoundly affected whereas, both above and below the lesion, the fibre preserved a perfectly normal appearance. In the diseased internodes he observed a granular destruction and subsequent disappearance of myelin, an increase in the amount of Schwann cell cytoplasm and multiplication of its nuclei. The continuity of axoplasm was, however, usually preserved. Gombault called this

condition "névrite segmentaire periaxile". If the poisoning had not been too severe, the affected internodes eventually recovered. A thin layer of new myelin was laid around the denuded axoplasmic core and gradually both axoplasm and myelin increased in thickness and attained the size they formerly had. After restitution however there were several short internodes on the segment previously occupied by one internode only.

The "névrite segmentaire periaxile" is not specific for lead poisoning. Gombault, Stransky (1903), and later workers found it in a variety of neurological diseases of very different origins.

The intercalated short internodes, as described by Rénaut and a few other authors in otherwise normal animals, seem to represent a phase of restitution after an inflammatory process which from one cause or another attacked these portions of fibres at an earlier period.

If such an interpretation of the occurrence of short internodes is correct, and it seems so according to the detailed description of the restitution given by Gombault, the intercalated short internodes offer a rare opportunity to study the reorganization of a stretch of adult fibre after selective destruction of myelin.

An account of some relations between various components of short internodes and neighbouring normal internodes is therefore presented in this paper.

MATERIAL AND METHODS

The "intercalated" internodes were encountered in fibres of dogs, rabbits and cats. The material presented here was obtained from various peripheral nerves and nerve roots of 15 adult cats. As it was not possible to know beforehand whether an animal will or will not contain the short internodes, they were measured when they were found in material surveyed for other purposes.

The measurements were made on teased fibres either freshly removed from the animal and kept in Ringer of fixed in buffered osmium tetroxyde. In order to avoid confusion with fibres which might have regenerated after Wallerian degeneration and which might not yet have reached their full size, the measurements were made only if the teased portion of the fibre contained at least one normal internode on each side of the intercalated segment.

The total diameters of internodes, both normal and short, were measured by the external border of myelin and the diameters of axoplasm by the internal border of myelin. The thickness of myelin was calculated from the

difference between these diameters. Each figure in the tables corresponds to a mean from at least 3 measurements taken along each internode.

The measurements of diameters were made on a ground glass projection of the microscopic image at a total magnification of 1000x, the readings being made to the nearest 0.5 mm. The length of internodes was read to the nearest 2 mm at a magnification of 250x.

The proximo-distal direction of the fibres was determined by the shape of juxtanodal bulbs in normal internodes (Lubińska 1954, Lubińska and Łukaszewska 1956).

RESULTS

The short internodes are encountered in some 20 per cent of apparently normal adult cats coming to the laboratory from various sources. They seem more frequent in older animals. At a rough estimation there is one short internode in several thousand of normal ones. It is not known whether the fibres containing short internodes are functional. The absence of trophic alterations outside the altered region seems to indicate that the function is also preserved. But as the presence of intercalated segments in any one preparation was unpredictable it was not possible to settle the question experimentally. The general behaviour of the animals was not affected but this would be so whatever the state of these fibres, owing to the extreme scarcity of lesions.

The data reported here were obtained from 32 fibres containing short internodes. They were undamaged during preparation and measured before marked deterioration set in.

The short internodes resemble in appearance the fibres regenerated after Wallerian degeneration in all respects except that the fibre continues peripherally by thick and long internodes indicating that no Wallerian degeneration ever took place there.

The thin segment always starts at a node and terminates at a node (Fig. 14, 15, 23—26). Sometimes it forms one short internode only but more frequently there is a succession of several internodes before the fibre resumes its normal diameter. Various degrees of maturation (in the sense in which the word is used for regenerating fibres) were observed in short internodes.

As it was the myelin which had been destroyed and newly deposited afterwards, it may be assumed that the thicker the myelin the more advanced is the restitution. So the internodes arranged according to the degree of myelination show also the evolution of other components of the fibre.

Table I

Normal and short internodes in fibres with several short internodes in succession. Internodes arranged in proximo-distal order. All dimensions are in microns

Nerve	Fibre serial No	Internode	Total diameter of the fibre	Axon diameter	Thickness of myelin	Location of the nucleus at	Length of the internode	Total length of the intercalated segment
Peroneal	1	I normal	19.8	11.7	4	—	—	
		II short	13.1	8.7	2.2	170	320	
		III short	13.3	8.7	2.3	230	455	
		IV short	13.1	9	2.1	175	345	1860
		V short	14.1	9.5	2.3	—	510	
		VI short	14.6	10.3	2.2	—	230	
		VII normal	20.6	12.7	4	880	1750	
		VIII normal	20.6	12.5	4	850	1600	
		IX normal	19.5	11.5	4	910	1850	
		X normal	19.8	11.7	4	860	1700	
Ventral root	2	I normal	20.8	11.7	4.5	—	1150	
		II short	17.5	11.7	2.9	210	400	
		III short	17.7	11.7	3	210	400	1180
		IV short	17.3	11.2	3	200	380	
		V normal	20.7	11.7	4.5	—	—	
Ventral root	3	I normal	20.6	13.6	3.5	—	—	
		II short	14	11.1	1.5	—	305	
		III short	14	11	1.5	—	235	845
		IV short	14.3	11.1	1.6	150	305	
		V normal	20	13.7	3.2	—	—	
Tibial	4	I short	15.2	11.8	1.7	—	510	
		II short	15.5	11.5	2	—	580	
		III short	15.3	12	1.7	—	380	1470
		IV normal	19	13	3	—	1530	
		V normal	19.3	14	2.7	—	1530	
Peroneal	5	I normal	17.7	11.7	3	—	—	
		II short	13.7	9.7	2	—	320	
		III short	14	10	2	230	470	1530
		IV short	13.3	9.3	2	—	470	
		V short	13.3	9.7	1.8	130	270	
		VI normal	17	10.7	3.1	—	—	

Table I continued

Nerve	Fibre serial No	Internode	Total diameter of the fibre	Axon diameter	Thickness of myelin	Location of the nucleus at	Length of the internode	Total length of the intercalated segment
Ventral root	6	I normal	22	14	4	—	1280	
		II short	15.7	11.7	2	—	410	
		III short	15.7	10.7	2.5	—	360	1250
		IV short	17.3	12.3	2.5	—	480	
		V normal	21	13.3	3.9	—	—	
Dorsal root	7	I normal	19	13.2	2.9	—	—	
		II normal	18.8	12.3	3.2	—	—	
		III short	14.7	10.7	2	—	640	1400
		IV short	14	10	2	—	760	
		V normal	18	10	4	—	—	
Peroneal	8	I normal	23	17	3	—	—	
		II short	16.7	13.7	1.5	—	320	
		III short	17	13	2	—	400	720
		IV normal	20	13.7	3.3	—	750	
		V normal	19.7	13.3	3.2	—	—	
Obturat.	9	I normal	18.3	10.7	3.8	—	—	
		II short	14.7	10.7	2	—	410	
		III short	16.7	12	2.3	—	530	1375
		IV short	16.3	11.7	2.3	—	435	
		V normal	23	15	4	—	—	
Peroneal	10	I normal	19	12.3	3.4	—	—	
		II normal	19	12	3.5	—	1500	
		III normal	19.7	13	3.4	—	1380	750
		IV short	16.3	12.3	2	—	280	
		V short	17.3	12.3	2.5	—	470	
Peroneal	11	I normal	15.3	8.7	3.3	—	—	
		II normal	14.7	7.7	3.5	—	—	
		III normal	16	8.7	3.6	—	—	
		IV short	12	8	2	220	440	1430
		V short	13	8	2.5	260	540	
		VI short	12.7	8.3	2.2	—	450	
		VII normal	15.3	8	3.6	—	—	

Table I continued

Nerve	Fibre serial No	Internode	Total diameter of the fibre	Axon diameter	Thickness of myelin	Location of the nucleus-at	Length of the internode	Total length of the intercalated segment
Obturat.	12	I normal	21.7	15.7	3	—	—	
		II short	17.3	14	1.6	—	150	
		III normal	24	17	3.5	—	1220	
		IV short	17	14	1.5	—	190	
		V normal	22	14.5	3.7	—	—	
Peroneal	13	I normal	12.2	7.5	2.4	—	—	
		II short	9	6	1.5	127	240	
		III short	9.1	6	1.5	120	200	805
		IV short	9	6	1.5	—	150	
		V short	9	6	1.5	—	215	
Median	14	I normal	18.7	11.7	3.5	—	—	
		II short	13.3	7.7	2.8	140	300	
		III short	13.7	7.7	3.0	230	470	1375
		IV short	13.3	7.3	3.0	145	305	
		V short	13.7	8.0	2.8	160	320	
		VI normal	19	11.3	3.8	—	1300	

The data concerning fibres with several short internodes in succession are collected in Table I, those of fibres with a single short internode, in Table II.

Length of intercalated internodes

When only one short internode is "intercalated" between the normal ones, its length usually oscillates between 50 and 150 μ, although sometimes internodes exceeding 300 μ are encountered. As the short internodes examined here were found in medium and large fibres where the length of normal internodes usually exceeds 1 mm, the small length of intercalated segments shows that only a fraction of the old internode had been affected. Gombault also mentions that the lesion is limited often to a small portion of an internode.

During the recovery a new node is formed between the unaffected part of the fibre and that in which the myelin has been destroyed.

When several short internodes in succession are "intercalated", the sum of their lengths usually corresponds to the length of one normal internode of the fibre, suggesting that a whole internode has been affected. The length of individual short internodes in such fibres varies most frequently between 300 and 500μ.

Schwann cells

In very immature intercalated segments a large amount of Schwann cell cytoplasm and several nuclei are seen between the axoplasm and the neurilemmal tube (Plate II, Fig. 26). As the maturation progresses the excess of cytoplasm disappears and, when the new internodes are formed, there remains only one nucleus in each internode. It is located, as in normal fibres, in the middle of the internode.

Axoplasm and myelin

The diameter of the axon and the thickness of myelin in short and normal internodes of the same fibres are shown in Fig. 1. In some instances the axon of the short internode is much narrower than that of normal internodes. The myelin is also very thin there. When the thickness of myelin in the short internode reaches some 70 or more per cent of that of the neighbouring normal internode, the axoplasmic cylinder usually approaches or attains the diameter of the axon in normal internodes (Pl. II, Fig. 25 and 26).

The configuration of myelin in the short internodes seems normal except for its thinness. The incisures of Schmidt-Lantermann are present. In the middle of the internode the axon is pushed away from the neurilemma by the nucleus and cytoplasm of the Schwann cell as in normal internodes.

The thickness of axon and myelin is sometimes different in successive short internodes, indicating that the process of restitution may not have started simultaneously in the whole length of the affected segment.

Nodes of Ranvier

The nodes at the site of transition between a normal and a short internode look very asymmetrical, as the normal internode ends in a large bulb whereas the short one either has none or only a very small one (Pl. I, Figs. 3, 5, 8, 11, Pl. II., Fig. 23). It is worthwhile to note that where only one short internode is present, at

Table II

Normal and short internodes in fibres with a single intercalated short internode. Internodes arranged in proximo-distal order.
All dimensions are in microns

Nerve	Fibre Serial No	Internode	Total diameter of the fibre	Axon diameter	Thickness of myelin	Length of the internode
Peroneal	1	I normal	12	6	3	—
		II normal	12	6	3	1200
		III short	6	4	1	50
		IV normal	12	6	3	—
Peroneal	2	I normal	14	7.7	3.2	—
		II normal	15	7.7	3.6	1160
		III short	10	6	2	110
		IV normal	14	7.7	3.2	—
Peroneal	3	I normal	18	11.7	3.1	—
		II normal	18	11.7	3.1	1310
		III short	10	7	1.5	85
		IV normal	18	11.3	3.3	—
Peroneal	4	I normal	16	9.3	3.4	1280
		II normal	17	10	3.5	—
		III short	10.3	7.3	1.5	340
		IV normal	16.9	9.3	3.9	—
Peroneal	5	I normal	15.7	9	3.4	—
		II normal	16	9.7	3.2	—
		III short	12	7.7	2.2	390
		IV normal	16	9.3	3.4	—
Median	6	I normal	19	12.7	3.2	—
		II short	11.4	8	1.7	640
		III normal	19	12.7	3.2	—
Ventral root	7	I normal	21	13	4	—
		II normal	20.3	13.3	3.5	—
		III short	15.7	11.3	2.2	170
		IV normal	22.7	14.7	4	—
		V normal	21.3	13.3	4	—
		VI normal	20.3	13	3.5	—

Table II continued

Nerve	Fibre serial No	Internode	Total diameter of the fibre	Axon diameter	Thickness of myelin	Length of the internode
Dorsal root	8	I normal	18	11	3.5	—
		II short	12	8	2	140
		III normal	18.3	11.7	3.3	—
Peroneal	9	I normal	15	8.3	3.3	—
		II normal	14.3	7	3.6	—
		III short	8	4	2	150
		IV normal	13	7	3	—
Peroneal	10	I normal	11.3	6	2.7	1180
		II short	8	5	1.5	140
		III normal	11	5	3	1100
Peroneal	11	I normal	15	9	3	—
		II short	11	7	2	240
		III normal	14.3	7.3	3.5	—
Peroneal	12	I normal	13.7	7.7	3	—
		II normal	13	7	3	1220
		III normal	13.7	7	3.3	1210
		IV short	7.5	5	1.4	100
		V normal	12.3	6.3	3	—
Peroneal	13	I normal	14	8	3	—
		II normal	13	7.3	2.9	1100
		III short	8.7	5.7	1.5	90
		IV normal	13	6	3.5	—
Tibial.	14	I normal	16.7	7.4	4.5	—
		II short	10.2	4.6	2.8	150
		III normal	16	7	4.5	—
Dorsal root	15	I normal	18.5	10	4.2	—
		II normal	18.7	11	3.9	1500
		III short	18	10.7	3.6	605
		IV normal	20	12	4.0	—
Ventral root	16	I normal	15	7.7	3.6	—
		II normal	14.5	7.3	3.6	960
		III short	10	6	2.0	95
		IV normal	14	7	3.5	—

least one of the large bulbs of the adjacent normal internodes is newly formed. It did not exist before the neuritic process intervened. The formation of this new bulb must occur fairly early during the recovery for it is visible even at a period when the short internode is only faintly remyelinated.

The bulbs at the ends of normal internodes adjacent to the short ones usually preserve their characteristic proximal or distal shape. A normal bulb proximal to the intercalated segment is shown

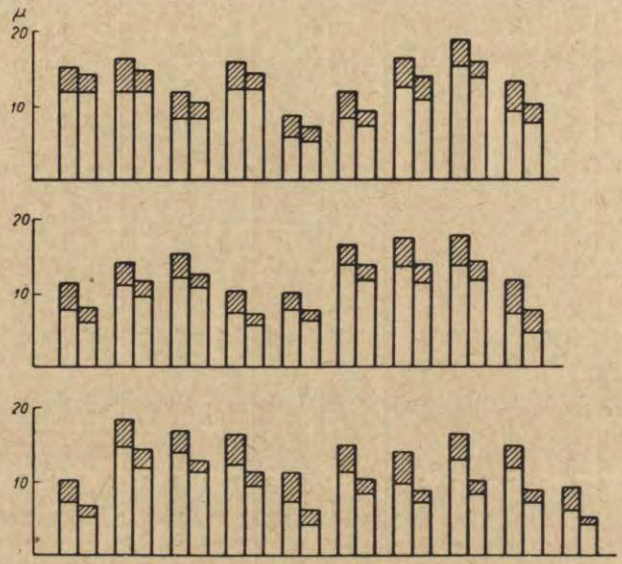

Fig. 1. Axon diameter and thickness of myelin in short and normal internodes of 28 fibres. Fixation in osmium tetroxyde. Fibres are arranged approximately according to the decreasing order of maturation. Left column-normal internode. Right column — short internode. Myelin — hatched. Axoplasm — blank. Ordinates — dimensions in microns

in Pl. I, Fig. 3 and the bulb distal to the intercalated segment in Fig. 5. Fig. 2 shows a portion of a normal, and Fig. 4 that of a short internode in the same fibre.

The shape of the nodes separating successive short internodes depends on the degree of their maturation. In internodes with thin myelin, the nodes are simply adumbrated by the interruption of myelin and the terminal portions of adjacent internodes are regu-

larly cylindrical. When the myelin becomes thicker, bulbous enlargements are formed at the nodes. These bulbs present a similar difference in shape and magnitude, according to their location at the proximal or distal side of the node as do the bulbs in the normal portions of the fibre. They may be used similarly for the determination of the initial orientation of the fibre in the body. A series of seven consecutive nodes of a fibre containing an intercalated segment is shown in Pl. II, Figs. 16—22. The first two are nodes between normal internodes, the third separates on the proximal side the normal internode from the intercalated segment. The three following nodes are between short internodes and the last marks the passage to the distal normal internode.

Perturbations in neighbouring internodes,

In view of Weiss' theory of axoplasmic flow it seemed important to determine whether the occurrence of an internode of reduced diameter would influence the diameter of the fibre above and below it. No unequivocal answer could be obtained. Most frequently the long internodes on both sides of the short one had similar diameters.

In some fibres the distal internodes appeared slightly thinner, in others they were thicker than the proximal ones (Table I and II).

In one instance Fibre 12 (Table I), two short internodes separated by one of normal length were observed. This long internode was markedly thicker than other internodes of the fibre. Such configuration is extremely rare. This fibre was taken from an old cat in which short internodes were very numerous and many abnormalities of myelin were seen on the long internodes too.

DISCUSSION

The short intercalated internodes are formed during recovery after a *local* neuritic process. Their study may therefore supplement the information concerning the maintenance of normal organization of nerve fibres obtained by analysis of Wallerian degeneration and subsequent regeneration.

A comparison of the two conditions brings out the following features.

1. When the myelin is destroyed selectively and the continuity of axoplasm is preserved, the fibre below the lesion does not degen-

erate, as it does after transsection of the axon, and the perturbation remains purely local.

2. The reaction of Schwann cells, that is the increase of the amount of cytoplasm and the multiplication of the nuclei, is similar in both situations, suggesting that in the Wallerian degeneration it is also the destruction of myelin and not that of axoplasm which leads to changes in the behaviour of Schwann cells.

3. The processes of remyelination and the recovery of initial diameters, as well as a permanent reduction of the length of newly formed internodes, are similar in both conditions.

Some of these relations call for a further comment.

The local reduction of the diameter of the axon

The diameter of the axon becomes reduced in the portion of the fibre where the myelin had been destroyed. It remains smaller than in the neighbouring normal internodes, both up and down the fibre, as long as the newly deposited myelin does not reach a certain critical thickness. The existence of a narrowed stretch of the axon should produce, according to Weiss theory, a piling up of

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PLATE I

Fibres with short internodes from peripheral nerves of cats. Fixed in buffered osmium tetroxyde. Scale — 20 μ

Figs. 2—5 various portions of a fibre. Fig. 2 — fragment of a normal internode. Fig. 3 — Transition to the intercalated segment, proximal side. Fig. 4 — fragment of a short internode, n — perinuclear region of the cell of Schwann. Fig. 5 — Transition to the normal internode on the distal side

Figs. 6—7. Fig. 6 — Node between a short and a distal normal internode Fig. 7 — node between two short internodes in the same fibre

Fig. 8—9. Fig. 8 — Node between a short and a distal normal internode. Fig. 9 — node between two short internodes in the same fibre

Figs. 10—11. Fig. 10 — Node between two short internodes. Fig. 11 — Node between the short and the proximal normal internode in the same fibre

Figs. 12—13. Fig. 12 — Node between two short internodes. Fig. 13 — Node between the proximal normal and the short internode in the same fibre

Fig. 14. An intercalated segment with three short internodes. In the upper part a portion of the proximal normal internode, in the lower, a portion of the distal normal internode

Fig. 15. Intercalated segment with a single short internode

PLATE I

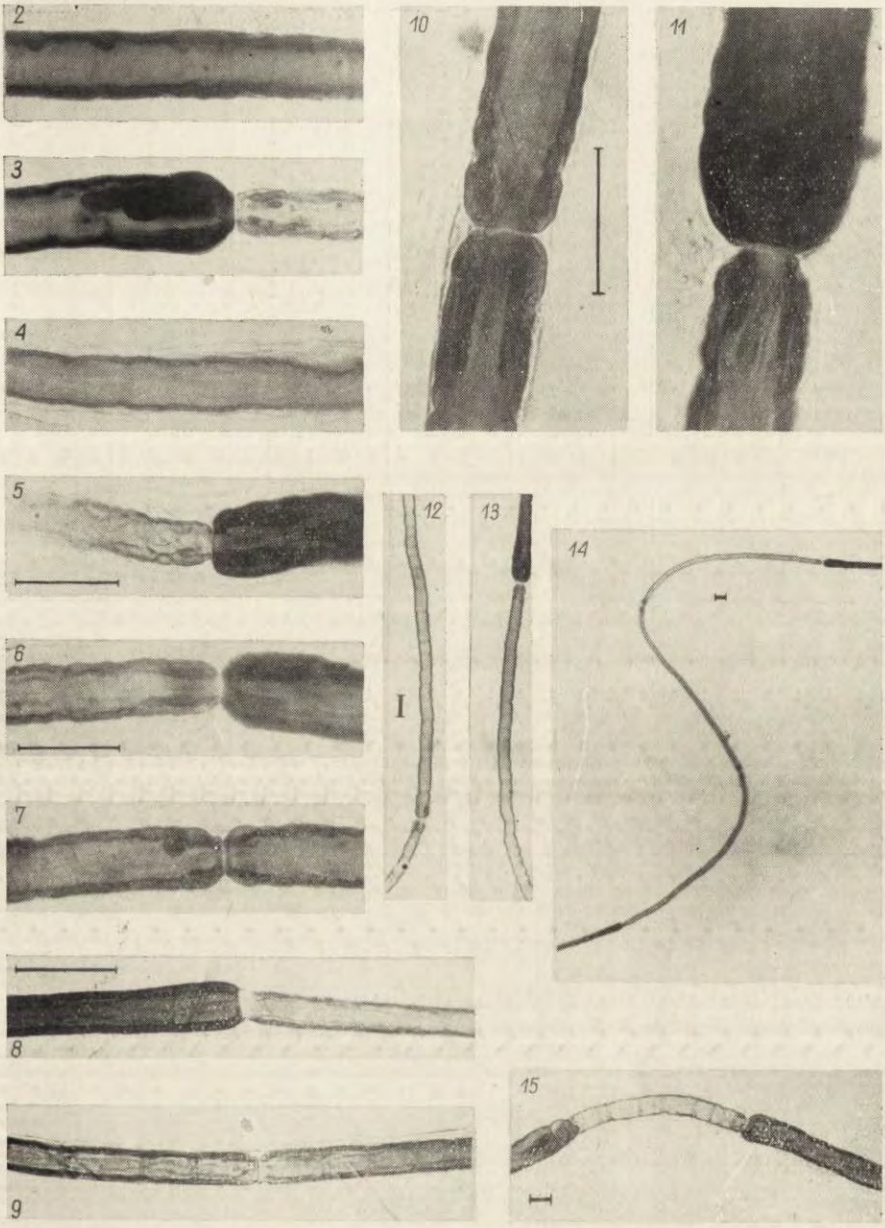
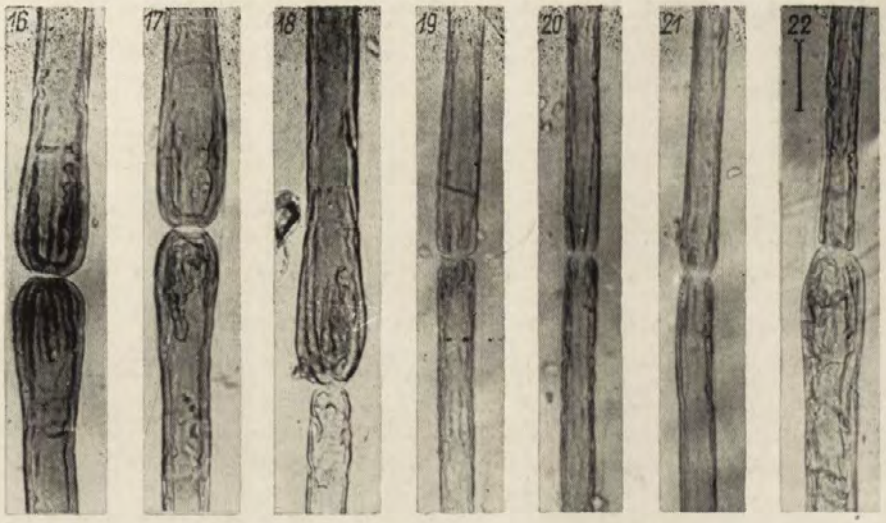
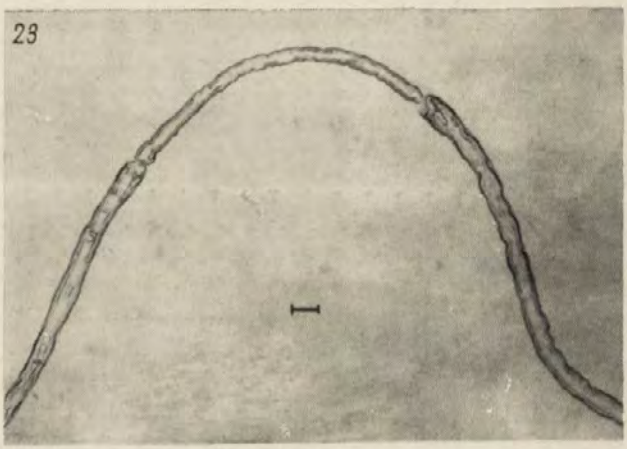
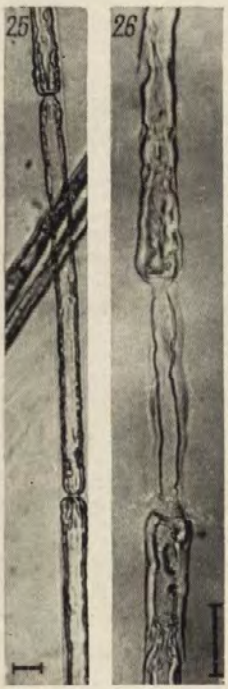


PLATE II







axoplasm above and a reduction of axon diameter below the narrowed portion. In the majority of fibres examined here, however, the presence of the narrow internode did not seem to influence the dimensions of normal internodes on either side.

The immature intercalated internodes are not the only places along a fibre where the diameter of the axon is reduced. At the very beginning of the fibre, beyond the axon hillock, the unmyelinated portion of the axon is very thin. It is from the point at which the axon becomes myelinated that its diameter increases. Strong (1906), who investigated this relationship in the axon of human motoneurons, found that when the myelin sheath appears abruptly, the increase in axon diameter is also abrupt. When, conversely, the myelin sheath gradually assumes its full thickness, the increase in axon diameter is likewise gradual.

Another reduction of axon diameter occurs when the fibre leaves the spinal cord and traverses the pial ring (Tarlow 1937). In this region the myelin is either absent or considerably thinned. After crossing the pial ring the caliber of the axon increases again.

These relations indicate clearly that it is possible for an axon to increase in diameter distally to a narrowed zone. It appears therefore that the permanent size deficit of regenerated fibres distal to constriction by an arterial sleeve, described by Weiss and Taylor (1944) and Weiss and Hiscoe (1948) is determined by some unelucidated conditions of the experiments, perhaps by the length of the compressed portion, and cannot be interpreted simply as a result of partial obstruction of the axoplasmic pathway.

PLATE II

Fresh fibres with short internodes. Cat nerves

Figs. 16—22. Successive nodes of a fibre. Figs. 16 and 17 — nodes between normal internodes. Fig. 18 — node between the proximal normal internode and the first short internode. Figs. 19—21 nodes between short internodes Fig. 22 — node between the last short internode and the next distal one

Figs. 23—26. Single short internodes. Fig. 25 — advanced degree of maturation. The diameter of the short internode attains that of normal internodes. Fig. 26 — An immature short internode with an excess of Schwann cytoplasm

It is perhaps important to note that Weiss nad Taylor observed a thinned myelin sheath in the distal parts of the fibres reduced in diameters. It is difficult to reconcile the above details of axonal morphology with Weiss' theory that the axoplasm moves proximo-distally in the fibre as a whole column and that "the size of the axon at any one level is the expression of a stationary equilibrium between the rate at which the axoplasm is delivered to that level and the rate at which it passes down to next distal level" (Weiss and Hiscoe 1948, p. 361). Such relationship certainly does not hold for branching fibres where the branches and sometimes even a single branch (Quilliam, 1956) may have a larger cross-section than that of the parent fibre.

The fact that an axon may increase in diameter distally to a local constriction seems to contradict the opinion that the nucleated portion of the neuron is the only source of axonal material at any one level of the fibre. If it were so, we would have to admit a greatly increased rate of flow in the narrowed portions of the axon. No evidence of such variable rate seems available at present. The variability of axon diameter along the fibre suggests, on the contrary, that the amount of some bulk substances, at least that of water, is regulated by local exchanges.

In fact at the nodes of Ranvier there are some indications that the region is less hydrated than the internode. So Nageotte (1921) and Hess and Lansing (1953) observed after fixation of fibres that the neurofibrils are both thicker and more closely packed in the node. De Renyi (1929) describes the nodal axoplasm as more rigid than elsewhere in the fibre. The increased rigidity is probably also due to the lesser hydration of the axoplasm in the node although other causes of this phenomenon are not excluded (Lubińska 1956 b). The other regions of reduced axonal caliber do not seem to have been examined in this respect.

In describing (1954) the asymmetry of the nodal region, consisting in characteristic differences of shape and size in the terminal bulbous enlargements of adjacent internodes, I interpreted the larger volume of the bulbs situated at the proximal side of each node as a result of damming, similar to that obtained above the artificial construction of nerve in Weiss experiments. This interpretation is completely invalidated by the relations found in the intercalated segments. At the boundary between a short internode

and the next distal normal one it is the bulb by which the normal internode begins, that is the bulb on the distal side of the limiting node, that is more voluminous than the bulb at the proximal side of the node terminating the intercalated segment (Pl. I, Figs. 5, 6, 8). Such configuration exists in spite of the fact that in this portion of the fibre the direction of the flow of axoplasm or of some of its constituents is from the narrow toward the wide internode. Thus the nodal polarity is certainly not due to damming only and itis origin must be sought elsewehre.

In all the instances discsussed above, that is in the slender initial portion of the fibre, in the region of the immature "intercalated" internodes, in the regenerating fibres distally to a constricting sleeve, as well as at every node, in fact everywhere where the diameter of the axon is reduced, a concomitant reduction or absence of myelin is observed. This close relationship suggests that the myelin is one of the important factors in the determination of the diameter of the portion of the axon ensheathed in it. Nageotte (1921) has already directed attention to what he called the "physiological oedema" produced in the axon under the influence of myelin. The mechanism of the interaction between these two components of the fibre in the absorption of water by the axon remains as yet unknown.

One could only speculate as to what extent the small caliber of non-medullated fibres is due to the failure of their Schwann cells to deposit myelin.

The cells of Schwann

During the local neuritic process, as well as in the Wallerian degeneration, there is a multiplication of nuclei and an increase in the amount of cytoplasm of the cells of Schwann. These phenomena seem released by the destruction of myelin and do not appear to be directly connected with the fate of the axon. In fact they seem as intense in the "névrite periaxile" in which the integrity of the axon is preserved as in the Wallerian degeneration where it is abolished.

Moreover, as shown by Joseph (1947), in the unmyelinated fibres the degeneration of the axon is not accompanied by any increase in the number of nuclei of the cells of Schwann. This shows that with those cells of Schwann which do not produce my-

elin, their equilibrium state is not influenced by the presence or absence of the axon in the tube*.

Abercrombie and Johnson (1942) and Abercrombie, Johnson and Thomas (1949) who investigated the outwandering of Schwann cells from normal and degenerating nerves as well as from nerves at various stages of regeneration, found that the migratory activity of these cells varies with the state of the nerve. The outwandering from a normal nerve and from a nerve degenerating for a few days was insignificant, whereas in later stages of degeneration the outwandering was intense. After 25 days of regeneration the outwandering from the proximal part of the reinnervated nerve was considerably smaller than that from the non reinnervated nerve. No such difference appeared with the distal portion of the reinnervated nerve at this period. When, however, the nerve was reinnervated for 100 days, the inhibition of outwandering was observed throughout the whole length of the reinnervated nerve. The time relations chosen for these experiments were such that at the first period the whole length of the nerve was reinnervated but the remyelination has not yet spread to the distal portion of the nerve, whereas at the second period the whole nerve was already remyelinated.

It is tempting to attribute the immobilization of cells of Schwann to the presence of myelin. When one considers the life-history of these cells, the migratory movements are present either before the formation of myelin, during development (Harrison 1910, Speidel 1932) or after its degeneration. In the last instance the cells of Schwann are mobile inside an intact neurilemmal tube (Holmes and Young 1942) or outwander from a sectioned one.

The striking contrast between the motility of the free cells of Schwann and their rigid confinement in a myelinated internode is easier to understand when one considers the mechanism of my-

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* Note added in proofs — in the abdominal vagus however containing only a few myelinated fibres. Abercrombie, Evans and Murray (1958) found recently an increase of nuclear population during degeneration. Rexed and Fredriksson (1956) also observed intense mitoses of Schwann cell nuclei in degenerating splenic nerve which is almost completely non-myelinated. Thus the nature of factors stimulating Schwann cells proliferation in degenerating nerves remains uncertain.

elination elucidated recently by Geren (1954) nad Schmitt and Geren (1956). In the developing fibres, when the surface of a cell of Schwann infolds in contact with the axon, a myelin layer appears between the apposing surfaces of Schwann cytoplasm. These apposing surfaces, and the layer of myelin between them, increase in length and form a spiral pattern by rotation of the Schwann cell around the axon. As the fibre matures, the number of turns of myelin increases and the cytoplasm is driven out from between them. It is conceivable that the resulting internal architecture of the cell and the limited space between the axon and the neurilemma, greatly restrict the motility of the cell of Schwann. If the myelin breaks down the cell become mobile again. Incidentally the mechanism of deposition of myelin described by Geren seems to provide an explanation for the very unusual morphology of Schwann cell cytoplasm in adult fibres. In the middle of the internode there is a collection of cytoplasm around the nucleus. Outside the perinuclear region, along the whole internode, only an extremely thin veil, fenestrated according to some authors, or continuous according to others, stretches up to the nodal region, where suddenly the amount of Schwann cytoplasm increases again (Gasser 1952, Hess and Lansing 1953). The increase at the latter place is perhaps due to the possibility for the excess of Schwann cytoplasm trapped between the lamellae to escape at their free edges when the turns of myelin become more closely packed during the maturation of the fibres. This hypothesis seems corroborated by observation of deteriorating explanted fibres in which the Schwann cytoplasm swells. It is easy to observe then that at the nodes it is located not only externally to myelin but also irregularly between the layers which become split and displaced near the nodal region by the swollen cytoplasm (Lubińska 1956).

Length of internodes

In intercalated segments the newly formed internodes are much shorter than the original ones. In this respect also they resemble the fibres regenerated after Wallerian degeneration (Vizoso and Young 1948). Vizoso and Young have also shown that the initial length of internodes in very young animals is of similar magnitude and their ultimate elongation is connected with the growth of the part of the body in which the nerves run.

In the intercalated segments the nucleated portion of the Schwann cell lies in the middle of the internode. This indicates myelin extends in the Schwann cell symmetrically on both sides of the nucleus, over a length roughly similar to that of normal myelination, that is some 150—250 μ on each side of the nucleus. If the length of the denuded fibre is larger, several Schwann cells occupy it and new nodes are formed between the adjacent cells. The last cell in the row may have only a very short segment left. In this short terminal portion the nucleus is also located in the middle.

SUMMARY AND CONCLUSIONS

The examination of "intercalated" short internodes, vestiges of a periaxile neuritis, shows the following relationships between various constituents of nerve fibres.

1. A segment of an adult fibre may survive and recover after destruction of its myelin, as shown by Gombault.

2. The diameter of the axon diminishes in the demyelinated portion in spite of the maintenance of normal dimensions of the axon in the neighbouring internodes both above and below the lesion.

3. In the course of subsequent remyelination, when the newly deposited myelin attains some 70 per cent of its normal thickness, the diameter of the affected portion of the axon usually returns to its normal value.

4. The selective destruction of myelin with preservation of axoplasmic continuity leads to the increase of the amount of cytoplasm of cells of Schwann and proliferation of nuclei. This suggests that a similar reaction of the Schwann cells during Wallerian degeneration is connected with the breakdown of myelin rather than with that of axoplasm.

5. During recovery several Schwann cells divide between them the denuded segment and deposit new myelin. The zone of myelination extends symmetrically on both sides of the nucleus. New nodes are formed and several short internodes appear on the stretch occupied previously by a single internode. The length of these short internodes is similar to that of fibres regenerating after Wallerian degeneration. In medium and large fibres the new internodes are usually 300—500 μ long. When the restitution is com-

plated and the axoplasm and myelin have resumed the normal thickness, the reduced length of intercalated internodes remains as the only premanent trace of the neuritic process.

6. Sometimes only a fraction of an internode is attacked and then new nodes are formed between the normal and the affected region. The short internode thus formed is usually very short. It rarely exceeds 50—150 μ.

I wish to thank Dr M. Abercrombie for communication of unpublished results and Prof. J. Konorski for reading the manuscript.

REFERENCES

ABERCROMBIE M., EVANS D. H. L. and MURRAY I. G. 1958 — Personal communication.

ABERCROMBIE M. and JOHNSON M. L. 1942 — Outwandering of cells in tissue cultures of nerves undergoing Wallerian degeneration. J. of Exper. Biol. *19*, p. 266—283.

ABERCROMBIE M., JOHNSON M. L. and THOMAS G. A. — 1949 — The influence of nerve fibres on Schwann cell migration investigated in tissue culture. Proc. Roy. Soc. B. 136, p. 448—460.

GASSER H. S. 1952 — Comments in the Neuron. Cold Spring Harbor Symp. Quant. Biol. *17*, p. 32.

GEREN B. B. 1954 — The formation from the Schwann cell surface of myelin in the peripheral nerves of chick embryos. Exper. Cell. Res. 7, p. 558—562.

GOMBAULT 1880—1881 — Contribution à l'étude anatomique de la névrite parenchymateuse subaigüe et chronique. Névrite segmentaire periaxile. Arch. Neurol. *1*, p. 11—38 and 177—190.

HARRISON R. G. 1910 — The outgrowth of the nerve fiber as a mode of protoplasmic movement. J. Exp. Zool. *9*, p. 788—848.

HESS A. and LANSING A. I. 1953 — The fine structure of peripheral nerve fibres. Anat. Rec. *117*, p. 175—200.

HOLMES W. and YOUNG J. Z. 1942 — Nerve regeneration after immediato and delayed suture. J. Anat. *77*, p. 63—96.

JOSEPH J. 1947 — Absence of cell multiplication during degeneration of non-myelinated nerves. J. Anat. *81*, p. 135—139.

LUBIŃSKA L. 1954 — Form of myelinated nerve fibres. Nature *173*, 867.

LUBIŃSKA L. 1956 — The physical state of axoplasm in teased vertebrate nerve fibres. Acta Biol. Exper. *17*, p. 135—140.

LUBIŃSKA L. and ŁUKASZEWSKA I. 1956 — Shape of myelinated nerve fibres and proximo-distal flow of axoplasm. Acta Biol. Exper. *17*, p. 115—133.

NAGEOTTE J. 1921 — L'organization de la matière dans ses rapports avec la vie. Alcan Paris.

QUILLIAM T. A. 1956 — Some characteristic of myelinated fibre populations. J. Anat., p. 172—185.

RENAUT M. J. 1881 — Recherches sur quelques points particuliers de l'histologie des nerfs. Arch. de Physiol. *13*, p. 161—190.

DE RENYI G. S. 1929 — The structure of cells in tissues as revealed by microdissection II. The physical properties of the living axis cylinders in the myelinated nerve fibre of the frog. J. Comp. Neurol. *47*, p. 405—425.

REXED B. 1942 — Über die Aktivität der Schwannscher Zellen bei der Nervenregeneration. I. Die Überbrückung neuritloser Nervenlücken. Ztschr. Mikr-Anat. Forsch. *54*, p. 177—205.

REXED B. and FREDRIKSSON T. 1956 — The frequence of Schwann cell mitoses in degenerating nerves. Acta Soc. Med. Upsaliensis *61*, p. 199—206.

SCHMITT F. O. and GEREN B. B. 1956 — On the significance of the Schwann cell in the structure and function of peripheral nerve. Protoplasma. *46*, p. 659—663.

SPEIDEL C. C. 1932 — Studies of living nerves. I. The movements of individual sheath cells and nerve sprouts correlated with the process of myelin-sheath formation in amphibian larvae. J. Exper. Zool. *61*, p. 279—317.

STRANSKY E. 1903 — Über diskontinuirliche Zerfallsprozesse an der peripheren Nervenfaser. Journ. f. Psychol. u. Neurol. *1*. p. 169—199.

STRONG S. O. 1906 — The mode of connection of the medullated nerve fiber with its cell body. J. Comp. Neur. *16*, p. 397—401.

TARLOV I. M. 1937 — Structure of the nerve root: I. Nature of the junction between the central and the peripheral nervous system. Arch. Neurol. Psych. *37*, p. 555—583.

VIZOSO A. D. and YOUNG J. Z. 1948 — Internode length and fibre diameter in developing and regenerating nerves. J. Anat. *82*, p. 110—134.

WEISS P. and HISCOE H. B. 1948 — Experiments on the mechanism of nerve growth. J. Exper. Zool. *107*, p. 315—395.

WEISS P. and TAYLOR A. C. 1944 — Impairment of growth and myelinization in regenerating nerve fibers subject to constriction. Proc. Soc. Exper. Biol. and Med. *55*, p. 77.

Acta Biologiae Experimentalis, Vol. XVIII, 1958

ON THE CONDITIONED REFLEX OF THE CESSATION OF THE ACT OF EATING. I. ESTABLISHMENT OF THE CONDITIONED CESSATION REFLEX

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(Received for publication 25 September 1957)

INTRODUCTION

The present study started from the assumption that a "neutral" stimulus when preceding the termination of an unconditioned stimulus acquires inhibitory properties (Konorski J., Lubińska L., Miller S. 1936). This assumption was the base for the hypothesis, put forward by Konorski J. (1948, p. 136), concerning the formation of inhibitory conditioned connections.

That a neutral stimulus can acquire inhibitory properties when applied during the action of unconditioned stimulus was also noticed by D. I. Solovejčik (1928), E. M. Kreps (1933), W. I. Pavlova (1933) and M. K. Petrova (1933), but they did not connect this phenomenon with signaling the end of the unconditioned stimulus. Many authors examined the properties acquired by neutral stimulus, when applied after the start of unconditioned stimulus, in connection with backward conditioning (for references see Razran G. 1956). As these investigations were mainly carried out on defensive reflexes (e. g. finger withdrawal, eyelid conditioning), and the neutral stimulus was applied after the unconditioned stimulus (electric shock, air puff), there could have been no question of the signalling of the end of the unconditioned reflex.

