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CONTENTS

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ON CONDITIONED REACTIONS OF PARAMAECIUM CAUDATUM TOWARDS LIGHT

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That *Paramaecium caudatum* does not react to light, even though the light be very strong, is a well known fact. Nevertheless there are some indications that this species is not totally indifferent to light, since the tempo of multiplication of the infusorian may be varied somewhat in accordance with the wave-length of the reflected light (D e m b o w s k a). The influence of light, however, is very slight.

B r a m s t e d t obtained in *Paramaecium* conditioned responses towards light and towards mechanical stimulation. He put a slide with a flat drop of the culture medium on a crystallizer filled with water and divided into two halves. One half contained water of 15°C, another of 42°. Under these conditions a single *Paramaecium* swimming in the drop remained practically exclusively in the cooler half and to every contact with the boundary between the two temperatures it responded with an avoiding reaction. At the same time the cold half of the drop was dark, the warm one strongly lighted. A contact of the protozoon with the area of higher temperature may be considered as a reinforcement of a conditioned stimulus (light) by the unconditioned (temperature). After 90 minutes, both halves of the crystallizer were filled with water at the same temperature of 15° and thermic stimulation no longer ensued from crossing the prohibited

boundary. Nevertheless, the infusorian continued to stay in darkness and to respond with an avoiding reaction to every light stimulation. Light was not absolutely avoided, but the test infusoria stayed about 33 times longer in the dark half of the drop than in the light one. After about 15 minutes, the acquired reaction ceased, and the infusorian swam indifferently in the whole drop. Under opposite conditions, when the light half was cool and the dark warm, the experiment failed. Bramstedt explains the fact by the „unbiological" combination of stimulants, since under natural conditions light is always connected with warmth, not with cold.

I shall not consider other experiments of Bramstedt, since for the moment we are interested in his fundamental result: the possibility of obtaining a conditioned reaction towards light. Grabowski pointed out that the investigations of Bramstedt involve some inexactitudes, his result being therefore not entirely proved. The fact that after some time there develops a negative reaction towards the light half of the drop remains true, however. But in control experiments, when the temperature of both halves of the drop was equal, Grabowski observed that the protozoon makes excursions towards the light half, gradually embracing greater and greater portions of the prohibited area. It becomes evident that the animal avoids not the boundary between light and darkness, but some other boundary, which is initially identical with the boundary of light, but which gradually shifts towards the light half of the drop. In fact under a temperature of 42°, bubbles of gas escape from the water, the solubility of gas diminishing with increasing temperature. Therefore in the control experiment the two halves of the drop differed not only in light conditions, but also in gas content i. e. in chemical properties, those properties being an unconditioned stimulus. Grabowski put under experimental conditions a drop of water, heating one half of it. After 90 minutes he equated the temperature of both halves and only then he put an infusorian into the drop. The protozoon began immediately to react negatively towards light, remaining mainly in the dark part, in spite of the fact that it was not conditioned.

Diebschlag objected that Grabowski heated his drop to 50° and not to 42°, thus causing an essential difference between the two halves. This argument does not seem to by very convincing, because a temperature difference of 27°, as given by Bramstedt, was amply sufficient to cause a marked difference in gas content of the liquid medium, which invalidates the fundamental result.

The whole problem was discussed by Alverdes, O. Koehler and Bramstedt at a special conference, but agreement was not reached.

Soest worked to a more exact method; in his experiments the light stimulus was followed by electric stimulation. He used a constant current of 220V as an unconditioned stimulus. I consider here only his experiments with *Paramaecium*. When the infusorian swam into the light half of the drop, it received a series of electric shocks until it turned back. After some time on the light boundary an avoiding reaction was observed, before the infusorian reached the prohibited line. *Paramaecium* stayed then in the dark half, escaping from the „punishment". Also in this case an opposite experiment did not succeed; it was not possible to compel the infusorian to remain constantly in the light half. As far as this last result is concerned it must be pointed out, that evidently Soest admitted an experimental error. In all his experiments the cathode of the constant current was in the dark half of the vessel. It must be remembered that the constant current causes a negative galvanotropism in *Paramaecium*. If the light area is prohibited, electric shocks compel the protozoon to swim towards the dark half, where the cathode is located. But if the dark half is prohibited, *Paramaecium* cannot swim towards the light half, because galvanotropism compels it to stay in the dark.

With a current of 220V and under constant location of electrodes a strong electrolysis is caused with accumulation in the dark half of the vessel of chemical substances, which are positive chemotropic stimulants. Under such conditions when the infusorian turns before receiving an electric shock, it does not necessarily mean that it has acquired a conditioned reaction, because both halves of the drop differed not only in light conditions, but also in chemical properties. Moreover those properties act as an unconditioned stimulus. Once more the basic result remains doubtful.

The method of Soest was improved by Wawrzynczyk. An infusorian came into a thin glass tube with sealed platinum electrodes on each end of it. Under the tube were colour filters; the tube was fastened horizontally to the stage of a simple microscope and it was strongly lighted from below. Experiments were carried out in a dark room. The unconditioned stimulus consisted in the inductive current causing no electrolysis. According to Statkewitsch, in an inductive field, *Paramaecium* swims towards the cathode of the current of interruption, thus the inductive current is a directive stim-

ulus. W a w r z y n c z y k used this property of the current for experimental purposes. When *Paramaecium* crossed the prohibited boundary between two adjacent light filters, it received a series of electric shocks, while the cathode of the interruption current was on the permitted side and the galvanotropism compelled the protozoon to turn back. This method makes it possible to keep the infusorian permanently in the middle parts of the tube and to avoid its making contact with both electrodes, which can cause difficulties.

With this method W a w r z y n c z y k obtained a series of conditioned reactions. After having turned many times under the influence of current, the infusorian begins to turn spontaneously, before the shock, and it remains for a long time on the permitted territory. The acquired reaction lasts for about 50 minutes, after which it gradually disappears. The light part of the tube may become a positive stimulus; it is not true that such a combination of stimulants is „unbiological". *Paramaecium* distinguishes light from darkness, moreover it distinguishes colours, as red, green and blue; it also distinguishes two intensities of dispersed light differing from each other to the extent of 10%. After the unconditioned stimulation has been suspended, the reaction obtained disappears, but it may be fully restored by a small application of electric shocks. In order to obtain this effect, it is necessary to ensure an exact synchronism of the induction shock with the moment of the crossing of the critical boundary. The conditioned reaction does not appear in very young specimens (till 2 hours after the last division) or in damaged ones.

Those results are of a great importance, since they offer new and very interesting possibilities of analysing conditioned responses. This circumstance makes it necessary to confirm exactly the basic facts, especially to prove, that in the control experiment there exists but one difference between the permitted and the prohibited area. The problem requires very careful experimenting, because *Paramaecium* is extremely sensitive to various accessory factors, which cannot be detected so easily.

The present paper deals with just this question.

METHODS

In all experiments a single specimen of *Paramaecium caudatum* came into a capillary tube, 42 mm in length and about 0,5 mm in internal diameter. The tube, containing tap water of room temperature, was

open at both ends and it was fastened horizontally to the stage of a preparation microscope. The experiments were caried out in daylight, the tube was lighted from below. On the capillary tube I put two closely fitting muffs of black paper, or of cellophane stained with solutions of malachite green, saffranine, methylene blue or crystalviolet. Into both ends of the tube were inserted platinum electrodes; the electrodes were connected with the secondary circuit of an induction coil. With an accumulator of 4 v and with a distance between the coils of 9 cm a current was obtained, which was harmless for the infusorian, but which stopped it immediately in its propagative motion. After a series of inductive shocks, the infusorian made some irregular movements and it directed itself towards the cathode of the current of interruption. The location of the cathode changed according to the movements of the protozoon.

Other particulars of method will be quoted in the description of the experiments.

OBTAINING THE BASIC EFFECT

The two black muffs on the capillary tube were each of 1 cm length; they were placed at distance of 1 cm from each other, the permitted area of the capillary being at the middle of the tube. Near the tube was a scala. I quote a typical experiment.

The protozoon, introduced into the capillary, swims freely along the whole tube, becoming slower in its movements near both electrodes . It comes between both muffs (permitted area), swimming towards the right. It crosses the right boundary of the area and it receives its first induction shock, the cathode of the current of interruption being on the left. The *Paramaecium* stops immediately; it makes several irregular movements at that spot, then it swims once more towards the right, receives a new series of electric shocks, the whole procedure being repeated 5 times. After this the infusorian turns definitively left and it swims along the whole permitted area. But after having crossed the left boundary of the area it receives a series of 7 shocks (the cathode on the right), it swims towards the right end of the area, and gets there 8 shocks etc. From the beginning of the experiment and over a period of about 10 minutes, there occur 47 such induced turnings; the number of shocks necessary for directing the infusorian towards the permitted area is from 1 to 16. After the 47-th turning the infusorian is on the left end of the area. It swims to the right, but before reaching the critical boundary it makes its first „spontaneous" turn. The protozoon stops at a distance of about 1 mm from the boundary, it makes some irregular move-

ments and it swims towards the left end without having received any electric punishment. But at the left end it crosses the prohibited boundary, receives 8 shocks, swims right and again it turns before having reached the end of the permitted area. This is repeated 6 times: on the left end it is necessary to stop the infusorian with an electric shock; on the right end the turning occurs „spontaneously". A few minutes later spontaneous turns begin also at the left end of the area. This occurs again at a distance of about 1-2 mm short of the end. After 12 succesive turns without punishment the reaction becomes somewhat irregular again. Of 17 succesive turns at both ends of our critical area, only 4 are spontaneous, all of them occuring at the right end; 13 turns are a consequence of electric stimulation applied from 1 to 15 times. Later there are 19 spontaneous turns at both ends followed by 3 induced turns on the left, then 31 spontaneous turns, 9 irregular reactions, 83 spontaneous turns etc. The behaviour of the protozoon is neither regular, nor rhythmic. But there are undoubtedly succesive periods of spontaneous turns and of induced ones with a gradual increase in the number of spontaneous turns.

The experiment described is so typical that there is no need to quote other examples. Repeating it many times with infusoria of different origin I observed on each occasion a very uniform picture. The basic effect may be considered as established: after some number of induced turns *Paramaecium* begins to turn near the prohibited boundary before having received an electric shock.

CONTROL EXPERIMENTS

We have now to prove that the cause of the spontaneous turns really consists in the passing from the light part of the capillary to the dark one, or that there exists only one difference between critical points of the capillary (i. e. both ends of the permitted area) and all other points of the permitted territory.

When, after 12-15 minutes of experimenting, there were only spontaneous turns at both ends of the area, I pushed both paper muffs right and left so as to make the length of the area between them equal to 2 cm. If the conditions of light determine the turns, then our infusorian may be expected to follow the position of the muffs and to react on both new boundaries. Instead of this the spontaneous

turns occur exactly at those same two points of the capillary, where the two ends of the area formerly were. It becomes evident that the infusorian turns not at the boundary of light and darkness, but at some other boundary, the nature of which is to be investigated. This conclusion is in agreement with the fact, already mentioned, that *Paramaecium* does not turn exactly at the very end of the area but rather anticipates it and turns at a point about 1-2 mm before the end.

The two points of the tube in which induced turns occur are those places in which the infusorian stays longer. In fact the protozoon swims without interruption along the whole permitted area, but it stops always at both ends of it, where it receives electric shocks and where it makes several movements on the spot. It is quite possible that precisely at those two points the infusorian leaves some material spur in the liquid medium. Probably some products of metabolism of the protozoon are accumulating in the water of the capillary, their concentration increases during the experiment and it reaches a threshold value, which causes the infusorian to react negatively towards it as towards an unconditioned stimulus. In other words we have to deal not with an acquired new reaction, but with the occurence of a new stimulus probably of chemotropic nature.

After some spontaneous turns the infusorian crosses again the prohibited boundary. This crossing may signify an extinction of the acquired reaction, which is not reinforced by the unconditioned stimulus. But the phenomenon may be explained simpler. When spontaneous turns are established, the movements of the infusorian become slow along the whole length of the area, the protozoon often turns in the middle parts of it without any visible cause and only seldom does it reach both ends of the area. The metabolites accumulated there diffuse, their concentration soon becoming under the threshold. There is then no hindrance to a free crossing of both boundaries of the permitted area.

In the experiment described above, the first spontaneous turn occured after 47 induced turns. Generally speaking, the number of induced turns fluctuate between 25 and 72. In the course of every experiment the number of induced turns decreases considerably, prior to the new series of spontaneous ones. The appearence of spontaneous turns may be interpreted as the return of conditioned reaction after renewal of unconditioned stimulation. And once more there exists a simpler explanation. When at both ends of the permitted

area the accumulated metabolites diffuse, and when their concentration decreases a short halt of the infusorian at the critical points may be sufficient to restore their threshold concentration. In a capillary tube, processes of diffusion are very slow.

On the whole the experiments quoted do not confirm the existence of conditioned reactions of *Paramaecium caudatum* towards light. The effect obtained may be sufficiently explained by accumulation and diffusion of metabolites in a capillary space. *Paramaecium* reacts negatively towards definite concentrations of those metabolites, its negative chemotropism being an uncoditioned reaction.

Nevertheless, our conclusion does not exclude the possibility of a conditioned reaction. It seems possible that such a reaction may be established at the same time as the threshold concentration of metabolites arises, but since chemotropism is very much stronger it dominates entirely the reaction towards light.

REACTION TOWARDS DARKNESS

In the foregoing experiments, the permitted area of the capillary tube was light, while the prohibited area was in the shadow of the muffs. According to Bramstedt and Soest, a reaction towards light as a positive stimulus is not possible. I carried on a series of experiments with an opposite arrangement: the permitted area was dark, the prohibited, light. Muffs of black paper proved to be inconvenient in this case, since the protozoon cannot be seen while remaining behind the muff. I used muffs of cellophane, stained red, blue, green or violet. A single muff, 1 cm long, covered the middle part of the capillary. As long as the infusorian remained inside the muff, it was not disturbed, but every crossing of both boundaries of the muff was punished with induction shocks.

The results of these experiments correspond very closely to the facts already known and it is not necessary to repeat all of them. After several induced turns at both ends of the muff *Paramaecium* begins its spontaneous turns, always first on one end of the area and only gradually on the another. At last the infusorian stays for a certain time permanently inside the muff, avoiding the electric punishment by turning a little before reaching either end of the muff. If at this period we remove the muff, the protozoon does not alter its behaviour, still turning at exactly the same two points of the capillary tube.

It was not observed that the colour of the muff influenced the result. On the average, the first spontaneous turn occured after the following number of forced turns:

Red muff	48
Blue	45
Green	43
Violet	46

The number of minutes from the beginning of the experiment until the first spontaneous turn fluctuates between 7 and 10. In the case of the green muff this time is a little shorter, but the difference is not statistically reliable. The identity of result in spite of the different colours of muffs is not surprising, since independently of the colour of the muff the infusorian always turns from any colour towards the diffused light, this latter being in all cases the same stimulus.

SUPERPOSING OF PLACE AND LIGHT

It never occurs that the infusorian begins its spontaneous turns at both ends of the area synchronically; practically always the turns begin at one end of the area, while at the other end induced turns are still observed. This fact is not in accordance with the presumed existence of conditioned reactions towards light. The decisive factor must be looked for in the change of light conditions, and therefore an equal reaction at both ends of the area is to be expected. But the fact may be easily understood under the assumption that the factor stopping the infusorian consists in accumulation at certain points of the tube of chemotropically active substances. We must consider one particular. It is quite possible to administer the electric shock exactly at the moment of crossing the prohibited boundary. But the difficulty is that the reaction of the infusorian towards inductive current is not always the same. Sometimes the protozoon stops at once and it turns back; in many cases, spontaneous turns occur with amazing exactitude at the same point of the capillary. But in the greatest majority of cases the infusorian stops at a certain length of the capillary, the point of stopping may be determined only approximately. Usually *Paramaecium* „jumps" over the prohibited boundary and the length of its jump depends on the number of punishments previously received, on the velocity of movement and on its direction (parallel to the axis of the tube or spiral). Very frequently it becomes

necessary to use several electric shocks in order to stop the protozoon. Under such conditions substances, excreted by the protozoon, accumulate not at a definite spot, but at some length of the tube amounting to several millimetres. It is utterly impossible to maintain an absolute equality of conditions at both ends of the permitted area and therefore an equality of results cannot be expected. If the space inside of which the infusorian stops is larger at one end of area that at the other, the excreted metabolites will obtain their threshold concentration at the narrower space sooner and therefore the first spontaneous turn will occur at the corresponding end of the area. The fact that the infusorian stops as a rule before the critical boundary is reached, once more points towards a somewhat diffuse character of the stimulus, which is but natural in case of the critical stimulus being a chemical factor.

Taking this into consideration I executed experiments, in which I superposed to one another the two possible forms of influence: the influence of the changed light conditions and the influence of a definite point of the tube, where metabolites accumulate in a sufficient quantity. At the edge of a muff both chemical stimulus and light stimulus are superimposed and it is to be expected that at this spot the effect will be more marked.

One end of the permitted area, fully exposed to light, was limited by the edge of a single black muff. The opposite end of the area was distant by 1 cm from the muff and it was only a geometrically definite point of the tube. In 14 experiments the muff was at the right side 7 times, and at the left 7 times. The spontaneous turns began 7 times at the edge of the muff, and 7 times at the opposite end of the area, the result being a purely random one. Should the influence of light be effective, it could be expected that the spontaneous turns would begin at the muff, where both kinds of influence are present.

REACTIONS IN ABSENCE OF MUFFS

Several experiments were performed in order to obtain a conditioned reaction simply at definite points of the capillary, marked only at the adjacent scala. The permitted area was 1 cm long and occupied the middle part of the tube. According to our previous reasoning, in absence of muffs spontaneous turns should begin somewhat later and they ought to require more prior induced turns.

In order to facilitate the comparison, I shall describe one experiment of this kind. The infusorian reaches the permitted area from the right. After having crossed its boundary, it receives 14 electric shocks, then it swims right, obtains 2 shocks, swims left, obtains 6 shocks etc. After 37 induced turns, the first spontaneous turn occurs at a point distant by 1 mm from the left end of the area. Now, the protozoon swims right, it receives 5 shocks and it directs itself left; there it turns exatly at the same point as previously and before a punishment is given, and this sequence of events reoccurs 5 times. The spontaneous turns occur only at the left side. Only later they begin to appear at the right end as well.

This description corresponds exactly with the former one and all experiments of this series gave a very uniform result. The number of induced turns preceding the first spontaneous turn is practically the same, as in cases where muffs were present. In 14 experiments, there were on the average 45 induced turns (47 in the first experiment). The time from the beginning of the experiment until the first spontaneous reaction was about 9 minutes (formerly 10 minutes). In spite of the absence of muffs, there is no sign of any weakening of the reaction.

DISCUSSION

In the experiments reported, everything possible was done in order to detect the slightest traces of a conditioned reaction of *Paramaecium* towards light. The effect was always negative. Under the conditions of the experiment there arises a strong chemical stimulus, which satisfactorily explains all the results obtained. If in our case the influence of light does exist, it becomes totally masked. The methods hitherto used proved to be insufficient to detect a conditioned reaction towards light.

But the result obtained does not exclude the possibility of such a reaction in Protozoa. I think only that *Paramaecium caudatum* is not suitable as an experimental object for those purposes. If this species is sensitive towards light, the influence of light is extremely weak as compared with the action of several other factor scarcely to be avoided.

One of the experimental possibilities consists in an artificial sensitising of *Paramaecium* towards light, which may be done with weak solutions of eosine and erythrosine (Efimoff, Metzner),

or with preparations of hypophysis (Bramstedt). Another and more promising method may consist in using for experimental purposes species of Protozoa possessing fotoreceptors.

The whole problem is of a great theoretical importance. It must be fairly admitted, that up to the present we do not have one single critically analysed case of learning in Protozoa. If the conditioned responses exist in unicellular beings, we have the important possibility of treating uniformly the evolution of behaviour in the whole animal kingdom. If such responses are impossible, then we shall be obliged to revise many of our views. In both cases the theory will have to follow critically established facts.

RESULTS

The present paper deals with the possibility of obtaining a conditioned response towards light in *Paramaecium caudatum,* as maintained by some authors. Single infusorians were put into a capillary tube with platinum electrodes, connected with an inductive coil. On the capillary two muffs of black paper were fixed; the muffs were distant by 1 cm from each other, the middle part of the capillary was open. This lighted part of the tube was the permitted area, but if the infusorian crossed the boundary between light and darkness, it received a series of inductive shocks till it turned back. This may be done by changing the position of the cathode of the current of interruption. Under those conditions the infusorian begins to perform „spontaneous" turns on the critical boundary before it has received an electric punishment. This result has been described by former authors as a conditioned reaction. But the control experiment proved that after the infusorian began its „spontaneous" turns and after the two muffs were shifted both ways, until the space between them became 2 cm long, the protozoon nevertheless turns exactly at the same two points of the capillary as before. The reaction has nothing to do with light. The same result may be obtained without any muffs, by simply stimulating the protozoon with inductive current at two random but constant points of the tube. The points at which the infusorian turns back correspond to those in which it stays the longest time during the experiment. It is probable that at these two points a material spur is left, perhaps in the form of metabolites excreted into the outer medium, and it is also probable that *Paramaecium* reacts negatively-chemotropically towards those products, the chemi-

cal factor being an unconditioned stimulus. In spite of performing diverse variations of the experiments, true conditioned responses in *Paramaecium caudatum* were never obtained. Perhaps this species is not a favourable experimental object, as far as light reactions are concerned.

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CIRCULATION OF FOOD VACUOLE IN PARAMAECIUM CAUDATUM

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The problem of circulation of the food vacuole in the body of *Paramaecium caudatum* has so far received but little attention. According to the old theory of Ehrenberg (1861) food vacuoles move along an invisible but preformed digestive tract. The existence of such a tract was challenged by Greenwood who investigated this question in *Carchesium polypinum*. At present all are agreed, that there is no permanent duct in protoplasma and the movement of food vacuole is caused either by plasmatic currents, or by a local contraction of protoplasma, similar to peristaltics. Nevertheless according to Kitching (1938) who investigated the circulation of food vacuole in *Peritricha*, there is in the cell a certain differentiated region of protoplasma, which not only conducts the food vacuole, but also sets it in movement. This region is connected with pharynx and it can move in direction of the protoplasma current.

The problem of circulation of the food vacuole in *Paramaecium caudatum* was fully dealt with by Nirenstein (1905). This author found that the food vacuole, after being torne from the pharynx bottom, passes quickly towards the hind end of the body. There a brief rest period occurs, lasting a few minutes, after which the food

vacuole begins to move towards the front, along the left side of the infusorian, and reaches a point situated at a level of 1/3 or 1/2 of the body length, rarely higher. There the food vacuole turns, it goes round the macronucleus and it returns again to the hind part of the body. This is the end of the so-called small circuit, which may be repeated several times. Later on the food vacuole moves again along the left side towards the front of the body, it passes the macronucleus, turns at the anterior end of the protozoon and finally it returns along the right side to the hind end, where it is defecated. The sequence of the small and great circuit is not constant. The complete circuit lasts from 2 to 4 hours.

Bills (1922) and Bragg (1935) corroborated these results though in a somewhat general way.

An interesting experiment was carried out by Dogiel and Issakowa-Keo (1927). If to the drop of liquid with infusorian some solution of m/32 of $MgCl_2$ with some india ink is added, then in the body of infusorian a food current is formed in the form of a ribbon twisted several times After a certain time this ribbon splits into a series of individual vacuoles. Coils formed by the ink ribbon correspond approximately to the small circuit of Nirenstein; this circulation can, in normal conditions, also be repeated several times.

It appears from this somewhat sketchy summary of the literature that the results obtained by Nirenstein were corroborated either only in general or indirectly. A closer observation of the circulation of food vacuole in *Paramaecium caudatum* points, however, to the fact that the regularity described does not always occur.

The object of the present paper is a detailed investigation of the tract of food vacuole from the moment of its formation until its defecation.

METHOD

The infusoria were bred on hay, lettuce or carrot infusion. Two series of experiments were carried out: with starved infusoria which were washed and which remained in pure water for 24 hours, and with nourished ones taken straight from cultures. The infusoria were given two kinds of medium:

Non feeding medium such as solutions of india ink, indigo, fuchsine, methyl blue and barium chromate.

Food suspensions such as yolk mixed with Congo red, bacteria from lettuce infusion and numerous species of bacteria developing on the embryonic extract of Carrel.

Our observations were made always on individual infusorians. The protozoon was put into the suspension investigated for from $^1/_2$ to 2 minutes which is a sufficient period of time to form one food vacuole. Then the infusorian was washed with pure water and moved to a drop of cherry glue. This glue was prepared by adding 5 g of an ordinary cherry glue to 100 cm^3 of twice distilled water. The mixture was boiled for 15 minutes. After cooling the glue was ready for use. In order to prevent the preparation from drying during the period of prolonged observation, the drop with glue containing one infusorian was placed on a glass slide on which a frame of thin celuloid (ca. 0,3 mm) was fixed with aceton. The frame was covered with a thin layer of vaseline, so that the cover glass fitted tightly. It is important that the drop with infusorian should not touch the celuloid. Although its cilia work strenuously, the infusorian moves very slowly in the glue, so that slight manipulations with the stage of the microscope make it possible to keep the protozoon permanently under observation. For a detailed determination of the position of the food vacuole along the body an ocular micrometer was used. The determination of depth in which the vacuole is lying is more difficult. For the sake of orientation a hexagonal prism was drawn into the scaled figure of *Paramaecium;* its facets were determined by letters (fig. 1). The real depth of the vacuole was determined with the micrometer screw, and every position of the vacuole could be defined by means of a pre-arranged letter. On figures the upper levels were drawn with a continuous line, the lower ones with a line of dashes.

The position of the vacuole was recorded every 5 minutes. The total length of the curve traced by the vacuole was measured with a Steinhaus longimeter. All manipulations, from the moment when the infusorian was placed in the observation drop and till the first adnotation of the position of the vacuole lasted from 2-4 minutes. The position of macronucleus was determined in the dark field by means of a paraboloid condensor.

THE COURSE OF FOOD VACUOLE IN THE BODY OF INFUSORIAN STARVING FOR 24 HOURS

The circuit of a vacuole containing non feeding substances (india ink, indigo, methyl blue, barium chromate, fuchsine) is chaotic and very variable, as may be seen from figs 2-3. Sometimes the vacuole remains almost stationary in the hind part of the body until its defecation (fig. 4b), sometimes it moves at a considerable speed along the whole body, covering every time a different route (fig. 2b). The weight of the substance taken has, it seems, no effect on the circulation; in any case the vacuoles with heavy barium chromate may circulate as quickly as those with other suspensions. The size of the vacuole is of no importance either. The time required for circulation is variable. For the same substance and under equal conditions this time fluctuates from 35 to 200 minutes.

Table I

Non feeding substances. Period of circulation in minutes and the total length of the route in microns.

Methylblue	Time	40	30	25	30	25	35	120	
	Route	113	112	146	374	190	255	636	
India ink	Time	70	70	50	95	115	65	200	35
	Route	129	166	238	255	1268	295	2329	250
Indigo	Time	200	40	150	180				
	Route	1093	274	980	790				
$BaCrO_4$	Time	105	95	110	125	45	90	170	
	Route	224	1225	625	774	363	640	1110	
Fuchsine	Time	20	25	25	40	70			
	Route	197	179	159	273	324			

The numbers of the Table I indicate that the length of the route covered by the vacuole is not proportionate to the total time of circulation, that is to say that the speed of vacuole movement is variable. The longest time required for circulation is 200 minutes.

A somewhat different result is obtained with substances assimilated by the infusorian. In the suspension of yolk with Congo red, the circulation of the vacuole lasted a very long time, from 6 to 10 hours. The prolongation of cyclosis does not depend on the fact that infusoria were observed in a viscous medium, because the control con-

taining one yolk vacuole and observed in pure water showed a similarly prolonged cyclosis (minimum 4 hours).

In all cases observed the great circuit was followed, that is to say the vacuole traversed the whole length of the body at least once. Its route, nevertheless, varies greatly and it does not suggest any marked regularity (Fig. 6).

In a suspension of yeast the circulation lasts up to 6 hours. The great circuit is always present, but a regularity in the configuration of the cyclosis is lacking (Fig. 7).

Bacteria from the lettuce infusion: circulation 1-4 hours, in general the great circuit is present (Fig. 8).

Bacteria from the embryonic extract. Special tests established that *Paramaecium caudatum* feeding on bacteria from the embryonic extract multiply very rapidly, which proves a considerable nutritive value of the extract. The food vacuole with these bacteria circulates in the body a very short time, 35-40 minutes, and it scarcely leaves the region of under macronucleus (Fig. 9).

Table II

Feeding substances. Time of circulation in minutes and the entire length of the route in microns.

Yolk	Time	390	340	480	615
	Route	362	377	511	1009
Yeast	Time	280	90	385	
	Route	544	382	725	
Lettuce	Time	110	105	60	230
	Route	1363	547	234	698
Embryonic extract	Time	35	45		
	Route	75	148		

A comparison of both series i. e. substances non assimilated and assimilated does not reveal any distinct regularity. In both cases the time required for cyclosis may fluctuate within very wide limits and so does the complete length of the route of the vacuole. But the speed of movement of the vacuole seems to be to some degree dependent on the nature of the suspension. We give in microns per minute the speed calculated on the basis of both tables of the movement of the food vacuole. The table III contains however the average speed while the real speed may be very different at different points of the route.

Table III

The speed of the movement of the food vacuole in microns per minute.

Methylblue	2.8	3.7	5.8	12.5	7.6	7.3	5.3	
India ink	1.8	2.4	4.8	2.7	11.0	4.5	11.6	7.1
Indigo	5.5	6.9	6.5	4.4				
$BaCrO_4$	2.1	12.9	5.7	6.2	8.1	7.1	6.5	
Fuchsine	9.8	7.2	6.4	6.8	4.6			
Yolk	0.9	1.1	1.1	1.6				
Yeast	1.9	4.2	1.8					
Lettuce	12.4	5.2	3.7	3.0				
Embr. extract	2.1	3.3						

In general the greater is the nutritive value of the content of the vacuole, the smaller the speed of its movement. The lowest speed is shown by a vacuole with yolk, which is the most valuable feeding substance.

In the relative literature the small circuit of the vacuole is said to be dependent on macronucleus, which produces digestive enzymes. We tried to ascertain whether there is a constant ratio between the time during which the vacuole remains close to macronucleus and the feeding value of the vacuole content.

The results of the Table IV are far from constant. They point nevertheless to a certain regularity. In the case of a feeding suspension the small circuit is always present. In the cases of nonnutritive suspensions, in 7 instances (out of 19 observed) the food vacuole did not approach the macronucleus at all.

THE ROUTE TAKEN BY THE FOOD VACUOLE IN THE BODY OF NOURISHED INFUSORIAN

When *Paramaecium*, taken directly from the culture, comprises many bacteria vacuoles, when one india ink vacuole is added to them, and when the infusorian is then placed in the glue — the circulation of the india ink vacuole is very short, lasting but 20-40 minutes. The vacuole remains generally under the macronucleus (Figs. 10a, b). If, on the contrary, the infusorian remained for some time in pure water and when it defecated a considerable part of its food vacuoles, then the route of the single india ink vacuole becomes different. The

Table IV

Time during which the vacuole remains in definite places of the body: a — The content of the vacuole, b — Total time of the cyclosis, c — Time during which the vacuole remains under the macronucleus, d — Time during which the vacuole remains in the region of the macronucleus itself, e — Time during which the vacuole remains over the macronucleus.

a	b	c	%	d	%	e	%
India ink	70	70	100	—	—	—	—
	70	50	71	20	29	—	—
	50	50	100	—	—	—	—
	95	10	10	85	90	—	—
	115	75	65	20	17.5	20	17.5
	65	65	100	—	—	—	—
	200	120	60	35	20	35	20
	35	5	14	25	72	5	14
Indigo	200	195	97.5	—	—	5	2.5
	40	40	100	—	—	—	—
	180	30	16.7	50	27.8	100	55.5
	150	90	60	40	26.6	20	13.4
$BaCrO_4$	105	105	100	—	—	—	—
	95	35	36.9	55	57.8	5	5.3
	110	65	59.1	30	27.3	15	13.6
	125	70	56	25	20	30	24
	45	45	100	—	—	—	—
	90	60	66.7	10	11.1	20	22.2
	170	30	17.6	35	20.7	105	61.7
Yolk	390	195	50	10	2.6	185	47.4
	340	120	35.3	70	20.6	150	44.1
	615	225	36.6	390	63.4	—	—
Yeast	280	70	25	190	67.9	20	7.1
	90	55	61.1	30	33.3	5	5 6
	385	70	18.2	105	27.3	210	54.5
Lettuce	110	90	81.8	20	18.2	—	—
	105	50	47.6	30	28.6	25	23.8
	60	15	25	40	66.7	5	8.3
	230	35	15.2	175	76.1	20	8.7
Embryo. extract	60	50	83.3	10	16.7	—	—
	35	25	71.4	10	28.6	—	—
	45	40	88.9	5	11.1	—	—

cyclosis lasts much longer, 2-4 hours, whereas the route of the vacuole is long and complicated (Fig. 11b); the great circuit is repeated several times and the vacuole returns repeatedly to the fore macronucleus region. The saturation of the infusorian has a marked effect on the character of the cyclosis.

THE CIRCUIT OF TWO FOOD VACUOLES

A different method of investigating the dependence of the circuit of the vacuole on its content is to observe two vacuoles of the same kind at the same time. In a medium containing bacteria *Paramaecium* forms food vacuoles without interruption. One individual of *Paramaecium* was put into a drop of bacteria suspension but with the addition of methyl blue. When after about one minute a single blue vacuole was formed, the infusorian was transferred for the same period of time into same bacterial suspension but coloured with fuchsine. Immediately afterwards the infusorian was placed in a drop of cherry glue. The result was that we obtained an infusorian containing two vacuoles of different colours but of the same bacterial content. Both, the time required for the circulation of these two vacuoles and the lengths of the route covered were very different, sometimes entirely opposite as may be seen from Fig. 12. In some later experiments infusorian with two identically coloured food vacuoles were observed. The observation in this case is more difficult but possible. Its result corroborated exactly the result of the former experiment: identical vacuole can circulate in protoplasma along quite different routes and the time of the cyclosis is often very different.

DISCUSSION

The hypothesis of Ehrenberg of a preformed track of the food vacuole is in conformity with his views on the problem of infusoria in general. Ehrenberg was of the opinion that the animals consisting of one cell possess in reality a very complicated morphological structure, invisible because transparent. This hypothesis could not be maintained and it was replaced by the hypothesis of a constant digestive track. In general, all authors are at one in stating that the food vacuole of *Paramaecium* moves in protoplasma along a definite, constantly repeated track. But at the same time it is admitted that the sequence both of the small and the great circuit can vary considerably and that those circuits may be repeated several times.

Such stability of the cyclosis assumes a necessity of the existence of a certain preformed plasmatic structure decisive as to the configuration of the movements of the vacuole. If there exist in the cell either protoplasmic currents having a constant configuration or

there are some local protoplasmic contractions, there remains still to be explained the problem of the stability of the route of the vacuole. Besides, as may be seen from Table III, the speed of vacuole movement in the central parts of the body may be relatively very considerable, and the plasmatic current of such a ratio could hardly fail to be observed.

It follows from the facts presented in this paper that the very problem which we are trying to solve, i. e. the cause of the constancy of the vacuole track, is rather doubtful, because the process of the circulation of the food vacuole in the body of *Paramaecium caudatum* does not correspond to the description given by Nirenstein. The route of the cyclosis is very variable and it does not show any regularity. We cannot speak of the existence of two regularly consecutive small and great circuits. The route of the vacuole is more or less casual.

The material here presented does not allow to decide whether the cyclosis is caused by casual plasmatic currents, or whether it depends, nevertheless, on a particular mechanism, varying parallel to the variation of the vacuole content, to the physiological state of the infusorian, to the number of vacuoles and many other possible factors. These questions can be solved only by special systematic experiments.

SUMMARY

According to the observations of Nirenstein, partly corroborated by other authors, the route of the food vacuole in the body of *Paramaecium caudatum* is constant. He described the small circuit round the macronucleus, and a great one throughout the whole body, drawing attention to the fact that the sequence of these two circuits may be different.

In the present paper this problem was investigated by a more exact method. An infusorian possessing one vacuole with the investigated substance was placed in a viscous medium to slow down its movements. The position of the vacuole was recorded every five minutes and it was determined by means of an ocular micrometer and the micrometric scale of the microscope. The experiments were carried out either with infusoria starving for 24 hours or with those taken directly from the culture. Food vacuoles contained either non feeding substances as india ink, methyl blue, fuchsine, $BaCrO_4$, in-

digo, or feeding suspensions like yolk, yeast, bacteria from a lettuce infusion, bacteria from embryonic extract. The routes of the vacuoles proved to be very variable, partly dependent on the content of the vacuole, partly on the degree of the saturation of the infusorian. Even so, two identical vacuoles in the same infusorian may be absolutely different, both as to the period required for circulation, and to the total length of the route. As a rule, the speed of vacuole moving in protoplasma is lower in the case of nutritive suspensions.

The hypothesis of N i r e n s t e i n cannot be sustained. The causes of vacuole circulation are much more complicated and to explain them requires special investigations.

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EXPLANATION OF FIGURES

Fig. 1. Schema of *Paramaecium*. Continuous lines correspond to the upper planes a, b, c. Lines of dashes — lower planes d, e, f.

Fig. 2. Infusorian starving for 24 hours. Circulation of an India ink vacuole. *a* — circulation lasted 95 minutes, the total route was 255 microns. *b* — circulation 200 min., route 2329 micr. *S* — starting point of the cyclosis.

Fig. 3. Indigo. *a* — circulation 200, route 1093. *b* — circulation 150, route 980.

Fig. 4. $BaCrO_4$. *a* — circulation 125, route 774. *b* — circulation 105, route 224.

Fig. 5. Methyl blue. *a* — circulation 40, route 113. *b* — circulation 30, route 112.

Fig. 6. Suspension of yolk with Congo red. *a* — circulation 390, route 362. *b* — circulation 615, route 1009.

Fig. 7. Yeast. *a* — circulation 90, route 382. *b* — circulation 280, route 544.

Fig. 8. Lettuce infusion. *a* — circulation 110, route 1363. *b* — circulation 60, route 234.

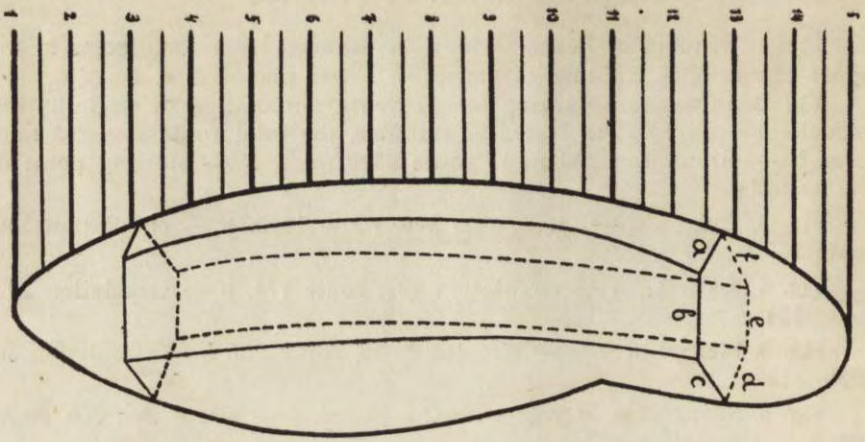
Fig. 9. Embryonic extract. *a* — circulation 35, route 75. *b* — circulation 45, route 148.

Fig. 10. Circulation of the vacuole in the body of a nourished infusorian. *a* — India ink. Circulation 35, route 374. *b* — circulation 20, route 243.

Fig. 11. *a* — methyl blue. Circulation 30, route 374. *b* — India ink. Bacteria vacuoles are already defecated. Circulation 210, route 1469.

Fig. 12. Simultaneous circulation of two vacuoles. *a* — methyl blue vacuole (continuous line) with circulation 25 and route 159, and fuchsine vacuole (line of dashes) with circulation 40 and route 273. *b* — methyl blue circulation 120, route 636, fuchsine circulation 70, route 324.

(The position of the macronucleus is recorded with line of dots).



