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CONTENTS

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ON THE PLASTICITY OF INSTINCT OF A GARDEN SPIDER (*Aranea diadema* L.) CONSTRUCTION OF A COBWEB

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The process of building a cobweb by a garden spider, like any other complicated function of lower animals, is usually classified as a manifestation of instinct. According to a classical definition by Ziegler an instinctive action is a stereotypic action of a blind mechanical character. More recent works draw attention to the fact that instinctive actions of animals, living in conditions widely different from normal, are adapted to new conditions and show distinct plasticity.

Experiments on the building of cases by caddis-fly larva (Dembowski, Teyrowski) show evident variations in the manner of repairing the case, in selection of building materials and in the character of the construction itself. The behaviour of the larva is obviously plastic. Similar results were obtained by Bierens de Haan with the caddis-fly larva *Limnophilus*. The plasticity of the process of case construction by *Nymphula nymphaeta (Pyralidae)* larva was established by Miklaszewska. Petrusewicz proved the plasticity of behaviour by spiders in cobweb construction. This author removed the spiders, in the act of building, from one cobweb to another which was in a different phase of construction: from cobwebs less advanced in construction to cobwebs more advanced and vice versa.

In this way it was proved that spiders are capable of changing their normal order of work. Spiders may jump over one phase of work or go back to an earlier phase, and can repeat an action already once executed. Such a behaviour of the spider can be understood only on the premise that the spider somehow controls its actions. A different, view, however, also has adherents. Fankhauser and Reik, for example, are of the opinion, based on experiments with larva *Neuronia,* that instinctive actions are not controlled. The plasticity of behaviour of animals is closely related to the plasticity of the nervous system. Bethe, Dembowski, Woitas and Thorner, in their research work, have amputated legs of several specimens of arthropoda in various combinations and then placed under observation the functional changes of the remaining legs. These authors established that each type of amputation evoked a corresponding modification in the coordination of movements. Legs which normally move alternately can move simultaneously and vice versa. There is a very large number of combinations of coordinated movements as substitutes. The authors come to the conclusion that the functions of the nervous system are plastic and that the nervous system is capable of adaptation to the changed situation. With the non-vertebrates the change of functions and the new coordination of movements appear immediately after the operation and there is no need of learning. An extirpation of the brain convolutions on the whole had no visible effect on the phenomena of substitution. In the opinion of the authors small disturbances which were observed were of a secondary character.

Experiments of Asratian on the amputation of dog's paws have proved the gradual learning of new substitute motions and the dependence of compensating activities on the cortex. He is therefore critical of the Bethe theory as applied to vertebrates. He agrees however that the participation of the cortex and the process of learning new substitute motions decrease as we mowe down the evolution ladder. The author did no experiments with non-vertebrates.

A study on the effects of amputation of spiders' legs was performed by Petrusewicz in 1938/9 at the Institute of Biology of the University of Wilno. This work was done just before the war and did not appear in print. The manuscript was lost. It has, however, been possible to peruse the notes of the author, who insists on the existence of substitute movements and the ability to construct

a regular web by spiders with amputated legs. Unfortunately, it has not proved possible to deduce from the notes what was the view of the author on the phenomenon of action compensation.

Like Petrusewicz, it was hoped to influence the process of construction of the cobweb through amputation of legs. Our aim was to ascertain whether actions of lower animals according to Ziegler's view, always correspond with the normal destination of their limbs, or whether in changed conditions new substitute actions are brought out. In the spinning of a cobweb the chief work is performed by legs, so their amputation, bringing confusion to the normal coordination of movements, should in a certain degree change the process of cobweb construction. Spider's legs serve not only to the stretching and pasting of the web but also to the measurement of distance. The last limb of spiders' legs is specially adapted to the manipulation of the thread and ends with a chitin claw and with resilient chitin thorns. In the normal process of web construction each pair of legs performs a specific function and the movements of the legs are strictly coordinated.

It was therefore necessary to ascertain whether operated spiders, with fewer legs active in the construction, can build a normal web. If actions connected with the process of cobweb construction are rigid and the nervous system shows no plasticity then the amputation of legs, usually active in construction, must lead to a disturbance in the construction. The capacity of operated spiders to build a regular web can be considered as a proof of their plastic behaviour and of the existence of substitute functions.

In connection with the first problem the question is raised as to how the missing legs are compensated for in the process of construction and what new coordinated movements must be evolved.

MATERIAL AND METHODS

Material for these experiments were young specimens of garden spider *Aranea diadema L.* For most of the experiments spiders, up to two months old were used. Only a few were performed on older spiders of about three months of age. The spiders were obtained from cocoons collected in autumn and preserved on frost or in a refrigerator. In January the cocoons were removed to the laboratory where the young spiders began to hatch.

For spinning the web the spiders were given square wire frames of two sizes — 8 cm. and 10 cm. supported vertically. A cobweb stretched on such a frame can be easily removed from place to place and makes observation, photography, and adequate lighting rather simple. In order to take a picture

the spider was chased with the aid of vibrating tweezers. Vibration makes the spider go down. In order to make the delicate threads of the cobweb visible they were spayed with powder.

The research proper consisted of observation of the process of web building by spiders with fewer legs than normal. Legs were cut off under a light ether anaesthetic. Spiders were placed for a few seconds in a jar with some cotton saturated with ether. As soon as the spider became motionless it was removed on a Petrie scale and the amputation performed immediately. A light ether anaesthetic is harmless and does not influence the character of the web, but a prolonged stay of the spider under ether may have a bad effect.

THE FIRST COBWEB

The cobweb of a garden spider belongs to the radiant type. The process of building the web was described precisely by Wiehle, Peters and Petrusewicz.

Young spiders, freshly hatched from the egg, do not spin a thread. After a few days comes a second moult and then the spider begins to spin a thread but is still unable to build a radiant web. We can see only a disorderly entanglement of dry web. It is caused by the spider's movements from a thread which it drags along. Separate spiders were placed on a wire frame and the web observed. With the exception of a scaffold which was like the letter „Y", which, by the way, was not very distinct, no element of a radiant web was ever observed nor radii or elements of a spiral.

After leaving the egg until the first moult is over the spiders remains in the cocoon and rest assembled. When the moult is over their behaviour undergoes a radical change. The young spiders leave the cocoon, rise in the jar to the lighted side, and disperse.

According to the observation made by Petrusewicz, the regular radiant web emerges suddenly, two weeks more or less, after the hatch. The first cobweb is just as perfect as the following ones. Undoubtedly there are no elements of learning in it.

COMPARISON OF COBWEBS BUILT BY NORMAL AND BY OPERATED SPIDERS

An analysis of cobwebs was carried out from photographs. Analyses were made of 212 cobwebs built by spiders with various types of amputation. For the sake of verification analyses were also made of 118 cobwebs built by normal spiders. The following factors were taken into consideration: the number of radii; the number of rows of the viscid spiral, the way the viscid spiral ended; the assymetry

of the cobweb and all deviations from the normal arrangement of the cobweb.

The cobwebs of the control spiders (which underwent no operation) were on the whole regular but showed large individual differences in the number of rows of the viscid spiral as well as in the number of radii. Control spiders from an old exhausted culture and one of a new culture produced irregular webs and this phenomenon came out in bulk. Evidently the bad state of the young culture and the exhaustion of the spiders lead to disturbances in the construction. Spiders from a new, strong culture did spin 12 and even 13 webs, one after another (one web daily), and then ceased to spin at all but there was no disturbance in the construction. All cobwebs were regular with no omissions or faults.

The webs of operated spiders show far greater individual variations. In a group of spiders with legs amputated in the same way there were regular webs side by side with completely entangled ones. The amputation of legs is of course connected with a loss of blood, so that the character of the web built by operated spiders is influenced not only by the lack of legs but also by the weakening of the spider, caused by the loss of blood. The analysis of the web was also made with a view to ascertaining whether spiders deprived of several legs, produce corresponding substitute movements of the adjacent legs, as a result of which the normal structure of the web is preserved. Normally each leg has a specific function to perform in the building of the web. The first and, in an auxilliary way, the second pair of legs, serve to appraise the dimensions. This can be seen in the process of stretching the radii and while stretching the viscid spiral. While pulling the radii the spider stands in the centre leaning the first and the second pair of legs against the radii. If the distance between the radii is large the strech between the legs is greater and in this place the spider lays an additional radius. While stretching the viscid spiral the spider measures the distance from the spiral guy line and from the preceding row of the viscid spiral with the first and second pair of legs. The fourth pair of legs serves for pulling and pasting the thread and to support it.

If the neighbouring legs would not substitute the functions of the missing ones, then the lack of legs would necessarily cause a disturbance in the construction. The fact that operated spiders build a regular web proves the existence of plasticity and of substituted movements.

Table I

A statement concerning the number of radii and the number of rows of the viscid spiral in webs built by operated and by control spiders.

Experiment No.	Type of operation	Number of analysed webs	Average number of radii	Min. number of radii	Max. number of radii	Average number of rows of viscid spiral	Min. number of rows of viscid spiral	Max. number of rows of viscid spiral	Number of regular webs	Number of irregular webs
1	1 (a)	14	17	11	30	13	2	20	6	8
2	2 (b)	20	15	7	27	13	6	23	10	10
3	3 (c)	22	22	11	26	21	10	28	15	7
4	4 (d)	12	23	16	27	20	11	23	8	4
5	1 + a	15	24	15	31	18	10	25	5	10
6	2 + b	12	24	19	30	21	13	32	12	0
7	4 + d	11	17	12	22	14	7	20	4	7
8	1 + 2	15	23	17	27	17	8	25	4	11
9	3 + 4	18	21	16	25	18	11	26	7	11
10	2 + 3	15	22	19	28	19	9	27	6	9
11	1 + 4	13	22	14	28	19	10	26	4	9
12	2 + 4	11	23	16	27	18	12	24	6	5
13	1 + b	14	22	14	29	19	13	28	5	9
14	3 + d	6	22	20	24	18	16	20	3	3
	total for all types	198	21	—	—	17	—	—	95	103
	Control webs	118	24	—	—	21	—	—	—	—

Several negative cases, where the spider did not build a web at all, were not taken into consideration. Several positive cases were also omitted, since the principal object was to ascertain, not whether every operated spider did build a regular web but whether it was within its bounds of possibilities to do so.

Starting from these premises a selection has been made, for illustrative purposes, of the most regular webs of the operated spiders, which do not differ essentially from the webs built by normal spiders, Fig. 1. (The number of regular webs is of course greater, but is not included in the picture). These illustrations show that spiders with one or two amputated legs in any combination are able to build a regular web, there must therefore be a change in the coordination of movements.

In irregular webs the deviations which occur most often can be brought down to the appearance of void places, to the reduction of the number of rows of the viscid spiral and to a certain assymetry of the web. Though no attempt was made to solve the problem in a statistical way, nevertheless some numerical data which give a picture of the normalcy of a cobweb is presented in Table I.

Webs of the control (non-operated) spiders had an average of 24 radii. The fluctuations in the number of radii were rather large, the smallest number being 9, the largest 35. These data are in accordance with those of Petrusewicz. According to the above author the initial cobwebs have fewer radii, the number increases with age, and the greatest number of radii are due to spiders of about 3 months of age. For young spiders, Peters gives the number of radii as about 50, but does not state the age. Our spiders at the age of about 2 months never built a web with such a large number of radii. All authors are unanimous that above this age the number of radii decreases.

Table II

A comparison of the number of radii in webs of operated and of control spiders.

Number of radii	Number of webs		% of webs	
	operated	control	operated	control
0 — 10	2	2	1%	2%
11 — 15	24	11	12%	10%
16 — 20	59	46	28%	39%
21 — 25	100	21	47%	17%
26 — 30	25	26	12%	22%
over 30	1	12	0%	10%
Total	211	118	100%	100%

The average number of radii for all types of operations in webs built by operated spiders amounted to 21 (Table I).

The scale of fluctuations of the number of radii was greater for the control spiders than for the operated spiders (Table II). In the control webs 10% of the total number of webs had more than 30 radii. In the webs of operated spiders 30 radii was the upper limit and only one web had over 30 radii. The analysed webs were divided into groups according to the number of radii. This showed that among the webs of operated spiders the most numerous group comprises those with a number of radii from 20 to 25. This group includes almost one half of all analysed webs built by spiders with ampu-

tated legs (47%). In the case of control spiders however the most numerous group comprised webs with a somewhat fewer number of radii, namely from 16 to 20. It should not be supposed however that there is an essential difference in the number of radii in webs built by operated spiders, as about 80% of webs built by control and operated spiders alike, have from 15 to 30 radii.

According to Peters the arrangement of the radii in the web corresponds to the arrangement of the spider's legs. In each cobweb the angles between the radii in the upper part of the web are larger than the angles between the radii in the lower part. Peters called attention to the fact that a spider lying in wait always sits head downwards, with forelegs resting on the lower radii and hind legs resting on the upper radii. The angles between the legs of the first and second pair are smaller than the angles between the third and the fourth pair of legs. The measurements made by this author seem to prove that angles between the radii of the lower and the upper part of the web differ in the same degree as the angles between the first and the second leg differ from angles between the third and the fourth leg. An amputation causes a parallel enlargement of the angle between the radii and in consequence a decrease of the number of the radii. According to the said author the amputation of forelegs causes a decrease in the number of radii in the lower part of the web and an amputation of hind legs a decrease in the number of radii in the upper part of the web.

With the help of our material a comparison was made between the average number of radii in the upper and lower parts of the web, and the differences between the number of radii in the upper and lower parts of the web are defined in percentages. This method differs from that employed by Peters in so far as the comparison is made between the average numbers of radii in webs of different spiders, while Peters compared the number of radii of the same spider before and after the operation. The control webs contained on the average 18% more radii in the lower part than in the upper part. In a group of spiders with the first pair of legs amputated the difference in the number of radii in the lower and the upper part amounted to 20%. Though the angle between the legs (the first pair of legs being missed) was enlarged there was no enlargement of the angle between the radii in the lower part of the web, just the opposite —the angle between the radii became a little smaller.

Five types of operation give data contradictory to these of Peters. Only one type of operation gave a diminution of the angle, agreeing with Peters, and only one type gave an enlargement of the angle. The average of the numerical data proves amputation alone does not lead to a decrease in the number of radii and does not disturb the process of their stretching.

Although the numbers, and the method of study used here, are different from those used by Peters, and our data are not comparable without reservation, yet they throw a certain light on the problem of dependence of the angle between the radii on amputation.

A numerical analysis of the rows of viscid spiral is shown on Table I. The webs of the control spiders have on the average 21 rows of viscid spiral. The number of rows of the viscid spiral in webs

Table III

A comparison of the number of rows of the viscid spiral in webs built by operated and by control spiders.

Number of rows of viscid spiral.	Number of webs		% of webs	
	operated	control	operated	control
0 — 10	20	5	9%	5%
11 — 15	47	8	22%	7%
16 — 20	88	27	42%	25%
21 — 25	48	43	23%	40%
26 — 30	8	22	4%	21%
over 30	1	2	0%	2%
Total	212	107	100%	100%

built by operated spiders fluctuated between the limits of 13 and 21 and on the average amounted to 17.

Among the webs built by operated spiders most numerous were webs of 16 to 20 rows (42%). Webs with 21 to 25 rows of viscid spiral comprised 23%. Webs of control spiders had mostly 21 to 25 rows of viscid spiral (40%) but still a great number (21%) had more than 25 rows. The upper limit in the number of rows of viscid spiral for control spiders can be taken as 30 (Table III). The numerical data seem therefore to indicate a certain decrease in the number of rows of viscid spiral in webs built by operated spiders. This may be due simply to the weakening of the spider as a result of the operation.

The character of the viscid spiral, in webs built by operated spiders, shows also, more often than the arrangement of radii, certain

deviation from normal. It is true that some webs of operated spiders had a perfectly regular viscid spiral, but beside such webs there were also webs with an entangled spiral, some with loops between the rows of the viscid spiral, others with an asymmetric spiral. In control webs, as well as in webs built by operated spiders, viscid spirals in most cases ended in the free zone. In a few cases webs of operated spiders had the free zone completely embroiled. In some webs which were built after operation a part only of the spiral guy line was cut off and some of its rows remained in the finished web.

On the whole our analysis does not show the amputation to disturb the building process. In each type of operation (after one or two legs were amputated) a certain percentage of webs had a regular structure, the deviations in all cases being explicable as a consequence of a general weakening of the spiders, caused probably by loss of blood. The substitution of the legs is evident. Moreover the large fluctuations with the control spiders indicate that the differences obtained are not statistically significant.

THE WAY SPIDERS WITH AMPUTATED LEGS BUILD A COBWEB

A comparison of a web built by operated spiders with a normal web indicates that spiders with one or two amputated legs may construct a regular web. It was therefore necessary to ascertain what auxiliary motions of their limbs enable the operated spiders to spin a regular web. In connection with this problem observations were made on a number of spiders, with one or two legs missing, in the process of building a web, in order to ascertain to what degree each separate leg participates in the building of the web and in what way the remaining legs perform the work of the missing ones. At the same time it was desired to clear up the source of loops and of asymmetric webs.

Observations were made on the process of construction used by some 50 spiders which had undergone various types of operation.

A spider with only the last limb of the leg amputated performs normal functions with the operated leg while building the web. The amputation of the last limb of the leg deprives the spider of claws. Judging from the construction of the claws it would seem that they are destined to stretch the web and that a spider deprived of its claws would paste itself to the sticky threads. Yet experience proves that spiders make use of the operated leg in manipulating the viscid silk and in measuring distances.

Legs of which one half are amputated do not participate in the work, and are substituted by a neighbouring leg. According to an observation made by Bethe spiders, insects and shellfishes change the arrangement of the legs after such an operation. Normally the first pair of legs is directed forwards, and the second a little to the side. The first leg being amputated, the second can take a position held previously by the first. (Fig. 2, drawing 1 ab). In case the first right and the second left are amputated the second right leg moves to the position of the first and movements of both legs while working are perfectly coordinated (Fig. 2, drawing 1 c).

The operated spiders perform the work of stretching the radii without any disturbance. Spiders, with one or two legs cut off in various combinations, stretched the radii always in a normal manner.

Troubles in construction appeared only when the spiral guy line was being stretched and chiefly when the viscid spiral was stretched. Evidently it is easier for the spider to pull the radii than to stretch the viscid spiral. The conclusion reached while observing the act of building identical with the one obtained by comparing various webs. In webs built by operated spiders the arrangement of the radii and their number were within normal limits, and in a certain number of webs the character of the viscid spiral and the number of the rows differed from normal.

The disturbances depend to a certain extent upon the type of operation. The amputation of one leg only is easily compensated for by the spider. While building the viscid spiral, legs of the first pair measure the distance from the spiral guy line on the one side and from the preceding row of the viscid spiral on the other. The second pair of legs plays an auxiliary role. Where a first leg is amputated a second leg takes its position and performs its functions. Functionally the work of the first pair of legs is now done by one first and one second and their movements are perfectly coordinated.

In case a second leg is amputated the arrangement of the legs remains unchanged and all functions of measuring are done by the first pair of legs.

The amputation of a third leg has the least influence on the process of building. It seems that the function of the third pair of legs is merely to keep the spider in balance while building the web. Small disturbances in the web built by spiders deprived of their third leg are probably due to secondary factors.

If a fourth leg is missing it can be substituted for by a third, if the latter is long enough, (as with old spiders). If the third pair is too short the spider pastes a thread by sitting with its spinnerets on the web. A symmetrical amputation of the first or of the second pair of legs causes no fundamental disturbance in the construction. All functions of the first pair may be done by the second and vice versa. (Fig. 2, drawing 2 ab).

Somewhat greater difficulties are caused to the spider by the amputation of the fourth pair of legs, chiefly in the process of fastening of the viscid spiral. Normally the spider pulls the thread from the spinnerets with its fourth leg and fastens it to the radius with a fourth leg of the opposite side. In case of amputation it pulls it from the spinnerets gradually while moving away from the previous point of attachment. A thread which is not upheld often splits. By sitting with the spinnerets on a radius the spider pastes the thread (Fig. 2, drawing 3 ab). Sometimes older spiders whose third pair is long enough help themselves with this third pair.

In case of an asymmetric amputation, i. e. cutting off the first right and the second left (or vice versa) movements of legs of different pairs which work together as substitutes become coordinated. When a second and a fourth leg are amputated then a first leg and the spinnerets work as substitutes, occasionally a third leg also helps. In case a second and a third leg are amputated the work is done by a first and a fourth leg. Spiders with a first and a second leg on the same side amputated were observed. When the spider was turned inward with the operated side measuring of the distance and the fastening of the viscid silk proceeded normally. When after a change of direction the spider turned the operated side outwards appeared loops and faults, in some sectors of the web. It is not always that the spider behaves so in such a situation. A spider was observed which first took the measure of the distance to the spiral guy line with the help of the first and of the second leg, then without change of direction turned the first leg outwards to the viscid spiral and took the measure to the preceding row of spiral (Fig. 2, drawing 2c). He repeated this action many times and the result was a pretty regular arrangement of the segment. Similar behaviour on the part of a spider during a normal process of building was never noted. This fact is worthy of special attention as a substitute movement was done not by a neighbouring leg, but by a leg from the other side, in addition to its normal function.

It is also interesting that the lack of one leg of a first or of a second pair on the side turned towards the viscid spiral usually causes greater confusion than its lack on the side turned toward the spiral guy line.

The question as to how the measuring of the distances between the rows of the viscid spiral is done, has called forth the attention of a number of authors. According to Hingston the distance between the rows of the viscid spiral is measured by the spider from the preceding row of the viscid spiral. Petrusewicz cites a number of observations which tend to show a more important role of the spiral guy line in the normal course of the viscid spiral. Peters calls attention to a whole number of other factors such as the eliptic course of the spiral, the appraisal of the relation of the width to the height of the segment which influence the course of the viscid spiral, etc.

We may suppose that the regular course of the viscid spiral depends on a whole assembly of factors but it can be partially changed depending on the situation of the spider.

It was not always possible to find the cause of faults and entanglements in the web. In the building of webs by spiders with one amputated leg, the arrangements of legs and the substitute motions performed by the neighbouring leg enabled the spider to appraise correctly the distance and to stretch the viscid spiral regularly, and yet these spiders built far worse than some spiders with two amputated legs. The construction of a regular cobweb depends not only on the arrangement of the legs, but on a number of other factors connected with the amputation.

DISCUSSION

An analysis of the webs built by spiders with amputated legs, shows the existence of substitutional motions. Under the influence of a post-operative shock and of anaesthesia spiders forego the spinning of a web immediately after the operation. Following the operation they were left for a whole night in a small jar and only the next day were they put on the frames. Spiders, in the first phase of cobweb building, do not improve in efficiency while manipulating. New coordinated movements appear at once. In the light of these observations a compensation of actions has a different course than with the vertebrates and comes without a previous period of learning.

Most of our experiments were concerned with amputation of one half of the legs. Legs which underwent operation did not participate in construction. Spiders with one or two amputated legs were capable of building a regular web. The number of radii and the number of rows of the viscid spiral in webs built by operated spiders did not differ substantially from numbers obtained for control webs. The differences lie within the limits of individual fluctuation and can be ascribed to the weakening of the spider.

The amputation of claws gave an unexpected result. It would appear that the claws which serve normally for manipulating the spider's thread are indispensable to the spinning of the web. It was proved however that spiders deprived of claws still use their operated legs to perform normal functions. It is true, though, that in such webs the viscid spiral is entangled more frequently.

One amputated leg is easily compensated for, the neighbouring leg serves as substitute, but the amputation of two legs creates greater difficulties, which are overcome by the spider in different way, depending on the type of amputation. The adjoining pair may work as substitute, as may the legs on the opposite side; and the function of thread-fastening can be done by the spinnerets.

Special attention attaches to the behaviour of the operated spiders during the process of web spinning. One spider which had one first and one second leg on the same side cut off presented a peculiar compensation of functions. While stretching the viscid spiral this spider took the measure of the distance on both sides from the viscid spiral and from the spiral guy line with the remaining legs of the first and the second pair. The one remaining leg of the first pair turned to the right and then again to the left. In the normal course of the process of construction of the web, the first and the second leg on the same side are in a way helping each other, they repeat the same functions, but not a single spider ever performs any functions on the right hand side with a left leg and vice versa. In this case the compensation of functions deviates mostly from the normal motional coordination. It proves at the same time a high plasticity of the nervous system, and broad possibilities in the spider's behaviour. Taking these facts into consideration we see that the process of web construction loses its absolute rigidity and that the behaviour of the spider overcomes the limits indicated by the conception of instinct.

The rigidity picture in the behaviour of lower animals has been derived as a consequence of surveying the behaviour of the animals independently from the conditions of their activity and from the medium in which they exist. The result was a rigid conception of instinct by Ziegler.

The functions performed in connection with the construction of a cobweb in normal conditions prove a very great individual versatility and they can be classified as monotype functions (Dembowski). The amputation of legs releases a number of new „catches" of the spider in the process of web construction, a number of new coordinations of movements acting as substitutes in connection with the lack of legs normally employed to perform certain specific functions.

The change in the behaviour of the spiders with the change of the conditions of the experiment can be illustrated by the following observation: While spinning the viscid spiral, spiders ar changing their position and are turning inwards alternatively with the left and with the right side. It was ascertained that spiders, which underwent an operation on one side only, lay the rows of the viscid spiral more regularly when their operated side is turned inwards (i. e. toward the spiral guy line) and their same side is turned toward the preceding row of the viscid spiral. It follows from the above that during the stretching of the spiral the correct evaluation of the distance from the viscid spiral is more important than that from the spiral guy line.

This problem has been raised in the literature by Hingston, by Peters as well as by Petrusewicz. But the method of research was different; the spiral guy line was cut off while the web was under construction. The results therefore cannot be directly compared with ours, and yet they all indicate a plasticity in the behaviour of the spider and their capacity to regulate when the normal points of judgment are missing as well as when they are deproved of their respective legs.

The difference in the results obtained in connection with the different methods of research indicate a definite interpretation. The behaviour of the spider is unbreakably connected with the conditions under which the experiments is performed. Due to the plasticity of the behaviour in one system of the experiment the most important element may be the viscid spiral, in another — the spiral guy line.

While building the web the spider does not act as a precision mechanism, which is capable of performing a certain definite function and no more. In reality the possibilities of the spider by far overcome the rigid limits established by the definition of instinct by Ziegler. A spider is a living organism possessing far greater possibilities than a mechanism. In new conditions its behaviour becomes multitypic and its capacities for new functions become apparent.

CONCLUSIONS

1. The amputation of a spider's legs creates a situation different from normal in the building a cobweb.
2. A spider can, while building a cobweb, make use of a leg deprived of claws in order to manipulate a viscid spiral as well as a dry thread.
3. Spiders with one leg amputated (to the middle or more) can build a regular cobweb without difficulty.
4. Spiders with two amputated legs can build a regular web with more or less effort depending on the type of operation.
5. The functions of amputated legs can be substituted by:
   a) neighbouring legs if they have a sufficient reach,
   b) legs on the other side: a spider can appraise the distance on both sides with one leg only,
   c) in place of a fourth leg spinnerets do the work of fastening.
6. Spiders with three or four amputated legs (extreme limbs only) can spin a web but the construction is greatly disturbed.
7. The spider's legs can, beside their normal functions, perform a number of auxiliary functions; a new activity brings forth a new coordinations of movements. These facts prove the plasticity of the nervous system.
8. The building of a cobweb by a garden spider is not a stereotypic function. Different capacities and various ways of adaptation to the changed situation are demonstrated.

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Fig. 1. Cobwebs by operated spiders.

1 A web built by spider No. 226 after amputation of one leg of the first pair
2 A web built by spider No. 133 after amputation of the first pair of legs
3 A web built by spider No. 250 after amputation of the second pair of legs
5 A web built by spider No. 192 after amputation of one leg of the second pair and one leg of the fourth pair, both on the same side.
6 A web built by spider No. 137 after amputation of the right leg of the first pair and of the left leg of the second pair.

Fig. 2. The arrangement of the spider's legs.