A more extensive study on this subject performed on alimentary and acid reflexes was published by N. A. Krestovnikov (1921). This author maintains that a neutral stimulus applied after the beginning of the act of eating does not acquire any conditioned properties. Krestovnikov, as well as the other previously mentioned authors from the Pavlov school (Solovejčik, Kreps, Pavlova, Petrova and others), in their investigations concerning the properties of simuli applied during the act of eating tested the stimuli during the intervals. Thus they presupposed that it was of no importance whether the stimulus was applied during eating or during the intertrial interval. However according to our results (Zbrożyna A. W. 1952, 1953) a stimulus applied during the act of eating is for a dog a completely different stimulus from one applied during an intertrial interval. And this even to such an extent that it is possible to elaborate a positive conditioned alimentary reflex to some stimulus applied during the intertial interval and a completely opposite reflex — the escape from food — to the same stimulus applied during the act of eating. On the other hand if a stimulus applied during eating does acquire some new (excitatory of inhibitory) properties, it can be hard to detect them against the background of the copious salivation evoked by food. Therefore in this research it was thought best to use as an indicator a motor effect of the cessation of eating, which is accessible for observation and registration (Zbrożyna A. W. 1954, 1957).

A stimulus applied before the withdrawal of food signals only the cessation of eating, if this withdrawal is not connected with nociception or any other unconditioned stimulus e.g. presentation of a new portion of food (Anochin P. and Streż 1934, Kasjanov V. 1949, Nemcova O. L. 1949). V. M. Kasjanov (1949) elaborated a conditioned reflex to a signal of withdrawal of food and found, that this reflex consisted in the quickening of the process of eating.

The possibility of conditioned reflexes being developed to the signal of withdrawal of food can neither be explained by reference to the law of effect (Thorndike E. L. 1911), nor to the principle of need reduction (Hull C. L. 1943), neither can it be based on the Pavlovian principle of the diversion of the excitation from the weaker to the stronger excited centres (Pavlov I. P. 1927, p. 385).

The aim of the present study was to elaborate conditioned reflexes based on the signaling of the cessation of the act of eating, and to investigate the properties of these reflexes.

METHODS

The experiments were carried out in a conditioned reflex chamber (Podkopaev N. A. 1952, p. 114). The experimenter was in the ante-chamber from where he observed the behaviour of the dog through a long narrow observation window. The action of the stimuli, the secretion of saliva, the movements of the dog's head, the movements of the lid shutting the food-bowl, were all registered on a kymograph. Later on the registration of

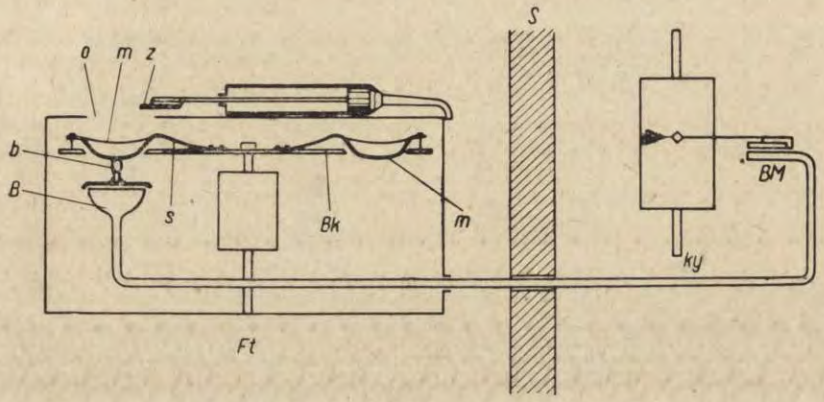

Fig. 1. Apparatus for recording the movements of the bowl
Ft — food-tray side view, Bk — revolving dise of the food-tray with bowls, m — bowl, o — opening in the foo-dtray through which the animal gets access to the bowl, s — spring to which the bowl is attached, b — pulley, on which the bowl is supported, z — sliding lid to shut the opening of the food-tray, S — wall dividing the reflex-chamber from the anti-chamber, ky — kymograph, BM — Marey tambour, B — a tambour with a small pulley fastened to it

the act of eating by means of registration of the movements of the bowl was also introduced. Salivation was registered by the method of W. Kozak (1950). The salivary fistula were prepared by the Gliński method (Podkopaev N. A. 1952, p. 90). Movements of the animal's head were recorded by a pneumatic relay (Zbrożyna A. 1953). The bowl movements were recorded by means of the following pneumatic apparatus (Fig. 1): each bowl was attached to the food-tray by a flat spring, allowing the bowl to move slightly, as the animal was eating. Underneath was a small tambour to the membrane of which was fastened a small pulley directly in contact with the bowl. The pressure caused by the dog while eating was transmitted through the pulley to the membrane, causing a change of pressure inside the

tambour. These changes of pressure were registered on the kymograph by means of Marey's tambour.

The food was given by sliding open the lid of the food-tray (see Fig. 2). When the animal had finished eating and lost all interest in the bowl, the lid of the food-tray was closed. Then the new bowl of food was moved in.

The food was withdrawn by sliding the lid shut very slowly and carefully so as not to hurt in the slightest the eating animal. Usually the very

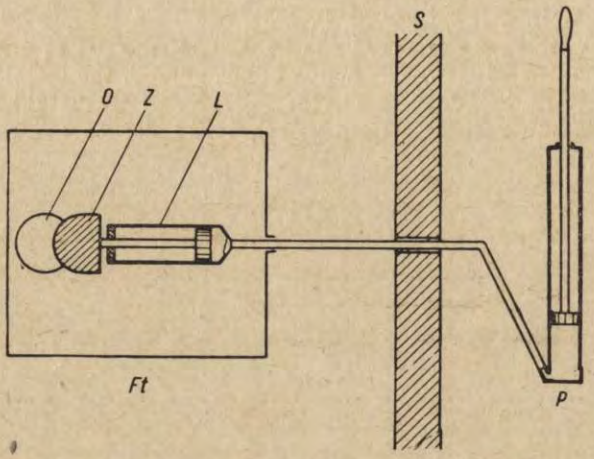

Fig. 2. Arrangement for the withdrawal of food

Ft — food-tray view from above, o — opening in the food-tray, giving access to the bowl, z — sliding lid, shutting the opening "O", L — syringe, S — wall dividing the reflex-chamber from the antichamber, p — motor cycle pump

sight of the sliding lid caused the dog to back away from the food-tray, so enabling it to be shut completely. The movements of the lid were recorded by means of a signal marker twice at the beginning of the lid movement and just before it was completely shut.

The food consisted of dried bread crumbs, moistened by broth, always in the same quantity. The portions of food in the bowls were also equal.

PROCEDURE

The experiments were carried out in two stages.

Ist stage — series of elaboration of normal positive conditioned alimentary reflexes followed by differentiation. Every experiment comprised on an average 6 trials, with the inhibitory stimulus applied at least in one or two of them. Usually positive conditioned reflexes and differentiation were elaborated to visual

stimuli (flashing light — positive, continuous weaker light — differentiation) and then reflexes to auditory stimuli were elaborated but only positive. The isolated action of the stimuli lasted 20 seconds. The quantity of food given to each dog was exactly the same in every trial.

2nd stage — after the positive conditioned reflexes and differentiation were fully established, the conditioned reflex of cessation of the act of eating elaborated. This was done in the following way. While the animal was eating, a stimulus, usually auditory, was applied and maintained for 5 to 10 seconds, and the the food was withdrawn (see — Method). The stimulus was applied about 20 to 30 seconds before the normal end of eating. This procedure of withdrawal of food was applied at the most twice in the course of one experiment. When applied too often the dogs tolerated it very badly, they refused to eat in the reflex chamber, became restless, barked, sometimes even tried to break lose from the leads. This was accompanied by a drop to zero of the salivary conditioned reflexes.

The animals were always fed after the experiments. They came fasting to the experiments, having last received food on the previous day.

RESULTS

The conditioned reflex of cessation of the act of eating, "cessation reflex", was elaborated in seven dogs, besides, in one of these dogs the development of this reflex was carried out simultaneously with differentiation; this latter elaboration is described in the next report of this series (A. Zbrożyna 1958).

Kacber a mongrel dog, weight about 9 kgs, was about 1 year old when the experiments started. The positive alimentary reflex to a buzzer and the differentiation to a bell had already been secured in this dog before the elaboration of the cessation reflex started.

The first cessation reflex was elaborated to a 200 Watts lamp. The light was switched on above the dog's head for 10 seconds, and then the food was withdrawn, (the process of eating lasted usually about 60 seconds). At first the light did not give rise to any visible changes in the animal's behaviour, but on seeing the lid of the bowl slowly sliding shut, he stopped eating and moved away

from the food-tray. During the third experiment, when the light was applied for the fourth time, the animal stopped eating and lifted his head out of the food-tray. This reflex, however, did not reappear in the next experiment. Only beginning from the 14th experiment of this series (16 lamp trials, reinforced by the withdrawal of food) did the cessation reflex to light stimulus become stable. The latent period of this reflex varied from 1 — 9 sec.

In the next series the cessation reflex was elaborated to the sound of bubbling applied from the 40th to 50th second of the act of eating. When the bubbling was applied for the first time no noticeable changes were observed in the animals behaviour; he ate without interruption up to the moment the food was withdrawn (Fig. 3, Exp. 88). When however the bubbling was applied in the

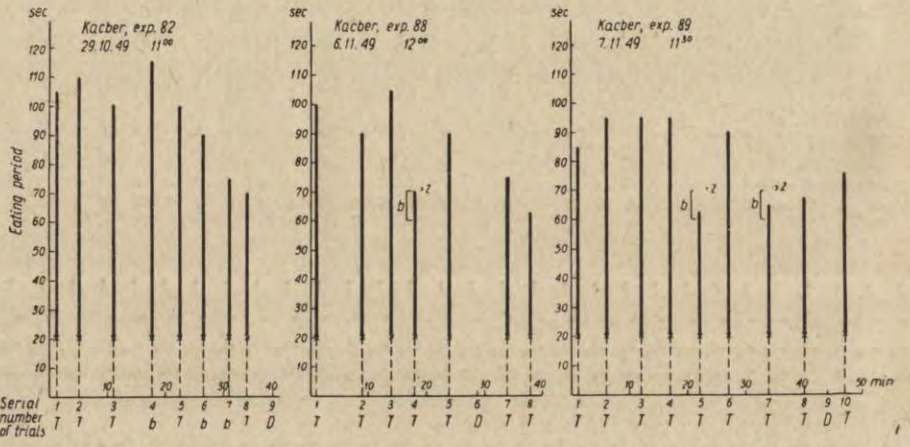

Fig. 3. The course of elaboration of the conditioned cessation reflex to bubbling noise (dog "Kacber")

The columns show the duration of the acts of eating. On the abscissae is shown time in minutes; on ordinates — time in seconds. Dotted lines — isolated action of conditioned stimulus, T — buzzer, b — sound of bubbling, D — bell, z — withdrawal of food, x — presentation of food

next experiment (2-nd bubbling trial, reinforced by the withdrawal of food) the animal stopped eating and withdrew from the food-tray during the action of the stimulus (Fig. 3, Exp. 89). Henceforth, this reflex always appeared to the sound of bubbling, although at first its latent period varied widely from 1 — 9 sec (just as in the case of the reflex to light), gradually stabilizing below 5 sec.

The conditioned cessation reflex did not in this dog change noticeably the secretion of saliva. The curves of the unconditioned secretion of saliva and secretory after effect during and immediately after the action of the bubbling stimulus run parallel to similar curves from other trials, where this stimulus was not applied.

B u r e k a mongrel, weighing about 12 kgs. When we started the elaboration of the conditioned cessation reflex, alimentary conditioned reflexes had already been established to the following stimuli: flashing light (60 Watts, frequency every second), noiseless propeller, rhythmic buzzer (interruption every second), metronomes with frequency 120 and 200 beats per minute (M_{120} and M_{200}) — positive reflexes, continuous lamp light (25 Watts) — differentiation.

The conditioned cessation reflex was elaborated to M_{200}. The act of eating lasted about 40 sec, the metronome was applied from the 15th — 25th sec after the start of eating. The series began

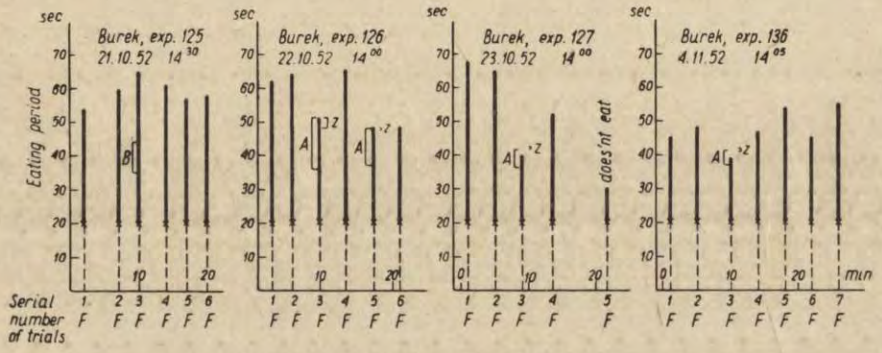

Fig. 4. The course of elaboration of the conditioned cessation reflex to metronome (dog "Burek")

The columns show the duration of the acts of eating. On the abscissae is shown time in minutes; on ordinates — time in seconds. Dotted lines — isolated action of conditioned stimulus, F — rhythmic flashes of light (60 W — 60 per min.), A metronome, 200 beats per minute, B — metronome, 120 beats per minute, z — withdrawal of food, x — presentation of food

with the application during the process of eating of M_{120} without the withdrawal of food. While M_{120} acted, the dog ate quietly without any interruption (Fig. 4, Exp. 125). Also the first trial with M_{200} passed without visible effect — the animal was eating quietly up to the moment of shutting the bowl lid (Fig. 4, Exp. 126). The conditioned effect of M_{200} appeared for the first time in the second

experiment of that series (third trial of M_{200} reinforced by withdrawal of food), the dog stopped eating and turned away from the food bowl. The latent period of this reflex was 3 sec (Fig. 4, Exp. 127). After this trial the interruption of eating appeared persistently, immaterial whether a metronome was applied, or not. In addition, the dog left untouched the last portions of food given during the experiment (he would not touch the 6th or even sometimes the 5th portion, whereas normally he could eat 7 without

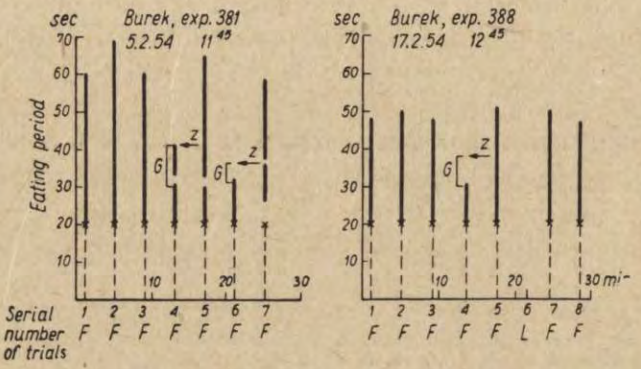

Fig. 5. The course of elaboration of the conditioned cessation reflex to the buzzing of a loud-speaker (dog "Burek")

The columns show the duration of the acts of eating. On the abscissae is shown time in minutes; on ordinates — time in seconds. Dotted line — isolated action of conditioned stimulus. F — rhythmic flashes of light (60 W — 60 per min.), G — buzzing of the loud-speaker, z — withdrawal of food, x — presentation of food

being fully satisfied). When the experiment finished, however, the animal would eat his normal daily ration of food. Because of this, during the next 9 experiments no stimuli of any kind were applied while the animal was eating, until the symptoms just described had disappeared. M_{200} applied after this period caused the cessation of the act of eating (Fig. 4, Exp. 136). Then the series of differentiating of the conditioned cessation reflex to M_{120} was performed (Zbrożyna A. 1958).

In one of the next series the conditioned cessation reflex to the buzzing of a loud-speaker was applied from the 10th — 20th sec of the act of eating. The reflex appeared in the very first experiment

of this series. In this first trial, the loud-speaker caused the immediate (0.5 sec) cessation of eating but the animal resumed eating when the stimulus had been maintained for 3 sec. However in the next trial the loud-speaker caused a full reflex: the dog drew back from the bowl and did not return (Fig. 5, Exp. 381). The latent period of this reaction was 1.5 sec. The food was withdrawn when the loud-speaker was in action for 10 sec. Since then the conditioned cessation reflex is permanent, regular, with the latent period about 1 sec (Fig. 5, Exp. 388). Interruption of eating without any stimulus appeared regularly in this dog: in both series, once or twice during every experiment the animal interrupted eating for about 1—2 sec, without any apparent reason, usually in the 10th — 15th sec of the act of eating i.e. at the time when the signals for withdrawal of food were usually applied.

The course of secretion of saliva during and immediately after the action of the conditioned stimulus of the cessation reflex is shown in Fig. 6. The curves of secretion from experiments performed before the establishment of the conditioned cessation reflex are compared there with those established after. The end of the act of eating is taken as the common point of these curves. Both groups of curves run parallel.

Nestor — a mongrel dog, weighing about 15 kgs. During the first stage a positive alimentary conditioned reflex was elaborated to flashing lamp light (60 Watts), to metronome (M_{120} and M_{200}), to a rhythmic buzzer and differentiation to continuous lamp light (25 Watts).

The conditioned cessation reflex was elaborated to M_{200}. Nestor normally ate for about 40 sec. Metronome was applied from 15th — 20th sec of the act of eating followed by the withdrawal of food. At first the dog did not react to the stimulus and ate quietly until the food was withdrawn. Only in the 6th experiment of that series did the animal stop eating during the action of the metronome (7 trials of metronome, reinforced by the withdrawal of food). From then on the conditioned cessation reflex appeared regularly, with a latent period of about 1 sec.

The curves of secretion of saliva during the action of the metronome in experiments before and after the elaboration of the conditioned cessation reflex are shown in Fig. 7. The curve of

experiment No 140, when the cessation reflex was already well established, runs a similar course to curves from the period before this reflex was yet elaborated (128, 129, 130). On the other hand

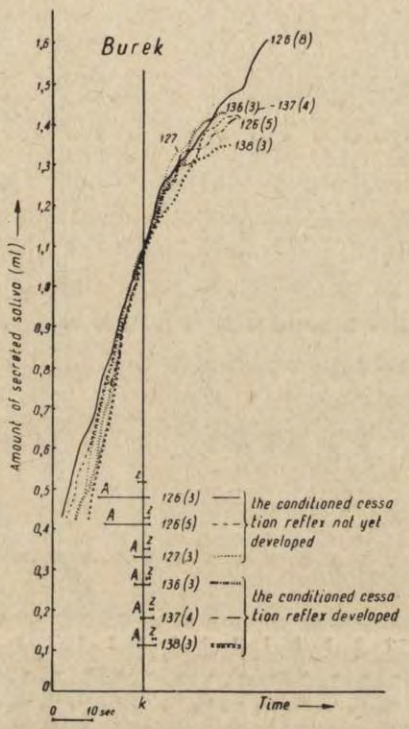

Fig. 6. Comparison of the salivary secretion curves in "Burek"

Series of elaboration of the conditioned cessation reflex to a metronome of 200 beats per minute (A). The numbers on the right of the curves and symbols of stimuli denote the serial number of experiment to which the curves refer; figures in brackets denote the serial number of trial in the given experiment. The curves start in the middle of the meal, underneath the period of action of the stimulus (A) and the moment of the withdrawal of food (z) are shown; k — the end of the act of eating (either as a result of withdrawal of food, or as a result of the action of the conditioned stimulus)

the curves 136 and 137, after the elaboration of this reflex, are sharply bent while metronome is in action.

K r u c z e k — a mongrel, weighing about 9 kgs. In the first stage a positive alimentary conditioned reflex to M_{120} and differentiation to M_{200} were elaborated.

The conditioned cessation reflex was elaborated to continuous light of a 200 W lamp. Kruczek normally ate for about 50—60 sec, the lamp was applied between 30th — 40th sec of the act of eating at the end of which time the food was withdrawn.

The first signs of reaction to the lamp appeared after 7 experiments (24 lamp-trials reinforced by food withdrawal). This reaction at first consisted in the dog trying to gulp the food quicker and more violently when the light was switched on. After 3 more experiments (comprising 10 lamp-trials, reinforced by the withdrawal of food) cessation of eating appeared when the lamp was lit. However this reflex disappeared after four further trials, in spite of the regular reinforcement of withdrawal of food, and since then, the dog stopped eating only when the bowl was being closed.

In the next series the conditioned cessation reflex was elaborated to a buzzer, aplied from 20—30 sec of the act eating. On the 10th sec of the action of the stimulus the food was withdrawn. During the first buzzer trial a very vivid orientation reaction appeared during the act of eating. In the next trial this reaction did not appear; the animal ate normally during the action of the stimulus, until the food was withdrawn. The conditioned cessation reflex appeared in the next experiment of this series, after two buzzer trials reinforced by the withdrawal of food. From then on this reflex became stabilized and very strong: when the buzzer started, the animal would stop eating and withdraw from the food-tray as far as the leash would allow it, leaving the rest of the food in the bowl. Also the lamp, which was applied from time to time at this period, produced regularly the conditioned cessation reflex.

In the next series, the conditioned cessation reflex was elaborated to a tactile stimulus (rhythmic stimulation of the back). The reflex to this stimulus was formed after only one application and it was also very strong, although it sometimes happened that the dog resumed eating after a few seconds, while the stimulus was still in action.

C a r b o, mongrel, weight about 20 kgs. In the introductory series a positive alimentary reflex was elaborated to flashing light of a 60 Watt lamp, then the differentiation to continuous light

of 25 Watts. After this differentiation was established, positive conditioned alimentary reflexes to the metronomes M_{120} and M_{200} were elaborated.

This dog differed from the other dogs in his way of eating. He gulped food in big mouthfuls often lifting his mouth from

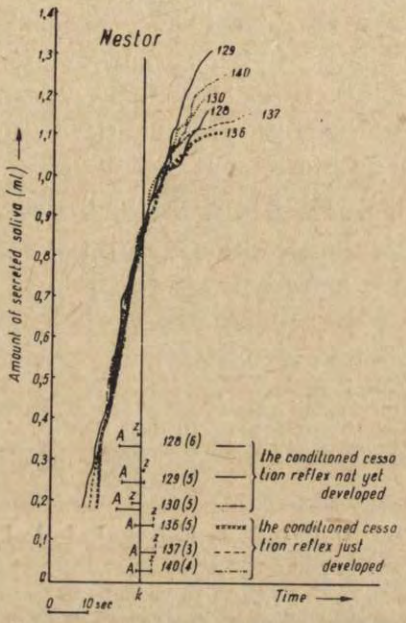

Fig. 7. Comparison of the salivary secretion curves in "Nestor"

Series of elaboration of the conditioned cessation reflex to a metronome of 200 beats per minute (A). The numbers on the right of the curves and symbols of stimuli denote the serial number of experiment to which the curves refer; figures in brackets denote the serial number of trial in the given experiment. The curves start in the middle of the meal, underneath the period of action of the stimulus (A) and the moment of the withdrawal of food (z) are shown; k — the end of the act of eating (either as a result of withdrawal of food, or as a result of the action of the conditioned stimulus)

the bowl. As a result his act of eating was not continuous, as with the other dogs, but was rather jerky, with interruptions of $^1/_2$—1 sec duration.

When the action of the stimuli during the act of eating was tested it appeared that continuous lamp-light did not produce any visible reaction, the auditory stimuli on the other hand (metronomes, loud-speaker) evoked a persistent orientation reaction, causing sometimes rather long interruptions in eating. At first, as the trials were repeated, this reaction increased in strength, so that in one of the trials the buzzer caused even a complete cessation of eating. Withdrawal of food was not applied at this period. A systematic extinction of this orientation reaction was carried out to one of the auditory stimuli (loud-speaker). This stimulus was applied for 5—10 sec beginning from the 10th second of the act of eating, the reflex became extinct after the stimulus had been applied 20 times in 5 successive experiments. When the orientation reflex was extinguished elaboration of the conditioned cessation reflex to M_{200} began. The reflex was elaborated after 3 metronome trials, reinforced by withdrawal of food. After then this reflex became stable: on the application of the metronome the dog lifted his head out of the bowl, and withdrew from the food-tray, but continued to look at the remaining food as if he wanted to resume eating; however this did not happen during the 18 trials that followed.

The curves of secretion of saliva before the conditioned cessation reflex was elaborated run parallel to those from the period after this reflex had been established. Curves showing a very abrupt change of course during the action of the stimulus appear both before the elaboration of the conditioned cessation reflex and after its elaboration. Also curves from both these periods appear showing no visible changes in course during the action of the stimulus signaling the withdrawal of food. The changes of rate of saliva secretion depend primarily on the amount of food remaining in the dog's mouth at the moment of cessation of eating.

Kiel, mongrel — wolf dog, weight about 20 kgs. In the introductory series the following reflexes were elaborated: to a flashing lamp (60 Watts), bell, buzzer, loud-speaker — positive conditioned alimentary instrumental reflexes (placing of the right foreleg on the food-tray); to continuous light (25 Watts) — differentiation. The conditioned cessation reflex was elaborated to the buzzing of a loud-speaker. Normally the act of eating lasted from 1—1½ minutes. The loud-speaker designed to evoke the conditioned cessation reflex was appiield from the 10th, 15th or 20th second of the

act of eating. The animal at first did not react to the loud-speaker and continued to eat quietly, stopping only when food was withdrawn. Often the animal would not lift his head when the lid was being closed over the food-tray, thus making it impossible for the food to be withdrawn. The conditioned cessation of eating to the buzzing of a loud-speaker appeared for the first time in the 5th experiment of that series, after 7 trials of this stimulus reinforced by the withdrawal of food. This however was not yet a full reflex — after a few seconds of the action of the loud-speaker the animal would resume eating, before the bowl was closed. The conditioned cessation reflex was at its best after a further 8 experiments (further 17 loud-speaker trials, reinforced by the withdrawal of food). After this the reflex showed a tendency to disappear: the action of the stimulus became gradually less and less effective.

As it is seen the conditioned cessation reflex has been established in all the animals used in our experiments. The reflex was elaborated to auditory, visual and tactile stimuli, during the action of which the food was withdrawn. This reflex consisted in the cessation of the act of eating during the action of the stimulus with the dog leaving the food uneaten, withdrawing or even completely turning away from the food-tray. The shutting of the bowl followed some seconds after the cessation of eating.

The elaboration of the conditioned cessation reflex took a different course on various dogs. In one it would appear after the first reinforcement of the stimulus by the withdrawal of food, in others however it was necessary to reinforce the stimulus several times before the reflex was established.

The elaboration of the conditioned cessation reflex to visual stimuli took a longer time and required a greater number of trials than the elaboration of this reflex to auditory stimuli. The elaboration of the reflex for the first time in an animal took longer than elaboration of this reflex to the subsequent stimuli.

In some dogs during the elaboration of the conditioned cessation reflex, cessation "without a stimulus" appeared. These animals would interrupt the process of eating, not only to the signal of withdrawal of food, but also without any apparent reason. Such interruptions appeared mainly at the moment when the signal of withdrawal of food was usually applied. This phenomenon usually disappeared after the reflex to the stimulus became established,

but in some animals it was maintained for quite some time. The stability of the described reflexes also varied in different dogs. In some of them the reflex was very stable and regular, in others unsteady, sometimes with a tendency to disappear completely. Generally the cessation reflex was most stable to auditory stimuli. A well established reflex was not subject to regression after a break of many months in the experiments.

The secretion of saliva gradually decreases when the animal stops eating, primarily because the amount of food in his mouth diminishes. We therefore tried to see if the signalling of withdrawal of food would influence in any appreciable way the secretion of saliva. In order to do this the secretory curves from before and after the elaboration of the conditioned cessation reflex were compared, with the end of the act of eating taken as the common point. As the conditioned cessation reflex became established and the latent period grew less, the time which elapsed between the start of the signal and the cessation of eating also got shorter. Comparison of the secretory curves showed that in the majority of cases the decrease in the rate of secretion after the end of the act of eating or after "unconditioned" cessation (to the withdrawal) was similar to the decrease after the conditioned cessation (to action of the signal). As a rule the signaling the withdrawal of food had no influence on the secretion. Although it should be noted that in one of the dogs described, we found a much more abrupt decrease in the rate of secretion after the signaled cessation of eating then either after the "unconditioned cessation", or after the normal end of the act of eating.

An interpretation of such curves is very difficult, mainly because of the difficulties in measuring the time and intensity of the action of the unconditioned stimulus (food) after the cessation of eating. It can however be said that although the signal of withdrawal of food can cause the cessation of the act of eating, it cannot influence in a visible way the course of the unconditioned salivary secretion.

DISCUSSION

As a result of the experiments just described a particular kind of conditioned reflex was obtained to stimulus applied during an unconditioned (alimentary) reflex, and preceding the cessation of it when it was at its height. The results however do not constitute

a sufficient base for asserting, that the development of such a conditioned reflex directly influences the course of the unconditioned secretion of saliva although undoubtedly it does so through the cessation of the act of eating.

Researches on the properties which may be acquired by a neutral stimulus applied during the unconditioned reflex were carried out by several authors of Pavlov's school. The first, who systematically investigated this problem was A. N. Krestovnikov (1921). The results of his experiments prove, that a neutral stimulus does not change its properties even when applied in a long series of experiments during the unconditioned alimentary or acid reflex.

Later researches of P. K. Anochin (1928), N. A. Podkopaev (1928), R. J. Rite (1928), D. J. Solovejčik (1928), E. M. Kreps (1933), K. J. Pavlova (1933), M. K. Petrova (1933), N. V. Vinogradov (1933) and Z. Nežданova (1940) seem to show that if the stimulus is applied only in a few trials during the unconditioned reflex, it acquires weak positive conditioned properties, whereas after more intensive training it becomes an internal inhibitor. This latter finding was explained on the basis of Pavlov's conception of cortical induction, the supposition being, that the inhibitory state induced by the excited center of unconditioned stimulus could be stabilized. In other words the neutral stimulus was as if "contaminated" by this inhibition.

The following procedures were used by these authors. In order to determine whether a neutral stimulus acquired positive conditioned properties a neutral stimulus was either applied several times during the act of eating and its effect were tested during the intertrial interval, or else after a series of overlappings a conditioned reflex was elaborated to this neutral stimulus by forward conditioning and the speed of this conditioning observed. To find out the possible inhibitory properties a neutral stimulus was applied repeatedly (up to several hundred times) during the act of eating; then a conditioned reflex was elaborated to this stimulus by forward conditioning and its magnitude and permanence was tested. In special trials the inhibitory after-effect of the hitherto overlapped stimulus was also tested. Finally series of experiments were carried out in which a positive conditioned stimulus was for

some time overlapped by the act of eating and afterwards its conditioned effect was tested.

A detailed analysis of these investigations however shows, that the conclusions drawn by the authors were not sufficiently founded. The results obtained are often contradictory. The experiments had been carried out before the improvements of technique were introduced by W. Kozak (1950), A. I. Makaryčev (1951) and by S. Sołtysik and A. W. Zbrożyna (1957). These authors have shown that serious errors result from Ganike or Ganike-Kupalov methods of estimation of saliva secretion and from fistulas performed by the method of Glinski. As the data concerning the problem under discussion show small variations of magnitude, it must be considered as falling within the limits of methodical error and no valid conclusions can be drawn from them.

An exact evaluation of the results obtained is made more difficult by the fact, that in these experiments the unconditioned stimulus (food, acid) was often applied without being preceded by the conditioned one. Such procedure and in some cases the lack of differentiations, produced rather copious secretion during the intertrial intervals as the result of the positive conditioning of the experimental environment. This secretion was especially increased during the action of extrastimuli (N. A. Krestovnikov 1921, N. V. Vinogradov 1933). Besides, there is often a lack of information about the level of salivation during the intervals and about the effect of extra stimuli. There is also lack of information about the behaviour of the dogs to stimuli applied during the act of eating. There is some evidence, which can be found in the cited papers (as well as the authors own observations) to show, that the animals, especially at the beginning of the training period, exhibit the orientation reaction to the stimulus applied during the act of eating: they interrupt the meal and return to it after the cessation of the stimulus. These conditions are sufficient to develop a positive conditioned reflex to this stimulus. All this seems to indicate that the conclusion drawn by some authors (E. M. Kreps 1933; V. I. Pavlova 1933, M. K. Petrova 1933, N. V. Vinogradov 1933), about the development of the positive conditioned reflex to a stimulus applied during the act of eating is rather unfounded. Where the papers on the acid reflex concerning this problem are concerned (N. A. Krestovnikov

1921, R. I. Rite 1928, Z. A. Nieżdanova 1940) the results obtained are contradictory. N. A. Krestovnikov (1921) applying the stimulus immediately after the introduction of acid did not observe any conditioning to this stimulus. Whereas Z. A. Nieżdanova (1940) succeded in elaborating in one dog a conditioned reflex by this procedure. On the other hand R. I. Rite (1928) obtained the extinction of the previously established acid conditioned reflex by the application of the conditioned stimulus just after the introduction of acid.

Equally unconvincing are the conclusions ascertaining the acquisition of inhibitory properties by the stimulus applied during the act of eating. These conclusions are based either on the inhibitory after-effect exercised by the previously overlapped neutral stimuli upon the positive conditioned reflexes, or on the resistance exhibited by such stimuli to the forward conditioning. In all these papers (N. A. Podkopaev 1928, V. I. Pavlova 1933, M. K. Petrova 1933) there is a lack of control tests and the experimental material is insufficient. In another kind of experiment the inhibitory influence of the application of a positive conditioned stimulus during the act of eating was examined (P. K. Anochin 1928, D. J. Solovejčik 1928, N. V. Vinogradov 1933, E. M. Kreps 1933, V. V. Stroganov 1940). Two of these authors (P. K. Anochin 1928 and V. V. Stroganov 1940) did not find any changes in the conditioned reflex to the stimulus applied during the meal. The cause of the decrease of conditioned reflex to the overlapped stimulus observed by the remaining authors is not quite clear. One of the factors which may have influenced it was the fact, that the examined stimulus was applied during the intervals without reinforcement. A certain role was probably also played by such factors as an unsuitable choice of stimuli (e.g. olfactory or thermic stimuli used by E .M. Kreps). Finally in these experiments was also lack of control tests. In addition, some authors (D. J. Solovejčik 1928, V. V. Stroganov 1940) obtained contradictory results. All these points do not permit these experimental results to be considered a base for the assumption that the application of conditioned stimulus during the act of eating really leads to the inhibition of the conditioned reflex.

Besides all these considerations it must be once more emphasized that the procedure of observing the effects acquired during

the act of eating in the intertrial intervals cannot in itself give the expected results, because as was shown (A. W. Zbrożyna 1952, 1953), the dog is unable to identify the same stimulus applied during the interval and during the act of eating.

It has been proved that when a suitable procedure is used, the conditioned reflex to the stimulus applied during the unconditioned reflex cannot only be evoked (P. K. Anochin and E. Streż 1934, V. Kasjanov 1949, B. I. Chodorov 1955) but also elaborated (J. Konorski, L. Lubińska and S. Miller 1936, O. L. Nemcova 1949). It has also been established that the application, during the act of eating, of a stimulus eliciting a strong orientation reaction, leads to the developement of a conditioned orientation reflex in which the act of eating itself becomes a conditioned stimulus (P. K. Anochin 1928, P. S. Kupalov and O. P. Jaroslavceva 1949). The animals at the very sight of food or after the first mouthful turn towards the source of the stimulus applied during the meal. A similar phenomenon is described in this paper: some dogs in the early period of elaboration of the conditioned cessation reflex showed this reflex "without any stimulus", as a rule at that moment of the meal, when usually the signal of withdrawal was applied. A reversal of the sequence of stimuli leads therefore to a similar reversal of conditioning. A. L. Bernstein (1934) observed, that the application of an acoustic stimulus after electric shock administered to the infraorbital skin surface produces the following interesting phenomenon: electric shock, besides its normal unconditioned wink, elicited also the wink characteristic for sound at the exact moment when the sound should have been applied.

An analysis of the researches concerning the problem of the so called "backward conditioning" of the defensive reflexes (J. Beritoff 1926, S. A. Switzer 1930, H. W. Wolfle 1930, 1932*, A. L. Bernstein 1934, H. Cason 1935, E. R. Hilgard and W. C. Biel 1937, J. M. Porter 1938, J. O. Narbutovič 1940, A. Spooner and W. N. Kellogg 1947, M. E. Fitz-

* E. R. Hilgard and D. G. Marquis (1940, p. 164) maintain that the reflexes obtained by this author should be considered as instrumental ones. This seems however to be the case only in the series with a sufficiently long isolated period. The matter requires a more detailed analysis.

water and M. N. Reisman 1952, G. Razran 1956) leaves also no doubt but that the conditioned reflex is developed only when the unconditioned stimulus is preceded by a neutral one, and only in this way does it become a conditioned stimulus.

Those papers which seem to prove the existence of the backward conditioning (J. Beritoff 1926, S. A. Switzer 1930, J. O. Narbutovič 1940) do not supply convincing experimental data in this matter. Beritoff (1926) observed conditioning in two pigeons with removed cerebral hemispheres. However in a previous series of experiments, carried out on the same animals, a conditioned defensive reflex to the same stimulus was elaborated by means of forward conditioning, and then extinguished. Besides the defensive reaction shown by these pigeons was, according to the author, very generalized and irregular. J. O. Narbutovič (1940) carried out experiments with backward conditioning on three dogs and got in one of them a transitory positive effect. S. A. Switzer (1930) is the only author who unequivocally observed backward conditioning and did not observe any pseudo-conditioning in the control group. However no other authors confirmed these results and the reflexes appearing sometimes in backward conditioning should be considered, in accordance with the results obtained by M. E. Fitzwater and M. N. Reisman (1952), as non-specific effects (pseudo-conditioning), which have nothing in common with conditioning proper (A. Spooner and W. N. Kellogg 1947). These phenomena are called by A. L. Bernstein (1934) and H. M. Wolfle (1932) "facilitation", "α - conditioning" by C. L. Hull (1934) and "sensitization" by E. R. Hilgard and W. C. Biel (1937).

The method of verbal learning used by many authors seems not to be suitable as a method for studying backward conditioning (G. Razran 1956). In view of the specific and complicated structure of speech it is impossible in these experiments or in experiments with nonsense sylables to verify the direction of the connections developed. The term "backward association" would be a much more suitable one (H. Cason 1924), although the very term "backward" is justifiably put in doubt (B. B. Murdock 1956).

Some authors (B. B. Murdock 1952, M. O. Nagaty 1951a, 1951b, M.A. Elšina, N. V. Zimkin and Z. E. Moreva 1955,

J. A. Barlow 1956) tried to find a solution to this problem using instrumental conditioned reflexes. The peculiar characteristics of these reflexes however were not aways taken into consideration. Clear information on that subject was obtained by J. A. Barlow (1956). This author used the instrumental reflex as indicator of the conditioned "quality" of the stimulus. The defensive conditioned reflex was established to a stimulus by means of forward conditioning in one group of rats, and in a second group the backward conditioning was used. Then in both groups the bar pressing method was applied in the following two situations: 1) the stimulus which was previously conditioned acted all the time except while the bar was being pressed, 2) this stimulus acted only when the bar was pressed. It appeared that the rats trained by forward conditioning and subjected to bar pressing method in the first situation pressed the bar more frequently, thus interrupting the action of the stimulus; in the second situation the opposite effect was obtained. On the other hand, the animals subjected to backward conditioning showed the following effect: in the first situation pressing of the bar followed by the interruption of the action of the stimulus appeared less frequent, in the second situation, however, the pressing of the bar connected with the action of the stimulus became more frequent. It can therefore be concluded that in "backward conditioning" the conditioned stimulus became the signal of the cessation of the defensive unconditioned stimulus (J. Wolpe 1954). In Barlow's experiments the instrumental movement was of the avoidance type, because it diminished the conditioned fear (L. J. Kamin 1956). This is especially interesting with regard to the backward conditioning problem because it could mean that a stimulus subjected to this procedure becomes the signal of the cessation of the unconditioned defensive stimulus.

The question arises to what type of reflex does the conditioned cessation of eating belong? On the one hand it could be considered as a classical conditioned reflex (conditioning by substitution) in view of the extraordinary similarity between the conditioned cessation reflex and the "unconditioned" one. It may be supposed that the withdrawal of food produces the excitation of the hypothalamic center inhibiting food intake (n. ventro-medial), just in the same way as the presentation of food excites its excitatory coun-

terpart (n. lateral). Consequently any stimulus which signals the withdrawal of food produces the conditioned inhibitory reflex, possessing the same effect as the unconditioned one. However, the curves of secretion of saliva presented in this paper do not substantiate this hypothesis; they show that the action of the conditioned stimulus signalling the withdrawal of food does not influence conspicuously the course of the secretion of saliva.

On the other hand, the reflex can be considered as belonging to the avoidance type. This reflex according to such interpretation would be based on the antagonistic connections between the alimentary and the defensive centres. By performing the conditioned movement of cessation of the act of eating the dog avoids the removal of food during the meal. The withdrawal of food as frustration may be connected with the increased excitation of the defensive centre. However none of these assumptions are based on what can be considered sufficient evidence.

SUMMARY

1. A conditioned reflex of cessation of the act of eating was established in seven dogs to a stimulus (visual, auditory or tactile) applied during the meal several seconds before the withdrawal of food.

2. The process of development of this conditioned cessation reflex is described and the curves of salivary secretion during the action of the stimulus signalling the withdrawal of food are compared.

3. The results of investigations concerning the problem of "backward conditioning" are discussed, and the probable mechanism of the conditioned cessation of eating are presented.

The author wishes to thank Prof. J. Konorski for his valuable advice and help with this work. I should like also to thank Mrs. A. Dunin for her cooperation in translating this paper.

REFERENCES

ANOCHIN P. K. 1928 — Vzajmodejstvie kletok uslovnogo i bezuslovnogo razdražitelej v tečenie primenenia posledniego. [The interaction of the cells centers of the conditioned and unconditioned stimuli during the action of the latter.] Trudy Fizjol. Lab. Pavlova. *2*, p. 95.

ANOCHIN P. & STREŻ E. 1934 — Izučenie dinamiki vyšej nervnoj dejatelnosti. Soobšč. 6. Charakteristika receptivnoj funkcji kory bolšich połu-

šarii v moment dejstvia bezuslovnogo razdražitela. [A study of the dynamics of the higher nervous functions. 6. Characteristics of the receptive function of the cerebral cortex during the action of the unconditioned stimulus.] Fizjol. Žurn. SSSR. *17*, p. 1225.

BARLOW J. A. 1956 — Secondary motivation through classical conditioning: a reconsideration of the nature of backward conditioning. Psychol. Rev. *63*, p. 406.