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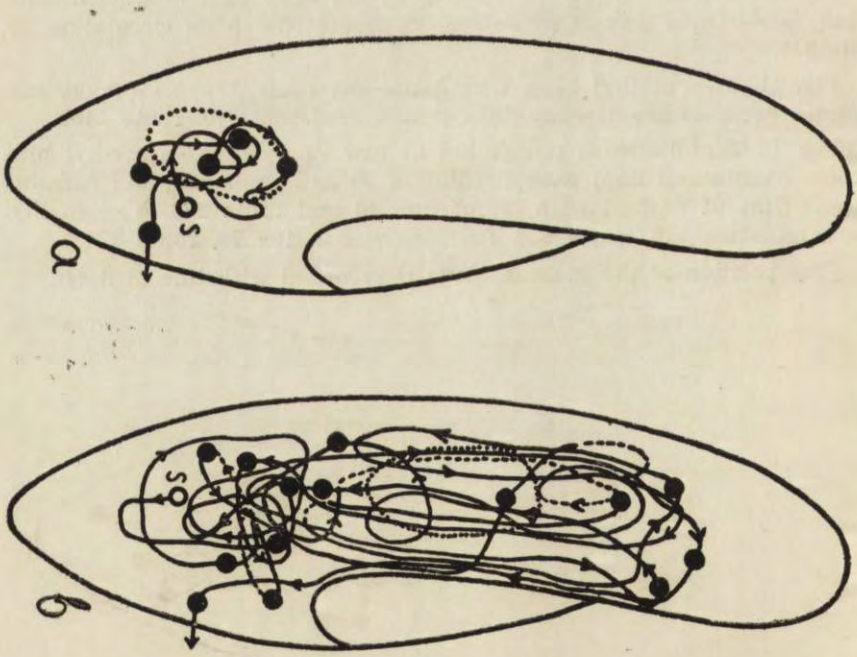

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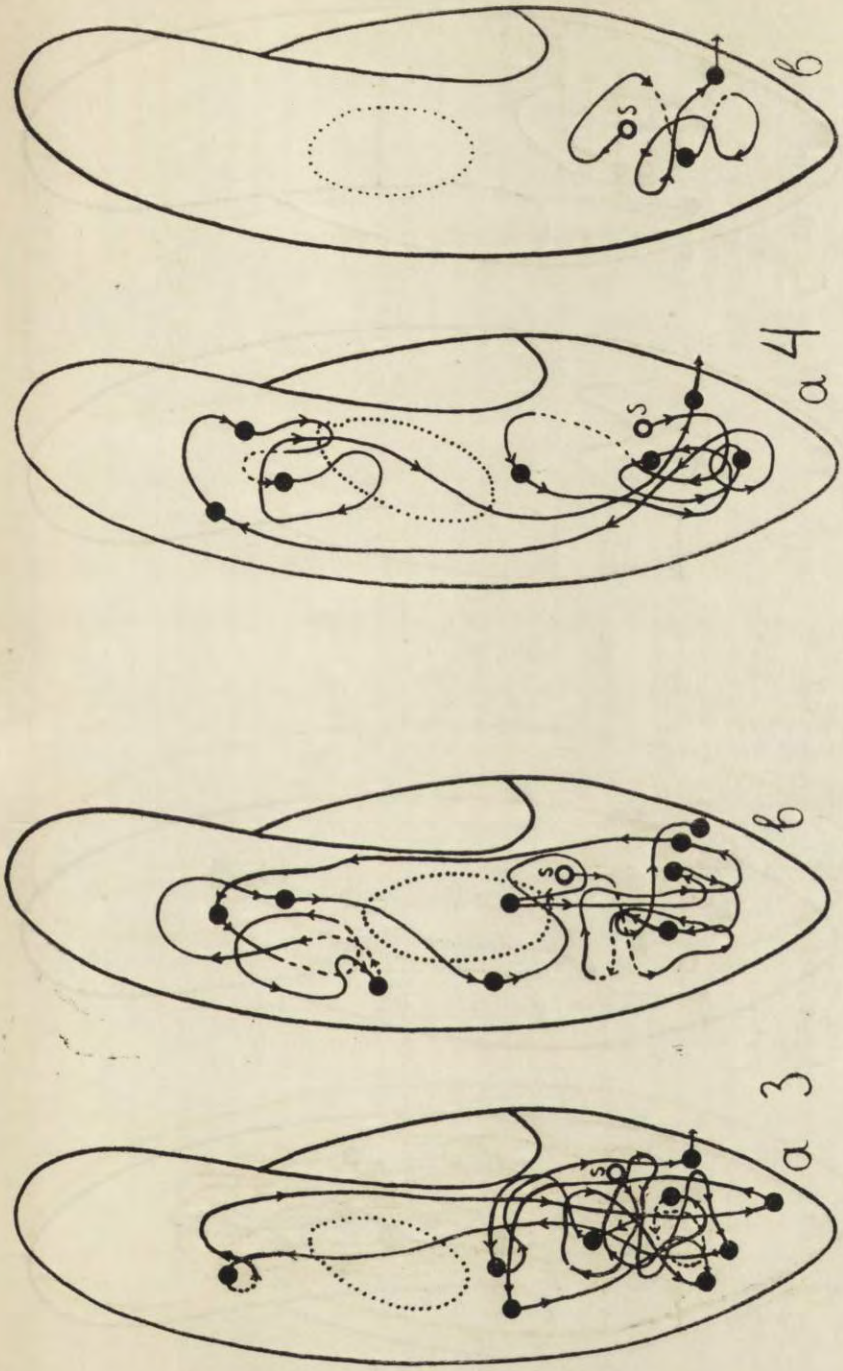

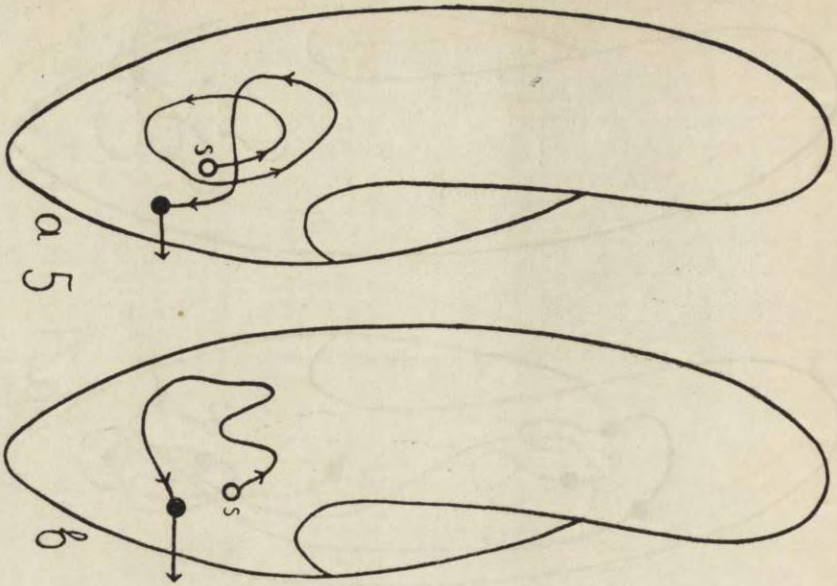

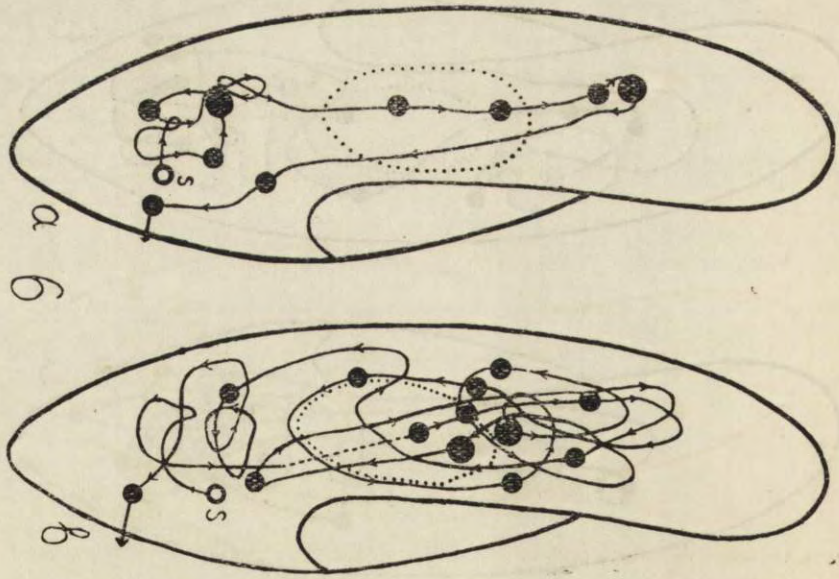

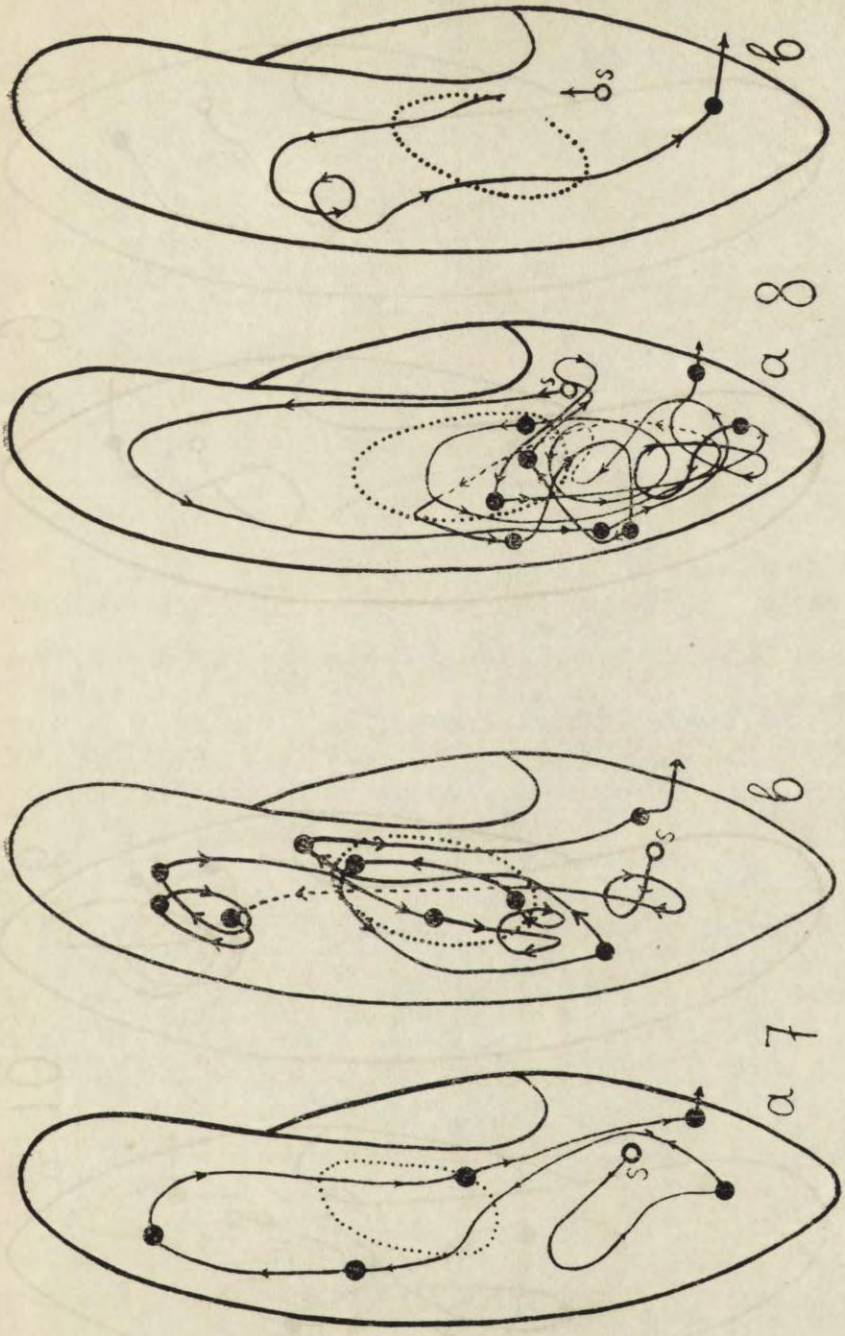

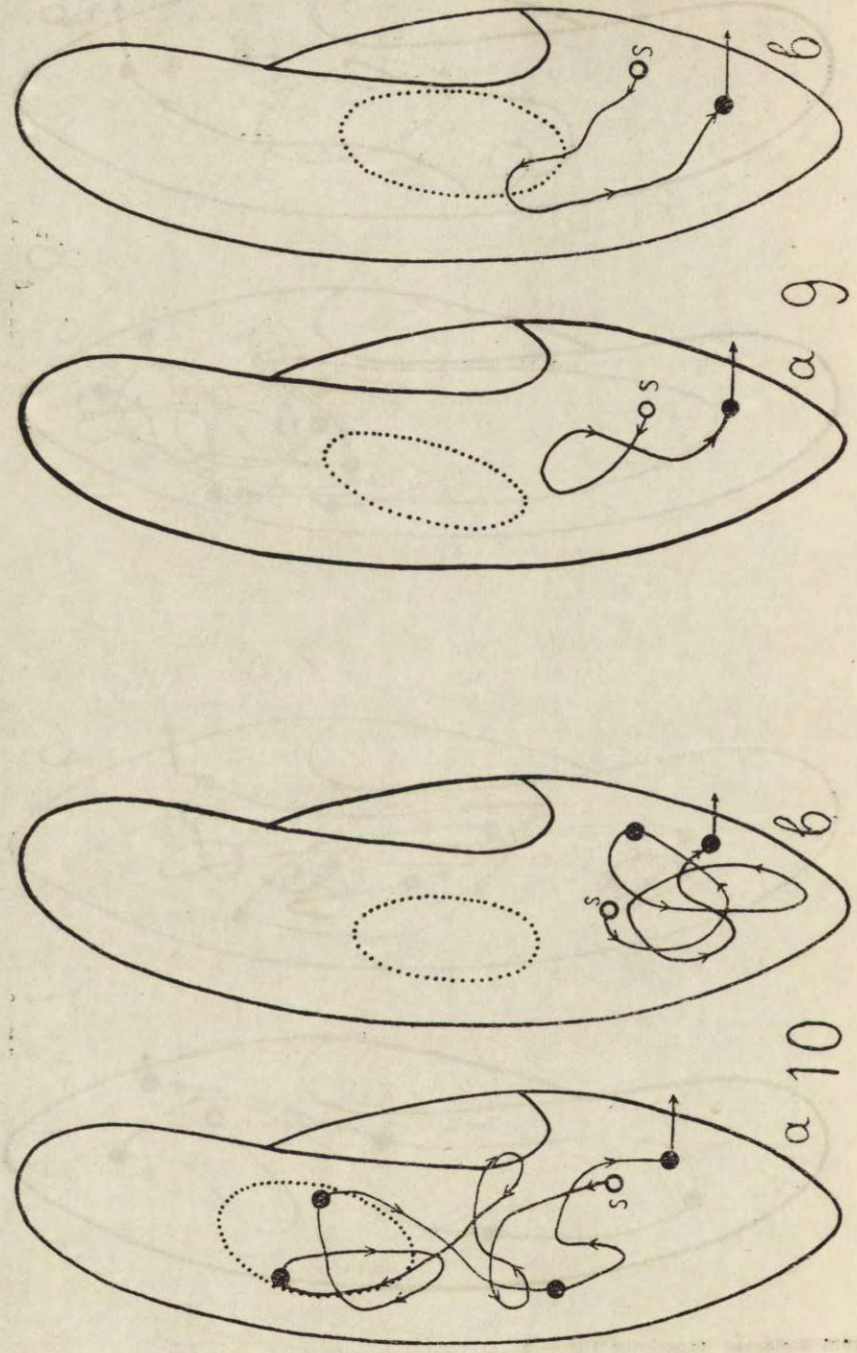

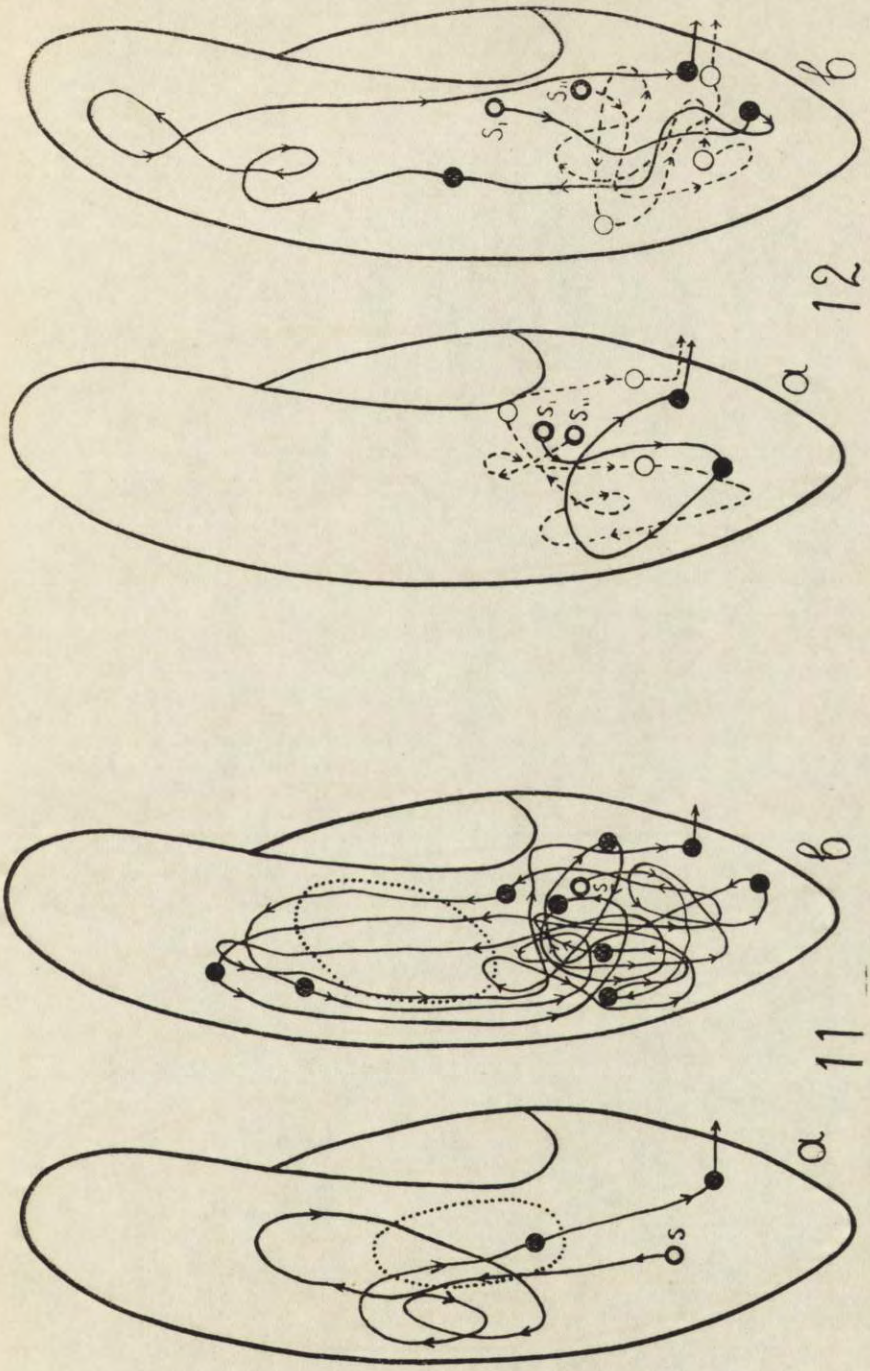

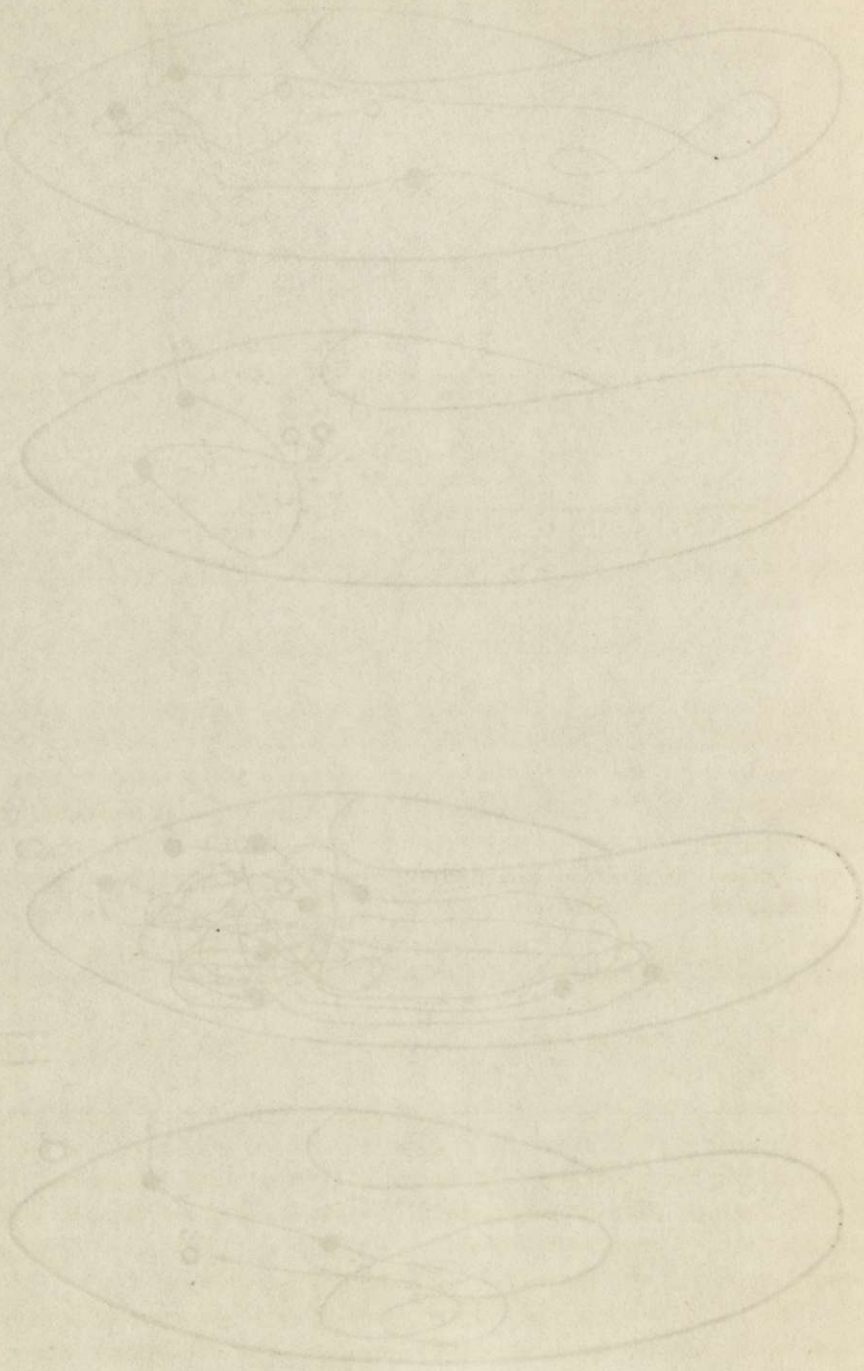

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AN ATTEMPT OF ANALYSING THE VARIABILITY IN THE BEHAVIOUR OF THE CADDIS-FLY LARVA *MOLANNA ANGUSTATA*.

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It is known from the previous researches, that the behaviour of the caddis-fly larva *Molanna angustata* is characterised by a high degree of individual variability. When larvae have a definite task to accomplish as for example to build a case, or to repair a damaged one, to choose the suitable building material or to turn, it practically never happens that two larvae will behave exactly alike. Suppose that the task may be accomplished in one of two ways more or less equivalent: for instance larvae have received building material composed of only two different kinds of grains and they can choose one or other, or when they have to turn over and they may do it equally well to the right or to the left. The larvae themselves differ individually and at the same time the exterior conditions are variable, and a minimal change of conditions at the moment of choice can decide about choosing one way or other. As the individual variability of the larvae depends on the variability of exterior uncontrolled conditions, we must conclude that in sufficiently numerous samples of larvae the distribution of frequency of two possible and equivalent solutions would approach the frequency of random distribution.

The situation becomes different when the same larva does the same work several times one after another. The conditions differ in

each case; our individual larva also is not a given constant, because surely the individual building its tenth case differs somewhat from the larva manufacturing its fifth case. But we can assume, that the changes apparent in an individual from day to day are smaller than the differences between separate individuals. If that is so, then the ways of behaviour of an individual larva ought to differ from a random distribution, as there exists a constant individual tendency. This is not a conclusion, but a problem which we are setting ourselves in the present paper.

Let us consider a simple example. To a larva a task is given which it can solve in one of two equivalent ways: *a* and *b*. The larva repeats its work ten times consecutively applying one of the following sequences:

1. a a a a a a a a a a or
2. b b b b b b b b b b or
3. a a a a a b b b b b or
4. a b a b a b a b a b or
5. a a b a a b a a b a

Each of these five systems gives some evidence of regularity which again permits us to assume the existence of some regulating factor. The systems 1 and 2 include only one of the two possible factors, in the system 3, *a* is repeated five consecutive times and *b* five times, in the system 4, we have a regular alternation, and in the system 5 the combination *aab* is repeated three times and *aba* three times. The probability of obtaining such sequences in the absence of some regulating factor is rather low and if only the systems of this type occured constantly in the work of *Molanna*, it would be an easy task to characterise them. But in practice the regularity, if existing, is approximate and it can be discovered only by statistical methods.

This reasoning explains our method. We set larvae different tasks and each individual had to solve them many times consecutively. Then we compared the behaviour of the larvae with the random distribution.

The sections one to five give facts of a somewhat qualitative character allowing for the formulation of certain hypotheses. In section VI we give the exact figures and their statistical meaning in relation to one experimental case. We are indebted to Prof. M. Olekiewicz from the Institute of Mathematical Statistics for the help in the statistical analysis.

MATERIAL AND METHOD

The experimental part of the work was carried on by Sulamita Staropolska in the years 1937 — 1938, partly in the Biological Institute of the Wilno University, partly on the Hydrobiological Station on the Wigry lake. The observations included the folloving larva activities:

1) Repairing the case in dependance on the manner and degree of injury and depending on the position of the larva in the case.
2) The selection of the grains of the building material of different shape according to size.
3) The selection of grains of the same shape according to size.
4) The selection of grains of the same size according to shape.
5) The process of turning when put on the back.

The individual larvae were put into crystallizers of 7 cm in diameter, the bottom of which were covered with a layer of building material. Observations on the lake Wigry were performed in almost natural conditions, the crystallizers with larvae being put into the lake at a depth of from 50 — 60 cm. Over 300 individuals were under observation and more that 2000 experiments were carried out with them.

I. REPAIRING THE CASE

Molanna repairs damaged cases in different ways depending on the nature and degree of damage. If the individuality of larvae is different, it would be expected that in conditions of constant damage to the case different ways of repairing would be found in definite individuals. The experiments were carried out in four series.

SERIES 1. The posterior half of the tube of the natural case was cut off, and the larva was put in the remaining part of the case in a normal position, its head directed forward (fig. 1).

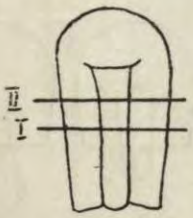

Fig. 1.

Scheme of the operation on the case performed in series 1 and 2 (I) and in the series 3 and 4 (II).

Each larva worked in a separate crystallizer, the bottom of which was covered by a layer of egg shells crushed and sieved. In this way the parts additionally built differed distinctly from the others. The result of each experiment was recorded after 24 hours. After this, all the parts which the larva had additionally built were removed with a preparation needle and the larva was put again in the same damaged case for the next 24 hours. This was repeated daily until the larva stopped working, probably exhausted. In each reparation the larva had to deal with exactly the same nature and degree of damage to the case. The experiments were made with 65 larvae, each of them carried out a repair from 2 to 21 times consecutively. There were altogether 488 repairs.

As always, the behaviour of different larvae as well as the behaviour of the same larva on different days varied greatly. Some of

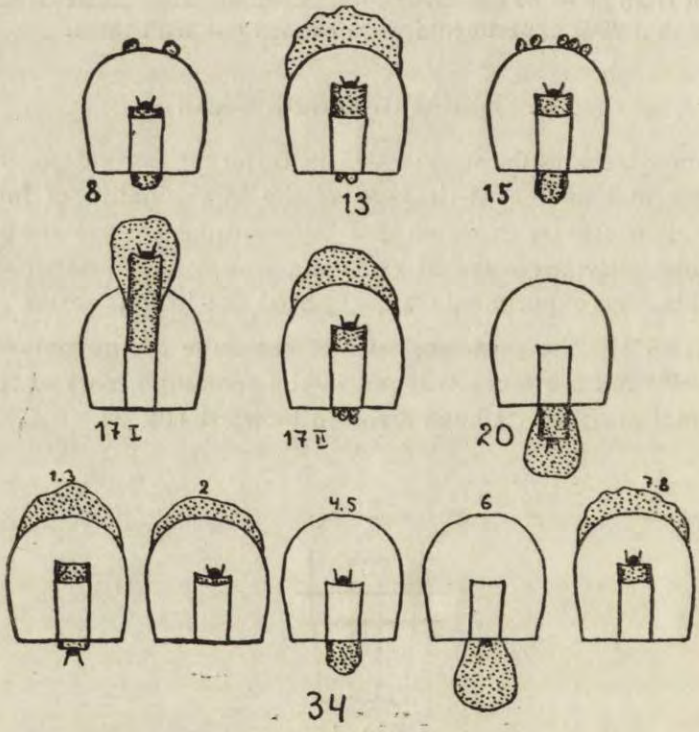

Fig. 2.

Examples of repairing cases. The additionally built part of the case is marked by dots.

the larvae rebuilt the case forward, others repaired it from both ends and in a different degree; some used indirect methods of repair, others at last abandoned the original case and built an entirely new one. In spite of this variability, in some cases there was observed an obvious inclination of some individuals to definite ways of behaviour. We give examples.

Larva Nr 8 was observed during 18 periods of 24 hours each, it made 16 repairs. In 12 instances the repair was of the same kind: the larva added a small collar of grains in prolongation of the anterior end of the tube, stuck an additional score of grains to the hind part of the tube and stuck 1 — 2 grains on the anterior end of the canopy In the remaining four cases there were no grains in the hind part of the tube, and in one case there were no grains at the edge of the canopy.

Larva Nr 12 was under observation during 6 days. Not a single time did it repair the case. Every day the larva was removed from the new case, built during the previous 24 hours, and put in the damaged natural case. During 6 days the larva manufactured 6 new cases, but it never made any attempt at repairing the old one.

Larva Nr 13 effected a repair 15 times. On 13 occasions the repair consisted of prolonging the tube cephalad and adding a large new canopy. In 9 instances the larva also added several grains to the posterior end of the tube. The last two repairs were rather poor.

Larva Nr 15 effected a repair 21 times. 17 times out of 21 the repair was on both sides: the larva added grains to the anterior and posterior end of the tube, and also it regularly added 2 — 6 grains to the anterior edge of the canopy.

Larva Nr 17 effected a repair 5 times, always building forward almost a whole new case, which consisted of a tube reaching far beyond the old canopy and of a large new canopy. In the last repair the larva had detached the new case from the remnants of the old one, using the old case only as a point of support .

Side by side with these examples of characteristic behaviour of individual larvae, many cases of great variability in the same larva were observed. For instance, for the first time larva Nr 34 added forward a piece of the canopy and prolonged the tube forward; it added several grains at the posterior end and settled in the case in a reversed position, with its head directed caudad. On the second occasion, it added grains at the anterior end of the tube and the canopy and it assumed a normal position. For the third time the larva

proceeded in a similar way as for the first. For the fourth and fifth time it added grains only at the hind part of the tube, the position of the animal in the tube being normal. For the sixth time, a large canopy was built at the posterior end of the tube and the larva assumed a reversed position. For the seventh and eigth time, the larva added the tube and the canopy forward, the position being normal.

In some cases the behaviour of some definite larvae was so specific, that one could foresee almost from day to day how the repaired cases would look. In section VI we shall give a statistical analysis of this series of experiments.

SERIES 2. The case was damaged in the same way, but the larva was put into the tube in a reversed position, head directed caudad. The experiment embraced 35 animals which repaired up to 18 successive times. The result was essentially the same: parallel with a great individual variability, there occured sometimes an obviously monotypic behaviour.

Larva Nr 17 repaired 6 times. It always built an additional piece of tube and canopy forward and added a few grains to the hind end of the tube. It settled in the case in a normal position. Fig. 2 — 17 II.

Larva Nr 20 repaired 14 times. Each time it built an additional piece of tube ending with a large canopy, both directed caudad. In 12 instances the position of the larva was reversed in relation to the norm i. e. it was congruent with the position of the new canopy.

SERIES 3. There was more damage to the case, the posterior two thirds of the tube were cut off and the larvae were put into the anterior section in normal position. The experiment embraced 43 larvae which effected from 5 to 15 repairs.

SERIES 4. The operation was the same, but the larvae were put in the reversed position. Neither series gave new results.

It is concluded from all these experiments that prevalent in the behaviour of *Molanna* seems to be an irregular variability. But in the work of some particular individuals, there is an obvious systematic preference for one way of repairing rather than another.

II. CHOICE OF THE GRAINS OF DIFFERENT SHAPE ACCORDING TO THEIR SIZE

The experiments included 22 larvae, each of which built successively 10 entire cases. After the larva was pushed out of its own case, it was put on the layer of egg shells differentiated by means of

two slightly different sieves. The flat surface of the grains varied within limits of 0,49 to 2,13 mm^2. After 24 hours the larva was pushed out of its new case and put again on the same layer of grains. This was repeated daily with each larva over a period of ten days. Out of the constructed cases three portions were cut: part of the canopy, a section of the ventral wall of the tube next to the fore opening, and a similar section of the dorsal wall. These parts of the case, laid on a level surface, were sketched under enlargement. The surface of the grains was measured on the drawing. In each section thirty adjacent grains were measured. In this way, 5 succesive cases of six larvae were taken into consideration, that is to say 5 . 6 . 30 . 3 = 2700 grains. Special care was taken to use larvae of as near similar length as posible. The tube length of the natural cases in this experiment was always 14 mm. Table I illustrates the result.

TABLE I.

Nr — number of the larva, A — number of the case, B — the average size of the grains of the canopy, C — the same for the dorsal wall of the tube, D — ventral wall of the tube of 5 successive cases of 6 larvae. The figures correspond to the average surface of grains in square mm.

No	A	B	C	D	No	A	B	C	D
	1	12.7	9.3	5.7		1	15.8	13.9	9.2
	2	14.6	12.0	5.7		2	16.6	12.6	10.0
1	3	13.4	11.0	6.6	4	3	11.7	11.7	7.0
	4	15.2	11.7	5.6		4	12 2	8.7	5.8
	5	—	—	—		5	16.4	11.0	8.5
	1	15.2	9.6	5.3		1	11.3	7.6	5.0
	2	14.0	9.9	5.8		2	14.2	10.3	7.2
2	3	13.0	12.3	12.0	5	3	13.7	11.2	6.3
	4	13.7	10.0	6.0		4	15.3	12.0	6.0
	5	13.9	11.0	5.6		5	16.5	15.0	9.1
	1	10.0	7.8	2.7		1	12.2	9.8	5.5
	2	9.8	8.0	2.6		2	11.7	10.5	5.5
3	3	10.5	8.8	3.3	6	3	15.1	11.0	7.2
	4	10.5	9.1	2.3		4	14.4	10.9	8.0
	5	10.0	7.8	3.0		5	13.4	9.2	6.9

This table contains interesting data. Larvae Nr 1, 2, 4, 5 and 6 worked somewhat monotonously. The grains are on the average bigger in the canopy than in the tube, especially its ventral side where the grains are the smallest. The relationship of the average surface

of the grains in the canopy to the surface of the grains in the dorsal section of the tube is in all six cases similar,and equal to 1,3. The relation of the grains of the canopy to the grains of the ventral section of the tube varies between 1,7 and 2,4. The original case of the larva Nr 3 was of the same size as in other larvae, nevertheless the proportions of its new cases are quite different. The grains of the canopy are markedly smaller to those of the other larvae, the grains of the dorsal section of the tube are proportionally smaller and the grains of the ventral section of the tube wall are comparatively very small and very inexactly chosen, as seen at Fig. 3.

The diversity of the structure of the cases of these two larvae is very apparent. The cases of larva Nr 3 all differ from the cases of Nr 2, and they are all exactly similar one to another.

In order to confirm that the smaller grains of larva Nr 3 are really its personal characteristic and not dependent on an accidental smaller size of available grains, this larva was put, together with another larva, into the same crystallizer. The result was in no way affected: the cases of both larvae were absolutely different.

III. CHOICE OF GRAINS OF SIMILAR SHAPE ACCORDING TO SIZE

In the experiments of this series, an artifical material was used in the form of squares cut out of film. The side of the square was either of 1 mm or 2mm. A graduated mechanical microscope stage was used to cut out the squares. In place of the objective a wooden block was screwed in, in which a sharp blade of hard steel was firmly inserted. The film was glued on a glass square which was put on the mechanical stage. With the help of the coarse adjustment of the microscope the steel blade was lowered until it touched the film, and by moving the stage with the help of the sidescrew a regular scratch on the film was obtained. After making several parallel scratches at distances of 1 or of 2 mm, the glass bearing the film was turned at right angles and again similar scratches were made. After washing the film with warm water to remove the glue, it was easy to divide it into separate squares.

To larvae removed from their natural cases a building material was given which consisted of a mixture of larger and smaller squares in the proportion of 1:4. Because of the similarity of the thickness

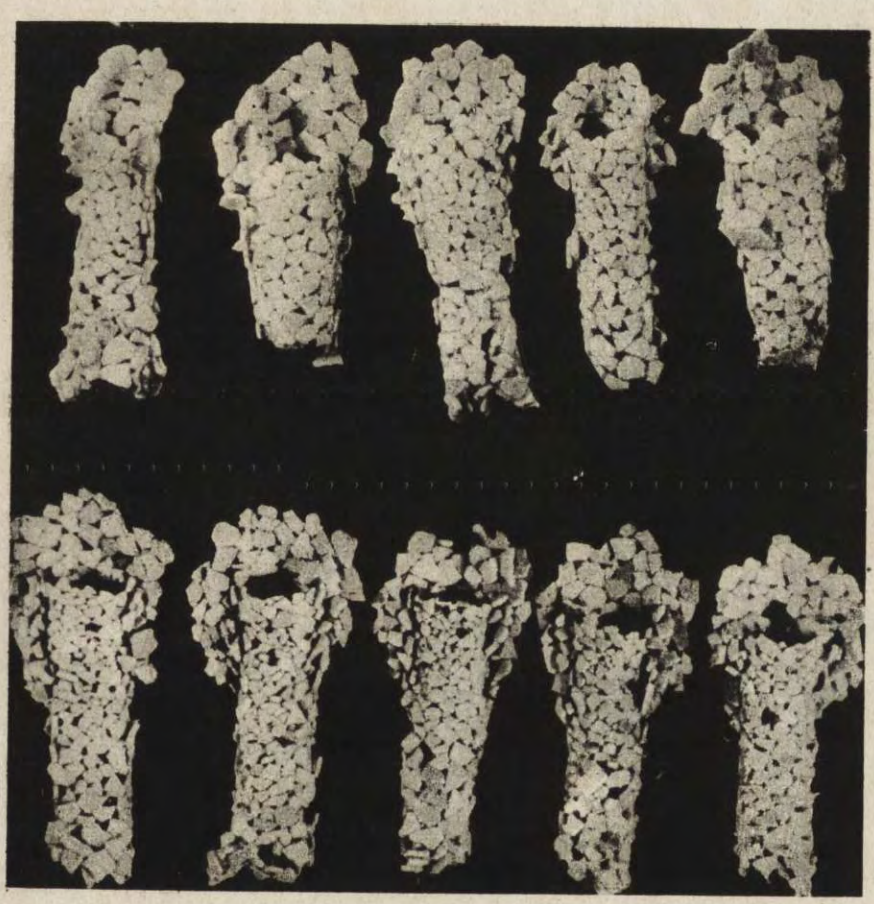

Fig. 3.

Five successive cases of the larvae Nr 2 and Nr 3.

of the squares it was possible to ascertain the proportion simply by weight. In the mixture, the sum total of the surface area of larger and of smaller squares was the same. The experiments were carried out with 10 larvae, each of them built 5 cases in five periods of 24 hours.

TABLE II.

The choice of film squares having 1 mm (B) and 2 mm (C) sides given to the larva in the ratio of 4:1. Five successive cases (A) of six larvae (Nr). The figures of the columns B and C give the absolute number of the squares in the case, and in the % column the percentage of the larger squares as compared to the smaller ones.

No	A	B	C	%	No	A	B	C	%
	1	110	12	10.9		1	139	22	15.8
	2	114	14	12.3		2	129	24	18.5
1	3	138	29	21.0	4	3	137	25	18.2
	4	144	33	22.0		4	133	20	15.0
	5	147	38	25.8		5	111	29	26.2
	1	117	9	7.6		1	141	7	4.9
	2	152	9	5.9		2	124	10	8.1
2	3	144	12	8.3	5	3	100	21	21.0
	4	133	8	6.0		4	97	20	20.1
	5	115	15	13.0		5	95	17	17.8
	1	136	28	13.2		1	217	9	4.1
	2	105	22	20.9		2	157	7	4.4
3	3	89	21	23.6	6	3	205	11	5.3
	4	93	26	28		4	159	10	6.3
	5	99	33	33.3		5	173	15	8.7

In Table II we give the result for 6 larvae. Considering especially the column containing the percentage of the larger squares in the whole case as compared to the smaller ones, we can see that the larvae Nr 2 and 6 choose less of the 2 mm squares than other larvae. The differences are systematic and they do not resemble a random distribution. There are also other characteristics, being individually specific.

In every instance, the larvae use the smaller squares for building rather than the larger ones; this can be explained by the fact, that by using smaller grains it is easier to obtain the regular shape of the whole case. This is because the grains in the case do not overlap but make contact at the edges, thus forming but one layer of

the case wall. The canopy is more flat than the tube and this is why bigger grains are used for building the canopy.

As hitherto, the larvae used for this experiment were as nearly similar in size as possible.

IV. SELECTION OF GRAINS OF SIMILAR SIZE ACCORDING TO THEIR SHAPE

Larvae removed from their natural cases were put on a layer of mixture consisting of celluloid squares and discs. To cut out the discs a special die was used. The diameter of the disc was 0,84 mm, the side of the square — 0,75 mm, so that the superficial area as well as the weight of both figures was approximately the same. The squares were mixed with the discs in a relation 1:1, determined by weight. Ten larvae were used and each of them built in 10 days 10 successive cases. In no one of the 100 newly constructed cases did the squares predominate numerically over the discs, but the percentage of squares as compared to discs varied considerably within limits from 14,3 to 56,5 (Table III).

TABLE III.

The choice from the discs (B) and squares (C) of equivalent superficial area given in the ratio 1:1. The table gives absolute total figures of squares and discs, forming part of 5 successive cases of six larvae, and in the % column — the percentage of squares as compared to discs.

No	A	B	C	%	No	A	B	C	%
	1	314	45	14.3		1	201	61	30.3
	2	290	47	16.2		2	197	55	27.9
1	3	272	54	19.9	4	3	181	62	34.2
	4	283	55	19.4		4	177	55	31.6
	5	274	47	17.2		5	174	63	36.2
	1	216	45	20.8		1	171	86	50.3
	2	212	38	17.9		2	178	88	49.4
2	3	200	46	23.0	5	3	141	79	55.3
	4	173	39	20.5		4	161	82	49.1
	5	167	38	22.7		5	167	78	46.7
	1	127	53	41.7		1	177	59	33.9
	2	153	72	47.1		2	171	56	32.7
3	3	154	85	55.2	6	3	167	49	29.3
	4	157	79	50.3		4	172	53	30.8
	5	152	86	56.5		5	153	46	30.0

Table III confirms the previous results. In spite of the high variability of behaviour, the preponderance of discs over squares is in larva Nr 1 three times greater than in larva Nr 5 (50,2:17,4). Both those larvae are characterised by their own specific „style" of work, obviously different.

The figures of the table III are only approximate; they give the total number of discs and squares making up the whole case, but they do not give the proportional relationship between particular parts of the same case. This problem requires special research.

V. THE TURNING OF LARVAE WHEN PLACED ON THE BACK WITH THE CASE

The observations were made on 25 individuals, remaining in their natural sand cases. The larvae were put on a layer of crushed egg shells of similar sizes (the grains were differentiated with help of two silk sieves of slightly differing mesh.). The larva together with its case was turned on its back and then it was observed how much time it required to assume the normal position. The commencement of reaction was taken to be the moment when the larva puts its feet, previously drawn inside, out of the tube. The manner of turning was also recorded.

As usual, the result varied considerably even with one and the same individual. For instance in the larva Nr 9 the speed of the reaction (the time from the moment of placing on the back till the commencement of the reaction) was in successive experiments:

2 min. 21 sec.
0 min. 15 sec.
0 min. 33 sec.
1 min. 32 sec.

The whole process of turning took in the same instances:

65 min.
3 min. 20 sec.
30 min. 39 sec.
16 min. 5 sec.

There is no regularity at all But with some larvae the reaction was obviously individual.

In larva Nr 2 the speed of reaction was successively:

	2"	4"	3"	5"
duration of reaction	49"	23"	34"	40"

In larva Nr 7 the speed of reaction was:

	1'15"	12"	17"	13"
duration	45'	65'	64'	55'

For each of both larvae the duration of reaction is peculiarly its own.

VI. THE STATISTICAL ANALYSIS

As has already been stated, the facts given allow the formulation of some hypotheses but it is difficult to generalize about them. If in a numerous group of larvae only one single individual shows some extraordinary faculties, it is legitimate to deduce that such faculties occur in the species of *Molanna angustata*. As we have seen, a few individuals really do behave in a peculiar way, but the behaviour of the great majority seems to be chaotic and unpredictable. Only calculation can solve this problem. In this section we propose to make a statistical analysis of one example: the behaviour of larvae while repairing the case (section I series 1 of this paper). The behaviour of the larva is variable and if strict criteria were applied, then each larva would constitute a class in itself. In order to make a statistical analysis possible, a somewhat primitive schematism is scarcely to be avoided. All the possible ways of repairing cases we subdivided into two categories only: repairing in which the prevalent direction was forward (m) and repairing in which the prevalent direction was to the rear (*p*). Each single repair was marked with one of these two letters.

Our task was to compare the work of larvae several times repairing the case with the random distribution of both factors *m* and *p*. The random distribution is defined theoretically and there is no need to realize it in practice. But an experimentally working biologist is accustomed to use data supplied by the experiment. If he obtained for comparison two sets of data: one arising from the work of larvae and the second obtained by using some mechanism, if the probability of *m* and *p* in both sets is clearly the same and if he can infallibly distinguish one set from another, then he has obtained the valid characteristic of his sets. In order to obtain sets showing the random

distribution, we used a revolving metal disc subdivided into four quarters. Two of them we marked with the letter **m**, two others with **p**. The disc revolved in a horizontal plane approximately 15 times per second. A few seconds after putting it in motion the disc was suddenly stopped and we noted within the limits of which quarter the line drawn on the immovable frame of the disc was situated. We made as many projections as there were instances of case repairing (488), and the sets obtained of the frequency of **m** and **p** we grouped in a way exactly similar to the groups of repairs. This was repeated twice. Then we compared three systems. One concerned the true behaviour of 65 larvae which had repaired their cases 488 times in all. The other two strictly random series we marked as Model 1 and Model 2. These full series are given on the page 54. We reproduce below the characteristics of these three groups of data.

1) For the 488 instances the frequency of **m** and of **p** is:

Larvae	254 m	234 p
Model 1	235 m	253 p
Model 2	246 m	242 p

Practically speaking the frequency of both factors is similar in all three sets and it is very near to a random distribution.

2) If the distribution of **m** and **p** is strictly haphazard, then each random group of these factors would contain as many **m** as **p**. Such a case is the most probable. It was determined how many times in 65 instances of our three series occurred the same number of **m** and **p**.

Larvae	5 times
Model 1	11 times
Model 2	10 times

Both models are markedly nearer to the random distribution then the larvae.

3) There were full sets (sequences) comprising only one letter **m** or **p**:

Larvae	20
Model 1	4
Model 2	3

Both models 1 and 2 are nearer to the random distribution than the larvae.

4) One letter was repeated the maximum number of consecutive times:

Larvae	18 times
Model 1	10 times
Model 2	5 times

The probability of the result obtained is for the larvae 1:262144, for the Model 1 — 1:1024.

5) When two consecutive letters of the set are identical (**mm** or **pp**), we call the reaction homotyp, when they are different (**mp** or **pm**) the reaction becomes heterotyp. In instances of random distribution the probability of both possibilities is equal. The calculation shows:

Larvae	338 homotyp	85 heterotyp
Model 1	206 homotyp	217 heterotyp
Model 2	206 homotyp	217 heterotyp

The structure of larval sets is basically different.

6) The larval sets are of widely different length: they contain from 2 to 21 repairs. The probability of the random occurrence of such differences is very small. In order to calculate it we applied the Chi square test (Cf. Cramer H. Princeton Univers. Public. 1946). It was found:

$$\chi^{\circ 2} = 137{,}225$$

or applying the Fisher's transformation into U°:

$$\chi^{0} = \sqrt{2\chi^{0\,2}} - \sqrt{2\nu - 1} = \sqrt{2.137{,}225} - \sqrt{2.63 - 1} = 5{,}38$$

This last value lies far outside the limits of allowable random variation of the Chi square. The probability of attaining this or an absolutely greater value in a single experiment is of the order 10^{-7}. It follows from it that the differences exhibited in the sample cannot be reduced to a mere display of random factors.

7) In each of the 65 sets, we calculated the proportion of the m's and then we applied the Chi square test. The result was:

$$\chi^{\circ 2} = 98{,}952.$$

The probability of attaining this or a greater value is of the order 10^{-9}. The dispersion in the larvae of *Molanna* is clearly supernormal. This can be illustrated by drawing the histograms of the proportions of the **m**'s from 0 through 0,2; 0,4; ... to 1,0. Simply from the appearance of the histograms it can be seen that the dispersion by the larvae is greater than by both models.

8) The same problem can be solved in a different way. We calculated the proportion of the **m**'s in two equivalent halves of the total working time of each larva. One of the halves consisted of odd days of work, the other of even. If the proportion of **m** is a random one, then the frequency of **m** in one half would be independent of the frequency in the other. But if in the work of the larva there exists a certain element of order, differentiating the distribution of **m** and **p** from a purely random one, then the two halves ought to reveal some common features in the proportions of the **m**'s. Or to speak more exactly, we had to calculate whether there exists a correlation between our two halves. We applied the t° test of Student. The coefficient of correlation is:

Larvae	0,768
Model 1	0,070
Model 2	0,094

There is a reliable correlation in larvae and a lack of correlation in the model.

Student's test:

(t°) for larvae	9,518
(t°) model 1	0,5
(t°) model 2	0,747

9) The differences in the length of the sets of individual larvae may be explained by the exhaustion of the animals occurring at different times. It is rather improbable however that endurance could vary within such wide limits. It is also difficult to believe that one of the two ways, **m** or **p**, is more difficult for the larva and leads to more rapid exhaustion. We have no proof that it is so. At the same time there is a very clear regularity in the frequency of **m** and **p** at the beginning of each set and at the end. We totalled all the **m**'s in the first half of all the 65 sets and in the second. The result was:

Larvae	First half 171	Second half 83
Model 1	First half 116,5	Second half 118,5

For the model the distribution is as expected strictly random one. But in the larval sets there is a very obvious preponderance of **m**'s in the first half of the sets and of **p**'s in the second. Calculating the proportions of **m**'s in both halves and applying the U — test we get the result

$$(U^{\circ}) = 10{,}38.$$

The probability of obtaining such a high value of (U°) by a simple display of random forces is extremely small. There cannot be any doubt about the fact that the proportion of **m**'s in the work of each larva diminishes with time.