1. Spider in the middle of the web:
   a) arrangement of the legs of an unoperated spider.
   b) first leg amputated, second leg takes the position of the mussing one,
   c) first right and second left legs amputated, first left and second right are cooperating.
2. Spider in the act measuring the distance to the viscid spiral and to the spiral guy line:
   a) normal position,
   b) first pair amputated, the second pair is measuring the distance,
   c) the first and the second legs on the right hand side being amputated, the spider takes the measure to the viscid spiral with the first left leg.
3. Spinder in the act of fastening the viscid spiral:
   a) with one leg of the fourth pair the spider fastens the thread and subtains it with the other.
   b) fourth pair of legs amputated, the spider fastens the thread by sitting on the radius with its spinnerets.

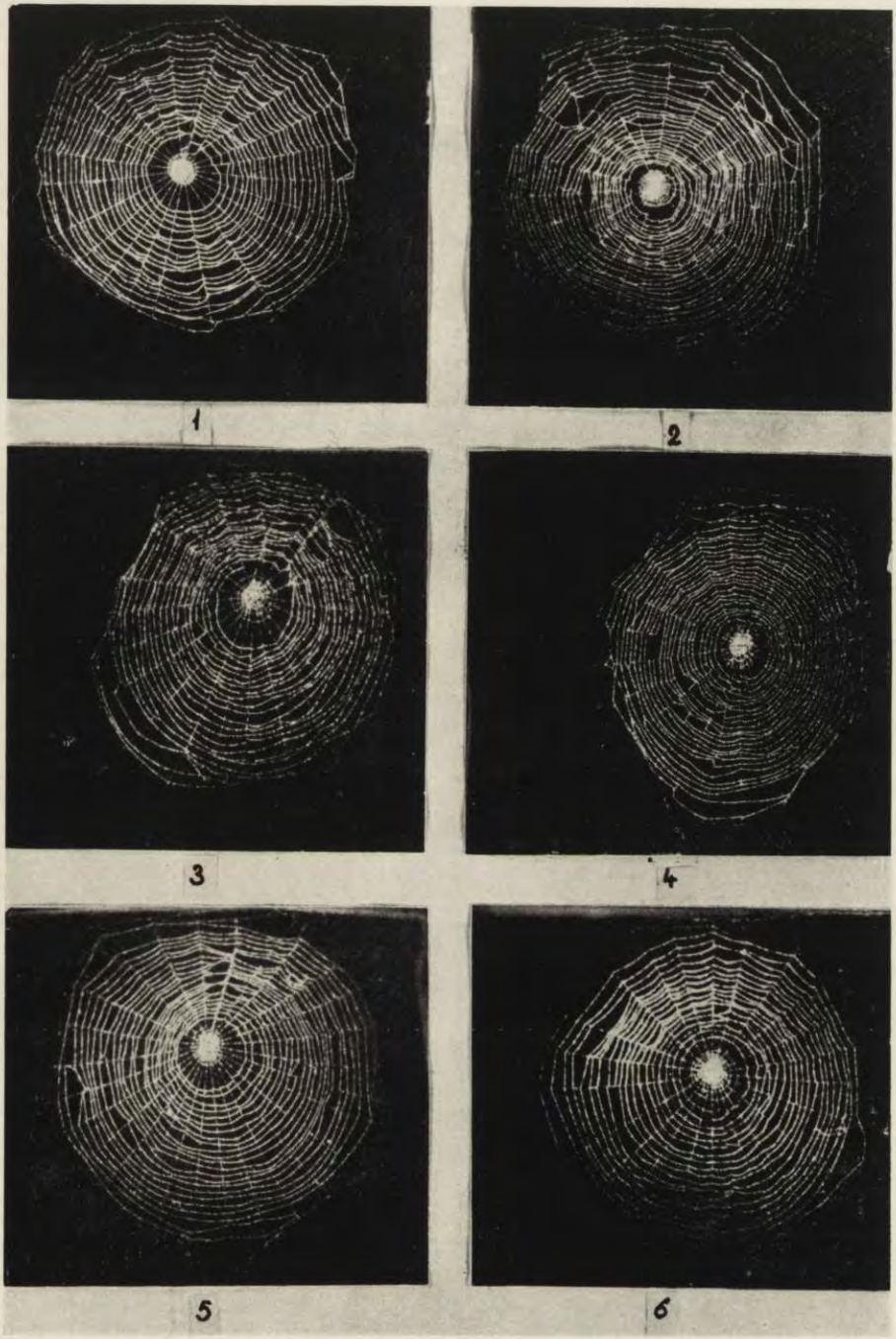

Fig. 1

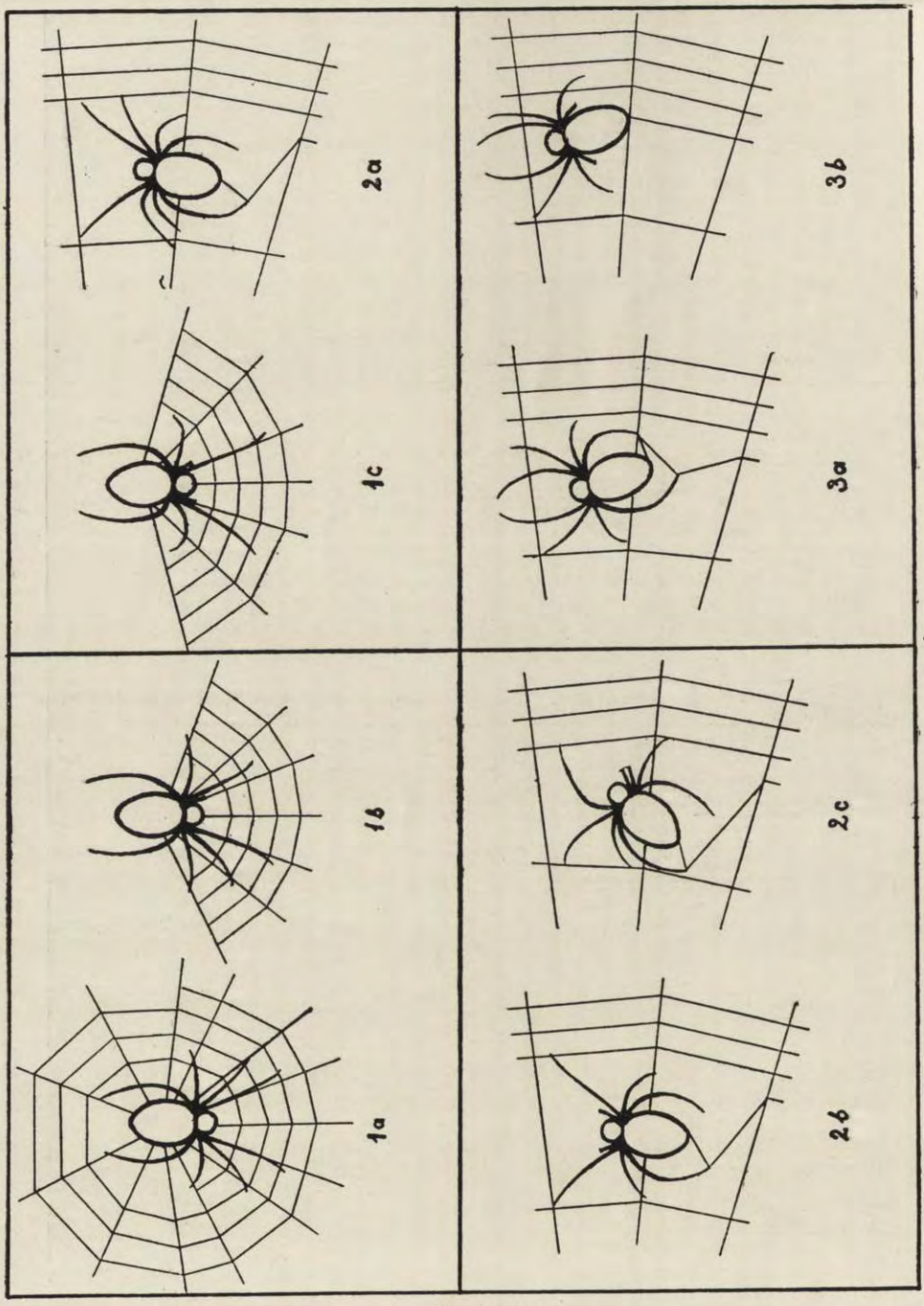

Fig. 2

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THE DEPENDENCE OF CHEMOTROPISM IN *Paramaecium caudatum* ON THE CHEMICAL CHANGES IN THE MEDIUM

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The phenomenon of chemotropism with Infusoria was most fundamentally studied by H. Jennings, and laws governing chemotropism described by him must be considered as basic. So far it was only known that oxygen is a factor chemotropically positive, while carbon dioxide, acids and bases are factors chemotropically negative. Massart (1889) considered Infusoria to be entirely insensible to the chemism of the substance and that only osmotic pressure caused an avoiding reaction. He proposed therefore to name the phenomenon itself „tonotaxis". It was, however, shown by Jennings (1897) that osmotic pressure plays no role of importance in this phenomenon and that the real active factor is the specific chemism of the substance. He divided chemical compounds into attractive (which cause positive chemotropism) and non-attractive substances (which cause an avoiding reaction). The intensity of the avoiding reaction as well as its appearance were not to be connected with a harmful character of the substance but were to depend on its chemical structure. In normal conditions Infusoria agglomerate in the diffusion zone of carbon dioxide produced by themselves and by bacteria respectively; in experimental conditions however we can observe a chemotropical agglomeration within the sphere of diffusion of the substance with a slight acid reaction. Jennings supposed the action of acids to be specific and to depend primarily on

anion and not on the hydrogen ion concentration. The action of salts was to consist in a complex action of anion and of cation whereby cations, uni- and bivalent, should cause a strong motor reaction in the form of an avoiding reaction described by him, and trivalent cations should cause no such motor disturbance. In connection with this, salts comprising uni-and bivalent cations were to cause negative chemotropism due to the preponderance of cation over anion, while salts comprising trivalent cations were to cause positive chemotropism due to the prevalence of anion over cation in their chemotropic reaction. In a series of analysed salts of common anion and of different cation concentrations Jennings found an increase in negative chemotropic action parallel with a decrease in atomic weight of cation.

Barrat (1905) critised Jennings's views. He considered positive chemotropism to be only one phase of negative chemotropism.

A special problem is posed by the question of the nature of chemotropism itself as it does not answer the conditions required by the classical theory of Loeb (1918). Phototropism, galvanotropism and geotropism conform with these conditions but chemotropism fulfills them only in part. A small diffusion zone around the introduced chemical substance may be considered to correspond to a direct stimulus, but the reaction of an animal to chemical stimuli is direct neither in the case of the substance having a positive nor a negative chemotropical reaction, as it consists in a performance of an indirect avoiding reaction mentioned above on the border of two mediums, in one case as well as in the other.

Kuehn (1919) divides tropisms into fobotaxis and topotaxis depending on movements they evoke. Both are due to the action of an external stimulus. Fobotaxis is due to a difference felt in the intensity of a stimulus with time. In such a case movement is not orientated in relation to the stimulus. Topotaxis is due to a sensed spacial difference of the stimulus, that is to the difference of stimulus felt at the same time. The movement of the animals is in this case orientated in relation to the stimulus and is therefore directional. According to this division chemotropism is classified as fobotaxis.

Alverdes (1922) considers that only animals of a higher body organisation are capable of a simultaneous reaction to stimuli, whereas he considers Protoza to be able to react only to stimuli acting in a certain sequence. With reference to chemotropism he was of the opinion that the Loeb theory of tropisms, as well as the theory of

local reaction, fails if we take into consideration the great variety of chemotropic movements that were observed, for the majority of which there is no place in the avoiding reaction described by Jennigs.

Łozina Łoziński (1920) points out the connection of the phenomenon of chemotropism in *Paramaecium caudatum* with the consumption of food. He noticed, in suspensions of certain dyes which cause a positive chemotropism, that the number of produced food vacuoles were greater than in suspensions which are not showing a positive chemotropic influence.

Kagan (1939) denies the existence of compulsion in the process of chemotropism and considers positive and negative chemotropism to be phases of the same phenomenon, which is conditioned by the existence of physico-chemical differences in the test mediums. Chemotropic value, that is the lowest electrolyte concentration which causes negative chemotropism or specific disturbances of Infusoria's movements, goes up, according to Kagan, with increase in valency of the cation, and, within a group of cations of equal valency, parallel to the size of atomic weight; while it seems to decrease with increase in valency of the anion.

The review of literature dealing with chemotropism in Protozoa reveals that many problems connected with this phenomenon require to be cleared up and to be critically appraised in view of the various opinions which lack persuasive force and differ essentially from each other. The aim of this work is therefore:

1. A critical appraisal of views prevailing to date on the part played by cations and anions in chemotropism.
2. To decide whether chemotropic agglomerations which can be observed in the area of acidified water medium are connected with a specific chemism of the substance or whether they depend on the hydrogen ion concentration of the medium.
3. To investigate whether adaptation plays any part in chemotropism.

METHODS

The material used in these studies was a pure line of the Infusoria *Paramaecium caudatum* reared at a temperature of 20—22°C. on a decoction of hay with tapwater lasting 48 hours. This culture was changed once a week. In this way were obtained thick cultures which, 48 hours before the experiment, were poured into a flask with a flat bottom of 1 litre capacity, filling it to 3/4 capacity; the remainder to the upper border of the flask's gullet, was

filled with tapwater at room temperature. By next day Infusoria formed thick agglomerations within the gullet of the flask. Then were transferred to a smaller vessel, such as a beaker, of about 50 ml. capacity. After a certain time Infusoria in thick agglomeration settle on the bottom of the vessel and partly form a thigmotropic ring beneath the free surface of the liquid. These thick agglomerations were taken up with a pipette and transferred to a beaker filled with tapwater. In this way some rather thick portions of Infusoria were obtained in the medium of tapwater with the smallest possible addition of culture. After some 20—24 hours Infusoria prepared in such a way were used for the experiments.

For simplicity sake we shall further call Infusoria so prepared „experimental Infusoria". We gave up entirely the method of rotation used to purify and to obtain a thick agglomeration of Infusoria, as it was ascertained that rotation has a harmful influence on the physiological condition of Infusoria and always leads to traumatisation, so that they become inclined to react in a non-typical way to chemical stimuli.

The electrolytic solutions were prepared in concentrations of 0.1 molar, and 0.1 normal respectively. These solutions were diluted with an appropriate amount of tap- or distilled water, depending on the experiment to be performed.

The pH of the solutions was determined with the aid of a pH meter of the Cambridge Instrument Co. with a precision of 0,01 pH.

The experiments were performed in the following manner. A glass plate of about 6 cm × 6 cm with a mat grinded border about 0.5 cm. in width was put on a magnifying glass table horizontally. A layer of liquid to which the Infusoria were to be introduced was laid down with the aid of the pipette the such a way that the liquid moistened the mat border of the glass. This rendered the flowing away of the liquid more difficult. The layer of liquid should be shallow, from 0.5 to 1 mm. in depth. To one corner of the plate were then carefully introduced 2 to 3 drops of the „experimental Infusoria". Diffusion of the introduced medium with Infusoria is insignificant, no more than within the limits of a radius of 1 cm. length. After some time the Protozoa were completely dispersed in the solution where they were introduced. The electrolytic solution whose chemotropic effect it was intended to demonstrate was then introduced from above into the middle of the glass plate with the help of a capillary pipette, adding some 5 drops of the solution and keeping the pipette just above the surface of the liquid. From this moment on observation of the phenomenon began. Any moving of the plate or of the table should be avoided during the course of the experiment. By a careful application of this simple method results were obtained which were in many respects more certain and more accurate than these obtained by the classic method of Jennings. Side by side with the above method was applied also the method of Jennings in order to compare results obtained by both methods. Further details of this method are given with the description of these experiments.

Experiments described in this work were repeated at least five times and each time the results were identical. It would be therefore superflous to describe the results of each observation separately.

From the same consideration the pH values of the applied solutions are given with a precision of only 0,1 pH; the chemotropic reaction of Infusoria in relation to the differences in the medium is so small that differences of less than 0,1 pH are of no importance.

DESCRIPTION OF EXPERIMENTS

Experiments which related to the part played by cations and by pH in chemotropism were performed in a medium of tapwater as well as in distilled water.

THE PART PLAYED BY CATIONS IN CHEMOTROPISM

The influence of cations on the phenomenon of chemotropism in *Paramaecium caudatum* is specific. This can be easily proved by analysing the influence of salts with different cations and a common anion. In such a case the lowest concentration of electrolyte which causes an avoiding reaction, i. e. a disturbance of movements of Infusoria characteristic for the said cation, can be defined for each cation. To this lowest concentration of electrolyte Kagan gave the name of „chemotropic value", and movements or turnings characteristic to the Protozoa made under the influence of contact with the tested salt solution were named by him „quality of chemotropic reaction".

The chemotropic value points at the same time, to the attractivenes of the said cation in relation to the remaining ones in its chemotropic reaction. It follows from the above that a grading of cations according to their chemotropic action may be established on an experimental basis in two manners:

1. by comparing the chemotropic value of the various cations,
2. by comparing the chemotropic reaction of Protozoa placed in a medium of electrolyte containing a definite cation, after analogous concentrations of electrolytes containing the remaining cations and one and the same anion are added.

Experiments were performed with the use of the following cations: $Li^{\cdot}$, $Na^{\cdot}$, $K^{\cdot}$, $Mg^{\cdot\cdot}$, $Ca^{\cdot\cdot}$, $Ba^{\cdot\cdot}$. In salts which were used for these experiments the common anion was Chlorine.

The reaction of Infusoria in a medium of tapwater differs greatly from a reaction to chemical stimuli in a medium of distilled water. It can be said in general that Infusoria in a medium of tapwater, resembling a natural one, are much less sensible to chemical stimuli than Infusoria placed in a medium of distilled water.

CHEMOTROPICAL VALUE OF: LiCl, NaCl, KCl, $MgCl_2$, $CaCl_2$, $BaCl_2$

Experiments conducted with the aim of defining the chemotropic value of the tested salts were performed in two series. In the first series of experiments the initial medium was tapwater and the medium experimented with was a solution of a corresponding salt in tapwater. In the second series the initial medium was distilled water and the medium experimented with was a salt solution in distilled water.

The results of the experiments are given in Table I.

Table I

Chemotropical value of: LiCl, NaCl, KCl, $MgCl_2$, $CaCl_2$, $BaCl_2$

Name of the salt	Chemotropic value in the medium	
	of tapwater	of distilled water
LiCl	from 8 mM to 10 mM	from 0,6 mM to 0,7 mM
NaCl	20 mM	from 3,5 mM to 4,0 mM
KCl	from 5 mM to 6 mM	from 0,5 mM to 0,6 mM
$MgCl_2$	20 mM	from 0,8 mM to 1,0 mM
$CaCl_2$	100 mM	100,0 mM
$BaCl_2$	0,1 mM	0,1 mM

Depending on the chemotropic value and according to the degree of reaction cations may be ranked as follows:

$Ca^{\cdot\cdot} > Na^{\cdot} > Mg^{\cdot\cdot} > Li^{\cdot} > K^{\cdot} > Ba^{\cdot\cdot}$ in relation to experiments performed in the medium of tapwater.

$Ca^{\cdot\cdot} > Na^{\cdot} > Mg^{\cdot\cdot} > Li^{\cdot} > K^{\cdot} > Ba^{\cdot\cdot}$ in relation to experiments performed in the medium of distilled water.

CHEMOTROPISM IN DEPENDENCE FROM CATION WITH APPLICATION OF ISOTONIC CONCENTRATIONS OF SALTS IN INITIAL AS WELL AS IN TESTED MEDIUM

The determination of chemotropic value enables us to rank cations according to the lowest concentration of electrolyte which causes a negative chemotropism. Theoretically the chemotropic value does not necessarily determine the attractiveness of cation. In order to verify the conclusions reached from previous experiments it was necessary to examine the chemotropic reaction in the light of cation attractivenes using isotonic concentrations of salts in the initial medium (containing Protozoa) as well as in the tested medium.

These experiments were performed, like the previous ones, in a medium of tapwater as well as in a medium of distilled water. Taking into consideration that the chemotropic value in the medium of tapwater is on the average many times higher than the same value in the medium of distilled water, the concentrations of salts which were applied to these experiments were in both cases different. The respective concentrations for experiments in medium of tapwater amounted to 20 mM, and for experiments in medium of distilled water amounted to 3.33 mM.

1. Experiments in the medium of tapwater.

In these experiments the medium into which it was intended to introduce Infusoria was prepared in the following way: in the first phase Infusoria were put by the usual method in clean tapwater and then 40 mM solutions of the respective salts were mixed with an equal volume of tapwater containing the Infusoria. In this way was obtained a 20 mM concentration of the said salts. It should be mentioned that the solutions were prepared with tapwater as solvent.

Infusoria placed in 20 mM solutions of LiCl and NaCl have a slackened movement, while in a solution of 20 mM KCl at first (for 2—3 minutes) we can observe a disturbance of movements in the form of a rotation around the diagonal axis of body, resp. in the form of a backward movement. After the lapse of 2—3 minutes the disturbance of movements described above is over and Infusoria begin to mowe forward at a decidedly slackened pace. In 20 mM solutions of $MgCl_2$ and $CaCl_2$ no disturbance of movements could be observed.

Especially violent disturbances of movements of Infusoria could be observed in a 20 mM solution of $BaCl_2$. They consisted in a backward movement going subsequently over to a movement around the diagonal axis of the body (Eisenberg 1932). In connection with such a violent disturbance of movements the chemotropic reaction due to electrolytes containing the remaining cations could not be determined, as Infusoria were not in a position to desert the medium containing $BaCl_2$.

The results put together in the Table II prove that the tested cations may be ranked in the following way, according to their dependence on a positive chemotropic reaction in isotonic solutions:

$$Ca^{\cdot\cdot}, Na^{\cdot} > Mg^{\cdot\cdot} > Li^{\cdot} > K^{\cdot} > Ba^{\cdot\cdot}.$$

Table II

(Solutions prepared in tapwater).

Infusoria placed in a solution of	The chemotropic reaction after the addition of salts					
	LiCl	NaCl	KCl	$MgCl_2$	$CaCl_2$	$BaCl_2$
LiCl		+	Ø	+	+	Ø
NaCl	—		Ø	—	·	Ø
KCl	+	+		+	+	Ø
$MgCl_2$	—	+	Ø		+	Ø
$CaCl_2$	—	·	Ø	—		Ø
$BaCl_2$	The chemotropic reaction is undefined					

Explanations: *the sign* + means positive chemotropism; *the sign* . means lack of chemotropic reaction; *the sign* — means negative chemotropism; *the sign* Ø means negative chemotropism with an „apparent ring" on the border of the introduced solution. The definition „apparent ring" means that Infusoria show a disturbance of movements in the form of rotation around the diagonal body axis as soon as they come in contact with the solution of salt which is introduced, due to which they form a ring around the drop which was poured in. It was observed such a disturbance of movements due only to the salts of barium and in a lesser degree to the salts of potassium. It is the expression of a specific strongly marked negative chemotropic reaction due to the above mentioned cations.

2. Experiments in the medium of distilled water.

In these experiments solutions of neutral salts: LiCl, NaCl, KCl, $MgCl_2$, $CaCl_2$, $BaCl_2$ were applied in 3,33 mM concentrations, that is in concentrations almost isotonic with tapwater.

From the medium of tapwater, Infusoria went over with great ease to a solution of $CaCl_2$, whereas to other solutions they went over not sooner than after 2—3 minutes. Such a comparatively long period needed by Infusoria to pass proves their gradual adaptation to the new chemical medium. A free passage of Infusoria to a solution of calcium chloride is easily understood when we take into consideration that Infusoria in normal conditions remain steadily in the medium of calcium salts. Infusoria do not pass freely to a solution of $BaCl_2$ but coming into contact with the above solution undergo a characteristic disturbance of movements in the form of a rotation around their diagonal body axis. In this one case it was necessary to change the method in such a way that Infusoria, moved over previously to distilled water, were mixed in an appropriate relation with a solution of $BaCl_2$. In a medium containing LiCl,

NaCl, and KCl movements of Infusoria were distinctly slackened while in a solution of $CaCl_2$ the movement was swift. The disturbance of movements characteristic for the solution of $BaCl_2$ makes it impossible to conduct experiments with the remaining electrolytes.

After the Infusoria remained for 1—2 minutes in a medium of a salt solution where it was intended to introduce them, the next step ot the experiment consisted in the addition of 5—6 drops of an appropriate solution of electrolyte to the middle of the glass plate in order to examine the chemotropic effect of the added solution.

The results of these experiments are given in Table III.

Table III

(Solutions prepared in distilled water. Explanations as in Table II).

Infusoria placed in a solution of	The chemotropic reaction after the addition of salts					
	LiCl	NaCl	KCl	$MgCl_2$	$CaCl_2$	$BaCl_2$
LiCl		+	—	—	+	Ø
NaCl	·		Ø	—	—	Ø
KCl	+	+		+	+	Ø
$MgCl_2$	—	+	Ø		+	Ø
$CaCl_2$	+	—	Ø	—		Ø
$BaCl_2$	The chemotropic reaction is undefined					

It may be seen from the above table that cations may be ranked in the following way in dependence on their positive chemotropic reaction in isotonic solutions:

$$Ca^{\cdot\cdot}, Na^{\cdot} > Mg^{\cdot\cdot}, Li^{\cdot} > K^{\cdot} > Ba^{\cdot\cdot}.$$

This ranking is almost identical with the results obtained in determining the chemotropic value and with the results obtained in analogous experiments conducted in the medium of tapwater.

THE PART PLAYED BY pH IN CHEMOTROPISM

The part played by pH in chemotropism has not been cleared up thus far. According to Jennings (1889) positive chemotropism under the influence of acidified tapwater depends upon a specific action of acids and is conditioned upon the existence of a definite concentration of anion, which enters in the compound of the added acid; the concentration of hydrogen ions has nothing to do with this phenomenon. It was explained by Jennings that a similar action of salt with an acid effect is caused by „small activity" of cation and, in consequence, the effect of action of such a salt depends solely on

anion and is therefore similar to the action of acid. We must admit that such a reasoning was suggestive, explained theoretically pretty well the chemotropic action of acids, bases and salts, and made a synthetic presentation of this phenomenon possible, with the result that an effort was made to show the dependence of chemotropic action on the valency and on the atomic weight of cations. According to such a conception chemotropism would depend only on a specific action of cation or anion, resp. on their mutual relation in the tested medium. Such a hypothesis did not take into account the part played by the hydrogen ion concentration, a change of which always accompanies all substances with a positive chemotropic action as soon as they are introduced in the midst of tapwater.

From data in literature it may be seen that tests on chemotropic effects of a substance were performed with solutions in which as solvent only tapwater was used. Such substances with a positive chemotropic action can be divided into three groups:

1) organic acids
2) non-organic acids
3) some salts (such as: NaH_2PO_4, $FeCl_3$, $K_2Cr_2O_7$).

It deserves to be mentioned that salts of the third group are marked by an acid action, a fact to which no special attention was given in literature where the example the most frequently mentioned of a substance with a positive chemotropic action is a 0.02% (3.3 mM) solution of acetic acid.

Quite independently, however, from the chemical structure of the substance, its addition to tapwater causes a chemical reaction consisting in the formation of carbonic acid from calcium bicarbonate contained in water. Carbonic acid produced in this reaction evaporates into the atmosphere in the form of carbon dioxide. In connection with this the pH of an acidified water differs when fresh, after the addition of acid, from the one some time later, when the surplus of carbon dioxide has evaporated into the atmosphere, as the pH of the solution in this case depends on the relation of the concentration of the ion HCO_3^- to the concentration of H_2CO_3. This change in the pH of the solution, which is evidently strictly connected with the evaporation of the surplus of carbon dioxide, may take place only when the surface of the liquid comes in contact with the atmosphere. This condition was fulfilled in the method applied in this work, as the experiments were performed with a broad access of atmospheric air, thanks to which the surplus of carbonic

acid could evaporate quickly in the form of carbon dioxide (within 2—3 minutes). The chemotropic experiments by the Jenning's method are performed however under entirely different conditions. Acidified water is introduced under the cover glass by means of a capillary pipette and therefore no contact with the atmospheric air is provided. It is evident that under such conditions the surplus of carbonic acid cannot evaporate in the form of carbon dioxide. The acidified solution which is introduced undergoes but little change of pH and only in so far as diffusion takes place on the border. The chemical composition of the acidified water as well as its pH are in this case almost constant, which fact makes it impossible to ascertain whether chemotropism which appears in the experiment is due to the chemical properties of the substance, to the carbonic acid in the solution or to a definite pH of the solution itself.