BERITOFF J. 1926 — Über die individuell erworbene Tätigkeit des Zentralnervensystems bei Tauben. Pflüg. Arch. f.d. ges. Physiol. d. Mensch. u. Tiere *213*, p. 370.

BERNSTEIN A. L. 1934 — Temporal factors in the formation of conditioned eyelid reaction in human subjects. Journ. Gener. Psychol. *10*, p. 173.

CASON, HUSLEY. 1924 — The concept of backward association. Amer. Journ. of Psychol. *35*, p. 217.

CASON, HUSLEY. 1935 — Backward conditioned eyelid reactions. Journ Exper. Psychol. *18*, p. 599.

CHODOROV B. I. 1955 — Dejstvie uslovnogo razdražitela pri primenenii jego na fonie bezuslovnogo refleksa. [Action of the conditioned stimulus applied against the background of the unconditioned stimulus.] Žurn. Vyšš. Nervn. Dejat. *5*, p. 61.

ELŠINA M. A. ZIMKIN N. V. and MOREVA Z. E. 1955 — Obrazovanie dvigatielnych oboronitelnych uslovnych refleksov u myšej pri predšestvovanii bezuslovnogo razdražitela uslovnomu. [The establishment of the motor defensive conditioned reflexes in mice by the application of the unconditioned stimulus before the conditioned one.] Žurn. Vyšš. Nervn. Dejat. *5*, p. 881.

FITZWATER M. E. and REISMAN M. N. 1952 — Comparisons of forward, simultaneous, backward and pseudoconditioning. Journ. Exp. Psychol. *44*, p. 211.

HILGARD E. R. and BIEL W. C. 1937 — Reflex sensitization and conditioning of eyelid responses at intervals near simultaneity. Journ. Gen. Psychol. *16*, p. 223.

HILGARD E. R. and MARQUIS D. G. 1940 — Conditioning and learning. Appl. Cent. Croft. Inc. N. Y.

HULL, C. L. 1934 — Learning. II. The factor of the conditioned reflex. In a handbook of general experimental psychology. (Ed. by C. Murchison.) Worcester. Mass. Clark Univ. Press. p. 382—455. Op. cit. after Hilgard E. R. & Biel W. C. 1937.

HULL C. L. 1943 — Principles of behaviour. An introduction to behaviour theory. D. Appl. Cent. Co. Inc. N. Y. London.

KAMIN L. J. 1956 — The effects of termination of the Cs and avoidance of the Us on avoidance learning. J. Comp. Physiol. Psychol. *49*, p. 420.

KASJANOV V. M. 1949 — Tormozit li bezuslovnyj razdražitel korkovyje kletki, k kotorym adresujetsa uslovnyj razdražitel? [Does the unconditioned stimulus inhibit the cortical cells to which is the conditioned stimulus addressed?] Probl. Vyšš. Nervn. Dejat. Anochina. Moskva, p. 163.

KONORSKI J., LUBIŃSKA L. and MILLER S. 1936 — Wytwarzanie się odruchów warunkowych w zahamowanej indukcyjnie korze mózgowej.

Elaboration des reflexes conditionnels dans l'écorce cérébrale à l'état d'inhibition induite.) Acta Biol. Exp. *10*, p. 297.

KONORSKI J. 1948 — Conditioned reflexes and neuron organization. Cambridge. Univ. Press.

KOZAK W. 1950 — Method of graphic registration of salivary secretion. Acta Biol. Exp. *15*, p. 185.

KRESTOVNIKOV A. N. 1921 — Suščestviennoe uslovie pri obrazovanii uslovnych reflexov. [The essential conditions for the formation of the conditioned reflexes.] Izv. Petr. Naučn. In-ta Lesgafta. *3*, p. 199.

KREPS E. M. 1933 — K voprosu o vozmožnosti obrazovania uslovnogo reflexa pri predšestvovanii bezuslovnogo razdražitela indifferentnomu razdražitelu. [The possibility of forming a conditioned reflex when the unconditioned stimulus precedes the indifferent stimulus.] Trudy Fizjol. Lab. Pavlova, *5*, p. 5.

KUPALOV P. S. and JAROSLAVCEVA O. P. 1949 — Vozmožnost obrazovania uslovnoj orientirovočnoj reakcji na funkcjonalnoje sostojanie kory bolšich polušarii. [The possibility of forming a conditioned orientation reaction to the functional state of the cerberal cortex.] Trudy Fizjol. Lab. Pavlova *15*, p. 53.

MAKARYČEV A. I. 1951 — Opyt niekotorogo usoveršenstvovania metodyki slunoregistracji pri issledovanii uslovnych refleksov. [An attempt to improve the method of registration of saliva secretion used in conditioned reflex experiments.] Žurn. Vyšš. Nervn. Dejat. *1*, p. 446.

MURDOCK B. B. Jr. 1956 — "Backward" learning in paired associates. Journ. Exp. Psychol. *51* p. 213.

MURDOCK B. B. Jr. 1952 — Forward and backward conditioning of the eyelid with a coordinated voluntary response. Amer. Journ. Psychol. *64*, p. 94.

NAGATY M. O. 1951a — The effect of reinforcement on closely following S-R connections. I. The effect of a backward conditioning procedure on the exinction of conditioned avoidance. Journ. Exper. Psychol. *42*, p. 239.

NAGATY M. O. 1951b — The effect of reinforcement on closely following S-R connections. II. Effect of food reward immediately preceding performance of an instrumental conditioned response on extinction of that response. Journ. Exp. Psychol. *42*, p. 333.

NARBUTOVIČ I. O. 1940 — Vozmožnost obrazovania uslovnogo refleksa pri predšestvovanii bezuslovnogo oboronitelnogo razdražitela indifferentnomu agentu. [The possibility of forming a conditioned reflex when the unconditioned defense stimulus precedes the indifferent stimulus.] Trudy Fizjol. Lab. Pavlova. *9*, p. 371.

NEMCOVA O. L. 1949 — O sposobnosti kory bolšich polušarii k recepcji v moment dejstvia bezuslovnogo razdražitela. [The ability of cerebral cortex to reception during the action of the unconditioned stimulus.] Probl. Vyšš. Nervn. Dejat. Anochina. Moskva. p. 186.

NEŽDANOVA Z. A. 1940 — Obrazovanie uslovnogo refleksa pri predšestvovanii bezuslovnogo razdražitela indifferentnomu. [The formation of conditioned reflexes when the unconditioned stimulus precedes the indifferent stimulus.] Trudy Fizjol. Lab. Pavlova. *9*, p. 353.

PAVLOVA V. I. 1933 — Obrazuetsa li uslovnyj refleks pri predšestvovanii bezuslovnogo razdražitela indifferentnomu? [Can a conditioned reflex be formed when the unconditioned stimulus precedes the indifferent stimulus.] Trudy Fizjol. Lab. Pavlova. *5*, p. 21.

PAVLOV I. P. 1927 — Conditioned reflexes. Trans. by Anrep. Oxford Univ. Press. H. Milford. 1Ed.

PETROVA M. K. 1933 — Preobladanie tormoznogo dejstvia bezuslovnogo razdražitela pri predšestvovanii jego indifferentnomu razdražitelu. [The prevalence of the inhibitory action of the unconditioned stimulus when it precedes the indifferent stimulus.] Trudy Fizjol. Lab. Pavlova. *5*, p. 49.

PODKOPAEV N. A. 1928 — Materialy k voprosu: čto delaetsa s kletkami indifferentnogo i uslovnogo razdražitelej vo vremia dejstvia bezuslovnogo razdražitela? [What happens in the cell centres of the indifferent and the conditioned stimuli during the action of the unconditioned stimulus.] Trudy Fizjol. Lab. Pavlova *2*, p. 205.

PODKOPAEV N. A. 1952 — Metodika izučenia usłovnych refleksov. [Method of investigation of conditioned reflexes.] Izd. Akad. Med. Nauk. SSSR.

PORTER J. M. 1938 — Backward conditioning of the eyelid response. Journ. Exp. Psychol. *23*, p. 403.

RAZRAN G. 1956 — Backward conditioning. Psychol. Bull. *53*, p. 55.

RITE R. J. 1928 — Vljanie bezuslovnogo refleksa na uslovnyj refleks. [The effects of the unconditioned reflex upon the conditioned reflex.] Trudy Fizjol. Lab. Pavlova. *2*, p. 212.

SOLOVEJČIK D. J. 1928 — Sostojanie vozbudimosti korkovych kletok vo vremia dejstvia bezuslovnogo razdražitela. [The excitability of the cortical cells during the action of the unconditioned stimuli.] Trudy Fizjol. Lab. Pavlova. *2*, p. 220.

SOŁTYSIK S. & ZBROŻYNA A. 1957 — The chronic fistula of shortened Stensen's duct in dogs. Acta Biol. Exp. *17*, p. 339.

SPOONER A. & KELLOGG W. N. 1947 — The backward conditioning curve. Amer. Journ. Psychol. *60*, p. 321.

STROGANOV V. V. 1940 — Vlianie centra bezuslovnogo razdrażenia na uslovnyj. [The effects of the center of unconditioned stimulation upon that of conditioned stimulation.] Trudy Fizjol. Lab. Pavlova. *9*, p. 360.

SWITZER S. A. 1930 — Backward conditioning of the lid reflex. Journ. Exp Psychol. *13*, p. 76.

THORNDIKE E. L. — Animal Intelligence. 1911. Macmillan. N. Y.

VINOGRADOV N. V. 1933 — Vozniknovenie novych sviazej v zatormożennych učastkach kory gołovnogo mozga. [The emergence of new connections in the inhibitory regions of the cortex.] Trudy Fizjol. Lab. Pavlova. *5*, p. 33.

WOLFLE H. M. 1930 — Time factors in conditioning finger-withdrawal. Journ. Gen. Psychol. *4*, p. 372.

WOLFLE H. M. 1932 — Conditioning as a function of the interval between the conditioned and the original stimulus. Journ. Gen. Psychol. *7*, p. 80.

WOLPE J. 1954 — Reciprocal inhibition as the main basis of psychotherapeutic effects. A. M. A. Arch. of Neurol. & Psych. *72*, p. 205.

ZBROZYNA A. 1952 — A phenomenon of "non-identification" of a stimulus acting against various physiological backgrounds in dogs. Acta Physiol. Pol. *3*, p. 70. Summaries of papers on III Congress of Polish Physiological Society 14—16 December 1952.

ZBROŻYNA A. 1953 — A phenomenon of non-identification of a stimulus operating against different physiological backgrounds in dogs. Lodz. Societas Scientiarum Lodzensis, *26*.

ZBROŻYNA A. 1954 — Conditioned interruption of eating and its properties. Acta Physiol. Pol. *5*, p. 502. Summaries of papers on IV (IV) Congress of the Polish Physiological Scciety 16—18 December 1954.

ZBROŻYNA A. 1957 — The conditioned cessation of eating. Bull. de l'Acad. Polon. des Sc. CI. VI. Vol. V. No 7—8, p. 261. Serie des Sc. Biol.

ZBROŻYNA A. 1958 — On the conditioned reflex of the cessation of the act of eating II. Differentiation of the conditioned cessation reflex. Acta Biol. Exp. *18*, p. 163.

Acta Biologiae Experimentalis, Vol. XVIII, 1958

ON THE CONDITIONED REFLEX OF THE CESSATION OF THE ACT OF EATING. II. DIFFERENTIATION OF THE CONDITIONED CESSATION REFLEX

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(Received for publication 23 October 1957)

In the course of further studies on the conditioned reflex of the cessation of the act of eating, the differentiation of this reflex has been undertaken in five dogs. This was done in two ways Either the differentiation was introduced after establishment of the conditioned cessation reflex, or it was carried out simultaneously with the elaboration of this reflex. In the first case, after the elaboration of the conditiond reflex of the cessation of eating, a second stimulus similar to that used to signal the removal of food was introduced into the experimental situation. This second stimulus was used at an analogous period of the act of eating as the stimulus signalling withdrawal of food, but was not reinforced by the actual removal of food. In this case the differentiation consisted in the extinction of the generalised conditioned reflex of the cessation of eating which appeared in response to the newly introduced stimulus. In the second case both stimuli which were to be differentiated were introduced together from the beginning of the experiments. Both stimuli were applied at similar periods during the act of eating, one of them being reinforced by removal of food, and the other not. In this case the differentiation consisted in the development of a conditioned reflex of the cessation of eating to one of the stimuli applied. The differentiation was produced by the

first procedure in three dogs (Kacber, Burek, Nestor) and by the second procedure in two dogs (As, Czang). The method used has been described in a previous paper (A. W. Zbrożyna 1958).

RESULTS

Kacber. When differentiation had begun in this dog, a conditioned reflex of the cessation of eating to bubbling noise had already been established (A. W. Zbrożyna 1958). As a differential stimulus a metronome giving 200 beats a minute, M_{200}, was introduced. It was applied at the 40th to the 50th second of the act of eating, in later series at the 30th to the 40th second. This stimulus was applied several times in each experiment, without reinforcement by the removal of food. In the first stages of these experiments bubbling noise was not applied at all. In the first experiment the metronome was applied four times and evoked cessation of eating every time (Fig. 1, Exp. 200). In the second experiment in five trials of the metronome the animal interrupted eating only

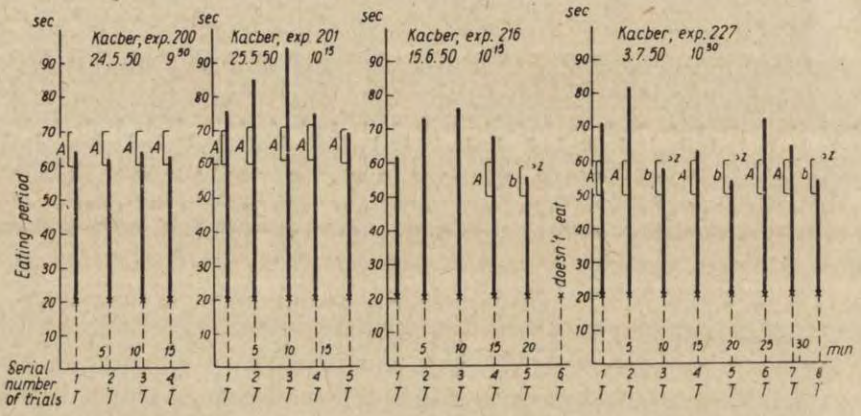

Fig. 1. The course of differentiation of the conditioned cessation reflex (dog "Kacber")

The columns show the duration of the acts of eating. Dotted lines — isolated action of conditioned stimulus. Abscissae — time in minutes; ordinates — time in seconds. T — buzzer, A — metronome, 200 beats per minute, b — sound of bubbling, x — presentation of food, z — withdrawal of food

in one trial, on the 9th second of the action of the stimulus (Fig. 2, Exp. 201). In the first ten experiments the dog ceased eating to the metronome only occasionally (once or twice in each session), after-

wards cessation reflex nearly disappeared. In this period the bubbling was reintroduced. The cessation reflex to this stimulus appeared to be regular and unaltered (Fig. 1, Exp. 216). On the other

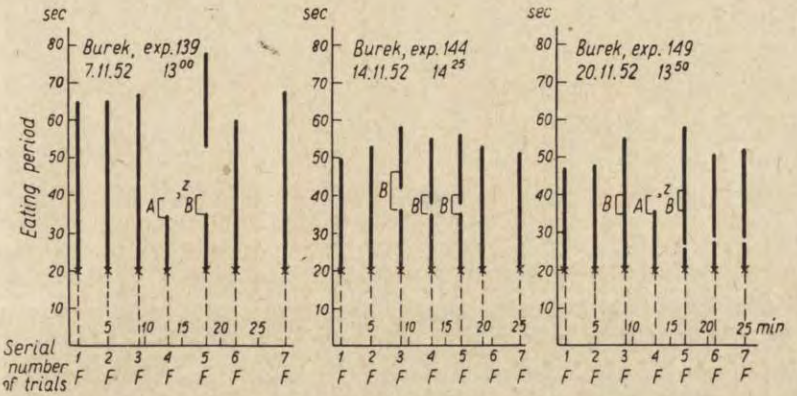

Fig. 2. The course of differentiation of the conditioned cessation reflex (dog "Burek")

The columns show the duration of the acts of eating. Dottes lines — isolated action of conditioned stimulus. Abscissae — time in minutes; ordinates — time in seconds. F — rhythmic flashes of light (60 W, 60 per minute), A — metronome, 200 beats per minute, B — metronome, 120 beats per minute, x — presentation of food, z — withdrawal of food

hand, the metronome remained without effect, the cessation reflex to this stimulus appearing only rarely (Fig. 1, Exp. 227). After an interval of three months this differentiation was maintained.

Burek. Differentiation in this dog had begun after elaboration of a cessation reflex to the action af a metronome (M_{200}), which was applied at the 15th to 20th second of the act of eating. The differential stimulus was a metronome giving 120 beats per minute, which was applied at a similar time during the act of eating, without reinforcement. The first trial with M_{120} produced a cessation of eating of over ten seconds (Fig. 2, Exp. 139). As the training continued, the interruptions of eating to M_{120} became shorter (Fig 2, Exp. 144) and appeared rather when the stimulus has ceased. Cessation of eating disappeared completely after 10 experiments of this series (which included 9 trials with M_{120}). The first such experiment is illustrated in Fig. 2, Exp. 149. At this stage the differentiation was not yet perfect: very short interruptions of eating in response to the M_{120} occurred from time to time, whereas the

M_{200} did not fail to evoke the cessation reflex. The interruptions of eating also occurred "without stimuli" at the moments when the metronomes were usually applied. This phenomenon gradually disappeared in the course of training. This series of experiments ended when the differentiation had been completely established.

The next series of experiments carried out in this dog consisted in the reinforcement of the M_{120} with a second portion of food. The M_{200} was accompanied by the removal of food as before. The M_{120} was applied, as in the previous series at the 15th to the 20th second of the act of eating and at the 20th second a new complete portion of food was given in place of the previously partially eaten portion. In the course of the first two experiments the response to both metronomes remained unchanged (8 trials of M_{120}). In the third experiment M_{120} began to elicit a cessation reflex accompanied by signs of expectation of the new portion of food. In the two following experiments this reaction of expectation began to appear also in response to M_{200}. Up to this moment this stimulus provoked turning away from the food-tray. After further ten experiments (8 applications of M_{200} and 20 of M_{120}) the reaction of turning away from the food-tray in response to M_{120} disappeared. To this latter stimulus the animal only lifted its muzzle slightly over the bowl in the last second before the new portion was placed before it. On the other hand in response to the M_{200} an ambivalent reaction appeared: the animal withdrew from the food-tray, sometimes turned its head away, after a few second came back to the food, looked at it and again withdrew, repeating this several times until the bowl was closed. This ambivalent reaction to the M_{200} disappeared after further three experiments (3 trials of M_{200} and 10 of M_{120}) and finally a definite cessation of eating reaction was established to this stimulus. The reaction to M_{120} remained unchanged. The ambivalent reaction to the M_{200} reappeared from time to time but eventually vanished completely.

In the next series of experiments with the same animal the cessation reflex to the humming of a loud-speaker was established (A. W. Zbrożyna 1958). After elaboration of this reflex differentiation to a buzzer was undertaken (Fig. 3, Exp. 386). In the course of forty experiments the buzzer was applied during eating 89 times and in each case it elicited the cessation of eating. In these experiments the loud-speaker was not used. At the beginning of this

series the interruptions of eating "without stimulus" also occurred (Fig. 3, Exp. 396), but they practically disappeared in the course of 10 experiments. On the other hand the extinction of the cessa-

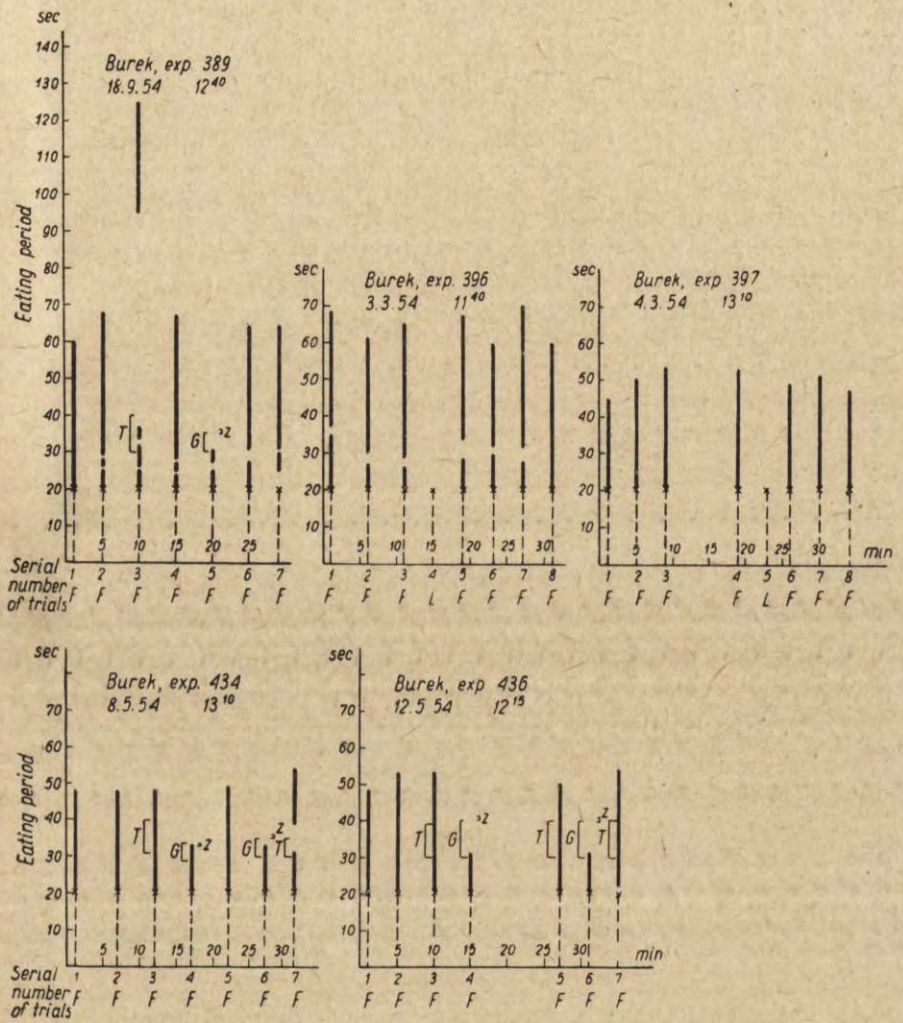

Fig. 3. The course of differentiation of the conditioned cessation reflex (dog "Burek")

The columns show the duration of the acts of eating. Dotted lines — isolated action of conditioned stimulus. Abscissae — time in minutes; ordinates — time in seconds. F — rhythmic flashes of light (60 W, 60 per minute), T — buzzer, G — buzzing of the loud-speaker, L — steady light (25 W), x — presentation of food, z — withdrawal of food

tion reaction to the buzzer took longer and was gradual: interruptions in eating which at the beginning lasted up to one minute (Fig. 3, Exp. 389), became much shorter and less frequent (Fig. 3, Exp. 434, 436). As soon as the cessation reaction to the buzzer was completely extinct the humming of the loud-speaker was reintroduced. At the first trial with this stimulus there was no interruption in eating: the animal ate until the moment the food was withdrawn (Fig. 3, Exp. 434). In the next trials however the cessation reflex to the loud-speaker reappeared. The introduction of the loud-speaker produced a transient disinhibition of the buzzer (Fig. 3, Exp. 434) but, after two experiments, the differentiation was completely restored (Fig. 3, Exp. 436). The differentiation was maintained for a whole year: in 200 trials with the buzzer very short interruptions occurred 33 times whereas the reaction to the loud-speaker was constant and regular throughout this period.

Nestor. Before the series of experiments with the conditioned cessation reflex, the positive conditioned reflex to a flashing light (60 W) and a differentiation to a continuous light was established. After the establishment of the cessation reflex to the metronome M_{200} (A. W. Zbrożyna 1958) the differentiation to the M_{120} was started. The first trial with M_{120} provoked a very marked cessation reflex: the animal ceased eating immediately and did not return to his food at all. In the following experiments however not only M_{120} but also M_{200} did not produce the cessation of eating. When the application of the M_{120} (not reinforced by removal of food) was discontinued the cessation reflex to M_{200} was restored almost immediately. After further fixation of this reflex (80 experiments, 57 applications of M_{200}) the M_{120} was introduced again. This caused serious disturbances in the beahviour of the animal: the dog refused to enter the experimental chamber, would not eat in the chamber and barked continuously during the experiment. The experiments were interrupted and the animal was given bromide (2 grams sodium bromide per os, each day). When the above symptoms disappeared the experiments were resumed without applying any stimuli during the act of eating. The introduction of M_{200} did not provoke at first any disturbances. After 34 experiments however these disturbances reappeared and the experiments were therefore discontinued.

As. In an introductory series of experiments positive conditioned reflexes to a flashing light, to M_{120} and to M_{200}, and differentiation to a continuous light were established. Then the two metronomes M_{120} and M_{200} were no longer applied before presentation of food and were given during the act of eating. The M_{200} was reinforced by withdrawal of food, M_{120} was not. The metronomes were usually applied 25 seconds after the presentation of food and they acted for 10 seconds. The first trials with the metronomes applied during eating produced no reaction, cessation of eating occured only to the shutting of the entrance to the bowl (Fig. 4, Exp. 155). In the third experiment (after 13 trials of M_{120}

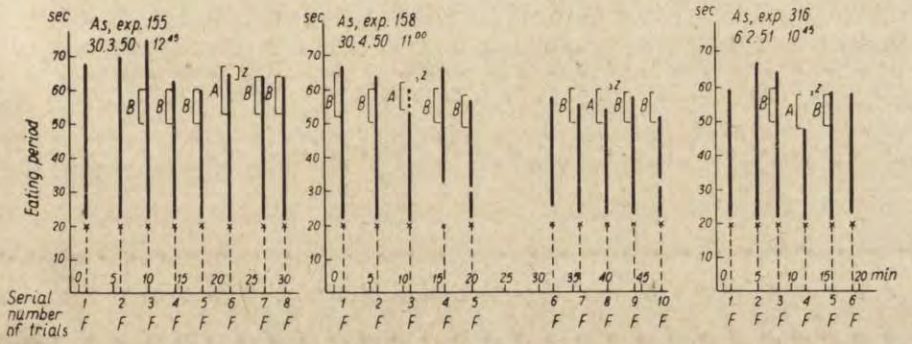

Fig. 4. The course of differentiation of the conditioned cessation reflex (dog "As")

The columns show the duration of the act of eating. Dotted lines — isolated action of conditioned stimulus. Abscissae — time in minutes; ordinates — time in seconds. F — rhythmic flashes of light (60 W, 60 per minute), A — metronome, 200 beats per minute, B — metronome, 120 beats per minute, x — presentation of food, z — withdrawal of food

and 5 of M_{200}) both metronomes began to evoke the cessation reflex (Fig. 4, Exp. 158). In the first phase of elaboration of this reflex its latent period was relatively long, 5 seconds or more, and the dog stopped eating for a few seconds, but returned to the food while the metronome was still in operation. Complete differentiation was achieved in the sixth and seventh experiments of this series; with M_{120} the animal ate without interruption whereas in response to M_{200} it stopped eating. This reaction became more and more striking and its latent period did not exceed 1—2 seconds. From time to time a cessation reflex appeared also to the M_{120}. This differentiation was maintained for some months (Fig. 4., Exp. 316) althoughs its difficulty for the animal was manifested by some

neurotic symptoms: the dog did not begin eating immediately after being given the food, left the last portions untouched, and finally refused to eat in the chamber altogether, so that the applicattion

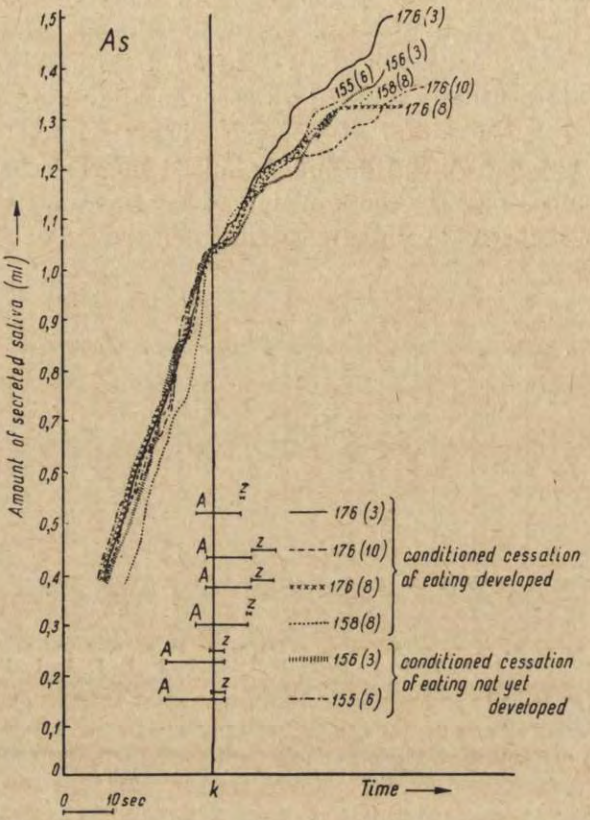

Fig. 5. Comparison of secretory curves in "As" Series of differentiation of the conditioned cessation reflex. A — metronome, 200 beats per minute, reinforced by the withdrawal of food (z). The numbers on the right of the curves and of the symbols of stimuli denote the serial number of experiments, figures in brackets denote the serial number of trials in the given experiment. The course of secretion from middle of the meal, up to the secretory after — effect is shown, underneath is shown the period of action of the stimulus (A), and the moment of withdrawal of food (z), k — the end of the act of eating (either as a result of withdrawal of food, or as a result of the action of the conditioned stimulus)

of stimuli during the act of eating had to be discontinued for some time. After a month, when the trials of metronomes during the act

of eating were reintroduced, the animal appeared in a normal condition.

In Figs. 5 and 6 the curves of the secretion of saliva in those trials in which the metronomes were applied during eating is shown. The M_{120} does not influence the course of salivary secretion during feeding, the reduction in secretion which occurs after

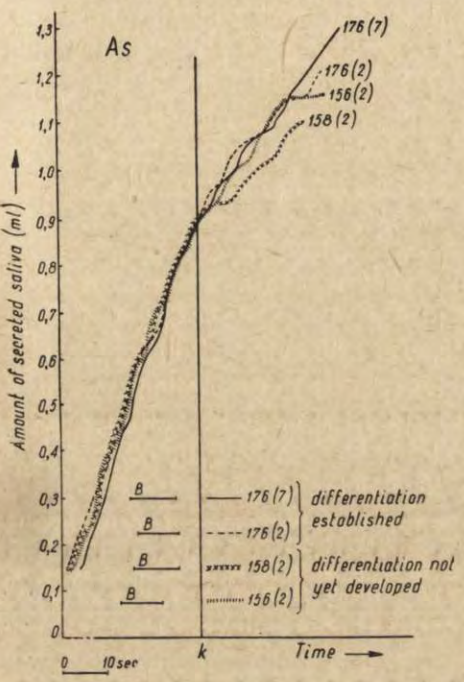

Fig. 6. Comparison of saliva secretion curves in "As"

Series of differentiation of the conditioned cessation reflex. B — metronome, 120 beats per minute, not reinforced by the withdrawal of food (z). The numbers on the right of the curves and of the symbols of stimuli denote the serial number of experiments figures in brackets denote the serial number of trial in the given experiment. The course of secretion from the middle of the meal up to the secretory after-effect is shown, underneath is shown the period of action of the stimulus (B), and the moment of withdrawal of food (z); k — the end of the act of eating

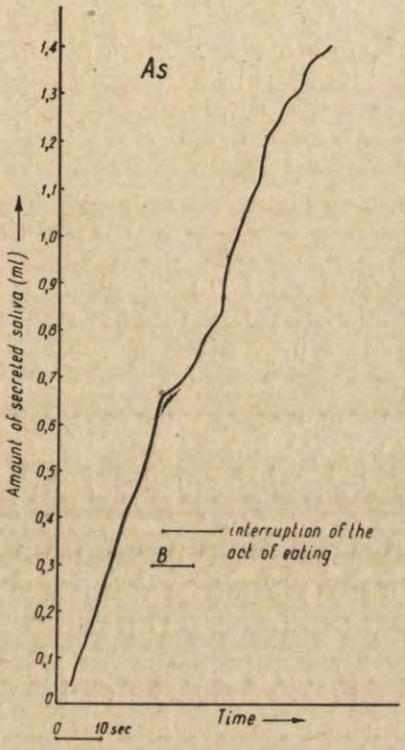

Fig. 7. The curve of saliva secretion in "As"

Interruption in the act of eating as a result of „disinhibition" to the metronome 120 per minute. Diminution of the speed of secretion appeared during the interruption. B — metronome 120 per minute

M_{200} is connected with the cessation of eating. Fig. 7 shows the curve of salivary secretion in a trial with M_{120}, which induced an interruption of eating, and here we observe a reduction in the rate of secretion.

Czang. With this dog the differentiation of the cessation reflex was carried out similarly as with As. From the very first experiment of this series two metronomes were used during eating: the M_{60} reinforced by withdrawal of food and the M_{200} without reinforcement. In the course of 45 experiments of this series (114 applications of M_{60} and 293 of M_{200}) the cessation reflex appeared randomly in response to both stimuli, and then gradually disappeared.

DISCUSSION

We see from the above results that the differentiation of the cessation reflex has been succesfully established in 3 out of the 5 dogs used in these experiments. This differentiation was easily established when the two stimuli were rather dissimilar (bubbling and metronome) whereas when they resembled each other (two frequencies of metronome) the training had to be much more prolonged. Differentiation after previous establishment of the cessation reflex turned out to be a simpler task than a simultaneous elaboration and differentiation of this reflex. Differentiation, i.e. introduction of a second stimulus which was not followed by withdrawal of food, was tolerated by the animals much worse than the elaboration of the cessation reflex, where only one stimulus was used always followed by the withdrawal of food. In ordinary conditioned reflexes in dogs the differentiation of such stimuli (e.g. metronomes of different rhythms) is not a difficult task and does not lead to neurotic states as was the case with one of the dogs described here. The reason for this is to be sought in the situation in which the differentiation was carried out. It seems that the act of eating itself introduces an additional difficulty in the process of differentiation, so that what is a relatively simple task in a normal situation often becomes unusually difficult during the act of eating. In any case from this point of view the dogs exhibited large differences in their behaviour: a differentiation which for one dog was simple led to serious nervous disorders in another.

In the differentiation described in the present experiments the differential stimulus is a "positive" one, in the sense that it permits the c o n t i n u a t i o n of the food intake. This "positivity" did not exist at first. Initially the stimulus interrupted the act of eating and it was only by careful training that the interruptions were suppresed. The supression was however not permanent. Sometimes the differentiation temporarily disappeared and the differential stimulus again interrupted the food intake. This indicates that the capacity to interrupt is potentially present in the stimulus. In this respect the relations are similar to those observed in ordinary differentiation.

When the differential stimulus does not interfere with the act of eating the course of salivary secretion also remains unaffected. In those experiments, however, in which the differential stimulus interrupts the intake of food the rate of secretion decreases (Fig. 7).

In one of the dogs a second differentiation was elaborated in which on the differential stimulus the animal expected an additional portion of food whereas on the signal of withdrawal of food it turned away from the bowl. It is noteworthy that the animal has been able to develop such complex differentiation during the act of eating.

The properties of the differential stimulus analysed here show that it differs from the ordinary differential stimuli in its positive character. This is due to new connections with the centre of food intake which are formed during the elaboration of differentiation in addition to the connections with the centre of inhibition of the act of eating. These new connections counterbalance the influence of connections with the inhibitory center so that the application of the stimulus no longer interrupts the act of eating. It seems necessary to assume therefore that such stimulus influences simultaneously both centres, the excitatory and the inhibitory.

SUMMARY

In order to analyse further the properties and organization of the conditioned reflex of cessation of eating, differentiation of this reflex was attempted in 5 dogs. In 3 of them a complete differentiation was obtained. The stimulus signalling the withdrawal of food evoked interruption of the act of eating and retirement from

the food-tray. The differential stimulus did not seem to influence the act of eating. In one of the dogs it was not possible to establish the conditioned reflex of cessation of eating and in another a severe neurosis developed during the differentiation.

The possible mechanism of this differentiation is discussed.

REFERENCES

ZBROŻYNA A. W. 1958 — On the conditioned cessation reflex of the act of eating. I. Establishment of the conditioned cessation reflex. Acta Biol. Exp. *18*, p. 137.

Acta Biologiae Experimentalis, Vol. XVIII, 1958

STUDIES ON THE EFFECTS OF THE CONDITIONED STIMULUS APPLIED AGAINST VARIOUS EXPERIMENTAL BACKGROUNDS

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(Received for publication 5 Mai 1957)

As is well known changes in the experimental environment in which a conditioned reflex has been elaborated disturb the effect of this reflex, and therefore a constant experimental background is needed to secure the regular responses to the conditioned stimulus. This was proved by Pavlov in the first years of his work on the higher nervous activity and confirmed by later investigators. Vatsuro (1948) found that changes of experimental setting cause a fall of conditioned reflexes in dogs and monkeys. Stroganov (1948) observed inhibition of the conditioned reflexes after transferring experiments from one experimental chamber to another. Beritov (1948) found that extraneous factors introduced into the usual situation might inhibit or completely change the normal conditioned motor reaction. Former studies of the author (Wyrwicka 1956) also have shown that an alimentary conditioned stimulus tested in another situation did not evoke the normal conditioned motor reaction connected with it in the first situation, but the reaction trained in this second situation.

On the other hand there exists some evidence showing that a conditioned stimulus may sometimes evoke the conditioned reaction connected with it in spite of a complete change of the experi-

mental situation. Lidell, James and Andersson (1934) found that a sheep in which a defensive conditioned reflex to the sound of a metronome had been elaborated, performed the conditioned movement also to the sound of a type-writer when the animal was accidentally in an office-room far from the laboratory. Beritov and al. (1935) established a defensive conditioned reflex to a tone reinforced by an electric shock in puppies in the usual experimental chamber. When the same tone was applied in a room quite different from the experimental chamber, puppies showed the general defensive reaction to it. A similar fact was observed in our laboratory; a dog in which an alimentary motor conditioned reflex had been established in a room, performed the same conditioned movement in the yard at the sight of the experimenter or the keeper if he had a bowl in his hands.

Some of the above facts prove that all changes of experimental background exert an influence upon the conditioned reaction to the given stimulus; other facts show, however, that a conditioned reaction established to a definite stimulus in a definite experimental circumstances may also appear to this stimulus outside the usual situation.

To obtain more facts concerning this problem the present study was undertaken.

MATERIAL AND EXPERIMENTAL PROCEDURE

Investigations were performed in 5 series of experiments on 14 male dogs, age 3—8 years.

In each series performed on at least 2 dogs, two situations different from each other were used as described below. At first an instrumental conditioned reflex (either alimentary or defensive) was elaborated and trained over a long period in each dog in one of the situations which was named by us "the usual situation". These reflexes were elaborated by the method of Konorski and Miller 1933 (see also Konorski 1948). The training experiments were performed daily; each of them consisted of 8—10 trials at intervals of 1—2 min: a conditioned stimulus was applied and then the instrumental reaction appeared immediately being reinforced by an unconditioned stimulus.

After the establishment of the conditioned reflex to the given stimulus in the usual situation the same stimulus was tested in another situation different from the usual one. This situation was named "the test situation".

The characteristics of the test situation in various experimental series was as follows:

1. In the first series the test situation was "an indifferent situation", i.e. a situation in which neither any unconditioned nor any conditioned stimulus was ever applied.

2. In the second series it was "a homogeneous situation I", i. e. a situation in which no instrumental conditioned reflex was elaborated but the same unconditioned stimulus as in the usual situation was applied.

3. In the third series it was "a homogeneous situation II", i.e. a situation in which an instrumental conditioned reflex, other than in the usual situation was elaborated but it was reinforced by the same unconditioned stimulus as in the usual situation.

4. In the fourth series it was "heterogeneous situation", i.e. a situation in which another instrumental conditioned reflex to another stimulus and reinforced by a different unconditioned stimulus than that in the usual situation, was elaborated.

5. In the fifth series it was "an alimentary homogeneous situation", i.e. a situation in which the same instrumental conditioned reaction as in the usual situation was trained but other conditioned stimuli were used; in this situation the inhibitory conditioned stimulus was tested.

RESULTS

Series I. The indifferent situation

A. Alimentary conditioned reflexes

The instrumental alimentary conditioned reflex of putting the right foreleg on the food-tray to the sound of a flute was elaborated in 5 dogs (No 1, 2, 7, 8, 9) in a usual experimental chamber (the usual situation). When it was firmly established we began experiments in the test situation; it was an empty room in which only a little table and a chair for the experimenter were placed. During several successive days, 1—2 hours after the daily experiment in the usual situation, each dog was brought for about 10 min to this situation where he was allowed to run freely; neither unconditioned nor conditioned stimuli were applied. After some days, when the orientation reflex to the new situation had disappeared, the sound of a rattle which was never used before and then the sound of a flute which had been used until now in the usual situation, were tested (protocol No 1).

As is seen from the protocol reactions of the dog to the indifferent stimulus and to the conditioned stimulus were quite different. The indifferent stimulus, the rattle, evoked only a slight orientation reflex while the conditioned stimulus, the flute, elici-

ted a general motor reaction, similar to the reaction of dogs outside the experimental room to the sight of the keeper who usually gave them food. As is known this reaction contains some elements of a general alimentary reaction. These elements were undoubtedly present in the reaction of the dog to the flute.

Dog No 1, 25.1.1957 Protocol No 1

Time	Stimulus	Reaction
	the dog runs freely around the room	
1 min 30 sec	Rattle for 15 sec	A slight orientation reflex to the place of the rattle, then the dog continues running
3 min	Flute for 15 sec	The dog comes to the flute, sniffs it, waves his tail, then he jumps putting his forelegs on knees of the experimenter

The reactions of other dogs were almost the same.

B. Defensive conditioned reflexes

An instrumental defensive conditioned reflex in the form of lifting the right hind leg to the rhythmic sound a whistle was elaborated in 2 dogs (No 3 and 4) in a usual experimental chamber. By lifting the leg the dog avoided an unconditioned reinforcement by an electric shock to the same leg. Normally the conditioned reaction of lifting the hind leg appeared immediately to the whistle and then the action of the whistle was interrupted and a shock was not applied.

When this conditioned reflex was firmly established the experiments in the test situation, analogous to those formerly described, were performed. First, the dogs were allowed to run freely in the second situation for about 10 min daily. Neither any conditioned nor unconditioned stimulus was applied. After some days a rattle (which was a quite new stimulus for these dogs) and then the whistle were tested (protocol No 2).

As we see from the protocol the rattle produced only a slight orientation reaction while the whistle elicited a general defensive reaction. The reaction of the second dog was very similar.