In Table IV we give the number of both factors **m** and **p** at the beginning of each set, in the middle and at the end, both for the larvae and for the models.

TABLE IV.

This table illustrates how many times **m** and **p** were found at the beginning of the sets, at the end and in the middle. The middle figures constitute the random distribution.

	Larvae		Model 1		Model 2	
	m	p	m	p	m	p
FIRST	54	11	38	27	35	30
MIDDLE	32	33	33.5	31.5	31	34
LAST	17	48	33	32	37	28

In the middle of the sets the distribution of **m** and **p** is a random one. When the posterior part of the case is off, the caddis-fly usually builds its case forward (**m**) and this seems to be the most natural method of repair. But why the larva has a strong tendency to change its method into **p** at the end of the set we do not know. In any case it is not probable, that the reason is a comparatively easier way of repair.

* * *

All nine criteria taken together make it possible to recognise unmistakably whether a given set corresponds to the work of the larvae or to some mechanism giving a random distribution. Not only do some chosen individuals indicate particular characteristics of behaviour, but also there can be demonstrated in the work of the mass of larvae some regulating factor, which differentiates methods of work employed by the animal from purely random series.

From the manner of behaviour of the caddis-fly larva observed in this work, we do not deduce any zoopsychological consequences, but we limit ourselves to recording facts.

SUMMARY

The aim of this paper is to investigate whether the activity of larva of the caddis-fly *Molanna angustata* has individual characteristics. When the larva has a task to perform, as building or repairing its case for example, and when this task can be accomplished in one of two equivalent ways, there arises the question whether the distribution of those two possible ways in the repeated work of one and the same larva is a random one, or whether it reveals any individuality, which would mean that the particular sequence of these two ways could be a characteristic of definite individuals.

The method consisted in giving the larvae the same task many successive times and then comparing the distribution of ways in which it was performed with the random distribution.

1) The posterior half or $^3/_4$ of the cases were removed and the larva put in the remainig forward part in a position normal or reversed. The manner of repair varied chaotically enough, but in some individuals one particular method of repairing was obviously prevalent.

2) In the building of a whole case from mixed material, the choice of grains according to size was observed. In spite of the fact that the larvae were of the same dimensions, great variation, clearly not a random one, was observed in the choice of material (Table I and Fig. 2, 3).

3) Observation of building from a mixture of artificial grains in the shape of squares of two dimensions showed the preponderance of smaller squares.

4) The same result was obtained when using a mixture of squares and discs of equal superficial area. In each of the last instances some individuals behaved in an individually different way.

5) Similar was the behaviour of larvae which were placed on their back and which turned to the normal position.

6) The experiments of series 1) were submitted to statistical analysis. Comparison was made using an apparatus consisting of a revolving disc which was stopped at random and provided the model of random behaviour. The various ways of repairing the cases were subdivided in two, **m** and **p**, depending on the direction of repair, and then the sequence was determined in which each larva used both possible methods during the repeated repairings.

65 larvae performed 488 repairs, in which almost the same number of both repairing types **m** and **p** was found. Several statistical tests such as Chi square test, U-test, t-test of Student, coefficient of correlation, all proved conclusively that the distribution of both methods of repair in the work of the larvae is not a random one, but has a certain regularity and differs notably from mechanical models. From this fact the authors do not at present deduce any zoopsychological consequences, considering that the material of facts available is not sufficiently extensive.

DISTRIBUTION OF M AND P
(section I series 1)

1. LARVAE

1. mmpmmpp **2.** pppppp **3.** mmmmmmmmpmppp **4.** mmmmp **5.** mm **6.** mmmmmm mpmpmppmpppppp **7.** mmmmmmmpmmmpmpp **8.** mpm **9.** mmmppp **10.** mpppppp **11.** mmmmmmmpmmmmpppppp **12.** ppppp **13.** mmmpppppppp **14.** mmmmmmmm mmmmmmmmmmmpp **15.** mmmmmpppppppppp **16.** mpppppp **17.** pppmmpppppppp ppp **18.** pmmmmmmmpmpmppp **19.** mmmmmppp **20.** mmmmppmmppp **21.** pppppp **22.** mpppppp **23.** pppp **24.** pppppp **25.** mpp **26.** mpppp **27.** mpppppppp **28.** mmmp **29.** mmmmmmmmppm **30.** mmmmpmmppp **31.** pppppmpp **32.** mpmpppppp **33.** m mpp **34.** mmmm **35.** mmmppp **36.** mmmmmmm **37.** mmmmmp **38.** mpppppp **39.** mm mmmpp **40.** mmpppp **41.** mmmmmp **42.** ppppppp **43.** mmmmmmm **44.** ppppp **45.** mmmmmm **46.** mmmmmm **47.** mmmppm **48.** mmmmmm **49.** mmmm **50.** mmmmmm **51.** mmm **52.** mmpppppp **53.** mmmmmp **54.** mpppp **55.** mpmpppp **56.** mmmmm **57.** mmmmpm **58.** mmmmmmm **59.** mpmpp **60.** mmmmp **61.** mpppppp **62.** mmmpppppppp **63.** mmmmmmpppm **64.** mmppmp **65.** ppppp

2. MODEL 1.

1. mppmmmm **2.** mmmmmm **3.** mpppppmpmppmpm **4.** mppppm **5.** pm **6.** mmpmmp pmmpppppppppppm **7.** ppppmpmpppmmmpp **8.** mmm **9.** pmpmmp **10.** ppmpmp **11.** mpmmpmpmmpppmpppppp **12.** mm pm **13.** mmppmpppmmpm **14.** mpmppmppmp mppmmppmmmm **15.** mppmppmmppppmpmp **16.** mppmmmm **17.** pppmmpmpppm ppmp **18.** mpmpmpmmmmpmmmm **19.** mpppmmpp **20.** mmpmpppmmpp **21.** pmm ppp **22.** mmmmmm **23.** pmpm **24.** mppmpm **25.** mmp **26.** pmppm **27** pppmmppp **28.** mppp **29.** pmpppmmpmmm **30.** mppmpmpppm **31.** ppmpmmmp **32.** ppmpppmm **33.** pmmp **34.** mmmp **35.** mpppmm **36.** mpmmppm **37.** pppmmp **38.** pmmmmm **39.** ppmpmmp **40.** mppmpp **41.** mpmppm **42.** mpmppmm **43.** mppmmmp **44.** ppmpp **45.** pmppmp **46.** mpmmmp **47.** mpmppp **48.** ppmmpm **49.** mppp **50.** ppmmpm **51.** pmp **52.** pmpmpmp **53.** mmpmpm **54.** pmpmm **55.** pmmpppp **56.** mpppm **57.** m mpppp **58.** mmmmmmm **59.** mpppp **60.** mppmm **61.** pmmmmpp **62.** mppmmmppmm **63.** pppppmmmp **64.** pmmppm **65.** mppmp

3. MODEL 2

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STUDIES IN THE BIOCHEMISTRY OF THE WAXMOTH *(Galleria mellonella).*

1. GROWTH OF THE LARVAE AND THEIR CHEMICAL COMPOSITION

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The first important works devoted to the physiology of the waxmoth *(Galleria mellonella)* come from Metalnikov (Metalnikov 1903, Sieber and Metalnikov 1904, Metalnikov 1908). As is generally known, the larvae of *Galleria mellonella* are parasites in the beehives and feed on the comb. Ingestion and oxidation of wax — (not met in other animals*) a substance very resistant to various chemical agents, must undoubtedly be connected with the specific character of many biochemical processes in the body of the animal and with the existance of a complex and specifically operating enzyme system. It is possible, moreover, that in these processes a lesser or greater role is played by some microorganisms which live with the larvae in a close symbiosis (Dickman 1933, Florkin 1949, and others).

It seems that the explanation of all these problems is possible only with the aid of a knowledge of the whole physiological and

*) As far as is known to us, besides *Galleria mellonella* only one other moth, *Achroea grisella*, feeds on wax.

biochemical process in *Galleria mellonella*, concerning which, in spite of somewhat numerous investigations, we have not adequate information so far.

The present paper is the first of a series of investigations which we intend to devote to the biochemistry of *Galleria mellonella*. It pertains to the chemical composition of larvae. (A preliminary account of this work has been given by Niemierko 1947a, and Niemierko et al. 1949).

Our knowledge on the chemical composition of *Galleria mellonella* is very limited. The earliest data come from Metalnikov (Sieber and Metalnikov 1904, Metalnikov 1908) who determined in larvae the content of dry substance, nitrogen, carbon and lipids. Manunta (1935) determined the content of fatty acids. Teissier (1931) in his extensive paper dealing with the growth of insects, investigated the chemical composition of larvae of *Galleria mellonella* of various sizes. He found that the percentage content of the dry substance and of the fats increases parallel with the growth of the larvae: the content of the fats increases from 7.5% to 23% in the fresh substance. The author determined also the percentage content of total phosphorus and came to the conclusion that its quantity changes parallel to the content of water in the body of the growing larvae and is about 0,4 — 0,5% compared to the quantity of water. Teissier determined also the content of ash, extractive substances and some other constituents. We possess, finally, data on the content of reducing substances in *Galleria mellonella* and on the transformation of these substances during the metamorphosis. (Crescitelli and Taylor 1935).

It is common knowledge that the growth of insects occurs in stages, separated by the moulting periods. The investigations of numerous authors have proved that during the particular stages very marked transformations of the chemical constituents occur in the body of the animal, and its composition is considerably changed (compare Heller 1928, Białaszewicz 1937, Niemierko 1947b). We observe moreover, the selective accumulation of certain substances in particular parts of the body (Heller 1949). It is essential, therefore, that when investigating the chemical composition of growing larvae we must fully determine and identify the stage of development.

Unfortunately, this process involves, in the case of *Galleria mellonella*, serious handicaps. The larvae burrow in the comb, in the corridors which they themselves have drilled; moulting in individual

larvae covers varying periods of time; the skin is shed gradually, not entire but in pieces; the number of moultings is not always the same; the weight of the body of the larvae which begin to construct cocoons and to pass through metamorphosis is often different. Teissier, who drew attention to all these facts, stressed also, that even to measure the length of the body (which in the case of very small individuals is the only possible way of recording their size) is not easy, because larvae of *Galleria mellonella* continually shrink and expand.

OBSERVATIONS OF THE GROWTH OF LARVAE*)

The data found in the literature referring to the growth of *Galleria mellonella* vary somewhat (Piepho 1940, Teissier 1931). This inclined us to investigate the number of moultings through which *Galleria mellonella* passes from the moment of hatching from the egg until growth ceases, the period of duration of particular stadia, and the changes of body weight during the different development stadia. The larvae were bred in the incubators at a temperature of 30°. The eggs laid by the female moth were gathered and, when the larvae were hatched, they were put separetely into small vessels, together with a minute quantity of comb. The moment in which the larva was hatched from the egg was noted, and the length and the diameter of the larval body were subsequently measured daily. Every moulting observed was registered. In some series of experiments the width of the head capsule was measured too. Bigger larvae, weighing more than from 2 — 3 mg were weighed every day.

It was observed that removing larvae from the vessels in order to measure or weigh them often has a harmful effect on the normal course of their development. There is a much higher mortality rate among larvae which have been measured than among those which have not been moved from the vessels. There exist in literature data to the effect that larvae of *Galleria mellonella* grow more slowly when bred separately than when reared en masse. This fact has been corroborated by our observations.

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*) The authors wish to thank Mrs. A. Wroniszewska for her valuable help in this part of investigation.

In order to ascertain the body weight of freshly hatched larvae, samples including some hundreds of individuals were weighed. The average weight of one larva was from 0,01 to 0,02 mg. On the next day after hatching the length of the larvae was from 1,2 to 1,4 mm and its diameter 0,16 — 0,26 mm. On the basis of this data it is calculated that the body weight of day-old larva is from 0,025—0,075 mg.

The number of moults generally amounted to 6 or 7. The first moulting was normally observed on the fourth or the fifth day after the hatching, and subsequent moultings at intervals of 3, 4, or 5 days.

In some series the width of the head capsule were measured after every moulting. One of the series of experiments performed on 17 larvae, of which 8 came to metamorphosis (9 died) gave the following results: in 32 days from the moment of hatching till the last moulting 7 moultings were noted. On the 35th and 36th days, the larvae constructed cocoons. The widths of the head capsule were as follows:

Moult	I	II	III	IV	V	VI	VII
Width of the head capsule (mm)	0.2	0.4	0.7	1.0	1.5	1.9	2.1

As has been already observed, the body-weight of different larvae of the same stadia varies considerably. This may be illustrated by the following comparison of observations, made with 16 larvae.

	3. moulting	4. moulting	5. moulting	6. and last moulting
Mean body weight	3.5 mg	9.5 mg	25.4 mg	63.1 mg
Deviation	(1. 5-4. 5)	(5-14)	(19-33)	(43-87)

In the majority of cases, the last moulting occured when the larvae weighed from 50 to 80 mg. The final body weight of the larvae when the animals stopped eating and began constructing cocoons, was generally 150 — 250 mg. In exceptional cases, we observed larvae amounting in weight to 300 or even 400 mg.

The successive ecdysis were most easily determined by means of weighing the animals every day and computing the changes of body weight in 24 hours either in mg or, (still more clearly) as $^0/_0{}^0/_0$ of the actual body weight. This kind of operation seems to be the most suitable when it is intended to trace and identify the stages of development in larvae of *Galleria mellonella*.

The approach of a moult is indicated by delay of the increase of body weight.

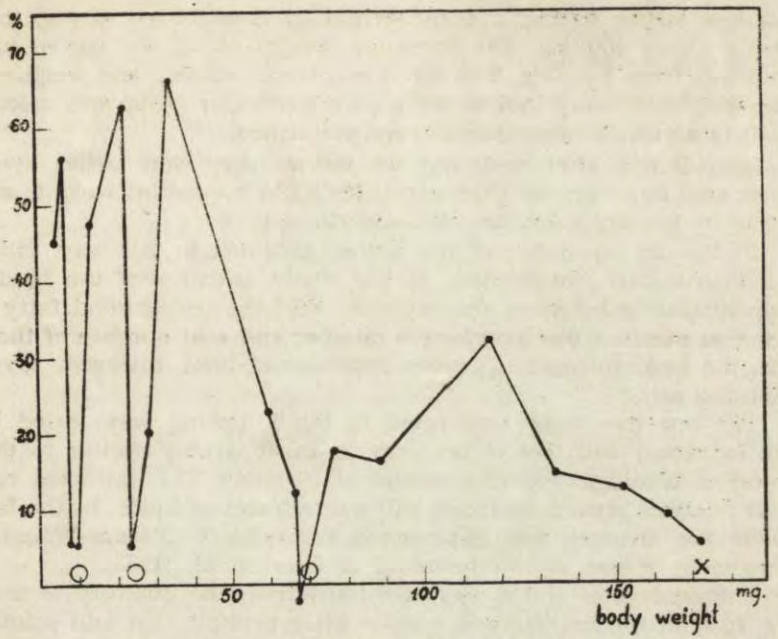

Fig. 1. Changes of body weight during 24 hours (as % of the actual weight).

• Data for successive days, o moults, x formation of cocoon.

Figure 1 illustrates measurements of this kind.

From the observations given above it appears that it is very difficult to accumulate larvae, in the same stadia of development, in sufficient numbers to perform various chemical analyses. We decided, therefore, that at least in the first stage of our investigations, we would subject groups of larvae to chemical analyses, every group consisting of larvae with a similar body weight. Chemical investigations into strictly determined stadia of development must be postponed to some future time when methods become available whereby individual organisms, can be analysed.

MATERIAL AND METHODS

Galleria mellonella were bred in a temperature of 30°, in glass vessels having a volume of one litre. In order to ensure the greatest uniformity in material, we took for particular experiments larvae bred in the same vessel. In this way the influence of minute differences of temperature, feeding and moisture were minimised. Three or four groups of animals of similar size were selected for chemical analysis. The first group

contained larvae having a body weight of from 10 — 20 mg, the last group — over 100 mg. The complete weight of all the larvae in each group was from 1 — 3 g. The specimens were counted, and weighed. The mean weight of every individual in each particular group was calculated. In all, 11 series of experiments were performed.

Immediately after weighing the larvae, they were dried, over sulphuric acid in a vacuum (Niemierko 1947c) to a constant weight, and the content of the dry substance was ascertained.

In the dry substance of the larvae, obtained in this way, following constituents were determined: 1) the whole quantity of the lipids, the unsaponifiable substances, the saturated and the unsaturated fatty acids, the iodine number, the thiocyanate number and acid number of the fatty acids, the lipid nitrogen; 2) other substances: total nitrogen, glycogen, chitin and ash.

The analyses were performed in the following way: dried larvae were extracted with hot chloroform in an apparatus similar to the apparatus of Kumagawa over a period of 10 hours. The extracted residue, as our analysis proved, included only some traces of lipids. In the fat free residue the nitrogen was determined according to Parnas-Wagner and glycogen by means of micromethod of Good et al. (1933).

The content of chitin was calculated from the quantity of nitrogen (N x 16.6) in the undissolved residue after precipitation and solution of the glycogen. The ash was determined by combustion in a platinum crucible.

The solution obtained after the extraction with chloroform was diluted to the mark in a volumetric flask and in samples of this chloroform solution the total amount of lipids and the different lipid constituents were determined. The unsaponifiable substances and the fatty acids were determined according to Kumagawa-Suto. The saturated fatty acids were determined by a micromodification of Bertram's method (Niemierko 1947d), and the amount of the unsaturated fatty acids was calculated from the difference. Iodine and thiocyanate numbers were determined according to Niemierko (1947e).

RESULTS OF ANALYSES AND DISCUSSION

Each series of our experiments included larvae of different sizes, beginning with a body-weight of about 10 — 20 mg and concluding with larvae at their final body weight, that is to say about 150 to 200 mg in general.

When we compare the chemical composition of growing larvae within the limits of one series, we can observe very distinct and similar changes for the whole range of chemical substances. This is shown by the curves illustrating the content of fatty acids throughout two series taken as an example (Fig. 2).

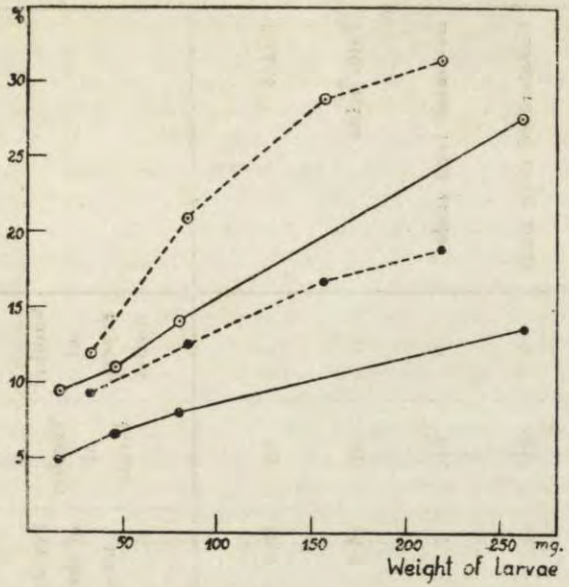

Fig. 2. Changes of fatty acids content during growth of the larvae. . saturated fatty acids, o unsaturated fatty acids, ——Series VI,- - Series VII

But the discrepancies as between one experiment and another in the content of the particular chemical constituents in the body of larvae of the same weight are often so great, that in order to compare mean values for the chemical composition of different larval bodies, we were obliged to divide them into three groups. The first group includes all the larvae of body weight below 50 mg, the second 50 — 100 mg and the third over 100 mg.

In conformity with our observations, the biological foundation of such a division is that in the first group there are larvae in the initial stages of growth, the second group comprises larvae directly before or after the last moulting, and in the third group there are larvae in their final stage of growth. Division into a greater number of groups, corresponding to the development stadia determined, could not be accomplished, because of the difficulties to which attention has been drawn above.

Reviewing the results obtained, let us consider first of all the content of the dry substance in the body of larvae. Mean values, given with the calculated „error of mean value" $\left(\varepsilon M = \pm\sqrt{\frac{\Sigma \triangle^2}{n(n-1)}}\right)$ are as follows for the three groups of larvae selected by us: I 26.5±0,6%,

Table I

Chemical constituents in growing waxmoth larvae

(M — mean value; ε M — error of mean value)

Size of larvae	< 50 mg			50 - 100 mg			> 100 mg		
	Number of experiments	Number of larvae	Percentage of the dry weight M ± ε M	Number of experiments	Number of larvae	Percentage of the dry weight M ± ε M	Number of experiments	Number of larvae	Percentage of the dry weight M ± ε M
Lipids	5	420	30.3 ± 2.5	5	161	38.6 ± 4.9	8	142	56.2 ± 1.2
Fatty acids	11	902	18.2 ± 1.1	13	421	27.9 ± 2.3	15	276	43.3 ± 1.4
Saturated fatty acids	8	617	7.5 ± 0.9	11	373	11.1 ± 1.1	15	276	14.4 ± 0.6
Unsaturated fatty acids	8	617	11.2 ± 0.7	11	373	18.5 ± 1.6	15	276	28.8 ± 1.0
Unsaponifiable substances	11	902	4.0 ± 1.1	13	421	4.1 ± 0.8	15	276	4.8 ± 1.0

II 30.4±4.1%, III 40.8±0.8%. Besides that, some analyses performed on a very great number of the smallest larvae, (that is to say of mean body weight 0.02, 0.04, 0.09, 3.4 and 5.4 mg) gave values of from 25% to 28% of the dry substance, that is to say a similar result to that which was obtained in the first group of the principal series.

We see, that parallel with the growth of larvae, the content of the dry substance increases. In the second group of larvae the error in the mean value of the dry substance is considerably greater than in the first and in the third groups. The same applies also to other constituents investigated. (Table I). This points to the fact that, in this stage of development, that is in the period when the larvae pass through the last moulting, there occurs the greatest deviation in the chemical composition of the body. The period of the last moulting is, as it were, a turning point in the formation and accumulation of these constituents of the body which occur in the final period of growth.

A rapid increase observed in the dry substance in the larvae of the body weight from 50 — 100 mg is connected with the accumulation of fats. This is illustrated by Table I, in which the mean values of the total quantities of lipids, saturated and unsaturated fatty acids, and unsaponifiable substances are given. It is observed that the content of all these constituents increase considerably.

The amounts of dry substance and lipids found by us agree with the data of Teissier (1931). Where our results differ from the results of this author is in the very marked discrepancies, demonstrated by us in the composition of the particular constituents in the body of growing larvae having the same weight.

The content of the unsaponifiable substances does not show any definite changes during the period of growth of the larvae. Fluctuations in the quantity of these substances in the body of larvae are in general much greater than those of other constituents. This depends first of all on a very great quantity of unsaponifiable substances in the wax on which the larvae feed.

Our next paper will deal fully with the problem of the metabolism of wax constituents (Niemierko and Włodawer, 1950).

The composition of fats accumulated by growing larvae does not undergo any considerable change. The relative proportions of saturated and unsaturated fatty acids does not reveal any great variations.

During the whole period of growth we observe a superiority of unsaturated acids. But the degree of desaturation of the fatty acids is, nevertheless, not very great.

Iodine and thiocyanate numbers determined are as follows:

Weight of larvae	< 50 mg	50-100 mg	> 100 mg
Iodine number	66 ± 1.2	63 ± 0.8	62 ± 1.0
Thiocyanate number	47 ± 1.8	54 ± 2.0	51 ± 0.8

The thiocyanate numbers obtained are relatively not much smaller than the iodine numbers. Applying Kaufman's interpretation (1935) to the thiocyanate number we may assume, that among the unsaturated fatty acids the acids of one double bond are prevalent. Since our analyses showed that the acid value of fatty acids is about 200, we may assume that the principal unsaturated acid in the *Galleria mellonella* larvae lipids is oleic acid. That is to say that these fats are different from the fats of the majority of other Lepidoptera, which as a rule contain a great quantity of highly unsaturated acids (Hilditch 1947).

In connection with the accumulation during the larval growing period of a great quantity of fats, the percentage content of protein decreases. This is as follows:

Weight of larvae	< 50 mg	50-100 mg	> 100 mg
% content of protein in the dry substance (Nx 6.25)	54.6 ± 2.6	48.5 ± 3.7	37.8 ± 0.5

The content of glycogen, ash and chitin, and of the lipid nitrogen may, on account of the small quantity of analysis and of the differences in the data obtained, have only a tentative character. We obtained for the glycogen values from 0.4 — 1.0%, for the ash 2.1 — 6.1% for chitin 2 — 4% of dry substance, for nitrogen in lipids 0.2 — 0.5%.

SUMMARY

1. After hatching from the egg, *Galleria mellonella* larvae have a body weight of about 0.01 to 0.02 mg and during growth pass through 6 or 7 moultings. Periods of moultings and the body weight during these periods point to very considerable individual differences.

2. The growth of larvae is connected with the percentage increase of the dry substance content from 26% to over 40%.

3. The changes in chemical composition of the body depend generally on the accumulation, during the period of growth of the larvae, of lipids. The quantity of unsaturated acids is greater than that of saturated. The degree of desaturation is not great. The principal unsaturated acids is, most likely oleic acid. The content of unsaponifiable substances shows great fluctuations.

4. Parallel to the accumulation of the fats, the relative content of proteins decreases.

5. The period of the last moulting seems to be a turning point in the accumulation of dry substance and fats in the body of larvae.

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STUDIES IN THE BIOCHEMISTRY OF THE WAXMOTH
(Galleria mellonella)

2. UTILIZATION OF WAX CONSTITUENTS BY THE LARVAE

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The peculiar ability of the waxmoth larvae to feed upon the comb, the chief constituent of which is the wax, has been known for a very long time. Earlier work on *Galleria mellonella* was summarized by Metalnikov (1908). It seems however that Dickman (1933) was the first to give evidence that the wax is not only ingested by the larvae but is really oxidized in the larval body. This author made the following experiment. Larvae were placed in flasks with weighed quantities of beeswax. The larvae were permitted to pupate and afterwards the wax was extracted from the residue of the flasks in the Soxhlet. The results showed a loss of about 50% of the wax. The loss could only be explained by the theory that some of the wax was digested.

Later on several other authors (Duspiva 1935, Manunta 1935) supposed that *Galleria mellonella* is able to utilize the fatty acids, the esters and the alcohols of the wax, but that it does not utilize the hydrocarbons. This assumption, however, has not been sufficiently prooved by experiments.

It seems that the problem of the utilization of the particular wax constituents can be elucidated only by means of balance experiments in which the amount of the fatty acids and the unsaponifiable substances of the wax must be determined in the food consumed by the larvae, in the larval bodies and in the excreta.

The aim of the present investigation was to perform such balance experiments. A preliminary communication of some of the results has been made by one of the authors (Niemierko 1947).

Several investigators were able recently to isolate bacteria from the digestive tract of the wax moth larvae. These bacteria cultured in the media which contained beeswax not only hydrolysed the wax (Dickman 1933), but also oxidized the fatty acids and esters (Florkin 1949). The part played by the microorganisms in the utilization of wax by *Galleria mellonella* has not been established so far. We do not deal with this problem in the present paper, as this will be the subject of our future studies.

MATERIAL AND METHODS

As in our previous study (Niemierko and Cepelewicz 1950) the wax-moth larvae which were taken for the investigations came from the breed at 30°C. The experiments were performed in the following way. 10 small larvae, weighing about 10 mg each, were placed in Erlenmeyer flasks over a weighed quantity (about 5 g.) of the comb. The control flasks contained only the comb. The flasks were kept at 30°C during a period of 10 — 15 days, until all the food was consumed by the larvae. The larvae, weighing at that moment usually about 100 mg each, were then carefully removed and the amount of the excreta was determined. The collected material, as well as the comb from the control flasks was dried in vacuum over conc. sulphuric acid, and afterwards extracted with hot chloroform in the apparatus of Landsiedl during several hours. The fat free residual which was left after extraction contained protein, carbohydrates, mineral constituents etc. The total amount of the fat free residual was determined by weighing.

The chloroform extract which contained all the lipids was evaporated to dryness and the residue weighed. The results gave the total amount of the lipids. The lipids were then dissolved in chloroform and the solution diluted to the mark in a volumetric flask. Samples of this solution were used for determinations of the different lipid constituents.

In the course of the analyses, especially in the case of the comb and the excreta, we encountered the following difficulties. The fatty acids which we tried to isolate from the wax as Ca or Ba salts by means of different methods described by Grün (1925) and „Witzöff" (1930) were

always contaminated with considerable amounts of the unsaponifiable substances and *vice versa*, the unsaponifiable substances were contaminated with the fatty acids. After many attempts we established the following procedure for the quantitative separation of these compounds.

Samples of the chloroform extract containing about 200 mg of lipids were pipetted into 80 ml centrifuge tubes. The solvent was evaporated, 10 ml of N alcoholic potash and 20 ml benzene were added and the mixture saponified under reflux. After 30 minutes heating, 20 ml of water were added and the boiling continued for another 15 minutes. The tubes were centrifuged and the benzene solution of the unsaponifiable substances poured into another centrifuge tube. To the alcoholic soap solution 20 ml of fresh benzene were added, the mixture boiled, centrifuged and the new benzene solution quantitatively separated. This operation was repeated three times the separated benzene solution being boiled each time with 50% alcohol to remove the admixtures of the fatty acids. Finally, all the benzene solutions were poured together, the benzene evaporated and the residual unsaponifiable substances dried to constant weight in a vacuum oven at 35°C.

To the separated soap solution conc. HCl was added and the liberated fatty acids were extracted with benzene using, as previously, centrifugation for the separation of the benzene solution. The benzene solution was washed with 5% NaCl and after evaporation of the solvent the fatty acids were dried in a vacuum oven.

The separated fatty acids were practically free from the unsaponifiable substances just as these were free from fatty acids.

The procedure described above gave far better results than the methods with Ca or Ba salts.

RESULTS

8 series of experiments were performed, the aim of which was to study to what extent the particular constituents of the comb are utilized by the waxmoth larvae. In these experiments the chemical composition of the fully grown larvae was compared 1) with the composition of the small larvae at the beginning of the experiments*), 2) with the composition of the food consumed and 3) with the composition of the excreta. In this way we were able to calculate the balances for the following substances: the dry substance, the fat free substance, the lipids, the fatty acids and the unsaponifiable substances.

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*) The total amount of the dry substance in these small larvae was inconsiderable, it did not exceed 0,025 g. We did not analyse the small larvae and took the necessary data from the paper of Niemierko and Cepelewicz (1950).

Table 1

Utilization of wax constituents by the waxmoth larvae

№ of experiment	Dry substance			Fat free substance			Lipids			Unsapsnifiable substances			Fatty acids		
	Initial quantity g	Final quantity g	Difference g	Initial quantity g	Final quantity g	Difference g	Initial quantity g	Final quantity g	Difference g	Initial quantity g	Final quantity g	Difference g	Initial quantity g	Final quantity g	Difference g
I	4.59	3.03	−1.56	1.44	1.36	−0.08	3.15	1.62	−1.53	1.86	0.85	−1.01	1.32	0.75	−0.57
II	4.80	3.46	−1.34	1.50	1.69	+0.19	3.30	1.72	−1.58	1.95	0.89	−1.06	1.39	0.79	−0.60
III	4.93	3.63	−1.30	1.54	2.01	+0.47	3.39	1.61	−1.78	2.00	0.83	1.17	1.40	0 73	−0.67
IV	5.08	3.67	−1.41	1.59	2.29	+0.70	3.49	1.37	− 2.12	2.06	0.74	−1.32	1.44	0.61	−0.83
V	4.85	3.62	−1.19	2.05	1.82	−0.23	2.76	1.80	− 0.96	1.64	1.00	−0.64	1.12	0.79	−0.33
VI	4.86	3.48	−1.38	2.07	2.00	−0.07	2.79	1.48	−1.31	1.65	0.81	−0.84	1.13	0.66	−0.47
VII	3.96	2.87	−1.09	1.20	1.24	+0.04	2.77	1.67	−1.10	1.67	0.93	−0.74	1.10	0.71	−0.39
VIII	3.97	2.68	−1.28	1.18	1.49	+0.31	2.97	1.20	−1.77	1.67	0.64	−1.03	1.10	0.53	−0.57
Mean	4.63	3 31	− 1.32 [−29%[	1.57	1.74	+0.17 [+11%]	3.08	1.56	−1.52 [− 49%]	1.81	0.84	0.97 [−54%]	1 25	0.69	−0.56 [45%]

The results of the determinations are presented in Table I. The average figures show that from 4.63 g of the dry substance which was present at the beginning of the experiments as much as 1.32 g disappeared, which amounts to 29%. On the contrary, the amount of the fat free substance shows changes in both directions. In some experiments this amount falls, in others it rises. The mean value show an increase amounting to about 11% .As this fraction is very heterogeneous the results obtained can have only a limited character. They suggest however that at least some of the constituents of this fraction may be formed from the lipids.

In all the experiments a very considerable diminution of the lipids takes place. The loss of these substances in the course of the experiments was from 35% to 61% of their initial quantity; the average value was about 50%. The diminution of the particular lipid constituents was very unequal. The analyses led us to a rather unexpected result. The unsaponifiable substances were utilized to a greater extent than the fatty acids: 54% of the former in comparison with 45% of the latter disappeared during the experiments. Since the wax contains larger amounts of the unsaponifiable substances than of the fatty acids, therefore the comparison of the absolute quantities of the determined lipid constituents which are lost gives a result which is even more pronounced: the amount of the unsaponifiable substances which are utilized by the larvae is almost twice as great as the amount of the fatty acids.

Table II shows the quantities of the unsaponifiable substances and of the fatty acids: 1) in the comb consumed, 2) in the grown larvae and 3) in the excreta. We see that the quantity of the lipids accumulated in the larval body in comparison with the amount excreted and the amount disappeared is very small. The grown larvae contain as a mean 0.17 g of the fatty acids and 0.03 g of the unsaponifiable substance*); the amounts of the fatty acids and of the unsaponifiable substances excreted were 0.52 g and 0.81 g respectively; the corresponding figures for the amounts lost were 0.56 g and 0.98 g.

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*) The results of our further investigations (Niemierko and Włodawer 1950) show that the unsaponifiable substances found in the larval body are chiefly the remainder of the ingested wax and are present mostly in the gut. Outside the gut their amount is insignificant since they form not more then 2—3% of the lipids.

The fate of these lipid constituents in the course of their utilization by the larvae is illustrated in fig. 1.

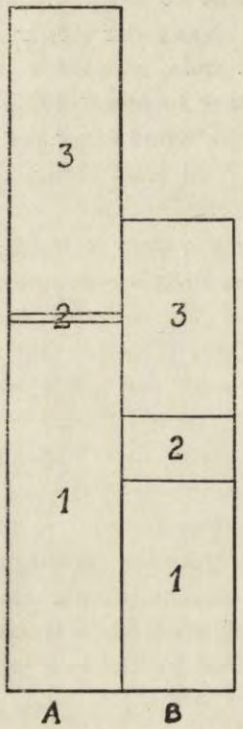

Fig. 1. Utilization of wax constituents by waxmoth larvae. A — total amount of unsaponifiable substances ingested by larvae, B—total amount of fatty acids ingested by larvae. 1 — amount disappeared. 2 — amount found in larval bodies, 3 — amount found in excreta.

From the data given in table I and II we can calculate the amount of lipids which must be utilized by the larvae for the formation of 1 mg of body lipids. The results of these calculations are presented in table III. We see that the formation of 1 mg of the body lipids by the growing larvae is connected with the ingestion of 9.5 mg of unsaponifiable substances and of 6.6 mg of fatty acids. 4.3 mg of the unsaponifiable substance and 2.8 mg of the fatty acids are excreted and 5.0 mg of the former and 3.0 mg of the latter are most probably oxidized.

Table II

Fate of lipid constituents of the ingested wax

No of experiment	Wax consumed		Grown larvae		Excreta		Lipids utilized	
	Unsaponifiable substances g	Fatty acids g	Unsaponifiable substances g	Fatty acids g	Unsaponifiable substances g	Fatty acids g	Unsaponifiable substances g	Fatty acids g
I	1.86	1.32	0.03	0.24	0.74	0.51	1.09	0.57
II	1.95	1.39	0.02	0.22	0.88	0.56	1.05	0.61
III	2.00	1.40	0.02	0.15	0.82	0.57	1.16	0.68
IV	2.06	1.44	0.02	0.16	0.72	0.46	1.32	0.82
V	1.64	1 12	0.03	0.18	0.97	0.60	0.64	0.34
VI	1.65	1.13	0.02	0.11	0.79	0.54	0.84	0.48
VII	1.67	1.10	0.03	0.18	0.90	0.54	0.74	0.38
VIII	1.67	1.10	0.03	0.13	0.62	0.40	1.02	0.57
Mean	1.81	1.25	0 03	0.17	0.81	0.52	0.98	0.56

The composition of the wax and of the excreta is shown in table IV. As we see, the differences in their lipid constituents are insignificant.

The acid value of the fatty acids was also determined. In the comb it was 162, in the excreta 148 and in the larval lipids 195. This last figure indicates that the fatty acids from the larval body contain about 18 C in the hydrocarbon chain, which is in agreement with the results obtained by Manunta (1935) and with our previous investigations (Niemierko and Cepelewicz 1950). The fatty acids of the wax contain in the mean 22 C in the hydrocarbon chain and those of the excreta — 24. We do not think however that these results are sufficient to justify the opinion that the relatively longchained fatty acids are utilized in smaller quantities than the acids with shorter chains.

Table III

Amounts of wax utilized for the formation of 1 mg of body lipids

No of experiment	Ingested		Excreted		Oxidized	
	Unsaponifiable substances g	Fatty acids g	Unsaponifiable substances g	Fatty acids g	Unsaponifiable substances g	Fatty acids g
I	6.8	4.9	2.7	1.9	4.0	2.1
II	8.1	5.8	3.7	2.3	4.3	2.5
III	11.7	8 2	4.8	3.3	6.8	4.0
IV	11.4	8.0	4.0	2.6	7 3	4.5
V	7.8	5.3	4.6	2.8	3.0	1.6
VI	12.6	8.7	6.0	4.2	6.4	3.7
VII	7.9	5.2	4.3	2.6	3.5	1.8
VIII	10.4	6.3	3.9	2.5	6.4	3.6
Mean	9.5	6.6	4.3	2.8	5.0	3.0

DISCUSSION

The results of our experiments show that the waxmoth larvae utilize about 50% of the ingested wax. These data are in agreement with the results of Dickman (1933) and Duspiva (1935). On the basis of the small differences in the acid values of the wax and of the excreta, Duspiva supposes that the fatty acids are better utilized than the esters. On the other hand, on the basis of the slightly higher percentage of the unsaponifiable substances in the excreta in comparison with the wax, Duspiva is of opinion that the hydrocarbons of the wax are not utilized. In our study, because of the methodical difficulties, the hydrocarbons were not determined separately. Hence, the isolated unsaponifiable substances contained both the alcohols and the hydrocarbons. Our balance experiments indicate very definitely that the unsaponifiable substances of the wax are not only

Table IV

Percentage of unsaponifiable substances and fatty acids in wax and in the excreta of waxmoth larvae

No of experiment	Excreta		Wax	
	Fatty acids %	Unsaponifiable substances %	Fatty acids %	Unsaponifiable substances %
I	38.8	61.0	41.0	59.0
II	39.3	61.2		
III	39.0	60.3		
IV	38.7	61.1		
V	38.2	61.3	40.5	59.5
VI	39.5	60.0		
VII	37.2	62.5	39.5	60.5
VIII	38.8	61.0		
Mean	38.7	61.1	40.3	59.7

really utilized by the larvae but that they disappear in quantities which are almost twice as great as the quantities of the fatty acids. The ability of *Galleria mellonella* to digest wax, which from the biochemical point of view distinguishes this animal from all other animals, seems moreover to be striking.

To explain this unusual preference for the unsaponifiable substances, which, it might be supposed, would be less accessible for the metabolism than the fatty acids, we advance the following hypothesis. In the course of their transformations in the larval body, the unsaponifiable substances, and principally the alcohols, are oxidized into the fatty acids. Owing to this process a continual formation of new amounts of the fatty acids takes place alongside their normal metabolic combustion.

We determine in our analyses not only the fatty acids originally present in the wax and in the larval bodies but also the acids newly

formed from the unsaponifiable substances. Therefore, the total amount of the fatty acids which are metabolized is in reality greater than the amount calculated from the balance experiments. Consequently, the utilization by the larvae of larger amounts of the unsaponifiable substances than of the fatty acids is, according to our view, apparent only.

In our further investigations we shall test the truth of this hypothesis.

SUMMARY

Balance experiments were performed in which the lipid constituents were determined 1) in the comb consumed by the waxmoth larvae, 2) in the larval bodies, and 3) in the excreta.

The larvae utilize about 50% of the ingested wax, they are able to oxidize not only the fatty acids but also the unsaponifiable substances of the wax.

In the course of their transformations in the larval body the unsaponifiable substances are probably oxidized into the fatty acids.

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STUDIES IN THE BIOCHEMISTRY OF THE WAXMOTH (*Galleria mellonella*)

3. OXYGEN CONSUMPTION OF THE LARVAE DURING STARVATION

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In connection with our previous studies in the biochemistry of the waxmoth there emerged the necessity of making experiments with the larvae during starvation (Niemierko and Włodawer, 1950 a).

It appeared, however, that any experiment on the metabolism of the starving larvae could cover only a very short period of time. After having been deprived of food before the last moult, the small larvae perish rapidly while the larvae which are in the last stadium of growth begin to construct cocoons immediately after being deprieved of food and usually pupate after only 24 hours.

It is well known (Wigglesworth, 1939) that in some cases it is possible to prevent the metamorphosis of insects by applying a ligature or by decapitating the larva. If the operation is performed prior to the so called „critical period" the insect usually shows no signs of metamorphosis and can survive for a considerable period. Experiments of this kind were carried out with the larvae of *Galleria mellonella* by Metalnikov (1937). The larvae remained alive for periods of time which varied in relation to the position at which the ligature was applied.

In our experiments, we put the ligature just behind the head. The larvae, which were kept at 30°C, usually remained alive for about 30 days, sometimes even for 40 days after the operation, and never showed either external or internal signs of metamorphosis. In this way we found the method of studying the metabolism of the larvae of *Galleria mellonella* during starvation.

In watching the behaviour of the larvae, we observed the following most interesting phenomenon. Immediately after ligation, the larva performs most violent movements for from 10 to 20 seconds, then it becomes completely motionless. It remains for a number of days in this state of absolute immobility and reacts only faintly to touch. Yet, after several days, usually from 6 to 15, an increased excitation of the larvae can be observed. Their reaction to touch becomes more and more visible. The most delicate touch suffices to cause the creature very violent movements. Somewhat later, there appear spontaneous movements. In still later stages even a slow creeping of the larvae can be seen, while sometimes they twist their whole body violently for as long as several hours without interruption. This state of augmented activity usually lasts until death by starvation.

It seemed interesting to investigate how far the rate of metabolism is influenced by the augmented activity of the larvae which is observed during starvation.

To pursue this end we systematically measured the oxygen consumption of the larvae during the whole period, from the moment of ligation until their death by starvation.

MATERIAL AND METHODS

The experiments were caried out with individuals of fully grown larvae which all came from the breed at 30°C (Niemierko and Cepelewicz, 1950) and which had a body weight ranging from 150 to 200 mg.

The larvae destined for experiment were separated from the general breed, individually weighed and kept in the thermostat at 30°. In a number of series of experiments the oxygen uptake was determined before ligation, at short intervals after ligation, and then every day, or every second day throughout the period of starvation until the death of the animal. After every measurement of the oxygen consumption, the larvae were weighed again and transferred to the thermostat at 30°.

The oxygen uptake was measured with the Warburg manometric method at 30° and in some experiments at 40°.

RESULTS OF THE EXPERIMENTS

The oxygen consumption of the larvae in their normal state, i. e. before ligation, was usually about from 3 to 5 ml per hour per 1 g of body weight at 30°C. Table I shows that individual differences are very great and vary between 2.3 ml and 8.6 ml.

Table I.

Oxygen consumption of non ligated larvae of *Galleria mellonella* at 30.0°C.

N° of larva	Weight (mg)	Oxygen consumption per hour	
		per larva (µl)	per gram of larval weight (ml/g)
3'A	235.0	1293	5.50
1/N	184.3	717	3.89
2/N	221.6	763	3.45
3/N	214.0	546	2.55
4/N	219.8	526	2.39
5/N	186.6	436	2.34
1/P	152	804	5.29
2/P	198	786	3.97
3/P	128	530	4.14
4/P	146.5	488	3.33
5/P	181.5	722	3.98
6/P	220	1742	7.92
7/P	185	1098	5.94
8/P	176	672	3.82
9/P	200	1721	8.6
10/P	197	1360	6.90
		mean value 4.63	

Immediately after ligation, there is a violent fall in oxygen uptake. On account of the method adopted the first measurement could not be made earlier than from 20 to 30 minutes after ligation. In that period the larvae showed only from 2 to 2.9 ml of oxygen consumption per hour per 1 g. During the next few hours the oxygen consumption continued to diminish, at first rather rapidly, then, gradually, more and more slowly; after 1—2 days it fell to from 0.5 to 1.0 ml per gram per hour. This is shown by Table II which illustrates the fall in the oxygen uptake during the first 24 hours.

Table II.

Oxygen consumption of the larvae during the first 24 hours after ligation at 30.0° C, in ml per gram per hour.

	No of larva:	1 P	2 P	3 P	4 P	5 P	6 P	7 P	8 P	9 P	10 P
	before ligation	5.29	3.97	4.14	3.33	3.98	7.92	5.94	3.82	8.6	6.90
after ligation	20'	.	.	.	.	.	2.49	2.65	1.91	2.9	2.22
	25'	2.86	2.32	2.39	2.17	2.47	.	.	.	.	.
	4 h	2.09	1.90	1.70	2.28	2.19	.	.	.	.	.
	4 h 15'	.	.	.	.	.	1.73	2.00	1.66	1.1	1.59
	8 h	1.79	1.48	1.56	1.53	1.63	.	.	.	.	.
	9 h	.	.	.	.	.	1.33	1.46	1.44	.	1.20
	11 h	1.47	1.39	1.41	1.30	0.68	.	.	.	.	.
	23 h	1.03	0.97	1.06	1.03	0.82	.	.	.	.	.
	26 h 25'	.	.	.	.	.	0.80	0.79	0.98	.	1.16

During the next few days the consumption of 0_2 remained still at this low level; in some cases it continued to fall gradually and its minimum is usually reached between the 3rd and 12th days after ligation. The lowest figure of oxygen uptake by a number of individual larvae was from 0.8 to 0.2 ml per gram per hour. This was only one fifth and in some cases as little as one tenth of the normal quantity, i. e. before ligation.