These difficulties are absent with the method used in the present work. The surplus of carbonic acid evaporates in the form of carbon dioxide into the atmosphere, the pH value of the solution undergoes a change in different degrees depending on the quantity of added acid, and the anion concentration characteristic for the said acid does not change while the experiment lasts. In such a way the conditions at the beginning of the experiment are identical with those of the Jennings's method, but after three minutes time, that is when the surplus of carbonic acid has been evaporated, the situation looks as follows:

1. The quantity of carbon dioxide dissolved in the acidified medium is identical with the one in the surrounding medium of tapwater.
2. The concentration of anion, characteristic for the added acid, remains unchanged from the beginning of the experiment.
3. The pH value of the solution is moving in various degrees in the acid direction, depending on the concentration of the added acid or salt resp.

This second part of the experiment, i. e. the observation of chemotropism after the surplus of carbon dioxide has been evaporated, makes it possible to establish what part is played by anion and pH respectively in chemotropism, depending on whether and what changes happen in positive chemotropism under the influence of various concentrations of acid or of salt added to the medium of tapwater. Theoretical reasoning leads to the conclusion that if the factor that causes positive chemotropism is the anion characteristic for the said acid or salt, then positive chemotropism, which appears

after the various concentrations of the tested substance are added should remain unchanged after a lapse of three minutes and even after a still longer period of observation, as the concentration of anion undergoes no changes during the whole experiment. If however in the second phase of the experiment it is ascertained that positive chemotropism is waning independently from the concentration of the added acid it would indicate a specific chemotropic action of carbonic acid and carbon dioxide respectively. The appearance of positive chemotropism in the second phase of the experiment, depending on the by pH value of the solution, would point to the predominant

Table IV

Chemotropism under the influence of various concentrations of HCl added to tapwater.

Concentration of HCl added to tapwater	before evaporation of CO_2		after evaporation of CO_2	
	pH	chemotropic reac.	pH	chemotropic reac.
3,66 mM	3,5	0	3,6	0
3,33 mM	4,5	0	5,8	+
3,0 mM	5,3	+	6,9	+
2,5 mM	5,8	+	7,5	·
1,66 mM	6,3	+	7,7	·
1,0 mM	6,6	+	7,9	·
0,33 mM	7,2	(+)	8,1	·

Explanations: the sign (+) means slightly positive chemotropism; the sign 0 means „chemotropic ring". The definition „chemotropic ring" means that Protozoa were assembled in that very narrow belt of the solution which shows an optimum of chemotropic pH and which appears as a result of diffusion of the added solution with the surrounding medium of water. Infusoria show then an avoiding reaction coming in contact with the added acidified solution whose pH is too low, as well as coming in contact with the initial medium of tapwater which shows pH from 8,0 to 8,1.

part played by pH in chemotropism and it should wane gradually in such low concentrations of the added substance in which pH approaches the initial value (previous to acidifying) and should stay on the same level in such concentrations in which pH of the solution is kept on a corresponding low level even after the surplus of carbon dioxide has been evaporated into the atmosphere.

It follows from the above that it is absolutely necessary to ascertain the pH of the tested solution immediately after its preparation and a second time after the surplus of carbon dioxide has been evap-

orated. The determination of pH in freshly prepared solution presents obviously no difficulty, but in order to eliminate the surplus of carbon dioxide from the solution it was necessary to shake the liquid in the flask for several minutes, or to leave it for a couple of hours in a container with a broad bottom. If the pH of the solution shows no changes after further shaking it can be taken for granted that the surplus of carbon dioxide has been indeed eliminated.

For these experiments tapwater was always used which was previously left in the laboratory for 24—48 hours. In such conditions constant pH value from 8.0 to 8.1, were obtained.

Table V

Chemotropism under the influence of various concentrations of acetic acid added to tapwater.

Concentration of acetic acid added to tapwater	before evaporation of CO_2		after evaporation of CO_2	
	pH	chemotropic reac.	pH	chemotropic reac.
4,0 mM	5,25	0	5,4	0
3,66 mM	5,4	0	5,7	+
3,33 mM	5,5	0	6,4	+
3,0 mM	5,7	+	7,1	(+)
2,5 mM	5,9	+	7,5	·
1,66 mM	6,3	+	7,8	·
1,0 mM	6,6	+	7,9	·
0,33 mM	7,6	(+)	8,1	·

The chemotropic effect of a number of substances were examined. The exact results of these experiments with the degree of concentration of the added acid and salt respectively, with the pH of the solution before and after evaporation of carbon dioxide and with the chemotropic reaction, are presented in the case of four substances: of hydrochloric acid, acetic acid, chromic acid, and potassium dichromate as the most typical representatives of the group of substances with a positive chemotropic action. The influence of the remaining acids and salts is discussed collectively with emphasis laid on those points which are worthy of attention. In order to compare the results and in order to reach some definite conclusions the experiments were performed with hydrochloric acid and with acetic acid by the Jennings's method as well. The results of the experiments are given in Table IV and V.

The results of experiments performed by the Jennings's method with hydrochloric acid and with acetic acid were identical with these presented above under the head „before evaporation of CO_2" with the exception of the two last items where acids were added in a concentration of 0.33 mM. In this last case positive chemotropism was not observed, due probably to a diffusion of a relatively weak solution, and could probably be connected with the way the tested solution was introduced.

Table VI

Chemotropism under the influence of various concentrations of chromic acid added to tapwater.

Concentration of chromic acid added to tapwater	before evaporation of CO_2		after evaporation of CO_2	
	pH	chemotropic reac.	pH	chemotropic reac.
3,33 mM	4,4	0	4,7	0
3,0 mM	5,2	0	5,6	+
2,5 mM	5,6	+	6,2	+
1,66 mM	6,1	+	7,2	(+)
0,83 mM	6,5	+	7,9	·
0,33 mM	6,8	+	8,1	·

It seems strange that in experiments performed with potassium dichromate chemotropic ring appears when solutions, whose pH was equal 5.9, 6.1, and 6.2, that is, pH values decidedly chemotropic positive, were applied. Yet this phenomenon may be easily explained if we take into consideration the results of experiments where potassium salts were applied and which were described previously. Solutions which show a concentration of potassium equal to 5—6 mM are already causing a decidedly negative chemotropism, which in relation to the solution of potassium dichromate is weakened by the positive chemotropic effect of the low pH. These two factors acting against each other cause Infusoria to agglomerate in a zone of a rather low pH and at the same time of a rather small concentration of potassium. (Table VII).

Conclusions of special interest are brought out by an analysis of a chemotropic reaction under the influence of a 3.33 mM solution of $K_2Cr_2O_7$. The ring that can be observed in the beginning passes after a lapse of 3 minutes into an agglomeration, that is in positive chemotropism. A change in pH after the surplus of CO_2 has been evaporated cannot be ascribed to such a phenomenon. A low pH is in this case a factor which enables the adaptation of Infusoria

Table VII

Chemotropism under the influence of various concentrations of potassium dichromate added to tapwater

Concentration of $K_2Cr_2O_7$ added to tapwater	before evaporation of CO_2		after evaporation of CO_2	
	pH	chemotropic reac.	pH	chemotropic reac.
5,0 mM	5,9	0	6,2	0
3,33 mM	6,1	0	6,4	+
2,5 mM	6,2	+	6,7	+
1,66 mM	6,3	+	7,3	·
0,83 mM	6,7	+	7,9	·
0,33 mM	7,0	(+)	8,0	·

to a medium containing a certain concentration of electrolyte with a negative chemotropic action. This question is discussed in detail in a chapter devoted to the part played by adaptation in chemotropism.

The results of experiments with hydrochloric acid, acetic acid, chromic acid and potassium dichromate point to the dominant part played by pH in chemotropism, as the chemotropic agglomerations in the second phase of experiments (i. e. after carbonic acid in the form of carbon dioxide has been evaporated into the atmosphere) can be observed only when the pH of the solution lies within the limits from 7.0—5.4. The waning of positive chemotropic reaction in lower concentrations of the said substances speaks against a specific part played by anion whereas the existence of such a reaction after an addition of higher concentrations of the substances speaks against a specific part played by carbonic acid. It must be therefore asserted that the factor which causes a positive chemotropism is a definite pH of the solution and not chemical properties of the substances mentioned above.

Apart from the above the chemotropic influence of the following substances were analysed: sulphuric acid, boric acid, tartaric acid, citric acid, malic acid, oxalic acid, picric acid, $FeCl_3$, NaH_2PO_4, KH_2PO_4, $AlCl_3$, $Na_2Cr_2O_7$. The chemotropic reaction of the above substances in principle does not deviate from what was said above in relation to hydrochloric acid, acetic acid, chromic acid and potassium dichromate.

The addition of oxalic acid to tapwater causes a precipitation of calcium oxalate.

Positive chemotropism under the influence of picric acid is only slightly marked in comparison with other substances, which is undoubtedly connected with its specific checking influence on a chemotropic reaction.

The addition of $FeCl_3$ to the medium of tapwater leads to the appearance of a brown sediment $Fe(OH)_3$ especially when low concentrations of salts are applied. Chemotropic reaction under the influence of ferric chloride is very characteristic as the Infusoria, immediately after swimming into the diffusion zone of ferric chloride, move very slowly and, after a further few seconds, remain entirely motionless showing a thigmotropic reaction. This is a specific reaction under the influence of cation $Fe^{\cdots}$. After some 2 or 3 minutes the Protozoa recover to a certain degree the freedom of movement and yet they move very slowly, disclosing at the same time an avoiding reaction when coming into contact with a medium of tapwater if the pH of the solution is kept within the limits 7.0—5.5, or they may swim freely in tapwater if the pH of the introduced solution reaches values above 7 at the time of evaporation of the surplus of carbonic acid in the form of carbon dioxide into the atmosphere.

The reaction of Infusoria under the influence of $AlCl_3$ is very much like the reaction under the influence of $FeCl_3$, as in this case also a distinct slackening of movements and even sometimes a complete stoppage of movements may be observed.

The slackening of movements of Infusoria under the influence of cations $Fe^{\cdots}$ and $Al^{\cdots}$ is undoubtedly due to, a specific action of cations. The avoiding reaction however which decides about the positive chemotropism depends on the changed pH of the medium as it wanes in low concentrations of the added salts when the pH of the solutions is raised to the initial value as soon as the surplus of carbonic acid in the form of carbon dioxide is evaporated into the atmosphere.

THE PART PLAYED IN CHEMOTROPISM BY DISTILLED WATER

The problem of the influence of pH is strictly connected with the role distilled water plays in chemotropism.

In laboratory conditions distilled water shows a weak acid reaction due to the diluted atmospheric carbon dioxide. It can be therefore considered to be a thinly diluted solution of carbonic acid. For freshly distilled water brought to a temperature of 20—22°C

pH values were obtained ranging from 5.4 to 5.3. Analogous pH values were ascertained in neutral salts dissolved in distilled water which were used in experiments.

For tapwater which was left for 24—28 hours in living room temperature (20—22°C) pH values = 8.1—8.0 were obtained.

The positive chemotropic influence of distilled water in relation to Infusoria staying in the medium of tapwater was known for a long time. Jennings (1905) supposed the source of positive chemotropism in this case to be due to a complete lack of salts in distilled water and that in exceptional cases only some such substances as acids and salts containing trivalent cations would show positive chemotropism in a certain small but definite concentration.

In examining the positive chemotropic influence of distilled water in relation to Infusoria staying in the medium of tapwater if was noticed that primarily Infusoria form a rather broad ring, and that a distinct agglomeration appears only after 2—3 minutes, which indicates a gradual adaptation of the Infusoria to the changed conditions of the medium.

It was decided to make a series of experiments in order to determine whether the chemotropic action of distilled water in relation to the medium of tapwater depends really on a complete lack of salts or on its low pH = 5.4 — 5.3.

With this object in view some experiments were performed where the initial media were various concentrations of calcium chloride prepared with distilled water as solvent. In these new conditions we analysed subsequently the chemotropic influence of distilled water. The application of neutral salts such a calcium chloride in this experiment permits the elimination of the influence of pH on chemotropism. We choose a calcium cation because of its presence in tapwater, that is, in a medium close to natural.

For our experiments we used solutions of calcium chloride in concentrations: 33.3 mM, 3.33 mM. 1.66 mM, and 0.83 mM. Independently from concentrations of calcium chloride the addition of distilled water caused always a negative chemotropism which was more or less strongly marked. We may deduce from these experiments that the attractivenes of distilled water in its chemotropic action in relation to tapwaer consists not on a lack of salt, but on a low pH.

Analogous to the experiments performed in the medium of tapwater the chemotropic influence was examined of acids and salts

with an acid action dissolved in distilled water using at the same time distilled water as initial medium. These experiments were done with hydrochloric acid, sulphuric acid, chromic acid, boric acid, acetic acid, tartaric acid, citric acid, malic acid, oxalic acid, picric acid and with salts $FeCl_3$, NaH_2PO_4, KH_2PO_4, $Na_2Cr_2O_7$ and $K_2Cr_2O_7$ in concentrations from 0.33 mM to 3.33 mM and the pH values of these solutions were within the limits from 4.5—2.5.

Individual Infusoria which swam into the diffusion zone of the tested substances perished almost instantaneously, and no positive chemotropic reaction was observed. The results of these experiments prove once more that positive chemotropism, under the influence of the substances mentioned above with the tapwater used as a solvent, depends solely, on a definite change of pH of the medium (within the limits 7.0—5.4) and is independent from their specific chemical structure.

CHEMOTROPISM IN DEPENDENCE ON THE pH OF THE INITIAL MEDIUM

The dominant part played by pH in chemotropism is due to many factors which were stated above. It was decided to examine further to what degree the chemoptropic reaction changes under the influence of solutions of various pH in dependence from the pH of the medium in which Infusoria are staying. Such an experiment was however difficult to perform in the medium of tapwater, whose pH is very changeable in connection with the chemical reaction which occurs under the influence of acidifying. It was possible to avoid these difficulties by using for the experiment salts with a buffer reaction. For these experiments salts of phosphoric acid namely NaH_2PO_4 and Na_2HPO_4 were selected. A solution containing the above salts in concentration 1 : 1 shows a pH = 6.8. By selection of various concentrations of the said salts solutions of various pH values were obtained and care was taken to see that the complete concentration of salts did not undergo larger deviations in order to avoid the appearance of an additional factor which could influence in an undesired manner the experiment.

After some initial experiments it became evident that Infusoria by themselves swim with great difficulty in the buffer solutions whose pH approaches the pH value of tapwater. A small addition of calcium salts to the solution (a solution of $CaCl_2$ in a concentration of 0.33 mM) removes this difficulty and has no influence on a change of pH value of the tested solution. As for some methodical consider-

ations, it was desired that the Protozoa enter the pure buffer solutions by themselves. These experiments were performed with solutions of sodium phosphate which contained an addition of calcium chloride in a concentration of 0.33 mM. Eight different buffer solutions were prepared whose pH was as follows: 8.8, 7.3, 6.8, 6.4, 6.0, 5.6, 5.4, 4.9. These solutions in consecutive experiments constituted a medium where Infusoria were staying, and at the same time advantage was taken of them to examine the chemotropic reaction. The total concentration of salts amounted in each case to ca. 6.66 mM. It was of course not possible to avoid a certain difference in the amount of sodium cation in the various solutions. This was however of no great importance as the sodium cation, in relation to calcium cation, is relatively best endured by Infusoria and its pres-

Table VIII

Solution 1	Na_2HPO_4	6,66 mM				pH = 8,0
Solution 2	Na_2HPO_4	5,0 mM	+	NaH_2PO_4	1,66 mM	pH = 7,3
Solution 3	Na_2HPO_4	3,33 mM	+	NaH_2PO_4	3,33 mM	pH = 6,8
Solution 4	Na_2HPO_4	1,66 mM	+	NaH_2PO_4	5,0 mM	pH = 6,4
Solution 5	Na_2HPO_4	0,66 mM	+	NaH_2PO_4	6,0 mM	pH = 6,0
Solution 6	Na_2HPO_4	0,33 mM	+	NaH_2PO_4	6,66 mM	pH = 5,6
Solution 7	Na_2HPO_4	0,15 mM	+	NaH_2PO_4	6,66 mM	pH = 5,4
Solution 8				NaH_2PO_4	6,66 mM	pH = 4,9

ence in the solution in small concentration has almost no influence on the chemotropic reaction. The chemical composition and the pH values of the various solutions are given in Table VIII and the results of experiments in Table IX.

The results of experiments performed with buffer solutions proves not only the very great part played by pH in chemotropism, but indicates the existence of a chemotropic pH optimum within the limits from 6.4 to 5.4. Infusoria placed in a concentration of buffer salts with a pH within optimum limits give no positive chemotropic reaction in case a solution with a pH higher or lower than the optimum is introduced.

Some additional experiments were performed whose aim was to adapt Infusoria to the buffer solutions with a pH = 4.9 — 5.0, and a pH = 5.4 — 5.6, in order to ascertain whether this will in some way cause slift of the chemotropic optimum of pH. Infusoria were placed in such a solution for some 10—14 days. During the

Table IX

Chemotropism in dependence on the pH of the initial medium.

Infusoria placed in a buffer solution	The chemotropic reaction of Infusoria after an addition of a buffer solution							
	pH 8,0	pH 7,3	pH 6,8	pH 6,4	pH 6,0	pH 5,6	pH 5,4	pH 4,9
pH = 8,0		·	+	+	+	+	+	0
pH = 7,3	·		·	+	+	+	+	0
pH = 6,8	—	·		·	(+)	(+)	(+)	—
pH = 6,4	—	—	·		·	·	·	—
pH = 6,0	—	—	—	·		·	·	—
pH = 5,6	—	—	—	·	·		·	—
pH = 5,4	—	—	—	·	·	·		—
pH = 4,9	—	—	—	(+)	(+)	(+)	(+)	

whole period of the experiment a constant pH of the solution was maintained, and yet no change of the chemotropic optimum of pH with the adapted infusoria could be noticed. We may deduce from these observations that an adaptation to a definite pH has no influence on a change in the chemotropic optimum of pH.

THE PART PLAYED IN CHEMOTROPISM BY ADAPTATION

Adaptive phenomena with the Infusoria may be examined from different points of view. Most often they are examined on the basis of the existence of these organisms in higher concentrations than usual of the given substance. Such an effect is obtained due to a gradual conveyance of Protozoa through increased concentrations of the tested substance. The adaptation to the changed conditions of the medium can also be appraised on the basis of the ability to regulate certain basic life functions such as the speed of the pulse of contractile vacuoles of Infusoria. By this method Górski (1938) ascertained that Infusoria kept for a long time in a temperature from 2 to 3°C, which in the beginning have shown a very marked slackening of the pulse of contractile vacuoles, after a certain time were able to regulate almost completely the disturbed life functions.

It was decided to examine whether the chemotropic reaction of Infusoria undergoes any change under the influence of adaptation to the changed (in a chemical respect) conditions of the medium. We observed a swiftly appearing adaptation to the changed condi-

tions of the medium in relation to distilled water as well as to the neutral salt solutions prepared with distilled water. A closer observation reveals that Infusoria penetrate into these solutions gradually, though primarily they show even an avoiding reaction in the border belt between the initial medium and the introduced solution.

A longer stay of infusoria in a medium whose pH = 4.9 — 5.0, resp. 5.4 — 5.6 had no influence on a change of the chemotropic optimum in the direction of more acid or more basic values. Having this in view it was decided to make a trial to adapt Protozoa to certain definite salt concentrations with a decidedly negative chemotropic action, in order to ascertain whether the staying of Infusoria in a higher concentration of salts will evoke a change of chemotropic reaction. Our choice fell on potassium chloride whose chemotropic value in medium of tapwater is from 5 to 6 mM. The addition of a 6.66 mM solution of KCl in tapwater to a medium of tapwater where Infusoria are staying causes a distinct negative chemotropic reaction. By reversing the conditions of this experiment, i. e. by placing Infusoria in a 6.66 mM solution of KCl and by introducing a few drops of clean tapwater we create a chemotropic agglomeration in the area it occupies. This simple experiment served to verify the influence of adaptation to some definite concentrations of KCl on a change of a chemotropic reaction of Infusoria. We performed analogous experiments with Protozoa placed in solutions: 13.32 mM, 26.64 mM and 53.28 mM of KCl, that is in concentrations 2, 4 and 8 times higher. In 13.32 mM solutions of KCl Infusoria in the first 2—3 minutes reveal a movement around the diagonal axis of the body which gradually wanes. In solutions 26.64 mM and 53.28 mM KCl Infusoria during the first 2 minutes move backwards and in the next 2 minutes perform a movement around the diagonal axis of body, but even these disturbances gradually dissapear and Infusoria begin to move very slowly. These experiments prove the existence of an adaptation to the changed conditions of the medium in the form of a change of motor reaction.

The method applied in these tests, which have to do with adaptation was very simple. A 0.1 mM solution of KCl was prepared in tapwater and was subsequently diluted in an appropriate relation depending on the experiment to be performed. In the same way were prepared appropriate solutions of salt with Infusoria in order to adapt them to the changed conditions of the medium. For this purpose a solution of 0.1 M KCl was diluted in an appropriate relation with

tapwater containing the Infusoria. These experiments were performed in the following way: Infusoria were placed in solutions of KCl at a concentration of 6.66 mM, 13.32 mM, 26.64 mM, and 53.28 mM and then at various moments of adaptation some drops of tapwater were added and the ensuing chemotropic reaction was examined. The tests of chemotropic reaction were performed in solutions 6.66 mM and 13.32 mM KCl into which were introduced Infusoria adapted just before the experiment.

Table X shows some results of experiments in which Infusoria adapted to various concentrations of KCl were placed in a 6.66 mM solution of KCl.

Table X

The chemotropic reaction by Infusoria adapted to various KCl solutions. Infusoria placed in a 6,66 mM solution of KCl just before the experiment.

Infusoria adapted to a soluton of KCl	Chemotropic reaction after an addition of tapwater		
	after 1 hour adaptation	after 3 hours adaptation	after 24 hours and a greater number of days adaptation.
6,66 mM	+	(+)	(+)
13,32 mM	(+)	(+)	·
26,64 mM	·	·	·
53,28 mM	·	·	·

Analogous tests were performed with adapted Infusoria placed in a 13.32 mM solution of KCl, that is in a concentration twice as high as the preceeding one. The results of these experiments are shown below in the form of an orientation table. (Table XI).

It may be mentioned that we consider the lack of chemotropic reaction as a proof of the adaptation of Protozoa to the salts of potassium. In the course of the experiment it was noticed that the movements of Infusoria in tapwater are regular and in solutions of KCl are slow in experiments performed both with adapted and with unadapted infusoria. It follows from the above that chemotropic reaction is not strictly connected with slow or more rapid movements of Infusoria under the influence of a given cation. We never saw Infusoria, whether adapted or not, put in a solution 26.64 mM KCl and higher, to bear a positive chemotropic reaction in relation to clean tapwater although the adaptation to solutions 26.64 mM and 53.28 mM KCl lasted for a period up to 3 weeks.

The phenomenon of chemotropic adaptation, if it can be so called, appears especially attractive if to the medium containing a 6.66 mM solution of KCl, resp. a 13.32 mM solution of KCl we introduce simultaneously Infusoria that were for a long time adapted to salts of potassium together with some unadapted. They can be easily distinguished from each other if they were previously raised in different culture mediums as for instance in a yolk medium and in a hay infusion. When tapwater is introduced the non- adapted Infusoria assembled in its area show a distinct avoiding reaction when coming into contact with the zone of potassium chloride diffusion, while adapted Infusoria swim freely in both mediums and show only some slackening of movements in solution of potassium chloride in relation to the speed they show in tapwater.

Table XI.

The chemotropic reaction by Infusoria adapted to various KCl solutions. Infusoria placed in a 13,32 mM solution of KCl just before the experiment.

Infusoria adapted to a solution of KCl	Chemotropic reaction after an addition of tapwater		
	After 1 hour adaptation	after 3 hours adaptation	after 24 hours and a greater number of days adaptation
26,64 mM	+	(+)	·
53,28 mM	(+)	·	·

The adaptation described above to solutions containing potassium salts wanes within 2 or 3 hours if Infusoria are placed again in the medium of clean tapwater.

Adapted Infusoria introduced i na medium of clean tapwater form chemotropic agglomeration after an addition of water acidified with acids resp. with salts with an acid reaction. This fact proves that adaptation to salt of potassium is specific and has no influence on a different reaction of Infusoria in relation to a changed pH medium.

An interesting light is thrown on the problem of adaptation by experiments performed with solutions of potassium dichromate in tapwater of a definite pH, i. e. after the surplus of carbon dioxide has been evaporated from the acidified medium. The addition of a 3.33 mM solution of $K_2Cr_2O_7$ (pH = 6.4) causes at first the formation of a ring, which however dissapears within 3 minutes then comes into being a distinct positive chemotropic agglomera-

tion. The pH value of the solution (6.4) cannot be the cause of such a reaction as the concentration of hydrogen ions on such a level is perfectly withstood by Infusoria and lies within the limits of the optimum chemotropic reaction of pH. The above reaction is undoubtedly due to the presence of potassium which at first causes a negative chemotropic reaction. As soon as the agglomeration of Infusoria is clearly visible the addition of further portions of a solution of potassium dichromate evokes no appearance of a ring which proves that Infusoria are completely adapted to the changed conditions of the medium.

A 3.33 mM concentration of $K_2Cr_2O_7$ converted in potassium corresponds to a 6.66 mM solution of KCl, that is to a solution which causes distinctive negative chemotropism. Undoubtedly as soon as a 3.33 mM solution of $K_2Cr_2O_7$ is added, potassium evokes at first an avoiding reaction and Infusoria agglomerate in a ring where the pH of the solutions is lower than 7.0 and the concentration of cation K is not too high. Gradually however under the influence of the low pH of the introduced solution comes an adaptation to the existing concentration of potassium salt and Infusoria form then a lasting agglomeration. In such a way Infusoria are in the beginning under the influence of two factors which seemingly react in opposite directions but finally the influence of the low pH of the solution prevails and the result is a positive chemotropic agglomeration in the diffusion zone of potassium dichromate. A further proof that the reaction of Infusoria described above in relation to the 3.33 mM solution of $K_2Cr_2O_7$ is connected indeed with adaptation is the fact that Infusoria adapted to higher concentration of potassium chloride (26.64 mM or 53.28 mM) and subsequently placed in a medium of tapwater immediately form an agglomeration not only in a 3.33 mM solution of $K_2Cr_2O_7$ (pH = 6.4) but even in a 5.0 mM solution of $K_2Cr_2O_7$ (pH = 6.2). A low pH of the medium undoubtedly influence therefore the adaptation of Infusoria to those components which normally evoke a negative chemotropic reaction. It is likely that adaptation, such as can be observed with Infusoria staying in the medium of tapwater in relation to the added distilled water and to solutions of some neutral salts in distilled water, is due to perfectly analogous phenomenon — the part of potassium is fulfilled by a changed chemical compound of the medium in relation to tapwater and the low pH of distilled water (pH = 5.4) is a factor which makes the adaptation possible.

GEOTROPISM AND CHEMOTROPISM

Dembowski (1929) has proved that an increased negative geotropism in pH from 5.3 to 5.1 of acetic acid may be observed with Infusoria. In concentrations of acetic acid of lower resp. higher pH values geotropism does not appear.

Górski (1938) confirmed the data of Dembowski but observed geotropism at some other pH values from 5.8 to 5.3. The intensity of reaction was greatest when pH = 5.3 which went together with a slackening of movements, with the liquefaction of plasm and with the increase in the volume of Protozoa and a decrease of the speed of contractile vacuoles.

These experiments on geotropism were repeated in various concentrations of acetic acid in order to determine whether this phenomenon has any connection with chemotropism. Our observations confirmed these made by Dembowski, although it was found that geotropism could be observed, at somewhat different values of pH, namely from 5.5 to 5.3 which was probably due to an application of a more precise method in defining the pH.