These results were confirmed afterwards on 2 other dogs (No 5 and 6) in which an instrumental defensive conditioned reflex of lifting the right hind leg to the flashing light of a lamp was elaborated; owing to this movement the dog avoided the electric shock applied to this left ear. When the same lamp was applied in the test situation the general defensive reaction appeared (see two first trials in the protocol No 5).

Dog No 4, 6.10.1956 Protocol No 2

Time	Stimulus	Reaction
	the dog moves freely	
5 min	Rattle for 15 sec	A slight orientation reflex in first seconds, then the dog continues walking around the room
7 min	Whistle for 15 sec	The dog almost immediately runs to the wall, hides his tail, then sits by the wall

Summarizing results described in A and B we may conclude that a conditioned stimulus, either alimentary or defensive, applied in an indifferent situation elicits a general alimentary or defensive reaction respectively, the specific instrumental movement however does not appear.

Series II. The homogeneous situation I

C. Alimentary conditioned reflexes

Experiments of this series were performed on 5 dogs used before in the series I, A, in which an alimentary instrumental conditioned reflex of putting the right foreleg on the food-tray to the sound of a flute was trained daily in a usual experimental chamber. The experiments performed in the test situation, ran as follows. The dog was moving freely around the room for about 10 min and received a piece of meat or bread every 20—30 sec. In experiments with dogs No 1 and 2 food was simply thrown on the floor, while dogs No 7, 8 and 9 received food in a bowl placed

on a beam lying along the wall of the room. After 2 days a new indifferent stimulus, a noise, and the conditioned stimulus, the flute, were applied in the test situation. The reaction of dogs of each group will be described separately.

Group I. Dog No 1, 30.1.1957 Protocol No 3

Time	Stimulus	Reaction
The dog moves freely, he receives pieces of food every 15—20 sec		
2 min	Noise for 15 sec	No distinct reaction
3 pieces of food are successively thrown on the floor		
3 min	Flute for 15 sec	The dog comes to the experimenter and performs the movement of begging

The dog No 2 showed similar reactions, however begging was not observed. Neither dog performed the conditioned movement of the right foreleg which was trained in the usual situation to the same flute.

Group II. Dog No 7, 9.10.1956 Protocol No 4

Time	Stimulus	Reaction
The dog is standing before the bowl, he receives pieces of food every 15—20 sec		
2 min	Noise for 15 sec	The dog looks at the experimenter
the dog receives successively 3 pieces of food		
3 min	Flute for 10 min	The dog puts his right foreleg on the beam besides the bowl after 2 sec

The reaction of putting the foreleg on the beam, i.e. the conditioned movement to the flute appeared in all 3 dogs of this group.

A comparison of protocols No 3 and 4 shows that the flute evoked only a general alimentary reaction if the food was thrown on the floor, and it evoked the specific instrumental movement if the food was given in the bowl standing on the beam, i.e. when

the test situation contained some elements similar to those of the usual situation.

D. Defensive conditioned reflexes

Experiments were performed on 4 dogs (No 3, 4, 5 and 6) which were used before in the series I, B.

1. In 2 of these dogs (No 5 and 6) the instrumental conditioned reaction of lifting the right hind leg to the flashing light of a lamp was trained in the experimental chamber (the usual situation). By lifting the hind leg the dog avoided an electric shock to the left ear. Then, 1—2 hours after the experiment in the usual situation, each dog was brought to the test situation where during

Dog No 5, 12.1.1957 Protocol No 5

Time	Stimulus	Reaction
1 min	Ratle for 15 sec	No distinct reaction
2 min 30 sec	Flashing light for 15 sec	Anxious movements, the dog shakes his head
4 min 10 sec	Electric shock on the ear, 2 times	General defensive reaction
4 min 35 sec	„ „ „	„ „ „
5 min 40 sec	Rattle 15 sec	No distinct reaction, the dog sits on the floor and looks at the door
6 min 30 sec	Electric shock	A slight lifting the right hind leg and general movements
7 min 15 sec	Flashing light, 15 sec	The dog touches his left ear with his paw and tries to remove the electrodes
8 min 30 sec	Flashing light, 15 sec	The dog crouches and runs behind the chair
A cuff is tied round the right hind leg of the dog		
9 min 50 sec	Flashing light, 15 sec	The dog lifts his right hind leg in the 3rd sec

about 10 min he was held in leash, 1.5 m in length, with electrodes attached to his left ear. After some days of habituation to the situation the test experiment was performed (prot. No 5).

As we see from the protocol, first a new indifferent stimulus (rattle) and the conditioned defensive stimulus (light) were tested, as described in series I, B. Then several electric shocks to the ear were given and both stimuli were again tested. As we see the reaction to the rattle was rather indefinite, similarly as in the first trial, while during the action of light the dog became restless and tried to remove the electrodes. Only when the cuff, used during the first period of the training in the usual situation, was tied round the dog's hind leg and the flashing light was again applied, the dog raised his hind leg, i.e. he performed the conditioned instrumental movement.

The reaction of the dog No 6 were similar, however the lifting of the right hind leg was never observed, even in spite of tieing the cuff round the leg.

2. The other 2 dogs (No 3 and 4) were trained to lift the right hind leg to the whistle thus avoiding an electric shock to the same leg. Then the dogs were brought to the test situation where they were held in leash and electrodes were attached to the right hind leg. After some days the electric stimulation and then the rattle (an indifferent stimulus) as well as the whistle were successively applied (protocol No 6).

Dog No 4, 12.10.1956 Protocol No 6

Time	Stimulus	Reaction
1 min	An electric shock to the right hind leg, 2 times	The dog whines, lifts his hind leg and looks at it
5 min	Rattle for 15 sec	The dog is sitting on the floor, in 10th sec. he lies quietly down
7 min	Whistle for 5 sec	The dog stands immediately up, he seems to be very anxious, after 3 sec he lifts his right hind leg energetically

As we see from the protocol the defensive conditioned stimulus, the whistle, evoked the instrumental movement of the hind leg when a shock had been applied to the same leg at the beginning of the experiment; the indifferent stimulus did not evoke such a reaction.

Experiments performed in the series II, C and D, have shown the following. If in the test situation an alimentary or defensive unconditioned stimulus, the same as in usual situation, has been applied and afterwards the respective conditioned stimulus is tested, then a distinct general alimentary or defensive reaction is evoked. The instrumental reaction however appears only when some factors facilitating this reaction are added (i.e. the bowl on the beam in alimentary conditioned reflexes, a cuff round the hind leg or an electric shock to this leg in defensive conditioned reflexes).

Series III. The homogeneous situation II

E. Alimentary conditioned reflexes

As these experiments were described in detail previously (W y r w i c k a 1955, 1956), we shall give here only their short summary. In 2 dogs the instrumental reaction of lifting the right hind leg (the movement r_1) to the metronome in the usual situation and the instrumental reaction of lifting the left foreleg in one dog and begging in another dog (the movement r_2) to the tactile stimulus in the test situation were trained. Both reflexes were reinforced by pieces of bread. When the metronome was applied in the test situation, the reaction r_2 and not the reaction r_1 (which had been trained to this stimulus in the usual situation) was elicited. The same was noted in the case of the tactile stimulus: when it was tested in the usual situation the reaction r_1 instead of the reaction r_2 was evoked. When in the following days these stimuli were used either in the usual or in the test situation they evoked always such reaction as was trained usually in the given situation, even when the experiments were performed successively on the same day.

F. Defensive conditioned reflexes

These experiments were analogous to those described in E. They were performed on 2 dogs (No 5 and 6) in which the reaction of lifting the hind leg (r_1) to the flashing light in the usual situation and the reaction of standing up on hind legs (r_2) to the sound of

a buzzer in the test situation were trained; both movements secured the animal against the electric shock applied to the left ear. When the flashing light was tested in the test situation the reaction r_2 instead of r_1 was elicited; the same was with the buzzer: when it was applied in the usual situation the reaction r_1 appeared instead of r_2 which had been trained to this stimulus in the test situtation.

The result obtained in experiments E and F prove that a given conditioned stimulus applied in the other situation, in which another instrumental reaction has been elaborated, evokes the reaction trained in this situation, i.e. the reaction connected with the experimental situation and not with the conditioned stimulus.

Series IV. The heterogeneous situation

3 dogs (No 10, 11 and 12) were trained alternately in 2 experimental situations. One of the situations named by us "the alimentary situation" was a normal experimental conditioned-reflex chamber and there the alimentary instrumental conditioned reflex of putting the right foreleg on the food-tray was established to the sound of a metronome in 2 dogs and to the sight of a moving black disk in the 3rd dog. Another situation, named by us "the defensive situation" was a room divided by means of a wooden partition into 2 parts, so that the dog was separated from the experimenter. During the experiment the dog was placed on a low stand and his movements were somewhat limited. Just in front of the dog's head a wooden wall, 1 m of height, was placed. In this situation an instrumental defensive conditioned reflex of lifting the right hind leg to the whistle was elaborated; owing to this movement the dog avoided the reinforcement in the form of an electric shock to the same hind leg.

Both types of experiments were performed alternately in short series lasting for several days. The cuffs needed for the registration of movements were always tied round all four legs in both situations.

When the conditioned reflexes were already well established, the defensive conditioned stimulus was tested in the alimentary situation and the alimentary conditioned stimulus — in the defensive situation.

G. The defensive conditioned stimulus applied against the alimentary background

In one of the experiments in the alimentary situation in which, as was mentioned, the dogs were putting the right foreleg on the food-tray to the sound of the metronome, the defensive stimulus (the whistle) was applied. In one dog (No 10) a low but distinct lifting of the right hind leg (i.e. the defensive movement) appeared and it was repeated after some seconds (see the kymographic record No 1); the dog whined and performed general restless move-

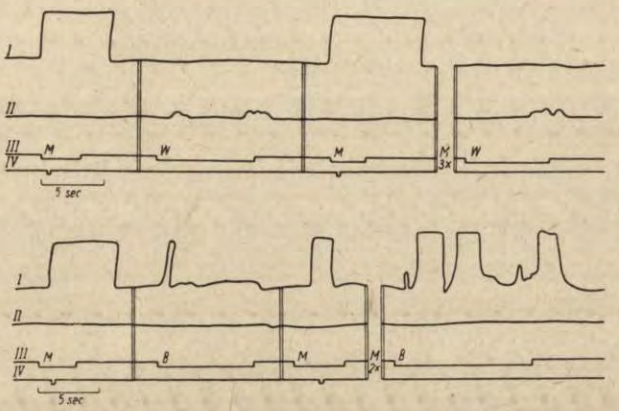

Fig. 1 and 2. Kymograms of experiments in which a defensive conditioned stimulus (fig. 1) and an indifferent stimulus (fig. 2) were tested in an alimentary situation

I — movements of the right foreleg; II — movements of the right hind leg, III — conditioned stimulus, IV — food reinforcement. M — metronome, an alimentary conditioned stimulus, W — whistle, a defensive conditioned stimulus, B — bell, an indifferent stimulus applied for the first time.

As we see whistle (W) evokes slight movements of the right hind leg, i. e. defensive conditioned movements (fig. 1), while bell (B) elicits movements of the right foreleg, i. e. alimentary movements

ments. In another dog (No 12) the whistle at first (in 1 sec) evoked a slight lifting of the right foreleg, but then shivering, bristling of hair and general fear reaction appeared, accompanied by a distinct lifting of the right hind leg. In the 3rd dog no definite reaction to the whistle was observed. The whistle was not reinforced by the electric shock. When the action of the whistle was

repeated, a distinct lifting of the hind leg appeared in all 3 dogs.

For the control new indifferent stimuli, the bubbling of water and the bell were tested in the alimentary situation. It was found that only a slight orientation reflex in the form of turning the head towards the stimulus appeared in one dog; two other dogs had put their right foreleg on the food-tray (i.e. they performed the

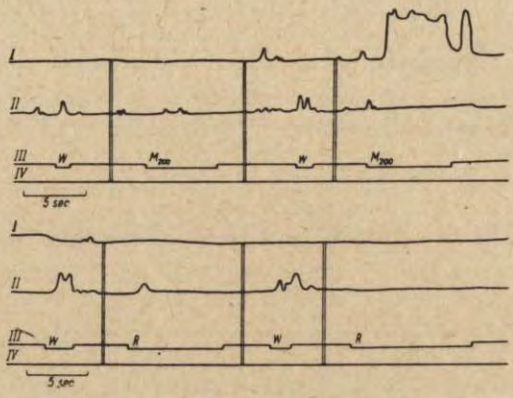

Fig. 3 and 4. Kymograms of experiments in which an alimentary conditioned stimulus (fig. 3) and an indifferent stimulus (fig. 4) were tested in a defensive situation

R — rattle, an indifferent stimulus applied for the first time; other explanations as for the fig. 1 and 2. As we see from fig. 3, an alimentary conditioned stimulus, metronome (M_{200}) evokes at first slight movements of the hind leg; a repetition of this stimulus however elicits lifting the right foreleg, i. e. an alimentary conditioned reaction. An indifferent stimulus, rattle (R), evokes small movement of the hind leg, i. e. a defensive reaction, only at its first application (fig. 4)

alimentary conditioned movement); this reaction was not reinforced by food. When the stimulus was applied again the reaction of putting the foreleg on the food-tray appeared in all 3 dogs. No defensive movements were observed (kymogram No 2).

H. The alimentary conditioned stimulus applied against defensive background

Similar experiment was performed in the stituation where the alimentary conditioned stimulus was tested. It was found that two dogs had not performed any movement to this stimulus, the third

one however made 3 slight movements of the right hind leg (i.e. the defensive movements). A repetition of the alimentary stimulus still did not produce any effect in first two dogs, but the third lifted slightly at first his hind leg and then his right foreleg and held it in such a position for some seconds (kymogram No 3); this movement was very similar to the movement trained in the alimentary situation.

When, for comparison, a new indifferent stimulus, the rattle, was tested in this situation, a small movement of the right hind leg. (i.e. the defensive movement) was evoked in all dogs already in the first trial (Fig. 4).

Summarizing the results of experiments G and H we may conclude that an alimentary or defensive conditioned stimulus applied in the second heterogeneous situation, i.e. defensive or alimentary situation respectively, evoked only a general conditioned reaction connected with the given stimulus or did not evoke any reaction at all; in few cases an adequate instrumental reaction was observed.

Series V. The application of an inhibitory conditioned stimulus in another homogeneous situation

I. Two alimentary situations were used

The first situation named by us "the situation I" was an usual conditioned-reflex chamber and another situation named by us "the situation II" was an office room situated in another building. In the situation II the dog standing on the floor was held in leash, about 1.5 m in length. The experimenter was sitting behind the table 1 m from the dog and gave stimuli and food from there.

2 dogs were used (No 13 and 14) in these experiments. In the situation I a positive alimentary instrumental conditioned reflex of lifting the right hind leg to the sound of the metronome and an inhibitory conditioned reflex to the rhythmic sound of a saw were established in both dogs. In the situation II the same alimentary instrumental reflex of lifting the hind leg to the sound of a rattle (named "rattle I") and an inhibitory reflex to the sound of another rattle (named "rattle II"), different from the first were elaborated in dogs. The training was performed alternately, several days in one situation and then several days in the other etc.

When these reflexes were well established the conditioned stimuli from the situation I were tested in the situation II and vice versa. The procedure was that at first the positive stimulus was introduced and only after 2 days the inhibitory stimulus was tested. When rattle II, i.e. the inhibitory stimulus from the situation II, had been applied during the experiment in the situation I it evoked a positive reaction in both dogs, however this reaction was rather weak and delayed. After a few repetitions of the stimulus the positive reaction disappeared. The same result was found when the inhibitory stimulus from the situation I, the saw, was tested in the situation II. For comparison the number of trial required to the chronic inhibition in the usual and in the test situation in both dogs is shown in the Table I.

Table I

Number of trials required for the chronic extinction of an alimentary conditioned reflex

No of the dog	Inhibitory conditioned stimulus	The usual situation	The test situation
		Number of trials required to the inhibition of the reflex	Number of trials required to the renewal of inhibition
3	Rattle II	120 (II)*	4 (I)*
	Saw	121 (I)	3 (II)
	Rattle II	103 (II)	3 (I)
4	Saw	82 (I)	4 (II)

* (I) — experiments were performed in the situation I; (II) — experiments were performed in the situation II.

As we see the number of trials required to inhibit the reaction is many times less in the test situation than in the usual situation.

To summarize, an inhibitory conditioned stimulus applied in another experimental situation, different from usual, evokes at first a transitory disinhibition, then the inhibitory reaction is restored.

The results obtained in all series of experiments may be summarized as follows.

It was found that the reaction evoked by a conditioned stimulus applied in other than the usual situation was changed. Namely, the conditioned stimulus applied:

1. In the indifferent situation — did not evoke a specific instrumental reaction but only a general reaction which contained some elements of alimentary or defensive conditioned reaction connected with this stimulus in the usual situation.

2. In the homogeneous situation in which no instrumental conditioned reflex was elaborated but the same unconditioned stimulus as in the usual situation had been given — evoked a general alimentary or defensive conditioned reaction and in some cases also instrumental conditioned movements decreased however and delayed.

3. In a homogeneous situation in which another instrumental reflex was previously trained — evoked this another instrumental reflex;

4. In the heterogeneous situation (i.e. an alimentary conditioned stimulus in a defensive situation and vice versa) in which another instrumental conditioned reflex was previously established — evoked a general conditioned reaction and in some cases also an instrumental reaction connected with it in the usual situation.

It was also found that an inhibitory conditioned stimulus (in relation to the alimentary reflex) kept its required inhibitory properties when it was applied in another homogeneous (alimentary) situation, different from the usual one.

The results obtained are presented in a Table II in which a cross shows the kind of conditioned reaction of the dog to a conditioned stimulus tested in various experimental situations.

DISCUSSION

As is seen from the Table II a conditioned stimulus applied in other situations elicited in all series the same general conditioned reaction as was established to it in the usual situation. This fact shows that in spite of the change of the experimental background the conditioned stimulus has the power to evoke its own conditioned reaction however incomplete. According to the scheme of the mechanism of the motor conditioned reaction (Fig. 5) put forward by us (W y r w i c k a 1952) we may conclude that the connections between the centres of the conditioned and the unconditioned stimulus (connection 1) remain active, independently of the situation in which the stimulus is applied.

In some cases the conditioned stimulus applied in the test situation evoked also the instrumental conditioned reaction. This occurred when: 1) the test situation possessed some components similar to those of the usual situation, e.g. the presence of the bowl on the beam (the series C, 2, prot. No 4); 2) the given instrumental movement was facilitated by tieing the cuff round the leg (series D, 1, prot. No 5) or by an electric stimulation of this leg (series D, 2, prot. No 6).

This shows that in order to evoke the instrumental reaction an increase of the excitation in its centre (R in the Fig. 5) was needed; this increase may be accomplished through the centre of conditioned

Table II

The kind of the test situation	The kind of the reaction			
	The general reaction connected with the given stimulus in the usual situation	The instrumental reaction (r_1) connected with the given stimulus in the usual situation	The instrumental reaction (r_2) connected with other stimuli in the test situation	The inhibitory reaction connected with the given stimulus in the usual situation
The indifferent situation	+			
The homogeneous situation in which only the unconditioned stimulus was applied	+	+*		
The homogeneous situation in which another conditioned reaction to another stimulus than in the usual situation was elaborated	+		+	
The heterogeneous situation	+	+*		
The homogeneous (alimentary) situation in the case of inhibitory stimulus	+			+

* The reaction was present only in some cases.

stimuli (CS), using the connection 3, or directly by the stimulation of the respective limb. The increase of the excitation of the centre of the unconditioned stimulus (UCS) by giving food or by electric shocks had no effect on the centre of the instrumental movement (R); in other words it was not possible to increase the excitation in the centre R through connection 2.

In some cases the instrumental conditioned reaction appeared also in the heterogeneous situation. This may be explained by the fact that our both heterogeneous situation had some common

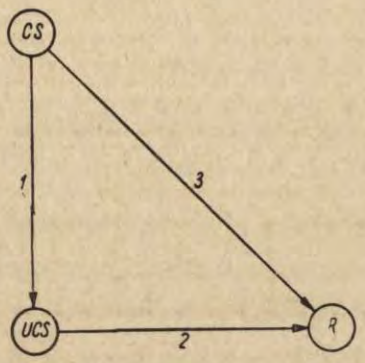

Fig. 5. The proposed scheme of the mechanism of the instrumental conditioned reaction

CS — the centre of the conditioned stimulus, UCS — the centre of the unconditioned stimulus, R — the centre of the instrumental conditioned reaction, 1, 2, 3 — connections between these centres acquired by training.

Centre CS sends impulses to the centre of the instrumental movement (R) as well as to the centre of the unconditioned stimulus (UCS) which in turn excites the centre R

elements, e.g. the separation of the dog from the experimenter during the experimental sessions, cuffs tied round his legs etc.

Now the appearance of another instrumental reaction in the homogeneous situation should be explained. A detailed interpretation of this fact in relation to alimentary conditioned reflexes was given previously (W y r w i c k a 1956). As in the present experiments a similar fact in relation to defensive conditioned reflexes was found, therefore the same interpretation may be applied to both kinds of reflexes. The explanation is in short as follows.

In the homogeneous situation II the brain centre of the same unconditioned stimulus as that in the usual situation and the centre of another instrumental movement (r_2) were excited subliminally owing to the conditioned connections existing between them and "the centre" of this situation. When the conditioned stimulus from the usual situation was applied not only the centre of movement r_1, connected with it by training, but also the centre of movement r_2 (through the centre of the unconditioned stimulus, by connections 1 and 2 in our scheme, Fig. 5) were excited. In con-

sequence the level of the excitation of the centre of movement r_2 (which had been already subliminal before the action of the conditioned stimulus) became higher than that of movement r_1 and this former was elicited, in spite of the fact that it was never connected with the conditioned stimulus from the usual situation.

An interpretation is required also with reference to the transitory disinhibition of the inhibitory conditioned stimulus when it was applied in the other alimentary situation (series V). There were not yet any conditioned connections between this stimulus and the new situation, therefore this might be the cause of the phenomenon (see the discussion of previous paper, Wyrwicka 1956). However the number of extinction trials required to inhibit anew the reaction in the test situation was much less than the number of trials required for the primary inhibition. It may be therefore concluded that this disinhibition was indeed the transitory one and the inhibitory conditioned stimulus had preserved its acquired properties in spite of the change of the situation.

Summarizing, we may draw the following conclusions. A conditioned stimulus tested in another experimental situation, different from the usual one, preserves its power to evoke a general conditioned reaction of the same sign; therefore it may be supposed that its connections with the unconditioned stimulus exist independently of connections with the situation. In conditions of our experiments an instrumental conditioned reaction, however, may be elicited in another situation only in the presence of some elements from the usual situation. It is, however, not evoked by mere increasing of the excitation of the unconditioned centre.

SUMMARY

Instrumental alimentary and defensive conditioned reflexes to various stimuli were established in dogs in an usual conditioned-reflex chamber. Then these stimuli were tested in various other experimental situations. It was found that each conditioned stimulus evoked a general conditioned reaction connected with it by training, but the instrumental conditioned reaction was elicited only when some elements from the usual situation were present in the test situation. The discussion of the phenomenon is given.

The author is grateful to Prof. J. Konorski for his interest in this work and valuable discussion. Thanks are due to Mgr C. Dobrzecka for helpful assistance in experiments of series I and II.

REFERENCES

BERITOV I. S. 1948 — Izmenčivost individualnych reflexov pod vlianiem vnešnich razdraženii. Obščaia fiziologia myšečnoi i nervnoi sistem. Chapter XI. Moscou-Leningrad.

BERITASHVILI J., CERETELI M. and AKHMETELI M. 1935 — O prisposoblenii molodych ščenkov k izmenenim obyčnoi obstanovki. Transactions of the Institute of Exp. Biology, State University of Tiflis.

KONORSKI J. 1948 — Conditioned reflexes and neuron organization. Cambridge.

KONORSKI J. and MILLER S. 1933 — Podstawy fizjologicznej teorii ruchów nabytych. Med. Dośw. i Społ. *16*.

LIDELL H. S., JAMES W. T. and ANDERSSON O. D. 1934 — The comparative physiology of the conditioned motor reflex. Comp. Psych. Monogr. Vol. *11*, no 1.

STROGANOV V. V. 1948 — Vlianie izmenenia obstanovki na vyssšuiu nervnuiu deiatelnost sobaki. Trudy fizj. labor. im. I. P. Pavlova. T. *XIII*.

VATSURO E. G. 1948 — Uslovno-reflektornaia ustanovka i vlianie ieie na tečenie uslovnych refleksov. Trudy fizj. labor. im. I. P. Pavlova. T. *XIII*, p. 21, 66, 95, 112.

WYRWICKA W. 1952 — On the mechanism of the motor conditioned reaction. Acta Biol. Exper. T. *XVI*, p. 131.

WYRWICKA W. 1956. — On the effect of experimental situation upon the course of motor conditioned reflexes. Acta Biol. Exper. T. *XVII*, p. 189.

Acta Biologiae Experimentalis, Vol. XVIII, 1958

INTERACTION OF MYOSIN SULFHYDRYL GROUPS AND PHOSPHORUS COMPOUNDS DURING CLEAVAGE OF ADENOSINE TRIPHOSPHATE

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(Received for publication 1 December 1956)

Little is known about the mechanism of interaction between myosin and adenosine triphosphate (ATP) which is followed by enzymic breakdown of ATP.

The presence of -SH groups in myosin is essential for its ATP-ase properties (Singer, Barron 1944; Bailey, Perry 1947; Weber, Portzehl 1954; Kielley, Bradley 1956). The blocking of myosin -SH groups causes complete inactivation of ATP-ase (Singer, Barron 1944). A partial blocking of myosin -SH groups produces a parallel decrease of enzymic ATP-ase activity and of its ability to form actomyosin (Bailey, Perry 1947) which suggest that the same centers take part in ATP-ase activity and in interaction with actin. Many authors show that the role played by -SH groups is extremely complex. Thus the presence of Ca ions myosin solutions, in which -SH groups are partially blocked by mercuric compounds, may bring about an increase of ATP-ase activity (Greville, Needham 1955; Kielley, Bradley 1956).

Some authors discovered in myosin and actomyosin the presence of phosphorus compounds (Bate-Smith 1938, Bailey 1942). Buchtal et al. (1949) established the existence of myosin bounds orthophosphates and nucleotides; the content of these compounds increased after addition of ATP. Certain authors sup-

posed that during enzymatic breakdown of ATP by myosin a phosphorylated protein may be formed (Weber 1955, Brahms 1956). Our present investigation is an attempt to elucidate some changes occurring in the myosin molecule in the course of enzymatic breakdown of ATP particularly the changes of the quantity of free -SH groups and of the amount of phosphorus compounds in myosin. A preliminary account of the work has already appeared (Brahms, Kąkol 1957).

MATERIALS AND METHODS

Preparation of myosin

Myosin was prepared from frog muscles (*Rana esculenta*) by the method of Portzehl and Weber (1950) with minor modifications. The following procedure was adopted. After keeping frogs for about one hour at 0°C, their spinal cords were destroyed and muscles prepared. The latter were placed for 20—30 minutes in Ringer's solution cooled to 0°C; they were then removed from Ringer's solution and refrigerated by means of liquid air and ground to a powder in a mortar. The ground muscles were mixed with Edsal-Weber solution cooled to 0°C (0.6 KCl, 0.01M K_2CO_3, 0.04M $KHCO_3$) using 4—6 ml of solution per one gram of fresh muscle. After 20—30 minutes, the suspension was centrifuged at 2000 revolutions per min at 0°C. The filtrate was diluted 14 × in bidistilled water (0°C) and kept at that temperature for 24 hours in order to precipitate myosin.

The myosin residue was either washed three times in 0.03 M KCl solution (crude myosin) or subjected to further purification by means of threefold sedimentation according to Perry's (1955) method (L-myosin). Before the experiment, the crude myosin and the purified L-myosin were dissolved by addition of KCl to a concentration of 0.5 M, and the protein content of the solution approximately determined by the biuret method according to Gornall et al. (1949). The exact protein content of the solution thus prepared was established by the determination of nitrogen according to Kjeldahl. The myosin solution was diluted to a definite protein concentration with glycine buffer at pH 7.

Determination of myosin-bound phosphorus compounds during enzymatic decomposition of ATP

For the investigation of the changes occurring in protein-bound phosphorus compounds during enzymatic reaction the principle adopted by Niemierko et all. (1957) was applied. In this work these phosphorus compounds which are found in protein residue had been accepted as protein-bound, protein being precipitated in the vicinity of its isoelectric point. These compounds pass into the solution when the protein residue is treated with 10 % trichloroacetic acid.

On the basis of this principle, the following procedure was adopted: 15 ml myosin solution (crude or L-myosin) was placed on large test tubes and ATP solution of known P_{in} and P_{15}* content was added. This was followed by incubation at 20°C for a determined period e.g. 5. 10. 20 and more minutes. In some experiments $CaCl_2$ or $MgCl_2$ solutions were added to the myosin solution prior to incubation. Final of $CaCl_2$ or $MgCl_2$ concentration was $5 \cdot 10^{-3}$M. The enzymatic reaction was interrupted by precipitating myosin with 15 ml acetate buffer at pH 4 and 0°C and subsequent cooling to 0°C.

After centrifugation and washing with buffer solution, the precipitated myosin was ground with 10% trichloroacetic acid (0°C) in order to liberate the associated phosphorus compounds. The content of labile phosphorus compounds was determined both in the filtrate after precipitating myosin with acetate buffer and in the extract obtained after the treatment of myosin residue with trichloroacetic acid. Phosphorus of labile phosphorus compounds (P15) was determined by the method of Fiske and Subbarow (1925) and ortophosphate according to Lohmann and Jendrasik (1926).

Quantitative determination of -SH groups

SH groups were determined by the method of amperometric titration according to Kolthoff, Stricks and Morren (1954); the enzymatic reaction and the determination of -SH groups was carried out in the same vessel. The procedure was as follows: 15 ml of the myosin solution under investigation (pH = 7; t = 20°C) were poured into the vessel, sodium salt ATP solution at pH = 7 was added from a burette connected with the vessel. While the myosin solution remained in the vessel (as well as during titration) purified hydrogen, obtained electrolytically was passed through the vessel. After a definite periode of time titration began with 10^{-3} M solution of mercuric acetate, the enzymatic reaction was thereby interrupted. According to Kolthoff and others, 1 ml of 10^{-3} M $Hg(CH_3COO)_2$ corresponds to 1 μM of -SH groups. This was corroborated in our experiments by a comparative determination of glutathione solution using the above mentioned amperometric method and the iodometric titration method according to Balachowski and Balachowski (1953). The quantitative data obtained concerning -SH groups were calculated per 1 gram of myosin.

RESULTS

The results of experiments with L-myosin solution are summarized in Table I. An increase of myosin-bound orthophosphate was noted as soon as the enzymatic cleavage of ATP began. In experiments where enzymatic ATP decomposition was carried to completion (as evidenced by the absence of further increase

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* P_{in} inorganic orthophosphate; P_{15} labile phosphorus of nucleotides, phosphate released after 15 min hydrolysis in 1 N H_2SO_4 at 100°C.

of free orthophosphate) a decline, was frequently observed after the initial increase of myosin-bound orthophosphate content. In

Table I

The content of myosin bound phosphorus compounds (P_{in} and P_{15}) during enzymatic decomposition of ATP

No myosin prepar.	Initial conc. of added ATP	Incubation period	Increase of free P_{in} in μg	Content of myosin bound		Increase of myosin-bound P_{in} in μM/1g myosin
				P_{in} μM/1g myosin	P_{15} μM/1g myosin	
LM-9	0	0		7.5	2.8	
	$3.8 \cdot 10^{-3}$ M	0′	0	11.6	9.8	4.1
	$3.8 \cdot 10^{-3}$ M	20′	11	15.3	21.5	7.8
	$3.8 \cdot 10^{-3}$ M	60′	24	13.7	15.5	6.2
	$3.8 \cdot 10^{-3}$ M	80′	54	12.2	15.7	4.7
LM-8	0	0		4.5		
	$3.8 \cdot 10^{-3}$ M	0′	0	4.5	12.9	0
	$3.8 \cdot 10^{-3}$ M	10′	17	6.7	17.6	2.2
	$3.8 \cdot 10^{-3}$ M	20′	24	8.9	10.8	4.4
	$3.8 \cdot 10^{-3}$ M	60′	64	14.5	15.6	10.0
LM-3	0	0		13.5		
	$4 \cdot 10^{-3}$ M	0′	0	13.5	41	0
	$4 \cdot 10^{-3}$ M	5′	20	40.5	68	27
	$4 \cdot 10^{-3}$ M	15′	20	40.5	68	27
	$4 \cdot 10^{-3}$ M	30′	65	37.1	69	24
LM-4	0	0		12.3		
	$4 \cdot 10^{-3}$ M	0′	0	12.7	23	0
	$4 \cdot 10^{-3}$ M	4′	40	22.6	32.6	9.9
	$4 \cdot 10^{-3}$ M	8′	65	28.3	36.2	15.6
	$4 \cdot 10^{-3}$ M	15′	85	27.3	—	14.6
	$4 \cdot 10^{-3}$ M	30′	95	15.4	18.1	2.7
	$4 \cdot 10^{-3}$ M	40′	95	14.0	11.9	1.3
LM-5	0	0		31.0		
	$4 \cdot 10^{-3}$ M	0′	0	34.2	17.7	3.2
	$4 \cdot 10^{-3}$ M	5′	7	35.4	—	4.4
	$4 \cdot 10^{-3}$ M	30′	30	39.2	16.4	7.2
LM-7	0	0	0	21	22	0
	$1.5 \cdot 10^{-3}$	0″	0	27	59	5
	$1.5 \cdot 10^{-3}$	40′	53	44.5	48	23.5

one series of experiments, after incubation with ATP in some samples of myosin solution, myosin was precipitated as usually, with acetate buffer at pH 4.6, whereas in some other samples myosin was precipitated by addition of an equal volume of ammonium sulphate solution of 90 per cent saturation in glycine buffer at pH 7. In both cases the increase of orthophosphate found in the precipitated myosin was very similar.

Table II

Changes in the content of myosin-bound orthophosphate during enzymatic decomposition of ATP (crude myosin t = 20°; pH = 7.0)

No myosin prepar.	Amount of myosin-bound orthophosphate in μM/1g myosin					
	Incubation period					
	0	25'	7'	10'	20'	40'
M—I	19.1	28.0	21.3	—	16.6	—
M—II	—	12.0	—	15.3	—	19.5
M—III	—	12.0	18.8	—	—	12.0
M—IV	12.0	13.0	—	14.1	16.1	—
M—V	—	16.1	—	24.2	21.3	17.4
M—VI	—	16.1	—	26.6	18.7	14.6
M—VII	—	10.9	—	13,9	14.6	14.5
M—VIII	—	11.1	—	13.1	15.3	18.6
M—IX	—	15.3	30.0	—	11.6	12.0
M—XIV	6.5	—	—	7.8	12.7	14.6
M—XVI	4.5	8.1	—	13.2	13.2	—
M—XVII	6.5	9.1	—	11.3	14.2	—

The data gained from experiments with "crude myosin" are presented in Table II. We can see that the character of the changes in the content of myosin-bound orthophosphate is similar to that observed in the case L-myosin (Table I).

Dealing with the data concerning both L-myosin and crude myosin it is to be noted, that each experiment was carried out with different myosin preparations derived from different frogs. Various preparations probably differed not only in ATP-ase activity but in other features as well. This might explain why the process of binding and subsequent liberation of orthophosphate, varied somewhat in different experiments.

The influence of Ca ions on the course of the enzymatic reaction is illustrated in Table III and. Fig. 1 which show results of one experiment. Along with the acceleration of enzymatic ATP decomposition by addition of Ca ions to a myosin solution, a more rapid increase is observed, as well as a decrease, in the content of protein-bound orthophosphate.

The presence of Mg ions, as shown in Table IV, does not accelerate the enzymatic reaction, as it does not bring about, a more rapid increase of free orthophosphate. It only seems to exert some influence on the increase in the content of myosin-bound orthophosphate at the beginning of the reaction.

As can be seen from Table I the precipitated myosin always contained different amounts of polyphosphate nucleosides (P_{15}).

The estimation of free -SH groups of crude myosin demonstrated that their content, during the course of enzymatic ATP de-

Table III

The influence of Ca ions on the content of myosin-bound phosphorus compounds during enzymatic decomposition of ATP (L-myosin, t=20°C pH = 7.0; $CaCl_2$ concentration in myosin solution = $5 \cdot 10^{-3}$M)

No of myosin prep.	Incub. period	Substances added to myosin solution	Increase of free P_{in} in μg	Increase of myosin-bound P_{in} in μM/1g myosin	Content of myosin bounds	
					P_{in} μM/1g myosin	P_{15} μM/1g myosin
LM-8	0	—	—		4.5	
	0	ATP	0	0	4.5	12.9
	10'	ATP	17	2.2	6.7	17.6
	20'	ATP	24	4.4	8.9	10.8
	60'	ATP	64	10.0	14.5	15.6
	0'	ATP + Ca^{++}	14	2.2	6.7	18.7
	10'	ATP + Ca^{++}	55	10.0	14.5	10.5
	20'	ATP + Ca^{++}	67	4.8	9.3	13.4
	60'	ATP + Ca^{++}	69	1.3	5.6	5.8
LM-6	0		0	0	15.2	
	30'	ATP	0	0	15.8	20.6
	15'	ATP + Ca^{++}	45	7.6	22.8	22.3
LM-9	0				7.5	
	20'	ATP	11	7.8	15.3	21.5
	20'	ATP + Ca	55	7.0	14.5	10.5

composition, initially decreases. After the minimum has been reached, this content increases, when the reaction is allowed to proceed (Table V).

Similar experiments were conducted with purified L-myosin. The results of the determinations indicate that the content of -SH groups in L-myosin from frog muscles fluctuated from 36 to 49μM per 1 gram of protein (Table VI). As in the case of crude myosin, the incubation of L-myosin with ATP causes a drop in the content of free -SH groups up to 33 per cent; but subsequently, when incubated for

Table IV

The influence of Mg ions on the content of myosin-bound phosphorus compounds during enzymatic decomposition of ATP (L-myosin, t = 20°C; pH = 7.0, $MgCl_2$ concentration in myosin solution $5 \cdot 10^{-3}$M)

No myosin prepar.	Incub. period	Substances added to myosin sol.	Amount of hydrolised P_{in} in μg	Increase of myosin-bound P_{in} during enzym.react. in μM/g protein	Content of myosin bounds	
					P_{in} in μM/g protein	P_{15} in μM/g protein
LM—8	0′	ATP	0	0	4.5	17.6
	20′	ATP	24	2.2	6.7	10.8
	0′	ATP+Mg^{++}	0	2.4	6.9	15.3
	20′	ATP+Mg^{++}	29	4.8	9.3	18.7
LM—9	0′	ATP	0	4.1	11.6	9.8
	20′	ATP	11	7.8	15.3	21.5
	60′	ATP	24	6.2	13.7	15.5
	20′	ATP+Mg^{++}	5	10.3	17.5	9.9
	60′	ATP+Mg^{++}	42	10.3	17.5	—
LM—3	15′	ATP	20	27	40.5	68
	30′	ATP	65	23.6	37.1	69
	15′	ATP+Mg^{++}	40	32.1	45.5	64
	30′	ATP+Mg^{++}	0	26.6	40.0	57
LM—5	5′	ATP	7	4.4	35.4	17.7
	30′	ATP	30	8.2	39.2	16.4
	5′	ATP+Mg^{++}	0	1.7	31.7	30.3
	30′	ATP+Mg^{++}	0	24.0	55.0	17.2

a longer period, the quantity of -SH groups increased in some experiments (Table VI).

Following the same procedure as in the experiments in which the influence of Ca and Mg ions upon the contents of phosphorus compounds in myosin during ATP decomposition was investigated, the influence of these ions upon the quantitative changes in free -SH groups of myosin was studied. It was shown that, during enzymatic breakdown of ATP, Ca ions accelerate both the reduction of the amount of myosin-free -SH groups and the subsequent increase of the content of these groups. These results were obtained with crude myosin as well as with L-myosin (Table VII).

Studies on the influence of Mg ions revealed that the decrease in the quantity of free -SH groups observed during enzymatic

Table V

Changes in the content of myosin -SH groups during enzymatic decomposition (crude myosin, t = 20°C; pH = 7.0)

No myosin prepar.	Initial concentration of added ATP	Incubation period	-SH groups content in μM -SH/1g myosin	Changes in -SH groups content	
				-SH in μM/1g myos.	in %
M — IX	$6 \cdot 10^{-3}$M	1′	44.7		
		10′	33.3	—11.4	—26.5
		20′	29.8	—14.9	—33.2
		40′	34.8	— 9.9	—22.1
M — X	$6 \cdot 10^{-3}$M	1′	38.7		
		10′	36.4	— 2.3	— 5.9
		20′	31.3	— 7.4	—19.1
		40′	37.2	— 1.5	— 3.9
M — XII	$6 \cdot 10^{-3}$M	1′	30.5		
		10′	25.4	— 5.1	—16.7
		40′	28.6	— 1.9	— 6.2
M — Va		0	31.0		
	$6 \cdot 10^{-3}$M	1′	31.0	0	0
		10′	27	— 3.0	9.7
		20′	29	— 2,0	6.5
		30′	30	— 1.0	3.3

reaction, occurs in the presence of Mg ions more rapidly than without them.