The respiratory metabolism usually remains for from 8 to 15 days at this low level, then a slow, although in some cases also quite a rapid increase in oxygen uptake is observed. This increase lasts almost to the instant of death of the larva. It can go as high as 2 ml, 3 ml or even 4.4 ml per gram per hour, which is nearly the same quantity as was shown by the larva before ligation. A small decrease in oxygen uptake can again be observed immediately before the death of the larva.

The results of these experiments are presented in Table III and fig. 1.

In a series of experiments, we decapitated the larvae after ligation. The operation had no influence whatever on the phenomenon studied and the oxygen uptake took the same course as before (Table IV).

Table III.

Oxygen consumption of ligated larvae during the whole period of starvation at 30°C, in mililitres per gram of actual weight per hour.

day after ligation	No of larva: 2 A	13/A	1/A	8/A	3/A	12 A	5/A	9/A	4/A	7/A	10/A	11/A	6/A
2.	0.58	.	0.67	.	0.54	.	0.80	.	0.99	.	0.71	.	.
3.	0.41	.	0 37	0.80	0.45	.	0.78	0 79	0.56	0.70	0.52	0.83	1.11
4.	0.57	.	0.32	0.77	.	.	0.70	0.72	0.46	.	.	.	1.11
5.	.	0.50	.	0.78	0.44	0.81	.	0.87	0.54	1.02	0.44	1.00	1.08
6.	0 56	0.53	0.20	.	0.46	0.72	0 67	.	0.43	.	0.37	1.39	1.02
7.	.	0.51	0.35	0.81	.	0.69	0.81	1.34	.	0.76	1.11	1.75	1.51
8.	0.46	0.49	.	0.69	0.35	0.61	0.80	1.36	0.42	0.72	1.67	2.92	1.26
9.	.	0.51	0 30	0.74	.	0.79	0.80	1.90	0.84	.	1.65	3.16	.
10.	0.36	0.50	.	0.98	0.40	0.77	0.78	2.46	0.80	0.72	1.25	2.08	1.52
11.	0.46	.	0.22	1.28	.	.	1.04	2.04	1.04	1.24	.	.	dead
12.	.	0.41	.	.	0.49	1.32	.	.	dead	dead	dead	dead	
13.	0.80	0.59	0.33	2.01	1.49	2.02	dead	dead					
14.	0.99	0.66	.	2.11	2.43	3.37							
15.	1.15	0.81	0.44	2.07	3.22	4.43							
16.	1.78	0.95	0.59	1.34	.	3.60							
17.	.	1.29	.	1.27	.	dead							
18.	.	.	.	1.40	dead								
19.	1.77	1.25	.	.									
20.	1.96	1.62	1.66	dead									
21.	1.99	1.44	2.88										
22.	1.71	2.04	2.18										
23.	2.16	1.31	dead										
24.	1 33	1.72											
25.	dead	dead											

During the period of starvation of the ligated larvae, the reduction of the body weight was considerable and usually reached 50 or even 70 per cent of the initial value. The oxygen uptake as shown in Tables III and IV was calculated in relation to actual body weight on the given day of starvation. If the rate of oxygen consumption per gram of initial body weight is calculated it is possible to study the change in the absolute rate of respiratory metabolism of individual larvae during starvation.

The results of these calculation show that the absolute 0_2 uptake during the last period of starvation rises, although the body weight has been considerably reduced.

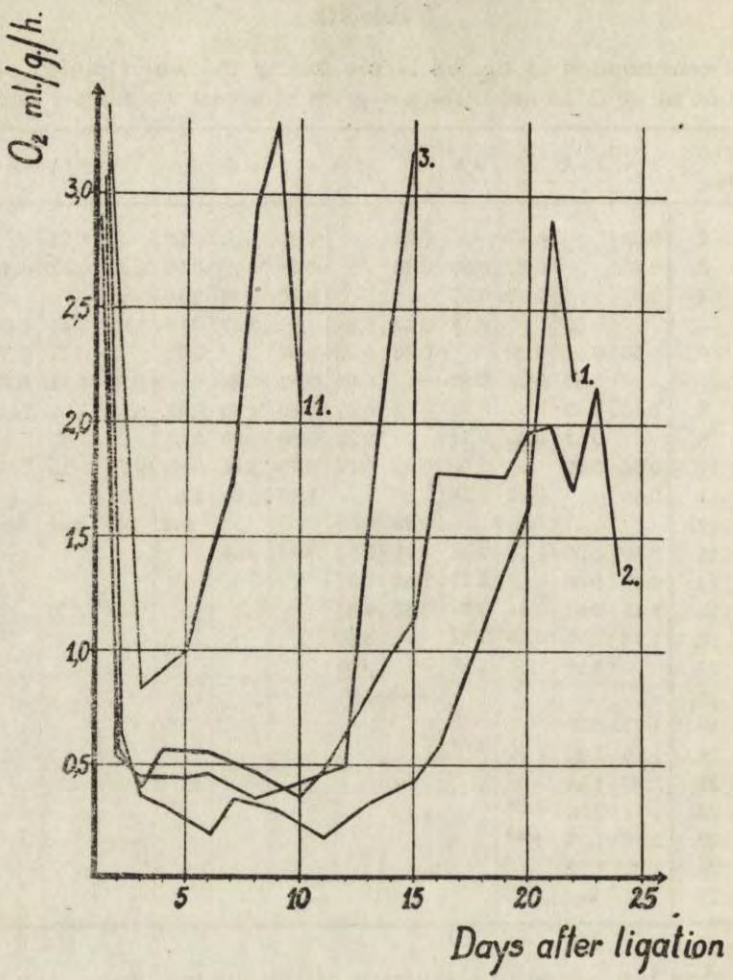

Fig. 1. Oxygen consumption of several larvae during starvation.

One of the series of experiments was carried through at 40°. It was observed that the rate of oxygen uptake at this temperature is somewhat higher while the general character of changes in the rate of respiratory metabolism during starvation is more or less identical with those at 30°. The drop, however, as well as the later rise in oxygen uptake, is not so strongly marked.

The researches of S. Niemierko (1950) have shown that during starvation of ligated larvae, and especially in its later stages, a con-

Table IV.

Oxygen consumption of the ligated and decapitated larvae at 30°C, in ml per gram of actual body weight per hour

	№ of larva:	1/G	16/G	8/G	4/G	10/G	7/G	15/G	5/G	6/G	11/G	12/G	2/G	9/G	13/G	3/G	14/G
day after ligation	2.	0.90	1.04	.	.	1.04	.	0.79	.	0.83	.	1.10	1.02	1.20	.	1.51	0.84
	3.	.	.	0.83	.	.	0.87	.	0.82	0.70	.	0.85	.	.	.	.	.
	4.	0.61	.	.	0.87	.	0.75	0.67	0.84	.	0.66	.	0.77	.	0.65	0.97	.
	5.	0.60	0.76	0.72	.	0.57	.	.	.	0.53	0.66	0.82	0.61	1.18	.	.	0.77
	6.	.	.	.	0.64	0.57	0.58	0.68	0.68	0.41	.	0.64	.	.	0.87	2.01	0.57
	7.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	8.	0.62	0.97	0.69	0.86	.	0.56	0.96	0.54	.	1.79	.	1.41	1.60	0.98	dead	dead
	9.	0.62	0.88	.	1.11	0.74	.	1.07	.	0.52	1.01	1.36	1.24	1.38	dead		
	10.	.	0.88	1.06	1.01	0.69	0.55	1.45	2.13	1.37	1.09	2.35	dead	dead			
	11.	0.77	0.84	.	1.68	0.75	1.07	2.91	2.52	1.91	1.45	.					
	12.	0.90	1.31	2.06	1.80	0.92	2.56	3.48	dead	dead	dead	dead					
	13.	1.45	1.88	1.56	1.44	1.39	1.35	dead									
	14.	.	.	.	.	.	dead										
	15.	3.08	1.94	dead	dead	dead											
	16.	dead	dead														

siderable reduction in the quantity of water in the body can be observed. The content of dry substance in normal larvae is from about 40 to 44 per cent. After 20 — 30 days of starvation it can reach from 50 to 60 per cent, and in some cases even 70 per cent.

We supposed that the increase observed in oxygen uptake bears some kind of relation to the considerable loss of water by the starving larvae and to the increase of the content of dry substance in their bodies. According to the work quoted, the loss of water shows immense individual variations and it is not possible to make a close study of this process within one and the same larva. We therefore made a series of experiments in which the influence of the content of water in the body of the larva upon the oxygen uptake could be examined indirectly. In pursuing this aim we kept the ligated larvae in a dry atmosphere over conc. H_2SO_4. Our analyses showed that these larvae lose water more rapidly than the controlled animals kept in conditions of normal humidity.

Table V.

Oxygen consumption of the ligated larvae kept in dry atmosphere at 30° C, in ml per gram per hour.

day after ligation	№ of larva: 14|A	16|A	15|A	19 A	22|A	21|A	18|A	17|A	20|A
1.	.	.	.	.	.	.	1.34	1.43	.
2.	0.80	.	.	.	0.49	0.66	0.61	.	.
3.	0.64	0.49	0.51	.	.	.	0.63	0.84	0.53
4.	0.58	.	.	0.73	0.62	.	0.63	.	.
5.	0.58	0.41	1.15	.	.	0.57	0.62	2.69	0.61
6.	0.89	.	.	.	.	.	.	.	.
7.	.	.	.	0.57	0.47	0.64	0.65	2.75	.
8.	2.28	0.44	2.67	0.60	0.51	0.54	1.33	5.13	2.04
9.	2.64	0.66	2.44	0.65	.	0.86	1.56	4.85	2.56
10.	2.63	.	1.83	0.76	0.52	.	2.49	3.17	3.11
11.	2.71	2.28	dead	0.98	0.64	0.78	3.33	dead	1.97
12.	2.80	3.46		1.31	0.72	1.06	2.51		dead
13.	.	3.20		.	.	.	dead		
14.	2.55	dead		1.82	2.19	2.92			
15.	2.14			2.09	3.20	3.62			
16	1.91			1.91	2.33	5.65			
17.	dead			dead	dead	dead			

The results of measuring the oxygen uptake of larvae which were kept in a dry atmosphere over H_2SO_4 are presented in Table V. We see that in general the rise in the oxygen uptake occurs earlier than in the case of the larvae previously studied. In one case, the oxygen uptake attained 5.6 ml per gram per hour, a level hitherto unrecorded. This quantity was more than ten times greater than that obtained with the same larva in the minimum stage, which occured on the 8th day of starvation.

Table VI records the days when an obvious rise in oxygen uptake was observed in the case of individual larvae kept over H_2SO_4, and others which were kept in conditions of normal humidity. We see that the increased oxygen consumption by the former takes place, on the average, on the 8th day, and that by the latter — on the 13th day.

Table VI.

Moment of the increased oxygen consumption of the ligated larvae kept in wet and dry atmosphere at 30°C.

a) larvae kept in wet atmosphere:

Nº of larva	day of increased oxygen consumption
1 A	16.
2 A	13.
3 A	13.
4 A	9.
5 A	11.
7 A	11.
8 A	10.
9 A	7.
10 A	7.
11 A	5.
12 A	12.
13 A	15.
mean 13.2	

b) larvae kept in dry atmosphere:

Nº of larva	day of increased oxygen consumption
14 A	6.
15 A	5.
16 A	10.
17 A	5.
18 A	8.
19 A	11.
20 A	8.
21 A	9.
22 A	12.
mean 8.2	

DISCUSSION

The rate of respiratory metabolism of the fully grown waxmoth larvae shows considerable variation. We observe that the oxygen uptake (at 30°) varies in the case of different individuals from 2 to 8

ml per gram per hour; the usual quantity, however, is from 3 to 5 ml. It seems probable that these variations depend to a considerable extent upon the varying muscular activity of individual larvae.

The process of oxygen consumption of ligated larvae can be divided into the following four periods: the first period, which begins immediately after ligation, probably lasts a very short time, 20—30 minutes at the most. A violent fall in the rate of respiratory metabolism of the larva is then observed. During this short period, the oxygen uptake sinks to about 2 ml per gram per hour. The larva is completely motionless and inert. It seems that the violent fall in oxygen uptake which is observed in that first period depends principally upon the compiete stop of muscle activity.

In the second period, which usually lasts from 1 to 2 days, a further, although small, decrease in the rate of respiratory metabolism is seen. The oxygen uptake usually falls to from 0.5 to 0.8 ml per gram per hour and is not much higher than the minimum observed in the next stage.

It is possible that the gradual decrease in oxygen uptake during the second period of starvation, according to our division, is connected with the forthcoming conclusion of the digestive process. The major part of the wax, as appears from the work of Niemierko and Włodawer (1950b) is being digested and assimilated by the larvae during this period of time.

During the third period, which lasts usually from 5 to 10 days according to individual larvae, the rate of metabolism remains at a very low level. Sometimes a further, although very small fall in oxygen uptake is seen, while in other cases there are variations in both directions. The lowest oxygen uptake observed was 0.2 ml per gram per hour.

The last period, which lasts almost to the death of the animal, again shows an increase in oxygen consumption. This increase is usually considerable and in some cases very violent. For instance, the oxygen uptake of one larva increased in two days from 0.5 to 2.4 ml per gram per hour. With other larvae this increase was more gradual. Different larvae showed the increased oxygen uptake on different days of starvation, the major of them between the 10th and 14th day. The rate of respiratory metabolism per hour and per gram of actual body weight would reach a figure not much lower than for normal larvae without ligation.

The process of respiratory metabolism that was observed in the case of starving larvae follows a characteristic „U" shaped curve. An identical character of curve for oxygen uptake was obtained as a result of research on the metamorphosis of *Galleria mellonella* (Crescitelli, 1935), of other Lepidoptera (Heller, 1928), and many other insects. Since, however — as has already been mentioned — the ligated waxmoth larvae never show any sign of metamorphosis, the increased demand for oxygen in the last period of starvation must be due to a different cause. The increased oxygen uptake usually occured in a period in which the larvae showed an increased excitation. It therefore seemed at first that the increased respiratory metabolism observed depends upon the intensity of the animals movements.

The increase of muscular activity, however, can be neither the sole nor even the decisive factor in the case. A notable increase of oxygen uptake was repeatedly observed in the case of completely motionless larvae. On the other hand, the larvae which showed most violent spontaneous movements sometimes had a comparatively low oxygen consumption.

Almost until the last day of their life, the ligated larvae retained quite a normal appearance in spite of the immense reduction of body weight which sometimes reached 70 or even 80 per cent. The normal appearance of the larvae argues against any infection as being the cause of the increased respiratory metabolism in the final period of starvation.

In seeking for an explanation of this phenomenon, special attention was devoted to the gradual dehydration of the body which accompanies the starvation of the waxmoth larvae and is specially intense in the final period of starvation. It appeared as possible that the increased oxygen uptake is due to some biochemical processes which are connected in that or some other way with the violent dehydration of the larval tissues.

This view finds some support in our experiments in which the ligated larvae were kept in a dry atmosphere over conc. H_2SO_4. Under such conditions the larvae become dehydrated much more rapidly. Parallel to this process is the definitely earlier period of increased oxygen uptake, while the rate of respiratory metabolism attains unusually high figures.

Further researches in that direction will seek to account for this phenomenon in greater detail and to reveal its mechanism.

SUMMARY

During starvation of the ligated larvae of *Galleria mellonella* oxygen uptake follows a characteristic U-shaped curve. It falls immediately after ligation and shortly becomes only $^1/_5$ and even $^1/_{10}$ of the normal value. After several days a slow or sometimes rapid rise in oxygen uptake is observed which lasts almost until the death of the animal.

The ligated larvae never pupate and no signs of metamorphosis can be seen. It seems probable that the rise of oxygen uptake may be due to some biochemical processes connected with a considerable dehydration of the ligated larvae which takes place in the late stages of starvation.

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STUDIES IN THE BIOCHEMISTRY OF THE WAXMOTH *(Galleria mellonella).*

4. METABOLISM OF TOTAL PHOSPHORUS DURING FEEDING AND DURING STARVATION OF THE LARVAE.

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In the literature, little information is given on phosphorus in the wax moth. Teissier (1930) determined the amount of P during the growth of *Galleria mellonella* and found in fully grown larvae about 0.5% of P. Lindsay and Craig (1942) investigated the distribution of radio-phosphorus in wax moth. They found that when the insects were fed P^{32}, as NaH_2PO_4 in their diet, P^{32} was most concentrated in the mid intestine epithelium, reproductive ducts and gonads.

In connection with the researches from this laboratory (W. Niemierko et al., 1949) in the biochemistry of *Galleria mellonella* it seemed desirable to study in detail the metabolism of phosphorus compounds in this animal.

The object of the present study is to investigate the metabolism of total P in the wax moth larvae during feeding and during starvation.

MATERIAL AND METHODS

As material for experiments were taken larvae from a breed kept in a temperature of 30° from the last period of growth. The larvae were ligated just behind the head in the manner described by Niemierko and Wojtczak (1950). The ligation absolutely prevented metamorphosis and permitted starvation for long periods of time. The ligated larvae were put in glas vessels in a thermostat, also in a temperature of 30°. The period of starvation of the larvae during separate experiments was up to 33 days. At intervals of one or several days the larvae were taken for examination and analysed individually.

After digestion with HNO_3 and H_2SO_4 total P determinations were carried out according to the method of Fiske and Subba Row (1935), applying amidol in the place of eukonogen. The colour was measured in the Spekker absorptiometer. In addition to the content of the total P in the body of larvae the amount of P excreted during the starvation period was also determined.

EXPERIMENTAL AND RESULTS

The examination started by determining changes in the amount of dry substance in single larvae during the starvation period. The results obtained are shown in Table I. From the data given it is seen

Table I.

Loss of body weight and content of dry substance during starvation of waxmoth larvae. (Mean values and standard deviation).

Day of starvation	No of experiments	Loss of body weight in % of initial value	Content of dry substance %
0	10	0	44 ± 0.7
2	14	4.5 ± 1.6	44 ± 2.9
6	10	9.7 ± 3.8	46 ± 2.4
11	9	20.9 ± 6.0	46 ± 2.7
17	9	37.8 ± 12	49 ± 2.4
21	6	52.9 ± 8.6	52 ± 4.6
27	5	65.2 ± 6.2	69 ± 8.8

that in the course of starvation the larvae are loosing large quantities of water and the percentage of the dry substance increases. This occurs especially in the later periods of starvation. The amount of dry substance in the body may reach 80%. The individual differences, as can be seen from the values of standard deviation, are very great.

The loss of body weight in different individuals during starvation also varies within very wide limits (Table I), and some of the larvae lose more weight during a shorter starvation period than others do during a longer period (Table III). During a 30 days starvation period, loss of weight up to as much as 70% of the initial value was observed.

As the starting point for resarches on P metabolism during starvation, the figures representing the amount of P in the body of the

Table II.

Content of total phosphorus in normal larvae.

Body weight mg	Content of P mg%
140	213
158	231
180	188
150	187
140	222
145	271
157	206
125	223
146	179
195	247
129	208
153	215
191	213
194	240
199	280
170	264
139	292
	Mean 228
	Standard deviation ± 33

non-ligated larvae were taken (Table II). The content of P in the fresh substance amounts on the average, according to 17 analyses made, to — 228 mg% (standard deviation ± 33.0). As in the case of the determination of the amount of dry substance, we can confirm, in this case also, considerable individual fluctuations. When calculating in relation to the dry substance which in feeding larvae

amounts to about 44%, the quantity of P is about 0.5%. Teissier (1930) in his researches obtained similar figures for total P.

The investigations on changes of the content of P in the body of larvae during starvation were carried on in three series of experiments. In the two first series, the larvae were starved for 31 and 32 days, in the third series for 9 days. At intervals of one or several days, 5 — 10 larvae were taken for analyses and analysed individually. The results obtained from these experiments are given in Table III. We can see that the content of P in the body of larvae calculated

Table III.

Content of total phosphorus in the larval body during starvation (Mean value and standard deviation).

Series	Day of starvation	No of experiments	Loss of body weight in % of initial value	Content of total phosphorus	
				mg% of actual body weight	mg% of initial body weight
I.	0	9	0	213 ± 23	213 ± 23
	6	5	11 ± 5	167 ± 8	147 ± 3
	11	5	22 ± 10	181 ± 12	141 ± 12
	17	5	50 ± 13	229 ± 50	111 ± 9
	21	4	59 ± 11	306 ± 21	121 ± 22
	27	3	74 ± 7	300 ± 21	90 ± 2
	32	3	64 ± 5	235 ± 33	83 ± 7
II.	6	6	10 ± 2	167 ± 8	149 ± 6
	11	8	23 ± 10	179 ± 18	135 ± 14
	31	6	59 ± 11	221 ± 41	90 ± 11
III.	0	5	0	225 ± 17	225 ± 17
	1	5	2 ± 0.4	220 ± 29	220 ± 30
	2	5	8 ± 4	239 ± 48	229 ± 39
	3	5	11 ± 9	222 ± 43	186 ± 32
	4	5	14 ± 12	202 ± 25	178 ± 25
	5	5	14 ± 4	196 ± 19	167 ± 10
	6	5	18 ± 6	187 ± 34	152 ± 27
	7	5	18 ± 3	203 ± 20	169 ± 15
	8	5	19 ± 4	228 ± 19	183 ± 13
	9	4	29 ± 4	220 ± 9	140 ± 36

in relation to actual wet weight increases during a prolonged starvation reaching in some cases 300 mg%. We can obserwe, however, a temporary loss which occurs usually about the sixth day of starvation.

In order to see what are the changes of the absolute quantity of P in the body of larvae during the starvation, the content of P was calculated in relation to the initial body weight of the individual examined. These data are also given in Table III. It can be seen that the content of P, which amounted to over 200 mg% in the fresh substance during the period before starvation, decreases within 32 days of starvation to above 80 mg% which is 40% of the initial value.

The experiments described convinced us that larvae lose very considerable quantities of P during starvation: about $^2/_3$ of the whole quantity in the body is excreted in about 30 days.

In order to examine the excretion of P during starvation and, partly at least, to eliminate the influence of individual discrepancies, the excreta of each larva were gathered throughout the starvation period. By determining the content of P in the body of the larvae at the end of starvation period and determining the quantity of P excreted during this time, it is possible to chart the content of P at the beginning of the starvation period and to calculate the losses of P for each larva in percentages of the initial value. Table IV repre-

Table IV.

Loss of body phosphorus during starvation (Mean value and standard deviation).

Days of starvation	No of experiments	Loss of body weight. % of initial value	Loss of P. % of initial value
6	10	11 ± 4	21 ± 7
11	13	20 ± 9	33 ± 4
17	4	50 ± 5	43 ± 5
27	4	74 ± 8	47 ± 5
32	8	63 ± 11	58 ± 6

sents the mean values of such calculations from two series of experiments. At the same time, there is given the percentage loss of body weight. It is seen that in the initial stages of the starvation period the percentage loss of the P surpasses the losses in body weight, but at later stages the loss of body weight is greater than the loss of P. The reason is probably this. During further starvation, as has already been mentioned, considerable loss of water occurs in the body of the larvae.

The amount of P excreted during 32 days of starvation amounts on the average to 58% of the initial content in the body. (The maximum loss amounted to 66%). The organism loses during the first 10 days of starvation about 30% of the P which it contained before starvation; in the later days of starvation the losses are already relatively smaller.

In order to give a detailed account of the curve of P excretion and of the changes in the content of P in the excreta, two simultaneous series of experiments were done. Fifteen larvae were used in each. The excreta were gathered every few days and the content of P in them determined. Such a method of performing the experiment allows for the curve of excretion of P in the same larvae to be followed exactly, contrary to the previous series, in which the results of analysis of the excreta of different larvae were compared.

Table V.

Excretion of total phosphorus during starvation of 15 larvae (Mean value calculated for one individual).

Series	No	Period of starvation Days	Mean body weight of 1 larva mg	Mean amount of excreta mg|24 h	Phosphorus excreted µg 24 h	Phosphorus content in excreta %
XXIV.	1	1	177	1.62	70.0	4.4
	2	2 — 4	160	0.72	32.0	4.2
	3	5 — 11	130	0.40	8.7	2.3
	4	12 — 15	116	0.32	3.0	0.86
	5	16 — 20	104	0.27	2.0	0.72
	6	21 — 25	89	0.23	2.4	1.06
	7	26 — 30	69	0.40	1.6	0.53
	8	31 — 33	59	0.36	3.3	1.11
XXV.	1	1	181	1.86	78.1	4.2
	2	2 — 4	162	0.68	26.2	3.9
	3	5 — 11	137	0.30	7.2	2.1
	4	12 — 15	128	0.21	2.7	1.4
	5	16 — 20	120	0.19	2.4	1.1
	6	21 — 25	114	—	—	—
	7	26 — 30	93	0.34	3.1	1.1
	8	31 — 33	79	0.35	3.3	1.0

The results of the analysis calculated for one larva and 24 hours are given in Table V and in Fig. 1. It is seen that after the first days of starvation the intensity of P excretion falls rapidly and after 12—15 days the curve of P excretion reaches an approximately constant level.

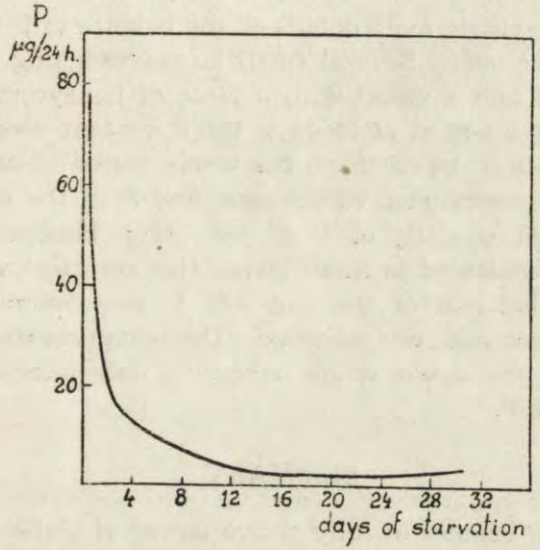

Fig. 1. Excretion of total phosphorus during starvation. Series XXIV.

The content of total P in the excreta during the feeding period is about 4.0%. The same content was found also in the excreta from the first day of starvation. In the later period of starvation, the amount of P slowly diminishes and from the twelth day it fluctuates around 1%. The individual differences are, in some cases, very great indeed; from another series of experiments it was found that the content of P in the excreta was, during the first five days of starvation, as much as 6% and in other cases only 2%. In the feeding larvae, 50% of the normal quantity of the excreta consists of lipids (Niemierko and Włodawer, 1950). Therefore the content of P in fat free excreta of feeding larvae can reach as much as 10% and even more.

In connection with the great quantity of P in excreta, especially during the period of feeding and the first days of starvation, it seemed important to analyse the food of larvae — the honeycomb.

It appeared in different samples of the honeycomb that the P content varies considerably, within limits from 0.24% to 0.97%. From 9 analyses the mean value was 0.57%.

High P content in the excreta, especially as compared with relatively small P content in the comb proves that the larvae of *Galleria mellonella* retain in their body only small quantities of the ingested P.

In order to obtain more details on the balance of P the following experiment was made. Several small larvae weighing about 20 mg each were put into a vessel with a piece of honeycomb previously weighed. After a period of 10 days, the P content was determined: 1. in the excreta of larvae from the whole period of experiment; 2. in the rest of unconsumed honeycomb, and 3. in the grown larvae. As we knew the quantity of P in the given honeycomb and the quantity of P contained in small larvae (the controls) we were able to calculate what part of the ingested P was assimilated by the larvae, and what part was excreted. The experiments proved that during growth the larvae retain only 15% and excrete about 85% of the ingested P.

SUMMARY

The total P content of fully grown larvae of *Galleria mellonella* amounts to about 200 mg%. Growing larvae retain in their bodies only about 15% of P, taken in with food, and they excrete about 85%. The content of P in the excreta is very considerable, the mean value is about 4%.

During 30 days of starvation larvae excrete about $^2/_3$ of their phosphorus. During the first several days of starvation, the excretion of P is especially intense.

The author is indebted to Miss A. Drabczyk for valuable technical assistance.

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STUDIES IN THE BIOCHEMISTRY OF THE WAXMOTH (*Galleria mellonella*)

5. ACID SOLUBLE PHOSPHORUS IN THE STARVING LARVAE.

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In the previous paper of this series (S. Niemierko, 1950) it was shown that as much as 50 — 60% of P can be eliminated from the larval body during starvation of waxmoth larvae. It appears from these observations that splitting of a large quantity of different phosphorus compounds must occur in the fasting larvae. The problem of the phosphate balance during starvation, therefore, deserves attention and it would appear that its examination can assist progress towards a characterization of the intermediary metabolism of phosphorus compounds in *Galleria mellonella*.

The aim of the present study was to investigate the amount of acid soluble P fractions in larvae starving for different periods of time. The acid insoluble P will be the subject of our further investigations.

There are few details about the metabolism of phosphorus in insects. The main data come from Heller and his collaborators (1949). They determined the amount of different acid soluble phosphorus compounds in the various stadia of *Deilephila euphorbiae*, in the

whole body and in intestine, blood, muscles, fat body etc. They affirmed an increase in the amount of inorganic P, during the period of metamorphosis: the adults have several times more inorganic P than the larvae, although considerable quantities of inorganic P are excreted in meconium.

Heller stated, moreover, the differences in the amount of phosphorus in relation to the sex of the insect. Barron and Tahmisian (1948) call attention to a similar phenomenon concerning other compounds (such as glycogen, cytochrome, DPN — diphosphopyridine nucleotide, DPT — diphosphothiamine in their examination of metabolism in the muscles of male and female cockroaches. In our experiments we were not able to take into account the possible influence of the sex of the insect upon the amount of the acid soluble phosphorus compounds which were studied, since the difference of sex can be established only in the late phases of pupae.

The determinations of acid soluble phosphorus were performed in the extract with trichloracetic acid at 0^0, the extraction being made three times (Umbreit et al., 1945). The quantity of particular phosphorus compounds was calculated with the use of a hydrolysis curve, according to Lohmann. In this way were determined the amount of inorganic P (P_{in}) before the hydrolysis, of phosphoarginine (hydrolysis during 1 minute of the extract with trichloracetic acid in 100^0), of phosphorus released in hydrolysis with N HCl in 7 minutes (P_7), of esters releasing P in hydrolysis in 3 hours (P_{180}) and finally esters nonhydrolysable in 3 hours (P_{nh}). Total acid soluble P was determined by digesting the extract with 2N HNO_3 and H_2SO_4.

In preliminary analyses we encountered the following difficulties. It appeared that analyses cannot be made on dry material. In comparison with analyses carried out on fresh substance, even vacuum drying (over H_2SO_4) caused a considerable increase in the amount of the inorganic P fraction at the expense of the partial decomposition of fractions which are easily and difficultly hydrolysable. The quantity of the total acid soluble phosphorus was unchanged during this process. We quote here as an example the results of several analyses: the amount of inorganic phosphorus determined in fresh substance was 13.9 mg%, determined in dry substance—83.5 mg% of wet weight, in chilled and then dried substance correspondingly — 39.0 mg%.

According to Heller (personal communication), the air drying of *Deilephila* even at normal room temperature causes no changes in the distribution of P. It seems as if the object of our experiments, *Galleria mellonella*, possesses an enzyme system which causes the decomposition of phosphorus compounds, even if drying is carried out in a vacuum, at a low temperature. This matter was not investigated in the present work and will be the subject of our further studies.

On account of the divergences in the results of the analyses made on dry and fresh substance, we carried out all the determinations of the acid soluble phosphorus fractions on fresh material only.

The experiments were made similarly to those described in the previous paper (S. Niemierko, 1950). About 100 ligated larvae were used in every series of experiments: they were weighed in groups of from 10 to 15 animals, placed in separate vessels at 30° and analysed every few days. In the further series of experiments, the larvae were kept and weighed individually and then analysed in groups of about ten animals on various days of starvation: the amount of phosphorus was too small to determine the phosphorus fractions in individual larvae. As in previous experiments, the period of starvation of the larvae lasted up to 33 days.

The preliminary analyses of dry material from 100 larvae showed that the amount of the total acid soluble P is 245 mg% in dry substance, while the amount of the total P in samples of the same material is 499 mg%. The acid soluble phosphorus constitutes, therefore, about a half of the total P. The mean content of the acid soluble P from other three series of analyses carried out on dry larvae was 237 mg%. The next determination made on fresh material and on groups of from 10 to 20 larvae each, gave as the mean of 15 series of analyses — 115 mg% ± 11.5, which is 266 mg% of the dry substance.

Table I.

Acid soluble P compounds in waxmoth larvae.

No of exper.	Mean body weight mg	Number of larvae	P in. mg%	P arg. mg%	$P_{7'}$ mg%	$P_{180'}$ mg%	P nh. mg%	P tot.ac.sol mg%
1	157	10	13.9	—	21.5	13.4	46.6	97.2
2	193	10	14.8	—	20.4	19.1	52.3	109.0
3	189	10	15.4	6.3	23.9	12.0	50.4	108.0
4	178	10	15.1	6.4	25.0	15.5	51.0	113.0
5	214	10	12.5	4.0	27.8	12.7	50.2	106.2
6	200	10	13.8	4.5	19.3	12.9	43.8	94.3
7	197	10	14.5	6 6	21.8	10.1	53.6	106.6
8	195	10	16 4	—	—	—	—	104.8
9	166	10	14.9	6.5	24.7	12.5	60.4	119.0
Mean	188		14.6	5.7	23.1	13.5	51.0	106.5
$\sigma = \pm \sqrt{\frac{\Sigma \Delta^2}{n-1}}$			±1.1	±1.6	±2.8	±2.7	±4.9	±8.0

Following these preliminary analyses, during which the amount of the total acid soluble phosphorus in the larval body was established — there began the estimation of the individual phosphorus fractions.

Table I shows the results of analyses of feeding larvae. We see that the amount of inorganic P is on the average 14.6 mg%, which is 13.8% of the total acid soluble phosphorus; the amount of P_{180} — 12.7%; while phosphorus of esters nonhydrolysable is the principle fraction, since it constitutes about one half of the total acid soluble phosphorus.

Analogous data for larvae after one day of starvation are submitted in Table II. In comparing the data in Table I and Table II, we do not state any essential difference. There is only a distinct decrease of the amount of P_7 in the larval body after 1 day of starvation — on the average from 23.1 mg% to 17.4 mg%.

Table II.

Acid soluble P compounds in waxmoth larvae after 1 day of starvation (calculated as mg% of actual body weight)

No of exper.	Body weight mg	Number of larvae	P in. mg%	P arg. mg%	$P_{7'}$ mg%	$P_{180'}$ mg%	P nh. mg%	P tot ac.sol. mg%
11	177	10	16.1	8.7	15.7	9.6	76.7	110.7
12	188	10	15 2	7.6	15.8	10 6	63 6	112.8
13	161	10	14.5	7.6	18.5	12.5	59.5	115 6
14	161	10	14.3	5.7	22 5	10.5	71.7	125.2
15	206	10	14.8	7 4	16.7	13.9	64.2	117.0
16	201	10	12.7	7.3	14.9	12.8	72.4	120.1
17	195	8	14.6	8.2	17.8	13.9	73.0	127.5
18	199	8	16.2	9.9	16.9	22.1	58.4	123.5
Mean			14.8	7.8	17.4	13.2	67.4	121.1
$\sigma = \pm \sqrt{\frac{\Sigma \Delta^2}{n-1}}$			± 1.2	± 1.2	± 1.2	± 3.9	± 6.8	± 6.4

The researches on the variation in the amount of different phosphorus fraction during the starvation of the larvae were carried out in nine series of experiments. It has already been mentioned in the previous paper (S. Niemierko, 1950) that there are great individual variations in the loss of body weight during starvation as well as in

Table III.

Content of different acid soluble P compounds in waxmoth larvae during starvation.

(Calculated as mg.% of initial body weight and per cent of initial value; $\sigma = \pm\sqrt{\frac{\Sigma \Delta^2}{n-1}}$)

Loss of body weight % of initial value	P in.		P arg.		P_7		$P_{180'}$		P nh.		P tot. ac. sol.	
	mg% σ	%	mg% σ	%	mg% σ	%	mg% σ	%	mg% σ	%	mg% σ	%
0	14.6 ± 1.1	100	5.7 ± 1.6	100	23.1 ± 2.8	100	13.5 ± 2.7	100	51.0 ± 4.9	100	106.5 ± 8.0	100
11 — 20	14.6 ± 1.8	100	6.9 ± 1.8	121	12.6 ± 1.3	55	11.7 ± 5.5	87	52.7 ± 12.2	103	98.7 ± 14.6	93
21 — 30	12 6 ± 2.1	86	6.8 ± 2.2	119	10.6 ± 2.5	50	8.9 ± 1.5	66	42.3 ± 13.3	83	79.9 ± 13.2	75
31 — 40	11.9 ± 0.8	81	4.2 ± 1.7	74	7.9 ± 1.5	34	9.9 ± 4.8	73	36.5 ± 10.9	72	69 9 ± 11.7	66
41 — 50	11.2 ± 1.5	77	4.0 ± 1.1	70	8.4 ± 3.7	36	6.4 ± 3.1	47	26.5 ± 11.7	52	56.5 ± 6.6	53
51 — 60	11.1 ± 1.4	77	4.9 ± 0.8	86	8.0 ± 1.9	35	6.8 ± 4.6	50	19.0 ± 14.5	37	50.2 ± 17.9	47
61 — 70	9.2 ± 1.5	63	4.5 ± 2.1	79	6.4 ± 2.9	28	7.9 ± 3.1	59	15.1 ± 7.9	30	46.5 ± 9.4	44

the amount of total phosphorus in the larval body. The same phenomenon occured in the experiments discussed in the present study. In order to make it possible to examine jointly the figures concerning the amount of various phosphorus fractions from different series of experiments, the results were calculated in relation not to the successive day of starvation, but to the loss of body weight. It seems that, to characterize the stage of starvation, this is a better method than the measurement of time from the moment when feeding was stopped, since some larvae lose much more of their weight than others in the same period of time.

The mean values of investigated P compounds were calculated from different series of experiments for ranges of each 10% of body loss during starvation. The calculation carried out according to this method (Table III) makes it possible to follow the course of changes

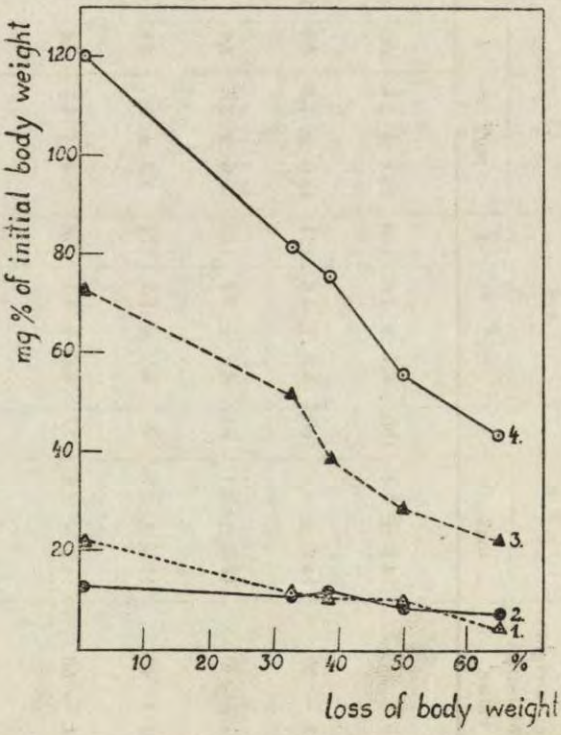

Fig. 1. Phosphorus compounds in larval body during starvation. 1 — P_T, 2 — P inorganic, 3 — P non hydrolysable, 4 — P total acid soluble.

in the amount of particular P compounds in the larval body, although there exist considerable individual variations. In comparison with other phosphorus fractions the decrease in the amount of inorganic P during starvation is the smallest; a diminution in the amount (in mg% of initial body-weight) appears only later, in the further stages of starvation. The quantity of compounds easily hydrolysable and nonhydrolysable falls during the 30 days long starvation in approximately the same ratio, but the considerable diminution in the amount of P_7 already appears in the early stages of starvation. In fact, as has already been mentioned, this diminution was observed after 1 day of starvation (Table II). The curve (Fig 1) for compounds difficultly hydrolysable (P_{180}) is parallel to the curve of total acid soluble P which has features in common with the curve representing the changes in total P.

Table III also present the relative changes of individual phosphorus fractions, the amount for the period of normal feeding was fixed at 100%. On the basis of these data one can see that when the larvae lose from 60 to 70% of body-weight, which occurs approximately between the 25th and 32nd day of starvation — the total acid soluble P reaches 44% of the initial quantity. In this period the amount of P_7 and P_{nh} falls to 30% of their initial quantity. To illustrate this relation Fig. 1 shows the curves representing the change in the amount of some phosphorus fractions in one of the series of experiments.

Table IV.

Content of P fractions during starvation of waxmoth larvae, calculated as percentage of total acid soluble P.

Loss of body weight % of initial value	P in. %	P arg. %	$P_{7'}$ %	$P_{180'}$ %	P nh. %
0	14	5	22	13	48
11 — 20	15	7	13	12	53
21 — 30	16	8	13	12	53
31 — 40	17	6	11	14	52
41 — 50	20	7	15	11	47
51 — 60	22	10	16	13	38
61 — 70	20	11	14	16	33

In order to emphasize the changes which occur in mutual quantitative relations between individual phosphorus fractions during starvation, a calculation was made of particular fractions as a percentage of total acid soluble P (its quantity was fixed as 100%). These results are shown in Table IV. We see that in the later periods of starvation the value of the fractions of nonhydrolysable esters is considerably diminished. During the period of normal feeding it constitutes 51% of the total acid soluble P, by the end of the period studied it falls to 33%. The decrease of the P_7 fraction occurs in the initial of starvation, while the value of fractions of the inorganic P and P_{180} is subject to a relative increase in comparison with other compounds.

From the results obtained, it is difficult to ascertain the transformations of the phosphorus fractions, which occur during starvation. It appears, however, from our experiments that the P fraction, whichs is nonhydrolysable in vitro under the influence of N HCl at 100° in three hours, is subjected to a definite decompostion in the organism. It is possible, that the remaining phosphorus compounds are also subject to transformations, since everything covered by our analyses might be a final stage, a resultant of a number of changes. It seems that only an investigation carried out with tracers could clarify this question.

In the present study the insoluble phosphorus compounds (i. e. lipid, protein, and nucleic P) were not examined. As has already been mentioned above, this will be the object of our further investigations. From a comparison of our analyses of total and acid soluble P it appears, however, that in the body of *Galleria mellonella* the acid soluble and acid insoluble P fractions are present in approximately the same quantities.

It seems quite probable that insoluble phosphorus takes an active part in metabolism of the wax moth larvae and that the turnover of insoluble phosphorus compounds overlap with the turnover of acid soluble P.

SUMMARY

The wax moth larvae contain about 220 mg% of total P. One half of this is the acid soluble P. Inorganic P amounts to 14 mg%, easily hydrolysable P — 29 mg%, difficultly hydrolysable P — 14 mg%, non hydrolysable — 51 mg%.

In the starving larvae, acid soluble P decreases gradually: after 25 — 30 days it falls to 50 — 60 mg% of initial body weight. Inorganic P diminishes only in the late period of starvation. The fraction hydrolysable in 7 minutes diminishes considerably at the very outset of starvation, it later decreases gradually to 6 — 8 mg% of initial body weight. The amount of non hydrolysable P compounds shows the greatest decrease during starvation.

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STUDIES IN THE BIOCHEMISTRY OF WAXMOTH
(Galleria mellonella)

6. METAPHOSPHATE IN THE EXCRETA OF *Galleria mellonella.*

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In our experiments on the metabolism of total phosphorus during starvation of larvae of *Galleria mellonella,* we established that the larvae lost a very considerable quantity of P; in a period of 30 days the loss of P could reach as much as 60% of the total P in the body. Our attention was then drawn to the fact that a high content of P is to be found in the excreta, especially during the feeding period and during the first days of starvation; this content sometimes amounts to 4% or even more (S. Niemierko, 1950).

It is known that nearly all phosphorus is excreted by animals in the form of orthophosphate. Our intention was to verify in what quantities other phosphorus compounds are excreted by *Galleria mellonella.* The analyses of the excreta gave an unexpected result. It appeared that only a small part, namely about 10 — 20% of total phosphorus, could be found in the excreta in the form of orthophosphate (determined by the Fiske - and Subba Row method).

We began therefore, systematic analyses of excreta in order to elucidate in what other form larvae of *Galleria mellonella* eliminate

P from their body. (Niemierko S. and Niemierko W., 1949). The analysis carried out convinced us, first of all that nearly all P is excreted in the form of acid soluble P, and only a very small percentage of total phosphorus is an acid insoluble P. In our various experiments it amounted in quantity to, at the most, 8% of the total P, and in many cases, was not found at all. In determining the fractions of acid soluble P we applied the same method as for the phosphorus fractions in larval body (S. Niemierko, 1950). Since initial analysis had proved that the fraction corresponding to P released after 1 minute hydrolysis of trichloracetic acid extract is extremelly small, we did not separate the fractions of easily hydrolysable P and we limited ourselves to the joint determination of P released after 7 minutes hydrolysis with N HCl in 100° (P_7). It appeared from the analyses that P_7 constitutes a considerable majority of total P excreted. Table I shows the results of three series of our experiments, with the excreta of larvae starving for different periods of time. We see from the results that P_7 constitutes from 65—90% of the total acid soluble P, inorganic P (P) from 2 — 23%, and the remaining fractions (P_{180} and P nonhydrolysable during the period of 3 hours — (P_{nh} are found only in small quantities and vary considerably in different experiments. In some experiments they were not found at all.