The most intensive geotropism was observed when the concentration was about 4 mM of acetic acid added to tapwater in pH from 5.4 to 5.3. The movements of Protozoa were at that time distinctly slackened. It follows that negative geotropism under the influence of tapwater acidified with acetic acid appears in higher concentrations than these which cause positive chemotropism. It is confirmed by the various pH values of the applied concentrations. It was ascertained that positive chemotropism appeared under the influence of acetic acid within the limits of pH from 7.0 to 5.7, while geotropism appeared when pH was 5.5 to 5.3.

On the basis of experiments with acetic acid it was not possible to ascertain a connection between these two phenomena. In order to reach some definite conclusions the influence of other acids should be examined.

THE PART PLAYED BY OSMOTIC PRESSURE IN CHEMOTROPISM

In order to appraise the part played by osmotic pressure in chemotropism experiments were performed with the use of the following organic substances: urea, glucose and glycerol. These substances were especially suited to these experiments as they do not change the pH of the medium. These experiments were performed in the fol-

lowing way. Protozoa were introduced into a medium of tapwater according to the method described above. Even the addition of quite considerable concentrations (from 0.1 M to 0.5 M) of urea, glucose or glycerol caused no chemotropic reaction and the Infusoria swam into solutions of the above mentioned substances without any motor disturbance although changes in plasma in the form of a disturbance with a typical contraction of the forepart of Infusoria were visible. It may be of interest that instantaneous death of Protozoa was observed in concentrations almost identical of the mentioned substances from 0.6 M to 0.7 M which proves that the cause of death was an excessive osmotic pressure of the added solutions and not a specific toxity of the substances. Just before their death Infusoria show a violent motor reaction of short duration in the form of a backward movement. This phenomenon has however nothing to do with chemotropism, as it arises as a result of heavy premortem physiological disturbances.

The results of these experiments confirm entirely the thesis of Jennings that osmotic pressure as such plays no part whatsoever in chemotropism.

DISCUSSION

According to the governing views anions were to cause positive chemotropism and cations negative chemotropism. The specific reaction of cations was supposed to depend on the valency and on the atomic weight of cation. According to Kagan the chemotropic value should increase parallel to the increase of the valency of cation and in a group of cations with equal valency — parallel to the magnitude of atomic weight. Kagan gives the following ranking of cations in edpendence on the size of the chemotropic value: ($H^{\cdot} >$) $Fe^{\cdot\cdot\cdot} > Cr^{\cdot\cdot\cdot} > Al^{\cdot\cdot\cdot} > Fe^{\cdot\cdot} > Ba^{\cdot\cdot} > Mn^{\cdot\cdot} > Sr^{\cdot\cdot} > Ca^{\cdot\cdot} > Mg^{\cdot\cdot} > K^{\cdot} > Na^{\cdot} > Li^{\cdot}$ which is only a little different from the liotropic rank of Hofmeister, defining the dependence between adsorption and cation. Such a mutual dependence was supposed to show the great part played by adsorption in chemotropism. Jennings indicated also the increase of negative chemotropic reaction among cations uni-and bivalent parallel to the decrease of atomic weight. He noticed however that potassium is an exception as its salts raise negative chemotropism in a lower concentration than salts of sodium although the atomic weight of potassium is higher than the atomic weight of sodium. It must be noted that there are more such exceptions. Experiments performed

in our laboratory with some uni-and bivalent cations have shown that in the group of bivalent cations the salts of barium cause a motor disturbance with Infusoria in a much lower concentration than salts of calcium and magnesium, although the atomic weight of the last two is considerably lower than the atomic weight of barium. The following rank of cations was ascertained depending on the positive chemotropic reaction and on the magnitude of chemotropic value.

$Ca^{\cdot\cdot} \geqslant Na^{\cdot} \geqslant Mg^{\cdot\cdot} > Li^{\cdot} > K^{\cdot} > Ba^{\cdot\cdot}$ in relation to experiments performed in the medium of tapwater.
(40) (23) (24) (7) (39) (137)

$Ca^{\cdot\cdot} \geqslant Na^{\cdot} > Mg^{\cdot\cdot} \geqslant Li^{\cdot} > K^{\cdot} > Ba^{\cdot\cdot}$ in relation to experiments performed in the medium of distilled water.

Dividing the above cations into uni-and bivalent we get the following ranking:

univalent cations: $Na^{\cdot} > Li^{\cdot} > K^{\cdot}$, bivalent cations: $Ca^{\cdot\cdot} > Mg^{\cdot\cdot} > Ba^{\cdot\cdot}$

No correlation could be observed between the chemotropic value and the valency of cations or between the atomic weight and the chemotropic value among cations of equal valency. Trivalent cations ($Fe^{\cdot\cdot\cdot}$, $Cr^{\cdot\cdot\cdot}$, $Al^{\cdot\cdot\cdot}$) added in the form of salts to the medium of tapwater cause its acidification, its action then is related to a corresponding change of pH of the medium and it is therefore difficult to deduct a competent conclusions as to its chemotropic reactions. Chemotropical agglomerations under the influence of salts solutions containing trivalent cations are undoubtedly due to a low pH of the medium although a characteristic reaction caused by $Fe^{\cdot\cdot\cdot}$ resp. $Al^{\cdot\cdot\cdot}$ in the form of a slackening of movements of Infusoria or their complete stoppage may be also observed. The change of pH of the medium caused by salts containing trivalent cations was not sufficiently appreciated in the literature and this is the reason for a faulty interpretation of the results of experiments. It was taken for granted that trivalent cations cause the least motor disturbance of Infusoria disclosed by a distinct slackening of movements, while bivalent cations raise no such distinct avoiding reaction as can be observed when univalent cations are added.

In the light of experimental facts established in this work we may assert that the chemotropic reaction is in no relation with the valency of the cation nor with the atomic weight of the corresponding element.

The problem of a specific influence of chemical properties of the substance on chemotropism was also improperly interpreted so far. There is no doubt as to the specific influence of cation as well as to the fact that osmotic pressure of the tested substance plays no role of importance in chemotropism, which was already properly emphasized by Jennings. Positive chemotropism under the influence of a substance with an acid action (acids and salts) depends on the pH value of the medium only, while the role of anion, resp. cation is to check in a greater or a smaller degree the positive reaction mentioned above. Thus breaks down the conception of a specific chemotropic influence of anion to the advantage of influence of pH, and the optimum of the chemotropic reaction of pH takes place within limits of pH = 6.4 — 5.4, independently of the chemical structure of the tested substance. The biological role of pH deserves to be specially emphasized if we take into consideration the fact that chemotropic agglomerations of *Paramaecium caudatum* in normal conditions arise in the first place under the influence of carbonic acid produced by Protozoa and by bacteria and which is undoubtedly the most important factor changing the pH of the natural medium.

The influence of pH of the solution deserves attention within the limits of optimal chemotropic reaction on the adaptation of Infusoria to these components of the medium in the face of which they normally show a negative chemotropic reaction. Such an adaptation can be of great biological importance as a change in pH and in the chemical compound of the medium in natural conditions undoubtedly appears.

It is worthy of attention that the influence of adaptation on a change of a chemotropic reaction was proved in an experiment with potassium salts, but it was impossible to obtain analogous results when trying such adaptation with solutions with a definite pH. The cause of this seemingly curious fact seems to be a basic difference between the reactions of pH and cations on the protoplasm of Protozoa. *Paramaecium caudatum* is able to live in conditions of various pH but Górski (1938) has proved that an endevour to adapt Infusoria to solutions with a pH lower than that normally encountered failed, as it was not possible to increase the resistance of Infusoria in relation to acids. Chejfec (1933) ascertained however that the duration of an acid reaction of food vacuoles is only in a slight degree dependent on the pH of an external medium. He observed also a prolongation of the acid reaction in proportion to

the alkalisation of the medium within the limits of pH from 5.0 to 9.0. Chejfec proved then that such a basic function for the life of Protozoa as digestion of food undergoes but small and indirect changes in relation to the pH value of food vacuoles under the influence of various pH values of the external medium. It seems improbable that a change of pH in the medium should have any influence on parallel changes of pH in protoplasm, as this would mean a complete change of biochemical processes in the cell. The influence of pH of the medium on the cell of Protozoa is probably limited to the ectoplasm and to the cilia apparatus of Infusoria and in no degree is changing the pH of protoplasm which, besides, has great possibilities to counteract the changes of pH, thanks to numerous buffer systems. In such, or in some other way, the organism is in a position to regulate to a great extent the pH independently from the pH value of the medium. The influence of cations such as potassium is of a different nature, as Infusoria are unable to cause a quantitative or a qualitative change of salts which penetrate gradually into the protoplasm. The „chemotropic" adaptation to salts of potassium lasts no longer than 3 hours and depends probably on the gradual rinsing of potassium salts from the cell in a medium devoid of potassium cation. On the basis of the above reflections it is possible to draw a conception that the organism of Infusoria is forced to adaptation in the first place when it is unable to change these factors of the medium which influence it directly.

The positive result of adaptation to definite concentrations of salts of potassium denies the phenomenon of compulsion in chemotropism, as the chemotropic reaction depends decidedly upon the „chemical past" of Protozoa. The existence of adaptation enables one to conclude that chemotropism depends upon physicochemical differences of the medium as well as upon a different physiological state of Infusoria.

SUMMARY

1. The influence of cations on the phenomenon of chemotropism is specific. For some uni-and bivalent cations the following ranking is established depending on the positive chemotropic reaction and on the chemotropic value:

$Ca^{\cdot\cdot} \geqslant Na^{\cdot} \geqslant Mg^{\cdot\cdot} > Li^{\cdot} > K^{\cdot} > Ba^{\cdot\cdot}$ in relation to experiments performed in a medium of tap-water.

$Ca^{\cdot\cdot} \geqslant Na^{\cdot} > Mg^{\cdot\cdot} \geqslant Li^{\cdot} > K^{\cdot} > Ba^{\cdot\cdot}$ in relation to experiments performed in a medium of distilled water.

Such ranking of cations does not indicate a dependence of chemotropism on the valency resp. on the atomic weight of cation.

2. Positive chemotropism appearing with *Paramaecium caudatum*, when an acidified medium of tapwater is added, is due to a change of the pH of the medium, and does not depend upon the specific chemical structure of the tested substances.
3. A trial to adapt Infusoria to solutions of low pH gave no results in a meaning of a removal of the optimum of a positive chemotropic reaction.
4. Osmotic pressure plays no role of importance in chemotropism.
5. The pH of the solution within the limits of optimum of a positive chemotropic reaction (pH = 5.4 — 6.4) has a great influence on the adaptation of Infusoria to these components of the medium in relation to which they usually show a negative chemotropism.
6. An adaptation to definite concentrations of potassium salts (KCl) in a medium of tapwater causes a change in the chemotropic reaction. Such an adaptation is specific and lasts no longer than 2 or 3 hours, if the Infusoria are put back in a medium of tapwater without an addition of potassium salts. It is due to the fact that Infusoria adapted to higher concentrations of potassium when put in a solution of 6.66 mM, resp. 13.32 mM of KCl after tapwater has been added, do not form in it a chemotropic agglomeration such as appears always in experiments performed with non adapted Infusoria.

The capacity of *Paramaecium caudatum* to adapt to the changed conditions of the medium and a change in the chemotropic reaction connected with it denies the existence of compulsion in chemotropism, and instead indicates a plasticity of the phenomenon.

The author wishes to express his thanks to Prof. J. Dembowski for his kind interest in this work.

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THE INFLUENCE OF THE STATE OF THE PERIPHERAL STUMP ON THE EARLY STAGES OF NERVE REGENERATION

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It was demonstrated in a foregoing paper (Lubińska and Olekiewicz, 1950) that the latent period of regeneration and the rate of elongation of regenerating fibres vary differently at different temperatures and it was concluded that the two phenomena must be determined by different factors. In order to disentagle this question other circumstances were sought in which the latent period would behave otherwise than the rate of elongation.

The present experiments concern the influence of the state of the peripheral stump, fresh or predegenerated, on nerve regeneration. It was found that the strong increase of the latent period of regeneration observed at low temperatures takes place only when the nerve has to regenerate into a fresh peripheral stump. It came out that when the regenerating fibres have to grow into a predegenerated path, the latent period is reduced and its variation with temperature is incomparably smaller. Thus, the duration of the latent period at different temperatures described previously, reflects actually the changes in the rate of degeneration of the peripheral stump with temperature. A rough evalution of the rate of degeneration of the peripheral stump at various temperatures was made

and it was found that its temperature course is similar to that of the latent period.

As to the rate of elongation of regenerating fibres, this does not depend on the state of the path, provided a certain degree of degeneration is attained.

MATERIAL AND METHODS

The experiments were performed on about 200 frogs, *Rana esculenta*. Their sciatic nerves were crushed aseptically high in the thigh and after allowance of definite periods for regeneration, the lenths of regenerated sensory fibres in both tibial and peroneal divisions were measured by a method described in detail previously (Lubińska and Olekiewicz, 1950).

During the regeneration the animals were kept in thermostats at 13°, 17° and 26°C. The variation of temperature in the thermostat did not exceed 0.5°C.

At each temperature three series of experiments were carried on.

I Standard series. Regeneration into a fresh peripheral stump. The nerves were crushed and the lengths of regenerating fibres were measured at 3, or more, different time intervals after the crush. The data thus collected served to determine the regression of the regenerated lengths on time and to calculate the duration of the latent period, as well as the rate of advance of regenerating fibres at the temperature studied. The results obtained in such series served as reference for the analysis of the results of following series, where the influence of predegeneration, and of the double crush was investigated.

II Series. Regeneration into a peripheral stump degenerated prior to the experiment. In this series the following procedure was adopted. The nerves were crushed and the animals were kept during 5 days at 26°C. In these conditions, as was established in preliminary experiments, the state of the peripheral stump allows a free advance of regenerating fibres along it.

After 5 days a new operation was performed and the nerve was crushed a second time, care being taken to make the crush as exactly as could be done at the site of the first crush. At this operation the fibres that regenerated during the 5-days interval were destroyed and all processes of regeneration had to start again at the temperature in which the animals were kept after the second crush. At definite periods after the second operation the lengths of regenerated fibres were measured and the latent period of regeneration as well as the rate of advance were calculated as in the standard series. The main difference between these experiments and those of the first series was that here the regenerating fibres had to follow a predegenerated peripheral pathway instead of a fresh one. A second difference was introduced by the double crush that produced a more extensive scar than that obtained by a single crush.

III Series. Regeneration after double crush into a non-predegenerated peripheral stump. In order to evaluate the possible rôle of the double crush in subsequent course

of regeneration, an additional series of experiments was performed. In this series the animals were kept for 5 days between the first and the second crushes at low temperatures of 13° or 17°C which were to be used later after the second crush. At these temperature the degeneration proceeds slowly and the peripheral stump is in a fairly good condition after 5 days. It may be thus admitted to a first appoximation, that this series differs from the standard one only by the character of the crush which is here double.

Things are different for the temperature of 26°C. Kept at this temperature the peripheral stump undergoes a marked degree of degeneration in 5 days. So, in order to get information on the rôle of the double crush with the pre-degeneration of the peripheral stump eliminated, the animals were kept at 13°C during the time between the crushes and transfered to 26°C after the second crush had been performed.

RESULTS

The results obtained are summarized in Table I and illustrated in Fig. 1. The straight lines in the figure represent the calculated regression lines of the lengths of regenerated fibres on the times of regeneration. The cirles represent the mean lengths reached by the regenerating fibres at various times after the crush.

Table I.

Rates of elongation and latent periods of regeneration in various series.

Series	13°C			17°C			26°C		
	Rates in mm/day	Confidence limits	Latent periods in days	Rates in mm/day	Confidence limits	Latent periods in days	Rates in mm/day	Confidence limits	Latent periods in days
I	0,56	0,47—0,66	7,5	0,76	0,68—0,85	4,4	2,2	2,0 — 2,3	2,9
II	0,54	0,45—0,62	2,9	0,72	0,64—0,80	3,0	2,1	1,8 — 2,5	1,2
III	0,63	0,47—0,79	9,2	0,79	0,62—0,97	6,0	2,3	2,0 — 2,6	3,0

As is seen from Table I, the rate of regeneration remains fairly constant at a given temperature and is not affected either by the state of degeneration of the peripheral pathway or the extent of the scar which is greater in the series II and III as compared to the standard series. The small variations of the mean rate are explained by the changes of experimental circumstances (unavoidable in these lenghty experiments) and fall well within the confidence limits, of each other. Comparison between mean lengths calculated (from regression equations) and observed for fibres regenerating at 17°C is given in Table II.

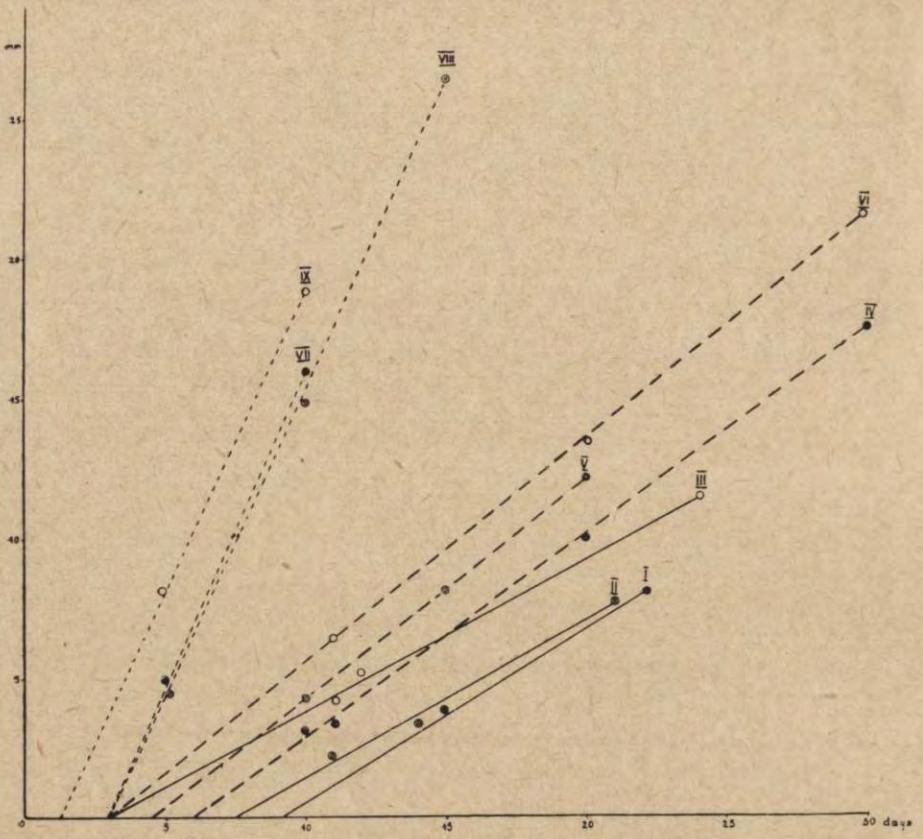

Fig. 1. Regression lines and mean lengths of regenerating fibres in various series.

13°C — I — double crush without predegeneration. II — single crush. III — double crush, predegenerated path.

17°C — IV — double crush without predegeneration. V — single crush, VI — double crush, predegenerated path.

26°C — VII — double crush without predegeneration. VIII — single crush. IX — double crush, predegenerated path.

As to the latent period of regeneration, this is, on the contrary, greatly affected by the state of degeneration of the peripheral stump. Predegeneration of the peripheral stump causes a marked decrease of the latent period at all temperatures studied. It falls from over 9 days to about 3 days at 13°C, and from 6 days to about 3 at 17°C. At 26°C, where the latent period after the double crush without predegeneration lasts about 3 days, this falls to 1.2 days as a result of predegeneration of the peripheral stump.

Table II.

Comparison between calculated and observed mean lengths of regenerating fibres in the 3 series at 17°C.

Series / Time in days	I		II		III	
	Calculated	Observed	Calculated	Observed	Calculated	Observed
10	4,3	4,3	2,9	3,0	—	—
11	—	—	3,6	3,4	6,3	6,4
15	8,1	8,2	—	—	—	—
20	11,9	11,9	10,1	10,0	13,5	13,4
30	—	—	17,3	17,3	21,4	21,4

At temperatures of 13°C and 17°C (Series III) the double scar produces an increase of the latent period of over one and a half day compared to the standard series at the corresponding temperature. At 26°C there seems to be no effect of the double crush.

Considerable influence of predegeneration on the curtailment of the latent period raises the question whether long latent periods at low temperatures may not be due to the slowing down of the processes of degeneration at these temperatures. The answer to this question was obtained from the protocols of the experiments in which the degree of impairment of neuro-muscular transmission in the peripheral stump was noted during the tests of regeneration. This degree was labeled as 1 — for slight impairment of transmission, 2 — for strong impairment (a very small muscular contraction on stimulation of the motor nerve) and 3 — for complete disappearance of neuro-muscular transmission. As these data were recorded but occasionally and embrace animals from different batches, scattered over several years, no claim can be made for more than a very rough evaluation of the influence of temperature on the rate of deterioration of the neuro-muscular transmission. This deterioration can be thought of as a rough measure of degeneration of the peripheral stump. No correlation with the histological appearance of degenerating fibres was made however. Moreover, the data obtained concern the degeneration of motor fibres while the tests of regeneration concerned sensory fibres. As, to our knowledge, no difference in the rate of degeneration of motor and sensory fibres was ever detected, it seems possible to utilize the information con-

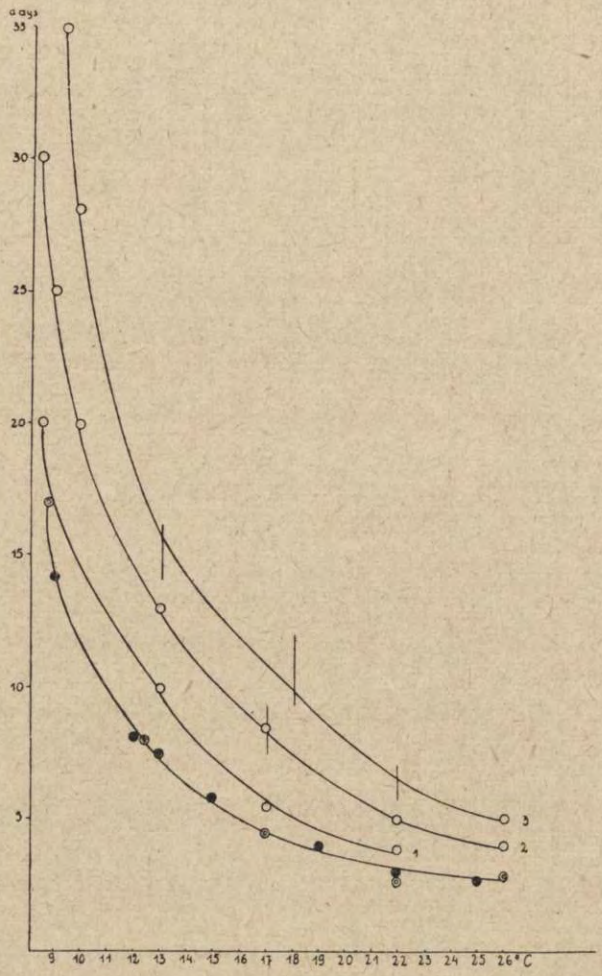

Fig. 2. Duration of latent periods and degrees of Wallerian degeneration at various temperatures.

Abscissae: temperature in °C
Ordinates: time in days

1. Small impairment of neuro-muscular transmission.
2. Strong impairment of neuro-muscular transmission.
3. Complete disappearance of neuromuscular trasmission.

Dotted circles — latent periods.

Black circles — times at which a certain degree of histological degeneration is attained (recalculated from Torrey).

cerning the impairment of neuro-muscular transmission as complementary to the main line of the present investigation.

The results are shown in Fig. 2, where the number of days after which a given degree of impairment of neuro-muscular transmission is reached in more than 80 per cent of nerves, is plotted against the temperature. The curve of latent periods at various temperatures is given in the same graph. The shapes of all curves are similar. It is to be noted that the latent period for each temperature is always shorter than the time needed for even the slightest deterioration of neuro-muscular transmission, labeled 1 in our protocols. In other words the regenerating, fibres may penetrate the peripheral stump before the neuro-muscular transmission is abolished. This fact was frequently observed directly during the tests of regeneration.

Besides the data obtained in the present experiments, the results reported by Torrey (1934) are recalculated and included in Fig. 2. This author investigated the temperature coefficient of nerve degeneration, measuring the time at which the same histological picture is obtained at various temperatures in the tibial nerve of *Rana pipiens*. As absolute numbers are not reported in his paper, the time of degeneration at 25°C is taken as unity and relative values for other temperatures are recalculated from temperature coefficients for various intervals. As it is seen from figure 2, they fit well the curve traced by the values of latent periods at various temperatures.

DISCUSSION

The main results reported here demonstrate that the curious temperature characteristics of the latent period of regeneration, reported previously, are determined by the state of the peripheral stump. The temperature course of the latent periods, so different from that of the elongation, is similar to the temperature course of the rate of degeneration of the peripheral stump. Everything seems to indicate that the regenerating fibres cannot enter the peripheral pathway before a certain degree of degeneration is reached. This degree may be very small since the regenerating fibres penetrate the peripheral stump when the old fibres have preserved their integrity to the extent that they are still able to conduct impulses. Thus the question as to what change in the physico-chemical properties of the peripheral fibres is necessary to permit the advance of new fibres is not clear. One is inclined to think that the turgor of a fibre

freshly separated from its perikaryon is so great as to constitute a mechanical obstacle to the penetration of the regenerating tip, and that this turgor diminishes progressively before the axon breaks down into ovoids. When the turgor falls down to a certain value, the regenerating tip is able to insert itself between the neurilemmal tube and the old fibre. Once it is there, it advances at a rate that is not influenced by the further breakdown and degeneration of the old fibre (probably because of the extreme thinness of the regenerating sprout during the phase of elongation).

It is interesting to note the similarity of the temperature course of Wallerian degeneration and of various kinds of cellular division (division of Amoeba, egg segmentation). Attention was drawn in a previous paper (Lubińska and Olekiewicz 1950) to similar temperature characteristics of latent periods of regeneration and of cellular division, though the similarity was not easy to understand. Now, as the duration of latent periods at various temperatures proved to be determined by the rate of degeneration of the peripheral stump, that is the segmentation of the continuous column of axoplasm into separate ovoids, the similarity with cellular division becomes more suggestive.

There seems to be no systematic study of the influence of predegeneration on the early phases of regeneration, in the literature. It is interesting to note, nevertheless, that Sanders and Young (1942), in comparing the regeneration into fresh and predegenerated autografts of rabbits, mention that the rate of elongation does not differ significantly in both cases, „though the shorter latent period shown for predegenerated grafts is suggestive". The point does not seem to have been investigated more fully, lying outside the main topic of the quoted paper.

The inability of regenerating fibres to enter a nerve pathway before a certain degree of degeneration is reached, accounts probably for the well known (Langley and Anderson, 1904 and others) fact that after suture of two central stumps no regeneration of one into another takes place.

The present investigation leaves open the problem, whether a true latent period, i. e. a time interval between the infliction of lesion and the start of elongation exists, or whether, apart from the obstacle caused by insufficient degeneration of the peripheral stump, the only initial delay is produced by a slower advance of regenerating fibres in the crushed region. The very short latent

period observed at 26°C with predegenerated peripheral stump seems to support the second possibility.

The interesting observations that at the very beginning of regeneration in the initial few millimetres, the rate of elongation is not constant (Wyrwicka, 1950) are not taken into account in the present investigation. In this paper, as in the preceeding one, the latent period is defined by the regression of regenerated length on time which is linear throughout except at the very beginning of the peripheral stump. The latent period corresponds to the value of the abscissae where it intersects the regression line.

SUMMARY

1. The duration of the latent period of regeneration is in a large measure determined by the rate of degeneration of the peripheral stump.

2. When the regenerating fibres are allowed to grow into a predegenerated stump, the latent period is greatly shortened.

3. The temperature course of degeneration of the peripheral stump is similar to that of the latent period of regeneration.

4. The degree of predegeneration necessary for the curtailment of the latent period is very small. It seems that the old fibres may still conserve their functional integrity and ability to conduct impulses over their entire length while the change in their state is sufficient to allow penetration of regenerating fibres.