Table VI

Changes in the content of L-myosin -SH groups during enzymatic decomposition of ATP (pH = 7.0; t = 20°C)

No myosin prepar.	Initial concentrat. of added ATP	Incubat. period	Content SH groups in μM -SH/1g myosin	Changes in -SH groups content	
				-SH in μM/1g myosin	%
LM—1	$3 \cdot 8 \cdot 10^{-3}$M	0	46.7		
		2′	31.2	—15.5	—33
		40′	46.7		
LM—2	0	—	49.3		
	$3 \cdot 8 \cdot 10^{-3}$M	0	49.3		
		4′	44.4	— 4.9	—10
		15′	39.0	—10.3	—21
		30′	42.8	— 6.5	—13
		40′	44.4	— 4.9	—10
LM—7	0	—	49.3		
	$4 \cdot 10^{-3}$M	0	49.3		
		4′	49.3		
		10′	42.2	— 7.1	—14
		30′	34.5	—14.8	—30
LM—8	$3 \cdot 8 \cdot 10^{-3}$M	4′	45.0		
		20′	42.3	— 2.7	— 6
		60′	34.7	—10.3	—23
LM—9	$3 \cdot 8 \cdot 10^{-3}$M	0	48.8		
		20′	43.3	— 5.5	—11
		60′	35.2	—13.6	—28
		80′	36.8	—12.0	—25
LM—8[A]	0	—	43.5		
	$3 \cdot 8 \cdot 10^{-3}$M	0	43.5		
		60′	29.2	—14.3	—33
LM—3	0	—	36.6		
	$4 \cdot 10^{-3}$M	0(15″)	36.6		
		5′	26.8	— 9.8	—27
		30′	52.0		

All the experiments described here indicate that during the course of enzymatic ATP decomposition, there is initially an increase in orthophosphate content of myosin, followed by a decrease. At the same time the changes in the content of free -SH groups

Table VII

The influence of Ca^{++} and Mg^{++} on changes in the content of myosin -SH groups during enzymatic decomposition of ATP (t = 20; pH = 7.0)

No myosin preparate	Substances added to myosin sol.	Initial concentration of added ATP	Incubation period	-SH groups content in μM/1g myosin	Changes in -SH groups content	
					-SH in μM/1g myosin	%
Crude myosin XVI	—	0	—	32.8		
	ATP	$6 \cdot 10^{-3}$M	10′	35.5	— 2.7	— 7
	ATP		45′	30.0	— 8.2	—22
	ATP + Mg^{++}		10′	30.0	— 8.2	—22
	ATP + Mg^{++}		45′	30.0	— 8.2	—22
	ATP + Ca^{++}		10′	30.0	— 8.2	—22
	ATP + Ca^{++}		35′	31.5	— 6.7	—17
	ATP + Ca^{++}		45′	33.9	— 4.3	—11
Crude myosin XV	—	0	—	31.3		
	ATP	$6 \cdot 10^{-3}$M	2′	27.9	— 3.4	—11
	ATP		20′	24.1	— 7.2	—23
	ATP + Mg^{++}		2′	24.1	— 7.2	—23
	ATP + Ca^{++}		2	24.2	— 7.1	—23
	ATP + Ca^{++}		20′	29.0	— 2.3	— 7
L-myosin 8	ATP	$3.8 \cdot 10^{-3}$M	4′	45.0		
	ATP		20′	42,3	— 2.7	— 6
	ATP		60′	34.7	—10.3	—23
	ATP + Ca^{++}		1′	34.7	—10.3	—23
	ATP + Ca^{++}		60′	38.2	— 6.8	—15
	ATP + Mg^{++}		1′	41.6	— 3.4	— 8
L-myosin 9	ATP	$3.8 \cdot 10^{-3}$M	0	48.8		
	ATP		20′	43.3	— 5.5	—11
	ATP		60′	35.2	—12.6	—28
	ATP		80′	36.8	—12.0	—24
	ATP + Ca^{++}		0	40.7	— 8.1	—17
	ATP + Ca^{++}		10′	44.0	— 4.8	—10
	ATP + Mg^{++}		0	45.0	— 3.8	— 8

take place in the opposite direction. The question may be put whether there is a quantitative relation between the changes in the content of myosin -SH groups and the changes in the amount of myosin-bound orthophosphate. The data obtained summarized in Table VIII and Fig. 2 indicate that an increase in orthophosphate content of myosin corresponds to a decrease of free -SH groups, whereas the decline of orthophosphate content observed at a later stage is related to the subsequent liberation of -SH groups. It fol-

Table VIII

Changes in the content of myosin -SH groups and myosin-bound orthophosphate during enzymatic decomposition of ATP (pH = 7.0; t = 20°C $MgCl_2$ concentration — $5 \cdot 10^{-3}$M; $CaCl_2$ concentration — $5 \cdot 10^{-3}$M)

No myosin prep.	Subst. added to myosin sol.	Initial concentration of added ATP	Incubation period	-SH groups content in μM -SH/1g myosin	Content of myosin bound P_{in} in μM/1g myosin	Changes	
						Δ -SH in μM/1g myosin	Δ P_{in} in μM/1g myosin
M—13	—		—				
	ATP	$6 \cdot 10^{-3}$M	1′	26.6	3.5		
	ATP		20′	23.2	6.7	— 3.4	+ 3,2
	ATP+Mg^{++}		20′	23.2	7.1	— 3.4	+ 3.6
M—14	ATP	$5.7 \cdot 10^{-3}$M	1′	32.2	6.5		
	ATP+Mg^{++}		10′	25.3	12.7	— 6.9	+ 6,2
	ATP+Ca^{++}		10′	25.3	14.6	— 6.9	+ 8.1
M—15	ATP	$6 \cdot 10^{-3}$M	1′	27.9	4.2		
	ATP		20′	24.1	7.6	— 3.8	+ 3.4
M—16		0	—	38.1	8.1		
	ATP+Mg^{++}	$6 \cdot 10^{-3}$M	10′	30.0	15.1	— 8.1	+ 7.0
	ATP+Ca^{++}		10′	30.0	15.4	— 8.1	+ 7.3
M—17	—	$6 \cdot 10^{-3}$M		33.0	6,5		
	ATP		40′	25.3	14.2	— 7.7	+ 7.7
LM—8	ATP	$3.8 \cdot 10^{-3}$M	1′	45.0	4.5		
	ATP		20′	42.3	6.7	— 2.7	+ 2.2
	ATP		60′	34.7	14.5	—10.3	+10.0
LM—3	ATP	$4 \cdot 10^{-3}$M		52.0	13.5		
	ATP		5′	26.8	40.5	—25.2	+27.0

lows from Table VIII that the quantitative changes found in -SH groups and in myosin-bound orthophosphate, expressed in μM, are equivalent.

DISCUSSION

The results submitted above point to the existence of a close correlation between the changes in the content of myosin -SH groups and myosin-bound orthophosphate. A decrease in the content of free -SH groups of myosin observed during enzymatic ATP dephosphorylation and a parallel increase of orthophosphate content in crude myosin and L-myosin, leads to the following supposition. Phosphorylated myosini may be formed as an intermediate product of enzymatic ATP splitting according to the reaction:

$$M-SH + ATP \rightleftharpoons M-S-P + ADP_i$$

(M-SH — myosin; M-SP — phosphorylated myosin).

The phosphorylated myosin subsequently splits liberating orthophosphate and regenerating free -SH groups

$$M-S-P \xrightarrow{H_2O} M-S-H + H_3PO_4.$$

The possibility of this reaction is suggested by the increase of the content of free -SH groups and the decrease of myosin-bound orthophosphate towards the end of the enzymatic process.

Te experiments with Mg and Ca ions are consistent with the supposition of the formation of phosphorylated myosin as an intermediate product during ATP splitting. The generally known role of Ca ions as an activator of ATP decomposition, catalysed by myosin, is reflected in our experiments in which in the presence of Ca ions the acceleration of orthophosphate binding with a simultaneous decrease in -SH groups was observed at the beginning as well as an acceleration of a reverse reactions during the further course of the enzymatic process.

Mg^{++} failed to activate the ATP-ase properties of myosin. An increase of myosin-bound orthophosphate and a marked decrease in -SH groups found at the beginning of the reaction seem to indicate that Mg^{++} activates the phosphorylation process of myosin.

In the course of enzymatic splitting of ATP besides the phosphorylation of myosin a combination of ATP with myosin may also take place. It appears that -SH groups are not engaged in this process as shown by the absence of changes in the contents of these

groups (by the method applied) at the moment of binding of ATP with myosin. It is possible that the process of binding of ATP with myosin constitutes one of the first stages of the enzymatic reaction and precedes the formation of phosphorylated myosin.

The formation and splitting of phosphorylated myosin in the muscle may be essential for the functioning of the muscle. One can suppose that phosphorylated myosin constitutes, next to ATP and phosphocreatine, one of the high energy compounds present in the muscle.

SUMMARY

Changes in the content of myosin-bound phosphorus compounds and myosin -SH groups were investigated during enzymatic cleavage of ATP by crude myosin and L-myosin.

1. As soon as the enzymatic reaction starts the content of myosin-bound orthophosphate increases and after reaching a maximum value, diminishes again.

2. A decrease in the content of free -SH groups of myosin during the process of ATP dephosphorylation could be observed, followed by a subsequent increase in the content of these groups.

3. There is a distinct relation between the content of myosin-bound orthophosphate and free -SH groups. Decrease of myosin SH groups corresponds to an increase in the content of myosin-bound orthophosphate, and vice versa. Quantitative changes in myosin -SH groups and myosin-bound orthophosphate expressed in molar quantities are equivalent. On the basis of these data, the opinion is advanced of phosphorylation of protein.

4. Ca ions accelerate both the phosphorylation and dephosphorylation of myosin, whereas Mg ions probably activate phosphorylation processes only.

The authors wish to thank Prof. W. Niemierko, head of the Department of Biochemistry, for his interest and criticism. and to Miss Cz. Rżysko M. A. for her valuable technical assistance.

We also wish to express our gratitude to Dr Z. Grabowski for his help in working out the amperometric titration method.

REFERENCES

BAILEY K. 1942 — Biochem. J. *36*, p. 121.

BAILEY K., PERRY S. V. 1947 — Biochem. Bioph. Acta *1*, p. 506.

BALACHOWSKIJ S. D., BALACHOWSKIJ J. S. 1953 — Metody Chimitcheskowo analiza krowi. Medgiz. Moskwa, p. 665.

BATE-SMITH E. C. 1938 — Report Food Invest. Ed.D.S.I.P. p. 1938, 221, cit. as per Buchtal et al.
BUCHTAL F., DEUTSCH A., KNAPPEIS G. G., MUNCK-PETERSEN A. 1949 — Acta Physiol. Scand. *16*, p. 326.
BRAHMS J. 1956 — Acta Biol. Experim. *17*, p. 277.
BRAHMS J., KĄKOL I. 1957 — Acta Physiol. Polon. *8*, p. 289.
FISKE C. H., SUBBAROW Y. 1925 — J. Biol. Chem. *66*, p. 375.
FLECKENSTEIN A., JANKE J., DAVIES E., KREBS H. A. 1954 — Nature *174*, p. 1081.
GORNALL A. G., BARDWILL CH. J., DAVID M. M. 1949 — J. Biol. Chem. *177*, p. 751.
GREVILLE G. D. 1956 — Biochim. Bioph. Acta *20*, p. 440.
GREVILLE G. D., NEEDHAM D. M. 1955. — Biochim. Bioph. Acta 16, p. 284.
KIELLEY W. W., BRADLEY L. B. 1956 — J. Biol. Chem. *218*, p. 653.
KOTTHOFF I. M., STRICKS W., MORREN L. 1954 — Anal. Chem. *26*, p. 366.
LOHMANN K., JENDRASIK L. 1926, Biochem. Z. *178*, p. 419.
NIEMIERKO W., DRABIKOWSKI W., DYDYŃSKA M., KĄKOL I., ZAŁUSKA H. — 1957, Acta Biol. Experim. *17*, p. 374.
PERRY S. V., in S. P. COLOVICK, KAPLAN N. O. 1955 — Methods in Enzymology Academic Press INC. N. Y. *II*, p. 582.
PORTZEHL H., WEBER H. H. 1950 — Z. Naturforschg. 5b, p. 123.
WEBER H. H. 1955 — III Congrés Intern. Biochimie, Conference et Rapports, Bruxelles, 356.

AMINO-ACIDS AND AMINO-SUGARS IN THE MOULTING FLUID OF THE SILKWORM (*BOMBYX MORI* L.)

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(Received for publication 1 April 1957)

The first morphological indication of moult in insect is separation of the hypodermis from the cuticle. A new cuticle forms on the surface of the hypodermis. The space between the old and the new cuticle becomes filled with so-called moulting fluid. The internal layers of the old cuticle gradually disappear. The dissolution of the endocuticle is probably due to enzymes present in the moulting fluid (Wigglesworth 1933, Jeuniaux and Amanieu 1955, Passonneau and Williams 1953).

Many authors have studied the origin, properties, composition and role of the moulting fluid. Passonnenau and Williams in 1953 published the results of their extensive studies on the moulting fluid of the silkworm *Platysamia cecropia*. These authors investigated the moulting fluid of pupae *Platysamia cecropia* at the beginning of formation imaginal organs (early moulting fluid) and at the end of the imaginal development (late moulting fluid). They showed the difference in chemical composition and enzymatic activity between the moulting fluids of these two periods. Early moulting fluid has a character of a gel, it contains considerable amounts of proteins and shows tyrosinase activity. Late moulting fluid is a sol, it contains much smaller amounts of protein, and more non-protein nitrogen; its N-acetylglucosamine content is several times greater and the proteolytic- and chitinolytic activity much higher (Passonneau and Williams 1953) than in early moulting fluid.

The purpose of this study was to investigate the chemical composition of the moulting fluid of the *Bombyx mori*. Special attention was paid to chromatographic identification of amino-acids and amino-sugars.

MATERIAL AND METHODS

The pupal moulting fluid from the prepupae of the *Bombyx mori* was investigated.

In order to obtain the material, a cut was carefully made in the legs, and the colourless exuding fluid collected by means of a capillary. The amount of moulting fluid obtained in this way was determined by weight, and reached 30 to 60 miligrams per individual. The fluid taken from 10 to 15 specimens was used to an analysis.

For deproteinization and conservation of the material 10 ml of ethanol were added to the sample. After several hours the fluid was centrifuged, the supernatant decanted and stored until used for analysis, usually for several months. Analyses were started by evaporation of ethanol from the samples under a low pressure at a temperature not more than 60°C, the residue dissolved in 0.02 ml of bidistilled water and analysed chromatographically. Due to the low solubility of amino-sugars in ethanol allowance must be made for certain losses of these substances.

For chromatographic separation of amino-acids present in the moulting fluid, two-dimensional ascending partition chromatography was applied [n-propanol-water, 7 : 3, and phenol-water, 7 : 3] on Whatman No 1 paper, and one-dimensional (described by Hackman 1956) for quantitative determinations on Whatman No 4 paper. In order do detect amino-acids besides ninhydrin and isatin solutions, tests characteristic for arginine (Acher and Crocker 1952), and for histidine and tyrosine (Frank and Petersen 1955) were also applied. The chromatograms, after spraying with ninhydrin solution, were dried at room temperature and the appearance af amino-acid spots was observed according to Opieńska-Blauth.

For separation of amino-sugars the one-dimensional ascending technique of Partridge (1948) on Whatman No 4 paper was used. Chromatograms were developed in phenol-water mixture (in the presence of a 1 per cent solution of NH_3) amino-sugars present in the material were detected by means of the Morgan and Elson (1933, 1934) method modified by Partridge (1948).

EXPERIMENTAL RESULTS AND DISCUSSION

Figure 1 is a diagram of a two-dimensional chromatogram developed in propanol-water and phenol-water reagents. The use of standard substances and application of ninhydrin or isatin made it possible to identify the following amino-acids: α-alanine, arginine,

asparagine, aspartic acid, phenylalanine, glycine, glutamic acid, leucine, lysine, proline, serine, threonine, tyrosine and valine. The presence of arginine was also confirmed by means of specific test (Acher and Crocker 1952). Spots x_1, x_2, and x_3 were not

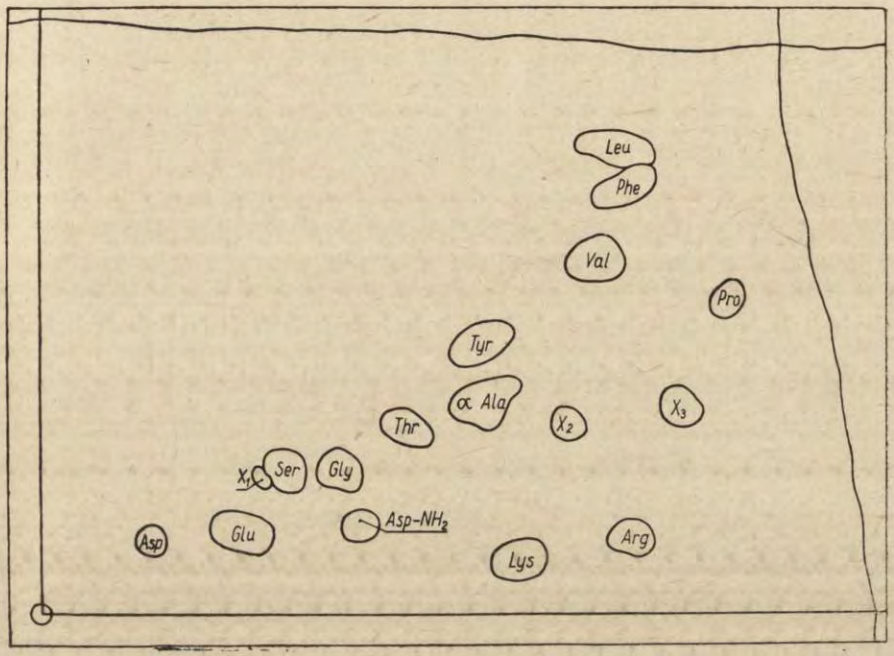

Fig. 1. Amino-acids in the moulting fluid from the prepupa of the silkworm *Bombyx mori* L.

Two dimensional technique on Whatman No 1. Chromatogram developed in n-propanol--water (7:3) and in phenol-water (7:3) with 0.2 per cent of cuprone. Sprayed alternatively with 0.2 per cent ninhydrin in acetone and developed without heating, or 0,2 per cent isatin in acetone with 4 per cent of acetic acid and heated in 105° for 10 min

identified. The position of spots x_2 and x_3 (Chen and Kühn 1956) suggest that they may correspond to, β-alanine nad γ-amino-butyric acid.

Figure 2 shows a two-dimensional chromatogram developed in n-propanol-water and tertiary isobuthyl alcohol-formic acid-water mixtures. By applying standard substances and various methods of development, it was possible to identify all spots visible on the chromatograms, corresponding to the following amino-acids: alanine, arginine, aspartic acid, glycine, glutamic acid, histidine, leucine

(with phenylalanine), proline, serine, threonine, tyrosine, valine, and traces of methionine. The last two amino-acids do not separate in propanol-water and phenol-water solvent mixtures and give one spot defined above as valine (Fig. 1). The presence of histidine was proved by developing chromatograms with diazotized sulphanilamide solution (Frank and Peterson 1955).

Figure 3 presents a one-dimensional chromatogram developed in a buthanol-acetic acid-water mixture and developed again in the

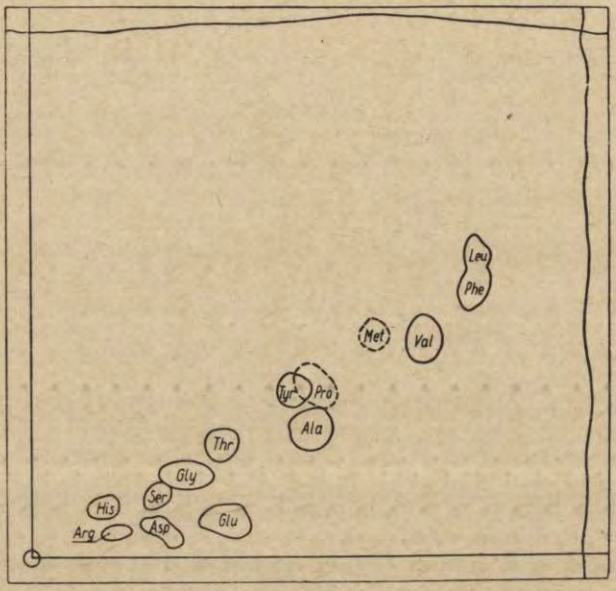

Fig. 2. Amino acids in the moulting fluid from the prepupa of the silkworm *Bombyx mori*

Two dimensional technique on Whatman No 1; chromatogram developed in n-propanol-water (7:3) and tert. butyl alcohol--acetic acid-water (75:15:10). Sprayed alternatively with 0.2 per cent ninhydrin in acetone and developed without heating (Opieńska-Blauth and all. 1956) or with 0.2 per cent of acetic acid and heated for 10 min in 105°

same mixture with ammonium molybdate and hydrogen perioxide (Hackman 1956). In these conditions methionine, according to Hackman — is oxidized into methionine sulfone, which travels more slowly than tyrosine. The spot correspoding to methionine sulfone appears between alanine and tyrosine. By means of this method it was possible to detect the presence of alanine, methionine

(traces), tyrosine, valine, phenylalanine (traces), and leucine in the moulting fluid. On this type of chromatograms, several spots of some other amino-acids appear below the alanine spot close to each other and are difficult to be distinguished. These aminoacids were separated by developing the chromatograms in phenol-water on Whatman No 4 paper saturated with a borate buffer at a pH 10.

Figure 4 shows a diagram of such a chromatogram. By means of this method the presence of aspartic acid, serine, threonine and alanine was found. In some chromatograms of this type a spot corresponding to cysteine was present.

Figure 5 is a chromatogram developed in 60 per cent aqueous acetone. The three sections of this chromatogram were sprayed with three distinct solutions: section one was sprayed with ninhydrin solution, the other two sections were sprayed alternatively with the isation and the diazotized sulphanilamide solution. This procedure confirms the occurrence of proline and histidine in moulting fluid.

Results obtained in the present investigation indicate that the following amino-acids occur in the moulting fluid of the *Bombyx mori* prepupa: α-alanine, arginine, asparagine, aspartic acid, cysteine (traces), phenylalanine, glycine, glutamic acid, histidine, leucine, lysine, methionine (traces), proline, serine, threonine, tyrosine and valine. The presence of tryptophane in the silkworm moulting fluid was not found. It is possible

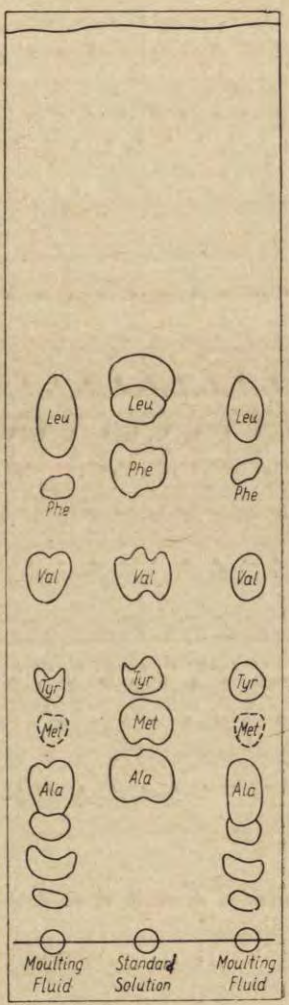

Fig. 3. Amino-acids in the moulting fluid from the prepupa of the silkworm *Bombyx mori*

One dimensional technique (H a c k m a n 1956) on Whatman No 4. Chromatogram developed in butyl alcohol-acetic acid water (77:6:17) and anew developed in the same mixture with addition of molybdate and perhydrol. Sprayed with 1 per cent ninhydrin in ethanol

that during the long period of storage or during evaporation of ethanol in which the moulting fluid was preserved, this amino-acid could be destroyed.

In addition to the analysis of the moulting fluid from the silkworm prepupa, moulting fluid from the fourth larval moult was investigated. In this stage of development collection of the moulting

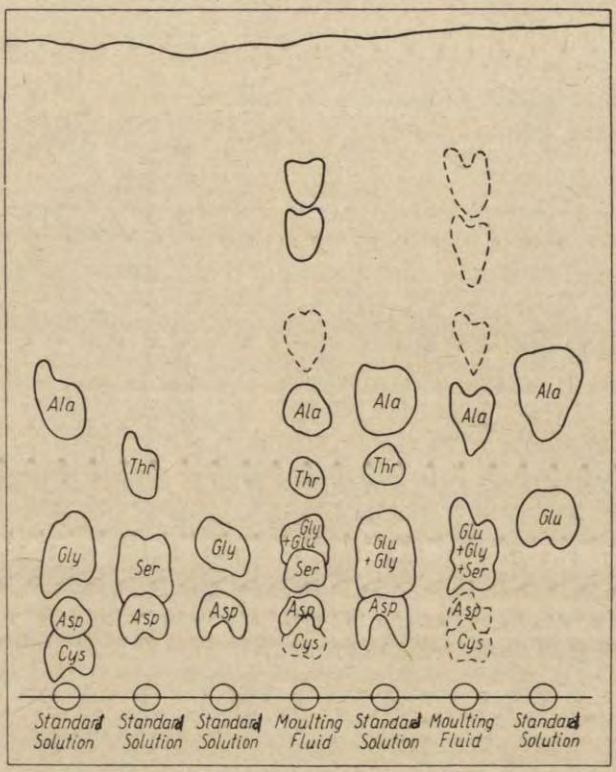

Fig. 4. Amino-acids in the moulting fluid from the prepupa of the silkworm *Bombyx mori*

One dimensional technique (Hackman 1956) on Whatman No 4 saturated with borate buffer solution at pH 10; chromatogram developed in phenol-water (7:3); Sprayed with 0.2 per cent ninhydrin in ethanol with 2 per cent of acetic acid

fluid was however very difficult. Only on several occasions was it possible to obtain small amounts of this fluid up to one microgram. Experiments were threrefore made on freshly cast and still moist sloughs (exuviae) from about 50 larvae. The sloughs were extract-

ed with distilled water, the extracts deproteinized with ethanol, evaporated under a low pressure and filtered, and the filtrate used for chromatographic analysis (two-dimensional technique, solvents: propanol-water and phenol-water). After spraying with ninhydrin, the same amino-acids could be detected which were found in the moulting fluid of prepupa.

Figure 6 shows a chromatogram developed in phenol-water mixture in the presence of 1 per cent NH_3 solution. Besides spots characteristic for N-acetylglucosamine and glucosamine unidentified spot x (section 1) appeared on the chromatograms sprayed with both reagents A and B of Partridge (1948). On the chromatogram sprayed with the reagent B only one spot characteristic for N-acetylglucosamine appeared (section 2).

Chromatographic analyses in an earlier investigation (unpublished) carried out on fresh samples of the pupal moulting fluid from prepupa and of the larval moulting fluid showed also the presence of N-acetylglucosamine and glucosamine as well traces of glucose. In addition a fluorescent substance was found in the moulting fluid. This probably represents one of the pteridines present in the larval integument of the silkworm (Hirata, Nakanishi and Kikkawa 1950; Zielińska and Klita 1957).

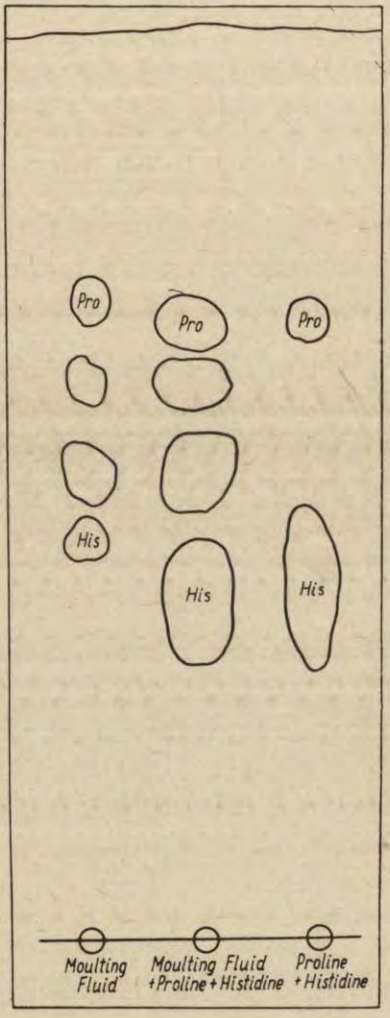

Fig. 5. Amino-acids in the moulting fluid from the prepupa of the silkworm *Bombyx mori*

One dimensional technique according to Hackman (1956) on Whatman No 4; chromatogram developed in 60 per cent aqueous acetone and sprayed with 0.2 per cent ninhydrin sol. in aceton

Some additional information about other chemical constituents of moulting fluid can be summarized in the following way*. According to the present investigation the content of protein and non-

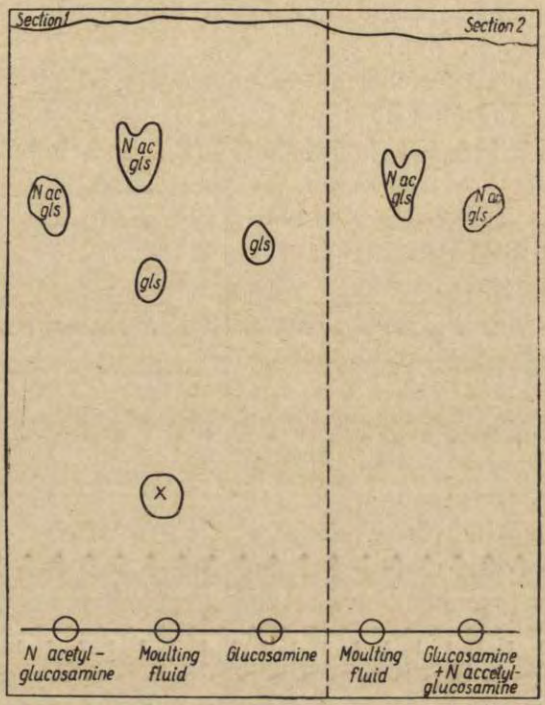

Fig. 6. Amino-sugars in the moulting fluid from the prepupa of the silkworm *Bombyx mori*
One dimensional technique on Whatman No 4; chromatogram developed according to Partridge (1948). Section 1 sprayed with reagents A and B, section 2 sprayed with reagent B only

protein nitrogen in the moulting fluid of the *Bombyx mori* prepupa (protein nitrogen about 3.5 mg/ml, non-protein nitrogen 2.5 mg/ml) is considerably higher than in the moulting fluid of the

* By means of qualitative reactions the presence of potassium, sodium (traces), calcium and chlorides, sulphates and phosphates was stated in moulting fluid from the prepupae and pupae of the *Bombyx mori* (Zielińska and Laskowska 1957), Passonneau and Williams also found potassium, calcium and chlorides in the moulting fluid from the cecropia silkworm, but sodium, sulphates or phosphates were not being investigated.

pupa (protein nitrogen about 2.5 mg/ml, non-protein nitrogen 0.5 mg/ml). According to the data obtained by Passonneau and Williams, the protein content in early moulting fluid of cecropia silkworm is considerably higher (protein nitrogen 4.7 mg/ml) than in the later moulting fluid (protein nitogen 1.1 mg/ml). The same authors show, that the activity of proteolytic and chitinolytic enzymes of the moulting fluid in *Platysamia cecropia* increases considerably towards the end of the imago development. At the same time a large part of the pupal endocuticle disappears, while the content of non-protein nitrogen increases from 0.4 mg/ml to 4.9 mg/ml. The content of N-acetyloglucosamine and its deacetylated derivative is several times higher in the late moulting fluid of cecropia (2.6 mg/ml and 0.8 mg/ml resp.) than in the early one (0.322 mg/ml and 0.145 mg/ml).

The moulting fluid of the *Bombyx mori* examined in these investigations was always collected several hours before the pupal moult, and was therefore always "late" moulting fluid. Chromatographic analyses of this fluid showed a number of amino-acids as well as acetylglucosamine and glucosamine.

The high proteolytic and chitinolytic activity of moulting fluid (Passonneau and Williams 1953; Jeuniaux and Amanieu 1955), as also the occurrence of protein (amino-acids) and chitin (amino-sugars) decomposition products which were found in the present investigation indicates that the moulting fluid of insects plays the role of a "digesting" factor in respect to internal cuticle layers during the process of moulting.

These investigations suggest that the following amino-acids are probably present in the protein of the pupal endocuticle *Bombyx mori:* α-alanine, arginine, asparagine, aspartic acid, cysteine (traces), phenylalanine, glycine, glutamic acid, histidine, leucine, lysine, methionine (traces), proline, serine, threonine, tyrosine, valine as probably also β-alanine and γ-amino-butyric acid.

Acid hydrolysate of the pupal cuticle protein of *Calliphora vomitoria* was investigated by Dennel (1956) and the following nine amino-acids were found: alanine, arginine, glycine, glutamic acid, leucine, lysine, proline, serine and valine. The present investigation makes it probable that the *Bombyx mori* cuticle contains not less than nineteen amino-acids enumerated above.

SUMMARY

The chemical composition of the moulting fluid from the fourth larval and from the pupal moult of the silkworm *Bombyx mori* was investigated. (Paper chromatography for detection of amino-acids and amino-sugars was used).

The occurrence of the following amino-acids was established: α-alanine, arginine, aspartic acid, asparagine, cysteine (traces), phenylalanine, glycine, glutamic acid, histidine, leucine, lysine, methionine (traces), proline, serine, threonine, tyrosine and valine.

The presence of N-acetylglucosamine and glucosamine was also found in the moulting fluid.

The high chitinolytic and proteolytic activity of the moulting fluid as also the presence of protein and chitin hydrolysis products indicate that moulting fluid plays the role of an "endocuticle digesting factor" of the old cuticle.

REFERENCES

ACHER R. and CROCKER CH. 1952 — Specific colour reaction for arginine and tyrosine after chromatography on paper. Acta Bioch. Biophys. *9*, p. 704.

CHEN P. S. und KÜHN A. 1956 — Vergleichende Untersuchung der freien Aminosäuren und Peptide während der Raupen-und Pupenentwicklung verschiedener Genotypen von Ephestia Kühnella. Z. Naturf. *11b*, p. 305.

DENNELL R. 1956 — Ortho-tyrosine in an Insect cuticle. Nature. *178*, p. 922.

ELSON and MORGAN. 1933 — A colorimetric method for the determination of glucosamine and chondrosamine. Bioch. J. *27*, p. 1824.

FRANK H. und PETERSEN H. 1955 — Eine Methode zur quantitativen Bestimmung von Histidin in Papierchromatogramen. Hoppe-Seyler's Z. *299*, p. 1.

HACKMAN R. H. and LAZARIUS M. 1955 — Quantitative analysis of amino-acids using paper chromatography. Austr. J. Biol. Sci. *9*, p. 281.

HIRATA Y., NAKANISHI K. and KIKKAWA H. 1950 — Xanthopterin obtained from the Skins of the Yellow Mutant of *Bombyx mori* (Silkworm). Science. *111*, p. 608.

JENIAUX CH. et AMANIEU M. 1955 — Propriétés chitinolytiques du liquide exuvial du ver à soie (*Bombyx mori* L.). Experientia. *11*, p. 195.

JENIAUX CH. 1955 — Propriétés chitinolytiques des extraits aqueux d'exuvies larvaires, prenymphales et nymphales de *Tenebrio molitor* L. Arch. internat. Physiol. Bioch. *63*, p. 114.

LOWER H. F. 1956 — Terminology of the Insect Integument. Nature. *178*, p. 1355.

MORGAN and ELSON. 1934 — A colorimetric method for the determination of N-acetylglucosamine and N-acetylchondrosamine. Bioch. J. *28*, p. 988.

OPIEŃSKA-BLAUTH J., KOWALSKA H. and PIETRUSIEWICZ M. 1956 — Nowy sposób identyfikacji aminokwasów na chromatogramach jedno i dwukierunkowych. Acta Bioch. Polon., *3*, p. 557.

PARTRIDGE S. M. and WESTALL R. G. 1948 — Filter-paper Partition Chromatography of Sugars. Bioch. J. *42*, p. 238.

PASSONNEAU J. V. and WILLIAMS C. H. 1953 — The moulting fluid of Cecropia silkworm. J. Exper. Biol. *30*, p. 545.

TREVELYAN W. E., PROCTER D. P. and HARRISON J. S. 1950 — Detection of Sugars on Paper Chromatograms. Nature. *166*, p. 444.

WIGGLESWORTH V. B. 1946 — Insect Physiology. London.

ZIELIŃSKA Z. M. and KLITA S. 1957 — Chromatograficzne badania barwników pterynowych. Acta Physiol. Polon., *8*, p. 584.

ZIELIŃSKA Z. M. and LASKOWSKA T. 1957 — Badania składu chemicznego płynu linkowego jedwabnika *Bombyx mori*. Acta Physiol. Polon., *8*, p. 582

Acta Biologiae Experimentalis, Vol. XVIII, 1958

THE BINDING OF ADENOSINETRIPHOSPHATE AND ORTHOPHOSPHATE BY PROTEINS *

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(Received for publication 15 August 1957)

Previous studies in this laboratory showed that the major part of adenine nucleotides present in muscles and also a certain fraction of the so called acid-soluble phosphorus compounds are not subject to extraction by buffer solution at pH of 4.6. This fact was observed both in acetone muscle-powder from frogs, (Niemierko et al. 1954, 1957 a) and also in the extraction of fresh, chilled muscles (Niemierko 1957 b). Results obtained show that in the above experimental conditions part of the phosporus compounds mentioned occurs in connection with muscle proteins. Earlier investigations by Straub and Feuer (1950) and later by other authors (Biro and Nagy 1955, Dubuisson 1950, Laki et al. 1950, Perry 1952) showed that actin contains certain strongly bound adenine nucleotides. A number of workers discovered the presence of adenine nucleotides and orthophosphate (Bailey (1942), Buchtal et al. (1949, 1952) Lajtha (1948) in myosin and actomyosin preparations, at the same time proving that these proteins can bind additional amounts of the compounds mentioned (Buchtal et al. 1949). It was even postulated that ATP and orthophosphate bind with myosin during the process of muscle contraction (Morales et al. 1955, Szent Györgyi 1947, 1953). Recently Brahms and Kąkol (1957) found that the amount of

* A preliminary communication of this paper was reported at 7th Congress of the Polish Physiological Society (Drabikowski 1957).

orthophosphate and ATP* bound with myosin is subject to change during enzymatic action.

All of the authors mentioned investigated the ATP and orthophosphate binding only with structural muscle protein on account of the eventual role of this binding in the process of muscle contraction. In connection with this problem, which was also investigated in our laboratory, the question arose as to whether the ability to form ATP and orthophosphate linkages constitutes a specific property of contracting muscle proteins, as assumed by Biro and Nagy (1955), or whether this is also true of other proteins. Already in 1932 Przyłęcki found that various ribonucleic acid derivatives such as nucleoside mono -3'- phosphates, nucleosides, purines, as also orthophosphate possess the ability to from combination with egg albumin. In recent years a similar problem was investigated by Klotz and Urquhart (1948)) who determined the binding energy of adenosine -3'- phosphate and adenosine with egg albumin. Investigation on this type off nucleotide and protein binding were however fragmentary, and did not relate to the binding of ATP and other nucleoside -5'- phosphates.

This study was aimed at a closer investigation of the binding of various proteins formed *in vitro* with adenine nucleotides and with orthophosphate. During the first stage of the investigations emphasis was put on the binding of these compounds by proteins as a function of the pH.

MATERIAL AND METHODS

The following proteins were used:

1. Non purified albumin from human blood serum (containing about 10% α-globulin and about 5% β-globulin).
2. Albumin from human blood serum, Cohns fractions No V (1950).
3. Egg albumin obtained according to Kekwick and Cannan (1936)).
4. Soluble muscle proteins — protein fractions extractable from muscles at a low ionic strength. [This fraction was obtained by extracting muscles with a phosphate buffer pH 7.7, $\mu = 0{,}15$ according to Dubuisson (1945). The extract obtained was dialysed in the presence of the same buffer at $\mu = 0.05$ and lyophilized].
5. Myosin — prepared by extracting frog muscles with Weber — Edsalll solution. Actomyosin was removed according to Portzehl et al. (1950)).

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* The following abbreviations are used: ATP — adenosinetriphosphate, ADP — adenosinediphosphate, AMP — adenosinemonophosphate, TCA — Trichloroacetic acid.

6. Casein.
7. Human blood γ-globulin.

Albumins were used in the form of solutions in distilled water. Casein in the form of a solution in 0,25% $NaHCO_3$, myosin in 0,5 M KCl, γ-globulins in 0,2 M NaCl.

ATP was obtained in our laboratory according to the Szent Györgyi (1947) changing it finally into a Ba salt; although partially a commercial preparation of a Na salt was used. ADP, AMP — a "yeast" adenilic acid (a mixture of AMP 3′ and AMP 2′), and adenosine constituted the commercial preparations. An acetate 0.2 M buffer was used.

The procedure used was as follows: a nucleotide or orthophosphate solution, or both together, were added to a protein solution (usually about 2%); this was mixed, and immediately an equal volume of a cooled buffer solution of various pH was added, and mixed again. In some cases coagulation of the protein took place after adding the buffer solution. Usually, however, coagulation of the protein took place after adding ethanol (usually one half of the volume) at 0°C, or after heating to 100°C. In this last case the solution was heated on a boiling water bath for several minutes until visible coagulation appeared, and then rapidly cooled. The precipitated protein was centrifuged, washed with a mixture of water and buffer solution eventually with an addition of ethanol). The precipitate was treated with 10% trichloroacetic acid at 0°C. The quantity of phosphorus compounds liberated by means of trichloroacetic acid was then determined in the filtrate obtained.

The following phosphorus fractions were determined: inorganic phosphorus (IP_o), labile nucleotide phosphorus liberated by 10 minutes of hydrolysis in 1 N acid at 100°C, (P_{10}), and total phosphorus of the TCA filtrate after digesting in the presence of H_2SO_4 and HNO_3 (P_t). Phosphorus was determined by means of the Fiske — Subbarow method (1925).

Beside phosphorus fractions, ribose was determined according to the Mejbaum (1939) method. The time of hydrolysis was 20 minutes; only in the case of AMP hydrolysis lasted 40 minutes in accordance with Albaum and Umbreit (1947).

Protein was determined by means of the biurette method according to Gornall et al. (1949), in which case the calibration constant was defined for each kind protein between the extinction value obtained and the protein concentration determined by means of Kjeldahl's method.

RESULTS

The relationship between ATP and orthophosphate binding and pH is presented in Table I*. As can be seen all of the proteins under study bind ATP. This binding ability depends markedly on the pH. The largest amounts of bound ATP are found in proteins

* Data relates to nucleotide amounts computed in micromols on the basis of average determinations for content of labile phosphorus, total phosphorus and ribose.