Table I.

Phosphorus compounds in excreta of *Galleria mellonella* larva (calculated as per cent of total acid soluble phosphorus in excreta).

Series	Period of starvation Days	P in. %	P_7 %	Other acid soluble P compounds %
XX.	7 — 9	14.2	85.5	0.3
XXVI	2 — 10	10.5	78.0	11.5
	11 — 14	5.9	79-1	15.0
	15 — 19	2.0	92.5	5.5
XXXII|1	1 — 14	15.0	79.2	4.8
XXXII|2	1 — 14	18.0	77.0	5.0
XXXIII	1 — 26	23.0	65.0	13.0

We may say, therefore, that in the excreta of starved waxmoth larvae the majority of total P is a labile phosphorus compound which

is converted to orthophosphate following 7 minutes hydrolysis at 100° in 1 N HCl, and that the amount of orthophosphate is only from 2 — 23% of the total acid soluble P.

Our first experiments in which the labile phosphorus compound was found in the excreta of *Galleria mellonella* were performed on the ligated starving larvae. Since it seemed that this might arise from some pathological processes connected with ligation and with long starvation, we carried out some analyses of excreta of normally feeding larvae. It appeared then that P_7 is a normal constituent of the excreta of the larvae. It is excreted during the period of feeding as well as during starvation. The absolute quantity of P_7 excreted by the feeding larvae is even much greater than during starvation period.

Since it was possible that the labile P compound found in excreta could be a compound present in food and not assimilated by *Galleria* we began an analysis of phosphorus compounds in the honey comb on which the larvae feed.

Analysis of honey comb samples, taken from various hives proved that all P in the comb is orthophosphate. The quantity of insoluble P shoved fluctuations within the limits of error of determinations. No fractions of P were found to be released after 7 minutes of hydrolysis. The content of orthophosphate deviated greatly and corroborated the data given by Niemierko S. (1950) that is from 0.3 to 0.9%.

Phosphorus ingested by larvae in the form of orthophosphate is, therefore, transformed in the body into an another compound and than it is excreted in the form of a labile P-compound.

In order to investigate the relative proportions between the fractions of P contained in the larval bodies and in their excreta, three series of balance experiments were carried out: the content of P fractions was determined — first in the body of larvae starving for a certain period of time, and secondly in the excreta from the same period. Having also the data pertaining to the content of P fractions in the larval body before the starvation, we may calculate the balance of the different P constituents. It appears from Table II, which contains the results of one of the series that total acid soluble P found in the excreta and in the body of larvae after the investigated period of starvation is some tens per cent greater than this same fraction in the body of larvae before the starvation. As might have been foreseen, excreted P must come also from the frac-

Table II.

P compounds in the larval body and P compounds excreted during starvation (calculated as mg% of the initial body weight). Series XXXII.

Days of starvation		0	14		25	
No of exper.		1	2	3	4	5
Loss of body weight %		0	34.1	36.7	49 5	64.8
P tot ac. soluble	in the body	105.7	81.5	75.0	55.6	43.7
	excreted	—	63.4	78.6	72.9	99.0
	the sum	105.7	144.9	153.6	128.5	142.7
P in.	in the body	12.7	11.3	11.5	8.9	7.5
	excreted	–	9.6	11.5	13.0	14.8
	the sum	12.7	20.9	23.0	21.9	22.3
P_7	in the body	22.2	11.7	10.9	9.8	4.2
	excreted	—	47.5	59.2	53.7	70 4
	the sum	22.2	59.2	70.1	63.5	74.6
Other acid soluble P compounds	in the body	85.2	58.5	52.6	36 9	32 0
	excreted	—	5.8	7.8	5.9	13.7
	the sum	85.2	64.3	60.4	42.8	45.7

tion of acid insoluble P of the body. Analogous calculations point to the fact that inorganic orthophosphate (P_{in}) and P_7 must originate from other P fractions. The amount of P_{in} found in the bodies and in the excreta after starvation is nearly twice and the amount of P_7 is several times greater than the quantity of these fractions in the larval bodies before starvation.

The fact that P in the excreta of *Galleria mellonella* is found chiefly in the form of an easily labile compound led us to make some further investigations in order to establish whether this compound is specific for the object of our experiments only, or whether it may be found in the excreta of other insects. We, therefore, analysed the excreta of Stock Insect *(Dixippus)*, Flower Moth *(Ephestia)*, and Silk Worm *(Bombyx mori)* and, we established that in all these cases, P was present only in the form of orthophosphate. Only in the case of *Achroea grisella*, an insect which has a similar way of life

and which feeds in the way as *Galleria mellonella,* that is on honey comb, did we find a labile P compound in the excreta. It was present in a quantity five times greater than the content of orthophosphate, similary as in the case of *Galleria*. The content of (P_{in}) in the excreta of *Achroea* was about 0.2%, P_7 — about 1%.

In connection with our determinations of P in the excreta of other insects we might add, that P content was there several times smaller than in either *Galleria* or *Achroea,* although the food of these insects contains nearly the same quantities of P as are found in the comb. For example, in the Stock Insect, which feeds on ivy, the content of P in excreta was 40 times smaller than in *Galleria.*

Excreta of insects are, as we know, a mixture of urine and faeces: the end of the Malpighian tubes is in hind part of the digestive tract. In the excreta are present the remains of the non — assimilated food, as well as the products of metabolism. Since P_7 is present also in the excreta of starving larvae it seemed likely that it was excreted through Malpighian tubes. Tentative experiments performed on the prepared tubes, together with the hind-gut (below the apertures of the Malpighian tubes) demonstrated in fact the presence of P_7.

In order to prove whether the excreted labile P compound is specific for the period of growth of the larvae, or whether it is excreted also in other stages of development, an analysis of meconium was performed. Meconium was collected either from the walls of the vessel, in which the moth were emerging from pupa previously placed there, or the moth was taken in the moment of eclosion and then the meconium was squeezed out of it.

Analysis of the meconium of 6 moths proved the presence of P_7 three times greater in quantity than P_{in}. It was found in meconium of one moth about 0.018 mg P_7 and about 0.006 mg P_{in}. It should be emphasised that the individual fluctuations are very considerable; in an other experiment, for instance, it was found that meconium included more P_{in} than P_7 (P_7 — 0.018 mg, P_{in} — 0.027 mg for one moth). Whether this arises from difference of sex, to which Heller (1938, 1949) and Barron et al. (1948) drew attention when they investigated the composition of male and female insect bodies, or whether it is simply a great individual deviation — it is difficult to say at present. Such an assertion requires further investigations. In any case, the analysis convinced us that P_7 is a compound which is present in the excreta of both feeding and starving larvae, and that it

is excreted also during the period of metamorphosis, that is to say during the time of essential histological and biochemical transformations.

The next problem which had to be examined was the chemical nature of this labile P compound. We established that it belongs to a barium insoluble fraction (analysis according to Umbreit, Burris et al., 1945). Qualitative tests did not give a reaction indicating the presence of sugar. It was established next that the P_7 is not only acid soluble, but also water soluble: the aqueous extract of the excreta contains the same quantity of P_7 as the extract with TCA. It suggested that the labile P compound present in the excreta of *Galleria mellonella* might be not an organic compound but a meta or pyrophosphate.

During the past few years the formation of metaphosphate from orthophosphate present in the medium was proved by Mann (1944) in *Aspergillus niger*, by Wiame (1949) as well as by Schmidt, Thanhauser and Hecht (1946), and by Juni et al. (1948) — in yeasts, by Houlahan and Mitchell (1948) in *Neurospora* and by Ebel (1949) in yeasts and in bacteria.

Wiame, to detect metaphosphoric acid, applied among others, the metachromatic test (1947, 1949) in vitro. The metachromatic reaction described by him consists in a change of colour of toluidine blue under the influence of the metaphosphoric acid. The dye changes from blue into purple. According to Wiame this is a specific test for metaphosphoric acid; other P — compounds are not liable to it*).

Before applying this test for detection of the possible presence of the metaphosphate in the excreta, we investigated the influence of pH on the change of color of toluidine blue. We proved that, if pH of the liquid is increased to 12 — 13, the purple color appears even when the metaphosphoric acid is not present. A lower pH does not change the color of toluidine blue. Since the aqueous extract of excreta has a pH of about 4, it was possible to use in our analyses the metachromatic test. We established also that the presence of salts (not only of KCl, as mentioned by Wiame, but of other salts also: NaCl, Na_2HPO_4, Na_2SO_4) inhibits to a considerable degree the metachromatic reation. It is inhibited also by strong acidification. The

*) This reaction appears also under the influence of some sulphuric esters of polymeric carbohydrates.

proved sensitivity of the metachromatic reaction to the metaphosphoric acid prepared by us, coincided exactly with the data given by Wiame that is to say 10^{-4}M.

Wiame performed the metachromatic reaction on biological material (yeasts) from which the interfering substances inhibiting the applied color test had formerly been removed. He isolated metaphosphoric acid from the TCA extract by precipitation with lead nitrate at pH = 4 and afterwards by precipitation of lead with H_2S. After dissolving the metaphosphate isolated in this manner he performed the test in the solution obtained.

In our analyses we used the aqueous extract instead of trichloracetic solution. Without special prior precautions it produced a positive metachromatic test. This was proved several times on the excreta of both feeding and starving larvae. After the hydrolysis with N HCl, when the compound investigated was converted into orthophosphate, the metachromatic reaction did not occur. (After the hydrolysis, the solution investigated was allways brought to pH=4, because, as we have already remarked, a too strong acid medium inhibits the test).

When the occurence of the positive metachromatic test directly in the water solution of the excreta of *Galleria mellonella* was established, we commenced the precipitation and purification of our labile P compound, with Pb acetate according to Wiame's method. We obtained the positive test with toluidine blue in this case also. The test was less pronounced than the one carried out directly; that may be because of the losses occuring during this procedure.

A known property of metaphosphoric acid is its ability to precipitate proteins. We performed this test in the manner descibed by Mann (1944): diluted animal serum (1:20) acidified with a drop of N acetic acid was treated with water solution of excreta. The precipitation of proteins was observed. But the extract hydrolised during 7 minutes with N HCl, and later properly neutralised, was inactive. It seems therefore, that this ability, as in the case of the ability to give a positive metachromatic reaction, is connected with the presence of the labile P compound.

Finally, the other property of metaphosphoric acid is its precipitation with Pb salts at low pH. This test was carried out at pH=2 (Mann 1944) by means of lead nitrate. We proved the presence in the precipitate of the labile compound which after 7 minutes hydrolysis changed into orthophosphate.

Because of difficulties connected with the separation of P_7 from the uric acid, which is present in the excreta in great quantity and which is also precipitated with Pb, Ba, Ag salts, the elementary analysis of the precipitated P_7 could not be carried out at the time. It will be the subject of our further investigations, which will try to resolve definitely our present hypothesis based on the data given above, that this is a metaphosphate. These data can be summed up as follow: in the excreta of *Galleria mellonella* is present a labile P compound, which is transformed after 7 minutes hydrolysis with N hydrochloric acid at 100° in orthophosphate; it is acid and water soluble; it is precipitated with Ba salts and precipitated with Pb salts at pH = 2; it gives a positive metachromatic reaction, and, finally it precipitates proteins.

* * *

Mann, proving the formation of metaphosphoric acid in *Aspergillus*, detected at the same time in this mould a metaphosphatase, which transformed metaphosphoric acid into orthophospate

It was of interest, therefore, to investigate whether, in the body of *Galleria mellonella* larvae, there is present enzyme or an enzyme system which would split metaphosphoric acid, found in the excreta of these larvae. With this view, we carried out the following experiments. We treated the water suspension of excreta with the brei of the larvae at a temperature of 40° for two hours, and then we checked the changes of the content of the P_7 fraction after the incubation. At the same time we performed the following control analysis: first— analysis of dry excreta in normal conditions, second — analysis of excreta mixed with water after two hours of incubation, and third — analysis of brei of larvae also after incubation. The water suspension of excreta kept in an incubator for 2 hours did not reveal any changes in the proportion of P constituents in comparison with the dry excreta before the incubation.

The results of the experiments in which the excreta were incubated with brei are shown in Table III. We see that the amount of P_7 diminishes and the amount of P_{in} increases.

The transformation of metaphosphate in excreta into orthophosphate under the influence of brei may be considered as an enzymic process, because, as we can see from Table III the brei heated first to a temperature of 100° became non active, and did not produce formation of orthophosphate. It was observed that a similar phenom-

Table III.

The enzymic breakdown of metaphosphate in excreta by the brei of *Galleria mellonella*

	Experiment with fresh brei		Experiment with boiled brei	
	P in. mg	P_T mg	P in. mg	P_T mg
Control brei after incubation	0 147	0.029	0.034	0.028
Excreta	0.044	0.336	0.031	0.199
Calculated sum	0.191	0.365	0.065	0.227
Amount found after incubation of brei with excreta	0.305	0 199	0.060	0.269
Difference	+ 0.114	— 0.166	— 0.005	+ 0.042

enon occured under the influence of brei of rabbit muscles. The results of the experiment are shown in Table IV. When, instead of the brei of larvae the homogenate was applied (using the glass homogenizer of Elwehjem), the phenomenon described above was, in general, corroborated.

Table IV.

The enzymic breakdown of metaphosphate in excreta by the brei of rabbit muscles.

	Experiment with fresh brei		Experiment with boiled brei	
	P in. mg	P_T mg	P in. mg	P_T mg
Control brei after incubation	0.107	0.064	0.073	0.029
Excreta	0.125	0.522	0.116	0.485
Calculated sum	0.232	0 586	0.189	0.514
Amount found after incubation of brei with excreta	0.369	0.406	0.210	0.515
Difference	+ 0.137	— 0.180	+ 0.021	+ 0.001

Experiments with the object of: a) a fuller investigation of the processes of transformation of metaphosphoric acid in the excreta under the action of enzymes found in the body of larvae, or in the tissues of other animals, b) choosing an optimum pH for this process, and c) detecting specific properties of these enzyme systems — will be dealt with in our further experiments.

* * *

Summing up results achieved so far in our investigations we can state, that the metaphosphate found by us in the excreta of *Galleria mellonella* is formed in the larval body in a very considerable quantity and is the chief phosphorus compound in the excreta. As was proved in our investigations on the phosphorus balance during the growing period (Niemierko S., 1950), the larvae of *Galleria mellonella* assimilate only less than 15% of the P present in the food, the rest is excreted. On the basis of our present investigations we can say that it is not a passive passage of phosphates through the digestive tract. Orthophosphate from the comb undergoes a transformation in the body of larvae and is excreted principally in the form of metaphosphate.

What the biological and biochemical significance of this process is — can be hardly stated at present. It seems likely, that it is connected with a specific manner of feeding of *Galleria* with wax, as only in the excreta of *Achroea*, feeding similarly to *Galleria* on honey comb, was the metaphosphate found too. On the contrary, in the excreta of other animals, this compound, as far as our information goes, has never been determined.

To answer the question whether, in the formation of metaphosphate, there participitate microorganisms, which, as some authors assume (Metalnikov, 1908, Dickman 1934, Florkin et al. 1949) are active during the digestion of wax, seems at present premature. Some of the results of our investigations are hardly in accord with such a view. The great quantity of P_7 in the excreta and a frequent total lack of insoluble P is quite amazing. Nearly all the nitrogen in the excreta of starving larvae is a non protein nitrogen, chiefly the nitrogen of uric acid (Zielińska, unpublished data). It seems, therefore, that the absolute number of intestinal organisms, which are permanently eliminated from the body must be almost negligible. How,

then, can we explain the enormous production of the metaphosphoric acid, which decreases but is not arrested even during the acute starvation of the larvae, where their digestive tract is almost entirely empty. Of course, non-intestinal micro-organisms could be taken into account here, particularly, since the number of microorganisms described living in the body of *Galleria mellonella,* is very considerrable (Steinhaus 1946).

In any case it seems, that it must have been a very close symbiosis of *Galleria mellonella* with eventual microorganisms, because the production of metaphosphoric acid is found, as described above, in different stages of growth and in different physiological states. The place where metaphosphoric acid is formed in the body of *Galleria mellonella* is also not known at present; as our tentative experiments proved, it appers in the Malpighian tubes, but where it is first produced, cannot yet be stated. We know, that its content in the excreta, as we proved by the analysis, is not changed after being preserved for one month in the laboratory, at room temperature. So it may be assumed that the process of formation of metaphosphate from orthophosphate may occur in the body of *Galleria,* and does not occur under the influence of the operation of microorganisms excreted outside the larval body.

We might suppose that the possible significance of the metaphosphate formation in the life processes of *Galleria* is the saving of the water released from orthophosphate through the transformation into metaphosphate. We know that insects in general, and *Galleria mellonella* in particular, are perpetually engaged in a struggle for water. Breeding *Galleria* in moist conditions gives good results: larvae of *Galleria* use in this case a smaller quantity of food per one mg increase in body weight, an analogous phenomenon was observed by Leclerc (1949) in the case of larvae of *Tenebrio.* Our tests, during which in the course of specially performed experiments we reared *Galleria mellonella:* first, in conditions of saturation with water vapour, on the permanently moistened comb; secondly: in conditions of normal moisture and thirdly: in a dry atmosphere over concentrated H_2SO_4 — revealed no variations in the quantity of the metaphosphoric acid production. It is possible, that it requires a more prolonged rearing of *Galleria* in moist conditions (perhaps through several generations) and possibly, an inversion of the biochemical processes in accordance with the new conditions.

But the significance of metaphosphate in the whole of the biochemical processes in *Galleria mellonella* may, neverthless, be much more complicated. It should be emphasized that the formation of metaphosphoric acid is connected with accumulation of energy, as this is a compound with energy rich bonds.

Our further investigations will seek to explain other problems which have been here suggested.

SUMMARY

1. The presence of labile P compound in the excreta of *Galleria mellonella* was proved. This compound is present in great quantities and constitutes about 60 — 90% of the total P excreted.

2. It is likely that this compound is a metaphosphate because:
   a. it is acid and water soluble,
   b. it is converted into orthophosphate after 7 minutes hydrolysis with N hydrochloric acid at 100°,
   c. it is a barium insoluble compound,
   d. it is precipitated by lead salts at pH = 2,
   e. it precipitates proteins,
   f. it gives the positive metachromatic reaction with toluidine blue.

3. This compound is found in excreta of both feeding and starving larvae as well as in the meconium of moth.

4. The presence of enzyme in the body of larvae, transforming the metaphosphate into ortophosphate, was proved.

5. Several hypotheses as to the significance and the role of this compound in the biochemistry of *Galleria mellonella* are suggested.

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THE RATE OF REGENERATION OF AMPHIBIAN PERIPHERAL NERVES AT DIFFERENT TEMPERATURES

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The rate of regeneration of peripheral nerves in mammals and in man was investigated by many workers by means of physiological, histological, and clinical methods.

The authors generally agree that the rate of regeneration in mammals, as determined by experimental methods, varies from 2 to 5 mm a day, depending on the character of the lesion and on various experimental circumstances. (For the moment we shall not consider the results obtained by clinical methods in man).

At present the interest of the workers is concentrated mainly on later phases of regeneration connected with the morphological maturation of the regenerating nerve fibre. The splendid quantitative work on these late phases of regeneration forms a basis of our present conceptions concerning the dynamics of the neuron. (For review cf. Young (1945), Young (1948), Weiss and Hiscoe (1948).

The work reported here returns to the study of the initial phases of regeneration — the latent period, and the elongation of regenerating fibres in the peripheral part of the nerve trunk. Our method of investigation is based on the ability of regenerating fibres to generate impulses on stimulation from the very beginning. There are

certainly marked differences between physiological properties of regenerating fibres and those of the corresponding normal fibres: the rate of conduction is smaller in the former (Berry, Grundfest and Hinsey (1944), Sanders and Whitteridge (1946); the electrical excitability is diminished (Holobut and Jalowy (1936), Erlanger and Schoepfle (1946); the mechanical excitability is greatly increased (Konorski and Lubińska (1946). These alterations in characteristics, together with the small diameter of regenerating fibres, give them a close resemblance to the mature fibres of C type (Lubińska 1947). Yet, in spite of this disparity in physiological characteristics, the impulses arising in the regenerating part of the fibre are conducted to the unaffected normal parts of the neuron, and, if we are dealing with afferent fibres, give rise to reflexes. Thus reflex reactions may be used for detection of regenerating fibres in the peripheral part of the nerve.

The experiments were performed on frogs and toads. The choice of this material made it possible to utilize a much greater number of animals than generally available in work on mammals, and on the other hand facilitated study of the influence of temperature on the rate of regeneration, a study which would call for complicated experimental procedures on Homoiothermes.

The investigation of the influence of temperature revealed the existence, in the early phases of regeneration, of two classes of processes marked by different thermic characteristics.

MATERIAL AND METHODS

The regeneration was studied on peripheral nerves of *Rana esculenta* and *Bufo bufo*. The toads were collected in early April, before they came back to land after the mating period, and were kept in the laboratory throughout spring, summer, and autumn. The frogs were collected between May and September, and the experiments carried during spring, summer, and autumn. There was no systematic study of seasonal influence, and the only observation of this kind was that no regeneration occurred in amphibians caught very early after the thawing of ice and investigated immediately afterwards, or in amphibians studied in late autumn (end of November, or December).

The regeneration was studied on sciatic nerves and their peroneal and tibial prolongations, and on the branches of these nerves.

The nerve was crushed with a strong linen thread in aseptic conditions under ether anaesthesia. The nerve was squeezed by the thread together with a small glass rod in order to facilitate the removal of the

thread after the crush had been made. The crush was generally made in upper part of the thigh.

At designate times (from 4 to 30 days after operation) the length of the regenerated fibres was determined by a method similar to the one described by Young and Medewar (1940), and Konorski and Lubińska (1944) for mammals. Slightly modified, it consisted in the following.

The medulla was transsected, and the posterior part of the animal skinned. The nerve was freed from its surroundings from the level of the crush towards the periphery at such a length as to ensure that the regenerating fibres did not reach the distal end of the exposed nerve. Then the nerve was pinched, millimetre by millimetre, with a fine forceps, from the periphery towards the centres. When the pinch fell at the region reached by the regenerating fibres, a reflex reaction appeared. This reaction was generally a flexion of the hind limb or limbs, sometimes a movement of the forelimb or of the head. If the pinches were continued toward the centres, reflexes became stronger, indicating that an increasing number of sensory fibres was affected. Afterwards the nerve was excised and the distance between the scar and the most distal point from which the reflex could be elicited was measured.This measure indicated the length of the fastest growing afferents evoking somatic reflexes.

The reflexes were very regular and constant in toads. They were less constant in frogs, i. e. sometimes a reflex appeared at a certain level, while the next pinch, more proximal, failed to elicit any reaction; on the third crush it appeared again, and so on. It proved possible to avert this difficulty by a subcutaneous injection of strychnine (Nitrate of strychnine 0,01 to 0,03 ml of solution of 1 mg/ml). In strychninized animals the reactions were very sharp and constant.

In some animals the crushing of the nerve was unsuccessful. A small number of sensory fibres escaped degeneration. They were easy to detect, since crushing of their most distal parts gave rise to reflexes. Such nerves were rejected.

Although the length of regenerated fibres was measured separately on tibial and peroneal nerves, the results were pooled together and regressions of regenerated length on time calculated.

The animals were divided into several groups kept during regeneration at various constant temperatures (9° to 26°C). No particular temperature changed by more than 1° to 1,5°C during the experiment. For each temperature the lengths of regenerated fibres were measured at various intervals after crushing. The number of these time intervals varied from 2 to 6. The given results are based on about 500 toad nerves and 500 frog nerves.

The method followed in this investigation has serious disadvantages. The length of regenerated segment can be determined only once on one nerve. The figures obtained come therefore from various animals, so that variation between individuals in addition to that within individuals is added to the changes caused by controlled factors such as time or temperature. Clearly, this augments the experimental error.

Another limitation of the method is that it can be applied only to afferent fibres, and involves nervous centres.

On the other hand the advantage of the method is that it is not based on the recovery of the function of the peripheral apparatus. Reinnervation of end-organs is a different process from the mere elongation of regenerating fibres along the nerve trunk. The former depends on a number of factors, such as the length of the period during which the end-organ was deprived of its innervation, and may have very different time characteristics. The assessment of the rate of nerve regeneration, based on observations of the recovery of function, is probably one of the reasons why clinical figures given for the rate of regeneration in man are generally smaller than figures obtained from experiments based on histological or experimental methods. This point has been more fully discussed elsewhere (Lubińska, 1944).

RESULTS

RANGE OF TEMPERATURE. The experiments were conducted under the range of temperature between 9° and 26°C. These limits were determined by two considerations: 1. Below 8° — 9°C regeneration does not occur. New fibres grow into the peripheral stump to a length of from 1 to 3 mm, and then the elongation stops. No further increase in length was observed in experiments conducted over a period of 40 days. 2. Above 26°C there is a high death rate under our experimental conditions, and a very wide fluctuation of results occurs in those animals that survive at this temperature. Here in addition to very long regenerates we observe quite short ones.

It is of interest to note that very similar limits of temperature were found for cellular division of *Amoeba* by Daniel and Chalkley (1932). Division of *Amoeba proteus* is possible at temperature from 4° to 35°C. But from 4° to 11°C divisions are rare and do not show normal course. The same applies to temperatures above 30°C. The highest proportion (97%) of divisions with normal course occurs within range from 11° to 30°C.

It will be demonstrated later that cellular division and the first stage of regeneration of peripheral nerves are subject to very similar changes under the influence of temperature.

INFLUENCE OF TEMPERATURE ON NERVE REGENERATION. Within the investigated range of temperature, the higher the temperature, the quicker the regeneration. It other words, at a given time after the nerve has been crushed, the length of the regenerated segment is greater when animal has been kept at a higher tempera-

ture. Quantitatively this relation is not simple and leads us to distinguish two categories of processes the rates of which are differently affected by temperature. If mean lengths of the nerves regenerated at a given temerature are plotted on y-axis against the time which has elapsed from the moment the nerves were crushed, a straight line can be fitted the slope of which defines the rate of nerve regeneration at this temperature. Figs. 1A and 1B show relations between the length of regenerated fibres and the time of regeneration at various constant temperatures in toads (A) and in frogs (B).

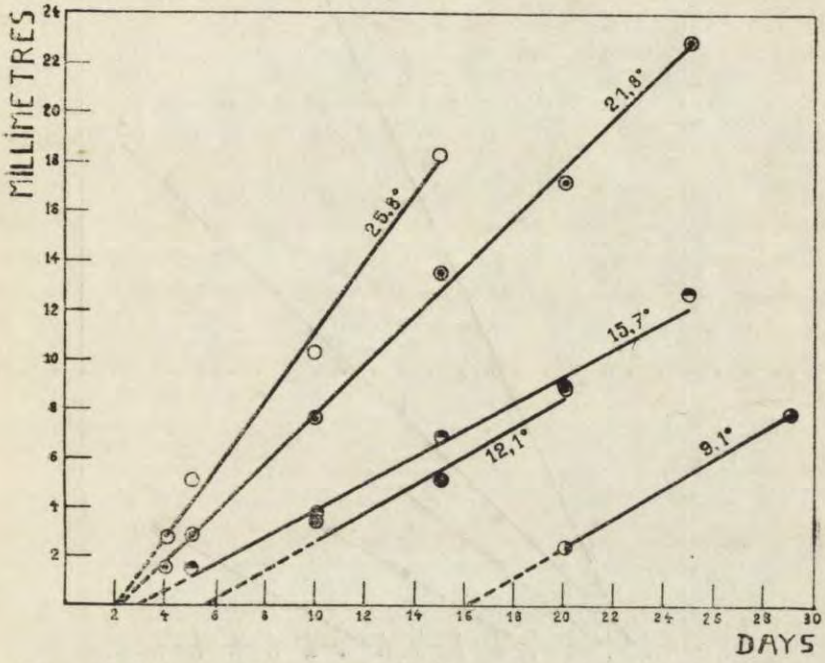

Fig. 1A. Lengths of regenerated fibres at various constant temperatures. *Bufo bufo*. Abscissae — time after crushing of the nerve in days. Ordinates — length of the regenerated fibres in mm.

We assume provisionally that the rate of regeneration is the same during the initial days of regeneration along the peripheral stump. This assumption could not yet be tested experimentally owing to technical difficulties (there are some indications that it is not entirely correct). By prolonging to the left the segments of straight lines up to their intersection with the abscissae, we obtain on the time axis periods during which the regenerating fibres do not appear yet in

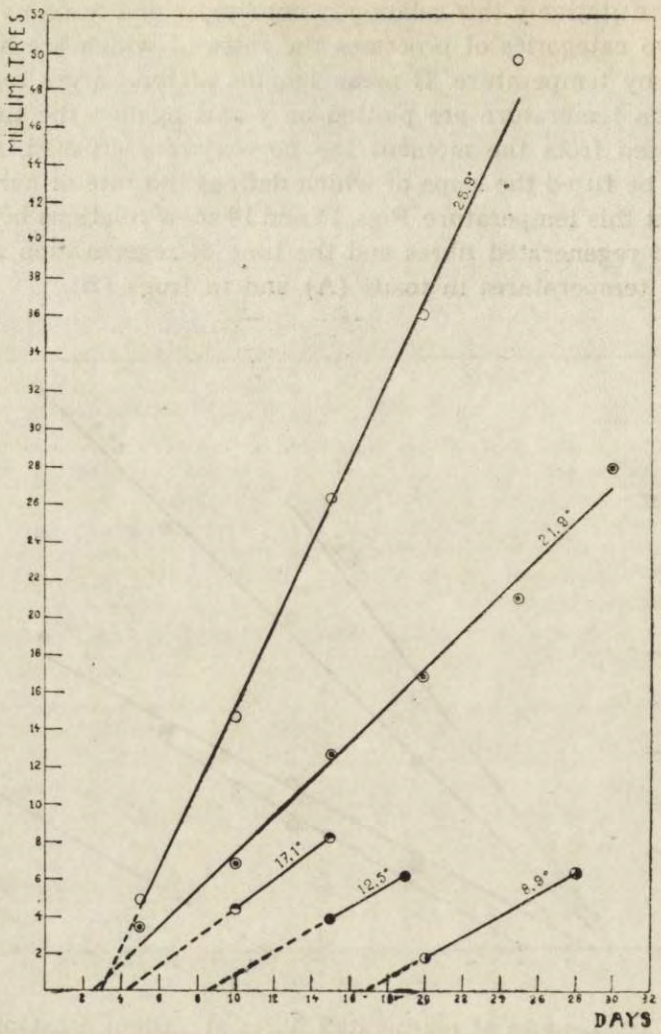

Fig. 1B. Lengths of regenerated at various constant temperatures. *Rana esculenta*. Abscissae: time after crushing of the nerve in days. Ordinates: regenerated fibres in mm.

the peripheral stump. We designate them, with other authors, as latent periods of regeneration.

From the inspection of Fig. 1 it is already apparent that the latent periods and the rate of elongation of regenerating fibres vary quite differently with temperature. We shall therefore consider separately the temperature relations of the two.

THE RATE OF ELONGATION OF REGENERATING FIBRES. At constant temperature the elongation of regenerating fibres proceeds at a uniform rate throughout the length of the nerve. It is independent both of the distance of regenerating tips from the perikaryon and of the time elapsed after the infliction of lesion. This conclusion is based on a series of experiments summarized in Table I.

Table I

Linear elongation of regenerating fibres at constant temperatures

Species	Bufo bufo		Rana esculenta		Rana esculenta	
Temperature in °C	21.8 °C		21.9 °C		25.9 °C	
Rate of regeneration in mm/day	1.0		1.0		2.2	
Latent period in days	2.2		2.6		2.9	
Number of nerves	124		147		151	
Time of regeneration in days	Mean length in mm		Mean length in mm		Mean length in mm	
	Observed	Calculated	Observed	Calculated	Observed	Calculated
4	1.6	1.8	—	—	—	—
5	2.8	2.8	3.4	2.4	4.9	4.5
10	7.6	7.8	6.9	7.3	14.8	15.3
15	13.5	12.7	12.6	12.3	26.2	26.1
20	17.2	17.7	16.8	17.2	36.1	36.8
25	22.7	22.6	21.1	22.1	48.7	47.7
30	—	—	28.0	27.1	—	—

In these experiments the lengths of regenerated fibres were examined at five or six time intervals after making the lesion, the longest interval corresponding to the period when new fibres have reached fine nerve branches in the foot. Thus the rate of regeneration was tested at various levels of the nerve, from the upper part of the thigh downwards.

The influence of temperature on the rate of elongation is presented in Fig. 2 (lower lines). Between 9° and 16°C the rate changes slowly, if at all. Its value is about 0,6 mm per day in the whole interval both in frog and in toad. Above 17°C there appears a marked increase which is faster than linear, and at 26°C the rate elongation attains 2.2 mm per day in frog and 1.4 mm in toad.

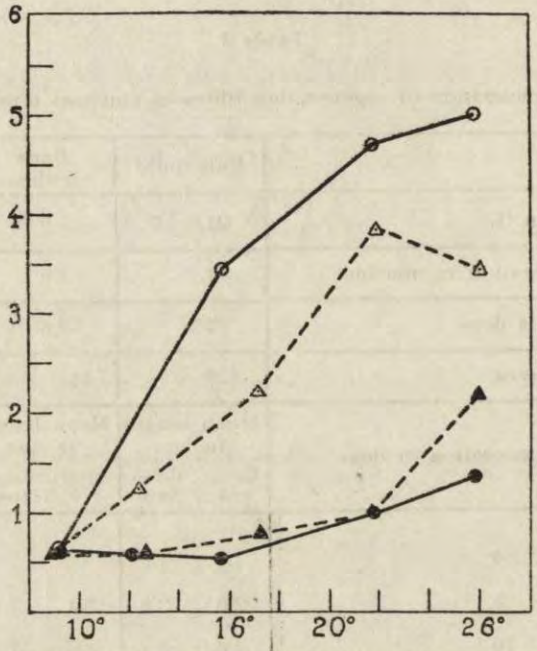

Fig. 2. Rate of regeneration and rate of processes underlying the latent period. Heavy lines — *Bufo bufo;* Dotted lines — *Rana esculenta.* Lower lines — rate of regeneration along the peripheral trunk. Upper lines — reciprocals of duration of latent periods. Abscissae — temperature in ° Centigrade.

THE LATENT PERIOD. The duration of the latent period is very great at the lower limit of temperature. At first it falls abruptly, then much slower with the increasing temperature. The latent period-temperature relation is shown in Fig. 3.

To estimate the rate of processes underlying the latent period and leading to the appearance of new nerve sprouts at the edge of peripheral stump, we calculated the reciprocals of duration of the latent period and plotted them against temperature in the same

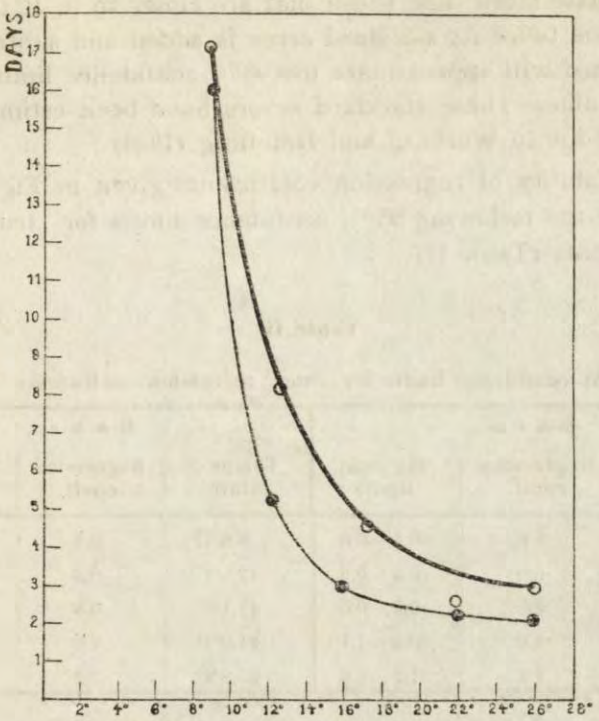

Fig. 3. Duration of the latent period at various constant temperatures. Abscissae temperature in ° Centigrade. Ordinates—latent period in days.

graph in which the rates of elongation were plotted (Fig 2, upper lines). It is remarkable that the temperature relations of the velocities of processes intervening during the latent periods are very different from those observed in elongation along the peripheral stump. The fastest increase of the former appears at low temperatures, where the rate of elongation along the stump is practically constant. At high temperatures, where the rate of elongation increases steeply, the changes in latent periods become insignificant.

The above disparity of the two curves suggests strongly that we deal with different processes during the latent period, and during the stage of elongation along the peripheral stump.

STATISTICAL ANALYSIS

REGRESSION. The regression values of Fig. 1 corresponding to different values of x (time) will not be affected equally by sampling error. The regression values placed further away from the mean of

x will fluctuate more than those that are closer to it. If to each regression value twice its standard error is added and subtracted, the limits obtained will approximate the 95% confidence limits for true regression values. These standard errors have been estimated from the formula due to Working and Hotelling (1929).

The reliability of regression coefficients given in Fig 2 can be judged from the following 95% confidence limits for „true" regression coefficients (Table II).

Table II

95% confidence limits for „true" regression coefficients

Bufo			Rana		
Temperature	Regression coeff.	95% conf. limits	Temperature	Regression coeff.	95% conf. limits
9.1°C	0.6	0.5—0.8	8.9°C	0.6	0.4—0.7
12.1°C	0.6	0.4—0.8	12.5°C	0.6	0.3—0.8
15.7°C	0.5	0.5—0.6	17.1°C	0.8	0.7—0.9
21.8°C	1.0	0.9—1.1	21.9°C	1.0	0.7—1.3
25.8°C	1.4	1.2—1.6	25.9°C	2.2	2.1—2.3

The important question whether rates of regeneration are constant can be answered by testing the linearity of regression. Since the linearity of regression can be tested when at least 3 values of x are available, the test could be applied only to temperatures 12°C, 16°C, 22°C, and26°C for *Bufo*, and 22°C and 26°C for *Rana*. The analysis of regression (Fisher, 1948) gives in all cases a very good agreement with linearity (insignificant values of F), except at temperature 12°C in *Bufo*, where regression is significantly non-linear (F = 9,60 with d. f. 1 and 24).

ANALYSIS OF VARIANCE. The analysis of variance with a view to testing the presence of individual differences in respect to regeneration has been made for each temperature. The results are shown in Table III.

The tilda placed over some values of F means that the „quotient" for „between individuals" is smaller than that for „within individuals". From this table it can be seen that near temperatures 9°C, 22°C, and 26°C both species exhibit highly significant individual differences in regeneration, and, besides, *Bufo* at 16°C. However, near

Table III

Analysis of variance

Bufo

Temperature	Source of variation	Degrees of freedom	Sum of squares	Quotient	F	One tail 5% point	One tail 1% point	Estim. components	In %
9.1°C	Between periods	1	326.1	326.1					
	Between individuals	12	53.2	4.43	7.91	2.10	2.87	1.26	69
	Within individuals	29	16.2	0 56				0.56	31
	Total	**42**	**395.5**					**1.82**	**100**
12.1°C	Between periods	2	214.3	107.13	~				
	Between individuals	14	61.4	4.38	1.19	2.34	3.40	0.00	0
	Within individuals	26	135.9	5.23				5.23	100
	Total	**42**	**411.5**					**5.23**	**100**
15.7°C	Between periods	4	1219.3	304.83					
	Between individuals	30	123.3	4.11	3.68	1.70	2,12	1.06	49
	Within individuals	64	71.5	1.12				1.12	51
	Total	**98**	**1414.1**					**2.18**	**100**
21.8°C	Between periods	5	5515.8	1103.16					
	Between individuals	32	644.7	20.15	2.44	1.58	1.93	3.65	31
	Within individuals	86	709.8	8.25				8.25	69
	Total	**123**	**6870.3**					**11.90**	**100**
25.8°C	Between periods	3	4153.0	1384·33					
	Between individuals	34	810.5	23.84	1.86	1.57	1.90	3.67	22
	Within individuals	76	974 3	12.82				12,82	78
	Total	**113**	**5937.7**					**16.49**	**100**

Rana

8.9°C	Between periods	1	381.0	381.04					
	Between individuals	25	144.7	5.79	3.72	1.73	2.18	1.50	49
	Within individuals	49	76.2	1.55				1.55	51
	Total	**75**	**601 9**					**3.05**	**100**
12.5°C	Between periods	1	39.65	39.65					
	Between individuals	13	31.98	2.46	1.27	2.35	3.40	0.00	0
	Within individuals	17	32.80	1.93				2.18	100
	Total	**31**	**104.43**					**2.18**	**100**
17.1°C	Between periods	1	167.70	167.70	~				
	Between individuals	10	3.51	0.35	1.89	2.69	4.23	0.00	0
	Within individuals	32	21.23	0.66				0 66	100
	Total	**43**	**192.45**					**0.66**	**100**
21.9°C	Between periods	5	9470.2	1894.05					
	Between individuals	29	804.6	27.74	4.50	1.56	1.87	5.14	45
	Within individuals	112	689.8	6.16				6.16	55
	Total	**146**	**10964.6**					**11.30**	**100**
25.9°C	Between periods	5	25885.4	5177.07					
	Between individuals	36	1348.6	37.46	4.90	1.57	1.89	8.30	52
	Within individuals	109	832.7	7 64				7.64	48
	Total	**150**	**28066.6**					**15.94**	**100**

temperature 12°C both in *Bufo* and *Rana* the presence of individual differences cannot be seen. Whether this is indicative of a specific effect of this temperature, or is a mere fortuitous coincidence (due perhaps to a smaller number of nerves), cannot be answered from the present data.

To compare the effects of individual differences with those of the remaining causes (outside of controlled factors), acting within individuals, the estimated population variances of these two components have been calculated. The estimated population variances „within individuals" are given as the corresponding „quotient" in the table. The estimated population variances „between individuals" have been calculated by the formula

$$\frac{a}{b} \cdot \frac{c - b \cdot d}{e}$$

a — Number of individuals
b — d. f. for „between individuals"
c — „sum of squares" for „between individuals"
d — estimated population variance „within individuals"
e — number of nerves.

It can be seen that individual differences, where they are significant, are generally more pronounced in *Rana* than in *Bufo*. On the other hand, the factors „within individuals" are more pronounced in *Bufo* than in *Rana*. The total effect, however, of these two sources of variation is very much alike in both species with somewhat larger values in *Bufo*. The relative contribution of individual differences constitutes from 22% to 69% of the total variation due to uncontrolled factors.

From *a priori* considerations the variation within individuals can be broken up into further 3 components due to variation (1) between nerves T (tibial) and P (peroneal), (2) between sides s. (left) and d. (right), and (3) of the remaining causes („error"). To make a complete analysis of variance into these components, the data containing measures on all 4 nerves were needed. Therefore only a part of the material could be used for the purpose.

This analysis has brought out that differences between tibial and peroneal are significant only at temperature 22°C in toad. Tibial there regenerates better than peroneal. For frog these diferences come nearest to the significant value at the same level of temperature, being at other temperatures quite insignificant. The estimated

variance of this component in toad at 22°C is about 1/3 of the variance „within individuals".

The differences between right and left sides in toad are at all temperatures insignificant, while in frog they are significant only at 26°C, giving the estimated variance of this component equal to about 1/3 of the variance „within individuals". Since the order of sides to test in the experiment had not been assigned strictly at random, and because of a greater susceptibility of frog to operational shocks, the last result may have a purely technical explanation.

DISCUSSION

The rate of nerve regeneration in adult amphibians has not, as far as we are aware, been determined hitherto. It might be interesting to compare the results reported here with the data given by Harrison (1910) concerning the outgrowing of the nerve fibres in culture *in vitro* of embryonic nerve tissue of amphibians, and by Williams (1930), who observed regeneration of sensory skin nerves in the tail fin of a tadpole *in vivo*. Harrison recorded a rate from 16 to 56μ an hour, i. e. from 0.4 to 1.3 mm in 24 hours. He does not state the temperature, but if his cultures were kept at room temperature, the figures are similar to those obtained by us in adult animals.

Williams, however, worked at a temperature of 20°C and recorded a mean rate of 7.1 μ per hour. Taking into account the latent period, observed by the author but disregarded in his calculations, the average rate is 12.3 μ per hour, which makes 0.29 mm in 24 hours This is much lower than the rate obserwed in our present experiments at the same temperature. Speidel (1935) gives a still lower figure of 0.15 mm a day for nerves regenerating after section in the tail fin of the tadpole.

It seems that the influence of temperature on the rate of regeneration of the peripheral nerves has been examined directly only by Deineka (1908), who kept rabbits with regenerating nerves in a thermostat at 30°C and observed an advance of 6 — 8 days in comparison with regenerating control animals. As the original paper is not available, we cannot discuss his results in detail.

The results here reported prove that temperature has a strong influence on the rate of regeneration in amphibians. In the light of these results it can be thought that some of the fluctuations in the

rate of regeneration in mammals, such as the seasonal difference in the rate of regeneration of cornea nerves in rabbits observed by Koechlin and v. Muralt (1945), could be attributed to the influence of temperature. The surface of the cornea is in permanent contact with the air and hence is probably subject to wide fluctuations of temperature between summer and winter.

It is also possible that the findings of Sunderland (1947), who observed marked differences in the rate of regeneration of peripheral nerves in man at different levels at which the regeneration actually took place, and independence of these rates from the level of lesion, are due mainly to the existence of a gradient of temperature along the limbs. The author attributes the diminution of the rate of regeneration towards periphery to the increase of distance from the perikaryon. Figures given by Sunderland are: 3 mm in 24 hours in the upper part of the forearm and thigh, and a gradual decrease of the rate to 0.5 mm in 24 hours in the palm and the foot.

It is indubitable that the observed decreasing rate can be attributed at least partly to temperature falling towards the distal parts of the limbs. When the surrounding temperature is 22°C, the skin of the foot is about 27°C, and the skin of the palm about 30°C. (Du Bois, 1949). If we consider that feet and palms, in contrast to arms and thighs, have very few thermally isolating tissues such as fat and muscles, the fall of nerve temperature between the upper and lower levels can be estimated from 5° to 12°C. In the denervated limbs these fluctuations are likely to be still greater.