5. Once the necessary degree of degeneration is attained, the rate of elongation of regenerating fibres is constant and independent of further progress of degeneration.

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ON THE ARREST OF REGENERATION OF FROG PERIPHERAL NERVES AT LOW TEMPERATURES

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It was mentioned in one of the foregoing papers (Lubińska and Olekiewicz 1950) that at 8°C, and sometimes at slightly higher temperatures, the frog peripheral nerves fail to regenerate. Actually, the regeneration does start, but new fibres enter very slowly the peripheral stump and do not advance there beyond a few millimeters. The further increase in their length with increasing time is very small, if any. Such behaviour is in marked contrast with an alternative behaviour observed at slightly higher temperatures where the regeneration does occur. Between 9°C and 16°C the regenerating fibres, having once entered the peripheral stump elongate there steadily at a rate of about 0.6 mm per day. At the borderline temperature either one or the other behaviour appears. No intermediate rate was ever observed.

The experiments reported here were performed in order to establish which of the two early stages, the latent period of regeneration, or the elongation of regenerating fibres along the peripheral stump, is affected by low temperature. It was demonstrated earlier that these two stages are differently influenced by temperature. It seemed interesting to elucidate for which of them the temperature of about 8°C is critical. The results obtained indicate that

it is the elongation of fibres in the nerve trunk that is inhibited at this temperature and that this is independent of the degree of degeneration of the old fibres in the peripheral path.

METHODS

The experiments were carried out on nerves of the hind limbs of the frog, *Rana esculenta*. The nerves were crushed in aseptic conditions and the lengths of regenerated fibres measured after various periods of regeneration by a method described in detail previously (Lubińska and Olekiewicz, 1950). After the crush the animals were kept in thermostats at temperatures just below those which allow for the normal regeneration (at about 8°C). The behaviour of regenerating fibres was studied under the following circumstances. 1. Growth into a fresh peripheral stump after a single crush of the nerve. 2. Growth into fresh peripheral stump after double crush. 3. Growth into a predegenerated peripheral stump, the nerve being crushed twice. 4. Further regeneration at low temperature after a start at 26°C.

RESULTS

Series 1. The animals were kept after the nerve crush at 8°C for 20, 30 and 40 days. This series illustrates the typical behaviour of fibres at a temperature preventing normal regeneration. The mean daily increase in length of regenerating fibres between 20 and 30 days is 0.07 mm. This is about ten times smaller than the slowest rate of regeneration observed at somewhat higher temperature in animals which do regenerate. No further increase is observed between 30 and 40 days.

It was shown previously (Lubińska, 1952) that at the lower end of the temperature range at which the regeneration does occur the latent period is greatly lengthened owing to the slow degeneration of the peripheral stump at these temperatures. It has been thought therefore, that what we are dealing with in the present experiments is a further protraction of the latent period, extending beyond the time intervals examined here. In order to test this hypothesis conditions were created for regenerating fibres to grow into a predegenerated peripheral pathway. Such a pathway could be obtained either by a preliminary section of the nerve and a suture after degeneration of the peripheral stump, or by making two crushes, the first to start the degeneration and the second, made at the same place as the first after an interval of time allowing for the desired degree of degeneration, to destroy the new fibres that have grown into the degenerating nerve during the interval. The second procedure was chosen and the following series of experiments were performed.

Series 2. In order to elucidate the possible influence of the second crush, an auxiliary series of experiments was performed in which two crushes were made at 5 days interval. The animals were kept at 8°C during the interval between the crushes. Under these conditions the degeneration of the peripheral path scarcely occurs (the neuro-muscular transmission is entirely conserved) and so this series resembles series 1 as far as the state of the peripheral path is concerned but differs from it in the sense that the regenerating fibres have now to cross a more extended crushed region. The lengths of regenerated fibres obtained in this series after 20, 30 and 40 days of regeneration were comparable to those of the first series, and we may conclude that the double crush by itself did not modify the conditions of regeneration at 8°C.

Series 3. In this series also two crushes were made at 5 days intervals, but the animals were kept between the crushes in a ther-

Table I.

Mean lengths in mm attained by „non regenerating" fibres.

Days	Series 1, 8°C, single crush			Series 2, 8° → 8°C, double crush			Series 3, 26° → 8°C, double crush		
	number of nerves	mean length in mm	Confidence limits	number of nerves	mean length in mm	Confidence limits	number of nerves	mean length in mm	Confidence limits
20	17	1,8	1,2—2,4	14	1,4	0,8—2,0	13	1,7	1,5—1,8
30	12	2,4	1,8—3,0	21	2,3	1,9—2,8	11	2,7	2,5—2,8
40	8	2,5	2,0—3,0	17	2,6	2,1—3,1	15	2,5	1,8—3,1

mostat at 26°C. At this temperature the peripheral stump undergoes a marked degeneration during 5 days. After the second crush which destroyed the fibres formed during the interval, the animals were transfered to 8°C. Thus, all processes of regeneration had to start anew at the same temperature. This series differed from the second series in the supply of a predegenerated peripheral pathway. It was by this very method that the influence of predegeneration on the duration of the latent period was found previously (Lubińska 1952), and it was expected that, here too, the predegenerated peripheral pathway would allow the advance of regenerating fibres. However, in the present experiments no influence of predegeneration could be detected in „non regenerating" nerves at 8°C. The

lengths obtained were exactly the same as in the series 1 and 2 with corresponding times, and never exeeded 3 mm in the peripheral stump.

The results of the three series are represented in Table I (and in Fig. 1). There appears no significant difference in the lengths of

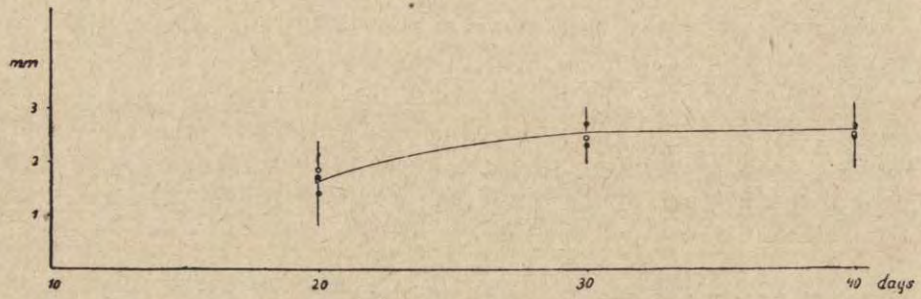

Fig. 1. Lengths attained by non-regenerating fibres. Abscissae — time in days. Ordinates — fibre length in mm.
Dots — series 1, circles — series 2, half filled circles — series 3.

Table II

Lengths of fibres regenerating at 26°C				Lengths of fibres regenerating at 8°C after initial 5 days regeneration at 26°C		
Days	Number of nerves	Mean length in mm	Confidence limits	Number of nerves	Mean length in mm	Confidence limits
5	20	5,5	5,3— 5,6	—	—	— —
10	40	14,8	13,9—15,7	—	—	— —
15	42	26,5	24,8—28,2	14	5,5	4,5—6,6
20	21	35,9	33,6—38,2	38	5,4	4,8—5,9
25	13	47,7	46,0—51,4	18	5,4	4,6—6,2

regenerated fibres, at the corresponding time intervals in these three series. Thus, one may conclude that the double crush does not modify the conditions of regeneration at 8°C and that the state of degeneration of the peripheral path is not the determining factor of the arrest of regeneration at low temperature.

The results of these experiments suggest strongly that it is not the processes connected with the latent period that are inhibited at low temperature, but the stage of elongation of regenerating fibres.

In order to verify this conclusion a 4th series of experiments was performed. This series was designated to eliminate the latent period altogether and to study the influence of low temperature on the phase of elongation only. This was obtained in the following way. The animals were kept after the crush for 5 days in a thermostat at 26°C. In preliminary experiments on 20 nerves it was determined that the regenerating fibres reach in these conditions about 5 mm in the peripheral stump during 5 days. (Mean length 5.49 mm, confidence limits 5.26 and 5.72). On the sixth day the animals were

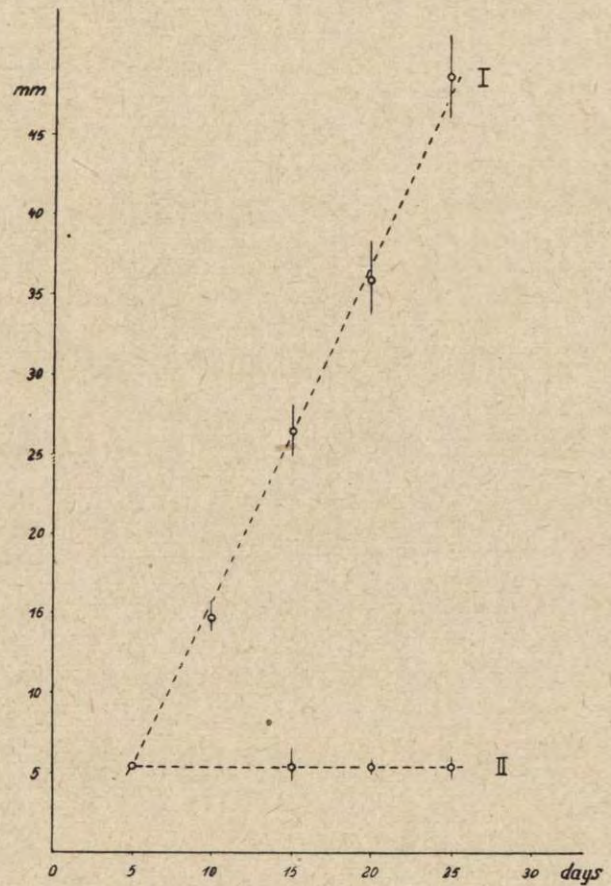

Fig. 2. Comparison of lengths of fibres regenerating at 26°C (I) and of fibres transfered to 8°C after initial 5 days at 26°C (II).
Abscissae — time in days. Ordinates — fibre length in mm.

transfered without any other treatment to a thermostat at 8°C and kept there for 10, 15 and 20 further days. Since the latent period and the first 5 mm of elongation took place at 26°C any further increase in length of regenerating fibres could be attributed to the regeneration at 8°C. The results of this series are represented in Fig. 2, lower line. The upper line represents the lengths of regenerated fibres in another series of experiments where the animals were allowed to continue their regeneration at 26°C. As is seen from figure 2, no increment over the initial length can be observed for animals kept at 8°C. The results of the series 4 indicate clearly that it is the phase of elongation of regenerating fibres that is inhibited at low temperatures.

DISCUSSION

The results reported here raise the following question. If the elongation of regenerating fibres is inhibited at low temperatures, how is it that the fibre grow nevertheless 2—3 mm into the peripheral stump? Since we are working on borderline temperature above which the regeneration does occur, the simplest supposition would be that small oscillations of temperature in the thermostat may produce this effect — when the temperature rises above the critical value, the elongation takes place, when it lowers again, the elongation stops. If this is so, then the length of regenerating fibres would be the sum of increments obtained during the short periods of heightened temperature in the thermostat. However a close inspection of experimental results seems to rule out such an explanation. The lengths attained in corresponding time intervals are very much alike in all three series (although these series were not carried out simultaneously) and the increases observed are largest during first 20 days, much smaller between 20 and 30 days and altogether doubtful between 30 and 40 days. This suggests that the elongation in the initial few millimetres of the peripheral stump is determined by other factors than those which determine the further advance along the peripheral pathway and that these initial mechanisms are not disturbed at the temperature of 8°C. It is perhaps interesting to compare these findings with the results obtained by Wyrwicka (1950) on white mouse and studied in more detail by Łukaszewska, (1952), on the frog. These authors found that in the initial few millimetres of the peripheral stump the rate of elongation is different from what it becomes later. The former is not constant and is smaller than

the rate of elongation in further, more distal portions of the peripheral stump at corresponding temperature. Moreover, the start of elongation takes place before the termination of the latent period as calculated in our previous papers on the basis of linear regression of regenerating tips in more advanced stages of elongation. These results seem to indicate that the „latent period of regeneration" as defined formerly may not be after all quite latent. During at least some part of it a small elongation may take place, though its time course is different from what is observed in later stages. Thus a period during which a slow advance of regenerating fibres takes place must be interposed between the true latency and the period of linear elongation. It is quite possible that the results reported here on the start of elongation in non-regenerating fibres belong to the same initial phenomenon.

SUMMARY

At a temperature of about 8°C the regeneration of peripheral nerves of the frog *Rana esculenta* does not follow the course it takes at slightly higher temperatures. At 8°C the process of regeneration does start but elongation practically stops after reaching 2—3 mm in the peripheral stump. This initial elongation is not linear with respect to time. It is independent of the degree of degeneration of the peripheral stump and reaches exactly the same length in fresh as well as in predegenerated peripheral stump.

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ELASTICITY AND DISTENSIBILITY OF NERVE TUBES

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In the present paper a series of observations and measurements on living unstained nerve fibres is reported. The observations concern both normal undisturbed fibres and fibres compressed over a small length (less than 0.5 mm) by a crush similar to that usually produced to start nerve regeneration. The analysis of deformations and of displacements of fibre contents observed in these circumstances permits some inferences to be drawn concerning mechanical properties of nerve fibres and their sheaths. These inferences are confirmed by additional observations on degenerating fibres and on the outflow of contents from the cut ends of fibres.

MATERIAL AND METHODS

Fibres from peripheral nerves of rabbits were used. The nerves (tibial, peroneal, median and ulnar) were removed under barbiturate anaesthesia and stored in Ringer solution at room temperature. Fresh fibres were examined within an hour after removal from the animal. Wallerian degeneration was studied in nerves kept in Ringer solution at about 22°C for three days; at this time almost all fibres are broken down into ovoids.

To obtain isolated fibres, both normal and degenerating nerves were ligated at one end, the ligature serving for fixation, stripped of their epineurium over a length of about 1—1.5 cm, and teased in Ringer solution with surgical needles. The preparation was then placed on a slide, the dissociated

fibres were spread out fanlike either in a drop of paraffin oil in Ringer solution and covered with a coverslip (sealed with paraffin wax to prevent dessication in the case of Ringer).

The crush of the nerve was made in following way. The nerve was placed longitudinally on a glass tube of about 12 mm diameter. A loop of nylon thread 0.25 mm in diameter was pressed against the nerve on the tube with a force of 1 kg. The direction of the thread was perpendicular to the long axis of the nerve (Fig. 1). The pressure was exercised during several seconds.

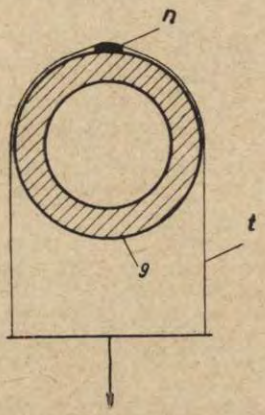

Fig. 1. Diagram of the position of nerve during the crush. g — glass tube, n — nerve, t — nylon thread.

Immediately after the crush the nerve was carefully stripped of epineurium and dissociated in Ringer solution, as were uncrushed nerves. The fibres were teased over a length of several millimetres comprising the region of the crush and adjacent parts of the fibre. Further away from the crush they remained in bundles.

On microscopic examination only those fibres were taken into account which were uninjured and could be traced over a length comprising the crushed region, the deformed neighbouring segments and the unaffected parts of the fibres.

The microphotographs serving for measurements were always taken from fresh preparations. Only fibres from 4μ upwards were measured. Smaller, faintly myelinated fibres are frequently very irregular in shape, their diameter varying markedly from place to place, and the evaluation of deformations produced by the crush is thereby much more difficult. The upper limit in diameter range was 22μ but occasionally fibres up to 24μ were encountered.

For the study of fibre diameters the microphotographs were taken at x 100 magnification and the plates projected at further x 5 magnification. The measurements were made from the projections. The external boundaries of myelin served to determine fibre diameters. At nodes and in the crushed regions devoid of myelin, the faintly visible limits of the fibres were measured, though

it is not possible to say whether the neurilemma or the surface of the axoplasm were visible. In degenerating fibres broken into ovoids, the neurilemma is sometimes faintly visible between the beeds and sometimes totally invisible. Since in any case it is a very thin tube, this imprecision is not likely to affect the results concerning deformation of the fibre.

RESULTS

I. NORMAL FIBRES. On unstained preparations only myelinated fibres were visible. They remained in good condition for about one hour. In such preparations the internodal segments presented optically empty axoplasm and, generally, regular cylindrical outline of myelin. From place to place a small invagination of myelin into the axoplasm, or an apparently isolated droplet of myelin in the interior of axoplasm, were seen. The incisures of Schmidt-Lantermann were narrow and clear cut. (Pl. I, Fig. 2 and 2-a).

The regular cylindrical shape of the internodal part of the fibre is modified in the vicinity of the nodes of Ranvier. On both sides of the node the fibre is enlarged and tapers gradually off to the normal diameter away from the node. The configuration of myelin at the node is generally modified over a length of 1 to 3 diameters of the fibre. There the myelin not only ensheaths the axoplasm but is seen on the optical section in the interior of the fibre forming irregular folds and ridges. These feature of nodes are discernible not only in isolated fibres. (Pl. I, Fig. 3 and 6) but, when the visibility is good, even in fibres remaining in bundles. (Pl. I, Fig. 4 and 5). It is to be noted that Nageotte (1922), who studied the nodes in teased fibres of young rabbits, states that the internode forms a regular cylinder terminating at nodes by hemispherical cupolae covered by a uniform layer of myelin up to the node itself, where the myelin almost comes into contact with the layer of myelin capping the neighbouring segment. He does not mention the terminal bulges of internodes, though they are visible on his microphotographs.

It is not possible to say whether the appearance of nodes in our preparations is an artefact. If so, it might be due either to a distorted chemical or osmotic balance of Ringer solution in which the fibres were teased and mounted or to mechanical trauma of dissociation. The first of these factors appears to be unlikely for control fibres teased and mounted in heparinized plasma of the same rabbit presented similar appearance. As to the mechanical lesion produced by direct contact of dissecting needles, it is immediately

visible: the damaged fibres are punctured or broken. But another kind of mechanical influence could be responsible for the modified appearance of the nodes. During dissociation one end of the nerve is fixed and the other floats freely in a drop of Ringer scolution. This floating end is teased by recurrent movements of the needle and each time the needle catches perineurium and tears it off the whole bundle is stretched by the pull and retracts quickly immediately afterwards. It is possible that the structure of the nodes is altered by these reiterated stretchings, but we were unable to avoid them. As this procedure does not alter visibly the structure of the internodal segments, it must be concluded either that enlargements and folds of myelin at the nodes exist in normal fibres or that the nodes are extremely labile and vulnerable structures liable to damage by factors which leave the internodal segments unaffected.

On ageing of the preparation the incisures widen more and more, the conical parts narrow into the lumen of the fibre and lamellae appear between the conical and the cylindrical parts of the incisure. (Pl. II, fig. 1, 2). An increase in the lenght of demyelinated fibre on either side of the node takes place. (Pl. II, Fig. 3—8). Later on droplets or loops of myelin isolated and apparently disconnected from the sheath may be observed in the interior of axoplasm even in the internodal parts. The striking feature of these ageing preparations is the extreme variability of resistance of diffrent fibres. Of two fibres of similar diameter running side by side on the slide, one preserves an almost geometrical regularity for many hours while the other soon develops deep structural changes. On a preparation about 24 hours old all degrees of deterioration may be seen, from almost unaltered fibres to typical ovoids of Wallerian degeneration.

II. CRUSHED FIBRES. The crush produces a characteristic alteration of the shape of the nerve fibre without rupture of the nerve tube.

The compressed part is thinned and the adjacent regions on both sides inflated. The inflation is visible over a certain length determined, among other things, by the size of the fibre. Further away from the crush, both proximaly and distally the fibre reverts to its normal structure and shape. The deformation of the fibre takes place during the few seconds the crush is applied. Owing to the great consistency of the fibre contents, such deformation persists in vitro, at room temperature almost unchanged for many hours.

In order to simplify the description of the results the following symbols were introduced to label different segments of the fibre: A — part of the fibre of normal appearance and diameter, proximal to the crush. B — widened part of the fibre, proximal to the crush. B' — proximal trunkated cone linking B to the compressed part. C — the compressed part. D' — distal enlarged part, E — normal part of the fibre, distal to the crush (Fig. 2). Only undamaged and

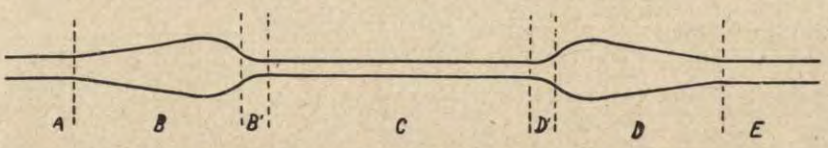

Fig. 2. Designations of various parts of a crushed fibre.
A — portion of normal undisturbed fibre proximal to the crush.
B — inflated portion proximal to the crush.
B' — trunkated conical portion linking B to the compressed part.
C — compressed part.
D — trunkated conical portion linking C to the inflated distal part
D — inflated portion distal to the crush.
E — portion of normal fibre distal to the crush.

unstretched fibres which could be followed on microphotographs over a length sufficient to include all these parts were measured. General views of preparations of crushed fibres are shown in microphotographs of Pl. III, Figs. 1, 2 and 3.

General appearance and dimensions of different portions of the crushed fibres

P a r t C. The width of the compressed region C is always less than that of the normal part of the fibre. This indicates that the fibre is not merely flattened by the crush, but that some degree of retraction of the tube diameter takes place. In small and medium fibres the part C seems nearly always to be a regular cylinder. In largest fibres the shape of C is frequently less regular, seems a little flattened but never exceeds or even reaches the normal tube diameter. The interior of the compressed part either appears empty or contains from place to place some remains of axoplasm. Sometimes a continuous core of optically empty axoplasm seems to occupy the full length of the compressed part and to pass without a phase boundary into the enlarged parts. In other fibres a phase boundary is visible between C and B' or D' and gives the impression that C is

entirely emptied of its contents. (Pl. IV, Fig. 3 right fibre). The myelin, perfectly visible in normal parts of the fibre, has been completely extruded from the crushed region.

The relation of the normal diameter to the diameter of the compressed part C was measured in over 500 fibres. The smallest C diameter was chosen in fibres whose part C was not quite cylindrical. The fibres were classified in groups differing by 2μ and the regression of compressed on normal diameter was calculated. The regression proved to be linear, the coefficient of regression being 0.488 ± 0.029. In other words fibres of all sizes are reduced by the crush to about half of their normal diameter. The regression line and

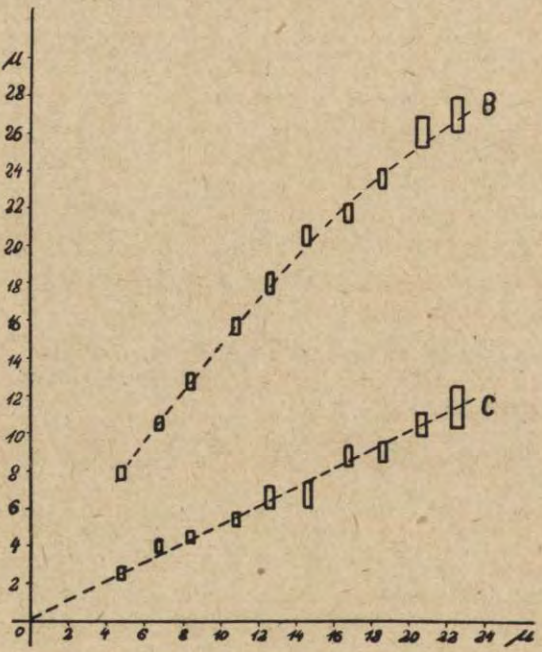

Fig. 3. Average diameters of constricted (C) and of inflated (B) regions of crushed fibres plotted against normal fibre diameters.

mean values of diameters C are represented in Fig. 3 (lower line). These results suggest that a normal nerve tube is inflated by its contents and tends to revert to an undeformed condition as soon as excess of pressure is removed by the crush, driving the axoplasm and myelin from the compressed region. Moreover, the linearity of regression indicates that fibres of various diameters are distended by their turgor pressure to a similar degree.

Parts B' and D'. The part C is linked to the adjacent enlarged parts by trunkated conical segments B' and D'. The external limits of these segments are drawn arbitrarily at points where their diameter is equal to that of the normal part of the fibre. Except for the diameter, the structure of these conical segments is similar to that of the inflated parts B and D. They are generally filled with droplets of myelin intermingled with axoplasm.

Frequently an optically empty core of axoplasm passes without phase boundaries directly from C through conical segments to B and D and may traverse them up to the normal parts of the fibre. (Pl. IV, Fig. 6 and 7). This central core of apparently higher consistency is surrounded by a layer of greater fluidity, of mixed axoplasm and myelin. In other fibres a phase boundary is visible between C and the conical part, or near the end of C. (Pl. IV, Fig. 3 right fibre).

Parts B and D. The enlarged parts B and D differ in shape in small and in large fibres. In small fibres they form a more or less regular spindle (Pl. IV, Fig. 5) the widest part lying approximately in the middle of the enlarged region. In large fibres the widening begins similarly on the sides adjacent to the crush, but away from them the diameters of the enlarged parts diminish very slowly and progessively and merge imperceptibly with the normal parts of the fibre. (Pl. IV, Fig. 2, 6). The interior of portions B and D over the greater part of their length is similar to that of portions B' and D', the normal structural arrangement of the fibre being completely disrupted.

In many fibres the morphologically disorganised contents do not occupy the full length of B and D. Towards the transitions to normal parts A and E, one may generally observe a portion of the fibre where the normal structure of nerve fibre is apparently preserved, the axoplasm clear and the myelin sheath with normal appearance, but with the diameter still enlarged (Pl. IV, Fig. 5). When a clear central core, which was previously alluded to, is seen, it may generally be traced over the full length of the enlarged part. In such cases no transverse phase boundary is visible either between C and B' or between B and A (Pl. IV, Fig. 6 and 7). In such fibres the disrupted contents seem to occupy the periphery of the lumen of the enlarged part and the impression is that the central core is more solid. Sometimes it appears contorted as a solid thread pushed into a more liquid medium. In other cases the lumen of the enlarged part is densely filled with droplets and loops of extruded

material, and the transition between B and A suggests that the central part of the lumen is filled with more liquid material than its periphery, for the myelin sheath in those intermediate parts retains its normal organisation and is continuous with the myelin sheath farther away from the crushed region, with the center of the lumen is occupied by a paraboloidal protrusion of disrupted material and many isolated droplets of this material are seen farther towards the normal part in the axoplasm. (Pl. IV, Fig. 3, left fibre, 8, 9, 13). As both pictures, that of the more liquid core and of the more liquid periphery of the fibre are frequently seen in the same preparation, they seem to indicate either that there are fibres differing normally in this respect, or that the consistency of axoplasm is variable and fluctuates easily from more liquid to more solid state.

The maximal diameters of the parts B and D are plotted in Fig. 3 (upper line) against the normal diameter of the nerve fibre. The relation is not linear. The increase is smaller in large fibres than in small ones.

This fact is probably due to the following circumstances: when the content of the part C is thrust by the crush into the adjacent regions, its pressure is counteracted both by the tension of the tube wall and by the resistance against longitudinal displacement of the column of axoplasm in front of the crush.

The first component varies as the tube radius, the second as the 4th power of the radius. Hence for smaller tubes the longitudinal displacement is small, because of the enormous resistance of the content against longitudinal displacement and the pressure is consumed mainly by the deformation of the wall. In larger tubes the resistance against longitudinal displacement falls and a greater part of the pressure is utilized to displace the content along the tube.

Beyond the enlarged portion the normally organised fibre is frequently slightly thinner than in regions distant from the deformation.

N o d e s. When a node of Ranvier occurs in the portions B or D, it scarcely exceeds the diameter of nodes in the normal parts of the fibre. The bulges on both sides of the node seem to retain also their normal maximal diameters, but they come generally closer to each other so that the outline of the node is modified (Pl. IV, Fig. 4, 11, 13). The internal structure of nodes is altered and they are filled, like the rest of parts B and D. with disorganised material extruded from the compressed part. In fibres of more liquid consist-

ency the lines of flow of material are rendered discernible by the obstacle created by the inextensible ring of the node (Pl. IV, fig. 4, 12, 13).