Table I

Combination of protein with

Exper. No	Kind of protein	pH	Amount present in the sample					
			protein		ortophosphate		ATP	
			mg	concentration in mixture in %	μM	μM/g protein	μM	μM/g protein
1	2	3	4	5	6	7	8	9
19	ovo-albumin	3.6	107	1.34	—	—	16.4	144
		3.6	107	1.34	—	—	16.4	144
		4.8	107	1.34	—	—	16.4	144
		4.8	107	1.34	—	—	16.4	144
		5.8	107	1.34	—	—	16.4	144
		5.8	107	1.34	—	—	16.4	144
18	serum γ-globulin	3.4	93.5	1.24	—	—	16.1	172
		4.8	93.5	1.24	—	—	16.1	172
		5.8	93.5	1.24	—	—	16.1	172
5	casein	3.8	445	1.78	53.5	121	29.8	67
		4.7	445	1.78	53.5	121	29.8	67
		4.7	445	1.78	53.5	121	29.8	67
		5.9	445	1.78	53.5	121	29.8	67
8	myosin	3.8	129	0.86	43.4	337	14.3	111
		3.8	129	0.86	43.4	337	14.3	111
		5.4	129	0.86	43.4	337	14.3	111
		5.4	129	0.86	43.4	337	14.3	111
		6.5	129	0.86	43.4	337	14.3	111
		6.5	129	0.86	43.4	337	14.3	111
11	soluble muscle proteins	3.8	117	0.52	238	2040	13.3	114
		4.8	117	0.52	238	2040	13.3	114
		5.8	117	0.52	238	2040	13.3	114
16	serum albumin	3.3	97	0.97	72.3	745	—	—
		4.6	97	0.97	72.3	745	—	—
		5.8	97	0.97	72.3	745	—	—

ATP or with orthophosphate

Method of protein coagulation	Amount found in the precipitate						
	protein	orthophosphate			ATP		
	mg	μM	μM/g protein	% of the total amount	μM	μM/g protein	% of the total amount
10	11	12	13	14	15	16	17
alcohol	85	—	—	—	8.8	103.5	53.8
heat	98	—	—	—	8.3	85.0	50.7
alcohol	57	—	—	—	2.8	49.0	17.5
heat	95	—	—	—	3.7	39.0	22.5
alcohol	26	—	—	—	0.4	15 4	2.4
heat	34	—	—	—	0.4	11.8	2.4
alcohol*	75	—	—	—	7.3	97.0	45.3
„	83	—	—	—	4.2	50.0	26.1
„	81	—	—	—	1.8	22.3	11.2
buffer only	445	1.8	4.0	3.4	15.3	34.5	51.2
„	422	1.0	2.4	1.9	4.1	9.9	14.1
alcohol**	411	1.2	2.9	2.2	4.1	10.0	14.1
„	331	0.6	1.1	1.1	2.0	6.0	6.7
alcohol	129	1.3	10.1	3.0	2.7	21.0	18.9
heat	129	1.2	9.3	2.8	2.5	19.4	17.5
alcohol	129	2.0	15.4	4.6	1.0	7.4	7.0
heat	119	0.8	6.7	1.8	0.4	3.4	2.8
alcohol	123	2.0	16.3	4.6	0.5	4.7	3.5
heat	120	0.8	6.7	1.8	0.4	3.4	2.8
alcohol	109	5.5	55.0	2.3	7.8	72.0	58.8
„	100	2.5	25.0	1.1	2.4	24.0	18.0
„	88	1.4	15.9	0.6	0.5	5.7	3.8
heat***	68	7.3	107.0	10.1	—	—	—
heat	92	5.2	56.3	7.2	—	—	—
heat	94	2.1	22.4	2.9	—	—	—

* 1 vol. alcohol.

** 2 vol. alcohol.

*** After heat action 3 vol. of ethanol were added and kept several days at room temperature.

Table II

Influence of ATP on orthophosphate binding by proteins

Exper. No	Kind of protein	pH	Amount present in the sample						Method of protein coagulation	Amount found in the precipitate						
			protein		orthophosphate		ATP			protein	orthophosphate			ATP		
			mg	concentration in mixture in %	μM	μM/g protein	μM	μM/g protein		mg	μM	μM/g protein	% the total amount	μM	μM/g protein	% of the total amount
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
13	serum albumin	4.7	105.5	1.01	—	—	16,7	165	heat	101	—	—	—	5.8	58.0	34.7
		4.7	105.5	1.01	70	695	—	—	„	99,5	3.9	39.0	5.8	—	—	—
		4.7	105.5	1.01	70	695	16.7	165	„	100	1.3	13.0	1.9	5.8	58.0	34.7

Table IV

Influence of diluting on the binding ability of ATP with proteins

Exper. No	Kind of protein	pH	Amount present in the sample				Method of protein coagulation	Amount found in the precipitate			
			protein		ATP			protein	ATP		
			mg	concentration in mixture in %	μM	μM/g protein		mg	μM	μM/g protein	% of the total amount
1	2	3	4	5	6	7	8	9	10	11	12
15	serum albumin	4.6	94	1.87	13	138	alcohol	86	4.9	57.0	37.7
		4.6	94	1.87	13	138	heat	90	5.7	63.5	43.8
		4.6	94	0.94	13	138	alcohol	78	4.2	54.0	32.3
		4.6	94	0.94	13	138	heat	83	4.8	57.8	37.0
		4.6	94	0.19	13	138	alcohol	74	3.3	44.7	25.4
		4.6	94	0.19	13	138	heat	76	2.6	34.4	20.1

precipitated in pH lying below their isolectric point. As pH rises, the amount of ATP declines; this dependence is illustrated in Fig. 1 based on numerical data from Table I. As can be seen the character of curves showing ATP binding is the same for all proteins although they differ in respect to their ATP binding capacity. From amongst the proteins under study, albumins bind the greatest

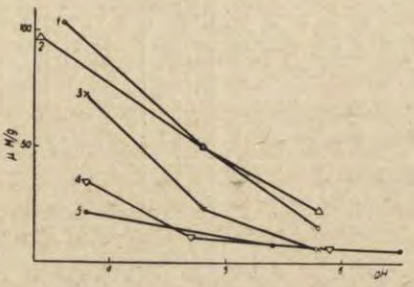

Fig. 1. Relationship between pH and the binding ability of ATP by different proteins

Experimental conditions: The mixtures of proteins and ATP were coagulated with 1/2 vol. of ethanol at various pH. (For experimental data see Tabl. I). Curve 1: eggalbumin, o—o. Curve 2: γ - globulin, Δ—Δ. Curve 3: soluble muscle proteins, x—x. Curve 4: casein, V—V. Curve 5: myosin, □—□. Ordinate: amount of bound ATP in μM/g proteins. Abscissa: pH

amounts of ATP, while the smallest amounts of this nucleotide were bound by myosin and casein. In the conditions of the experiment more than 100 μM ATP per gram of protein were bound in the case of albumin at a pH below the isoelectric point; in the case of myosin and casein only up to about 35 μM per gram of protein was bound at more or less the same initial concentrations of protein and ATP within the same pH range. A value of 50—60 μM per gram of protein was obtained for albumins in the isoelectric point, while in the case of myosin and casein only around 10 μM ATP per gram of protein was bound.

The binding capacity of orthophosphate depends, as can be seen from Table I, similarly as in the case of ATP, on the pH. Only in the case of myosin it was not possible to observe such a distinct dependence. However orthophosphate is bound by proteins in a much lesser degree than ATP; binding took place only, when the amount introduced was large.

Furthermore a mutual influence of orthophosphate and ATP on binding capacity with proteins was also proved. Protein was subjected in the same conditions to coagulation with orthophosphate or ATP, and with a mixture of both of these compounds. Results are presented in Table II. As can be seen from the table, orthophosphate in the presence of ATP is subject to binding in a still smaller degree than in its absence. On the other hand orthophosphate in an even

Table III

Relationship between concentration of ATP and its binding ability with proteins

Exper. No	Kind of protein	pH	Amount found in the sample						Method of protein coagulation	Amount found in the precipitate				
			protein		orthophosphate		ATP			protein		ATP		
			mg	concentration in mixture in %	μM	μM/g protein	μM	μM/g protein		mg	% of the total amount	μM	μM/g protein	% of the total amount
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
12	serum albumin	4.6	291	1.17	—	—	8.7	30	alcohol	190	63	3.8	20.0	43.7
		4.6	291	1.17	—	—	8.7	30	heat	281	96.5	3.6	12.8	41.3
		4.6	291	1.17	—	—	17.4	60	alcohol	198	68	6.9	34.8	39.6
		4.6	291	1.17	—	—	17.4	60	heat	286	98	6.8	23.7	39.0
		4.6	291	1.17	—	—	34.8	119	alcohol	244	86.5	13.8	56.6	39.6
		4.6	291	1.17	—	—	34,8	119	heat	278	96	14.4	51.7	41.3
		4.6	291	1.17	—	—	52.2	179	alcohol	270	92.5	19.4	72.0	37.0
		4.6	291	1.17	—	—	52.2	179	heat	280	96.5	19.4	69.5	37.0
		4.6	304	1.18	—	—	69.6	237	alcohol	280	92.5	22.8	83.0	32.7
		4.6	304	1.18	—	—	69.6	237	heat	298	98.5	25.8	86.5	37.0
		4.6	304	1.18	—	—	—	—	alcohol	80	26.5	—	—	—

much greater concentration has no influence on the binding of ATP. It therefore seems that the affinity of orthophosphate for proteins is weaker than that of ATP.

The fact that at the most only several percent of the introduced P_o is subject to binding while in the case of ATP about ten times this amount is bound, confirms the above. The later value depends partially upon the proportion of ATP to protein in the initial

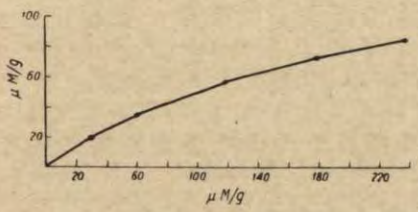

Fig. 2. Relationship between concentration of ATP and its binding ability with protein

Experimental conditions: The mixtures of serum albumin with various amount of ATP were coagulated with 1/2 vol. of ethanol at pH 4.6 (For experimental data see Tabl. III). Ordinate: amount of bound ATP in μM/g protein. Abscissa: amount of ATP in initial mixture in μM/g protein

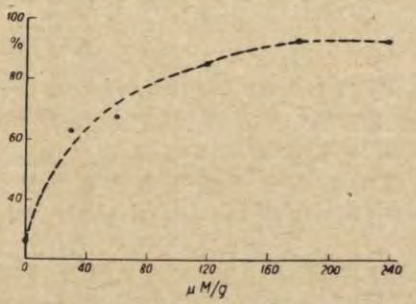

Fig. 3. Relationship between coagulation of protein and concentration of ATP

Experimental conditions: The mixtures of serum albumin with various amount of ATP were coagulated with 1/2 vol. of ethanol at pH 4.6 (For experimental data Tabl. III). Ordinate: % of precipitated protein. Abscissa: amount of ATP in initial mixture in μM/g protein

mixture. The dependence between the last mentioned and the amount of bound ATP is illustrated in Table III and in Fig. 2. It can here be seen that when the amount of added ATP is increased, its content in the precipitated protein increases also. It is however characteristic, that percentage of bound protein is still, irrespective of its concentration, more or less the same.

The absolute amounts of nucleotide subject to precipitation with protein both in the case of heat denaturation, as well as precipitation with ethanol, were very similar. However the content of ATP in the protein precipitated with ethanol was higher at lower initial concentrations of the nucleotide, as — in contrast to heat denaturation — not all of the protein was subject to precipitation. The amount of protein coagulating under the influence of ethanol in the isoelectric point or below it depends distinctly upon the ATP concentration. This dependence is illustrated in Fig. 3 which shows

Table V

The binding of various nucleotide — derivatives by proteins

Exper. No	Kind of protein	pH	Amount present in the sample					Method of protein coagulation	Amount found in the precipitate			
			protein		nucleotide-derivative				protein	nucleotide-derivative		
			mg	concentrat. in mixture in %	kind	μ M	μ M/g protein		mg	μ M	μ M/g protein	% of the total amount
10	casein	3.7	214	1.52	adenosine	32	149	buffer only	192	1.8	9.4	5.6
		3.7	214	1.52	„	32	149	heat	198	0.7	3.5	2.2
		3.7	214	1.52	AM-3-P	31.6	148	buffer only	194	2.5	12.8	7.9
		3.7	214	1.52	„	31.6	148	heat	199	2.4	12.1	7.6
		3.7	214	1.52	ADP	32	149	buffer only	199	5.2	26.1	16.1
		3.7	214	1.52	„	32	149	heat	199	3.6	18.1	10.2
		3.7	214	1.52	ATP	31.3	147	buffer only	203	7.0	34.7	22.4
		3.7	214	1.52	„	31.3	147	heat	205	5.7	27.8	18.3

Table VI

Influence of some salts on the binding ability of ATP with proteins

Exper. No	Kind of protein	pH	Amount present in the sample						Method of protein coagulation	Amount found in the precipitate			
			salt		protein		ATP			protein	ATP		
			kind	concentration in M	mg	concentrat. in mixture in %	μ M	μ M/g protein		mg	μ M	μ M/g protein	% of the total amount
20	ovo-albumin	3.6	—	—	121	1.21	16.4	129	alcohol 1 vol.	99	8.3	89.5	50.5
		3.6	$CaCl_2$	0.75	121	1.21	16.4	129		116	3.0	25.7	18.3
		3.6	„	0.50	121	1.21	16.4	129		119	3.9	32.8	23.7
		3.6	„	0.25	121	1.21	16.4	129		121	4.5	37.3	27.4
		3.6	NaCl	0.75	121	1.21	16.4	129		108	3.1	27.8	18.9
		3.6	„	0.375	121	1.21	16.4	129		112	4.8	42.8	29.3

the data relating to the amounts of precipitated proteins from Table III. As can be seen ATP considerably increases the percentage of precipitated protein in these conditions.

Binding of ATP by protein is also influenced to a certain extent by the degree of dilution of the initial mixture of protein and nucleotide. This relationship is presented in Table IV. It will be observed that as the solution is diluted, the amount of ATP found in the precipitated protein somewhat declines.

Comparisons were made between the binding capacity of ATP and of other ralated compounds such as ADP, AM 3'P, and adenosin. It was shown that both at the isoelectric point, as also below it, ATP is subject to binding with proteins in a greater degree than the remaining substances (Table V). On the basis of the results obtained it can be assumed that the binding capacity with proteins decreases as the number of phosphate groups in a nucleotide molecule decreases.

On the basis of results relating to comparison of the amounts of ATP bound by protein coagulated with ethanol and precipitated by heating, it can be assumed that the linkages are fairly stable at a temperature of 100°C, however after a longer period at heating partial dissociation takes place. Thus for example after 30 minutes of heating on a water bath at a temperature of 100°C the concentration of ATP in the precipitate declines from 66,3 μM/g to 49,4 μM/g, or by 20 %. On the other hand, however, protein precipitated by means of heat is capable of binding with ATP. A solution of serum albumin and 15,3 μM ATP was heated at pH 5,4. As results from the data given above, the precipitate did not contain much ATP. The whole was then brought to pH = 4,6 and shaken out for 30 minutes. The precipitate then contained 6,0 μM of ATP. In the control sample precipitated at once at pH = 4,6 in the presence of the same amount of ATP, 6,3 μM of ATP was found. The next sample was coagulated by heat at pH = 4,6 after adding ATP, and then brought to a pH of around 5,8. In these conditions only 2,3 μM of ATP was found in the precipitate.

Investigations were also conducted on the influence of some inorganic salts on the binding of ATP with proteins. As can be seen from data presented in Table VI, the presence of $CaCl_2$ or NaCl decreases the amount of nucleotide bound with proteins.

Binding of nucleotides with proteins probably takes place immediately, as no difference was found in the ATP concentration in

protein precipitated with ethanol directly after mixing, and protein precipitated 45 minutes after adding the nucleotide.

DISCUSSION

The majority of studies hitherto conducted and relating to the affinity of ATP to proteins were devoted principally to the binding of ATP with contractile muscle proteins, or with the role of such binding in the process of muscular contraction. As can be seen from these studies, the formation of protein linkages with nucleotides is not an exceptional property of muscular proteins. Przyłęcki and his co-workers in their studies on various symplexes were also somewhat interested in the problem of nucleotide and protein linkages. Their studies, however, related only to the binding of products of nucleic acid degradation and were somewhat fragmentary.

Przyłęcki (1932) observed the relation between the binding of nucleotide and orthophosphate by proteins, and the pH. Of the nucleoside-3′-phosphates investigated by him, adenosine 3′phosphoric acid constituted the only exception which was subject to binding with proteins to the same extent irrespective of the pH. In the present study the relation between the amount of bound ATP and orthophosphate, and the pH was very marked in all of the proteins under investigation. Only the binding of orthophosphate by myosin does not show such a distinct relationship. Explanation of this phenomenon will be the subject of further studies.

This dependence of binding ATP by proteins upon the pH seems not to apply to actin, as shown by data obtained by Straub and Feuer (1950) and Laki et al. (1950). As proved by these authors, the binding between actin and ATP is very inconsistent at the isoelectric point.

The amount of ATP discovered previously in our laboratory (Niemierko et al. 1957 a, 1957 b) bound with muscular proteins at pH = 4,6 (up to around to 70 μM/g of protein), corresponds to the amounts of this nucleotide found in this study in proteins precipitated at the asoelectric point. On the other hand, the concentration of ATP and P_o disclosed by various authors (Bailey 1947, Buchtal et al. 1949, Laitha 1948) in myosin and actomyosin preparations, as also found in these proteins after adding ATP, were considerably lower than the amounts undergoing binding as a result of the procedure applied in the present investi-

gations. Thus, for example, Buchtal et al. found 25 μM of nucleotides per gram of actomyosin. These differences might result from the fact that the preparations of muscular proteins mentioned were investigated in a neutral or slightly alkaline medium, and not in an acid one, in which — as shown by these investigations — the binding capacity of ATP increases considerably.

Data of the above mentioned authors, and results reached by us, do not agree with the results of Szörenyi and Czepinoga (1946). These authors, in investigating ATP binding and phosphorus liberated from ATP as a result of the enzymatic action of myosin, proved that these compounds shown an exceptional binding capacity in an alkaline medium. Experiments conducted by these authors showed that 1 g of myosin bound 370 μM of ATP and 490 μM P_o at pH = 9,1 i.e. considerably more than was found in this study in proteins below their isoelectric point.

Madsen and Cori (1957) recently showed by means of ultracentrifuging that phosphorylase preparations contain bound AMP in amounts of 4 M per 1 M of protein. The amounts of ATP bound with protein as a result of the procedure applied in the present study are of the some order. Hence for example blood serum albumin (assuming its molecular weight to be 69 000) binds around $\frac{1}{3}$ M ATP per mol at pH = 4,7.

It seems that the ability to bind ATP and orthophosphate is a universal property of all proteins. This fact, however, and the precise relationship between it and the pH does not allow any conclusions to be drawn concerning the binding of these compounds with proteins in the muscle *in vivo*.

Brahms and Kąkol (1957) submitted ATP to the enzymatic action of myosin which was then precipitated from the mixture by bringing it to the isoelectric point, and investigated the content of P_o and labile phosphorus. On the basis of the results obtained, the authors presume that a correlation exists between the enzymatic action of myosin and the amount of phosphorus bound, wich was separated from the ATP during dissolution. To what extent these results relate to our data, showing an interrelation between binding of phosphorus compounds and pH, is not yet clear.

It is difficult at present to state anything definite as to the nature of the bonds between nucleotides and proteins. Experiments

carried out by using various amounts of ATP seem to indicate that at least within the limits of the concentrations under study, a state of equilibrium is reached between that part of the nucleotide bound, and that part remaining in the supernatant irrespective of the amount of ATP. Thus for example around 40 % of the ATP was subject to binding at the isoelectric point of blood serum albumin (Table IV).

Our investigations showed a distinct difference in the binding of ATP and orthophosphate. Orthophosphate binds to proteins to a much smaller extent than nucleotides. Furthermore its affinity to proteins seems to be much weaker, as the presence of ATP considerably decreases the amount of bound P_o. These results are not in agreement with data from studies by Szörenyi and Czepinoga (1946) who did not find any mutual influence of ATP and phosphorus originating from the dephosphorylation of ATP during binding of these compounds with myosin; they hence assumed that both of these compounds link at different points of the protein molecule.

It seems that binding of proteins with nucleotides takes place at once. The fact that proteins are subject to coagulation in the presence of ATP is possibly here of some significance, for as was demonstrated by Przyłęcki (1932), protein denatured in the presence of nucleotides binds these compounds slowly so that fairly long stirring of the mixture is necessary. Temperature has a certain influence on stability of the binding. Inorganic salts also lower the binding capability of ATP with protein. A similar phenomenen was observed by Szymona (1955) in investigating the binding of ATP with proteins in microorganisms.

Morawiecki (1954) as also Mandl et al. (1952) found that ATP protects protein against denaturation above the isoelectric point. Morawiecki only mentions the fact that ATP below this point exerts an opposite effect, and does not give any numerical data. It was definitely proved in the present study that ATP shows a coagulating effect at a pH below the isoelectric point of proteins (Table III and Fig. 3). This effect is so strong, that when a protein solution (for example serum albumin) is mixed in an acid medium, in which this protein is not coagulated, with ATP of a relatively high concentration, precipitation takes place. At an initial concentration of 111 μM of ATP per gram of serum

albumin, coagulation did not yet take place at a pH = 3,6 neither after adding alcohol, nor after heataing. At a concentration of 170 μM ATP/g and a pH = 3,8, addition of alcohol caused partial precipitation notwithstanding that heating still showed no action. It was, however, possible to evoke coagulation by means of heating at an initial concentration of 340 μM ATP per gram of protein.

At present it is impossible to state whether ATP binds with native protein or only with denatured protein, whether nucleotides are already bound with proteins in the solution or whether they bind at the moment of coagulation. It is known that heat denaturation does not break these bonds, and that proteins denatured in the absence of nucleotides possess as already showed Przyłęcki (1932), the capacity of binding nucleotides. This is confirmed by our results relating to the reversible binding of nucleotides in changing the pH. Protein denatured above the isoelectric point binds additional amounts of ATP after acidifying. A similar phenomenon was observed by Niemierko et al. (Dydyńska and others, 1957), with fresh frog muscle. Muscle protein after grinding with and acetate buffer at a pH 5,8 binds small amounts of ATP. If, however, the whole homogenate is brought to a pH of 4,6, then the protein precipitate binds additional amounts of ATP and P_o.

On the other hand, a number of data (investigation by Przyłęcki, 1932, Buchtal and co-workers, 1952, Szörenyi and co-workers, 1946, and Klotz, 1948) obtained by applying dialysis and ultrafiltration indicate, that nucleotides and orthophosphate are also bound with native proteins. Przyłęcki notes, however, that native protein binds less nucleotides than denatured protein.

This problem will be the subject of future investigations.

I wish to extend my thanks to Drs Z. May and J. Malec from the Institute of Haematology for preparations of serum albumin and gamma globulins, and for lyophilizing protein preparations, as also to Mrs A. Jurowska for technical assistence.

SUMMARY

It was shown that the ability of proteins coagulated by means of heat denaturation or by an addition of ethanol at 0°C to bind ATP and orthophosphate depends markedly on the pH. The great-

est amount of both nucleotide and orthophosphate is bound at a pH lying below the isoelectric point of the investigated proteins. As the pH increases, the amount of bound ATP and P_o declines. Orthophosphate is subject to binding to a much smaller degree than ATP.

The ATP binding capacity of various proteins is different. Of the proteins under study, albumins bound greater amounts of ATP than myosin and casein.

As the concentration of ATP in the solution increases, its content in the precipitated protein also increases. In comparison with other adenine derivatives, ATP is subject to binding to the greatest extent. The presence of inorganic salts ($CaCl_2$, NaCl) exerts a negative influence on the binding of ATP with proteins.

REFERENCES

ALBAUM H. G., UMBREIT W. W. 1947 — Differentiation between ribose--3-phosphate and ribose-5-phosphate by means of the orcinol-pentose reaction. J. Biol. Chem. *167*, p. 369.

BAILEY K. 1942 — Myosin and Adenosinetriphosphatase. Biochem. J. *36*, p. 121.

BIRO N. A., NAGY B. 1955 — Vergleichende Untersuchungen über das Strukturgebundene Adeninnukleotid des Muskels. Acta Physiol Acad. Sci. Hung. *8*, p. 313.

BRAHMS J., KĄKOL I. 1957 — Interaction of myosin sulfhydryl groups and phosphorus compounds during decomposition of adenosinetriphosphoric acid. Acta Biol. Exper., in press.

BUCHTAL W. F., DEUTSCH A., KNAPPEIS G. G., MUNCH-PETERSEN A. 1949, 1952 — Phosphorylation and adenine nucleotide uptake of actomyosin and actin-free myosin. Acta Physiol. Scand. *16*, p. 316, *24*. p. 368.

COHN E. J. et al. 1950 — A system for the separation of the components of human blood: quantitative procedures for the separation of the protein components of human plasma. J. Am. Chem. Soc. *72*, p. 465.

DRABIKOWSKI W. 1957 — The ability of various proteins to bind ATP (in Polish). Acta Physiol. Polon. *8*, 314.

DUBUISSON M., JACOB J. 1945 — Recherches sur les proteines musclaires. III. L'electrophorèse des protéines des muscles striés normaux et fatigués de la grenouille. Bull. Soc. Roy. Sci. Liege. *14*, p. 145.

DUBUISSON M., MATHIEU L. 1950 — Sur la polymerisation de la G-actine. Experientia. *6*, p. 103.

DYDYŃSKA M., KĄKOL I., KOWALSKI T., STRZELECKA H., NIEMIERKO W. 1957 — Combination of nucleotides with proteins of the muscles and some other organs of frog (in Polish). Acta Physiol. Polon., *8*, 316.

FISKE W. C., SUBBAROW Y. 1925 — The colorimetric determination of phosphorus. J. Biol. Chem. *66*, p. 375.

GORNALL A. G., BARDANILL C. J., DAVID M. M. 1949 — Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. *177*, p. 751.

KEKWICK R. A., CANNAN R. K. 1936 — The hydrogen ion dissociation curve of the cristalline albumin of the hen's egg. Biochem. J. *30*, p. 227.

KLOTZ J. M., URQUHART J. M. 1948 — The combination of adenine, adenosine and adenylic acid with serum albumin. J. Biol. Chem. *173*, 21.

LAJTHA A. 1948 — The P-content of myosin. Hung. Acta Physiol. *1*, p. 134.

LAKI K., BOWEN W. J., CLARK A. M. 1950 — The polymerisation of proteins. ATP and polymerisation of actin. J. Gen. Physiol. *33*, p. 437.

MADSEN N. B., CORI C. F. 1957 — The binding of adenylic acid by muscle phosphorylase. J. Biol. Chem. *224*, p. 899.

MANDL J., NEUBERGER C., GRAUER A. 1952 — Solubilisation of insoluble matter in nature. I. The part played by salts of adenosinetriphosphate. Bioch. Bioph. Acta *8*, p. 654.

MEJBAUM W. 1939 — Ueber die Bestimmung kleiner Pentosemengen insbesondere in Derivaten der Adenylsäure. Z. Physiol. Chemie *258*, p. 117.

MORALES M. F., BOTTS J., BLUM J. J., HILL T. L. 1955 — Elementary processes in muscle action: an examination of current concepts. Physiol. Rev. *35*, p. 475.

MORAWIECKI A. 1954 — Influence of adenosine triphosphate on the thermodenaturation of albumins (in Polish). Acta Biochim. Polon. *1*, p. 47.

NIEMIERKO W. 1954 — Freies und gebundenes ATP im ruhenden Froschmuskeln. Acta Physiol. Acad. Sci. Hung. *6*. Suppl, p. 64.

NIEMIERKO W., DYDYŃSKA M., DRABIKOWSKI W., KĄKOL I., ZAŁUSKA H. 1957 — Investigation of free and protein-bound nucleotides in acetone-chloroform-dried muscle powder. Acta Biol. Exper. *17*, p. 373.

NIEMIERKO W., DYDYŃSKA M., KĄKOL I. 1957b — Investigation of some protein-bound phosphorus compounds in fresh muscle. Acta Biol. Exper. *17*, p. 388.

PERRY S. V. 1952 — The bound nucleotides of the isolated myofibril. Biochem. J. *51*, p. 495.

PORTZEHL H., SCHRAMM G., WEBER H. H. 1950 — Aktomyosin und seine Komponenten. I. Mitt. Z. f. Naturforsch. *5b*, p. 61.

PRZYŁĘCKI S. J., GRYNBERG M. Z. 1932 — Ueber die Bindung der Biokolloide. VII. Mitt. Eiweiss und Nukleinsäure sowie deren Abbauprodukte. Biochem. Z. *251*, p. 248.

STRAUB F. B., FEUER GY. 1950 — Adenosinetriphosphate — the functional group of actin. Bioch. Bioph. Acta. *4*, p. 455.

SZENT-GYÖRGYI A. 1947 — Chemistry of muscular contraction. Acad. Press N. York.

SZENT-GYÖRGYI A. 1953 — Chemical physiology of contraction in body and heart muscle. Acad. Pres. N. York.

SZÖRENYI E. T., CZEPINOGA O. P. 1946 — A new specific property of myosin (in Russ). Ukr. Bioch. Żurnał. *18*, p. 179.

SZYMONA M. 1955 — Private communication.

Acta Biologiae Experimentalis, Vol. XVIII, 1958

STUDIES ON THE BIOCHEMISTRY OF THE WAXMOTH (*GALLERIA MELLONELLA* L.). 18. SUCCINOXIDASE SYSTEM IN METAMORPHOSIS

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(Received 1 October 1957)

It was shown in a previous paper of this series (Wojtczak 1952) that the activity of succinoxidase in waxmoth pupa, as measured in the whole homogenate, decreased rapidly immediately after pupation and, later on, it rose considerably during the metamorphosis; its maximum coincided with the emergence of the adult insect. The present paper describes more detailed investigations on the succinoxidase system of waxmoth pupa and is particularly concerned with the two main components of this system, i.e. with succinic dehydrogenase and with cytochrome c oxidase.

MATERIALS AND METHODS

Fully grown larvae of the waxmoth, *Galleria mellonella* L. were placed separately in small vessels, without food, at 30° C. They pupated usually within two days. In order to establish the time of pupation, the cocoons were checked every few hours by looking at them in transmitted light, and in one series of experiments the cocoons were observed continuously, which enabled to establish the moment of pupation with greatest precision.

Pupae of known age were removed from cocoons and homogenized at 0° C with ice-cold 1/15 M phosphate buffer of pH 7.2 for the determina-

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tion of succinic dehydrogenase and succinoxidase, and of pH 7.4 for the determination of cytochrome c oxidase*. The buffer was taken in such volume that the resulting homogenate contained 100 mg. fresh pupal weight per milliliter, except in the case of the measurements of succinic dehydrogenase activity, when homogenates containing 200 mg. of fresh pupal weight per ml. were used.

The activity of succinic dehydrogenase was measured by the Thunberg method. The incubation mixture contained: 25 μmoles of sodium succinate, 0.25 μmole of methylene blue, and 0.5 ml. of homogenate containing 27 μmoles of phosphate buffer (pH 7.2) and 100 mg. of homogenized pupae. The total volume was 1.0 ml. Samples were incubated at 30° C, and the time needed for complete decolorization was noted. Controls were run parallel without succinate. The activity of succinic dehydrogenase was expressed by the formula:

$$\text{Activity} = 1/a - 1/b,$$

where: a, time (minutes) of decolorization of the mixture incubated with succinate; b, time (minutes) of decolorization of the control (without succinate).

The activity of cytochrome c oxidase was determined manometrically essentially according to Schneider and Potter (1943). For inactivation of phenoloxidase (tyrosinase), which interferes in the oxidation of ascorbate, thiourea was added to a final concentration 0.01 M. Each Warburg vessel contained: 0.08 μmole of cytochrome c prepared according to Keilin and Hartree (1945), 34 μmoles of sodium ascorbate, 80 μmoles of phosphate buffer (pH 7.4), 30 μmoles of thiourea, and homogenate containing from 10 to 40 mg. of fresh insect material. Total volume was 3.0 ml. Readings were taken for 20 minutes at 5 minute intervals at 30°C. Autooxidation of ascorbate was calculated by extrapolation.

Succinoxidase activity was determined manometrically in a Warburg respirometer. Each vessel contained: 0.08 μmole of cytochrome c, 80 μmoles of phosphate buffer (pH. 7.2), μmoles of thiourea**, homogenate corresponding to 40 mg. of fresh insect material, and 34 μmoles of Na succinate added from the side arm of the vessel. Total volume was 3.0 ml. Readings were taken at 5 minute intervals at 30° C for 20 minutes. Controls without succinate were run parallel. The activity of succinoxidase was expressed as the difference in O_2 uptake by the samples with and without succinate.

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* Such pH values had been determined as optimal in a preliminary series of experiments.

** Thiourea is not necessary in measurements of succinoxidase activity. It was used, however, in these experiments in order to keep the composition of the mixture as similar as possible to the mixture for the determination of cytochrome c oxidase. Thiourea in concentration used here is almost without effect on the activity of succinic dehydrogenase, succinoxidase and cytochrome c oxidase.

RESULTS

It was observed that a considerable decolorization of methylene blue by the homogenate took place even in the absence of any added substrate. This "spontaneous" dehydrogenase activity, expressed in Fig. 1 (lower curve) as the reciprocal of decolorization time,

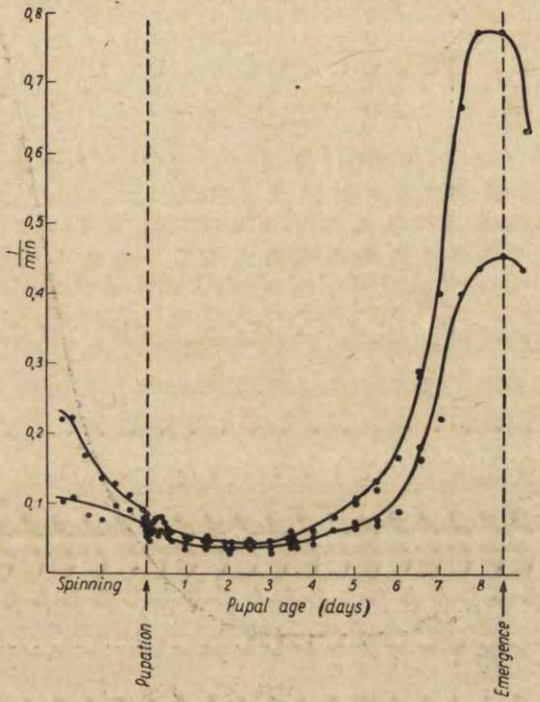

Fig. 1. Rate of methylene blue reduction by homogenates of waxmoth pupae; lower curve, spontaneous reduction; upper curve, reduction in the presence of Na succinate. The rate of methylene blue reduction is expressed as the reciprocals of decolorization time in minutes

varied according to a U-shaped curve during the metamorphosis. The time of decolorization was shortened when succinate was added to the incubation mixture (Fig. 1, upper curve). The difference of reciprocals of these two values was a measure of succinic dehydrogenase activity. Its variation during the metamorphosis is shown in Fig. 2. It is apparent that a decrease in the activity of succinic dehydrogenase begins at the period of cocoon spinning by the larva. Shortly after pupation, a small rise in the activity of

succinic dehydrogenase was observed, which lasted a few hours. Later on, the activity decreased again and remained on a low level for some two days. Starting at the 4th day of pupal life, the activity increased rapidly and attained its maximum at pupal age of 8 days, i.e. a few hours before emergence of the adult insect. An

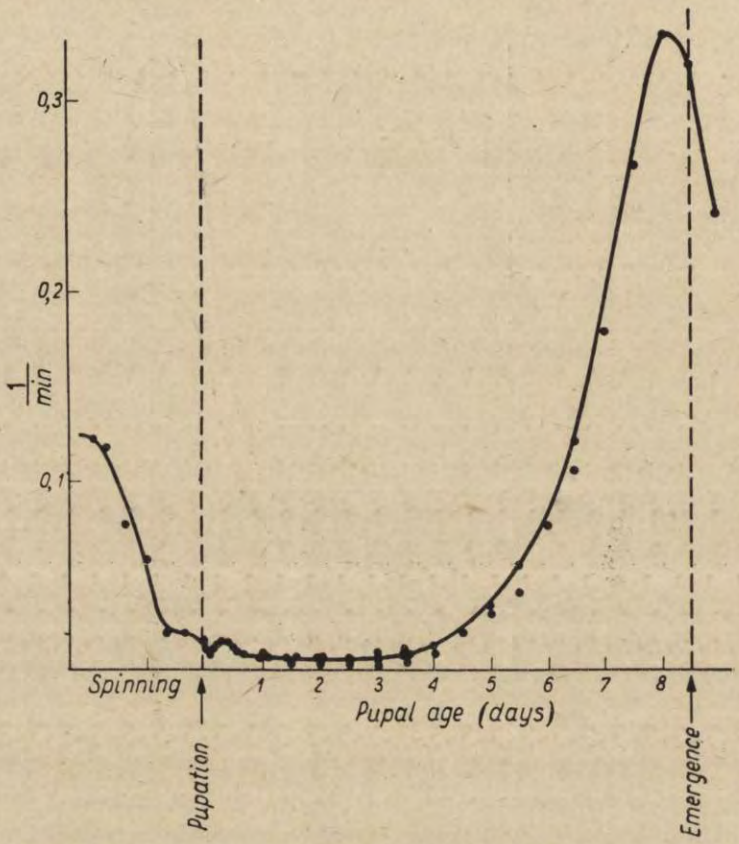

Fig. 2. Variation in activity of succinic dehydrogenase during metamorphosis of the waxmoth. Activity is expressed according to the formula: Activity = 1/a − 1/b (for explanation see text)

almost 70-fold increase of succinic dehydrogenase activity occurred from the 3rd day of pupal age till the emergence (see also Fig. 4).

Similar patterns are followed by the variation in cytochrome c oxidase activity (Fig. 3, upper curve), with the two exceptions: 1) the maximum of the activity appears about one day before

emergence, and 2) the maximal activity to minimal activity ratio is only about 12 (Fig. 4).

The succinoxidase activity also follows a U-shaped curve (Fig. 3, lower curve) with a small and transient rise at about 10

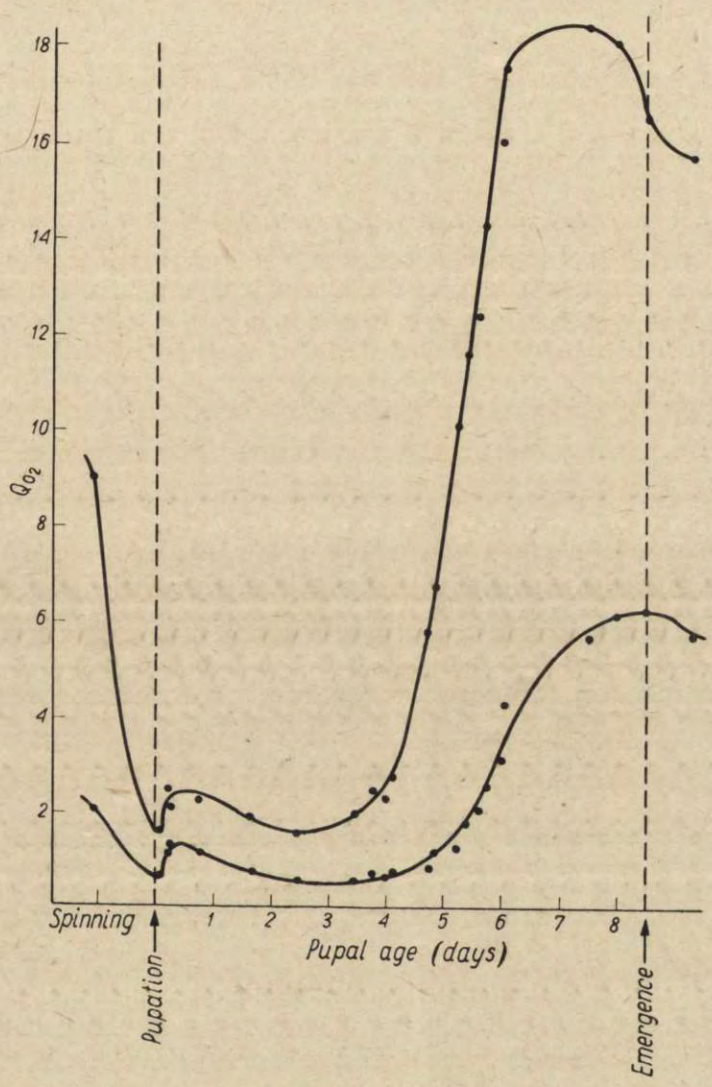

Fig. 3. Variations in activity of cytochrome c oxidase (upper curve) and of succinoxidase (lower curve). Activity is expressed in ml. O_2 per hour per gram fresh insect material (QO_2)

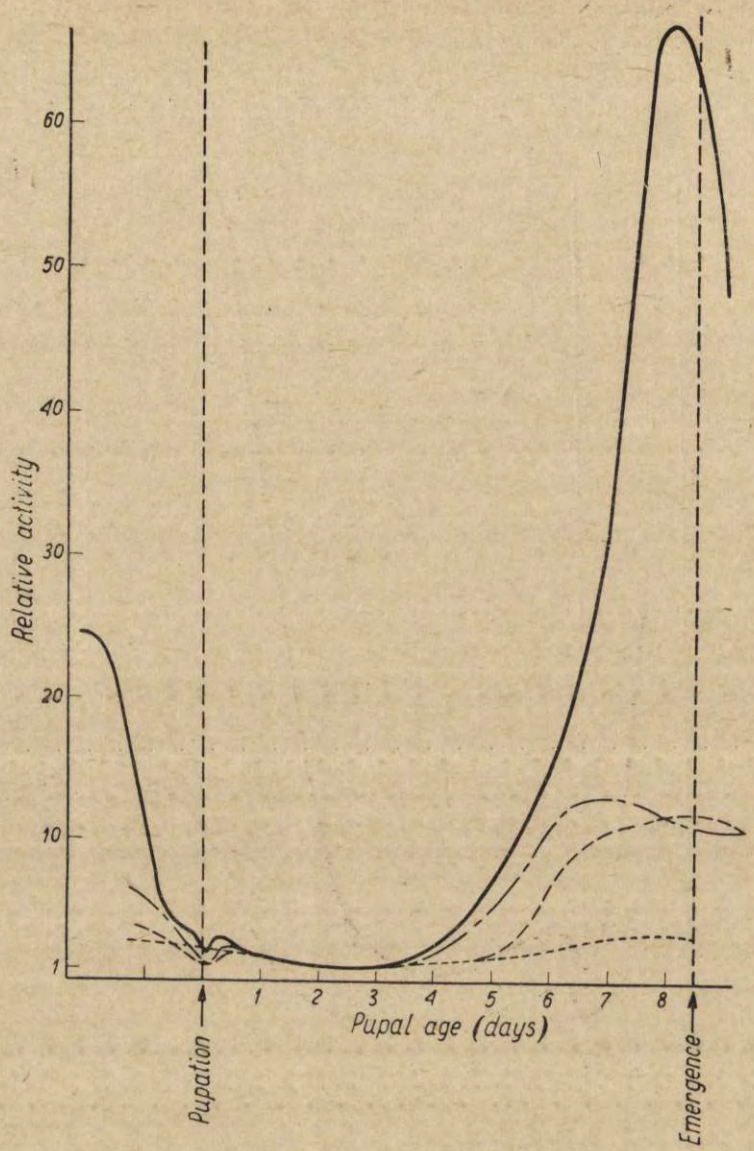

Fig. 4. Acitivities of succinic dehydrogenase (———), cytochrome c oxidase (— · — · — ·) and succinoxidase (- - - - - -), and oxygen uptake (. . . . . .) by intact pupae (according to W o j t c z a k 1952) in the course of metamorphosis, expressed in relative units, activity at pupal age of 3 days being taken as unity

hours after pupation and a maximum which coincides with the emergence of imago. Maximal activity to minimal activity ratio amounts to 12, as is the case of cytochrome c oxidase (Fig. 4).

The ratio of cytochrome c oxidase activity to succinoxidase activity is approximately constant throughout the pupal life and amounts to about 3 in the state of minimum enzyme activity as well as at the end of pupal development.

Fig. 4 illustrates the variation in activity of succinic dehydrogenase, cytochrome c oxidase and succinoxidase expressed in relative units: the activity of all these enzymes at their common minimum (3 days after pupation) being taken as unity.