If we assume that the Q_{10} of regeneration in man is the same as in frog at approximately 22 — 26°C (7.3), the differences recorded by Sunderland can be entirely explained by the fall of temperature in proximo-distal direction.

It is possible, nevertheless, that the coefficient of temperature of nerve regeneration in man, within the limits of temperatures mentioned, is smaller, and does not entirely explain the observed differences. At any rate, it considerably limits the operation of other factors in the proximo-distal diminution of rates of regeneration reported by Sunderland.

In our experiments on amphibia, as well as in experiments on guinea pigs, rats (Zbrożyna, 1950), and rabbits (Konorski and Lubińska, 1945), the rate of regeneration is shown to be independent of the

distance from the perikaryon, and constant. But these are small animals, and such a factor as distance from the centres can possibly be more easily detected on the long nerves of man.

It would be of interest to determine whether the results here reported could be correlated in any way with influence of temperature on the properties of neurons, metabolic and functional. So far no striking analogy has been found, except perhaps for the spike height (Gasser, 1931), and injury potential (Bremer and Titeca, 1946). The temperature relations of these phenomena and of the rate of processes connected with the latent period of regeneration are somewhat similar.

Our experiments reported in this paper reveal the existence, in the initial stages of regeneration of peripheral nerves, of two distinct kinds of processes, presenting different thermic characteristics.

On the one hand there are processes underlying the latent period, and on the other hand — processes of elongation of the regenerating fibre along the peripheral stump.

One might be inclined to think that the latent period is connected with the phenomena occuring in the perikaryon after the axon amputation, and that a synthesis of a certain amount of new protoplasm is necessary to make fibres sprout, and, therefore, the influence of temperature on the duration of the latent period might be, to a cerain degree, a reflection of the effect of temperature on the synthetic processes taking place in the perikaryon.

But all changes observed in the perikaryon during the axon reaction, both morphological (Barr and Hamilton, 1948) and chemical (Bodian, 1947, Hyden, 1947), occur very slowly. The regressive phenomena develop during a period of from 2 to 4 weeks and later on, recovery is observed to be very slow and gradual. These results were obtained in mammals, and it is quite probable that in Poikilothermes the cycle of events connected with axon reaction takes place still more slowly. Since in the present experiment the nerve regeneration was investigated over a period not exceeding (as a rule) 4 weeks, we may assume that the initial phases of peripheral nerve regeneration occur to a certain degree autonomically, and that, at any rate, they are not closely connected with the whole of the processes described as axon reaction.

This conclusion is corroborated by a very interesting work of Gutmann and Sanders (1934), and Sanders (1948), who studied the

temporal evolution of diameters of regenerating axons both proximally and distally to the crush. From a calculation of the volume of the axon in the proximal part, and in the regenerating part of a rabbit nerve, these authors conclude that during the first 90 days after crushing the whole protoplasm of the regenerating part comes exclusively from the outflow from the proximal segment. Only later on, when the diameters of the fibres begin to expand both in the proximal and in the distal segment, does the protoplasm freshly synthetized in the perikaryon contribute to the volume of regenerating fibres.

We do not possess corresponding information concerning the evolution of diameters of regenerating fibres in amphibians. We have grounds for assuming, nevertheless, especially on account of the brevity of the examined periods, that the initial periods of regeneration are here also to a large extent determined by the events taking place in the axonal part of the neurone. Therefore it is in the properties of the axon itself that we should look for the elucidation of the processes involved in regeneration.

In summarizing we have to consider in the initial stages of regeneration after the nerve crush the following circumstances. Assuming with Weiss (1948) and others that in a normal neuron there is a constant movement of axoplasm in the proximo-distal direction, a crush of the axon gives rise to the following phenomena.

1. A new interface is created in the place where was a continuous phase of axoplasm.

In the experiments here recorded the crush produced a displacement of axoplasm (and of myelin) in both directions (central and peripheral), leaving the neural tube empty at a length of about 0.3 to 0.5 mm.

2. At this new interface the proximo-distal convection of axoplasm is arrested for days, and at low temperatures even for weeks.

The only property of this arrest which could be measured in our experiments is its duration. We know that the course of events resulting in the appearance of new fibres at the edge of peripheral stump develops very slowly. The necessary time is very long as compared with the time used to pass a similar segment of 0.3 — 0.5 mm in the peripheral trunk. Weiss thinks that the delay is due simply to the narrowing of neural tubes at the site of the crush impeding the advance of regenerating tips. But if it were so, the temperature

coefficient of slower progression along narrower path should be similar to that of the faster progression along undisturbed peripheral stump. Since temperature relations of the latent period and of peripheral advance are so markedly different, we have to admit that distinct kinds of processes occur during these two stages of regeneration. The mere crossing of the scar occupies a relatively small part of the latent period, its bulk being determined probably by preparatory events determining the sprouting of new fibres. It is impossible as yet to say whether these events are linked simply with the overcoming of new interface, or whether they are connected with the

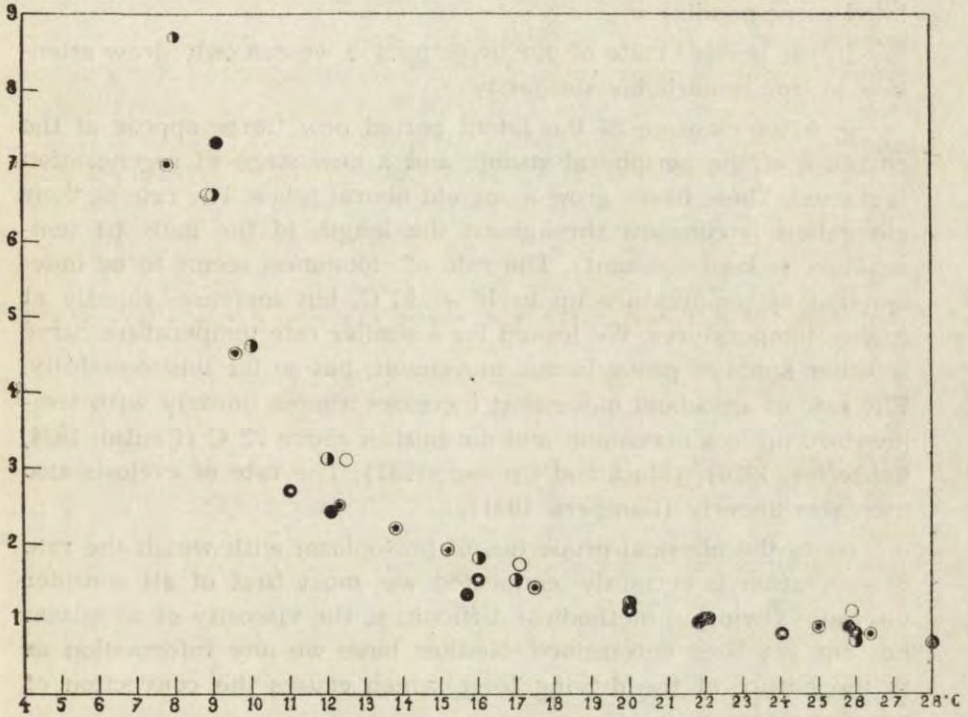

Fig. 4. Comparison of the latent period of regeneration of peripheral nerves and of the duration of cellular division at various temperatures. Abscissae — temperature in ° Centigrade. Light circles — latent period of regeneration in *Rana*. Heavy circles — duration of cellular division in *Amoeba*. Half-filled circles — latent period of fertilization of sea-urchin egg. Black circles — latent period of regeneration in *Bufo*. Encircled dots — duration of cellular division in a Ciliate. For explanation see the text.

reaction of the neurone to such a powerful stimulus as the crush. In any case it is worthwile to point out to the striking resemblance between the behaviour of the latent period of regeneration and duration of processes of cellular division at different temperatures. We represented in Fig. 4 the temperature ralations of the time elapsing between insemination and the first segmentation of sea- urchin Arbacia- egg (Loeb, 1915), the duration of division in *Amoeba* (Daniel and Chalkley, 1932), the duration of division in a *Ciliate* (Phelps, 1946), and the latent periods of regeneration in frog and toad. To facilitate the comparison, the duration of each phenomenon at 22°C was taken as unity and the original data for other temperatures were recalculated correspondingly.

In the present state of our investigation we can only draw attention to this remarkable similarity.

3. After elapsing of the latent period new fibres appear at the entrance of the peripheral stump, and a new stage of regeneration is started. These fibres grow along old neural tubes. The rate of their elongation is constant throughout the length of the path (if temperature is kept constant). The rate of elongation seems to be independent of temperature up to 16 — 17°C, but increases rapidly at higher temperatures. We looked for a similar rate temperature curve in other kinds of protoplasmic movement, but so far unsuccessfully. The rate of amoeboid movement increases almost linearly with temperature up to a maximum and diminishes above 22°C (Pantin, 1924, Schaeffer, 1926), (Mast and Prosser, 1932). The rate of cyclosis also increases linearly (Lambers, 1931).

As to the physical properties of protoplasm with which the rate of elongation is certainly connected we must first of all consider viscosity. Owing to methodical difficulties, the viscosity of axoplasm has not yet been determined. Neither have we any information as to the nature of the driving force which causes the convection of axoplasm.

The viscosity of other kinds of protoplasm investigated at various temperatures behaves differently in different cells, and is even variously estimated by different authors in the same objects. In general it decreases with temperature within range of temperatures similar to that used in the present experiments. Some authors found, however, a maximum viscosity at about 15°C (Heilbrunn, 1927 and 1943). Almost all workers record a considerable decrease in viscosity

of protoplasm in the interval between 8°C and 17°C (Costello, 1934, Fauré-Fremiet, 1913, Murphy, 1940). But judging from the reported regeneration rates, the viscosity of axoplasm should not present considerable variation in this range.

SUMMARY

1. The rate of regeneration of amphibian peripheral nerves was studied during the early stages after the crush (up to 30 days). The temperature range was from 9° to 26°C.

2. The regeneration was notably accelerated by the rise of temperature within the studied range.

3. As the crushed nerves were allowed to regenerate at various constant temperatures, it appeared that the latent period on one hand, and the rate of regeneration along the peripheral nerve on the other reacted very differently to temperature changes. It is assumed, therefore, that different processes underlie these two phases.

4. The latent period diminishes with increasing temperature. The curve falls rapidly at low temperatures, then the slope becomes less abrupt, and beyond 22°C the decrease of latent period is insignificant. It is noteworthy that the temperature relations of the latent period of nerve regeneration are almost identical with those of the latent period of fertilization in sea-urchin eggs, and with duration of cellular division in *Protozoa*.

5. The rate of regeneration along the peripheral path shows very small changes between 8° and 16° — 17°C. At higher temperatures the rate of regeneration increases rapidly. At constant temperatures the rate of regeneration is constant at all levels of the nerve up to the terminal branches.

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ON THE RATE OF REGENERATION OF THE SCIATIC NERVE IN WHITE MOUSE.

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The problem of regeneration of the peripheral nerve in amphibians and in mammals has received considerable attention. The investigation was generally started some days after the moment of the cutting or crushing. The earlier period attracted only very slight interest.

The present paper seeks to make a detailed investigation of the rate of elongation of the regenerating sciatic nerve in white mouse, with special reference to the initial period, covering from a few hours until three days after the moment of crushing.

The results obtained point to the fact that the phenomenon of regeneration can be observed even a few hours after the crush. The rate of elongation, very slight at first, increases gradually and achieves an approximately constant value after from 4 — 5 days. Statistical analysis of the results enables us to state the curvilinear regression.

MATERIAL AND METHODS

Experiments were performed on over 100 mice, their mean weight being about 18 g. Some specimens weighed 10 — 11 g. as well as more than 25 g. We endeavoured to ensure that the animals had the same

living conditions throughout the duration of the experiments. These conditions were similar to the normal manner in which other laboratory animals live. The mice were fed on a grain mixture; every few days they were given a small quantity of milk.

The investigations were performed according to the method of L. Lubińska (1950). This is as follows:

Every mouse was operated under aseptic conditions in ether anaesthesia. During this operation the sciatic nerve was crushed a little distally to trochanter major, by means of a strong silk thread. A thin glass rod was introduced between the knot and the nerve to facilitate the removal of the thread after the crushing. If the knot of the thread is pulled tight enough the crushed part of the axon is destroyed, and the neural tube preserved. The fibre, when regenerating later, can move along the old path.

If the length of regeneration was to be tested after several days (more than three) the site of the crush was marked by a fine black silk, threaded through the nerve and loosely tied. Thus the scar was easily found later.

Both the sciatic nerves vere crushed.

After the designate lapse of time (from a few hours to nine days), the mouse operated on is put under the somnifene anaesthesia (intraperitoneal injection of somnifene-„Roche"). On account of the small weight of the mouse, the dosage of narcosis is very difficult. In general 0.01 ccm of somnifene was given. Persistence of reflexes — on pricking the skin for instance — was the proof that the narcosis was adequate and not too deep. When the sleep was too deep and when there were no reflexes, the experiment was considered a failure, and the results of it were not taken into consideration.

After the injection, the mouse is reopened and the sciatic and tibial nerve are isolated. After being cut from the periphery the end of the nerve is held by a forceps and suspended in such a manner, that the entire legth of the nerve including the scar hangs loosely in the air. Then, with a second fine forceps the nerve is pinched successively at short segment (0,3mm), moving from the periphery towards the centre; care must be taken not to pull on the nerve — if that is done, the part of the nerve situated above the scar might be stimulated and make the result uncertain. The first reaction, obtained by pinching is as a rule small: the subsequent pinching towards the centre gives increasingly strong reflexes. The nerve is cut in that place in which the pinching evokes the first clear reflex reaction. We must now cut the nerve above the scar, place it on a slide and measure, by slipping a gauge underneath, the distance between the level at which the first reaction was evoked and the scar.

The accuracy of measurements was to 0.5 mm. In the case of very slight distances from the place of crush, the measurements were made to 0.25 mm. approximately, in such a way that, for instance the interval found to be between 0.5 mm and 1 mm was recorded as 0.7 mm. In the

initial period of regeneration (first 48 hours) special care is needed, on account of the proximity of the scar, to get exact data. Not only is a very fine forceps required here, but also a certain precision of movements.

RESULTS

Over 100 mice were treated in the manner described above. But not all the operations were successful; sometimes, for instance, the nerve, instead of being crushed, was torn; sometimes the crush was not complete, some fibres remaining intact. They were detected by elicitation of reflexes from the most distal part of the fibre; sometimes the mice died (for various reasons) between the operation and the test. Finally, results from 168 regenerating nerves in 106 animals were obtained.

Gathered in the table I are figures showing the length of the regenerating nerve segment in millimetres at different time intervals after the operation. Such indications as 1, 2, 3, 4 days and so on are to be understood as the lst, 2nd, 3d, 4th etc. 24 hours plus a few hours (from 0 to 6). In the majority of cases, the tests were performed after the completion of the period of 24 hours plus 2 — 4 hours.

The curve of the fig. 1 illustrates the results obtained.

As is seen from the graph, the line joining the mean length of the regenerating nerve segments is not a straight line. The initial segment does not have a regular course: within 6 — 8 hours after the crushing of the nerve, reflex reactions were evoked by pinching the nerve with a fine forceps at a distance of 0.5 mm below the crush. After from 18 — 20 hours, the regenerated segment are already of about 1 mm; the results obtained after 24 hours are not, as a rule, higher. It is possible that a relatively too small number of measurements accounts for the fact that marked differences in the lengths of the regenerating segment in these short periods are not observed.

After two days, the regenerating segment is generally about 1.8 mm long. We do not know whether a small decrease of rate of elongation has already occured here, or whether it is caused by some technical reason.

After two days, a new period in the course of regeneration is begun: that is, the rate of elongation of the nerve is doubled and becomes 2 mm a day. This period lasts for more or less three days. Then a rate of 3 mm a day is reached, and this lasts for 8 days.

Table I.

Mean length of regenerating nerve fibres in millimetres at different time intervals after the crushing.

Time (days) after the crushing of nerve	0,25	0,8	1	2	3	4	5	6	7	8	9
Number of nerves	6	12	23	20	23	14	18	16	13	14	9
Length of regenerating nerve fibres in mm. (Mean value)	0,5	1,0	1,0	1,8	4,0	6,0	8,3	11,8	14,7	17,3	19,1
Range	0,3-0,7	0,7-1,3	0,5-1,3	1-2,5	2-6	4-7,5	5-12,5	10-16	7-23	11,5-22	15,5-23

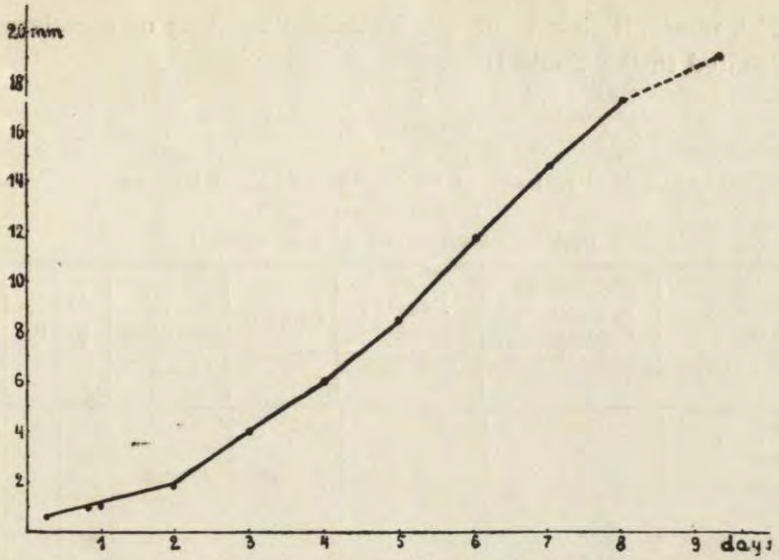

Fig. 1. The rate of regeneration of the sciatic nerve in white mouse. Abscissa: days after crushing of the nerve. Ordinate: length of regenerating nerve-fibres in mm.

The regenerating tibial nerve reaches, after a period of from 7—9 days the sole; in the region of the heel, the tibial nerve splits into thin branches, which makes it difficult to ascertain the end of the regenerating fibre. When such a thin branch was tested, a distinct reaction was sometimes evoked, duly increasing as a result of a gradual pinching of the nerve centralwards; but sometimes, when an absence of reflex was noted from the thin nerve branches, suddenly, with no normal gradation, a strong reaction was evoked by pinching the nerve in the place where the branches united into a thick trunk near the sole. This would indicate that in the individual nerve branches the regenerating fibres already exist, but their number is too small to evoke the reaction of the animal. And it is only when several such branches unite in a common trunk and are pinched simultaneously that the reflex appears. Our method fails in these circumstances.

The results obtained for the ninth day of the regeneration are, therefore, not sufficiently certain, and so this apparent decrease in the rate of the process is subject to some reservations. Therefore the corresponding part of the curve is drawn in the fig. 1 by a dotted line.

The numeral results of the statistical analysis of our data are represented in the Table II.

Table II.

Analysis of variance of regeneration of the sciatic nerve in white mouse.
Test for non-linearity of regression.*)

Variation	Number of Parameters, Columns, and Observations	Degrees of Freedom	Sums of Squares	Quotients	F	$F_{.05}$	$F_{.01}$
Linear regression	2	1	4517,06	4517,06			
Deviations of means of columns from linear regression	10	8	89,72	11,22	3,21	2,02	2,67
Residual within columns	125	115	401,99	3,50			
Total	—	124	5008,77	—	—	—	—

*) The period of 0,25 days was not included. If included, it would make the non-linearity of regression still more significant.

DISCUSSION

The experiments reported here show that several hours after the crush the process of regeneration has started. This conclusion is based on the fact that pinching of the nerve about 1 mm below the crush gives rise to reflex after such time interval. The rate of this process, at first very low, increases in the subsequent days, reaching after 4 or 5 days a maximum and more or less constant rate of about 3 mm in 24 hours.

The fact that the reflexes are already evoked within a period of less than 20 hours after the crush seems to be of great interest. It

means that the processes of the outflowing of axoplasm from the central part of the fibre through the crushed place to the distal part of the nerve start almost immediately after the crush. We cannot affirm, however, that such early start occurs in all fibres of the nerve.

It is possible that the mechanism of regeneration in these early periods is different from that of subsequent phase of elongation.

SUMMARY

The rate of regeneration of the sciatic nerve in white mouse was investigated from a period of 6 hours to ninth days after the crushing of the nerve trunk in the region of the hip. The investigations were made on 100 animals at different time intervals. It was proved that the rate of regeneration, during the first two days is of about 1 mm in 24 hours and increases, reaching a maximum of 3 mm in 24 hours between the 4th and 5th day. This rate remains constant up to 8 days. The statistical analysis of the rate of the regeneration of the sciatic nerve points to a curvilinear regression.

I should like to express my thanks to Dr L. Lubińska for much helpful advice. I should like also to thank Prof. M. Olekiewicz for the statistical analysis of the results obtained.

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CHRONIC EXTINCTION AND RESTORATION OF CONDITIONED REFLEXES.

I. EXTINCTION AGAINST THE EXCITATORY BACKGROUND.

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1. INTRODUCTION

The present paper is devoted to the detailed study of processes which occur in the course of elaboration of inhibitory conditioned reflexes and their transformation back into active conditioned reflexes.

It is well known that the investigations of internal inhibition have been chiefly concerned either with acute inhibition, which constituted the extinction of conditioned reflexes, or with the chronic inhibition, which constituted differentiation in the broadest sense of the word.

The production of an acute extinction is inappropriate for our purpose because it involves changes in the excitability of the unconditioned centre, and it strongly disturbs the normal course of the conditioned reflex experiment. On the other hand, the elaboration of a differential inhibition by the non-reinforcement of a stimulus similar to the conditioned stimulus, which is reinforced, has the defect of depending on the degree of similarity of both the stimuli and therefore its course cannot be considered as „standard".

For the above reasons we decided to study such a form of internal inhibition as would not possess any of the defects specified. This form is the chronic extinction of a conditioned reflex. For, firstly, when we apply an extinguished stimulus once or twice amongst positive stimuli during the experimental session, we do not disturb the normal course of the experiment and we can examine accurately the gradual process of the transformation of this stimulus into the inhibitory stimulus. Secondly, the course of the elaboration of an inhibitory reflex is in this case not disturbed by the circumstance that the stimulus more or less similar to the inhibitory stimulus is reinforced. Thirdly, the starting point of the elaboration of the inhibitory reflex is strictly determined: we know both strength of the conditioned reflex to be extinguished and the degree of its fixation. The same applies to the reverse process, i. e. the restoration of the chronically extinguished stimulus by its application with the reinforcement among other stimuli.

The most important results of this paper can be summarised as follows: while the chronic extinction is a very slow process and its rate depends on the character of the stimulus and the degree of its fixation, the restoration of the inhibited reflex is a rapid process, which occurs almost from day to day and is practically the same for all stimuli.

In addition to these experiments the repeated extinction and restoration of one and the same stimulus was tested. Interesting results which were here obtained require further examination.

2. METHOD

In our experiments, the classic method used in Pavlov's school for the investigation of alimentary conditioned reflexes was applied (Podkopaiev 1936).

In order to make the results more exact, we introduced a modification, which consisted in replacing air by water in the system of tubes leading from the salivary fistula to the manometer (Kozak 1950). In all this system only lead tubes and hard rubber tubes were used in order to minimise in the results falsification caused by the movements of the animal's head.

Dogs used in these experiments were fed once a day between 4 and 5 p. m. The experiments were carried out every day (except holidays) between 8 and 11 a. m. Experimental sessions lasted about 20-40 minutes.

As conditioned stimuli, various auditory and visual stimuli were applied. The order of the application of stimuli was not constant, the intervals between trials were usually 4 — 7 minutes. The duration of the isolated period of conditioned stimuli was in all experiments (except in those at the beginning of the training) 20 seconds. The food was given in small bowls presented to the dog by means of a mechanical device. The food consisted of dried minced bread, moistened in regular, constant quantities with broth.

3. RESULTS

Two dogs (mongrels) were used in the experiments; their weight was from 10 — 12 kg, their age — 2 — 3 years. One of them„Bobik" was very lively and voracious; his conditioned reflexes were considerable and regular. The other dog, „Cygan", was less excitable, his conditioned reflexes were not so regular and tended to diminish towards the end of the experiment.

In both these dogs, conditioned reflexes to the following stimuli were established: a beat of a metronome, the ringing of a bell (strong stimuli), the sound of a buzzer, bubbling in water, a whistle (weaker stimuli), and an electric lamp (the weakest stimulus).

A. THE COURSE OF CHRONIC EXTINCTION OF ACTIVE CONDITIONED REFLEXES

The chronic extinction of various stimuli was carried out on „Bobik". It was conducted always in more or less the same standard way. In every series of experiments only one stimulus was subjected, by applying it without reinforcement among other stimuli usually on the third or fourth place, to extinction. At the beginning of the first series, the stimulus subjected to extinction was applied twice during each experiment; afterwards we found it more practical to apply it only once. To illustrate the course of an experiment we adduce one of our protocols (Table I).

After each series with extinction another series with the restoration of the extinct stimulus followed, and only when the restoration was fully accomplished a new series with the extinction was conducted.

Table I.

A typical course of experiment with chronic extinction.

„*Bobik*" 12 th March 1949, Nr 228.
(The seventh day of extinction of lamp).

Nr of trial	Time in min.	Conditional stimulus	Salivary conditioned reaction			Rein-forcement	Unconditioned reaction	
			first 10 sec.	second 10 sec.	total		first 10 sec.	subsequent 20 sec.
1	1	Bell	14	21	35	+	37	73
2	6	Metronome	18	20	38	+	42	73
3	11	Lamp	14	8	22	—	4-6-5-1 (every 10 sec)	
4	16	Metronome	12	18	30	+	35	75
5	20	Bell	18	25	43	+	40	72

The following series of extinctions were performed („Bobik"):

13th April 1948 — 3rd May 1948, the first extinction of the metronome,
7th September 1948 — 5th October 1948, the second extinction of the metronome,
23rd October 1948 — 18th January 1949, the third extinction of the metronome,
3rd March 1949 — 11th March 1949, the extinction of the lamp (a weak stimulus firmly established),
7th April 1949 — 11th May 1949, the extinction of the whistle (a fresh strong stimulus),
13th June 1949 — 6th October 1949, the extinction of the bell (a strong stimulus firmly established).

The course of extinction of various stimuli is represented in figs 1 and 2. These graphs were constructed in the following way: each curve represents the course of extinction of the conditioned reflex to a particular stimulus. From every three successive experiments the mean magnitude of the conditioned reflex to the stimulus, which was being extinguished, and the mean value of the conditioned reflex to a strong positive conditioned stimulus were taken. (As a standard strong stimulus we took the bell, and in experiments with the extinction of the bell — the metronome). Then the first of these values was taken as a percentage of the second. Since the magnitude of conditioned responses during the first 10 seconds of the isolated period of a conditioned stimulus fluctuated in accordance with the

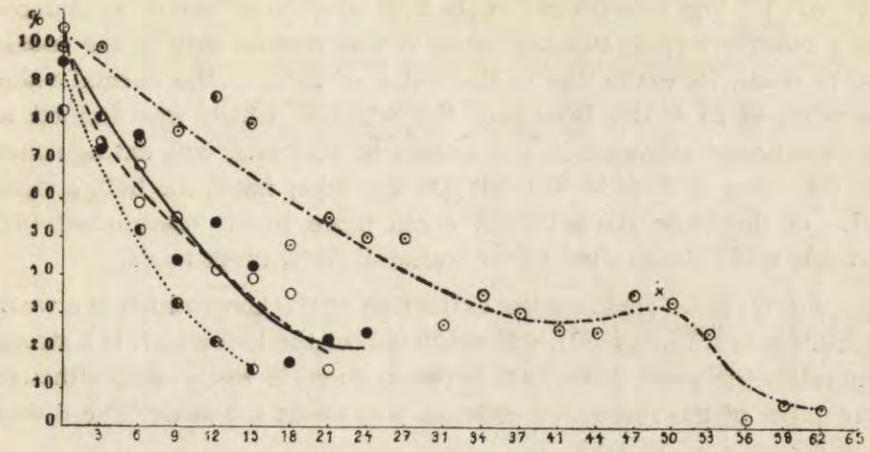

Fig. 1. Chronic extinction of conditioned reflexes to metronome, whistle, bell and lamp in „Bobik".

Abscissae: successive days of experiments.

Ordinates: magnitude of reflexes as a percentage of standard positive reflex.

———————— metronome
— — — — — — — whistle
— . — . — . — . — bell
. . . . . lamp
x, reinforcement given in error.

length of the intervals and with the spontaneous salivation just before the trials and so on, we used for the construction of the curves only the values of the responses to the second 10 seconds of the isolated period of a conditioned stimulus; these values were much more stable. Yet it should be mentioned that the curves constructed on the basis of all the values of the conditioned reflexes (during 20 seconds) did not differ notably from the curves represented here. The values of the standard conditioned reflex to the first stimulus in an experiment, and to a stimulus applied just after an inhibitory stimulus were not taken into account, as they were irregular and somewhat diminished.

As is seen from fig. 1 there are great differences in the rate of chronic extinction of various stimuli: 1° the extinction of firmly established conditioned stimuli is slower than that of fresh stimuli; 2° so called strong stimuli are extinguished more slowly than weak stimuli.

Ad 1°: The metronome in its first extinction can be considered as a relatively fresh stimulus since it was trained only in the course of 74 trials. Its extinction to the value of 20% of the normal value occured in 23 trials. Similarly the whistle, which was trained as a conditioned stimulus in the course of 16 trials, was extinguished to the value of 20% in 20 trials. On the other hand, the bell, a stimulus of the same strength but much more firmly established (667 trials), was extinguished to the value of 20% in 42 trials.

Ad. 2°: If we compare the extinction of the lamp which is a weak conditioned stimulus with the extinction of the bell which is a strong stimulus, the great difference between them is easily seen although the „age" of the respective reflexes was about the same. These data are represented in table II.

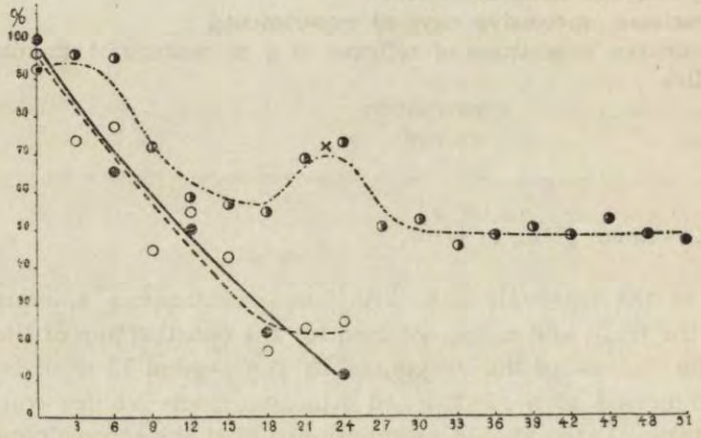

Fig. 2. Multiple chronic extinction of conditioned reflex to metronome in „Bobik".

Abscissae and ordinates as in fig. 1.

——————— the first extinction

— — — — — — — — the second extinction

— · — · — · — · — · — the third extinction.

x, as in fig. 1.

The process of the multiple extinction of the metronome deserves special consideration. As is seen from fig. 2, in the first extinction the reflex to the metronome was extinct to 20% after 23 trials.

Afterwards this stimulus was reinforced only in the course of 10 trials and then it was re-extinguished.*) This time the extinction to 20% occurred also in 23 trials. After the second extinction, the stimulus was again applied with reinforcement 10 times and then re-extinguished again. This time it was impossible in spite of 60 trials to extinguish the metronome below the value of 50% of the value of the bell.

Table II.

The number of trial in which a conditioned stimulus was extinguished to 20% of a standard value.

	Metronome	Lamp	Bell	Whistle
The number of reinforced trials before extinction	74	495	667	16
The number of unreinforced trials till 20% of a standard value achieved	23	14	42	20

How much a single reinforcement of an extinguished stimulus can disturb the course of extinction is seen from the „hump" in the third curve of extinction (x). This hump was caused by one reinforcement given in error. Its effects lasted for five days and only then did the values of the reflex return to the previous level (50% of the bell). A similar hump caused in the same way is seen in fig. 1. (x).

B. THE RESTORATION OF THE EXTINGUISHED CONDITIONED REFLEXES

When the inhibitory conditioned reflex to a given stimulus was established, we changed the experimental procedure in such a way that the inhibitory stimulus was again reinforced by the presentation of food. The first restoration of the conditioned reflex to the metronome both in „Cygan" and „Bobik" was conducted rather „acutely", i. e. we applied the metronome with reinforcement several times during an experimental session. In the following series it ap-

*) It is worth mentioning that after 10 reinforcements there was a 53 days interval in experiments. After this interval the reflex to the metronome was fully preserved.

peared that it is more correct to employ „chronic" restoration of the extinct reflex (by applying the restored stimulus once per session), i. e. to act exactly in the same way as in the case of chronic extinction.

Appended are protocols of the experiments on „Bobik" and „Cygan", representing „acute" restoration of the conditioned reflex to the metronome (Table III and IV).

Table III.

The course of acute restoration of metronome.

"Cygan," 6 th July 1948, Nr. 120.

Nr. of trials	Time in min.	Conditioned stimulus	Salivary conditioned reaction			Reinforcement	Unconditioned reaction	
			first 10 sec	second 10 sec	total		first 10 sec	subsequent 20 sec
1	3	Lamp	5	9	14	+	33	71
2	8	Bell	13	15	28	+	41	78
3	13	Metronome	1	4	5	+	33	80
4	18	Metronome	3	7	10	+	33	80
5	24	Metronome	3	11	14	+	40	79
6	29	Metronome	1	9	10	+	38	78
7	34	Metronome	1	3	4	+	40	73
8	39	Bell	9	18	27	+	44	75

"Cygan," 8 th July 1948, Nr. 122.

1	2	Lamp	8	10	18	+	33	77
2	7	Bell	19	27	46	+	40	78
3	12	Metronome	15	22	37	+	47	71
4	17	Metronome	11	14	25	+	44	83
5	22	Metronome	7	21	28	+	47	79
6	27	Metronome	5	11	16	+	50	79
7	32	Metronome	9	21	30	+	45	83
8	36	Bell	11	25	36	+	41	84

Consider first the protocols concerning „Cygan". We see that in the first experiment with the reinforcement of the metronome (Nr 120) the reflex to this stimulus behaves in a peculiar way: at the beginning, it increases (5 — 10 — 14) and achieves a half value of the reflex to the bell, then it falls rapidly (14 — 10 — 4). That this fall concerns only the stimulus just restored is seen from the fact that to apply the bell after it gives a normal effect. In the experiment Nr 121, the metronome was not applied, while in the experi-

ment Nr 122 this stimulus produced at once very great effect (37), fluctuating in successive trials (25 — 28 — 16 — 30)

Table IV.

The course of acute restoration of metronome

„Bobik", 13 th July 1948, Nr 105.

Nr of trial	Time in min	Conditioned stimulus	Salivary conditioned reaction			Rein-forcement	Unconditioned reaction	
			first 10 sec.	second 10 sec.	total		first 10 sec,	Subsequent 20 sec.
	1	Lamp	10	12	22	+	34	64
	6	Bell	21	26	47	+	39	72
	10	Lamp	11	16	27	+	39	74
4	14	Metronome	4	1	5	+	34	76
5	18	Metronome	5	10	15	+	38	75
6	22	Metronome	7	7	14	+	39	78
7	27	Metronome	8	11	19	+	38	79
8	32	Bell	16	17	34	+	38	69

„Bobik", 14 th July 1948, Nr 106.

1	1	Lamp	15	16	31	+	39	75
2	6	Bell	21	25	46	+	40	76
3	11	Lamp	15	22	37	+	40	78
4	15	Metronome	15	24	39	+	40	79
5	19	Metronome	16	22	38	+	40	81
6	23	Bell	16	24	40	+	40	80
7	28	Metronome	10	20	30	+	41	80
8	33	Lamp	10	12	22	+	38	82

The course of the restoration of the reflex to the metronome in „Bobik" was very similar. In the first day of restoration the reflex increases and stops on the value of about 50% of the bell (5 — 15 — 14 — 19) without subsequent fall as in the case of „Cygan". The next day, the reflex to the metronome is at once fully restored being almost equal to the reflex to the bell.

So we see that the acute restoration of a conditioned reflex is interrupted by another process, opposite to that of restoration, which prevents the reflex from appearing to its full extent. The analysis of this second process will be given later; here we shall note that it was its existance which urged us to apply only chronic restoration in the following series of experiments.

To conclude the considerations of the protocols adduced, it is worth mentioning that in both dogs the unconditioned reflex produced by the first reinforcement of the metronome was slightly diminished.

Table V.

The restoration of extinguished conditioned reflexes in *„Bobik".*
The value of the second 10 sec. of the stimulus as a percentage of standard stimulus.

Successive days of exper.	Metronome (second restoration)	Lamp	Whistle	Bell	Means
0	20	23	17	0	
1	58	63	47	65	58
2	83	110	60	90	86
3	88	96	80	90	89
4	90	91	90	100	93
5	96	100	80	68	86
6	94	85	91	130	100
7	95	100	91	110	99
8	95		100		98

The results of the chronic restoration of reflex in „Bobik" are represented in Table V. As this table shows, in all cases of the restoration, the conditioned reflex grew rapidly from day to day. While immediately before restoration the extinct reflex amounted on the average to 20% of the reflex to a strong stimulus (with the exception of the metronome extinguished for the third time which was inhibited only to 46%), a single reinforcement was sufficient to increase the reflex to 58%, and the second reinforcement — to 86%.

In the subsequent few days the reflex reached its normal value. And so the process of restoration of a conditioned reflex occurs much more rapidly than the process of its suppression; it seems as if the dog throughout the period of extinction were expecting the moment when the stimulus would be reinforced, and reacted to this change immediately.

C. THE INFLUENCE OF THE ALIMENTARY EXCITABILITY ON THE MAGNITUDE OF THE EXTINGUISHED AND RESTORED CONDITIONED REFLEXES

In a few experiments we examined the magnitude of conditioned reflexes in the course of extinction and restoration against the background of the diminished alimentary excitability, which was caused by feeding the animal before the experiment.

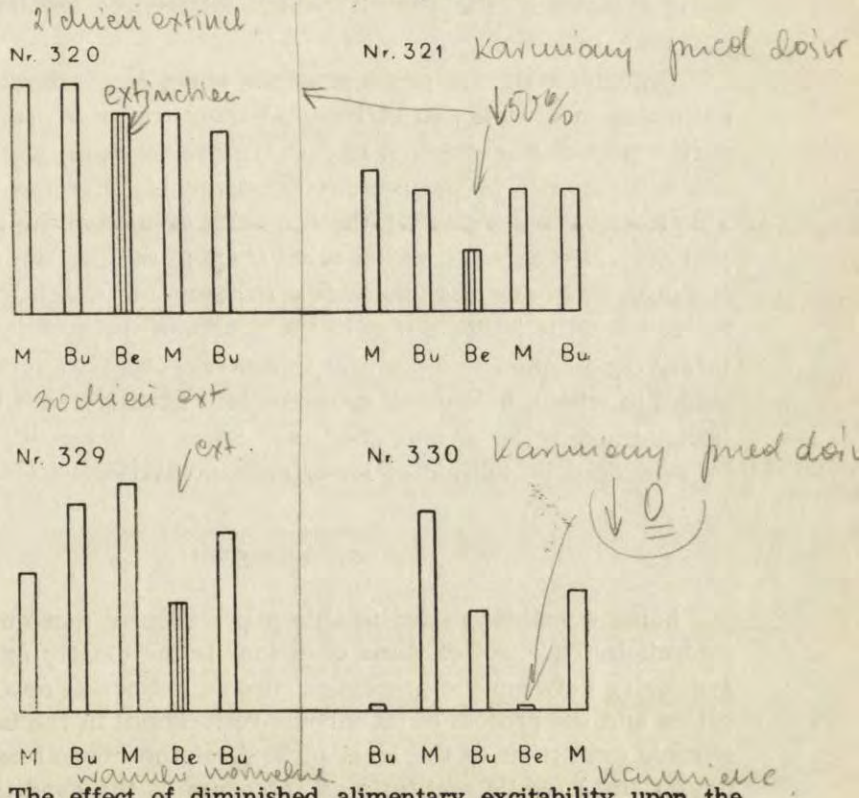

Fig. 3. The effect of diminished alimentary excitability upon the magnitude of the reflex in the course of extinction (M — metronome, Bu — bubbling, Be — bell).

As examples, let us cite experiments in which the effect of the diminished excitability on the reflex in the course of extinction was examined.

Appended are two successive protocols from the series of experiments in which the reflex to the bell was being extinguished (fig. 3). In the experiment Nr 320 (21st day of extinction) the reflex to the bell was still equal to other reflexes, while in the next experiment, when the alimentary excitability was diminished, it amounted only to a half of their value. Similarly, in the experiment Nr 329 (30th day of extinction) the reflex to the bell still amounted to more than a half of the reflex to the metronome, while in the experiment Nr 330, against the background of the diminished alimentary excitability, the bell had a nil effect. It can be said that the diminution in alimentary excitability makes clear the differences between various states of the stimuli, which differences are not visible in „normal" condition.

Unfortunately, the problem of the effect of the diminished excitability upon conditioned reflexes in their transitory stages (i. e. in earlier periods of extinction or just after restoration) was not worked out satisfactorily in the present experiments. Therefore we are not able to say at what stage in the course of extinction the phenomenon just described appears, and at what stage in the course of restoration it vanishes. In any case those few experiments which we have performed seem to indicate that in the course of transformation of excitatory conditioned reflexes into inhibitory, and vice versa, there are stages in which, in spite of apparent lack of differences between the transformed reflexes and „normal" reflexes, these differences can be evidenced by the aid of lowering alimentary excitability.

4. DISCUSSION

Some questions raised in this paper require more detailed consideration. The first of these questions is the clearly expressed dissymmetry between the process of chronic extinction of a conditioned reflex and the process of its chronic restoration. In the best case, the chronic extinction to the level of 20% of the normal value lasts 14 days (lamp), while *restitutio ad integrum* of the conditioned reflex with its re-reinforcement lasts in all cases 3 — 4 days.

The problem arises as to what may be the cause of this dissymmetry. First, it is clear that it does not depend (or it depends only to an insignificant degree) on the relation between the duration of the excitatory and inhibitory training of given stimulus. The best proof of this is the extinction of the whistle. This stimulus was trained as an active conditioned stimulus for only 8 days, while as an inhibitory stimulus it was trained for 24 days, and nevertheless, as soon as this stimulus was again reinforced, its return to the active state was almost instantaneous. And so the cause of the phenomenon in question must be sought elsewhere.

The following factors can here be taken into account: 1°. The strength of the reinforcing unconditioned stimulus. It is commonly accepted that the stronger the reinforcing stimulus, the easier and more rapid is the process of elaboration and the slower the extinction of the conditioned reflex. In our experiments, the reinforcing stimulus was undoubtedly very strong. The dog came to our experiments after a day's starvation, his alimentary excitability was very high, and he received very tasty and attractive food.

2°. Our manner of conducting experiments. Our experiments were always carried out against a strong „excitatory" background. During an experimental session, each dog was given several excitatory conditioned stimuli and only one or two inhibitory stimuli. (This was done in order to keep excitatory conditioned reflexes on a high level). When, afterwards, the inhibitory stimulus was transformed into excitatory, all the trials in the experiments became reinforced. So it is possible that it is much more difficult for an animal to extinguish a single unreinforced stimulus given among other stimuli which are reinforced, than to „equalize" the so far unreinforced stimulus with other stimuli.

As far as the rate of extinction of various stimuli is concerned, our data are similar to those, obtained by other authors (Babkin 1904, Fedorov 1949, Jaroslavceva 1940, Solovieitchic 1940, Stroganov 1929) in conditions of acute extinction. Reflexes to strong stimuli and to firmly established stimuli are extinguished more slowly than reflexes to weaker or less established stimuli. As a new fact there must be considered the unusual difficulty of the extinction in the case when a given stimulus was several times transformed from excitatory to inhibitory and vice versa. We found that in this case the reflex can be extinguished only to a certain degree (in our experiments about 50% of the normal value). It seems as if the stimulus

were unable to become wholly inhibitory. This important fact requires further investigation.

As to the restoration of the extinguished conditioned reflexes, it was impossible, because of the great rapidity of restoration, to grasp whether this process depends on the strength and the fixation of the inhibitory reflex or not. In our experiments the restoration occurred at the same rate after both a short and a long inhibitory training. It is possible that if the training lasted many months the restoration of the reflex would be slower.

There remains the problem whether or not our experimental data can be considered as a direct and accurate indication of the learning process, both in the case of elaboration of inhibitory reflex and its transformation into the excitatory reflex. Our experiments with a diminution of the alimentary excitability seem to show that they cannot. The fact that during the extinction of a reflex there is a stage when its value is, under normal conditions, not diminished, but the diminution can be manifested with the decrease of alimentary excitability, seem to indicate that in the „structure" of the reflex some changes occurred which could not be manifested against the background of high alimentary excitability. On the contrary, if, during the transformation of an inhibitory reflex into the excitatory one, there is a stage when in „normal" conditions the magnitude of the reflex seems to be equal to the normal reflexes but with the diminished alimentary excitability it is lower, this fact again indicates that the „structure" of the reflex was not fully identical with the structure of the other reflexes.

And so the important question arises whether the dissymmetry of the two observed processes (that of inhibition and that of restoration) is not purely apparent and caused only by the excitatory background of our „normal" experiments. We hope to be able to answer this question in following papers.

For the problem concerning the mechanism of the process of restoration of conditioned reflexes those experiments in which the reflex was restored „acutely" in one experimental session seem to be significant. We stated that the reinforcement several times in succession of the extinguished stimulus does not produce the same growth of the reflex as is seen when the stimulus is applied only once daily; in „Cygan" instead of an increase a subsequent diminution of the reflex then occurred, but the next day the reflex to this stimulus became fully restored.

A comparison with our own experience comes to mind, when we learn some text in the evening and although we do not apparently succeed in memorising it, the next day we find that it is wholly assimilated.