Volume of displaced content of the fibre. In order to elucidate whether there is some leakage of fibre material over the surface of the tube during the crush, careful measurements of diameters and lengths of various parts of the fibre were made in some fibres, and corresponding volumes calculated.

The procedure used was as follows: The mean diameter of normal parts A and E was calculated from 8—10 measurements. Then the lengths of B and D were determined from points where the diameters started to exceed systematically the mean value up to a point, towards C, where it again passed the mean value to diminish farther .The diameter of enlarged parts was measured every 25μ on the length of the fibre and the volume of the portion was calculated from these measurements. The calculation of volume C was somewhat more complicated, as some parts of the compressed regions appear empty and others contain remains of axoplasm. The volume of parts apparently empty was taken therefore as zero, and the volumes containing remains were calculated as usual.

Errors of measurements were determined by measuring the same microphotograph several times at intervals of several weeks. The mean diameters of parts A and E differed in successive measurements within limits of about 1%, the mean diameters of part B, and D by about 2—3%.

The results obtained on 11 fibres of various diameters are collected in Table I, from which it is clear that the material extruded from the portions B', C and D' is entirely accommodated within the adjacent parts of the nerve tube which, after the crush, remains impervious to the fibre contents. (This conclusion refers to the bulk of material only, no attempt having been, been made to ascertain possible alteration of permeability on ionic or molecular level). The table shows also the progressive increase in length of the portions B and D with increasing diameter of the fibre.

While a great number of microphotographs was inspected during the course of the present investigation, the impression was gained that the displacement of fibre contents is not symmetrical on both sides of the crush and that more substance is driven in a peripheral rather than a central direction. The phenomenon seemed particularly conspicuous in the larges fibres. A similar prevalence of peripheral displacement was detected by Causey (1948)

Table I

Lengths and volumes of distorted portions of crushed fibres. Comparison with volumes of the corresponding segments in normal fibres. Measured from projections at linear magnification × 500.

Fibre	Mean normal diameter in μ	Length of parts			Volume of parts			Sum of volumes of distorted portions of the fibre	Volumes of corresponding segments of normal fibres	Difference between volumes of distorted portions and volume of normal segment of fibre
		B in μ	D in μ	B′+C+D′ in μ	B in μ^3	D in μ^3	B′+C+D′ in μ^3	in μ^3	in μ^3	in %
3/246	5,9	60	72	252	$3{,}0\times10^3$	$3{,}4\times10^3$	$3{,}8\times10^3$	$1{,}02\times10^4$	$1{,}06\times10^4$	— 3,8
3/343	7,3	101	120	304	$8{,}7\times10^3$	$9{,}5\times10^3$	$4{,}0\times10^3$	$2{,}22\times10^4$	$2{,}19\times10^4$	+ 1,5
2/343	7,6	101	98	260	$8{,}1\times10^3$	$8{,}9\times10^3$	$3{,}8\times10^3$	$2{,}08\times10^4$	$2{,}11\times10^4$	— 1,5
6/246	7,7	106	122	252	$8{,}3\times10^3$	$7{,}6\times10^3$	$5{,}7\times10^3$	$2{,}17\times10^4$	$2{,}23\times10^4$	— 2,8
1/ 81	13,9	170	144	236	39×10^3	34×10^3	$13{,}2\times10^3$	$8{,}62\times10^4$	$8{,}41\times10^4$	+ 2,5
1/104	14,9	348	306	512	94×10^3	90×10^3	$26{,}7\times10^3$	$21{,}1\times10^4$	$20{,}4\times10^4$	+ 3,4
5/227	17	392	376	402	108×10^3	97×10^3	$55{,}2\times10^3$	$26{,}0\times10^4$	$26{,}4\times10^4$	— 1,5
1/283	17	280	330	458	93×10^3	109×10^3	$43{,}3\times10^3$	$24{,}6\times10^4$	$24{,}2\times10^4$	+ 1,7
2/ 86	18,5	320	304	402	119×10^3	103×10^3	54×10^3	$27{,}7\times10^4$	$27{,}6\times10^4$	+ 0,4
5/283	20,6	372	444	522	178×10^3	180×10^3	$78{,}5\times10^3$	$43{,}7\times10^4$	$45{,}0\times10^4$	— 2,9
1/327	24,3	556	328	368	518×10^3	315×10^3	$56{,}1\times10^3$	$88{,}9\times10^4$	$88{,}4\times10^4$	+ 0,6

when the nerve *in situ* was compressed. It was thought therefore at first, though it seemed improbable, that a similar phenomenon might occur in explanted nerves. Suspicions as to such source of asymmetry was aroused by two facts: 1. While in some preparations the asymmetry of displacement was conspicuous and visible on large fibres even without measurements, in other preparations it was manifestly absent, the predominance appearing sometimes in one direction, sometimes in another, in different fibres of the same preparation. 2. In those preparations where asymmetry was manifest, the diameter of undisturbed parts of fibres seemed to increase slightly, but systematically, towards the periphery. These facts seemed to point to an artefact related to the experimental procedure. Their cause proved to be the following. In the initial part of the investigation the nerves were always ligated by their central end, the thread serving for fixation of the nerve during dissociation of fibres. The distance between the ligature and the crushed part was never measured exactly. It varied from about 5 to 15 millimetres. It was supposed that the presence of ligature or the direction of teasing might be the cause of asymmetry of displacement and not the original position of the nerve in the body. A number of preparations was therefore made in which the nerve was ligated at its peripheral end instead of at the central and the volumes displaced peripherally (part D) and centrally (part B) were measured in about 100 fibres of various diameters. A slight but statistically significant difference (at the 0.05 level) was found in favour of central displacement. It was similar as to the extent and significance, but opposite in direction, to the difference found in nerves ligated by their central end. The conclusion to be drawn from these experiments is that the ligature compressing the nerve may produce a gradient of pressure along the fibres between the ligature and the free end. In these conditions, when a crush is applied between the ligature and the cut end, more resistance against displacement of the fibre contents is encountered on the side adjacent to the ligature than on the side away from it and, consequently, more of the material passes in the direction away from the ligature. The phenomenon is apparent only when the crush is applied near the ligature, but no quantitative investigation of this point has been made.

III. CUT ENDS OF FIBRES. When the nerve has been cut with a sharp razor blade, the free ends of the teased fibres in the preparation show initially a plane surface of section. Soon afterwards a convexity appears, and in about one hour a discernible outflow of material from the cut end takes place. At the same time the terminal part of the tube narrows progressively and the orifice becomes manifestly smaller than the normal diameter of the tube (Pl. V, Fig. 1—5). The phenomenon is greatly accelerated in the fibres in which the crush was made a few millimeters above the section of the nerve. Presumably the increased pressure in the inflated part D is responsible for this acceleration. The quantitative analysis of the phenomenon is very difficult for several reasons. The rate of exit is variable and the outflow of the contents is generally so slow

that changes in physico-chemical state of the fibre intervene before an appreciable amount of material has escaped. The shape of the outflowing portion is very irregular and does not allow of the calculation of volume. Moreover the outflowing material is poorly visible and seems to disperse in the new medium.

IV. DEGENERATING FIBRES. The tendency of the nerve tube to collapse every time the internal pressure diminishes, as well as its capacity to inflate under increased pressure, is well seen also in degenerating nerve fibres. When the nerve fibre breaks into completely separated ovoids, the nerve tube is faintly visible between them. Its diameter is strongly retracted in these space intervals. It is inflated over the central portions of the ovoids, probably by the tendency of the ovoid to assume a spherical shape under the influence of surface tension. As the parts of the fibre near nodes degenerate later and conserve a cylindrical shape when the middle of internodal segments is fragmented into ovoids (Lubińska 1952), it is possible to determine in the same fibre its normal diameter, and as well as the minimal diameter between, and the maximal diameter over the ovoids. The diameter of the tube in the intervals between ovoids is then similar to the diameter of parts C in crushed fibres of similar sizes. A microphotograph including a cylindrical fragment of the fibre, an ovoid and half emptied portions of the tube on both sides of the ovoid is reproduced in Pl. V, fig. 6.

DISCUSSION

Some information may be gathered from the present experiments concerning the state of the fibre contents. The appearance of the crushed fibre shows that both axoplasm and myelin are driven from the compressed region into adjacent parts of the fibre and remain immiscible in spite of the disruption of the normal structure. The inflated parts are filled with intermingled droplets of axoplasm and myelin with sharp phase boundaries. In the experimental conditions reported here the displacement of fibre contents is practically irreversible, indicating a high consistency of the extruded material. It is nevertheless probable, that at body temperature the fibre contents might be much more fluid and a return flow to the compressed region could take place. Even at room temperature some outflow of material takes place at the cut ends of fibres, where the resistance against the flow is diminished.

The observation of lines of flow which appear under the rapidly applied crushing force seems to indicate that in some fibres a more solid central core exists in the axoplasm whereas in others the peripheral portion of the axoplasm appears less fluid than its centre.

Somewhat more inferences may be drawn from the present experiments concerning the properties of the nerve tubes. The deformations observed in various parts of a crushed fibre indicate that the nerve tube is extensible and elastic. Under normal conditions it is distended by the fibre contents and tends to revert to an unstretched diameter in circumstances lessening the internal pressure in the fibre. Thus, the removal of the contents from a compressed region of the fibre results in a diminution of the diameter of this part, which retains its cylindrical shape. The diameter of the shrunken parts falls to about half of its value in normal fibres. This relation holds for fibres of all sizes, indicating that in normal life all nerve tubes are distended by their contents to twice their „unstretched" diameter.

The normal inflation of nerve tubes does not attain the limits of their extensibility. In many circumstances one may observe a stronger distension of the fibre, beyond normal diameter, without rupture of the tube. Such additional inflation was observed in the course of the present investigation over the ovoids of degenerating fibres. It was probably produced by the tendency of ovoids to assume a spherical shape. Similarly the regions of fibres adjacent to the compressed part were also overinflated by the excess of material thrust into them by the crush. In spite of additional distension, the tubes remained impermeable to the bulk of the contents and the total volume of material extruded from the crushed region was found in these inflated neighbouring portions of the fibre.

The elasticity of the tube is manifested also by the retraction of tube diameter near the cut ends of fibres, when outflow of material takes place. The elastic reaction of the tube wall is moreover probably the main cause of the outflow of axoplasm from an excised fibre against capillary forces.

In addition to its elasticity in a radial direction the nerve tube seems elastic also in a longitudinal direction, for a stretched nerve fibre retracts quickly on release of tension. The diminution of fibre diameter behind the inflated regions points also to longitudinal elasticity of the tube. Longitudinal elasticity of the whole nerve is well known.

While the nerve tube in the internodes is extensible and elastic it seems, under the pressures applied here, inextensible in the nodes. Nothing can be said therefore of elasticity of the parts of the tube overlying the node.

The observations on degenerating fibres suggest that some properties of the fibre are different in the internodal parts and in the vicinity of nodes. At a stage of degeneration when the internodes are completely broken into ovoids, segments of undivided fibre, retaining the original outline are seen on both sides of the node.

It is interesting to note that some of the facts reported here were already noted by Ranvier (1878) on nerves fixed in osmic acid. Thus he observed that when a nerve is flattened by compression the individual fibres are thinned but retain the cylindrical shape. He attributed this fact to the liquid or nearly liquid state of the substance immersed in a liquid medium. In a controversy with Key and Retzius concerning the existence of so-called „incomplete nodes", where the myelin passes without discotinuity from one segment to another, Ranvier demonstrated that myelin may be forced through the node by compression of the neighbouring segment of the fibre. He concluded that incomplete nodes are artefacts produced by displacement of myelin caused by mishandling of the nerve. As the fact was observed on preparations stained with osmic acid, Ranvier does not consider the fate of axoplasm in these circumstances.

The shrinkage of degenerating nerve tubes is a well known phenomenon. It was recently reinvestigated by Sunderland and Bradley (1950) who studied the evolution of mean tube diameter over long periods of degeneration. The tendency of the nerve tubes of degenerating fibres to shrink wherever the turgor pressure of their contents diminishes, and to be distended by increased internal pressure, is manifest also on fixed and stained material. The following description by Glees (1943) corresponds closely to what was observed here on living unstained fibres.

„The endoneural sheath ... is dilated wherever digestive chambers have been formed. In those parts of the endoneurial tubes where no digestive chambers have developed, the endoneurial tube is reduced in diameter and appears to have collapsed. The formation of Schwann bands leads to the result that the endoneurial tubes, emptied of their former contents, almost regain their initial diameter".

Such behaviour of nerve tube during degeneration may also be regarded as a manifestation of two features of nerve fibres: the

elasticity of the tube, and the fact that in normal conditions the tube is distended by its contents. It returns to its unstretched state every time the excess of the contents is removed from a portion of the tube — by an external pressure as in the crush, by outflow of material from a cut end, or by formation of ovoids during degeneration.

Thus, the normal diameter of nerve fibres seems to be determined by an equilibrium between the turgor pressure of fibre contents and the elastic tension of the tube wall.

It is premature to discuss the role of the constant pressure exercised by the deformed tube wall on the fibre contents in processes of regeneration and, possibly, of degeneration of nerve fibre. One point may nevertheless be mentioned. Hammond and Hinsey (1945) and Simpson and Young (1945) recorded the restrictive influence of small tubes on the final diameter of large fibres regenerating into them. This fact is easy to interpret in the light of the present findings.

The turgor pressure of the fibre develops an elastic tension of the tube wall. This elastic tension is proportional to the degree of inflation, i. e. to the difference between the tube circumference as seen in living fibres and the circumference of the unstretched tube. It was shown that the normal degree of inflation is similar in fibres of all sizes. This relationship indicates, if we admit the same coefficient of elasticity for various fibres, that the wall tension under normal conditions is similar in all fibres. It means that the normal turgor pressure prevailing in a neurone counteracts a determined tension. When an axon regenerates into a smaller tube than the original one, the turgor pressure characteristic for the neurone will be insufficient to stretch the smaller tube to the diameter of the old. To obtain such result a stronger pressure would be necessary. Therefore, in spite of the extensibility of the tube, a large axon regenerating into a small tube is bound to remain undersized.

SUMMARY

1. Deformations produced by the nerve crush observed on living unstained nerve fibres of rabbit are described.
2. The diameter of the crushed region of the fibre from which its contents was driven diminishes, the compressed part conserving generally its cylindrical outline.

3. For fibres of various sizes a constant relationship between normal and compressed diameter is maintained. Each tube is reduced in the crushed region to about half of its original diameter.
4. On both sides of the crushed portion the fibres are distended by the accommodation of the additional volume of axoplasm and myelin extruded from the compressed region.
5. The diameters of the inflated parts of the tube increase with the fibre calibre. This increase is smaller in large fibres than in small and medium ones.
6. Both compressed and inflated portions of the tube remain impervious to the fibre contents so that there is no leakage of material over the surface of the tube.
7. When the fibre content flows out from the cut ends of the fibre, the diameter of the terminal part of the tube diminishes.
8. In degenerating fibres broken into ovoids the nerve tube is inflated over the ovoids and retracted in the intervals between them to a half of their initial diameter.
9. It is concluded that the nerve tube is elastic in the internodal parts of the fibre and, under normal conditions, distended by its content. It tends to revert to an undistended state, in every circumstance lessening the internal pressure in the tube.
10. The normal diameter of the fibre seems thus to be determined by the balance between the turgor pressure of the axoplasm and the elastic tension of the tube wall.

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PLATE I. NORMAL FIBRES

Fig. 1. (B — 16). A group of teased fibres. Mounted in paraffin oil. Magnification × 128. n — nodes of Ranvier, partly demyelinated.

Figs. 2. and 2-a. (B — 86 and B — 87). Internodal portions of fibres. Mounted in Ringer. Magnification × 583. i — incisures.

Fig. 3. (B — 35). Node of Ranvier in an isolated fibre. Mounted in Ringer. Magnification × 583.

Figs. 4 and 5. (FB — 5/5 and B — 32). Nodes of Ranvier in undissociated bundles. Mounted in Ringer. Magnification × 583. n — nodes.

Fig. 6. (B — 31). Node of Ranvier in an isolated fibre. Mounted in Ringer. Magnification × 583.

PLATE II. AGEING PREPARATIONS

Figs. 1 and 2. (B — 45, B — 43). Internodal segments of ageing preparations. Widening of incisures. Lamination and loops of myelin. Photographed 8 hours after preparation. Mounted in Ringer. Magnification × 583.

Figs. 3 and 4. (B — 13, B — 15). Nodes of Ranvier. Photographed 8 hours after preparation. Teased fibres stained with toluidine blue. Mounted in paraffin oil. Magnification × 583.

Fig. 5. (B — 34). Node of Ranvier. Unstained, mounted in Ringer. Magnification × 583.

Figs. 6 and 7. (B — 47, B — 44). Nodes of Ranvier. Unstained, mounted in Ringer. Photographed 8 hours after preparation. Magnification × 583.

Fig. 8. (B — 40), Node of Ranvier. Unstained, mounted in Ringer. Photographed 4 hours after preparation. Magnification × 647.

PLATE III. CRUSHED FIBRES

Fig. 1. (B — 39). Crushed fibre. Mounted in Ringer. Magnification × 216.

Fig. 2. (343). A group of crushed fibres. Mounted in paraffin oil.

Fig. 3. (54). A group of crushed fibres. Mounted in paraffin oil. Magnification × 100.

The designations are the same as in Fig. 2 in the text.

PLATE IV. DETAILS OF CRUSHED FIBRES

Fig. 1. (B — 72). Portions A and B of crushed fibre. Mounted in paraffin oil. Magnification × 384.

Fig. 2. (B — 50). Portions A, B and a fragment of C of a crushed fibre. Mounted in heparinized plasma of the same rabbit. Magnification × 583.

Fig. 3. (FB — 3/1). A group of crushed fibres. Portions A, B and partly C. Mounted in Ringer. Magnification × 384.

Figs. 4, 6 and 7. (B — 37, B — 57, B — 55). Parts B of crushed fibres. Mounted in heparinized plasma of the same rabbit. Magnification × 384. A central core of less disorganised axoplasm is visible in these fibres.

Fig. 5. (B — 41). A small fibre. Portion A and B. Mounted in Ringer. Magnification × 583.

Figs. 8 and 9. (FB — 3/7 and FB — 3/8). Transitions from B to A in crushed fibres. Mounted in Ringer. Magnification × 583.

Figs. 10 and 11. (B — 30, B — 68). Portions A and B of crushed fibres. Mounted in Ringer. Magnification × 384.

Fig. 12. (B — 67). Transition from A to B in a crushed fibre. Mounted in Ringer. Magnification × 583.

Fig. 13. (B — 22). Node of Ranvier in the part B. Mounted in paraffin oil. Magnification × 384

The designations are the same as in the fig 2 in the text.

PLATE V.

Figs. 1, 2, 3, 4 and 5. (B — 73—75, FB — 5/7). Outflow of material from cut ends of the fibres. Mounted in paraffin oil. Magnification × 583.

Fig. 6. (B — 76). A fibre degenerating in vitro for 3 days. Mounted in Ringer. Magnification × 583. On the right an unbroken portion, near the node (unshown). In the middle an ovoid. Retracted parts of the tube with remains of contents on both sides of the ovoid.

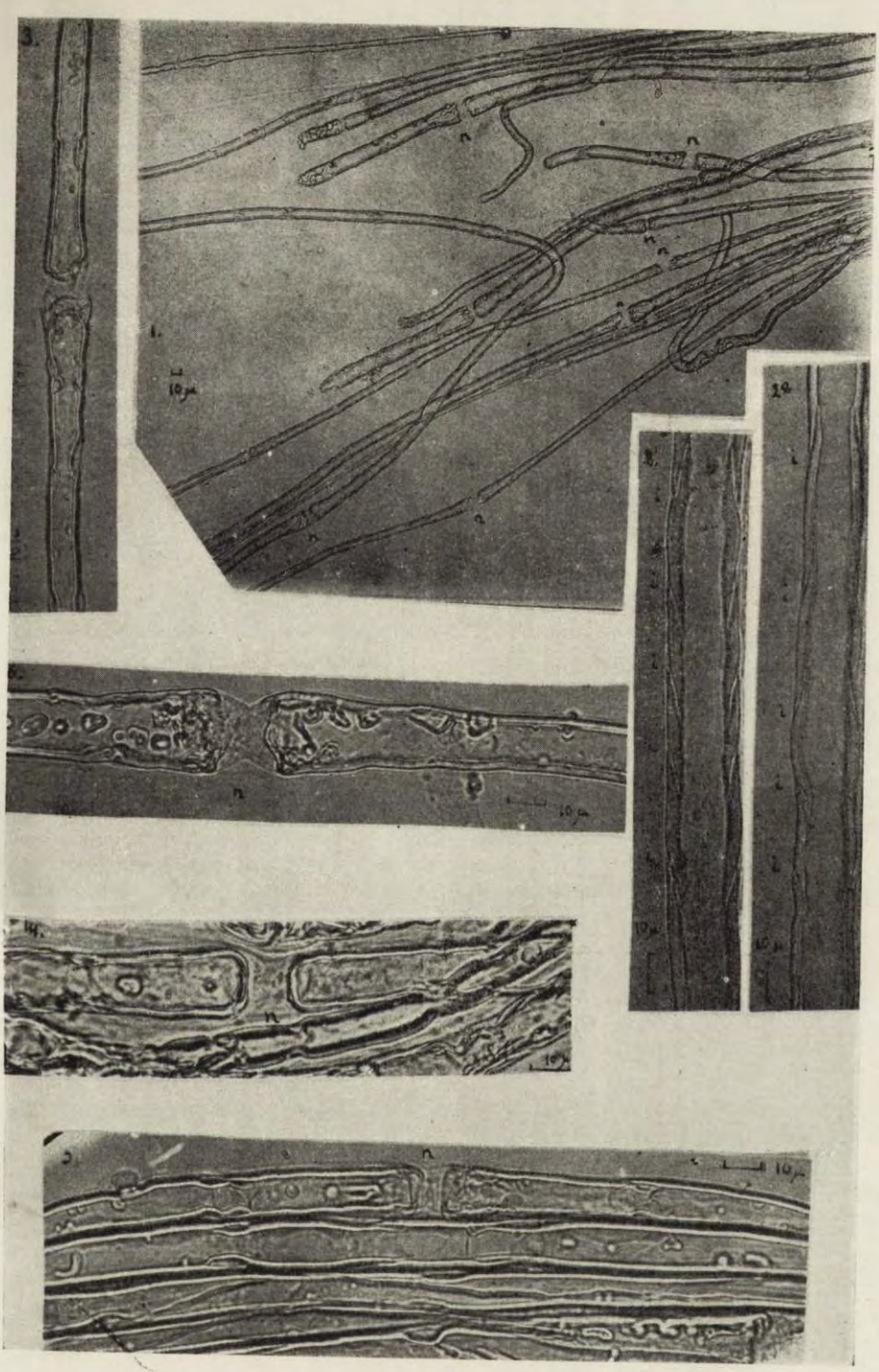

PLATE I.

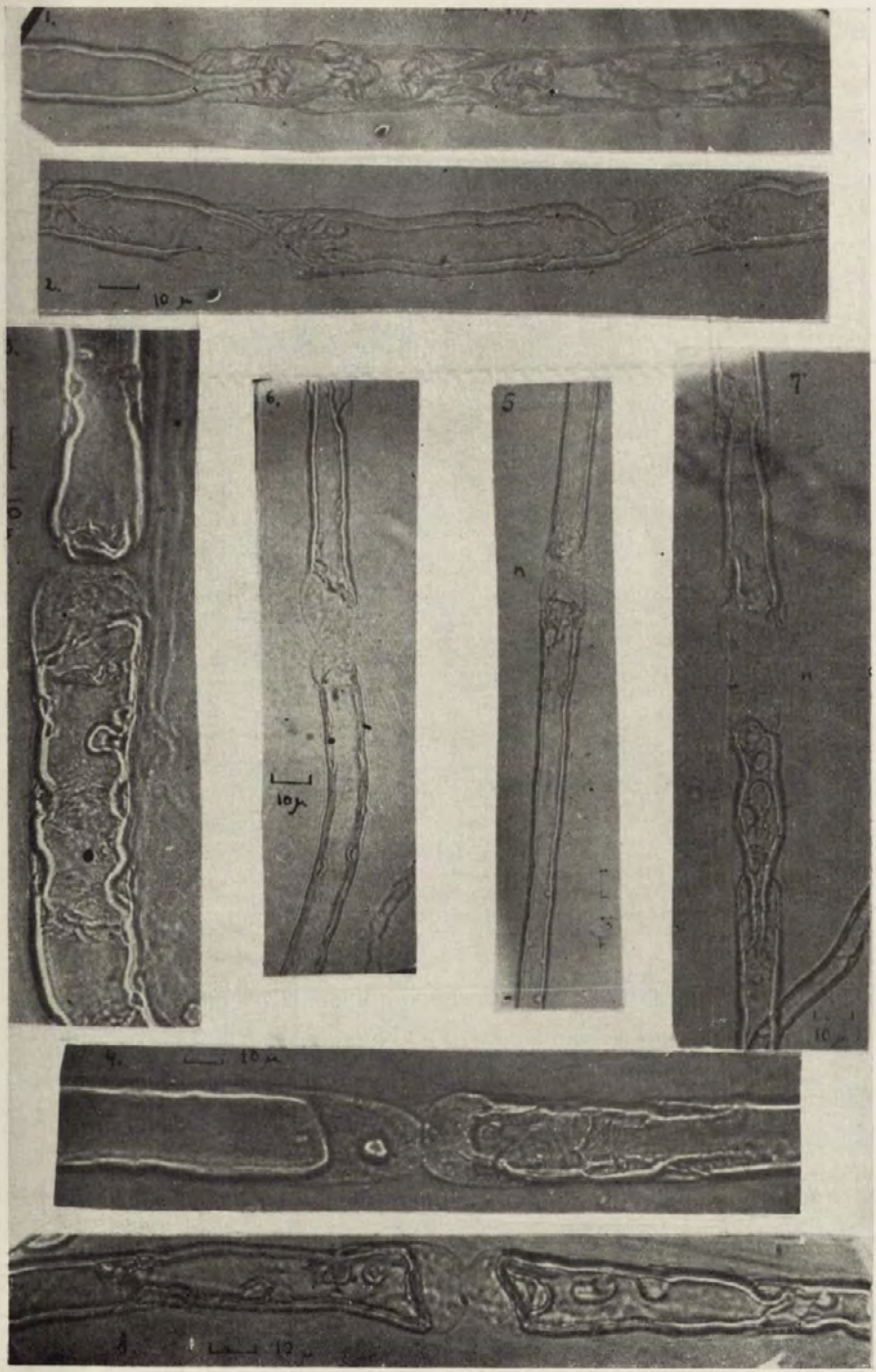

PLATE II.