DISCUSSION

The present experiments support the view that the activities of most respiratory enzymes follow U-shaped curves during the pupal development of holometabolous insects. Succinic dehydrogenase and cytochrome c oxidase have been studied in a number of insect species (Agrell 1949, Sacktor 1951, Bodenstein and Sacktor 1952, Ludwig 1953, Ito 1955, Ludwig and Barsa 1956) and results similar to those of the present experiments have been obtained. Most of the investigations cited above are, however, concerned with either the dehydrogenase or the oxidase activity. So far as we know, there are only two publications (Ito 1955, Ludwig and Barsa 1956), which like the present paper, describe both the succinic dehydrogenase and the cytochrome c oxidase activities in the same species.

The present investigations make it possible to compare the relation of activities of the components of the succinoxidase system in the course of metamorphosis. It is apparent that cytochrome c oxidase of the waxmoth pupa is not "saturated" with the succinic dehydrogenase, since the activity ratio of succinoxidase to cytochrome c oxidase is far below unity. Furthermore, it seems that succinic dehydrogenase is not the rate limiting factor of the succinoxidase system, because the variation in activity of the system is not proportional to the variation in succinic dehydrogenase activity. Thus, at the end of pupal life the activity of the succinic dehydrogenase increases about 70 times as compared with the minimum activity in young pupae, whereas the activity of the whole system increases only 12 times. In the present measurements of

succinoxidase the incubation mixture was supplemented with exogenous cytochrome c, it follows therefore that the level of cytochrome c in the pupa had no influence on the results of the experiments. Hence it seems probable that a component of the succinoxidase system, other than succinic dehydrogenase, cytochrome c oxidase and cytochrome c, is the rate limiting factor of the activity of the whole system in the pupa. This component is situated in the electron transport chain between the succinic dehydrogenase and cytochrome c.

It would be of interest to compare the activity of cytochrome c oxidase with the level of oxygen consumption by intact pupae, although no definite evidence is available as yet that the efficiency of cytochrome c oxidase in electron transport is of the same magnitude in vitro as in vivo. Oxygen uptake by the living pupa of *Galleria mellonella*, as studied by Taylor and Steinbach (1931), Crescitelli (1935) and Wojtczak (1952), follows during the metamorphosis a U-shaped curve and amounts to 0.5 ml. per gram fresh weight per hour at its minimum (about 3 days after pupation) and 1.6 ml. at its maximum, 7.5 days after pupation (Wojtczak 1952). As compared with these values, the in vitro oxygen uptake by cytochrome c oxidase amounts at the age of 3 days to 1.5 ml./g./hour, which is 3 times greater than the O_2 uptake by living pupa, and at the age of 7.5 days it amounts to 18.5 ml./g./hour, which is more than 11 times greater. Fig. 4 illustrates the relative increase in oxygen consumption by the developing pupa which is about 3-fold, and in the activity of cytochrome c oxidase which is about 12-fold. Thus, it seems that the potential efficiency of cytochrome c oxidase present in the body surpasses the actual needs of respiring pupa to a greater extent at the end of pupal life than in young pupae. This fact may be explained by the formation of imaginal muscles which are very rich in cytochrome c oxidase and have a rather low resting respiratory metabolism (Kubišta 1957).

It was shown in the present investigations that a small and transient increase in the activity of all the enzymes studied occurred shortly after pupation. A similar increase in activity of some dehydrogenases has been observed by Agrell (1949) in *Calliphora erythrocephala*. No explanation of this phenomenon is possible as yet.

It is well known that the succinoxidase system is strictly bound to the mitochondria, and it would be of particular interest to study the changes which occur in insect mitochondria in the course of metamorphosis. This is, however, a separate problem.

SUMMARY

The activity of succinc dehydrogenase, cytochrome c oxidase and succinoxidase was measured throughout the pupal stage of the waxmoth, *Galleria mellonella* L. Succinic dehydrogenase, cytochrome c oxidase and the whole succinoxidase system decrease in activity during the cocoon spinning of the larva and at the prepupal stage. They continue to decrease after pupation unitil a pupal age of about 3 days. Starting at this age, a considerable increase in activity of all the enzymes studied occurs. Maximal activity is attained at the moment of the emergence of adult insect or shortly before.

REFERENCES

AGRELL I. 1949 — The variation of apodehydrogenases during insect metamorphosis. Acta Physiol. Scand. *18*, p. 355.

BODENSTEIN D. and SACKTOR B. 1952 — Cytochrome c oxidase activity during the metamorphosis of *Drosophila virilis*. Science *116*, p. 299.

CRESCITELLI F. 1935 — The respiratory metabolism of *Galleria mellonella* at different constant temperatures. J. Cell. Comp. Physiol. *6* ,p. 351.

ITO T. 1955 — The physiology in the metamorphosis of *Bombyx mori*. II. Succinoxidase system in the pupal stage. Annot. Zool. Jap. *28*, p. 1.

KEILIN D. and HARTREE E. F. 1945 — Purification and properties of cytochrome c. Biochem. J. *39*, p. 289.

KUBIŠTA V. 1957 — The influence of carbon monoxide and cyanide on the resting metabolism of insect muscle. Acta Soc. Zool. Bohemslov. *21*, p. 126.

LUDWIG D. 1953 — Cytochrome oxidase activity during diapause and metamorphosis of the Japanese beetle (*Popillia japonica* Newman). J. Gen. Physiol. *36*, p. 751.

LUDWIG D. and BARSA M. C. 1956 — Variations in the activity of the succinoxidase system during the development of the mealworm, *Tenebrio molitor* Linnaeus (Coleoptera, Tenebrioidae). Ann. Entomol. Soc. America *49*, p. 103.

SACKTOR B. 1951 — Some aspects of respiratory metabolism during metamorphosis of normal and DDT-resistant house flies, *Musca domestica* L. Biol. Bull. *100*, p. 229.

SCHNEIDER W. C. and POTTER V. R. 1943 — The essay of animal tissues for respiratory enzymes. II. Succinic dehydrogenase and cytochrome oxidase. J. Biol. Chem. *149*, p .217.

TAYLOR I. R. and Steinbach H. B. 1931 — Respiratory metabolism during pupal development of *Galleria mellonella*. Physiol. Zool. *4*, p. 604.

WOJTCZAK L. 1952 — Studies on the biochemistry of the waxmoth (*Galleria mellonella*). II. Respiratory enzymes in development. Acta Biol. Exper. *16*, p. 223.

Acta Biologiae Experimentalis, Vol. XVIII, 1958

INTRACELLULAR LOCALIZATION OF ENZYMES IN THE WAXMOTH, *GALLERIA MELLONELLA* L.

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(Received for publication 10 April 1957)

The development of the cell fractionation technique has greatly increased our knowledge of the intracellular distribution of enzymes, especially in mammalian tissues. Investigations on the localization of enzymes in insect cells are, however, few in number. There remain still many problems to be elucidated and more work must be done to build up a more general picture of enzyme distribution in the insect body.

The intracellular localization of cytochrome c oxidase, of succinic dehydrogenase and of the succinate-cytochrome c reductase system has been investigated in insect muscles by Watanabe and Williams (1951) and by Sacktor (1953b) and in insect embryos by Bodine, Lu and West (1952). These enzymes were found to be concentrated in the mitochondrial fraction, but neither cell nuclei nor the soluble cytoplasm were examined more closely.

There is little information on the distribution of phenoloxidase (polyphenoloxidase, tyrosinase) in insect cells. Some data point to cell granules as the site of this enzyme in insects (Danneel 1943). In mammals, phenoloxidase was found in microsomes of the malignant tissue, melanoma (Lerner et al. 1949).

Alkaline and acid phosphatases were found in insects by Drilhon (1943) and later on investigated by a number of authors

(Fitzgerald 1949, Drilhon and Busnel 1945, Denucé 1952, and others). The localization of phosphatases in insect tissues was studied by histochemical methods by Bradfield (1946) and Day (1949). However, no precise information is available on intracellular localization of these enzymes in insects.

Adenosinetriphosphatase (apyrase) has been investigated in insects by Gilmour (1948), Gilmour and Calaby (1952), Sacktor et al. (1953), Maruyama (1954), Sakagami and Maruyama (1956) and others. In insect muscles this enzyme is chiefly bound to mitochondria (Gilmour 1953, Sacktor 1953a).

Insect pyrophosphatase has been extensively investigated by McElroy et al. (1951), Denucé (1952), Niemierko and Wojtczak (1952) and others. This enzyme was found in the soluble fraction of the housefly preparation (Sacktor 1953a).

The purpose of the present experiments is to investigate the problem of intracellular localization of enzymes in the larva of the waxmoth, *Galleria mellonella* L., which is the object of many studies of this Laboratory. In addition, further information on properties of the enzymes studied were obtained.

METHODS

Tissue fractionation

Fully grown larvae of the waxmoth, *Galleria mellonella* L. were chilled on ice and homogenized with nine volumes of 0.25 M sucrose. In this way a 10 per cent homogenate was obtained, one milliliter of which corresponded to 100 mg. of fresh tissue weight. In order to remove unbroken cells and fragments of cuticle and tissues, the homogenate was centrifuged for 5 minutes at low speed (about 100 × g) and the resulting "clean" homogenate was fractionated as follows. The homogenate was centrifuged for 15 minutes at 600 × g. The sediment, which consisted mainly of cell nuclei, was washed with 0.25 M sucrose, recentrifuged, and dispersed in 0.25 M sucrose so that the final volume was equal to the volume of the homogenate used, thus yielding the "10 per cent nuclear suspension". The supernatant was further centrifuged for 15 minutes at about 20,000 × g. The sediment was washed with sucrose, recentrifuged, dispersed in 0.25 M sucrose and adjusted with additional sucrose to the volume of the initial homogenate. This "10 per cent mitochondrial suspension" appeared, on microscopic examination, to consist of mitochondria-like granules of various size and shape. The final supernatant fluid was once more centrifuged for 15 minutes at 20,000 × g. Some sediment, which was sometimes formed, was discarded

and the clear supernatant was used for further experiments as the soluble or cytoplasmic fraction. It contained, however, microsomes, which were not isolated by this procedure.

All steps of the fractionation were carried out at 0° to +2°C.

Enzyme assays

Unless otherwise stated, all fractions and homogenate were used in "10 per cent" concentration.

Phenoloxidase activity was measured manometrically essentially as described previously (Wojtczak 1952). Each vessel of the Warburg respirometer contained 2.0 ml. of the investigated cell fraction and 0.5 ml. of substrate solution: 0.1 M p-cresol or 0.1 M catechol. The temperature was 30°C.

Cytochrome c oxidase activity. This was measured manometrically by a modification of the method of Schneider and Potter (1943), in which thiourea in a final concentration of 0.01 M was used to inhibit phenoloxidase, because of the interference of this enzyme in the oxidation of ascorbate. In this concentration thiourea affects cytochrome c oxidase to a negligible degree (L. Wojtczak, unpublished). Each respirometer vessel contained: 0.04 μmole cytochrome c, 20 μmoles thiourea, 0.08 μmole $AlCl_3$, 30 μmoles sodium ascorbate (added from the side arm), 0.05 ml. to 0.30 ml. of the cell fraction diluted to 5 per cent concentration, and 0.06 M phosphate buffer, pH 7.4, to a final volume of 2.0 ml.; 0.1 ml. of 10 per cent KOH was present in the center well. Readings were taken for 40 to 60 minutes at 10 min. intervals at 30°C. Autooxidation of ascorbate was calculated by extrapolation.

Succinate-cytochrome c reductase was determined by measuring spectrophotometrically the rate of cytochrome c reduction in the presence of succinate. The reaction mixture, per milliliter, contained: 0.02 μmole cytochrome c, 5 μmoles KCN, 12.5 μmoles sodium succinate, about 45 μmoles phosphate buffer, pH 7.2, and 8 to 32 μl of the homogenate or the investigated cell fraction. Final volume was 0.8 ml., the light path 2.0 cm. Readings were taken (at 550 mμ) every 30 seconds at room temperature about 20°C.

The activity of acid phosphatase was measured by the rate of glycerophosphate hydrolysis in the following incubation mixture (per milliliter): 30 μmoles sodium glycerophosphate, 10 μmoles $MgSO_4$, 100 μmoles acetate buffer, pH 5.0, and 0.25 ml. of the homogenate or the cell fraction. Total volume was 4.0 ml. The mixture was incubated for 60 minutes at 37°C, the reaction was stopped by trichloroacetic acid, and orthophosphate was determined by the method of Fiske and Subbarow (1925).

Alkaline phosphatase was measured with the same reaction mixture, except that in these experiments carbonate buffer, pH 10.0, was used.

In experiments on the influence of pH on the enzymatic hydrolysis of glycerophosphate similar incubation mixtures were used, except that in

this case varying pH values were obtained by means of the following buffer solutions: acetate, pH 3.6 to 5.6 (Walpone 1914), veronal, pH 6.3 to 9.2 (Michaelis 1930), glycine-bicarbonate (0.3 M glycine plus 0.15 M $NaHCO_3$), pH 7.2 to 9.0, and carbonate-bicarbonate, pH 9.2 to 10.7 (Delory and King 1945). All pH values were measured with the glass electrode.

Pyrophosphatase activity was determined with a medium, which contained, per milliliter, 4 μmoles sodium pyrophosphate, 5 μmoles $MgSO_4$, glycine-bicarbonate buffer, pH 8.3 (75 μmoles glycine and 37.5 μmoles $NaHCO_3$), and 0.2 ml of 1.25 per cent cell fraction or homogenate. Total volume was 5.0 ml., incubation time 15 minutes, temperature 37°C. The reaction was stopped by the addition of trichloroacetic acid and orthophosphate determined.

Adenosinetriphosphatase (ATPase) activity was determined with the following incubation mixture, per milliliter: 1 μmole ATP*, 1 μmole $MgSO_4$, 100 μmoles veronal buffer, pH 8.0, and 0.33 ml. of 2.5 per cent cell fraction or homogenate. Final volume was 3.0 ml, incubation time 10 minutes at 37°C. The reaction was stopped and orthophosphate determined as mentioned above.

Other methods. ATP was obtained from rabbit muscles (Meshkova and Severin 1950).

Cytochrome c was prepared according to Keilin and Hartree (1945). Its concentration was measured by the increase in optical density at 550 mμ on reduction with ascorbate. The extinction coefficients of 8.8×10^6 and 28.3×10^6 cm^2/mol for the oxidized and reduced forms of cytochrome c (Theorell and Åkesson 1941) were used.

RESULTS

The localization of phenoloxidase was studied with two substrates: p-cresol and catechol. The results are shown in Tables I and II, from which it is apparent that the distribution patterns of phenoloxidase activity are different for the two substrates used. It appeared that p-cresol was almost exclusively oxidized by the soluble fraction, whereas catechol was oxidized by both the soluble and mitochondrial fractions. Some catecholase activity was also found in nuclei, but this result may be due to contaminations. Cresolase activity of mitochondria is very low. It is noteworthy that the cresolase:catecholase ratio amounts to 1 in the whole homogenate and in the cytoplasmic fraction, whereas it is about 0.24 in mitochondria.

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* The following abbreviations are used: ATP, adenosine triphosphate; ATPase, adenosinetriphosphatase; EDTA, ethylendiaminetetraacetate; DNP, 2,4-dinitrophenol; PCMB, p-chloromercuribenzoate.

Two possible explanations of this might be taken into account: 1° two different phenoloxidases of various cresolase:catecholase ratios may be present, or 2° the same enzyme may be distributed among mitochondria and cytoplasm, and its higher cresolase:catecholase ratio observed in the cytoplasm may be attributed to some stimulatory effect on the cresolase activity of some compounds that are present in the soluble fraction. The second possibility might be supported by observations that small amounts of ortho-diphenols (Kertesz 1947) and ascorbic acid (Kendall 1949) stimulate monophenol oxidation.

Table I

Intracellular localization and substrate specifity of waxmoth phenoloxidase. Enzyme activity in the homogenate is expressed in µl. O_2 per hour per mg fresh tissue weight; activity in cell fractions is calculated for the amount of fraction, which corresponds to one mg. fresh tissue. The data are means of several experiments

Cell fractions	Activity		Cresolase to catecholase ratio
	with p-cresol	with catechol	
	means and standard deviations		
Whole homogenate	4.40 ± 0.62	4.50 ± 0.48	0.96
Nuclei	0.17 ± 0.10	0.46 ± 0.25	
Mitochondria	0.23 ± 0.12	0.95 ± 0.24	0.24
Cytoplasm (plus microsomes)	3.60 ± 0.23	3.86 ± 0.92	0.93

To elucidate this question experiments were performed, in which small quantities of catechol (1 µmole for each vessel), or of ascorbic acid (10 µmoles for vessel), or of the soluble fraction, thermally inactivated, (1.0 ml for vessel) were added to the mitochondria shaken with p-cresol as substrate. Oxygen consumed by the oxidation of the substances added was subtracted and the net oxygen consumption by p-cresol oxidation was compared. It became apparent that neither catechol nor ascorbic acid stimulated p-cresol oxidation by the mitochondria. Thermally inactivated soluble fraction had no effect as well. Thus, it seems that high cresolase:catecholase ratio of the soluble fraction is not due to stimulatory effect of some compounds present in it; the possibility may be taken into account that two different phenoloxidases exist in mitochondria and in the cytoplasm.

Experiments on cytochrome c oxidase showed that this enzyme was located chiefly in mitochondria, but some activity was always found in the soluble fraction as well (Table II). In spite of thorough removing of mitochondria by duplicate centrifugation, the supernatant fluid contained some oxidase activity, which sometimes amounted to 16 per cent of the activity in the whole homogenate.

Table II

Intracellular distribution of enzymes in waxmoth larva. Absolute activities of the enzymes in the homogenate are calculated per mg. fresh tissue weight. Activities of the enzymes in cell fractions are expressed in per cent of their activities in the whole homogenate. The last column gives the sum of activities in cell fractions as per cent of activities in the homogenate. All values are means of 3 to 8 experiments

Enzyme	Homogenate	Nuclei	Mitochondria	Cytoplasm and microsomes	Recovery
	absolute activities	percentage values			
	means and standard deviations				
Phenoloxidase: cresolase activity	4.40 ± 0.62 µl O_2/mg/hour	3 ± 2	5 ± 3	83 ± 19	91
catecholase activity	4.50 ± 0.48 µl O_2/mg/hour	10 ± 6	21 ± 5	86 ± 19	117
Cytochrome c oxidase	15.6 ± 1.7 µl O_2/mg/hour	4 ± 2	62 ± 4	10 ± 6	76
Succinate-cytochrome c reductase	0.0031 ± 0.0010 µ M red. cyt./mg/min	5 ± 3	42 ± 7	0	47
Acid phosphatase	1.06 ± 0.18 µg P/mg/hour	0	35 ± 6	65 ± 7	100
Alkaline phosphatase	2.50 ± 0.85 µg P/mg/hour	19 ± 3	47 ± 5	37 ± 5	103
Pyrophosphatase	36 ± 6 µg P/mg/15 min	4 ± 2	18 ± 2	80 ± 4	102
ATPase	2.0 ± 0.3 µg P/mg 10 min	12 ± 4	67 ± 5	19 ± 3	98

Succinate-cytochrome c reductase was found exclusively in the mitochondrial fraction (Table II). In nuclei and in the soluble fraction only trace amounts of the activity could be detected. In the soluble fraction a rather intense endogenous non-enzymic cytochrome c reduction was observed, but it was neither malonate-sensitive nor was it enhanced by the addition of succinate. The succinate-cytochrome c reductase of mitochondria was inhibited by about 95 per cent by 1.25×10^{-2} M malonate (at succinate concentration 1.25×10^{-2} M). Only about 50 per cent of the activity of homogenate was recovered after fractionation (Table II); a similar fact han been also observed in mammalian liver (De Duve at al. 1955). In our experimental conditions the reduction of cytochrome c by succinate followed zero order kinetics in respect to cytochrome c, i.e. the reduction rate was independent of cytochrome c concentration. Only below the concentration of about 10^{-6}M, cytochrome c became the rate limiting factor (Fig. 1). This is in contrast to first order kinetics found for succinate-cytochrome c reductase of mammalian tissues (Cooperstein et al. 1950) and of insect muscles (Sacktor 1953b).

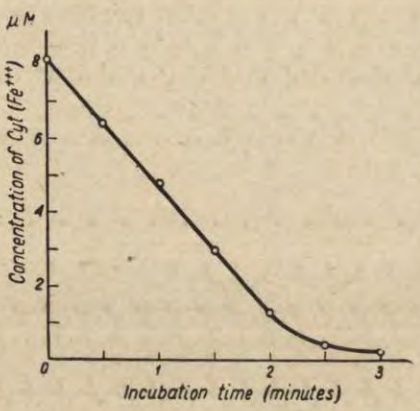

Fig. 1. Enzymic reduction of cytochrome c by succinate as function of time. The incubation mixture contained 25 μl mitochondrial suspension

Phosphatases. In preliminary experiments on the whole homogenate the effect of pH on phosphatase activity was studied. Two optima were found, one in the acid range at pH 5.0 to 5.5, and the other in the alkaline range at pH about 10.0 (Fig. 2). All subsequent experiments were carried out only at these two optimal pH values.

Distribution patterns of the two phosphatases are shown in Table II, from which it is apparent that the acid phosphatase is located in mitochondria and cytoplasm, whereas the alkaline phosphatase is present in all three fractions.

The effect of a number of substances on phosphatase activity was examined. These experiments are summarized in Tables III and IV for acid and alkaline phosphatases respectively. It was found that the acid phosphatase of the soluble fraction was acti-

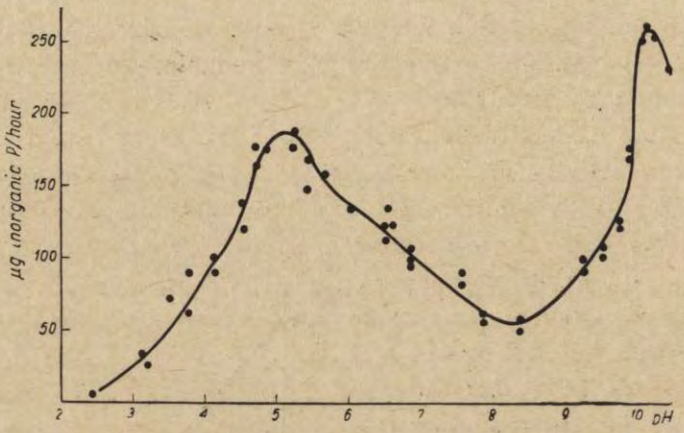

Fig. 2. Glycerophosphatase activity at different pH values. The incubation mixture contained 1.0 ml. of 10 per cent homogenate

vated by Mg^{++} and Co^{++}, whereas in mitochondria these ions had no influence. An inhibitory effect of Zn^{++} was more pronounced on mitochondrial acid phosphatase than on the phosphatase of the soluble fraction. The enzyme in both fractions was strongly in-

Table III

Activation and inhibition of acid phosphatase. Activity in the absence of any substance tested was taken as 100

Substance tested	Concentration (M)	Homogenate	Mitochondria	Cytoplasm
		per cent activity		
Mg^{++}	0.005	109	100	145
Co^{++}	0.005	165	100	185
Ca^{++}	0.005	72	100	97
Mn^{++}	0.005	100	120	100
Zn^{++}	0.005	67	55	90
F^{-}	0.001	25	15	25
Cysteine	0.001	81	100	84

hibited by 0.001 M fluoride and slightly by cysteine. From these experiments the question arose as to whether these differences in susceptibility to activatory and inhibitory effects of the mitochondrial and of the soluble phosphatases might point to the existence

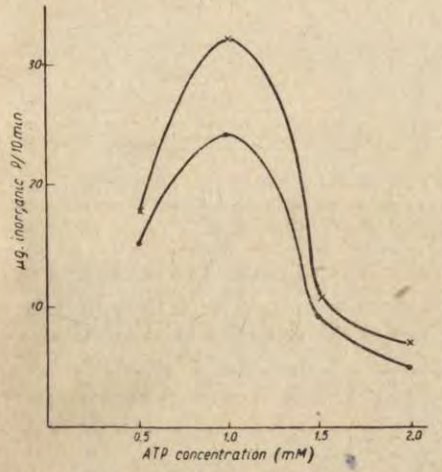

Fig. 3. Effect of ATP concentration on ATPase activity. The incubation mixture contained 1.0 ml. of 1.25% homogenate (lower curve) and 1.0 ml. of 2.5% homogenate (upper curve)

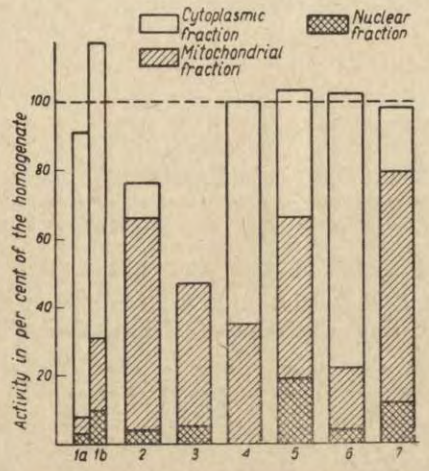

Fig. 4. Intracellular distribution of enzymes in the waxmoth larva
1 — phenoloxidase: cresolase activity (a) and catecholase activity (b), 2 — cytochrome c oxidase, 3— succinate-cytochrome c reductase, 4 — acid phosphatase, 5 — alkaline phosphatase, 6 — pyrophosphatase, 7 — ATPase

of two acid phosphatases or whether the differences could be attributed to some secondary effect, e. g. to the structure of mitochondria.

For this reason experiments were performed, in which mitochondria were destroyed by pretreatment with hypotonic medium, and the properties of acid phosphatases were studied. It appeared that the effect of Mg^{++}, Co^{++} and Zn^{++} was the same independently of whether intact or damaged mitochondria were examined.

The alkaline phosphatase of the waxmoth differs from the acid phosphatase by being insensitive to 0.001 M fluoride (Table IV). The enzyme is not activated by Mg^{++} and Co^{++} and is strongly inhibited by Zn^{++} (0.005 M) and by cysteine (0.001 to 0.0025 M). It is noteworthy that neither 0.001 M glutathione nor butyl mercaptan (in saturated water solution) have any effect.

Pyrophosphatase activity, as shown in Table II, is chiefly found in the soluble fraction. The enzyme is inactive in the absence of Mg^{++}. A pronounced inhibitory effect of Zn^{++} (0.005 M) was observed. No inhibition by 0.001 M monoiodoacetate could be observed, while about 25 per cent inhibition took place under the influence of 0.0001 M p-chloromercuribenzoate (PCMB). Glutathione in concentration 0.0002 M was without effect.

Table IV

Activation and inhibition of alkaline phosphatase. Activity in the absence of any substance tested was taken as 100

Substance tested	Cocentration (M)	Homogenate	Nuclei	Mitochondria	Cytoplasm
		per cent activity			
Mg^{++}	0.005	110	112	111	109
Co^{++}	0.005	91	100	100	100
Ca^{++}	0.005	100	[illegible]	100	100
Mn^{++}	0.005	100	100	100	100
Zn^{++}	0.005	9	38	27	0
F^{-}	0.001	95	101	95	90
Cysteine	0.001	31			
	0.0025	25	0	16	0
Cystine	0.001	81			
Glutathione	0.001	100			
Buthyl mercaptan	saturated water solution	90			

More detailed studies on properties of waxmoth pyrophosphatase were published elsewhere (Wojtczak 1956).

Adenosinetriphosphatase. Some properties of waxmoth ATPase were studied on the whole homogenate. Preliminary experiments proved that there was no close relationship between the amount of orthophosphate liberated and the amount of homogenate used. Moreover, the rate of ATP hydrolysis was not constant in the time interval of from 5 to 30 minutes. These irregularities were perhaps, caused by interference of adenylate kinase. Therefore, results of experiments described below have only a tentative character. In these experiments it was shown that the high-

est activity was obtained at the substrate concentration of 0.001 M (Fig. 3); higher ATP concentrations were inhibitory. Full activity was achieved when magnesium ions were present. The pH optimum appeared to lie between 7.2 and 8.0. All subsequent experiments were carried out at pH 8.0 (veronal buffer).

Table V

Effect of various substances on the activity of ATPase. Acitivity in the absence of any substance tested was taken as 100. In all experiments 0.001 M Mg^{++} was present

Substance tested	Concentration (M)	Homogenate	Mitochondria	Cytoplasm
		per cent activity		
Ca^{++}	0.005	54	35	
Mn^{++}	0.005	43		
Zn^{++}	0.005	20	10	
F^{-}	0.001	95	100	90
PCMB	0.0001	68		
NaN_3	0.001	71	25	
Cysteine	0.005	100		
Glutathione	0.0002	100		
DNP	0.001	100	100	100

The ability of various cell fractions to hydrolyze ATP is shown in Table II. It is apparent that the highest ATPase activity is associated with mitochondria. The ATPase of the waxmoth is strongly inhibited by divalent cathions: Ca^{++} Mn^{++} and Zn^{++} (Table V); fluoride is without effect; PCMB and sodium azide are inhibitory. No activation by 2,4-dinitrophenol was observed.

Figure 4 represents graphically intracellular distribution of the enzymes in waxmoth larva.

DISCUSSION

The present investigations give some new informations on intracellular distribution and properties of enzymes in an insect. It must be pointed out, however, that since homogenates of whole larvae of the waxmoth were fractionated in the procedure described above, figures obtained are a resultant of distribution patterns of enzymes in various tissues and organs of the insect studied.

It is known from the papers of W a t a n a b e and W i l l i a m s (1951) and S a c k t o r (1953b) that in insect muscles, like in mammalian tissues, cytochrome c oxidase is located exclusively in mito-

chondria. In our experiments on waxmoth larvae, some activity of cytochrome c oxidase was also observed in the so called "soluble fraction". It may be supposed that some small mitochondria-like particles exist in tissues of the larva; these particles are not sedimented at 20 000 × g and may account for cytochrome oxidase activity present in our "soluble fraction".

The present experiments demonstrate that in the waxmoth, like in muscles of the house fly (Sacktor 1953b), the succinate--cytochrome c reductase is strictly bound to mitochondria. This is in accordance with numerous observations on vertebrate tissues as well. The present findings disagree, however, with the results of Sacktor (1953b) with insect muscles and of Cooperstein et al. (1950) with mammalian tissues as far as kinetics of cytochrome c reduction are concerned: a zero order rate was observed in our experiments, while first order kinetics are reported by the above mentioned authors. This discrepancy is, in our opinion, not a result of some differences in experimental conditions, since when we examined mitochondria of rat liver in identical conditions as mitochondria of the waxmoth (experiments not reported here) a typical first order rate of cytochrome c reduction by succinate was observed.

As far as phenoloxidase is concerned, the present investigations are the first attempt to examine its localization in insect cells by fractionating centrifugation technique. The results are in accordance with the observations of Lerner et al. (1949) for mammalian phenoloxidase and point to the cytoplasm or microsomes as the principal sites of this enzyme in the insect cell. Earlier experiments of Danneel (1943) pointed, however, to some not-filtrable structures of insect "brei" as sites of polyphenoloxidase.

As found in the present experiments, some phenoloxidase activity was bound to mitochondria. The enzymic activity of the latter differs, however, from that of the soluble fraction by being far more intense wth polyphenols than with monophenols. It seems that mitochondrial phenoloxidase possesses other properties than the enzyme from the soluble fraction.

The present results indicate that the general features of the intracellular distribution of dephosphorylating enzymes in the waxmoth are similar to those found in mammals and in insects so far studied.

Acid phosphatase was found in the waxmoth larva in both mitochondria and the cytoplasm (plus microsomes). Similar results were obtained by Palade (1951) and by Waked and Kerr (1955) for mammalian tissues, and the opinion has been expressed (De Duve et al. 1955; Thomson and Moss 1956) that this enzyme is bound to some kind of intracellular granules of a size intermediate between mitochondria and microsomes.

The distribution of alkaline phosphatase in the waxmoth among cytoplasm, mitochondria and nuclei resembles to intracellular distribution of this enzyme in mammalian tissues (Emery and Dounce 1955, Waked and Kerr 1955).

Some properties of waxmoth phosphatases are similar to those of mammals: inhibition of the acid phosphatase by fluoride and inhibition of the alkaline phosphatase by cysteine (Morton 1955). On the other hand, the alkaline phosphatase of the waxmoth is strongly inhibited by zinc ions, whereas in mammals it is activated by Zn^{++} (Hoare and Delory 1955, Hofstee 1955).

Some differences in acitvation and inhibition of the acid phosphatase in mitochondria and in the soluble fraction suggest the possibility that two different enzymes are present in the waxmoth. This point needs, however, further investigation.

It was shown by the present investigations that pyrophasphatase in waxmoth cells is located in the cytoplasm, similarly to what had been found for the house fly (Sacktor 1953a) and for mammalian tissues (Swanson 1952, Weked and Kerr 1955).

The present experiments on localization and properties of waxmoth ATPase agree with earlier results of Gilmour (1953) and Sacktor (1953a) who have investigated ATPase in insect muscles. The following properties seem to be common to insect mitochondrial ATPases: activation by Mg^{++}, inhibition by Ca^{++}, and insensitivity to flouride. Also an inhibitory effect of excess substrate, as reported in the present experiments, has been observed by Gilmour and Calaby (1952) for the ATPase of insect muscles. Insect ATPase resembles the ATPase of mammalian liver (Swanson 1951, Novikoff et al. 1952) by being bound to mitochondria, but contrary to mammalian ATPase, it is insensitive to fluoride.

Some new evidence is given in this study that the general features of intracellular localization of enzymes in insects are similar to those found in mammals, although some peculiarities of enzyme distribution in insect cells have been noticed.

SUMMARY

Intracellular localization of enzymes in larva of the waxmoth, *Galleria mellonella* L., was studied by the cell fractionation technique. The three following cell fractions were investigated: nuclear, mitochondrial, and cytoplasmic or soluble fraction; the latter contained microsomes, which were not separated in this procedure.

Succinate-cytochrome c reductase was found to be exclusively present in mitochondria. Also a great part of cytochrome c oxidase was present in mitochondria, but about 10 per cent of the total of this enzyme was always found in the cytoplasmic fraction. Phenoloxidase was mainly found in the cytoplasm. Acid phosphatase was present in mitochondria and in the cytoplasm, and alkaline phosphatase in all three fractions. Pyrophosphatase was confined to the cytoplasm and adenosinetriphosphatase mainly to mitochondria.

Some properties of these enzymes in the waxmoth are described.

REFERENCES

APPELMANS F., WATTIAUX R. and DE DUVE C. 1955 — Tissue fractionation studies. 5. The association of acid phosphatase with a special class of cytoplasmic granules in rat liver. Biochem. J. *59*, p. 438.

BODINE J. H., LU K. H. and WEST W. L. 1952 — Succinic dehydrogenase in mitotically active and blocked embryonic cells. Physiol. Zool. *25*, p. 109.

BRADFIELD J. R. G. 1946 — Alkaline phosphatase in invertebrate sites of protein secretion. Nature *157*, p. 876.

COOPERSTEIN S. J., LAZAROW A. and KURFESS N. J. 1950 — A microspectrophotometric method for the determination of succinic dehydrogenase. J. Biol. Chem. *186*, p. 129.

DANNEEL R. 1943 — Melaninbildende Fermente bei *Drosophila melanogaster*. Biol. Ztbl. *63*, p. 377.

DAY M. F. 1949 — The distribution of alkaline phosphatase in insects. Austr. J. Sci. Res. B. *2*, 31.

DELORY G. E. and KING E. J. 1945 — Biochem. J. *39*, p. 245; cited by G. Gomori in "Methods in Enzymology" edited by S. P. Collowick and N. O. Kaplan. Academic Press Inc., New York 1955. Vol. *I*, p. 138.

DENECÉ J. M. 1952 — Recherches sur le système phosphatasique des glandes séricigènes chez le ver à soie (*Bombyx mori* L.). Experientia *8*. p. 64.

DRILHON A. 1943 — Sur la présence et l'activité des phosphatases chez les insectes. C. R. Soc. Biol. *137*, p. 390.

DRILHON A. et BUSNEL R. G. 1945 — Recherches sur les phosphatases d'insectes, Bull. Soc. Chim. Biol. *27*, p. 415.

DUVE C. de, PRESSMAN B. C., GIANETTO R., WATTIAUX R. and APPELMANS F. 1955 — Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. Biochem. J. *60*, p. 604.

EMERY A. J. and DOUNCE A. L. 1955 — Intracellular distribution of alkaline phosphatase in rat liver cells. J. Biophys. Biochem. Cytol. *1*, p. 315.

FISKE C. H. and SUBBAROW Y. 1925 — The colorimetric determination of phosphorus. J. Biol. Chem. *66*, p. 375.

FITZGERALD L. R. 1949 — The alkaline phosphatase of the developing grasshopper egg. J. Exper. Zool. *110*, p. 461.

GILMOUR D. 1948 — Myosin and adenylpyrophosphatase in insect muscle J. Biol. Chem. *175*, p. 477.

GILMOUR D. 1953 — Localization of the magnesium-activated apyrase of insect muscle in the sarcosomes. Austr. J. Biol. Sci. *6*. p. 586.

GILMOUR D. and CALABY J. H. 1952 — The Mg-activated apyrase of insect muscle. Arch. Biochem. Biophys. *41*, p. 83.

HOARE R. and DELORY G. E. 1955 — The effect of zinc and magnesium ions on the activity of kidney alkaline phosphatase. Arch. Biochem. Biophys. *59*, p. 465.

HOFSTEE B. H. J. 1955 — Alkaline phosphatase. I. Mechanism of action of Zn, Mg, glycine, versene and hydrogen ions. Arch. Biochem. Biophys. *59*, p. 352.

KEILIN D. and HARTREE E. F. 1945 — Purification and properties of cytochrome c. Biochem. J. *39*, p. 289.

KENDAL L. P. 1949 — The action of tyrosinase on monophenols. Biochem. J. *44*, p. 442.

KERTÉSZ D. 1947 — La théorie de la monophénoloxydation enzymatique. Enzymologia *12*, p. 254.

LERNER A. B., FITZPATRICK T. B., CALKINS E. and SUMMERSON W. H. 1949 — Mammalian tyrosinase. Preparation and properties. J. Biol. Chem. *178*, p. 185.

MARUYAMA K. 1954 — Studies on adenosinetriphosphatases of various insect muscles. J. Fac. Sci. Univ. Tokyo, Sectio IV. *7*, p. 231.

MESHKOVA N. P. i SEVERIN S. E. 1950 — "Practicum po biochimii životnych". Moskva.

MC ELROY W. D., COULOMBRE J. and HAYS R. 1951 — Properties of firefly pyrophosphatase. Arch. Biochem. Biophys. *32*, p. 207.

MICHAELIS J. 1930 — J. Biol. Chem. *87*, p. 33; cited by G. Gomori in "Methods in Enzymology" edited by S. P. Collowick and N. O. Kaplan. Academic Press Inc. New York 1955. Vol. *I*, p. 138.

MORTON R. K. 1955 — The substrat specifity and inhibition of alkaline phosphatase of cow's milk and calf intestinal mucosa. Biochem. J. *61*, p. 232.

NIEMIERKO S. and WOJTCZAK A. 1952 — Meta and pyrophosphatase in the body of waxmoth larvae. Acta Physiol. Polon. Papers of III Physiological Congress, p. *217*. In Polish with English summary.

NOVIKOFF A. B., HECHT L., PODBER E. and RYAN J. 1952 — Phosphatases of rat liver. I. The dephosphorylation of adenosinetriphosphate. J. Biol. Chem. *194*, p. 153.

PALADE G. E. 1951 — Intracellular distribution of acid phosphatase in rat liver cells. Arch. Biochem. Biophys. *30*, p. 144.

SACKTOR B. 1953a — Investigations on the mitochondria of the house fly, *Musca domestica* L. I. Adenosinetriphosphatases. J. Gen. Physiol. 36, p. 371.

SACKTOR B. 1953b — Investigations on the mitochondria of the house fly. *Musca domestica* L. II. Oxidative enzymes with special reference to malic oxidase. Arch. Biochem. Biophys. *45*, p. 349.

SACKTOR B., THOMAS G. M., MOSER J. C. and BLOCH D. I. 1953 — Dephosphorylation of adenosine triphosphate by tissues of the American cockroach, *Periplaneta americana* (L.). Biol. Bull. *105*, p. 166.

SAKAGAMI S. F. and MARUYAMA K. 1956 — Aktivitätsänderung der Adenosintriphosphatase im Flügelmeskel der Bieneanarbeiterinnen während ihres ersten Imaginallebens. Z. Vergl. Physiol. *39*, p. 21.

SCHNEIDER W. C. and POTTER V. R. 1943 — The essay of animal tissues for respiratory enzymes. II. Succinic dehydrogenase and cytochrome oxidase. J. Biol. Chem. *149*, p. 217.

SWANSON M. A. 1951 — Phosphatases of the liver. II. The so called adenosinetriphosphatases. J. Biol. Chem. *191*, p. 577.

SWANSON M. A. 1952 — Phosphatases of the liver. III. "Neutral" pyrophosphatase. J. Biol. Chem. *194*, p. 685.

THEORELL H. and ÅKESSON A. 1941 — J. Am. Chem. Soc. *63*, p. 1804, 1812; cited by S. O. Nielsen and A. L. Lehninger 1955. J. Biol. Chem. *215*, p. 555.

THOMSON J. F. and MOSS E. M. 1956 — The intracellular distribution of a bound acid phosphatase of rat liver as studied by gradient centrifugation. Arch. Biochem. Biophys. *61*, p. 456.

WAKED N. and KERR S. E. 1955 — The distribution of phosphomonoesterases and pyrophosphatase in the particulate fractions of dog cerebrum J. Histochem. Cytochem. *3*, p. 75.

WALPONE G. S. 1914 — J. Chem. Soc. *105*, p. 2501; cited by G. Gomori in "Methods in Enzymology" edited by S. P. Collowick and N. O. Kaplan. Academic Press Inc. New York 1955. Vol. *I*, p. 138.

WATANABE M. I. and WILLIAMS C. M. 1951 — Mitochondria in the flight muscles of insects. I. Chemical composition and enzymatic content. J. Gen. Physiol. *34*, p. 675.

WOJTCZAK A. B. 1956 — Studies on the biochemistry of the waxmoth (*Galleria mellonella* L.). 15. Pyro- and polyphosphates of the excreta of larvae and their enzymatic hydrolysis. Acta Biol. Exper. *17*, p. 235.

WOJTCZAK L. 1952 — Studies on the biochemistry of the waxmoth (*Galleria mellonella*). 10. Respiratory enzymes of the larva. Acta Biol. Exper. *16*, p. 199.