How can this fact be explained? It seems that two alternative explanations can be offered. On the one hand, as we pointed out earlier, the diminution of alimentary excitability makes more manifest the difference between a „fresh" and an „old" conditioned stimulus. As at the end of an experimental session, the alimentary excitability may be lowered, this may be the cause of the low value of the freshly restored reflex. On the other hand, if we assume that the restoration of a conditioned reflex is due to the establishment of new interneural connections between the conditioned and unconditioned centre, and that fresh connections are more readily fatigued than the old ones, then the diminution of the new reflex after several repetitions of the stimulus could be explained by reference to the liability to fatigue of the new connections. The question deserves a more detailed experimental analysis.

SUMMARY

1. The chronic extinction of an alimentary conditioned reflex occurs much more slowly than its restoration.

2. The rate of the chronic extinction depends on the fixation of a conditioned reflex and the strength of a conditioned stimulus.

3. The full restoration of the extinct reflex requires only several trials.

4. A reflex extinguished and restored several times seems to become resistant to full extinction.

5. The diminution of alimentary excitability may disclose the partial inhibition of a conditioned reflex which in „normal" conditions is concealed.

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THE CHRONIC EXTINCTION AND RESTORATION OF CONDITIONED REFLEXES.

II. THE EXTINCTION AGAINST AN INHIBITORY BACKGROUND.

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In our previous paper on the chronic extinction and restoration of conditioned reflexes (Konorski and Szwejkowska 1950) we established that the restoration of an extinct reflex occurs much more rapidly than the extinction.

We thought that the cause of this incongruity is to be sought in, among other places, our experimental procedure. Our method of conducting experiments consisted in applying, once in an experimental session among several other stimuli which were reinforced, a stimulus subjected to extinction without reinforcement. The food given to the animal during the experimental session was very attractive and his alimentary excitability was high. So, the whole experimental situation, including the smell of food in the experimental chamber, the sight of the food tray etc., represented as a matter of fact a strong conditioned alimentary stimulus; in other words, the animal brought to the experimental chamber had a strong expectation that he would obtain food, and indeed he did obtain it several times in a session, except only in one inhibitory trial.

In order to explain what significance the above described technique of conduction experiments had on the course of extinction and restoration of a reflex we changed our procedure in such a way that a stimulus subjected to extinction was applied not among excitatory conditioned stimuli but in separate experimental sessions in which the food was not given at all. Such sessions were interspersed with „normal" sessions, in which positive conditioned stimuli were applied. In this way we created a situation in which, on the one hand, we eliminated a factor (that is the application of the inhibitory stimulus among positive stimuli) which, in our view, could hamper the elaboration of the inhibitory conditioned reflex, but on the other hand we did not permit the experimental situation as such, and not the proper unreinforced stimulus, to become an inhibitory stimulus.

In the present series of experiments, we established that the new variation of our experimental procedure did not affect the course of restoration of the inhibited reflex, i. e. the incongruity between the course of extinction and restoration was fully preserved.

As the above short account shows experimental sessions in this series of experiments were of two kinds, either purely inhibitory or purely excitatory. So the problem arose as to how an excitatory stimulus would behave against the inhibitory background and contrariwise, how the inhibitory stimulus would behave against the excitatory background. This point was also elucidated in the present paper.

METHOD

We used the classic Pavlovian method of conditioned reflex experiments, with some modifications which were described in our previous paper (Konorski and Szwejkowska).

RESULTS AND DISCUSSION

I.

The experiments were carried out on two dogs, *„Cygan"* and *„Nepek"*, in which several conditioned reflexes (to the sound of a bell, a metronome, a bubbling, and a tremulous string, and to an electric lamp) had previously been established. The isolated period of conditioned stimuli was 20 seconds. One of these stimuli, the bell, was subjected to extinction in such a way that it was applied with-

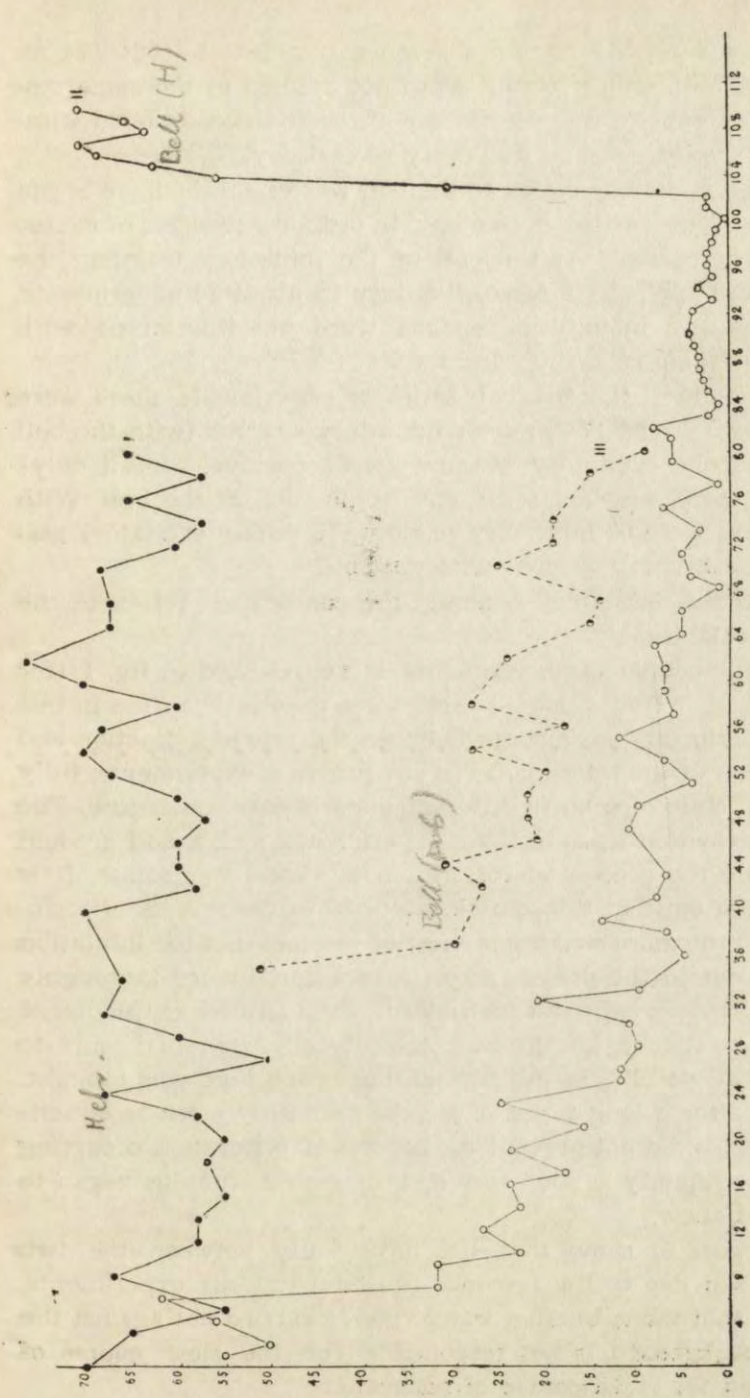

Fig. 1. The whole course of experiments with *Nepek*. Abscissae: successive experimental sessions. Ordinates: amount of conditioned salivation in grades of scale (1 grade = 0,01 ml). Curve I, metronome. Curve II, the bell against the inhibitory background. Curve III, the bell against the excitatory background. In the 102nd day the first reinforcement of the bell.

out reinforcement twice during a given experimental session at an interval of 6 min. Other stimuli were not applied in the same session. Ordinary experimental sessions with positive conditioned stimuli were conducted more or less every second day.

When the inhibitory reflex to the bell was established, we began to apply this stimulus also, once a day, in ordinary sessions, of course without reinforcement. At the end of the inhibitory training, the bell alone was applied on successive days in about 20 experiments, and these purely inhibitory sessions were not intermixed with ordinary experiments.

In the whole of the present series of experiments there were conducted with *„Nepek"*: 63 purely inhibitory sessions (with the bell alone), 17 purely excitatory sessions (with positive stimuli only) and 20 excitatory sessions with one application of the bell. With *„Cygan"* there were 66 inhibitory sessions, 18 purely excitatory sessions and 20 excitatory sessions with the bell.

After all this inhibitory training, the conditioned reflex to the bell was re-established.

The whole course of experiments is represented in fig. 1 (for *„Nepek"* and fig. 2 (for *„Cygan"*). Both these graphs show that in this series of experiments incongruity between the rate of extinction and the restoration of the reflex is, as in our previous experiments, fully preserved, in spite of a quite different experimental procedure. The process of extinction is, as in those experiments, a slow and gradual process, while the process of restoration is almost immediate. It is worth pointing out that this „immediateness" of the process of restoration is here the more striking in view of the fact that the inhibition was carried out in the present experiments much more thoroughly than in our previous experiments. Indeed, while a reflex extinguished against the excitatory background was usually inhibited only to the value of about 20% of the normal, the reflex here was brought, particularly after a long series of purely inhibitory sessions, practically to nil. This did not prevent the process of restoration occurring with extreme rapidity as soon as the extinguished stimulus began to be reinforced anew.

All this goes to show that the incongruity between the two processes is not due to the previous procedure of our experiments, i. e. the fact that the extinction was formerly carried out against the excitatory background is not responsible for the slow course of extinction and the rapid course of restoration.

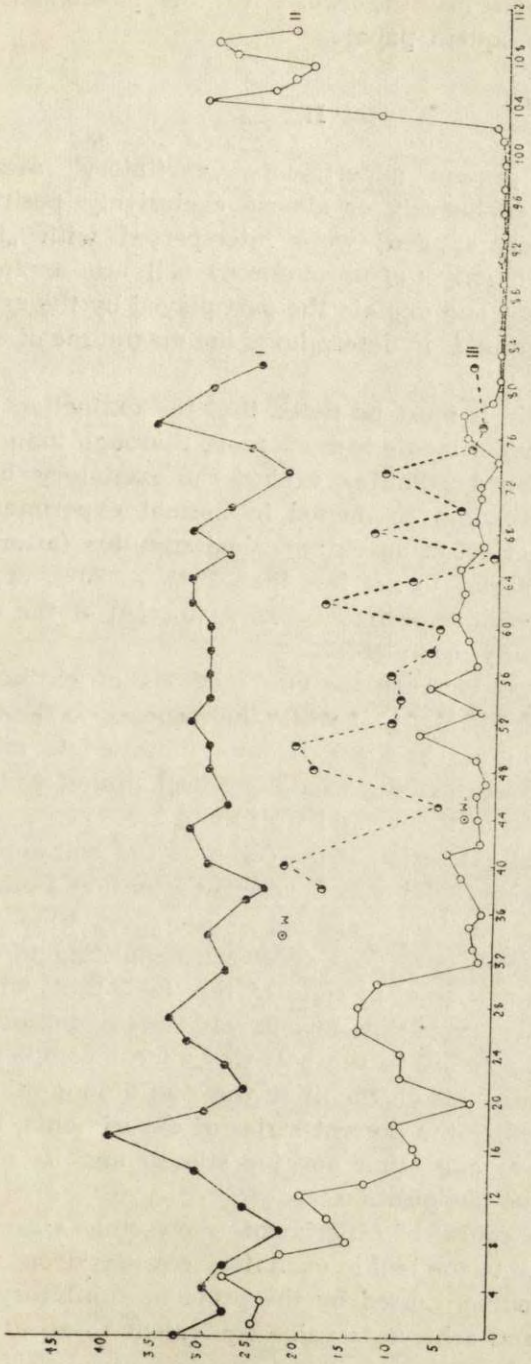

Fig. 2. The whole course of experiments with *Cygan*. The same designations as in fig. 1.

Further research into the causes of this phenomenon will be dealt with in subsequent papers.

II.

Since in our present experiments, „excitatory" sessions (i. e. those, in which exclusively or almost exclusively positive conditioned stimuli were applied) were interspersed with „inhibitory" sessions (in which only the unreinforced bell was applied) it was possible to examine and explain the part played by the excitatory or inhibitory „background" in determining the magnitude of conditioned reflexes.

To begin with, it must be noted that the extinction of the bell in this series of experiments is much more thorough than the extinction of a conditioned stimulus against the excitatory background. Whereas the extinction conducted in normal experimental conditions brings the effect of an extinguished stimulus (after a training of the same duration) to not less than 20% — 30% of its normal value, in the present experiments the extinction of the conditioned reflex is, practically, complete.

The difference between the two methods of extinction is also clearly seen from the fact that while the response to the bell applied against the inhibitory background was inhibited to nil, the same stimulus applied against the excitatory background gave an effect not less than 20% of the normal (fig. 1 and 2).

The course of extinction of the reflex to the bell applied against the excitatory background calls for careful attention. Both figs (1 and 2) show that the stimulus, applied for the first time against this background, gave in both dogs an effect amounting to 80% of the effect of the metronome, in spite of the fact that when applied against the inhibitory background it was already inhibited to 10%. So the inhibitory reflex to the bell was almost completely disinhibited. Undoubtedly this disinhibition was due, among other things, to the fact that, before the present series of experiments, the bell was always applied among other positive stimuli and its meaning was then not negative but positive.

The further course of experiments shows that after a few repetitions the reflex to the bell in excitatory sessions drops very rapidly (which is, of course, caused by the previous inhibitory training of this stimulus) and achieves a value equivalent to about 30% of the

effect to the metronome, while in inhibitory sessions its value is not more than 10%. So the difference of 20%must be attributed to the background against which the respective experiments are performed, i. e. to the relative „tonus" of the alimentary centre, which is high in excitatory sessions and low in inhibitory sessions.

In order to examine this point more thoroughly let us turn to the experiments on *Nepek*, since the data obtained on this dog are more suitable for a detailed analysis. This is because his salivary reflexes were both regular and abundant and he salivated copiously in the intervals between stimuli (a conditioned reflex to the experimental situation), which gave a direct measure of the permanent excitation of the alimentary centre during the experiment.

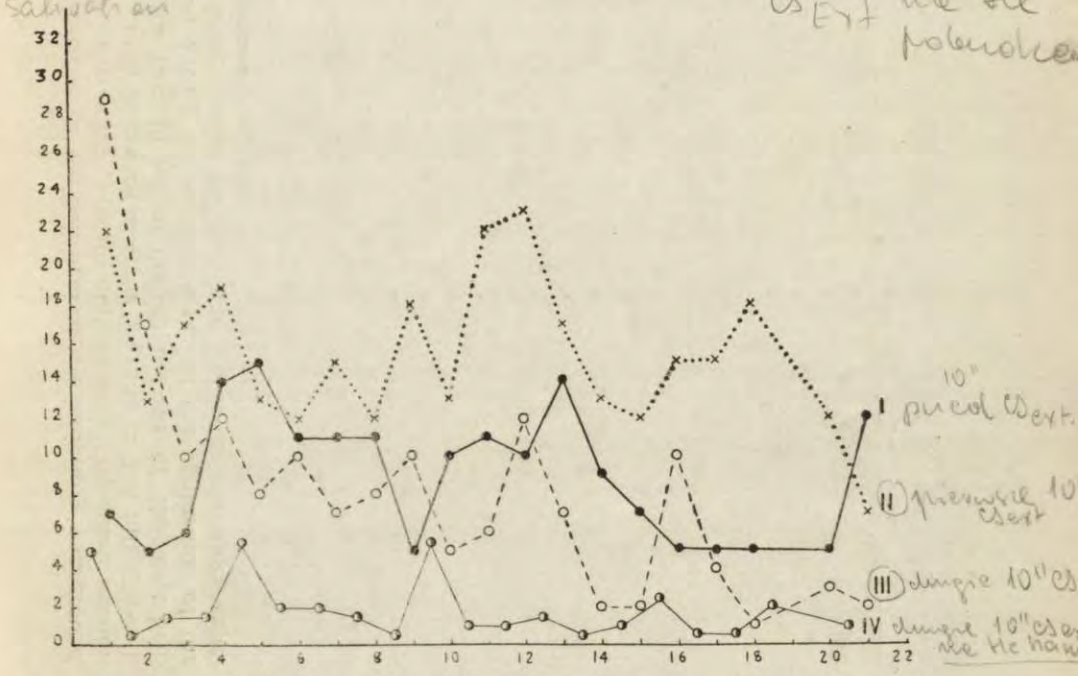

Fig. 3. The development of the inhibitory reflex to the bell against the excitatory background in *Nepek*. Abscissae: successive experimental sessions. Ordinates: amount of conditioned salivation in grades of scale (1 grade = 0,01 cm^3). Curve I, salivation in interval during 10 sec. just preceding the application of the bell. Curve II, salivation in the first 10 sec. of the action of the bell. Curve III, salivation in the second 10 sec. of the action of the bell. Curve IV, salivation during the second 10 sec. of the action of the bell applied in inhibitory experiments (for comparison).

Fig. 4. The extinction of the bell against the inhibitory background in *Nepek*. Abscissae: successive experimental sessions. Ordinates: amount of conditioned salivation in grades of scale (1 grade = 0,01 ml). Curve I, salivation during the first 10 sec. of the application of the first bell. Curve II, salivation during the second 10 sec. of the application of the second bell. The arrow denotes the day from which the inhibitory experiments were performed every day.

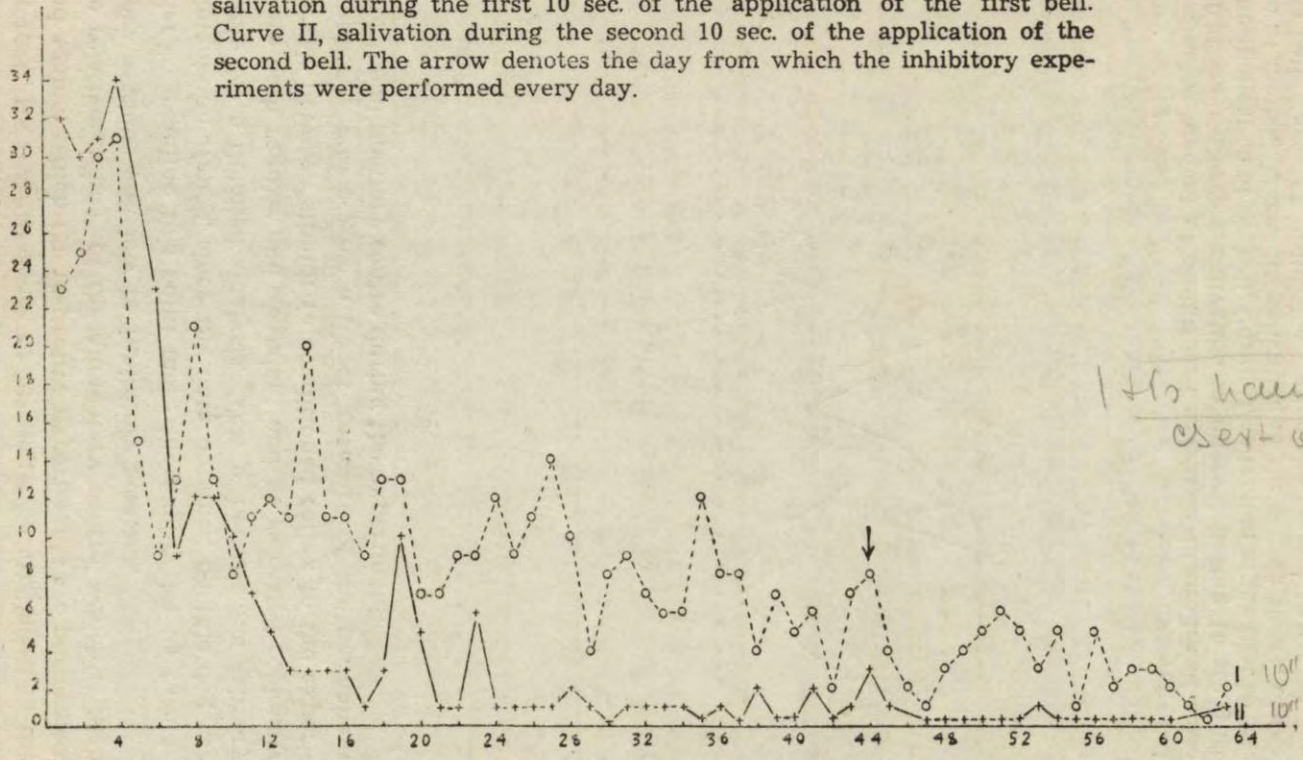

Fig. 3 represents the development of the inhibitory reflex to the bell aplied against the excitatory background. Curve I denotes the salivation in intervals during 10 sec. just preceding the application of the bell, curve II represents the salivation in the first 10 sec. of the action of the bell, curve III the salivation in the second 10 sec. of the action of the bell. (Curve IV is given for comparison and it represents the salivation in the second 10 sec. of the action of the bell taken from adjacent inhibitory experiments; each point of this curve denotes the mean value of the effect of the first and the second application of the bell in inhibitory sessions).

We see that salivation in the first phase of the action of the bell slightly exceeds the salivation in the interval, but in the second phase the reflex falls, sinking below the value representing the background salivation. If we assume that the extinguished stimulus causes the bombardment of the alimentary centre both by excitatory and inhibitory impulses, it is not difficult to explain this picture. In the first phase of the action of the bell, this stimulus being applied amongst other auditory stimuli has, thanks to generalisation, a slightly positive effect, which means that excitatory impulses outstrip inhibitory impulses. But gradually the individual, strongly inhibitory character of the stimulus itself takes the upper hand over the excitatory influences and the reflex falls even below the normal salivation in intervals.

Let us now examine the reflex to the bell in „inhibitory" experiments. As we see in fig. 1, this reflex, although very minute, does not vanish completely. The reason of this is evident. Fig. 4 represents the salivation during the first 10 sec. of the application of the first bell (curve I) and the salivation during the second 10 sec. of the application of the second bell (curve II) in inhibitory sessions. In addition we give here some protocols of typical experiments (Table I).

We see that the salivation during the first 10 sec. of the action of the first bell is relatively high, amounting to about 20% — 30% of the salivary effect produced during the corresponding period of a positive stimulus. But later the reflex to the bell falls rapidly and at the end of the experiment drops to nil.

These relations can be understood in the following way. After a number of experiments, the dog has elaborated a sort of differentiation between excitatory and inhibitory sessions, and, as these sessions are interspersed somewhat irregularly, the dog coming to an

Table I.

A typical course of experiment with chronic extinction against inhibitory background.

Nepek, 15 th June 1950, nr 282|45*)

Nr. of trial	Time in min.	Condit. stim.	Salivary condit. react.			Reinforc.
			first 10sec	second 10sec	total	
1	3	Bell	9	2	11	—
2	8	Bell	2	1	3	—
Nepek, 31 th September 1939, nr. 311|73						
1	2	Bell	7	1	8	—
2	7	Bell	2	0	2	—

*) The first figure denotes the general number of the experiment performed with the dog, the second figure denotes the number of the experiment of this series.

experimental session does not „know" what type of experiment he has to look for. This is determined during the first seconds of the action of the first stimulus. Therefore it is understandable that while the reflex to the first 10 sec. of the action of the bell is relatively high (amounting to about 10 grades of our scale), the reflex to the first 10 sec. of the action of a positive stimulus in excitatory experiments of this series is, on the contrary, relatively low (amounting to 15 — 25 grades while formerly it always amounted to about 30 grades, cf. the protocols below). And so during the first 10 sec. of the action of the first stimulus the dog, so to speak, „orientates himself" as to what he has to look for this day, and he reacts accordingly in the further course of the experiment.

When, at the end of this series, we began to perform daily inhibitory experiments (without interspersing excitatory ones), the reflexes to the bell diminished still further, at the cost of course of the first 10 sec. of the first bell. This is not surprising, if one takes into account that the salivation at the beginning of the first application of the bell is primarily due to the weak excitatory background

existing at the beginning of every experiment, and that this excitatory background gradually subsides, as inhibitory sessions are conducted on successive days.

It should be noted that the above relations, constantly and systematically occurring in *Nepek*, were not obserwed in *Cygan*, probably because in this dog the salivary reaction to conditioned stimuli was generally less abundant and began after a long latent period, and because he did not salivate in intervals.

Both in *Nepek* and in *Cygan* the inhibitory reflex to the bell, even when elicited against the excitatory background, was unusually strong, in any case much stronger than inhibitory reflexes trained in these dogs by the usual procedure. This again is best seen in Nepek and is manifested in the very prolonged and strong inhibitory after-effect. In fact, we repeatedly elaborated various inhibitory reflexes in this dog (by means of chronic extinction and differentiation) but we have never seen such a strong inhibitory after-effect as in this series of experiments. To show this we give the following protocols (Table II).

Table II.

A course of experiment with chronic extinction against excitatory background.
***Nepek*, 30 th August 1949, nr. 310|72.**

Nr. of trial	Time in min.	Condit. stim.	Salivary cond. reaction			Reinforc.
			first 10 sec	second 10 sec	total	
1	3	Bubbl.	17	25	42	+
2	8	Metron.	32	38	70	+
3	14	Bubbl.	33	32	65	+
4	19	Bell	15	10	25	—
5	24	Bubbl.	15	28	43	+
6	28	Metron.	30	30	60	+
Nepek, 16th September 1948.						
2	8	Metron.	24	32	56	+
3	13	Bell I	26	29	55	+
4	18	Bell II	12	3	15	—
5	23	Bell I	24	27	51	+

The first of the protocols adduced is from the present series. It shows that the stimulus applied after the inhibitory bell was inhi-

bited to $^2/_3$ of the normal. The second protocol is one of the previous series. No inhibitory after-effect can be detected here after the application of the inhibitory stimulus.

The inhibitory after-effect is much stronger still when the positive metronome was applied in an inhibitory session after the twofold application of the bell. The corresponding protocols are given in tab. III.

Table III.

A course of experiment with positive metronome against the inhibitory background.

Nepek, 1 th June 1949, nr. 277|40

Nr. of trial	Time in min.	Condit. stim.	Salivary condit. reaction			Reinf.
			first 10 sec.	second 10 sec.	total	
1	5	Bell	9	2	11	—
2	10	Bell	3	1	4	—
3	15	Metron.	16	23	39	+
The normal effect to the metronome from adjacent experiments:						
		Metron.	34	37	71	+
		Metron.	36	40	76	+
Cygan, 1 th June 1949, nr. 271|42						
1	4	Bell	1	9	10	—
2	10	Bell	0	0	0	—
3	16	Metron.	0	7	7	+
The normal effect to the metronome from adjacent experiments:						
		Metron.	13	17	30	+

Even more instructive are those experiments performed on *Cygan* in which we applied a quite new auditory stimulus, a whistle, both against the excitatory and the inhibitory background (table IV).

The whistle applied against the excitatory background evoked, owing to generalisation, a considerable conditioned response, while the response to the same stimulus applied against the inhibitory background was almost nil.

Table IV.

A course of experiments with a whistle against the excitatory and the inhibitory background.

Cygan, 13 th May 1949, nr. 264|35.

Nr. of trial	Time in min.	Condit. stim.	Salivary condit. reaction			Reinf.
			first 10 sec.	second 10 sec.	total	
1	2	Bubbl.	6	15	21	+
2	6	Lamp	13	20	33	+
3	12	*Whistle*	9	13	22	−
4	17	String	8	14	22	+
5	23	Metron.	14	14	28	+
Cygan, 13 th June 1949, nr 274|45.						
1	4	Bell	1	2	3	−
2	9	Bell	0	1	1	−
3	14	*Whistle*	0	3	3	−

All facts adduced indicate very clearly that in the conditioned reflex experiments conducted by the classic method a conditioned reflex is a resultant of two agents: the first is the individual character (excitatory or inhibitory) of the conditioned stimulus, a character resulting from its previous training; the second agent is the background against which the stimulus is applied, which background is, so to speak, made and shaped by the whole of the given experiment and a series of precedent experiments. This general principle is, of course, well known to those workers who are trained in using the Pavlovian method in the study of conditioned reflexes. It found its clearest expression in experiments concerning the so called „stereotype" (Asratian 1938, Skipin 1938 and others).

Owing to the fact that in our experiments the inhibitory stimulus, instead of being applied among the excitatory stimuli, was, so to speak, excluded from them and applied separately in special inhibitory sessions, the influence of the background on conditioned reflexes manifested itself with a particular clarity.

SUMMARY

1) The chronic extinction of a conditioned reflex conducted against an inhibitory background occurs more rapidly and is more thorough than when it is conducted against an excitatory background.

2) The restoration of the reflex, extinguished against the inhibitory background, occurs very rapidly and achieves a normal value after a few reinforcements.

3) Neither a method of extinction nor the duration of the inhibitory training affected the rate of the restoration of the reflex.

4) A stimulus extinguished against the inhibitory background and transferred then to the excitatory background is at first largely disinhibited; then the reflex to this stimulus rapidly decreases, but it does not achieve such low values as are achieved by the reflex to the same stimulus applied against an inhibitory background.

5) A positive reflex elicited against the inhibitory background is very strongly inhibited.

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METHOD OF GRAPHIC REGISTRATION OF SALIVARY SECRETION.

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In the course of experiments on conditioned reflexes there arises a necessity to determine the amount of saliva secreted during the operation of extraneous, conditioned and unconditioned stimuli as well as during the intervals between them.

It is of considerable importance, also, to determine the secretion during the short intervals of time (minutes and fractions of minutes) and the evolution of this phenomenon during the periods of days and weeks.

With this in view, a chronic salivary fistula was performed on the animal. In 1894 Pavlov described Glinski's method of fistula whereby the opening of the parotid duct with the surrounding fragment of mucous membrane was transplanted to the cheek, and sutured to the skin.

A funnel was sealed over the duct opening when the wound had healed, and then the falling saliva drops were counted. An observer remained in the dog chamber. Nevertheless, on account of the discontinuity of the drops and the difference in their size — depending on many factors, such as the rate of secretion, the viscosity of saliva, the size of the opening, and, finally, the movements of the animal — the count of drops does not give a true picture of secretion.

When the investigation of conditioned secretion was initiated in Pavlov's school, the amount of saliva was observed in this way. And only when there arose the necessity to isolate the dog acoustically and visually from the observer, did Ganike elaborate a method of determining the amount of saliva from a distance. In this method the funnel sealed over the duct opening was replaced by a so-called salivary capsule with a small glass window and two tubes: the capsule was connected by means of a long rubber tube with an open, horizontal, graduated glass tube placed on the other side of the soundproof wall of the animal chamber. This glass tube contained a coloured liquid, which was displaced as the dog salivated. From time to time the saliva in the tube leading from the salivary capsule had to be removed.

This disandvantage was eliminated by Kupalov (1929) by fixing a washing bottle close to the dog. The water from the washing bottle was drawn into the salivary capsule through a connecting tube. In this manner the saliva secreted from the duct opening was mixed with the water in the capsule and urged the water towards the washing bottle. That caused an increase in the pressure of air above the water level in the washing bottle and this increase was transmitted through the long tube to the other side of the wall of the chamber. There, the air pushed the horizontal column of the coloured liquid along the scale. The movements of the liquid were either read by the experimenter or registered by an electric recorder noting the drops of the liquid on a kymograph.

It seems that the method described below, by means of which it is possible to obtain a direct recording of salivary secretion on the kymographic paper, removes the causes of error set up by the great compressiblity of air in the tubes and washing bottle.

DESCRIPTION OF METHOD.

I. THE DEVICE. The device consists of 1) a system of tubings in the dog chamber, 2) a recording system in the experimenter chamber, 3) a tube connecting both systems; this tube runs, two meters high, through the wall of the chamber (fig. 1, RO).

The dog has a salivary fistula of parotid gland and the opening of the duct is transplanted to the cheek. The „salivary capsule" is

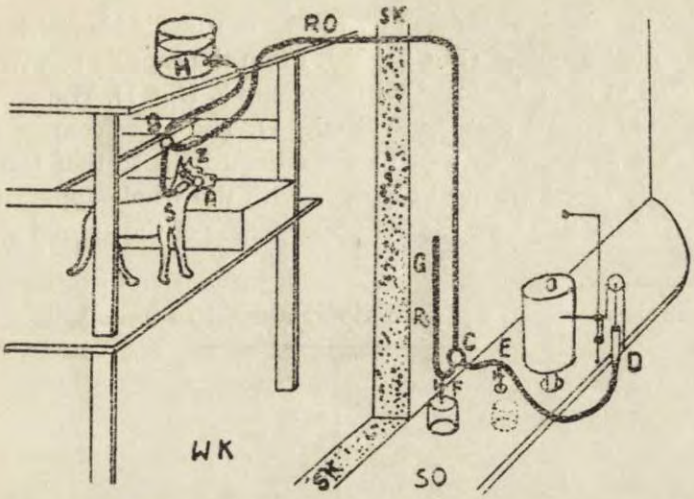

Fig. 1. SCHEME OF CONNECTIONS. **A** „salivary capsule", **Z** clamp, **S** glass bulb 20 ml, **B** three-way tap, **RO** lead tube 7 mm ∅ external, 4 mm ∅ internal, **C** three-way tap, **RS** glass tube, **E** clamp, **D** Tuberculin glass syringe, **G** opened end of the glass tube, **F** tap, **H** water reservoir, **SO** experimenter's table, **SK** chamber wall, **WK** inside of chamber.

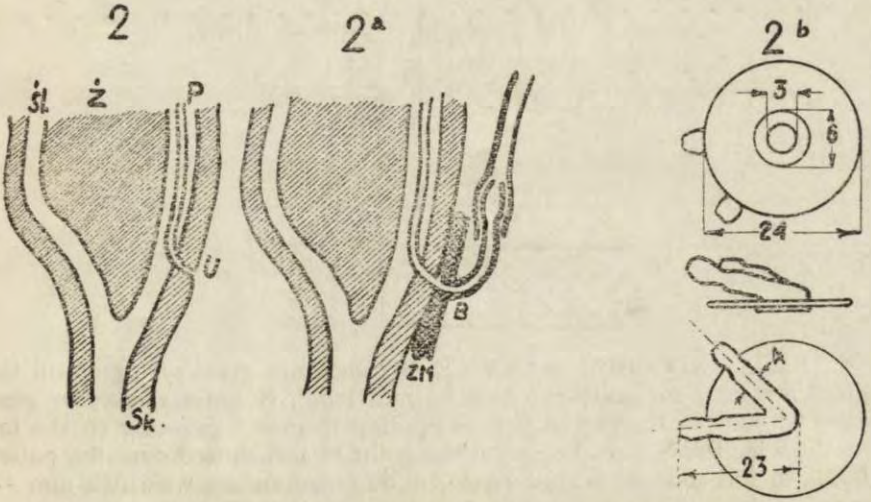

Fig. 2. SCHEMATIC SECTION OF THE CHEEK OF THE DOG. **P** parotid duct, **U** its opening, **SK** skin, **SL** mucous membrane, **Ż** masseter muscle. 2a) The same as in fig. 2, at the opening of the duct the capsule **B** is sealed with Mendeleev wax **ZM**. 2b) **„Salivary capsule"**, nickeled **brass.**

sealed to the cheek of the dog by means of so-called Mendeleev wax*) over the duct opening. (Fig. 2a, 2b). From the capsule run two rubber tubes. One of them is closed with a clamp Z (fig. 1). The second tube is connected with a glass bulb S (fig. 1). The tubes coming from the capsule are sealed with wax to the animal hair, so that the dog can comfortably move his head together with the whole apparatus. From the head of the dog towards the upper frame of the animal stand runs

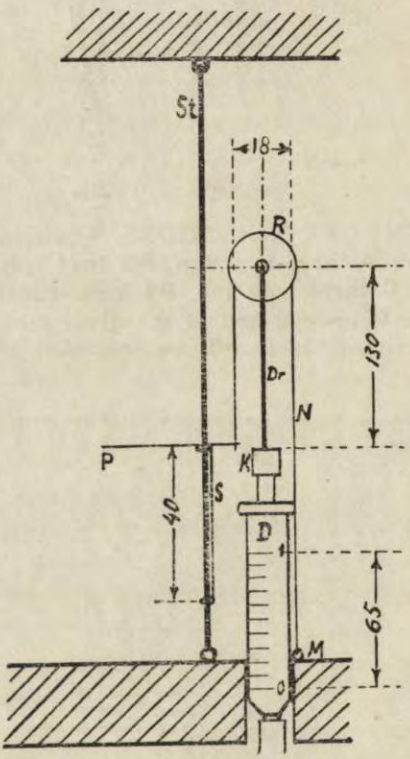

Fig. 3. RECORDING DEVICE. **D** tuberculin glass syringe with the glass piston (1 ml approximately 60 mm long), **K** brass sleeve, **Dr** steel wire 0.8 mm ∅, **R** grooved bakelite pulley 18 mm∅, pivoting on the top of the wire **Dr**; **N** thread fixed at the point **M** and shifted over the pulley **R** up to the slide **S**; **S** slide made of thin aluminium wire (0.5 mm ∅) moving along the taut string **St**; **P** writing point fixed to the slide **S**.

*) Colophony 6.0 parts, red lead 2.4 parts, bees wax 1.5 parts, by weight. For every 10 dkg of the wax there must be some drops of linen oil; the whole to be melted together (Podkopaev 1936).

a loose rubber tube; it connects the tube S on the head of the dog with a three-way tap B (fig. 1). One branch of the tap B is connected with the water reservoir H, the other one with the leaden tube RO.

The tube RO runs two meters high through the wall to the experimenter's chamber.

There the tube RO is connected with a three-way tap C (fig. 1). One branch of this tap is connected with a T- tube, which, in its turn, connects with the tap F above the beaker and with a vertical glass tube RS; the upper end G of which is opened at the level of or higher than the head of the animal.

The second branch of the tap C is connected with a T-tube, the lower end of which is connected with a rubber tube having a clamp E (above the beaker). A side branch of the T-tube is connected to a tuberculin glass syringe D (fig. 1, 3). The lower end of the syringe D is placed at the level of the clamp E (fig. 1) but lower than the head of the dog in the chamber. The syringe is fixed on a stand.

On the glass piston of the syringe there is fixed a sleeve K (fig. 3) with a vertical steel wire Dr (fig. 3), approximately 130 mm long. On the top of the wire Dr is placed a light bakelite pulley R, pivoting on a horizontal axis. The thread N (one end of which is fixed at point M) is shifted over the pulley; the other end of the thread is attached to slide S, which during the upward movement of the piston of the syringe, is pulled by the thread in the same direction; that causes its writing point to draw a line on the kymographic paper.

II. PROCEDURE. The tap B (fig. 1) is turned to connect H with RO. The tap C is turned in such a way that it joins RO with the tap F, which is opened. The water from H fills the tubes and runs over into the beaker under the tap F. When the air is forced out of the tubes we close the tap F. The tap C is now turned so that it connects RO with D. When the water has filled the syringe D we close the tap C. Then we press gently the moistened piston of the syringe D against the surface of the water at the opening of the syringe. Next we open the clamp E until the piston falls to the bottom of the syringe: the clamp E is then secured. We introduce water into the tube RS to the level of the syringe. Then we close the tap B, and turn the tap C so that it connects RO with RS.

The dog is placed on the animal stand. We cut round the fistula the hair of the dog and seal there the salivary capsule (fig. 1, 2b) in the manner demonstrated in figure 2a. The sealing is done with

slightly heated (about 50°C) Mendeleev wax. We turn the tap B so that it joins H with A (fig. 1) and fill the tubes and the capsule until the water pours out through the other tube of the capsule. We close the tap B and seal with wax the tubes connecting the capsule to the hair of the dog. Then we close the clamp Z on one of the tubes and turn the tap B so that it connects A with RO (fig. 1).

If the sealing was tight and if there is no air in the tubing system, the water level in RS remains constant (the dog does not eat during this time); otherwise, the level rises suddenly. When everything is in order we connect the tube RO with the syringe D by means of the tap C.

Now, if the saliva is secreted by the dog, the piston of the syringe will rise, recording with the writing point a curve on the revolving drum. When the piston reaches the top of the syringe, we let off the water from the syringe by releasing the clamp E. An

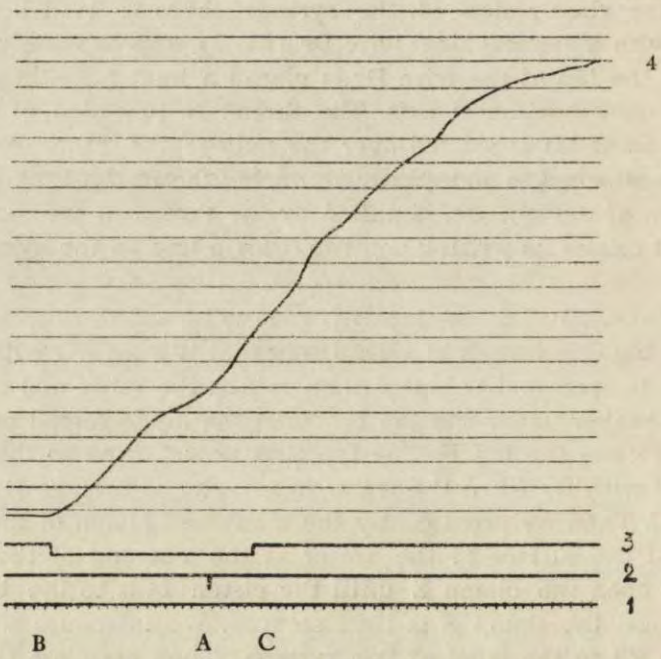

Fig. 4. RECORD OBTAINED BY OUR METHOD. 1 — line of time (in seconds), 2 — line of unconditioned stimulus (in A the food was presented), 3 — line of conditioned stimulus (in B the stimulus was given, in C it was stopped), 4 — record of salivary secretion (horizontal parallel lines were drawn on the kymograph paper to facilitate the reading of the amount of saliva secreted).

example of such a recording is shown in fig. 4. The ordinates of the record are strictly proportional to the volume of secreted saliva. In our device the coefficient is 120 mm/ml.

III. ANNOTATIONS. The glass bulb S is introduced into the circuit in such a way that the saliva is not admitted into further tubes (fig. 1). Nevertheless the tubings should be frequently washed by passing through them the water from the reservoir H. To obtain an exact record, the opening of the capsule must be small (2 mm ∅), the rubber tubings must be rigid and thick-walled (6 mm ∅ external, 3 mm ∅ internal), and wherever possible, rubber must be replaced by lead and glass tubes. This is important because of the movements of the animal which, if the tubes are elastic and if the capsule has a large aperture, falsify the recordings of the device. Still more important is an exact elimination of the air bubbles from the tubing system.

The tuberculin glass syringe D (1 ml approximately 60 mm long) must be carefully washed and placed absolutely vertical. When it is ascertained that the glass piston is pushed smoothly by the water, it must not be touched and turned any more: it must be protected against dust and drying. The difference of levels between the head of the dog and the syringe must be such as to make a slightly negative pressure of the liquid in the salivary capsule. Then, when the clamp Z at the capsule is released a rising of the piston should be observed.

RESULTS

The method described above removes some of the causes of error found in the methods formerly employed, and, moreover, presents some additional advantages.

First of all, we obtain a continuous record, a full curve of secretion. Every ordinate of the curve denotes the amount of the saliva secreted from the beginning until that moment. And the slope of the curve indicates at its every point the intensity of secretion at the moment given. The movements of the head of the animal have a very slight effect on the record.

The margin of error in the saliva volume so recorded is approximately 0,01 — 0,02 ml. If the movements of the animal are exceptionally strong, this error is slightly increased.

SUMMARY

A method of continuous recording of salivation in the dog is described. It eliminates some of the errors of methods previously employed. The kymographic curve exactly reflects the course of secretion.

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RESEARCHES INTO CONDITIONED REFLEXES OF THE SECOND TYPE.

1. TRANSFORMATION OF CONDITIONED REFLEXES OF THE FIRST TYPE INTO CONDITIONED REFLEXES OF THE SECOND TYPE.

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In earlier publications of one of the authors (Konorski and Miller 1933, 1936; Konorski 1939) the following facts were established. Suppose that in a dog an alimentary conditioned reflex of the first type is elaborated to a certain stimulus, which means that this stimulus is simply reinforced by food and evokes an alimentary conditioned response; then to another stimulus a conditioned alimentary reflex of the second type is established, which means that to t h i s stimulus the dog must perform a definite motor reaction (as e. g. the raising of the leg) in order to get a food reinforcement. If now we apply the conditioned stimulus of the first type it does not, as a rule, evoke the conditioned response of the second type, but this response is easily elicited by this stimulus whenever it is not reinforced by food.

In our present experiments we transformed firmly established conditioned reflexes of the first type into conditioned reflexes of

the second type. In the course of this transformation we encountered an unexpected phenomenon. It was found that to those stimuli which were formerly conditioned stimuli of the first type the conditioned reflex of the second type was elaborated with great difficulty and resistance; that is to say that the former function of a stimulus forms a very strong obstacle to the formation of a new reflex to this stimulus. Even in the stages when, apparently, the motor reflex to the old stimulus of the first type does not at all differ from the „normal" conditioned reflex of the second type, it can be shown that this is in fact not so, and the „past" of the stimulus is not „lost" and determines the strength of the motor reaction of the second type evoked by this stimulus.

To describe this condition is the object of the present paper.

METHOD

The experiments were performed on dogs in an ordinary conditioned reflex chamber. Salivation from the parotid gland (the opening of the duct being transplanted to the outside) was recorded by means of a Ganike-Kupalov manometer (Podkopaiev 1926) slightly modified so that the aerial part of the system was reduced to a minimum, a modification which considerably increases the precision of the measurements. The manometer was joined with a Luer syringe placed vertical and having its piston connected with the writing point, so that the rate of salivation was recorded on the kymograph. The way in which movements of the right fore leg were recorded is shown in fig. 1.

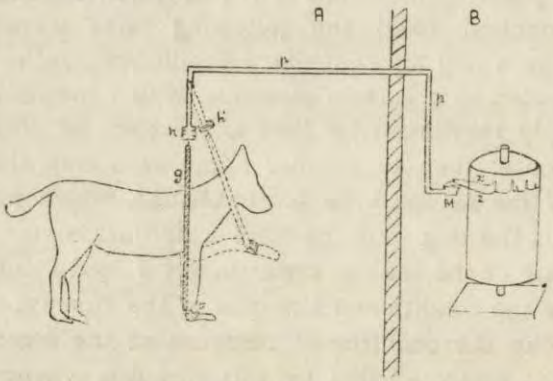

Fig. 1. The device recording the movements of animal's leg (schematic). A — The experimental chamber, B — The experimenter's room, z, z' — Writing point, M — Marey tambour, p — Airduct, h, h' — Rubber harmonicon, g, g' — Rubber thread, o — Band.