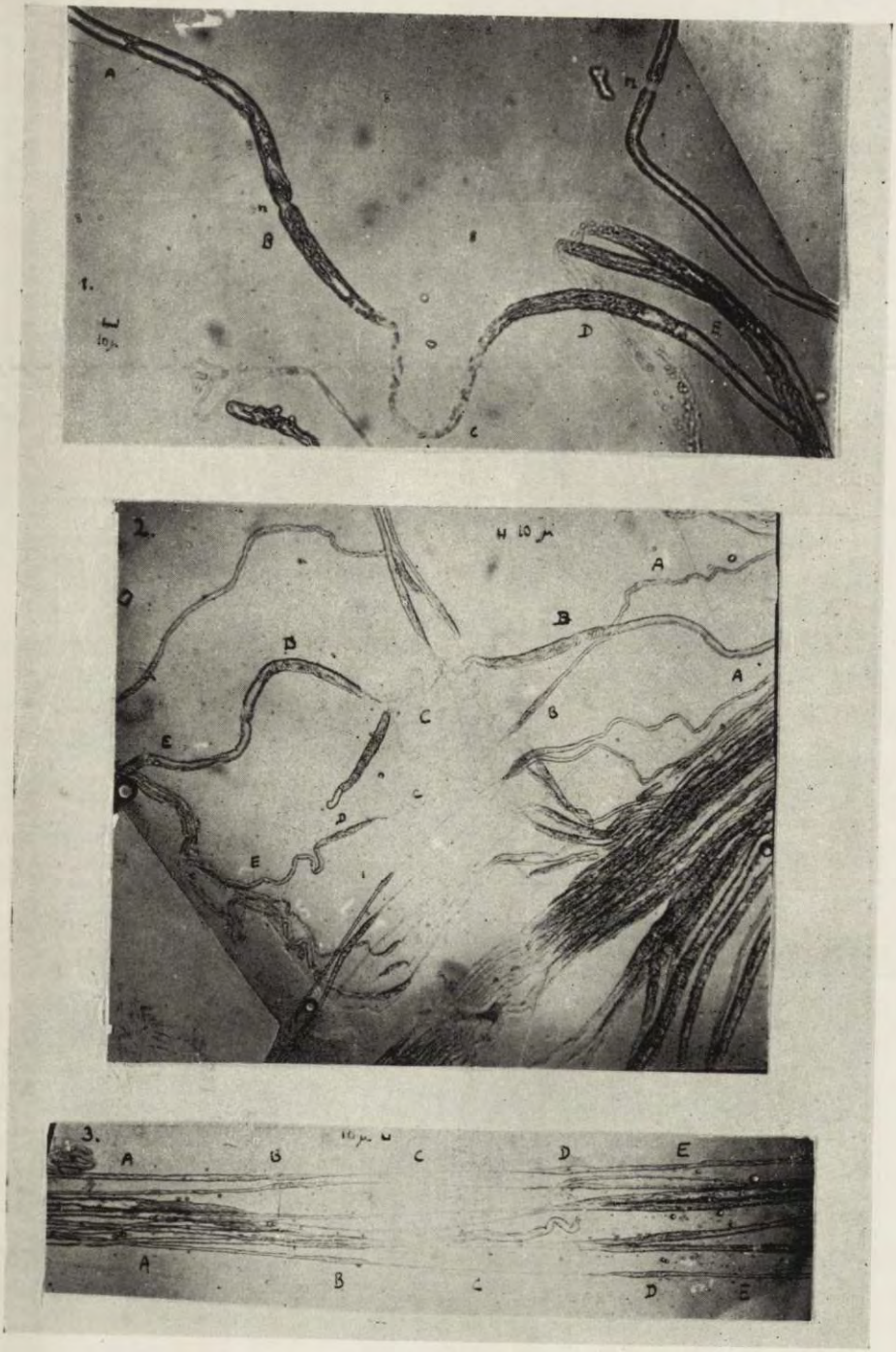

PLATE III.

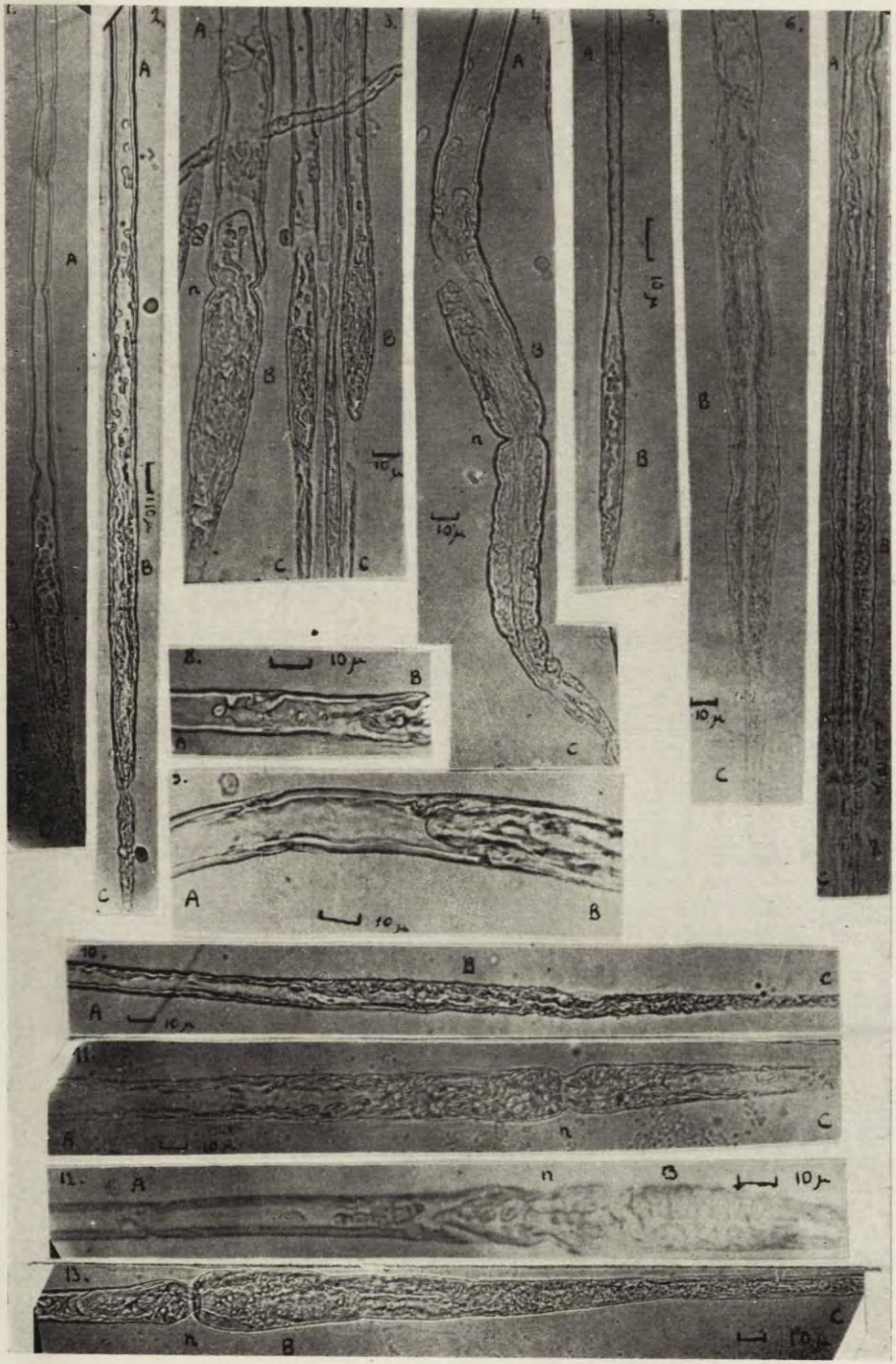

PLATE IV.

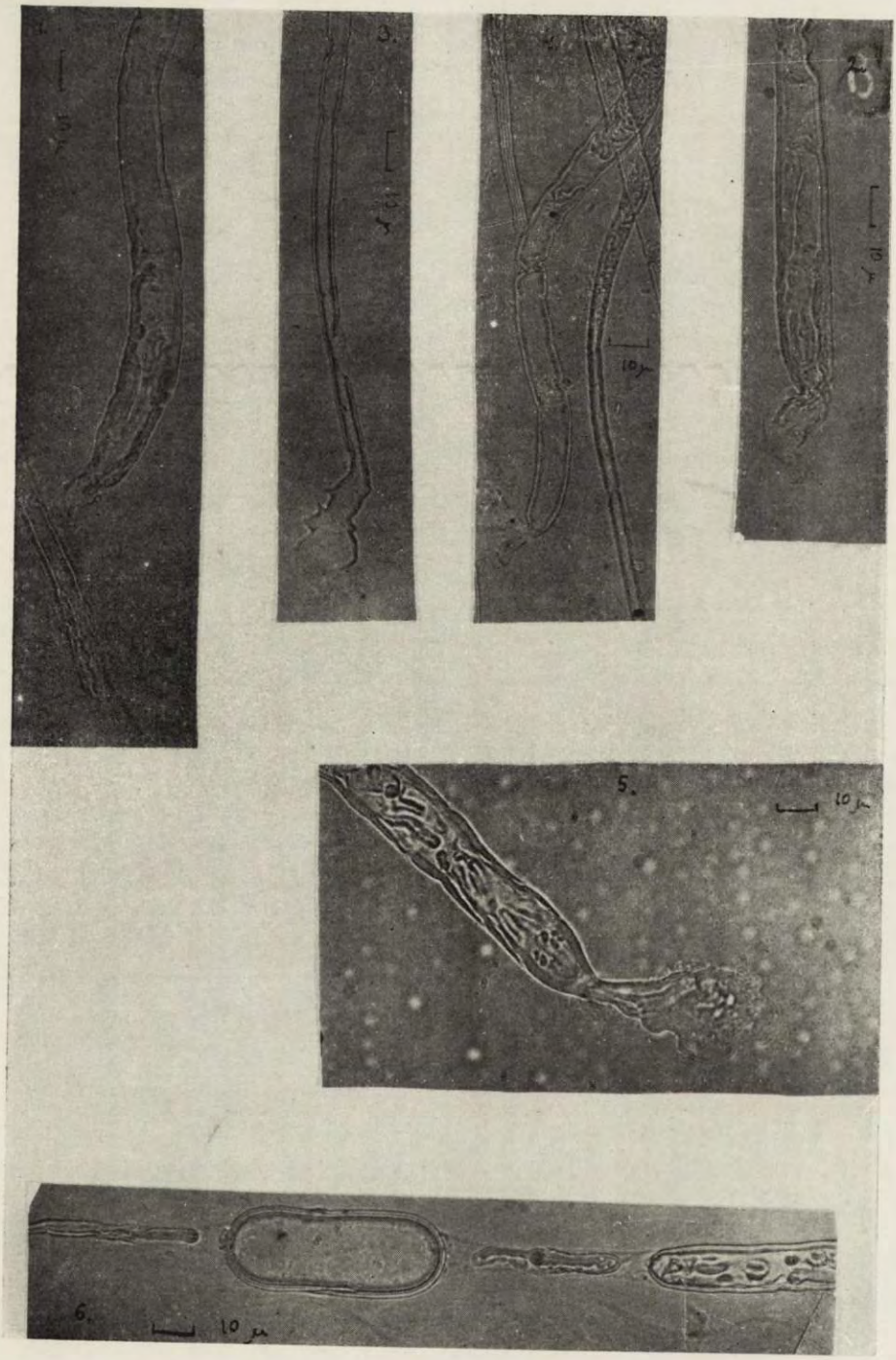

PLATE V

Acta Biologiae Experimentalis Vol. XVI, Nr 6 1952

CHRONIC EXTINCTION AND RESTORATION OF CONDITIONED REFLEXES

III. DEFENSIVE MOTOR REFLEXES

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(Received 21 March 1952)

In our previous paper (J. Konorski and G. Szwejkowska, 1950) it was shown that, as far as alimentary conditioned reflexes are concerned, the process of their extinction is much more protracted than the process of their restoration which occurs almost immediately. The problem arises whether this „dissymetry" in the course of two processes might be attributed to the biological role of alimentary reinforcement, namely to the fact that this reinforcement brings about a „satisfying state of affairs", while its withdrawal is connected with some sort of frustration. In consequence the animal would rather „reluctantly" elaborate an inhibitory reflex which is directed, so to say, against its interest, while it would very easily restore the positive reflex as soon as the extinct stimulus becomes again reinforced.

In order to test this hypothesis it was necessary to see what would be the course of extinction and restoration when instead of alimentary conditioned reflexes defensive reflexes be used. The present paper deals with this problem.

METHOD

In dogs there were elaborated defensive motor conditioned reflexes by means of an electric shock (a single condenser discharge, 2μF, ca 150 V) applied to the leg, as reinforcement. The electrodes were attached to the upper part of the foot. The conditioned stimulus preceded the unconditioned one by 3 sec. Every experimental session consisted of 9—10 trials performed in intervals of 1.5—3 min. The motor reaction of the leg as well as the respiration were recorded on a kymograph.

RESULTS

In two dogs a defensive conditioned reflex was established to two stimuli. In one dog, „Cygan", conditioned stimuli were: a rotating toymill, and the sound of rustle; as unconditioned stimulus served an electric shock applied to the hindpaw. In the second dog, „Bobik", conditioned stimuli were: an oscillating black disk, and the sound of whistle, for unconditioned stimulus an electric shock applied to the forepaw was used.

In the preliminary training which took for Cygan 35, and for Bobik 54 experimental sessions, the conditioned reflexes were satisfactorily fixed, and they were manifested by a high lifting of the respective leg, and also by distinct changes in respiration (Fig. 1a). In a following series one of the conditioned stimuli (rustle for Cygan and whistle for Bobik) was applied without reinforcement. It was given 3 times in every experimental session and was always intermingled with the control stimulus (toymill for Cygan and disk for Bobik). The course of an experiment at the beginning and at the end of this series is represented in Fig. 1b and 1c respectively.

After 28 such experiments in Cygan and 24 experiments in Bobik the experimental stimulus was again reinforced by shock.

The course of extinction and restoration of the conditioned reflex is represented in Figs. 2a and 2b. We see that the process of extinction is slow and gradual. At the beginning of extinction the dog reacts positively to each application of the unreinforced stimulus, while at the later stage there is a period of mixed reactions, and finally the inhibitory reflex becomes wholly established. The process of restoration, on the contrary, is very prompt: after a few days the positive reflex to the extinct stimulus was in both dogs fully restituted. And so, while during the course of extinction the number of „faulty' responses (i. e. excitatory instead of inhibitory) was 50 in

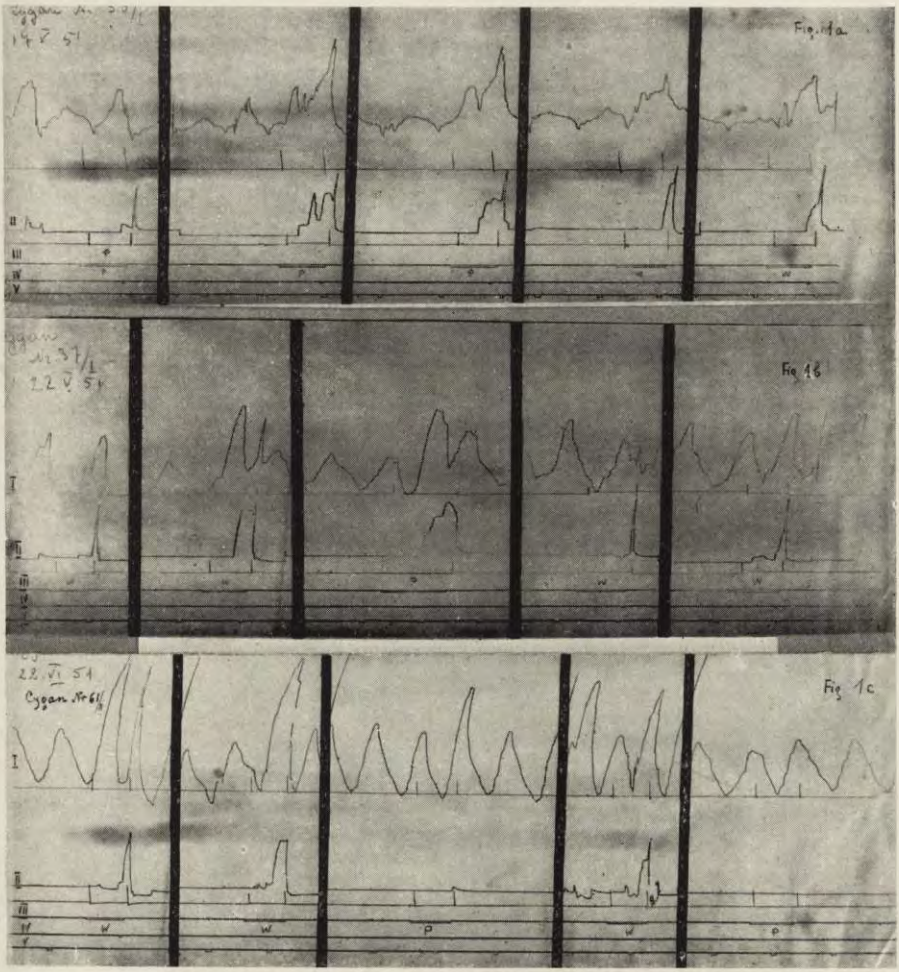

Fig. 1. Extinction and restoration of the defensive conditioned reflex in Cygan.

a. a typical course of an experiment in the preliminary training of positive conditioned reflexes (exp. Nr. 33, 17/V/51).

b. the course of an experiment at the beginning of chronic extinction (exp. Nr. 37, 22/V/51).

c. the course of an experiment at the end of the chronic extinction (exp. Nr. 61, 22/V/51).

I, respiration, II, motor reaction of the leg, III, conditioned stimulus (W, toy-mill, P, rustle). IV, electric shock, V, time in 5 sec. Note a strong inhibitory after-effect following the application of unreinforced stimulus in b. Note also the marked correspondence between motor and respiratory reaction.

Cygan and 36 in Bobik, during the course of restoration of the reflex the number of „faulty" responses (i. e. inhibitory instead of excitatory) was respectively 3 and 2. These numbers give a best evidence to show how difficult it is for the dog to extinguish the positive conditioned reflex, and how easy it is to restore it.

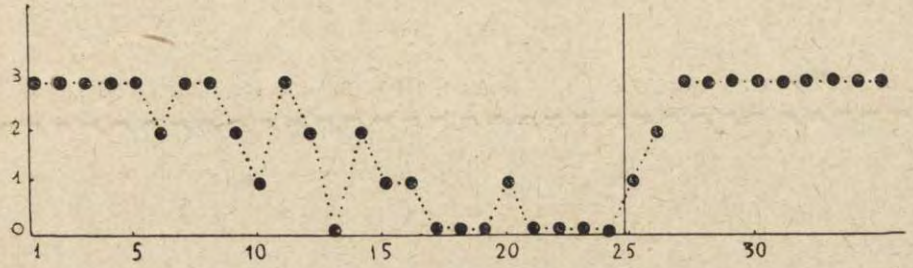

Fig. 2a

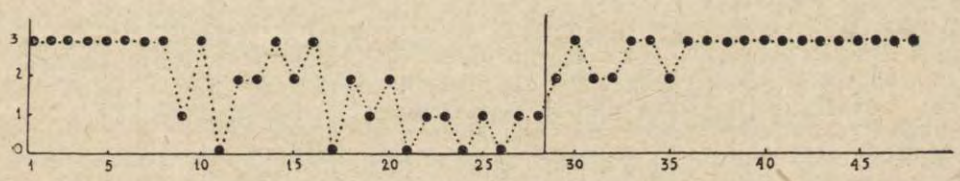

Fig. 2b

Fig. 2. The course of extinction and restoration of the conditioned defensive reflex in Cygan (a) and Bobik (b).

Abscissae: successive experimental days.

Ordinates: numbers of positive reactions to the experimental stimulus. The latter is not reinforced up to and including 24 th day in Cygan, and up to and including 28th day in Bobik. Starting from 25th or 29th day the stimulus is again reinforced.

DISCUSSION

As it is seen from these experiments the „dissymetry" in the course of extinction and restoration of conditioned reflexes was also found to hold in the case of defensive reflexes. Thus, the hypothesis stated above, according to which this dissymetry would be attributed to the biological role of food as a „positive" reinforcing agent, cannot be held. This dissymetry seems to be rather a general rule concerning all types of reflexes, and the problem of its origin is still to be elucidated.

SUMMARY

In defensive conditioned reflexes, like in alimentary reflexes, the chronic extinction of a conditioned reflex develops slowly and gradually while its restoration occurs almost immediately after a few reinforcements.

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Acta Biologiae Experimentalis Vol. XVI, Nr 7 1952

CHRONIC EXTINCTION AND RESTORATION OF CONDITIONED REFLEXES

IV. THE DEPENDENCE OF THE COURSE OF EXTINCTION AND RESTORATION OF CONDITIONED REFLEXES ON THE „HISTORY" OF THE CONDITIONED STIMULUS. (THE PRINCIPLE OF THE PRIMACY OF FIRST TRAINING)

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In the previous papers of this series the following facts concerning the transformation of excitatory into inhibitory conditioned reflexes, and vice versa, were established.

1) The chronic extinction of an alimentary conditioned reflex is a gradual and slow process while its restoration is very prompt (Konorski and Szwejkowska 1950).

2) This difference between the course of both processes is not due to the fact that the extinction and restoration are conducted against the excitatory background (i. e. when the experimental stimulus is applied among positive stimuli), since the same difference is seen when the extinction occurs against the inhibitory background (Szwejkowska 1950).

3) This difference is not due to the fact that the food reinforcement represents for the animal a positive value, since the same

holds when instead of alimentary conditioned reflexes defensive motor conditioned reflexes are used (Konorski and Szwejkowska 1952).

The present paper tries to elucidate the puzzle of this difference in the course of extinction and restoration of conditioned reflexes from the point of view of the history of the conditioned stimulus.

METHOD

The typical method of salivary food conditioned reflexes adopted in this series and described elsewhere (Konorski and Szwejkowska 1950) was used.

All experiments with chronic extinction and restoration of conditioned reflexes were conducted in the same standard manner. An „experimental" stimulus (i. e. stimulus designed to a particular sort of transformation) was applied once in each experimental session, usually in the third or fourth place. A well established and strong positive conditioned stimulus was used as a control stimulus, and it was applied 3—5 times in a session.

The course of experiments in which acute or subacute transformations of conditioned reflexes were performed is described in further text.

RESULTS

The experiments were performed on 3 dogs. As their course was not quite identical we shall consider them separately.

1) „Bekas", a male dog, of the age of 4 years, weight 17 kg.

Series I. Adaptation to the stand and experimental situation, 8 days.

Series II. Elaboration of an alimentary conditioned reflex to the sound of Metronome. 10 experiments, 42 trials.

Series III. Introducing of an unreinforced stimulus, Lamp. While continuing to apply Metronome with reinforcement, a new stimulus — the rythmic lightening of a Lamp — was introduced, this stimulus from the very beginning not being reinforced. It is worth noting that this stimulus, applied for the first time, evoked a salivary reaction which amounted to 40% of the reaction to Metronome. In following trials this reaction rapidly diminished, attaining the mean value of interval salivation, and thereafter decreasing beneath this value. It should also be noted that the application of Lamp, especially if it was given twice or thrice in succession, produced a more or less pronounced inhibitory after-effect. All this goes to show that Lamp has acquired a strong inhibitory character. Altogether this series included 79

experiments, in which Metronome was applied 290 times and Lamp 185 times.

Series IV. Elaboration of a positive conditioned reflex to Lamp. Starting from exp. No. 90 to No. 119, Lamp was applied once daily (usually in the third or fourth trial) w i t h r e i n f o r c e m e n t. The course of elaboration of the conditioned reflex to this stimulus is shown in Fig. 1 (compared with the elaboration of the conditioned reflex to a new stimulus in this dog, cf. also Fig. 6). It is seen that this process is very slow, and the conditioned reaction to Lamp

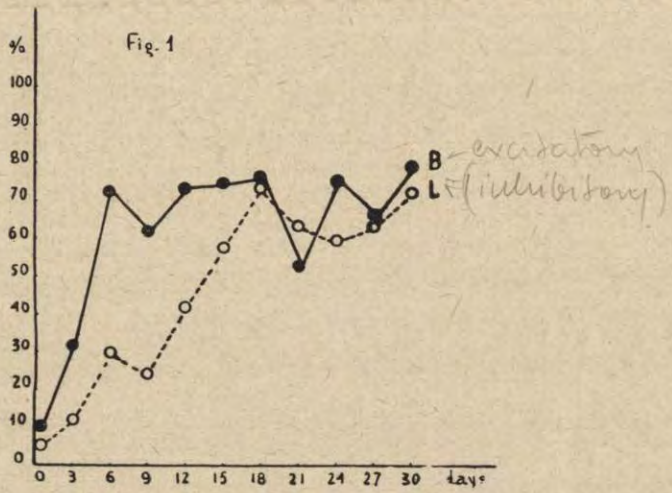

Fig. 1. The elaboration of the conditioned reflex to a primarily inhibitory stimulus (Lamp) and to a new stimulus (Bell) in „Bekas".
Abscissae: experimental sessions. Ordinates: the magnitude of a conditioned reflex in percentage of the magnitude of the reflex to the control stimulus (M). Circles, reflex to Lamp, dots, reflex to Bell. Each point represents mean value of three successive experimental sessions.
The reflex to the inhibitory stimulus grows much slower than that to a new stimulus.

does not reach the value of the conditioned response to Metronome, although the „strength" of both these stimuli (as judged from the evidence obtained in other dogs) is more or less the same.

Series V. Chronic extinction of the conditioned reflex to Lamp. Starting from exp. No. 120 and on, Lamp continued to be applied once daily among the applications of Metronome, but now again u n r e i n f o r c e d. The course of this extinction is shown in Fig. 2a. It is seen that the process of extinction occurs rapidly (in

6 experiments the reflex drops to 1/3 of its initial value), but the response to Lamp does not reach its bottom it had before Series IV. While previously the salivation to Lamp amounted only to 3—6% of the salivation to Metronome, now it dropped no lower than to 15—25% of the response to Metronome.

Series VI. Introducing a new conditioned stimulus, Bell. A new excitatory conditioned stimulus — the sound of a bell — was introduced. The elaboration of the conditioned reflex to this stimulus was

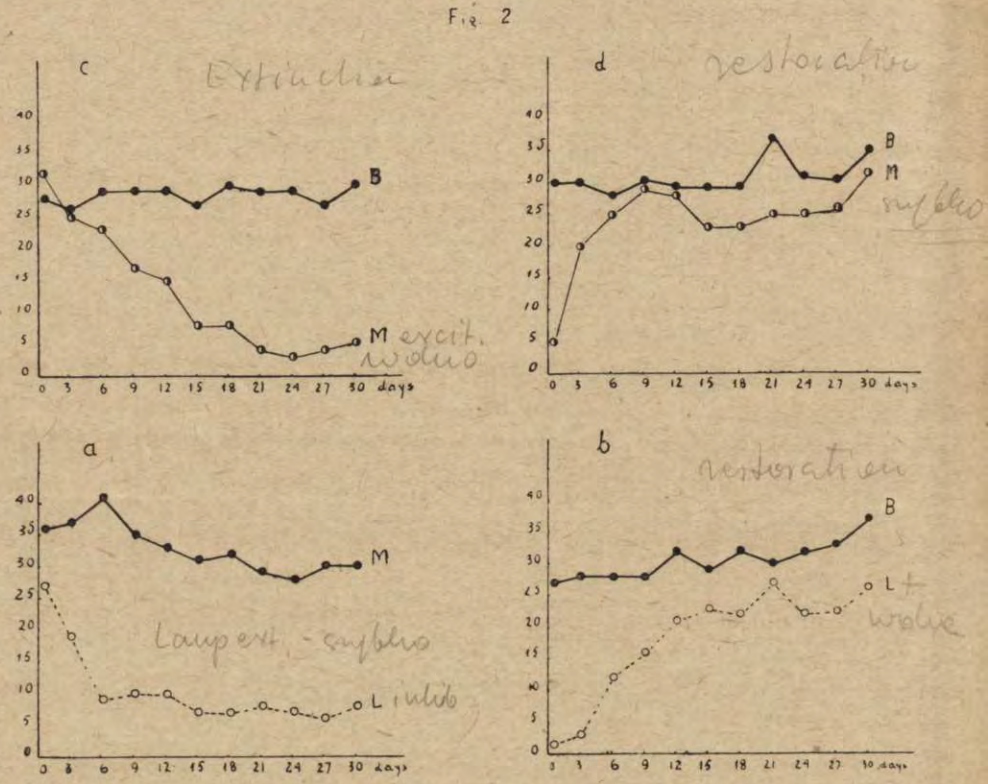

Fig. 2. Chronic extinction and restoration of conditioned reflex to a primarily excitatory stimulus (Metronome) and to a primarily inhibitory stimulus (Lamp) in „Bekas". Abscissae: experimental sessions. Ordinates: magnitude of conditioned reflexes in grades of the manometer. Circles, experimental stimulus, dots, control stimulus. a, extinction of Lamp, Metronome being a control, b restoration of Lamp, Bell being a control. c, extinction of Metronome, Bell being a control. d, restoration of Metronome, Bell being a control. The extinction of the primarily inhibitory stimulus is rapid, its restoration slow, the extinction of the primarily excitatory stimulus is slow, its restoration rapid.

relatively slow (cf. Fig. 1 and 6) but finally it reached the same value as the reflex to Metronome. This series will be discussed later.