Acta Biologiae Experimentalis, Vol. XVIII, 1958

STUDIES ON THE BIOCHEMISTRY OF WAXMOTH (*GALLERIA MELLONELLA* L.). 19. CYTOCHEMICAL INVESTIGATION OF POLYPHOSPHATES IN THE INTESTINAL TRACT OF WAXMOTH LARVAE

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(Received for publication 15 July 1957)

Niemierko and Niemierko (1950a, 1950b) were able to show the presence of very large quantities of polyphosphates in the excreta of waxmoth larvae. These authors suggest a connection between the formation of polyphosphates and the unique ability of the larvae to assimilate wax. More detailed studies on the nature of polyphosphates of the excreta of *Galleria mellonella* were performed by Niemierko and Wojtczak (1952) and Wojtczak (1956).

In the present investigation, in an attempt to throw some light on the role of these compounds, their distribution in the intestinal tract of the larvae under different physiological conditions has been examined by the use of cytochemical methods. Some of the results have been briefly reported at the III International Biochemical Congress (Niemierko, Włodawer and Przełęcka 1955).

MATERIAL AND METHODS

Last instar larvae of *Galleria mellonella* bred on honey comb at 30°C were investigated. The investigations included: 1) normally feeding larvae coming from a common stock, 2) larvae feeding on pure wax, 3) larvae starved for few days, 4) larvae starved experimentally one to three days

and refed after the period of starvation during 1, 2, 3 or 4 hours*, 5) larvae which have just begun to spin the cocoon.

The prepared intestines were fixed for 1—2 hours either in Bakers formol — calcium (Baker 1947) or in pure 4% formalin and cut on a freezing microtome. To detect polyphosphates the following methods were used:

1. PbS reaction. The sections were treated with 10% lead nitrate buffered solution and the precipitated lead phosphate was made apparent with ammonium sulfide (Wachstein and Pisano 1950, Ebel 1952). The reaction was performed not only at pH 4.5 (in acetate buffer), as in Wachstein and Pisano test, but also at pH 2.5 (using citrate buffer). As a source of ammonium sulfide a 0.5 M water solution of thioacetamide boiled with an equal volume of concentrated ammonia was used. Control sections were hydrolysed for 5 min. with 0.1 N hydrochloric acid at 30°C and afterwards treated as described above.

2. Staining reaction with aniline basic dyes, methylene blue — tripaflavin (MT) (Windisch, Stierand and Haehn 1953). To avoid a mistake due to the presence of nucleic acids, especially RNA, which can also be stained with the basic dyes, control experiments were performed in which sections were extracted with 10% TCA at 4°C for 1/2 to 2 hours. Such an extraction removes the polyphosphates leaving RNA (Stich 1953). The staining with basic dyes of the precipitated lead-polyphosphate complex was also applied. The negative result of this reaction enabled to distinguish polyphosphates from RNA (Stich 1953).

3. Metachromatic reaction with toluidine blue (TB) was performed according to Wiame (1947a). The dye was applied not only to sections. In some experiments it was added to the food of the larvae. In this case the distribution of metachromatic granulations was observed microscopically in fixed intestines without other additional treatment.

EXPERIMENTAL

By means of the cytochemical tests described above the epithelial cells of all parts of the intestine were investigated. When the Wachstein and Pisano test was applied to the intestinal tract of feeding larvae, deposits of lead precipitate were observed only in the midgut epithelial cells. The quantities of this precipitate varied considerably in various larvae.

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* For each particular series of experiments about 30 specimens after the last moulting period, weighing each ca 100 mg were taken. Starving larvae were kept in separate vessels, their excreta being removed. It was necessary to select carefully the specimens taken for experiments as the well grown larvae of much greater weight than 100 mg, when deprived of food, begin cocoon spinning and undergo metamorphosis.

Sections treated with lead acetate at pH 2 and 4,5 gave the same results. Treatment of control sections with 0,1 N HCl for some minutes — which should hydrolyse the polyphosphates to orthophosphates, prevented any PbS staining at all. Three kinds of precipitate could be seen: 1) small black granulations of at most one micron diameter (Plates I 3, 4, 7, II 5, 6, 7), 2) larger agglomerations of granules having a few microns diameter (Plates I 6, II 8), 3) a diffused colouring of the whole cytoplasm (Plate I 1, 2).

In all cases the polyphosphate deposits were seen in the cell cytoplasm. The cell nuclei — in normally feeding larvae — remained unstained. The polyphosphates granules seemed to appear both in cylindrical (Plate I 4) and in goblet cells (Plate I 5). Only midgut epithelial cells gave the positive PbS reaction. Other tissues of the intestine (like connective tissue, the muscles surrounding it, the epithelium of trachea) were always completely devoid of any staining effect (Plate I 2, 7). The characteristic inclusion — "granulations fuchsinophiles" — (so called after Tschang Young Tai 1929) of the *Galleria intestine* never showed any positive staining (Plate I 5, III 2a, 5) but some of the granules contained in the goblet cells sometimes gave a positive PbS reaction (Plate I 5). As was mentioned above, the polyphosphate granules were dispersed throughout the cell cytoplasm. In some intestines they seemed to be coagulated into larger forms (Plate I 6, II 8, III 1). When studied microscopically under high resolution the granulated structure of these agglomerations could be observed quite distinctly (Plate III 2a, 5). These two kinds of polyphosphate deposits, small granules and larger agglomerations were sometimes accompanied by a yellow diffused colouring of the whole cytoplasm (Plates I 4—7, II 7, 8, III 1, 5), and sometimes not (Plates I 3, II 5, 6). In some cases only the diffused staining could be obtained after the PbS test (Plate I 1, 2). It would appear that it could not be the result of insufficient washing out of the reagents used as sections were observed including both midgut and hindgut epithelium, showing a distinct yellow diffused colouring of the cytoplasm of midgut epithelial cells only. Other tissues — intestine muscles, connective tissue and hindgut epithelial cells—remained completely unstained (Plate I 1).

At present, we do not know what causes the appearance of these three different forms of polyphosphate deposit. Perhaps this depends on circumstances that polyphosphates may exist either in

combination with some cell proteins or in a free diffused form in the cytoplasm. The microscopically observed differences in the degree of staining, from black through brown to diffused yellow, seem to be caused by the degree of condensation of the polyphosphates detected in the cells: the lower this condensation, the less dark the colouring obtained.

In the cells of larvae fed either with the normal food, the honey comb, or with pure wax similar pictures could be seen (Plate I 8).

In addition, in several experiments the intestines were examined by means of staining by the MT and TB methods mentioned above. No disagreement between the results obtained by these methods and by the Wachstein and Pisano lead nitrate test was observed (Plate II). One noticed quite often, on sections from the same intestine, a good agreement in the general distribution of polyphosphate compounds given by the PbS and the MT test (Plate II 1 and 5, 2 and 6). In control sections, extracted with cold TCA, neither MT

Abbreviations

gc — goblet cell
cc — cylindrical cell,
mg — midgut,
hg — hindgut
gr — granulations of polyphosphate deposits
ag — agglomerations
dc — diffused colouring
ct — connective tissue
m — muscles
tr — trachae
gf — „granulations fuchsinophiles"
mchg — metachromatic granules
exp. 10/1 — the first number — experiments number
the second number — number of specimen in each experiment

All are frozen sections (10—15 micron thick) of the alimentary tract of waxmoth larvae

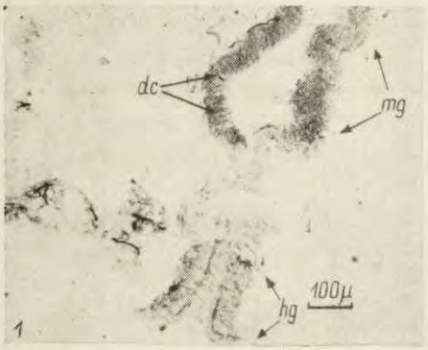
PLATE I

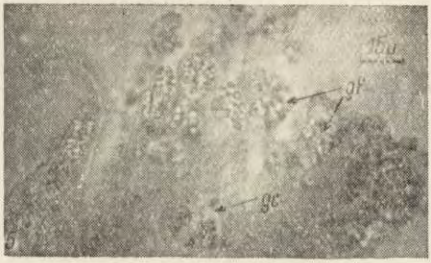
Wachstein and Pisano — PbS — test. Figs. 1—7 — larvae fed on bees comb. Fig. 8 — larva fed on pure wax

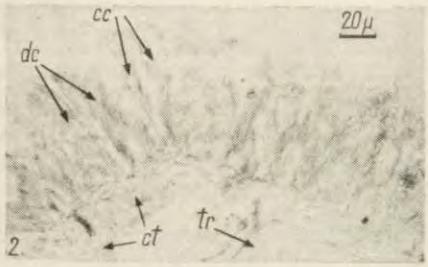
All Figures — except of Fig. 1 present midgut. Fig. 1 — both midgut and hindgut

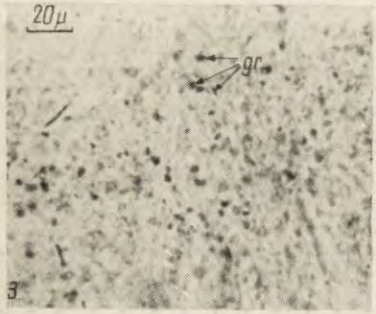
1 — exp. 19/14 (40 x)
2 — exp. 25/2 (400 x)
3 — exp. 22/1 (450 x)
4 — exp. 19/1 (400 x)
5 — exp. 13/2 (200 x)
6 — exp. 13/3 (400 x)
7 — exp. 13/1 (600 x)
8 — exp. 11/5 (400 x)

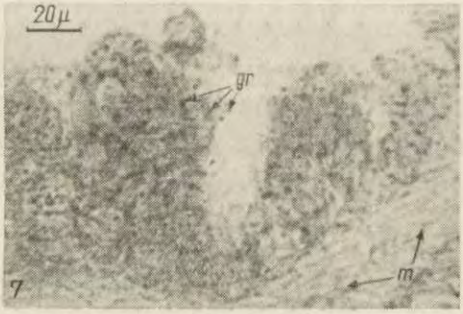
PLATE I

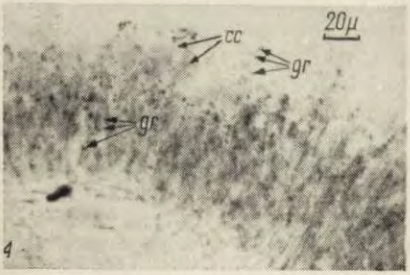












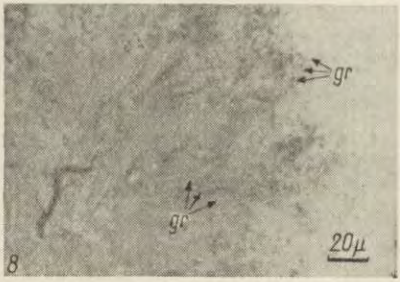
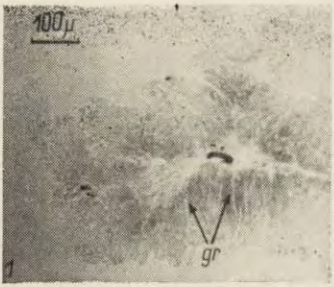
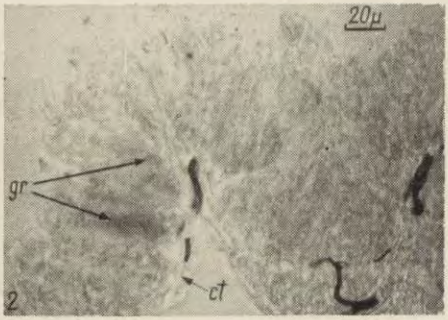
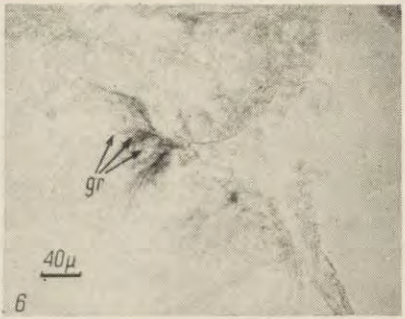
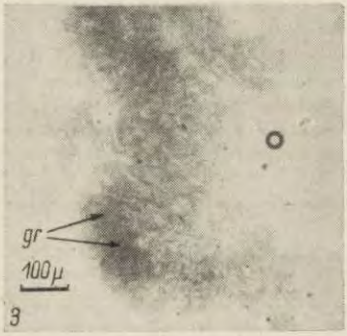


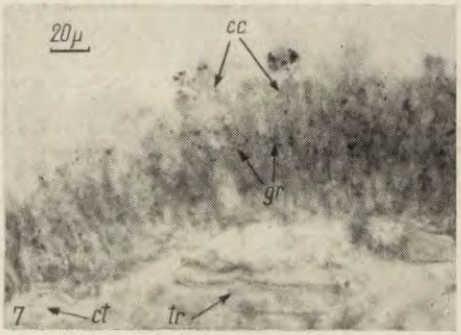
PLATE II

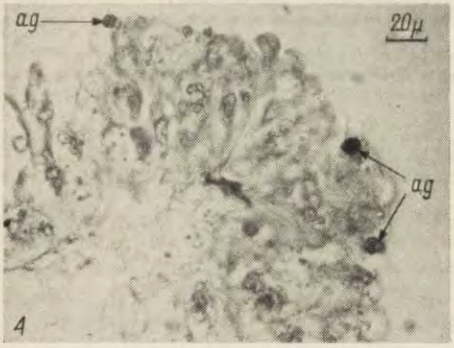


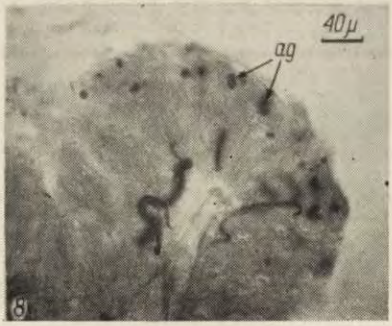












stained granules nor lead precipitates could be observed. When staining of sections was performed after lead nitrate treatment no coloured granules could be found.

In certain series experiments larvae were fed with honey comb which was sprayed with a solution of toluidine blue. In this case the sections of their intestines were examined, after fixation, without any additional staining and a quantity of metachromatic granules could be detected in the midgut epithelium (Plate III 3, 4, 6—8). As in the case of PbS reaction the stained granulations were observed in the cylindrical (Plate III 3, 7) as well as in the goblet cells (Plate III 2b, 4, 6, 8). The "granulations fuchsinophiles", when visible, remained unstained. All other tissues were also colourless.

After one to three days of starvation of the larvae the effect of the PbS reactions on their intestines varied immensely from that on the intestines of feeding larvae. In most cases on large deposits of polyphosphates could be detected. The midgut cells were either completely deprived of any microscopically detectable lead precipitate (Plate IV 2, 5), or only a few small black granules dispersed throughout the cytoplasm were seen (Plate IV 1, 6, 7). No inclusions were seen in the goblet cells (Plate IV 4, 8). In some cases one obtained only a pale diffused colouring of the whole cytoplasm of the epithelial cells (Plate IV 3, 4).

In the intestines of the larvae which were fed after a short period of starvation the polyphosphates deposits were again visible in the midgut epithelial cells after Wachstein and Pisano test. In most cases as soon as after one hour of refeeding, the intestines of the larvae investigated showed a much greater quantity of polyphosphates in their cell cytoplasm as compared with the starv-

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PLATE II

Larvae fed on bees comb. A comparison of results obtained by the MT (methylene blue and tripaflavine) and PbS test. Figs. 1, 2, 3, 4, — MT test. Figs. 5, 6, 7, 8, — PbS test

1 — exp. 27/1 (100 x) 4 — exp. 5/13 (400 x) 7 — exp. 19/1 (400 x)
2 — exp. 27/2 (400 x) 5 — exp. 27/1 (200 x) 8 — exp. 27/3 (200 x)
3 — exp. 27/5 (100 x) 6 — exp. 27/2 (200 x)

ed larvae (Plate V 1, 5 — compare with Plate IV 2, 3, 5, 6; Plate V 2, 3, 7 — compare with Plate IV 4, 8, and Plate V 6 — compare with Plate IV 7). The same three forms of polyphosphate deposits, already observed in normally feeding larvae, were also seen here. Most often the diffused form of the lead precipitated polyphosphates appeared in the first or second hour of refeeding (Plate V 1, 5). After about two hours usually the granulated form was visible (Plate V 2, 6). The large agglomerations of polyphosphates were detected only later (Plate V 3, 4, 7, 8).

The intestines of larvae which have ceased to feed and begun to spin cocoons appeared similar to that of starved larvae. Only a small quantity of polyphosphates granulations could be detected in their midgut cells.

DISCUSSION

We have shown that in the midgut epithelial cells of feeding larvae considerable quantities of polyphosphates can be found after application of the Wachstein and Pisano test. The same results were obtained by means of the metachromatic reaction with toluidine blue and after staining the tissue with basic aniline dyes. The first question to discuss is the specificity of the tests used.

Investigations connected with the cyto and biochemical detection of polyphosphates deal chiefly with their distribution in bacteria and lower plant cells. It has been shown by biochemical methods that the metachromatic granules already well known from cytological observations evidently contain polyphosphates (Wiame 1946, 1947a, 1947b, Ebel 1949, 1951, Lindegren 1951, Lindegren and Townsend 1954, Albaum and co-workers 1950, Mann 1945, Sall and co-workers 1955, 1956). The cytochemical investi-

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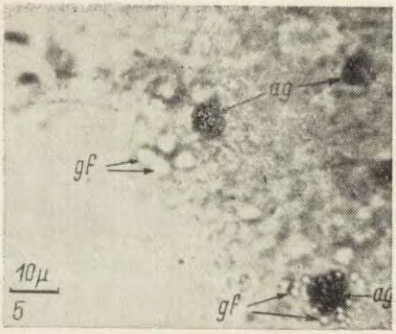
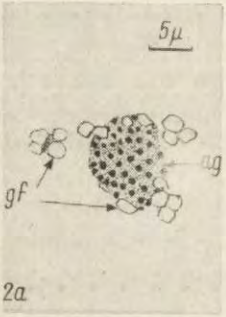
PLATE III

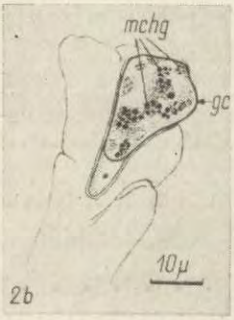
Figs. 1, 2a, 5, — larvae fed on honoey comb. PbS test. Figs. 3, 4, 6, 7, 8, — larvae fed on honey comb sprayed with a solution of toluidine blue

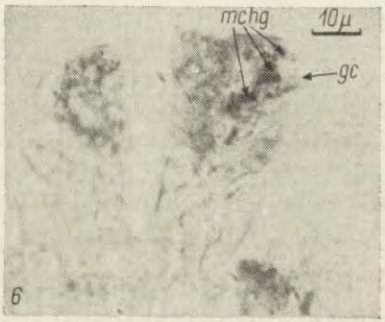
1 — exp. 27/7 (400 x)
2a—exp. 27/7, 2b—exp. 28/3
3 — exp. 28/1 (200 x)
4 — exp. 28/2 (400 x)
5 — exp. 27/7 (1000 x)
6 — exp. 28/3 (1000 x)
7 — exp 28/3 (400 x)
8 — exp. 28/4 (400 x)

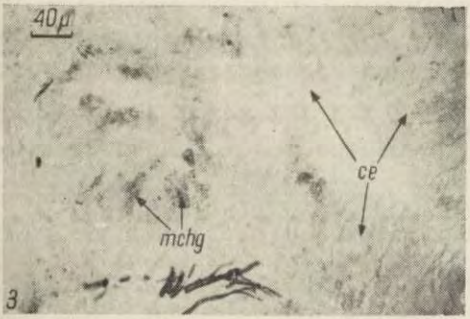
PLATE III

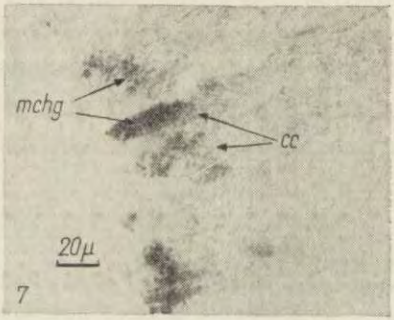


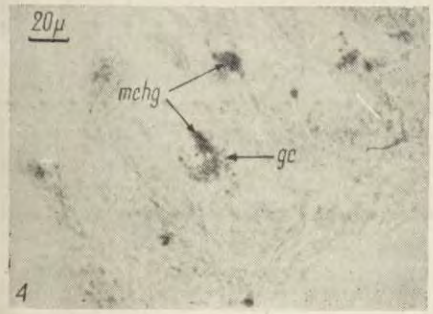












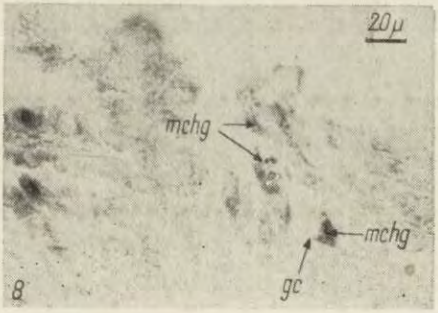
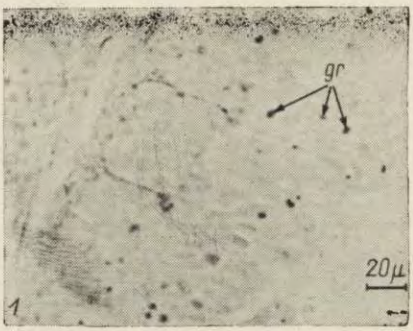
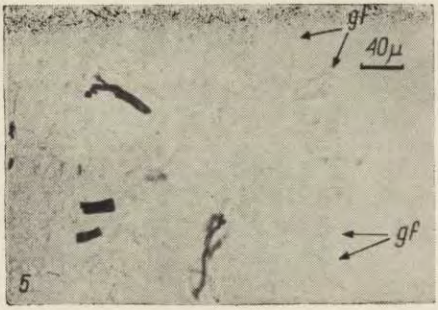
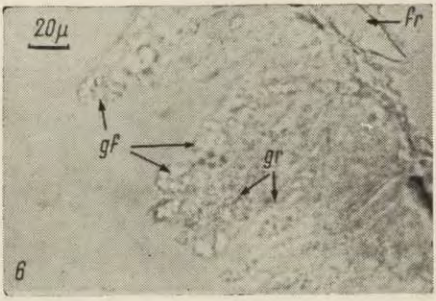
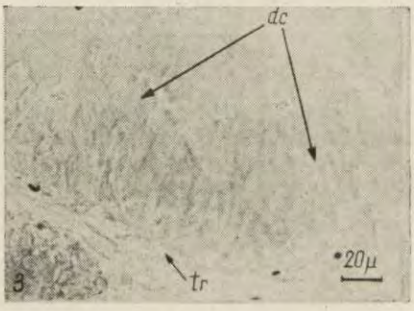


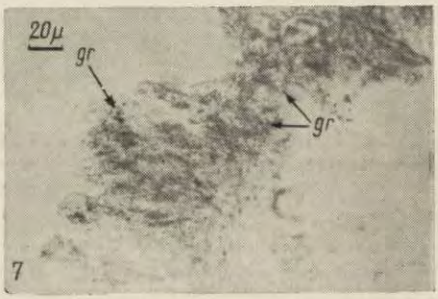
PLATE IV

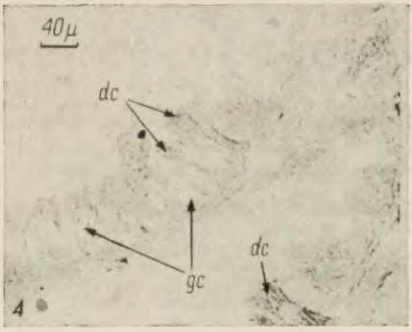


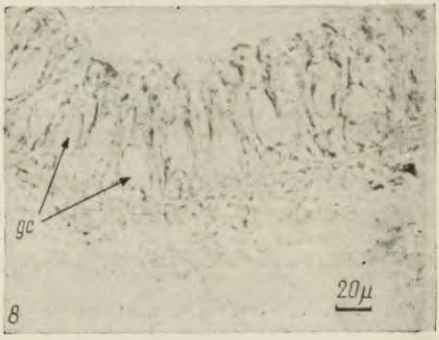












gations also throw some light on the subject. König and Winkler (1948) established by means of basic dyes staining reactions and by chemical analysis of TCA cell extracts, that the metachromatic granules of *Corynebacterium* contained phosphorous compounds and calcium ions. These authors gave however no exact data allowing us to establish the nature of these compounds. According to Bringmann (1951) they can be regarded as metaphosphate complexes. In 1950 Wachstein and Pisano found that these cell inclusions selectively bind $Pb^{\cdot\cdot}$ ions. Similar results were obtained by Macary (1951) who indentified these granules of Corynebacterium as polyphosphates using the metachromatic reaction and a method of precipitating with salts of $Co^{\cdot\cdot}$ or $Fe^{\cdot\cdot}$. Stich used staining methods in his investigations on distribution of polyphosphates in *Acetabularia mediterranea* (1953, 1956). The chemical analysis of cell extracts compared with an analysis of synthetic compounds confirmed the polyphosphate nature of the stained granulations. Investigations by the same author with labelled P^{32} gave another proof that the metachromatic granules in *Acetabularia* are sites of intensive phosphorus metabolism (1955).

All reactions used in the present investigations were, in our preliminary experiments, first tested on yeast cells which, as well known from the literature, contain polyphosphates. It was established that formalin fixation (Bakers' formol-calcium or 4 % formalin) does not prevent the positive effect of staining or lead precipitation reactions. The treatment of cell smears by hydrochloric acid — causing a hydrolysis of polyphosphates to orthophosphates — prevents any positive staining. After such treatment, no polyphosphates could be detected in the phosphorus enriched yeast cells, neither by staining reactions nor by the Wachstein and Pisano test.

PLATE IV

Larvae starved one to three days. PbS test

1 — exp. 8/14, 24 hrs of starv. (400 x),
2 — exp. 25/1, 48 hrs of starv. (200x)
3 — exp. 25/5, 48 hrs of starv. (400 x)
4 — exp. 17/8, 72 hrs of starv. (200 x)
5 — exp. 25/4, 24 hrs of starv. (200 x)
6 — exp. 25/4, 24 hrs of starv. (400 x)
7 — exp. 19/7, 48 hrs of starv. (400 x)
8 — exp. 17/8, 72 hrs of starv. (400 x)

As is well known, the orthophosphate lead salts are soluble in acid medium (Harms and Jander 1936). Therefore one can suppose that at pH 2—3 used in our modification of Wachstein and Pisano test the orthophosphates do not precipitate and that only polyphosphates give the insoluble lead salts (Schmidt 1951, Spiegelmann and Kamen 1947).

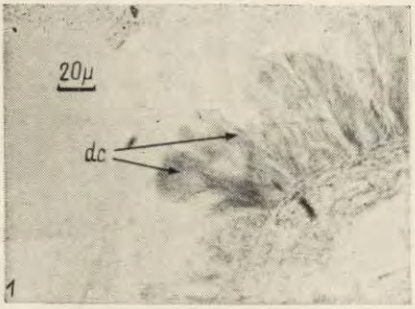
As to the staining reactions it must be added that, according to Wiame (1947b, 1948), only highly polymerized phosphates shown the metachromasia. Windisch and his co-workers (1953) are of the opinion that only compounds with more than two acidic groups can be stained with methylene blue following tripaflavine. It seems, however, that by means of the cytochemical tests now available it is impossible to determine the exact nature of these compounds and the degree of their polymerisation. As yet, we have also not been able to distinguish higher polyphosphates from pyrophosphates. In any case, we can suppose, that orthophosphates are not responsible for the positive results of the reactions used in the present investigations. As was mentioned above, the hydrolysis of polyphosphates to orthophosphates prevents any positive staining or lead precipitation. An additional proof was the application by us, in a few cases, of the reaction of Serra and Feig for inorganic orthophosphates (after Pearse 1956). When the test was applied to the formalin fixed frozen sections no positive staining effect was obtained, whereas the Wachstein and Pisano test applied to the same sections afterwards gave a distinctly visible polyphosphate deposit.

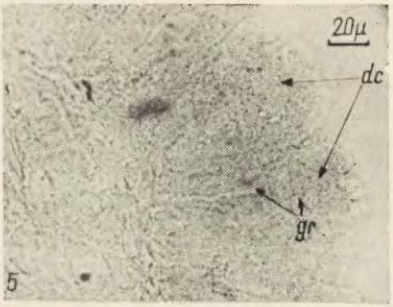
PLATE V

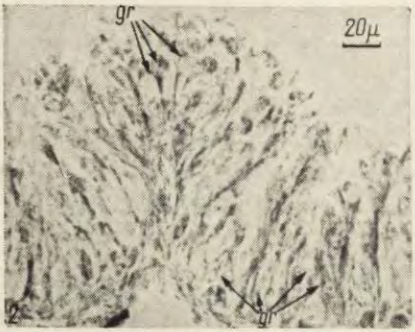
Larvae fed after a short period of starvation. PbS test

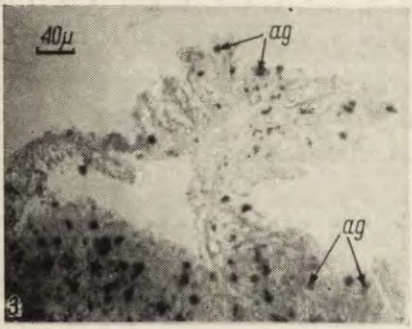
1 — exp. 25/2, 48 hrs of starv. — 1 hr of refeeding (400 x)
2 — exp. 17/18, 72 hrs of starv. — 2 hrs of refeeding (400 x)
3 — exp. 17/10, 72 hrs of starv. — 4 hrs of refeeding (200 x)
4 — exp. 7/5, 72 hrs of starv. — 24 hrs of refeeding (400 x)
5 — exp. 25/6, 48 hrs of starv. — 1 hr of refeeding (400 x)
6 — exp. (19/19, 48 hrs of starv. — 3 hrs of refeeding (400 x)
7 — exp. 17/10, 72 hrs of starv. — 4 hrs of refeeding (400 x)
8 — exp. 17/16, 72 hrs of starv. — 4 hrs of refeeding (400 x)
More detailed explanations in text

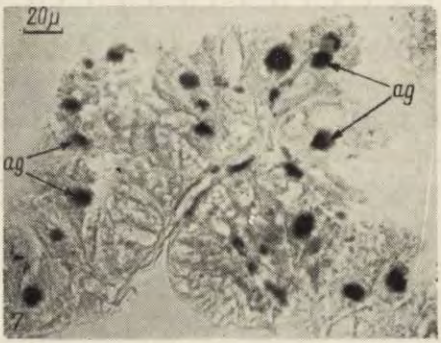
PLATE V

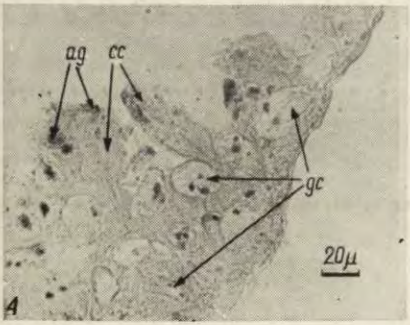


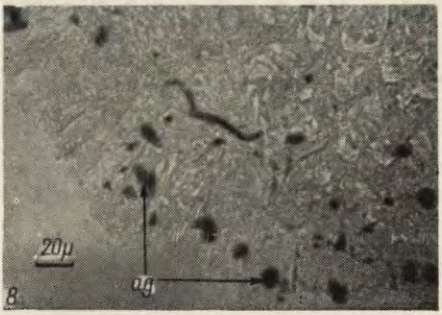












It is also known that not only inorganic polyphosphates but also some other acidic compounds present in the cell, especially nucleic acids, can be responsible for staining reactions with basic dyes. We know that in *Galleria mellonella* intestinal epithelial cells RNA are present in large quantities. The negative results obtained by us after two control tests: the cold TCA extraction and the staining of precipitated lead complex with methylene blue, seem to give further evidence that the detected compounds are polyphosphates and not ribonucleic acids (Stich 1953).

As was mentioned above, polyphosphates were detected in quantity in midgut epithelial cells of larvae feeding either on honey comb or pure wax. Similar results were obtained on the whole intestinal tracts by Niemierko (1957) by means of chromatographical methods. At the same time a considerable amount of phospholipids (Przełęcka 1956) and in particular of acetalphosphatides (Przełęcka and Gołaszewski 1957) could be found in the intestinal cells of feeding larvae. In starving larvae the microscopically detectable deposits of these substances, i.e. the polyphosphates, the phospholipids and acetalphosphatides, diminish quite distinctly. Very soon after refeeding, the investigated compounds are observed by means of cytochemical methods to return to their previous level. This correlation seems to support the opinion of Niemierko and Niemierko (1950a, 1950b) on the possible connection of polyphosphate formation in the larval body with wax metabolism.

Further investigation of these compounds in different organs of the same organism gave some additional data on this subject (Wroniszewska and Przełęcka 1957). During the early period of metamorphosis polyphosphates could be found in tissues and cell organelles which, as observed microscopically, were completely free of these compounds in the larval stage. For example polyphosphates were present in cell nuclei and nucleoli of tracheal, intestinal and of spinning gland epithelium of young pupae, where as a rule they were never observed in feeding larvae. After a very long period of starvation (20—30 days) the polyphosphates were easily found in the spinning glands, the protein content of which undergoes resorption in such conditions (Zielińska 1957). It seems therefore not impossible that the function of polyphosphates in *Galleria mellonella* may be various.

Their appearance in organs which undergo resorption suggests that they are perhaps metabolically connected not only with processes of utilization of wax, but also with catabolic processes which dominate during the long experimental starvation and metamorphosis.

Heller and al. (1950, 1956) found pyrophosphates and small amount of polyphosphates in *ductus ejaculatorius* of *Celerio euphorbiae*. Stich and Grell (1955) found the metachromatic stainable granules of polyphosphate nature in the Malpighian tubes of *Culex pipiens*, in larvae nearing pupation. Finally we have some own preliminary data about the appearance of the polyphosphates detectable by the PbS test in young pupae of *Bombyx mori* and *Tineola* sp. All these facts seem to indicate that formation of polyphosphates in insect body may be a more general phenomenon and that these compounds may be of great importance in insect metabolism.

SUMMARY

Polyphosphates were detected by means of cytochemical methods in the midgut epithelial cells of waxmoth larvae. A study was made on the influence of various physiological states of the larvae (feeding, starvation, cocoon spinning) on the appearance of polyphosphates. The observed correlation between the digestion and absorption of wax and the presence of polyphosphates in intestinal cell cytoplasm seems to support the opinion on the role of polyphosphates in utilization of wax by the larvae.

The appearance of polyphosphates was also detected in larvae undergoing metamorphosis or starved for a long period. The polyphosphates were found in those tissues and cell organelles in which in normally feeding larvae they never could be found by means of cytochemical methods.

ACKNOWLEDGEMENTS

The authors wish to express their sincere thanks to Doc. S. Niemierko and Prof W. Niemierko for their kind criticism, valuable advice and many helpful discussions.

Thanks are also due to Mrs. A. Jurowska for her able technical assistance.

REFERENCES

ALBAUM H., ACHATZ A., HUTNER S., HIRSCHFELD A. 1950 — Phosphorylated compounds in Euglena. Arch. Biochem. *29*, p. 210—218.

BAKER J. R. 1946 — The histochemical recognition of lipine. Quart. J. Micr. Sci. *87*, p. 467—478.

BRINGMANN G. 1951 — Licht- und elektronenmikroskopische Untersuchungen über die zytologische Natur der Granula von *Corynebacterium diphteriae*. Zentralblatt f. Bakteriologische Parasitenkunde u. Infektionskrankheiten u. Hygiene. I Abt. *156*, p. 493—502.

EBEL J. P. 1949 — Participation de l'acide metaphosphorique à la constitution des bactéries et des tissus animaux. C. R. hebd. Seances Acad. Sci. *228*, p. 1312—1313.

EBEL J. P. 1951 — Recherches chimiques et biologiques sur les poly et métaphosphates. Thèses de l'Université de Strasbourg. No 108/E.

EBEL J. P. 1952 — Recherches sur les polyphosphates contenus dans diverses cellules vivantes. IV. Localisation cytologique et role physiologique des polyphosphates dans la cellule vivante. Bull. Soc. Chim. Biol. *XXXIV*, p. 5—6, 498—504.

HARMS J. and JANDER G. 1936 — Angew. Ch. *49*, p. 106, quot. by K. KLEMENT 1953 — Handb. d. Anal. Chem. Bd Va β. Berlin.

HELLER J. 1956 — Pyrophosphates in the hawk-moth, *C. euphorbiae*. I. Tentative fractionation of phosphorus P_7. Bull. Acad. Polon. Sci. Cl. II, *10*, p. 341—344.

HELLER J., KARPIAK ST. and ZUBIKOWA J. 1950 — Inorganic pyrophosphate in insect tissue. Nature *166*. p. 187.

HELLER J., Piechowska M. 1956 — Pyrophosphates in the hawk-moth. *C. euphorbiae*. II. Transfer of pyrophosphates during copulation. Bull Acad. Polon. Sci. Cl. II. *10*, p. 345—349.

KÖNIG H. und WINKLER A. 1948 — Über Einschlüsse in Bakterien und ihre Veränderung im Elektronenmikroskop. Naturwissenschaften. *35*, p. 136.

LINDEGREN CARL C. 1951 — The relation of metaphosphate formation to cell division in yeast. Explt. Cell Res.. Vol. *II*, p. 2.

LINDEGREN CARL C. and TOWNSEND F. 1954 — Methods for distinguishing the spindle, the centrochromatine and the chromosomes of the yeast cell. Cytologia. *19*, p. 2—3, 104—109.

MAGARY J. 1951 — Des corpuscules métachromatiques des corynebactéries. Ann. Inst. Pasteur. *81*, p. 657—662.

MANN T. 1945 — Studies on the metabolism of mould fungi. 2. Isolation of pyrophosphate and metaphosphate from *Aspergillus niger*. Biochem. J. *38*, p. 339—345.

NIEMIERKO S. 1957 — unpublished data.

NIEMIERKO S. and NIEMIERKO W. 1950a — Metaphosphate in the excreta of the waxmoth, *Galleria mellonella*. Nature. *166*, p. 268.

NIEMIERKO S. and NIEMIERKO W. 1950b — Studies on the biochemistry of waxmoth (*Galleria mellonella*). 6. Metaphosphate in the excreta of *Galleria mellonella*. Acta Biol. Exper. *15*, p. 111.

NIEMIERKO S. and WOJTCZAK A. B. 1952 — Meta and pyrophosphatase in the body of waxmoth larvae. Acta Physiol. Polon. Papers of III Physiological Congress, p. 217 (in Polish with English summary).

NIEMIERKO W., WŁODAWER P. and PRZEŁĘCKA A. 1955 — Role of some phosphorus compounds in the utilization of wax by the waxmoth larva. II Congress Intern. de Biochimie. Bruxelles.

PEARSE A. G. E. 1954 — Histochemistry. Theoretical and Applied. London.

PRZEŁĘCKA A. 1956 — Studies on the biochemistry of waxmoth (*Galleria mellonella*). 14. Cytochemical study of phospholipids in the intestinal tract of waxmoth larvae. Acta Biol. Exper. *17*, p. 231—234.

PRZEŁĘCKA A. and GOŁASZEWSKI T. 1957 — unpublished data.

SALL T., DAVIS J. C. and MUDD S. 1955 — Metachromatic granules as index of metaphosphate storage in *Corynebacterium diphteriae*. J. Hist. Cytoch. *3*, 384—385. (Proc. of the VIth Ann. Meeting Histoch. Soc. Philadelphia 1955).

SALL T., MUDD S. and DAVIS J. C. 1956 — Factors conditioning the accumulation and disappearance of metaphosphate in cells of *Corynebacterium diphteriae*. Arch. Biochem. Bioph. *60*, 130—146.

SCHMIDT G. 1951 — The biochemistry of inorganic pyrophosphates and metaphosphates. Symposium on phosphorus metabolism. Baltimore. *1*, p. 443—476.

SPIEGELMANN S. and KAMEN M. 1947 — Some basic problems in the relation of nucleic acid turnover to protein synthesis. Cold Spring Harbor Sympos. Quantit. Biol. *12*, p. 211—223.

STICH H. 1953 — Der Nachweis und das Verhalten von Metaphosphaten in normalen, verdunkelten und Trypaflavin — behandelten Acetabularien. Z. Naturforsch. *8*, p. 36—44.

STICH H. 1955 — Synthese und Abbau der Polyphosphate von Acetabularia nach autoradiographischen Untersuchungen des P^{32} — Stoffwechsels. Z. Naturforsch. *10b*, p. 281—284.

STICH H. 1956 — Änderungen von Kern und Polyphosphaten in Abhängigkeit von dem Energiegehalt des Cytoplasmas bei Acetabularia. Chromosoma. *7*, p. 693—707.

STICH H. and GREL M. 1955 — Incorporation of phosphorus 32 into the malpighian tubes during the metamorphosis of *Culex pipiens*. Nature. *176*, p. 930—931.

TCHANG YUNG-TAI 1929 — L'histogenèse et l'histophysiologie de l'épithélium de l'intestin moyen chez un lépidoptère (*Galleria mellonella* L.). Bull. Biol. France et Belg. Suppl. *12*.

WACHSTEIN M. and PISANO M. 1950 — A new staining technique for the polar bodies. J. Bacteriol. *59*, p. 3, 357.

WIAME J. M. 1946 — Basophilie et metabolism du phosphor chez la levure. Bull. Soc. Chim. Biol. *28*, p. 552—556.

WIAME J. M. 1947 a — Etude d'une substance polyphosphorée, basophile et métachromatique chez les levures. Biochim. Bioph. Acta (Amsterdam). *1*, p. 234.

WIAME J. M. 1947b — The metachromatic reaction of hexametaphosphate J. Am. Chem. Soc. *69*, p. 3146—3147.

WIAME J. M. 1948 — Metaphosphate and metachromatic corpuscules in yeast. Rev. Ferm. Aliment. *3*, p. 83—86, quot. by SCHMIDT G. 1951 — Symp. on phosphorus metabolism. Baltimore. *1*, p. 443—476.

WINDISCH F., STIERAND D. and HAEHN H. 1953 — Über den Nachweis der Zellphosphate und die Unspezifität bisher gebräuchlichen Methoden zur histochemiscen Lokalisierung der Ribonukleotide. Protoplasma. *42*, p. 346—364.

WOJTCZAK A. B. 1956 — Studies on the biochemistry of waxmoth (*Galleria mellonella* L.) 15. Pyro and polyphosphates of the excreta of larvae and their enzymatic hydrolysis. Acta Biol. Exper. *17*, p. 235—253.

WRONISZEWSKA A. and PRZEŁĘCKA A. 1957 — Cytochemical study of polyphosphates in diferent organs of *Galleria mellonella*. Papers of IV Congress of the Polish Physiological Society.

ZIELIŃSKA Z. M. 1957 — Studies on the biochemistry of the waxmoth *Galleria mellonella*. XXX. Nitrogen metabolism in the tissues during starvation. Acta Biol. Exper. *17*, p. 351—371.