EXPERIMENTAL PART

Experiments were conducted on three dogs: Azor, Rex and Bimbek.

Azor: male, mongrel, weight 20 kg, about three years old. Experiments on conditioned reflexes were begun in the middle of October 1948. There were then established conditioned reflexes of the first type to the metronome, the bell and the lighting of the lamp, which were trained for three and a half months.

Rex: male, mongrel, weight 14,5 kg, about two years old. Experiments on conditioned reflexes were begun in the middle of April 1948, and were continued at intervals until the middle of March 1949. Conditioned reflexes of the first type to the metronome, the bell and the lighting of the lamp were established and trained during this time.

Bimbek: male, mongrel, weight 11 kg, about two years old. Experiments with conditioned reflexes were begun in the middle of April, 1948, establishing conditioned reflexes of the first type to the metronome, the bell and the lighting of the lamp. Experiments of this kind were continued until the beginning of March, 1949.

More or less at the same time (February — March 1949) there were elaborated in all three dogs alimentary conditioned reflexes of the second type: the sound of bubbling of water — raising the right fore leg. The method of establishing this reflex was the same as has already been described (Konorski and Miller, 1933). It was as follows: at the sound of bubbling, the dog's leg was lifted mechanically (passive movement) and this combination was reinforced by food; from time to time the bubbling took place with no lifting of the leg and with no reinforcing. When the dog started slightly to raise the leg, this movement was assisted and brought to a greater height (semi-active movements).

After the training, which, in Azor and Rex took some days, and in Bimbek rather longer (on account of some difficulties which need not here be described) the conditioned reflex of the second type to bubbling has been established.

When the training of the conditioned reflex of the second type to bubbling was completed, and the dogs were regularly raising their legs to this stimulus, while standing quietly or almost quietly in the intervals between stimuli, we commenced the essential series of experiments, that is to say the former conditioned stimuli of the

first type were again applied, and later they were transformed into the conditioned stimuli of the second type. This transformation was conducted in such a way that the action of stimulus was prolonged until the dog raised his leg, and then it was immediately reinforced. In other words, the method was that always employed when forming a conditioned reflex of the second type, that is: external stimulus + the movement are reinforced, but the external stimulus alone is not reinforced.

The course of the experiments will be described for every dog individually.

Azor: From 9th March 1949, in addition to the conditioned stimuli of the second type (bubbling) conditioned stimuli of the first type, viz. the metronome, bell and lamp, were applied. Here are extracts of the reports of the first two experiments (9th and 10th March).

Experiment on 9 th March, 1949.

Time of experiment in minutes	Stimulus	Amount of saliva	Lift. of the leg	Reinforcement
9′	Bubbling 8″	13,8″	2″ 3″ 5″ 7″	+
13′	Metronome 20″	9/10″ + 11/10″	—	+
17′	Bubbling 8″	10/8″	1″ 2″ 4″ 7″	+
21′	Bubbling 4″	1/4″	2″	+
26′	Lamp 20″	10/10″ + 7/10″	—	+
30′	Bubbling 13″	?	1″ 2″ 4″ 7″ 11″	+

Experiment on 10 th March, 1949.

Time of experiment in minutes	Stimulus	Amount of saliva	Lift. of the leg	Reinforcement
10′	Bubbling 13″	16/13″	4″ 6″ 9″ 12″	+
14′	Bell 20″	9/10″ + 14/10″	—	+
18′	Bubbling 7″	2,7″	2″ 6″	+

As we see from these reports, whereas bubbling immediately or almost immediately produces a raising of the dog's leg and the frequency of these raisings is more or less one raising every 2 seconds,

neither metronome nor the lamp or bell evoke, during 20 seconds of their isolated action one single movement of the leg. To these stimuli the dog displays only an alimentary reaction, looks persistently into his bowl, but does not raise the leg.

With the next experiment a systematical training of the conditioned reflex of the second type to metronome was begun, this stimulus being applied until the dog raised his leg to the height required and then immediately reinforced. As a control the bubbling was applied at the beginning and at the end of each experiment. This training lasted for 8 days during which the metronome was applied 33 times.

The dog's behaviour at the sound of the metronome is very different from his behaviour during the bubbling. Whereas at this latter stimulus, the dog almost immediately raises the leg very high and very decidedly, and very often puts it on the food tray, and these mevements are repeated every one to three seconds until the dog receives food, the picture of his behaviour to the metronome is quite different. First: the movements are sometimes very slight, much lower than to bubbling. Second: the movements are much less frequent than to bubbling, there are long intervals, sometimes 10 — 20 seconds in between; during this time, the dog shows a distinct direct reaction towards his food bowl. With regard to salivation, distinct differences between the conditioned reflex to bubbling and to metronome are not observed (see fig. 2, where the 7th day of the training of metronome is shown).

Proceeding, the bell and the lamp were introduced into the experiments, and during a further 7 experiments all 3 stimuli were applied, bubbling being also applied for control. The picture is as a rule the same. To the bubbling the motor reaction is vehement and energetic, to other stimuli much weaker, and these latter evoke a strong, direct, alimentary reaction. For the sake of illustration, let us cite the record of the seventh experiment (fig.3). As we see from the record, the lamp evokes the weakest motor reaction. Is this so because it is, physiologically, the weakest stimulus, or because it acts on the visual analyser, whereas other stimuli (bubbling included) belong to the auditory analyser — this we cannot say.

We had to make sure whether the fact described, consisting in a distinct and indubitable disproportion between the motor reflex to bubbling on the one hand, and the motor reflexes to the metro-

nome, bell and lamp on the other, depend, in fact, on the „past" of these latter stimuli. With this object, we tried a new stimulus: whistling. On its first application, this stimulus evoked an orientation reaction, but in spite of that, as early as the 4th second the dog raised his leg very high. Immediately, the reinforcement was given. During the following trials the orientation reaction decreased, and the latent period of raising the leg was shortened. In the fourth trial, the reinforcement was postponed to the fifth second. This produced a very energetic raising of the leg and putting it on the food tray till the moment of reinforcement, these movements being in no way different from those evoked by the bubbling. In the sixth trial the bell was applied for control. After the first stronger movement there are decreasing ones, separated by intervals of several seconds (fig. 4).

So we see that the motor reflex to whistle is formed immediately (of course due to generalization) and that from the very beginning it takes exactly the same form as the reflex to bubbling. During some following experiments, this reflex even increases, whereas the reflexes to three stimuli with the „past" remain in a dwarfish condition, and are much less strong (fig. 5).

From this time on, the experiments with conditioned motor reflexes with Azor continued without any distinct change. It is true that the motor reaction to the stimuli referred to tend to be the same as the reflexes to bubbling, especially when they are trained in this respect. But in all cases, in which we have to do with a general decrease of motor excitability — as for instance on the extinction of the reflexes or the decrease of alimentary excitability — the difference between the motor reflex to the bubbling and the reflexes to the stimuli with the „past" can be distinctly observed. These matters will be described more fully in subsequent papers.

Rex: The course of the experiments with Rex was almost the same. The difference here was that the motor excitability of the dog was very great: at the sound of bubbling the dog immediately raised his leg and repeated this movement with a maximum frequency till the presentation of food. When after a prolonged training with bubbling the bell was applied for the first time, the dog immediately raised his leg as in the case of bubbling. But then the movements ceased and the dog became almost quiet for the rest of the action of the stimulus (fig. 6).

Then there was commenced an intensive training of motor conditioned reflex to the bell, metronome and lamp. The result was just the same as in case of Azor. Even after many experiments these stimuli did not evoke such a strong motor reaction as did bubbling, though to all auditory stimuli salivation was approximately the same. The most usual behaviour of the dog to metronome or bell is as follows: during the first few seconds of stimulus the dog raises his leg rather frequently. If food is not given, the raising of the leg stops and the dog displays a direct alimentary reaction — his eyes are fixed on the bowl and he remains in a position of „waiting". Only later on, if food is still withheld, there is a series of new rather slight raisings. This picture is absolutely different from the one seen with bubbling, when the dog very vigorously raises all four legs, most frequently the right fore leg.

As in Azor's case, a motor reflex to two new stimuli was formed in Rex: the second bubbling (the higher pitch) and rotating of a small propeller before the dog's eyes. The motor reflexes to these stimuli were easily established and in a short time became stronger than to the metronome, the bell and the lamp (fig. 7).

The defficiency in Rex of motor reflexes to the bell, metronome and lamp had also a permanent character, which appears distinctly with the decrease of alimentary excitability.

The course of the experiments with B i m b e k was analogous to those described above, so we shall not give here any more details, only attaching two records, to illustrate the difference between the action of the stimuli referred to (fig. 8a and b). The first of these experiments took place on the 13th day of the training with the metronome, bell and lamp (as the stimuli of the motor reflexes), the second after a month of training. As we see on the records, the situation shows but little change, although more than twenty experiments were made in between.

DISCUSSION

The results obtained in all three dogs are so clear and similar that they leave no room for doubts. They all teach us that:

1. The elaboration of alimentary conditioned reflexes of the second type to the stimuli, which before were conditioned stimuli directly signalling food (that is conditioned stimuli of the first type),

encounters as a rule great difficulty and is much less easy than elaboration of such reflexes to quite new stimuli*).

2. A complete equalising of those stimuli with the stimuli which from the beginning were conditioned stimuli of the second type does not occur in spite of many months of prolonged training.

Let us consider these two points separately. With regard to the first fact, i. e. the difficulty of elaboration of the motor conditioned reflex to the stimuli which hitherto have been conditioned stimuli of the first type, the explanation does not present much difficulty and is suggested simply by observation of the dog during the experiment. As we know, the motor reaction of the dog to conditioned stimulus of the first type, which simply announces the food, is the directing of the head and of the whole body towards the place where the food normally appears and subsequently quick turnings of the head from the stimulus to the bowl. The whole reaction may be described as „waiting" for an alimentary unconditioned stimulus. In the conditioned reflexes of the second type, this direct reaction of „waiting" is disturbed by the necessity of performing a definite acquired movement.

As results from our experiments, both these reactions are antagonistic in their mutual relations and the first of them has to be inhibited to make the second possible.

The antagonism between these two reactions is clearly seen from the behaviour of our dogs in the experiments. To the stimuli which have hitherto been ordinary conditioned stimuli, and which are then transformed into stimuli of the second type, the dog behaves in the following way: sometimes from the very outset of the stimulus, the animal shows a direct alimentary reaction and only later on, when „waiting" is too long and the food is not given, the dog begins to raise his leg, first very low, afterwards higher and higher; direct reaction is here replaced slowly by the acquired motor reaction. Sometimes there is a difference: at the beginning of the stimulus, when the moment of the reinforcement is still remote (because in

*) Of course, we are considering the elaboration of conditioned reflexes of the second type the motor reaction of which is already established. It is quite clear that the first motor reflex (in our experiment to bubbling) may be established much more slowly, because this process is connected with the production by the dog of a characteristic motor coordination, not hitherto performed by him.

experiments with conditioned reflexes of the first type isolated period of the conditioned stimulus lasted 20 seconds) the dog „is capable" of a stronger or weaker motor reaction; afterwards, a clear alimentary reaction is displayed — the movements of the leg stop, the dog looks into the bowl — and only if the food is not given, the new series of movements is started. Sometimes, finally, direct reaction simply suppresses more or less the acquired motor reaction which becomes weakened and less frequent.

How strong is the direct reaction of „waiting" for food is proved by the fact that, during the training experiments of transforming conditioned reflexes of the first type into the conditioned reflexes of the second type, the dog for 20 seconds, and sometimes even for a longer time, looks persistently into the bowl, not raising his leg, though the raising could provide him with the food already during the first seconds of the action of the stimulus.

As results from our experiments, a conditioned reflex of the second type is formed to the n e w stimulus almost immediately. This happens partly because of the generalization (new stimuli were of the same analyser as the former), but principally because of the general attitude of the dog during the experiments, resulting from the fact that at every trial the dog m u s t perform the acquired movement to receive the food. It is likely, that if we trained, during a series of experiments, both types of reflexes in parallel, that is if we simply reinforced some stimuli by food without the necessity of performing any movement to them, generalization would be directed not towards the acquired motor reaction but towards the direct alimentary reaction. It would be so, also, if the new stimulus were similar to one of the conditioned stimuli of the first type.

In our experimental situation, where the acquired movement is a n e c e s s a r y condition of receiving the food, the elaboration of a new reflex tends towards a conditioned motor reflex. The fact that this reflex is very quickly established to the new stimulus and that the dog quite easily and immediately to this stimulus performs the movement in the manner required, is the best proof that the difficulty of the performance of this same movement to the conditioned stimuli of the first type is connected with the inhibition, the character of which was explained above.

There is now a second question to be considered — why is this „dwarfness" of the conditioned reflexes of the second type to the old stimuli of the first type so persistent. It seems that this fact may also

be explained. According to the existing evidence (Pavlov 1949) all relearning is based not on a kind of disappearance of interneuronic connections already formed but only on the superposition of new connections, depending on new experience, which connections determine the new type of reaction of the animal. The disappearance of the old connections, being a process dependent on „forgetting" is a very slow process, and if a particular conditioned reflex is firmly established, it practically cannot take place.

And so the conditioned stimuli of the first type which were transformed into the stimuli of the second type should for a long time, and may-be for ever, betray their past, because their primary reaction can be to a greater or a lesser degree suppressed and masked by the reaction newly established to these stimuli but it can never be effaced.

It should be added that in our experiments there existed certain additional conditions which to some extent favour the first reaction. The given stimulus had not ceased to be an alimentary conditioned stimulus; as before, on its occurence the dog received the food, though he must perform also an acquired movement. And so the direct alimentary reaction to the stimuli applied is, though disturbed by the other reaction, always produced. Hence the frequent returning to the old type of reaction, when only favourable conditions appear.

It is interesting to speculate whether in the case when our conditioned reflex of the first type were transformed into a defensive reflex, the difference between the stimuli „with a past" and „without a past" would also appear for the same long period and equally distinctly.

SUMMARY

The elaboration of alimentary conditioned reflexes of the second type (motor) to the stimuli which formerly were ordinary (signalling) conditioned stimuli is very difficult; and even after some months of training of these reflexes they are not so strong as the reflexes elaborated to quite new stimuli. This phenomenon is explained by the fact that the direct alimentary reaction evoked by the conditioned stimulus of the first type is antagonistic to the acquired motor reaction (of the second type) and therefore inhibits its elaboration and occurence.

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Fig. 2. The comparison of the motor conditioned reflex to the metronome with the reflex to bubbling (From the exp. with Azor 21st March 1949): I. movements of the right foreleg, II. food reinforcement, III. conditioned stimuli: M, metronome, B, bubbling, IV. time (5 sec.), V. salivation (increases downwards), vertical arcs comprise conditioned reaction. The motor reaction to the metronome is much weaker than to bubbling.

Fig. 3. The comparison of the motor conditioned reflex to bubbling with the reflex to lamp, bell and metronome (From the exp. with Azor, 1st April 1949). I. motor reaction, II. VI. to show the isolated periods of conditioned stimuli in respect to motor and salivary reaction, III. food reinforcement, IV. conditioned stimuli: B. bubbling, L. lamp, Dz. bell, M. metronome, V. time (5 sec.), VII. salivation (increases downwards). The motor reaction to bubbling is much stronger than to the other stimuli.

Fig. 4. The elaboration of the motor conditioned reflex to a new stimulus (whistle) (Exp. with Azor 2nd April 1949). I. motor reaction, II. food reinforcement, III. conditioned stimuli: Gw. whistle, Dz. bell, IV. time (5 sec.), V. to show isolated periods of conditioned stimuli, VI. salivation. Whistle from the very beginning evokes a stronger motor reaction than does the bell.

Fig. 5. Comparison of the motor conditioned reflex to bubbling and whistle with the reflex to bell (From the exp. with Azor 3rd May 1949). I. motor reaction, II. VI. to show isolated periods of conditioned stimuli, III. food reinforcement, IV. conditioned stimuli: B. bubbling, Dzw. bell Gw. whistle, VII. salivation. The whistle evokes as strong motor reaction as bubbling and much stronger than the bell.

Fig. 6. The comparison of the motor reflex to bubbling with the reflex to the bell (From the exp. with Rex 22nd March 1949). I. motor reaction, II. food reinforcement, III. conditioned stimuli: B. bubbling, Dz. bell. IV. time (5 sec.), V. salivation. The bell applied for the first time after the motor conditioned reflex to bubbling was established evoked the motor reaction at the beginning of its action.

Fig. 7. The comparison of the motor conditioned reflex to bubbling and rotating object with the reflex to the bell (From the exp. with Rex, 24th May 1949). I. motor reaction, II. VI. to show isolated periods of conditioned stimuli, III. food reinforcement, IV. conditioned stimuli: W. rotating object, B. bubbling, Dz. bell, V. time (5 sec.), VII. salivation.

Fig. 8. The comparison of the motor conditioned reflex to bubbling with the reflex to bell, metronome and lamp (From exp-s with Bimbek, 19th May 1949 (a) and 24th June 1949 (b). I. motor reaction, II. VI. to show isolated periods of conditioned stimuli, III. food reinforcement, IV. conditioned stimuli: Dz. bell, B. bubbling, M. metronome, L. lamp, V. time (5 sec.), VII. salivation. In both these experiments bubbling evokes a much stronger motor reaction than the other stimuli.

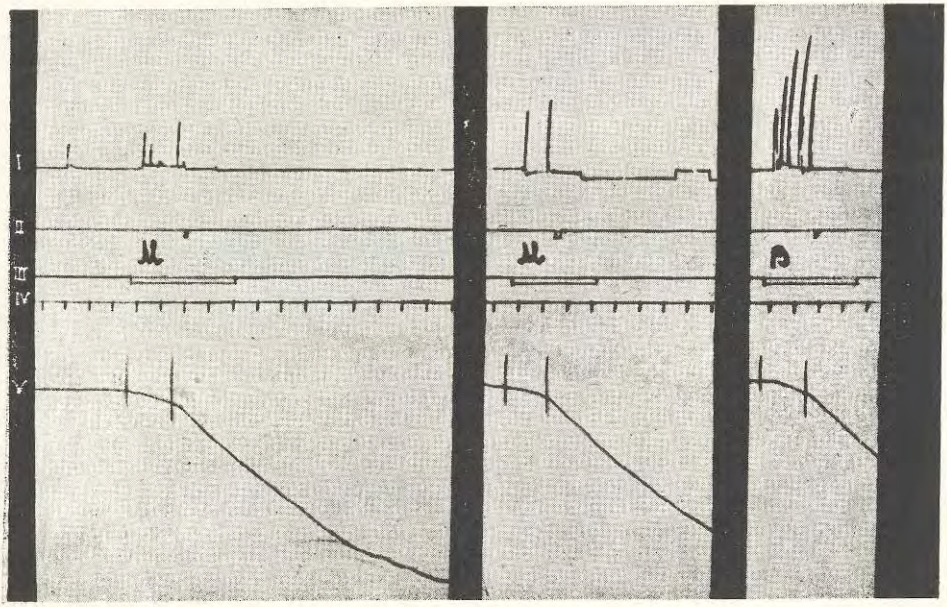


Fig. 2.

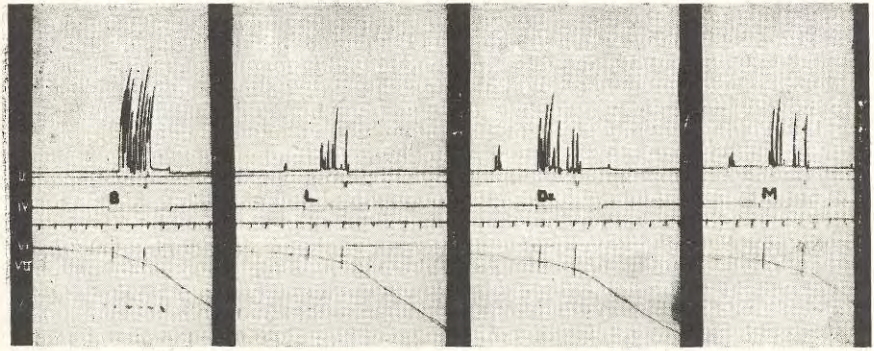
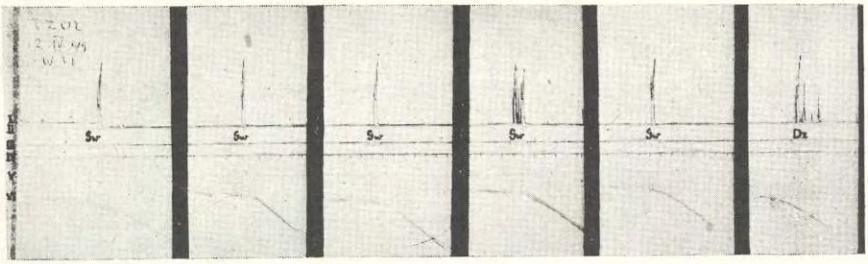


Fig. 3.



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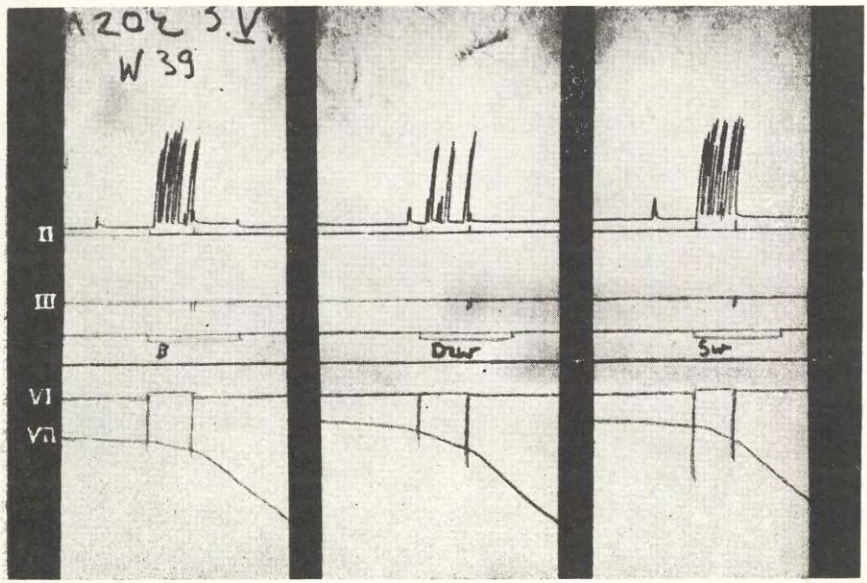

Fig. 5.

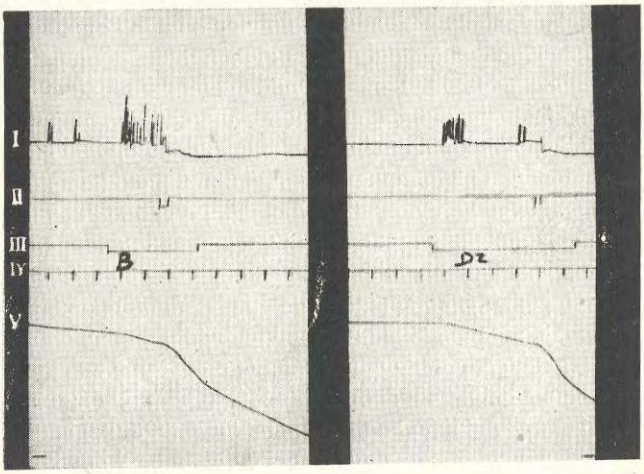

Fig. 6.

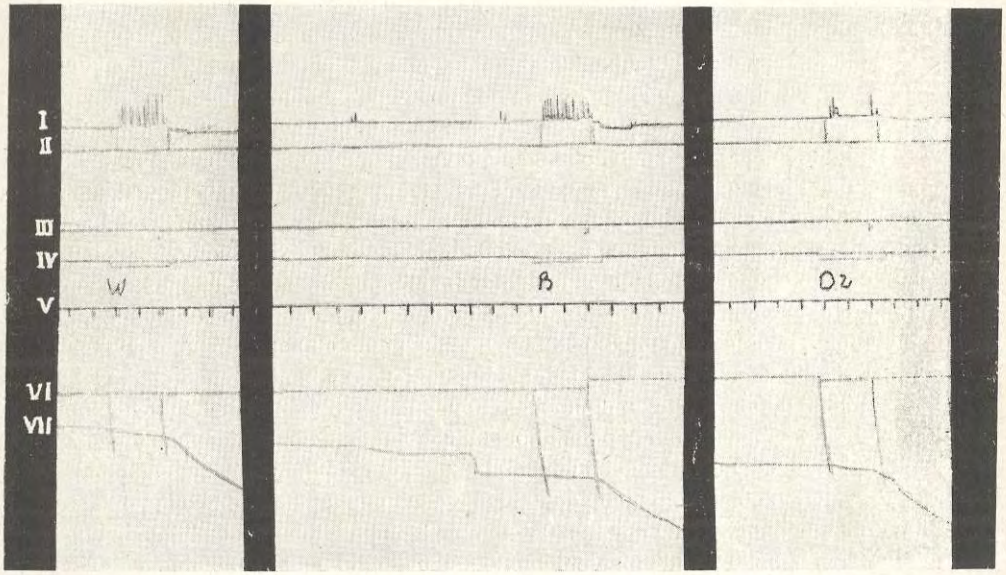

Fig. 7.

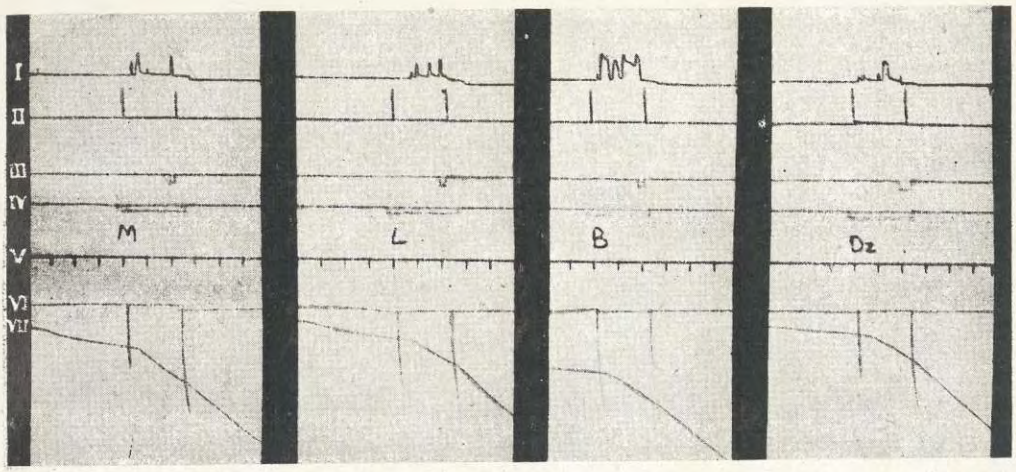

Fig. 8a.

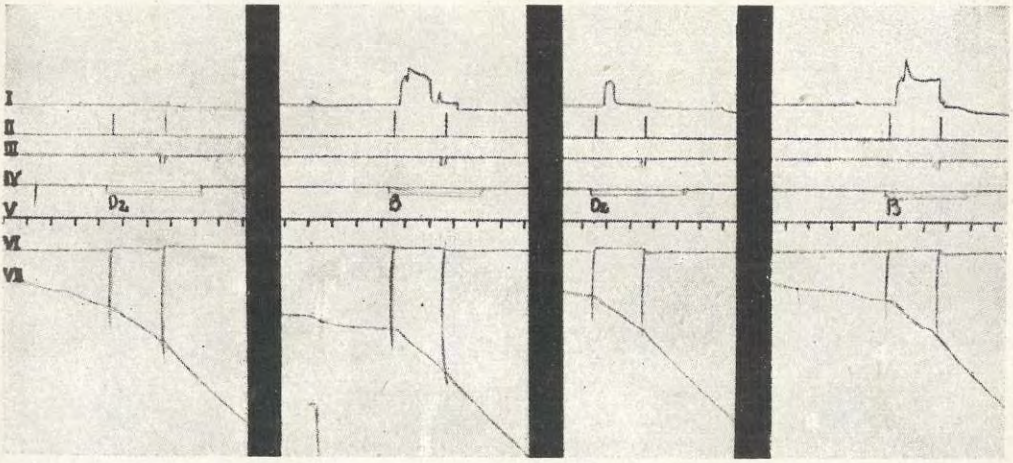

Fig. 8b.

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RESEARCHES INTO CONDITIONED REFLEXES OF THE SECOND TYPE

2. THE EFFECT OF THE DIMINISHED ALIMENTARY EXCITABILITY UPON CONDITIONED REFLEXES OF THE SECOND TYPE

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The influence of the diminished excitability of the alimentary centre on the conditioned reflex of the first type and the general behaviour of animals has repeatedly been the subject of research. Rikman's experiments, which were performed in Pavlov's laboratory (Pavlov 1938) showed that the reduced excitability of the alimentary centre in dogs caused a considerable decrease of the magnitude of conditioned reflexes; Rikman found in these conditions the phase of equalization and the paradoxical phase on a low level; that means that reflexes to strong stimuli were more diminished than reflexes to weak ones. The later experiments made by Skipin (1941) did not confirm Rikman's results. Skipin found that with a diminished excitability of the alimentary centre, the salivation in dogs decreases considerably but the decrease of the reaction is the same to all stimuli, or there is a stronger fall of the reflexes to weak stimuli than to strong stimuli. Skinner's experiments (1938) on the extinction of

habits in rats which had received food immediately before the experiment also showed the fall of the conditioned reactions and a greater facility of extinction in the same day.

The present paper is concerned with the investigation of changes in conditioned reflexes of the second type caused by the diminishing of the alimentary excitability. The results (which are more or less analogous to those obtained by Skipin and Skinner) are: 1) a general diminishing of reaction and the tendency to a greater fall of the response to weak stimuli than to strong ones, especially in reference to the motor reaction, and 2) an accelerated course of acute extinction. Besides this, a certain mutual independence of motor and salivary reaction was found.

METHOD

The experiments were made on dogs in an ordinary conditioned reflex chamber. Salivary and motor reaction of the animal was recorded by means of the method given in the former paper (Konorski and Wyrwicka 1950). Besides the automatic record there was a direct registration of salivation in the scales of manometer and also the observation of the general behaviour of the dog.

EXPERIMENTAL PART

The experiments were performed on three dogs: Azor. Rex and Bimbek; all are males, mongrels, varying in weigth from 11 kg (Bimbek) to 20 kg (Azor), and in age 2 — 3 years.

The experiments were made in two series. The first in May and June 1949 and the second in January and February 1950.

When the first series of the experiments was begun all dogs had well established conditioned reflexes of the second type to the sound of bubbling of water, the metronome (120/sec.), the bell and to the lighting of the lamp. Rex had a supplementary reflex to the object rotating before him. The motor reaction (of the second type) consisted in lifting the right foreleg. The dogs performed this movement either immediately after the outset of the stimulus or with the latency of a few seconds, and usually repeated it until the food was given. Simultaneously a copious salivation was observed. The isolated action of all stimuli lasted about 10 seconds. The salivary reaction was approximately the same to the bubbling, metronome and bell, but there was a lesser reaction to the lamp. On the other hand

the motor reaction was the strongest in all dogs to the bubbling, a little weaker (or frequently equal) to the metronome, but much weaker to the beli and lamp. The reason of this was that the bubbling was the conditioned stimulus of the second type from the beginning, and the metronome, bell and lamp were used formerly as conditioned stimuli of the first type. Likewise the rotating object, which was used for Rex from the beginning as a conditioned stimulus of the second type, caused a stronger motor reaction than did the bell and lamp.

The ordinary experiment in which the above mentioned stimuli were applied was repeated daily and was the background for special experiments performed every few days. These special experiments differed from the ordinary ones simply that just before going into the experimental chamber, the dog received the half of, or the whole of his daily supply of food, or also, besides this ration, a supplementary portion of food of which he was allowed to eat as much as he could or wanted to eat. In this way the alimentary excitability of the dog became lower than normal when he came to be experimented on in a hungry state.

The results of special and adjacent normal experiments are computed and represented on diagrams. The strength of the motor reaction is expressed in the reports by a number of crosses (4 crosses mean the maximal motor reaction of each dog), this strength being assessed according to the frequency and the height of the movements of the leg and their latent period. The salivation is measured in the scales of the manometer.

Let us consider in turn the adduced diagrams.

Fig. 1 shows two experiments with Bimbek. We see that after receiving the half of his daily ration before the experiment began, the salivation is reduced nearly equally to all stimuli and it diminishes gradually towards the end of the experiment. On the other hand the motor reaction in the first part of the experiment is more or less normal to bubbling and metronome but it is fully abolished to the bell. In the second part the motor reaction is greatly reduced to metronome and bubbling and there is no reaction to the lamp. When Bimbek was completely satiated with food before the experiment (fig. 2) there is observed a considerable fall in the salivation, the effect to the bubbling, metronome and bell being more or less equal, and to the lamp — nil. In spite of this slight salivary reaction the

dog does not take food during the whole experiment. On the other hand the movements appear only to the stimuli which are strongly „motogenic", i. e. the bubbling and metronome, but to the bell and lamp there is no reaction.

In fig. 3, which shows the experiments with Rex, it is observed that on receiving his whole daily ration before the experiment, both the salivary and the motor conditioned reaction is considerably reduced to all the stimuli. But again, the salivation is more or less equally reduced to all the stimuli and tends to diminish towards the end of the experiments. The motor reaction is more reduced to the metronome than to the bubbling.

After Rex had been satiated with food (fig. 4) the conditioned secretion of saliva ceased entirely. The dog did not take food (except at the first trial) during the whole experiment, but still it displayed motor reaction: to the bubbling a little stronger and to the metronome and rotating object a little weaker, but there was no reaction at all to the bell and lamp, which were the weakest motogenic stimuli.

Similar results were obtained in experiments with Azor (fig. 5).

After the above described experiments, conditioned reflexes of the second type were established to new stimuli: whistle and a tactile stimulation, and besides, the motor reaction of the lifting of the right foreleg was changed to the putting of the right foreleg on the food-tray. Generally, the dog having placed his foot on the tray did not take it down until it received reinforcement. In anticipation of food, depending on the strength of the stimulus, he lifted his food more or less frequently from the tray and hit it. For instance the whistle,strongly motogenic stimulus, caused (especially in Rex and Azor) a frequent tapping of the paw on the food-tray; on the contrary the weakly motogenic, tactile stimulus, caused quiet laying down of the foot on the tray without lifting it again. The size of the motor reaction was assessed mainly according to its latent period and intensity of the movement.

In the following we shall consider a series of experiments in which the bubbling, whistle and tactile stimulus were applied, i. e. only those stimuli which were trained from the very beginning as conditioned stimuli of the second type.

Fig. 6 and the report No 142 and 143 show two experiments with Bimbek: an ordinary and a special experiment before which

the dog received his ration of food for the whole day. We see that with the diminished alimentary excitability the salivation is reduced almost equally for each stimulus. As to the motor reaction it is observed only to strong stimuli: bubbling and whistling; the movement to the tactile stimulus appears only once in the beginning of the experiment and is very tardy (in 32") in comparison with the movement to whistle (in 7,5" and 10") and bubbling (in 12").

BIMBEK 23. II. 1950

Report No. 142

The dog did not receive any food before the experiment

Time	Stimulus	Latent period	Motor reaction	Salivary reaction	Reinforcement
1′	Whistle 10″	3.5″	++++	37	+
5′	Tactile stimulus 10″	9″	++	27	+
9′	Bubbl. 10″	4″	++++	28	+
13′	Whistle 10″	3″	++++	28	+
17′	Tactile stimulus 10″	6″	++	25	+
21′	Bubbl. 10′	6″	++	23	+
25′	Tactile stimulus 15″	12″	+	16 10″,10 5″	+

BIMBEK 24. II. 1950

Report No. 143

The dog received the whole of its daily ration of food before the experiment.

Time	Stimulus	Latent period	Motor reaction	Salivary reaction	Reinforcement
1′	Whistle 10″	7.5″	++	13	+
5′	Tactile stimulus 20″	32″	±	8|10″,4|10″	+
9′	Bubbl. 10″	12″	+	12|10″,8,3″	+
13′	Whistle 11″	10″	+	5	+
17′	Tactile stimulus 20″	—	—	3|10″,3|10″	—
21′	Bubbl. 15″	22″	+	9|10″,8,5″	+
25′	Tactile stimulus 20″	—	—	2,10″,2|10″	—

The experiments with Rex (fig. 7 and the report No 141 and 142), having received the whole of his daily ration of food just before the experimental session shows the same picture: both the salivary and the motor reaction are more or less equally reduced to all the stimuli.

Only at the end of the experiment the salivary reaction drops almost to nil and the motor reaction to the tactile stimulus appears after the cessation of the stimulus.

REX 23. II. 1950

Report No 141

The dog did not receive any food before the experiment

Time	Stimulus	Latent period	Motor reaction	Salivary reaction	Reinforcement
1′	Bubbl. 10″	<1″	++++	17	+
5′	Whistle 10″	<1″	++++	22	+
9′	Tactile stimulus 10″	1,5″	+++	12	+
13′	Bubbl. 10″	1″	++++	7	+
18′	Whistl. 10″	1″	++++	12	+
22′	Tactile stimulus 10″	1,5″	+++	7	+
26′	Bubbl. 10″	2,5″	++++	13	+

REX 24. II. 1950

Report No 142

The dog received the whole of its daily ration of food before the experiment

Time	Stimulus	Latent period	Motor reaction	Salivary reaction	Reinforcement
1′	Bubbl. 10″	1,5″	+++	6	+
5′	Whistle 10″	4,5″	+++	9	+
10′	Tactile stimulus 10″	4″	++	6	+
14′	Bubbl. 10″	4″	+++	3	+
18′	Whistle 10″	4,5″	+++	8	+
22,5′	Tactile stimulus 20″	26″	±	0|10″,3|10″	+
26,5′	Bubbl. 10″	5″	++	1	+

The experiment with Azor (fig. 8 and the report No 153 and 154) confirm the above results: the salivation is considerably and rather equally reduced to all stimuli; on the contrary the motor reaction is much more lessened to the tactile stimulus than to other stimuli, where an equal fall is observed.

Besides these experiments we performed two experiments with Azor and Bimbek in which the acute extinction of a conditioned reflex was conducted after the dog had received the whole of his daily portion of food just before the experiment. The results of the

AZOR 20. II. 1950

Report No. 153

The dog did not receive any food before the experiment

Time	Stimulus	Latent period	Motor reaction	Salivary reaction	Reinforcement
1′	Whistle 10″	3″	++++	12	+
5′	Bubbl. 10″	6″	+++	16	+
9′	Tactile stimulus 10″	9″	+	8	+
13′	Whistle 10″	4″	+++	13	+
17′	Tactile simulus 10″	9″	+	10	+
21′	Bubbl. 10″	8.5″	++	13	+
25′	Whistle 10″	4″	+++	10	+

AZOR 21. II. 1950

Report No 154

The dog received the whole of its daily ration before the experiment

Time	Stimulus	Latent period	Motor reaction	Salivary reaction	Reinforcement
1′	Whistle 10″	8″	++	0	+
5′	Bubbl. 15″	11″	+	0|10″, 1|5″	+
9′	Tactile stimulus 25″	22″	±	1|10″, 1|10″	+
13.5′	Whistle 10″	7″	++	3	+
17.5′	Bubbl. 30″	15″	+	1|10″,8|10″.10|10″	+
23′	Tactile stimulus 30″	29″	±	0|10″,3|10″,3|10″	+
27′	Whistle 10″	5″	+++	4	+

acute extinction against the background of the reduced alimentary excitability were compared with the results of the acute extinction of the same stimulus (whistle) in normal conditions.

As we see in fig. 9, which shows the course of extinction in both cases in Azor, in the state of a reduced alimentary excitability the secretion of saliva is much lessened from the beginning and speedily falls almost to nil. On the other hand the movements are kept to almost the same level in the first four trials; and in further four trials the motor reaction is reduced, while the salivary reaction has entirely ceased.

The comparison of the reports of experiments with the extinction of the conditioned reflex to whistling with Bimbek (fig. 10) gives a still more impressive picture. In the state of a lessened alimentary

excitability, the salivation from the beginning is largely reduced and it falls to nil after 5 trials, while in normal extinction it does not vanish even after 14 trials. The motor reaction disappears in the 7th trial in the state of diminished alimentary excitability, while in normal conditions it is still seen in the 14th trial.

CONCLUSIONS AND DISCUSSION

The results of the above described experiments can be summarized as follows:

1. The reduction of the alimentary excitability causes the decrease in both the salivary conditioned reaction (of the first type) and the motor conditioned reaction (of the second type).

2. This decrease is either equal for all stimuli or the reaction to weak stimuli is more reduced than to strong stimuli. This last is more discernible in the motor reaction.

3. The acute extinction of the conditioned reflexes of the second type in the state of low alimentary excitability proceeds quicker than in normal conditions.

4. In the state of a strongly reduced alimentary excitability the motor reaction to strong motogenic stimuli can still be observed although it is greatly diminished, whilst at the same time the secretion of saliva has ceased and the dog does not take food any more.

The first conclusion is evident even by a superficial examination of the reports and diagrams of experiments. The salivation as well as the movements are diminished more or less proportionally to the state of the satiation of the dog. This is well seen if we compare the experiment with Bimbek after having given the dog the half of its daily ration of food (fig. 1) to the experiment after having plentifully fed the dog (fig. 2). The same is observed in the 3rd and 4th diagram representing the experiments with Rex. The more he was satiated the less was the motor reaction and secretion of saliva. This principle in reference to the salivation is generally known to all students of conditioned reflexes of the first type; it is manifested in the fact that in the beginning of the experiment (with alimentary reflexes) the dog always shows a greater conditioned reaction than afterwards, when he becomes more and more satiated.

The second conclusion calls for a wider discussion. As far as the salivary reaction is concerned, it is reduced more or less equally to

all the stimuli and only here and there we can observe a greater fall of salivation to weak than to strong stimuli (fig. 1 — lamp, fig. 7 — tactile stimulus). The situation is somewhat different in regard to the motor reaction. When the alimentary excitability is lowered not very much the motor reaction to strong motogenic stimuli (bubbling, whistle) is only slightly reduced (cf. fig. 1 first part. fig. 7 first part); when the dog is more satiated the reaction is more reduced but it is never completely abolished. On the other hand, the motor reaction to weak motogenic stimuli is much more „sensitive" and drops down very early. So in nearly all our experiments of the first series the motor reaction to the bell and lamp is fully suppressed and in the second series the same is observed in regard to the tactile stimulus. And so in the state of a diminished alimentary excitability there is much greater discrepancy between strong and weak motogenic stimuli than in normal condition.

Let us turn to the next conculusion. The acute extinction conducted against the background of reduced alimentary excitability proceeds more quickly than in normal conditions. This is easy to understand in view of the fact that the stimulus to be extinguished elicits a diminished conditioned reaction from the very beginning of the experiment. It looks as if the beginning of the extinction conducted against the decreased alimentary excitability correspond to the later stages of the normal extinction. So the beginning of the extinction in Azor, after the dog was fed, corresponds more or less to the 4th extinction trial in normal conditions (fig. 9) and the first extinction trial in Bimbek, after the dog was fed, corresponds more or less to the 6th trial of normal extinction (fig. 10). In reference to the motor reaction the situation is somewhat different. In Azor the motor reaction is extinguished much more slowly than the salivary reaction and persists when the salivation is already abolished. In Bimbek extinction of the two reactions occurs more paralelly which is due to the fact that this dog in general had a very copious salivation in comparison with other dogs.

The earlier disappearence of the salivary reaction in comparison with the motor reaction is met very often in the state of a strongly reduced alimentary excitabillity (our 4th conclusion). As is seen in fig. 4, after being satiated Rex displayed no salivary conditioned reaction at all and did not take food (with the exception of the first trial) throughout the experiment and nevertheless he raised his leg to all stronger stimuli. Just the same is seen in an analogous experi-

ment with Azor (fig. 5): the salivary conditioned reaction is nil (except slight salivation in the 4th trial), while the motor reaction to the strongly motogenic stimulus still persists. It seems that the motor reaction is less directly dependent upon the excitability of the alimentary centre than is the salivation and therefore it can be still elicited when the salivary reaction is fully suppressed. It is also possible that the motor reaction to strongly motogenic stimuli after a time acquires some „automatic" character and is displayed to these stimuli somewhat independently of whether the dog „wants" food or not.

SUMMARY

The influence of a reduced alimentary excitability upon the conditioned reflexes of the second type was examined in three dogs. The reduction of both salivary and motor reaction was observed which was more or less proportional to the degree of satiation. Generally the reduced excitability affected more strongly the reflexes to weak than to strong stimuli, the difference being particularly well expressed in regard to motor reaction. The acute extinction of the reflex was accelerated in conditions of reduced alimentary excitability in comparison with the extinction in normal conditions. With very strongly diminished alimentary excitability, in spite of the full suppression of salivary reaction and the refusal of the dog to take food, the motor reaction to strongly motogenic stimuli could still persist.

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Fig. 1 — 10. Each picture represents the course of two experiments: a normal (control) experiment, and special experiment (in the state of a reduced alimentary excitability).

Abscissae denote successive trials in an experimental session. The letters below denote the names of stimuli: B — bubbling, M — metronome, Dz — bell, L — lamp, W — rotating object, Gw — whistle, D — tactile stimulus.

Ordinates represent the magnitude of a conditioned reaction. In regard to salivation the figures denote the amounts of saliva secreted during the action of the conditioned stimulus in 0.01 ml. In regard to the motor reaction 20 units in the case of Azor and Rex, and 40 units in the case of Bimbek, represent the maximal motor reaction (in reports 4 crosses).

The motor reaction is expressed by rectangles. The higher (uncheq-uered) rectangles represent the magnitude of the motor reaction in contrai experiments, the lower (chequered) rectangles represent the magnitude of the motor reaction in experiments with a reduced alimentary excitability. Columns below the abscissa (fig. 5) denote the motor reaction immediately after the cessation of the stimulus.

The salivary reaction is expressed by broken line. The upper line represents the magnitude of conditioned reactions in control experiments, the lower line represents the magnitude of conditioned reactions in experiments with a reduced alimentary excitability.