Series VII. Chronic extinction of Metronome. The new conditioned stimulus, Bell was now taken as a control and Metronome was submitted to chronic e x t i n c t i o n in a usual way (Fig. 2c). The process of extinction was slow, the reflex to Metronome dropped to 1/3 of its value only after 15 experiments.

Series VIII. Restoration of the reflex to Metronome. After 30 extinction experiments Metronome was again applied w i t h reinforcement (Fig. 2d). The process of restoration was rapid, and in a few days Metronome regained its previous value.

Series IX. Restoration of the reflex to Lamp. Now the last series was performed in which the excitatory reflex to Lamp was re-established, while, as in the previous series, Bell was taken as a control (Fig. 2b). The process of restoration was slow and, as in Series IV, the reflex to Lamp reached only 70% of the control value.

Summary of the course of experiments on „Bekas".

In this dog Metronome was trained from the beginning as an excitatory conditioned stimulus, while Rythmic Lamp was trained from the very beginning as an inhibitory stimulus. It appeared that while the process of extinction of the reflex to Metronome was slow and its recovery rapid, the transformations of the reflexes to Lamp took quite a different course: both the first elaboration of the excitatory conditioned reflex and its restoration after extinction were very slow, while its extinction was more rapid than the extinction of Metronome. The elaboration of the conditioned reflex to a new stimulus, Bell was more rapid than the formation of the conditioned reflex to the primarily inhibitory stimulus, Lamp.

2) „Dudek", a male dog, age 3 years, weight 12 kg.

As the course of experiments performed on this dog was similar to those performed on „Bekas", we shall report them more concisely. Here, too, after the elaboration of a positive conditioned reflex (to Bell) a new stimulus (Whistle) was introduced and it was applied without reinforcement. After a lapse of time it became a strong inhibitory stimulus. Then, conditioned reflexes to two new stimuli (Lamp and Metronome) were established. Finally, in the last series of experiments Whistle was transformed into an excitatory conditioned stimulus.

The course of the formation of conditioned reflexes to Metronome and Whistle is represented in Fig. 3. It shows that in spite of the quite equal „strength" of both these stimuli the conditioned reflex to Metronome is formed much more rapidly and attains much higher value than the reflex to Whistle.

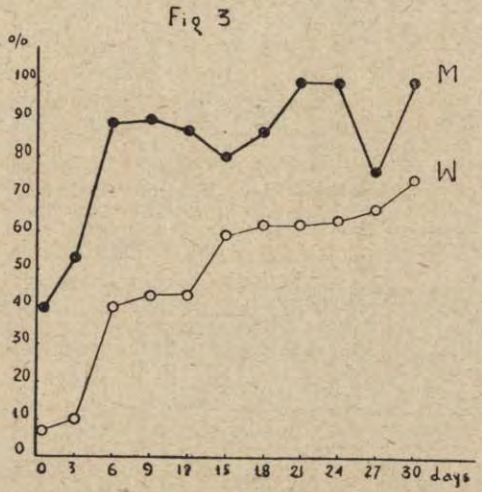

Fig. 3. The elaboration of the conditioned reflex to a primarily inhibitory stimulus (Whistle) and to a new stimulus (Metronome) in „Dudek". Abscissae: experimental sessions. Ordinates: the magnitude of conditioned reflex in percentage of the reflex to the control stimulus (Bell). Circles, the reflex to Whistle, dots, the reflex to Metronome.

3) „Romus", a male dog, age 3 years, weight 18 kg.

As the course of experiments conducted on this dog was somewhat different from that performed on „Bekas" and „Dudek" we shall report them in more detail.

Series I. Adaptation to the experimental situation — 4 days.

Series II. Elaboration of a conditioned reflex to Metronome. 28 experimental sessions, 124 trials.

Series III. Introduction of a new stimulus, Bell, applied without reinforcement. There were 48 experiments, 84 applications of Bell, 200 applications of Metronome. Alike in „Bekas" and „Dudek" the unreinforced stimulus evoked at the beginning of its application a quite significant salivary reaction (ca. 50% of salivation to Metronome), which gradually subsided. It also produced a marked inhibitory after-effect.

Series IV. Introduction of two new stimuli with reinforcement. This was a long series of experiments in which, in addition to the old conditioned stimuli (Metronome — excitatory, Bell inhibitory), two new stimuli (Whistle and Rhythmic Light) were introduced. Conditioned reflexes to these stimuli were formed with great dif-

Table I.

Acute elaboration of conditioned reflexes to a new stimulus (Bub) and to the inhibitory stimulus (Bell)

Elaboration of conditioned reflex to Bub.

No. of exp. / No. of trial	168	169	170	171	172	189	190	
1	37*	35	43	34	30	43	43	
2	8	45	51	36	37	34	44	
3	33	46	54	40	38	36	40	
4	28	54	46	48	37	28	38	
5	32	54	48	39	37	41	35	
6	35	45	45	40	37	37	28	
Sum of 6 trials	153	279	287	237	216	219	228	1619

Elaboration of conditioned reflex to Bell

No. of exp. / No. of trial	178	179	180	181	182	192	193	
1	31*	15	32	25	22	23	30	
2	4	23	36	35	41	38	34	
3	24	23	34	30	38	31	34	
4	30	26	36	33	30	29	24	
5	32	21	27	24	26	30	33	
6	20	22	31	26	26	27	36	
Sum of trials	141	130	196	173	183	178	191	1192

* The conditioned reflex to the control stimulus (M).

ficulty, and they never attained the value of the reflex to Metronome. Moreover, the dog became very restless, the reflexes were more and more irregular and all symptoms of an experimental neurosis were mainfest. On the assumption that the new stimuli were resposible for the disturbance in conditioned reflex activity they were

withdrawn and only Metronome continued to be applied. In fact, this measure restored the normal state in a few days: conditioned reflex activity became again regular, the reflexes became high, and the animal was quietened down.

Series V. The comparison of elaboration of the conditioned reflex to a new stimulus and to the inhibitory stimulus, Bell. When the normal conditioned reflex activity was fixed, a new stimulus (the sound of bubbling of water) was introduced. It was applied 6 times daily during 5 successive experimental sessions without any other stimuli intervening. Thereafter it was withdrawn, and a number of control experiments with Metronome followed. After that, Bell was applied w i t h r e i n f o r c e m e n t in just the same way

Protocol No. 1. „Romus". Exp. No. 194, 16/7/51.

No. of trial	Min.	C. s.	Its isol period	C. r.	Reinf.	Remarks.
1	2′	Bub.	20″	44	Food	Vivid alim. reaction to Bub.
2	6′	Bub.	20″	41	Food	The same.
3	10′	Bell	20″	33	Food	Very poor alim. reaction to Bell, eats rather slowly and as it were „cautiously"
4	14′	Bell	20″	31	Food	The same. After eating restless.
5	18′	M	20″	39	Food	Very vivid alim. reaction to M, eats without interruptions.
6	22′	M	20″	42	Food	The same.

as Bubbling. The comparison of the formation of the conditioned reflex to Bubbling and Bell is shown in Table I. It will be seen that there is a great difference between the two processes. The reflex to Bubbling is formed promptly and soon achieves high value. The elaboration of the reflex to Bell is more irregular and remains much below that to Bubbling and Metronome. The series ended with an experiment in which all three stimuli were given (see protocol No. 1). It is seen that although Bell is applied in the „best" place (i. e. in which conditioned reflexes are usually the greatest) it gives the weakest conditioned reaction, while the reaction to Bubbling and to Metronome is much stronger.

Series VI. Subacute extinction of Bell and Metronome.

After a series of experiments in which both Metronome and Bell were applied with reinforcement, and the two reflexes were more or less equalized, the extinction of the reflex to Bell and to

Metronome was carried out. The extinction of Bell was conducted in a following way. Each experiment consisted of 7—9 trials. At first Metronome, with reinforcement, was applied several times, then three applications of the unreinforced Bell followed, and finally several applications of Metronome terminated the experiment. In such a way 10 experimental sessions were conducted. After a lapse of time in which Metronome and Bubbling was used a similar series with the extinction of Metronome was performed, Bubbling being used as a control. The comparison of the two series is represented in Fig. 4. It is easy to observe that the process of extinction of the reflex to Bell is much more rapid than that to Metronome.

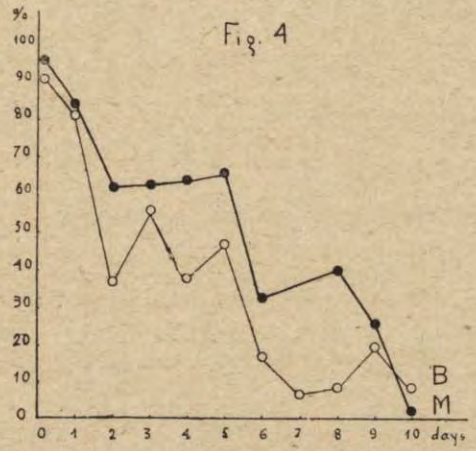

Fig. 4

Fig. 4. Subacute extinction of the conditioned reflex to a primarily inhibitory stimulus (Bell) and to a primarily excitatory stimulus (Metronome) in „Romus". Abscissae: experimental sessions. Ordinates: magnitudes of conditioned reflexes in percentage of the reflex to a control stimulus. Circles, the reflex to Bell, dots the reflex to Metronome. Each point represents the mean value of the reflex of a given day (3 trials).

The extinction of the reflex to Bell is more rapid than that to Metronome.

Summary of the experiments on „Romus".

In „Romus" as in „Bekas" and „Dudek" one stimulus (Metronome) was trained from the beginning of experiments as an excitatory conditioned stimulus and another stimulus (Bell) as an inhibitory conditioned stimulus. The properties of the Bell were investigated. It was established that: 1^0 its „acute" transformation into a positive conditioned stimulus (by applying this stimulus exclusively, time after time) was slower and more incomplete than the formation

of the conditioned stimulus from a new stimulus; 2^0 its „subacute" extinction (by applying it again without reinforcement each day several times in succession) was more rapid than the similar extinction of the normal positive stimulus (Metronome). It was also observed that the elaboration of conditioned reflexes to new stimuli, just after an intensive training of the inhibitory conditioned reflex to Bell, was rather difficult and produced a temporary experimental neurosis in the dog.

DISCUSSION

Kosteneckaya (1949) has first established the following facts. If a dog is trained in such a way that from the very beginning of the conditioned reflex training the food is given without any signalling stimuli, and various „indifferent" stimuli are applied in intervals between food presentations, then these stimuli adopt a strong inhibitory character, and moreover, this first inhibitory training leaves its marked impression on the whole later conditioned reflex activity. So, it was shown that conditioned reflexes to new stimuli are elaborated with much greater difficulty than they are elaborated normally, that they remain weak and irregular, and improve only after the inhibitory stimuli are withdrawn.

These findings were fully confirmed in our laboratory. In one of our dogs (unpublished experiments of W. Kozak) the preliminary training was just the same as in Kosteneckaya's experiments, and afterwards it was observed that the elaboration of all conditioned reflexes in this dog occurred with exceeding difficulty, and they remained always relatively weak in comparison with the unconditioned reflex (cf. Fonberg 1952).

In this series of experiments the procedure was somewhat different. First, the conditioned alimentary reflex to a given stimulus (say, stimulus A) was normally established, and then another stimulus (say, stimulus B) of the same or another analyser was introduced, and it was for a long period applied without reinforcement. It was demonstrated that the stimulus B has acquired quite different properties than the stimulus A. While stimulus A behaved much in the same way as those stimuli we dealt with in our previous papers (Konorski and Szwejkowska 1950, Szwejkowska 1950), viz. the process of its extinction was protracted and its restoration was prompt and complete, stimulus B could be transformed into a positive conditioned stimulus with great difficulty, its effect remained weaker,

than the effect of stimulus A, and its extinction occurred relatively rapidly, while its restoration was very protracted. And so, the problem put forward in our previous papers, why there is such a distinct difference between the rate of extinction and restoration of conditioned reflexes, seems now to be elucidated. It is shown to depend on the previous „history" of the stimulus. If this stimulus has been, from the very beginning of its conditioned reflex „career", applied with reinforcement, then it is difficult to transform it into an inhibitory stimulus, while it is very easy to restore its previous excitatory character. When, on the contrary, the first training of a given stimulus has been inhibitory, then its properties are quite reverse; now it is difficult to transform it into an excitatory stimulus, while it is easy to render it again inhibitory. We shall call this principle „the principle of the primacy of first training".

Many experimental data obtained in Pavlov's laboratories concerning various forms of transformation of excitatory conditioned reflexes into inhibitory, and vice versa, may be adduced to support this principle.

A number of authors (Rikman, cit. after Pavlov 1940, p. 302 ff, Pawłow, 1951, str. 254, Jakovleva 1938, 1944, Maiorov 1938, Timofeeva 1948, and others) have found that a well established differential inhibitory stimulus as a rule can be transformed with a great difficulty into an excitatory conditioned stimulus. Such a stimulus, even after many reinforced trials, gives much smaller conditioned responses, compared to a control excitatory stimulus; furthermore, these responses are often irregular, and sometimes a more or less pronounced disturbance of the conditioned reflex activity (experimental neurosis) results from its application (Rikman). Moreover, even in those cases, when after being transformed it has reached the same effect as the control stimulus, its „latent" inhibitory properties may be shown by submitting it to the acute extinction with the subsequent restoration; its extinction occurs much faster, while its restoration occurs much slower, than the respective processes of the control stimulus (Iakovleva 1944). All this goes to indicate that differential inhibitory stimuli must be classed as belonging rather to the group of our B stimuli than to the group of our A stimuli. In fact, such stimuli are generally from the very beginning of their application being given without reinforcement, and so their first training is inhibitory.

But it must be stressed that the more „similar" is a differentiated stimulus to its excitatory counterpart (i. e. the greater is generalisation to be overcome), the more excitatory elements it carries along from the beginning of its training, and consequently cannot be regarded as 100% inhibitory as are regarded those stimuli that are quite unlike their excitatory counterparts. In order to illustrate this point we shall adduce the following series of experiments.

In one of our dogs the Bell (S_1) was an excitatory conditioned stimulus. To this a differentiation was established by two stimuli: another Bell (S_2), very similar to the first one, and the sound of a Buzzer (S_3), rather unsimilar to S_1. Both these stimuli were applied the same number of times, and after the inhibitory reflexes had been firmly established, they both started to be reinforced by food. The course of the formation of inhibitory and then excitatory conditioned reflexes to these stimuli is represented in Fig. 5. We see that stimulus S_2 exhibits at the beginning a strong generalization to stimulus S_1, and the course of its transformation into a positive

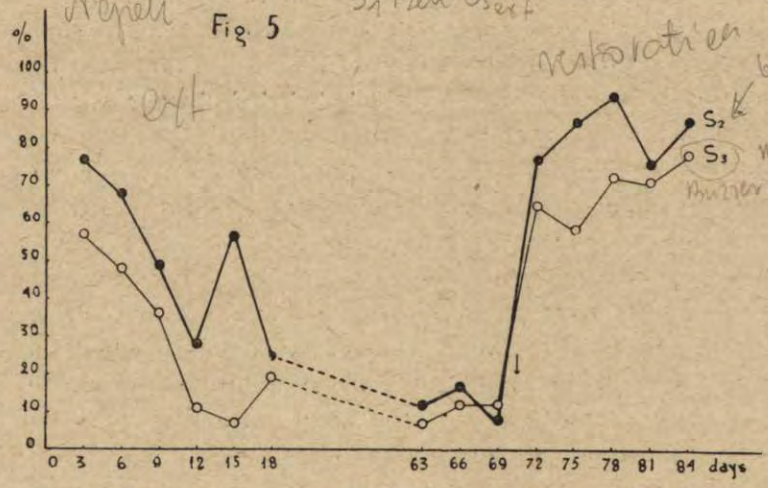

Fig. 5. The elaboration of an inhibitory conditioned reflex and its transformation into an excitatory conditioned reflex to a more (S_2) and less (S_3) similar stimulus in respect to the primarily conditioned stimulus (S_1) in „Nepek". Abscissae: experimental sessions. Ordinates: magnitudes of the reflex in percentage of the reflex to control stimulus. Circles, the reflex to S_3, dots, the reflex to S_2. At arrow S_2 and S_3 start to be reinforced. The inhibition of the reflex to S_3 is more rapid than that of the reflex to S_2, while its restoration is slower and more incomplete.

stimulus is very prompt. In other words it comes closer to our A group. In contrast to it, the stimulus S_3 exhibits much a lesser generalization to stimulus S_1, consequently it is more inhibitory since the beginning of training than S_2, and therefore its transformation into an excitatory stimulus is more difficult and incomplete. In other words it comes closer to our B group. Thus, various differentiated inhibitory stimuli may be placed in between the A and B groups, closer to the one or the other, according to the subtleness, resp. coarseness of differentiation.

Similarly, it is well known that if a stimulus is for a long time reinforced in an ordinary manner (i. e. after 20—30 sec. from its outset), and thus a short-delayed reflex to it is well established, then it is very difficult, and sometimes practically impossible, to transform it, by protracting its isolated action to 2—3 min., into a long-delayed reflex. But if a stimulus is trained with a delayed reinforcement from the beginning of its practice, then a delayed reflex is formed relatively easily, and in this case it is difficult, and may be even impossible, to transform it into a normal reflex by moving the time of reinforcement closer to the beginning of the conditioned stimulus (Pavlovian Wednesdays 1949, I p. 120, 129, III p. 378 etc., Frolov cf. after Pavlov 1940 p. 229, Pawłow 1951 str. 193, Jakovleva 1944, Timofeeva 1948 and others). Experimental neuroses may result from such transformations (Timofeeva 1948).

Furthermore it must be emphasized that the nature of the first training determines not only the properties of that stimulus to which it is applied, but extends partially to the whole of the conditioned reflex activity of the dog, influencing also the properties of other stimuli submitted to other forms of training. So, in all dogs of our first group, as reported in previous papers of this series, the first training was purely excitatory, and no permanent inhibitory reflexes were formed at all. In consequence not only the restoration of all extinguished conditioned reflexes proceeded in them very rapidly, but also all conditioned reflexes to new stimuli developed very readily, and after a few reinforcements they achieved the same magnitude as the old and well established reflexes. In contradistinction to this group, we noticed that our second group of dogs, in which after the first excitatory training (to stimulus A) the inhibitory training (to stimulus B) was carried out, displayed quite different properties. Conditioned reflexes to new stimuli (elaborated after the inhibitory training) grew much slower than in the first group, and

often did not achieve maximal values. Similar facts were reported by Kosteneckaya (1949).

Accordingly, in Fig. 6 three curves of elaboration of new conditioned reflexes are shown.

Curve I represents the mean rate (taken from 2 series of experiments) of the elaboration of conditioned reflexes to new stimuli in those dogs which have been hitherto submitted almost exclusively

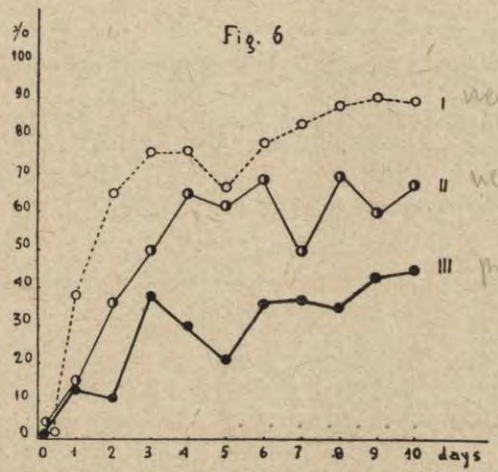

Fig. 6. The elaboration of conditioned reflexes to new and to inhibitory stimuli in various series of experiments. Abscissae: experimental sessions. Ordinates: magnitudes of conditioned reflexes in percentage of control stimuli. Further explanation in text.

to an excitatory conditioned reflex training (these dogs served in experiments reported in our previous papers of this series, Konorski and Szwejkowska 1950, Szwejkowska 1950).

Curve II represents the mean rate (taken from 5 series of experiments) of the elaboration of conditioned reflexes to new stimuli in those dogs which were submitted at the beginning of experiments to both excitatory and inhibitory training (the respective series of experiments were reported in the present paper, cf. Fig. 1 and 3)

Curve III represents the mean rate (taken from 2 series of experiments) of the elaboration of conditioned reflexes to those stimuli which were trained from the very beginning as inhibitory conditioned stimuli (cf. Fig. 1 and 3).

It is seen that while the elaboration of conditioned reflexes according to curve I proceeds very rapidly, and the elaboration of

conditioned reflexes according to curve III develops very slowly, the curve II takes the medium course, being not so rapid as in the first instance and not so slow as in the third. Generally speaking (as Kosteneckaya has pointed out) the more excitatory conditioned reflexes are already formed and the less inhibitory stimuli are applied, the more rapid is the elaboration of new conditioned reflexes.

Incidentally it should be pointed out that all three curves represented in Fig. 6 exhibit a marked collapse on the fifth day of elaboration of conditioned reflex. We are signalling this fact without going into its further comment.

To sum up we can say that the properties of a given stimulus depend first of all on its individual training; however, they are also affected by the training of all other stimuli in the animal.

But it should not be thought that the properties of a stimulus acquired in its first training are quite unalterable and that this stimulus preserves its primary character irrespectively of its later fate. The fate of the primarily inhibitory stimulus — Rythmic Lamp — in „Bekas" is in this respect very instructive. The salivation to this stimulus after its first inhibitory training was almost nil. But after a monthly excitatory training this stimulus being re-extinguished did not attain such a low level as before, and it continued to elicit a slight salivation which persisted even after many trials (cf. Fig. 2a). Thus, not only the first training left its permanent imprint on the character of the stimulus (which was amply discussed above), but the second training succeeded to modify somewhat its character, and this modification was also enduring and could be traced during succeeding trainings. Similar results were obtained by Iakovleva (1938) and Timofeeva (1948).

The problem arises whether the above formulated principle, stressing the primacy of the first training for the properties of a given stimulus, is restricted only to the transformations of excitatory reflexes into inhibitory ones, and vice versa, or whether it has a more general application. Undoubtedly there is a body of evidence to show that its application is in fact more general and that it bears also upon transformations of heterogeneous conditioned reflexes.

A striking example may be taken from „Pavlovian Wednesdays" (1949 I p. 313) in which an interesting experiment by Rikman is reported. This author elaborated a defensive conditioned reflex (an electric shock to the leg being used as reinforcement) to a musical tone, and then trasformed it into a conditioned alimentary reflex.

This transformation was very difficult for the dog and was accomplished within a year, after hundreds of trials. But it sufficed to apply another musical tone only once but reinforced by an electric shock to completely abolish the alimentary conditioned reflex to the previous tone and restore the defensive reflex.

Another example is presented by Frideman's experiments (Pavlov 1940 p. 227, Pawłow 1951, p. 193). This author transformed a conditioned alimentary reflex into a defensive acid conditioned reflex. „The complete replacement of the one conditioned reflex by the other required about 30 reinforcements by the new unconditioned stimulus. After a considerable practice of the conditioned reflexes to acid the conditioned stimuli were once more transformed back again into alimentary ones; the transformation occurred rapidly, and only a few reinforcements were needed. This indicates that the original alimentary connection was still preserved in spite of the establishment of a new connection with the reflex to acid".

We may also refer to our own experiments (Konorski and Wyrwicka 1950) in which the transformation of the classical alimentary conditioned reflex (of the first type) into a motor alimentary conditioned reflex (of the second type) was much more difficult than the elaboration of such a reflex to a quite new stimulus.

All this goes to show that the principle of the primacy of first training has a general application and is valid, *mutatis mutandis*, in respect to all types of transformations of conditioned reflexes. It is not difficult to indicate very many instances from the life of animals, as well as of man, where the operation of this principle may be easily found.

The important question is: what may be the physiological mechanism through which this principle works? If we accept after Pavlov that the elaboration of conditioned reflexes is founded on the acquisition of new connexions in the cortex (so called „temporal connexions"), then why is it that the first connexions that a definite stimulus forms with some other stimulus are much „stronger" than new connexions formed by the stimulus, and why is it that those first connexions determine to a large extent its later properties?

Of course, our knowledge of this subject is insufficient to give a full and unequivocal answer to this question. But tentatively we may guess that the following mechanism may be involved here. When a given „indifferent" stimulus is combined with an unconditioned stimulus, there are no substantial obstacles for the respective

„temporal connexions" to be formed between the relevant cortical „centres". But when the established conditioned reflex to that stimulus is transformed into an antagonistic reflex (e. g. an excitatory reflex into an inhibitory one, or an alimentary reflex into a defensive reflex), then the acquired character of the stimulus and the reaction it produces prevents the new connexions with the antagonistic centre to be formed, and this makes the process of such transformation much more protracted than it would be, if this very kind of training were conducted at first.

According to Pavlov's principle of „stratification" of conditioned connexions (Pavlovian Wednesdays, I p. 296, 313, II p. 494, 527, III p. 378), which states that freshly elaborated connexions do not annihilate the old ones but are, so to say, „superimposed" on them, the old connexions survive the antagonistic training and, as soon as this training is discontinued and the old training is resumed, they easily outbalance the new and weak connexions, and so the old reflex is restored.

It remains to consider why is it that a particular training of one stimulus not only determines the properties of that stimulus but is also partly manifested in the properties of other stimuli subjected to different forms of training. The answer to this question must be sought in the integrative character of the conditioned reflex activity. According to it no particular stimuli presented to the animal are independent from one another, but are in some sense interconnected and generalized, forming a whole in which any component puts its share. And so, quite different is the „attitude" of a dog in whom every stimulus hitherto applied in the experimental situation was always reinforced by food, and the „attitude" of another dog, who from the very beginning of its training has learned that there are not only stimuli that announce food but also other stimuli that announce nothing.

Both in our experiments and in experiments of other authors it was often observed that difficult forms of training, such as the transformation of a „purely" inhibitory conditioned reflex into an excitatory conditioned reflex, or the extinction of a firmly established excitatory conditioned reflex, result in more or less severe disturbance of conditioned reflex activity: the dog becomes restless, refutes to take food in the experimental situation, conditioned reflexes are weak and irregular, etc. This fact is easy to understand, if

we take into account that the reinforcement of a strongly inhibitory stimulus leads to a severe „conflict" between the excitatory and the inhibitory processes, which, as is well known from the investigations of Pavlov's school, is the main source of experimental neuroses.

SUMMARY

1. When an „indifferent" stimulus is subjected, from the very beginning of its application, to the inhibitory training in respect to a given reflex (i. e. if this stimulus is applied without reinforcement in a situation in which a definite unconditioned reflex is elicited), then it is much more difficult to transform it thereafter into an excitatory conditioned stimulus than it would be if this stimulus were subjected from the beginning to the excitatory training.

2. The extinction of such a stimulus (after it has been made an excitatory conditioned stimulus) occurs more rapidly than the extinction of those stimuli which are excitatory from the beginning of their training, whereas its restoration is more protracted.

3. When in addition to excitatory conditioned reflexes subsequent inhibitory reflexes are established, then excitatory conditioned reflexes to n e w stimuli are not formed so easily as in the case when the dog is subjected only to the excitatory training.

4. On the basis of these facts „a principle of the primacy of first training" is formulated and its general validity is emphasized.

5. The possible physiological mechanism of this principle is discussed.

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CHRONIC EXTINCTION AND RESTORATION OF CONDITIONED REFLEXES

V. REPEATED EXTINCTION AND RESTORATION OF CONDITIONED REFLEXES

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In one of the papers of this series (Konorski and Szwejkowska, 1950) it was noticed that if a conditioned reflex is repeatedly extinguished (each extinction being performed chronically) the succeeding extinctions become more and more difficult.

In view of the great interest that this fact arouses it was decided to study it more carefully, using not only alimentary but also defensive conditioned reflexes.

METHOD

In experiments with alimentary conditioned reflexes the standard technique (Konorski and Szwejkowska 1950) was used. The experimental stimulus (i. e. the stimulus submitted to repeated extinction and restoration) usually was applied once daily among excitatory conditioned stimuli. In experiments with defensive conditioned reflexes the method was the same as in a previous paper of this series (Konorski and Szwejkowska 1952). The experimental stimulus was applied thrice daily among positive stimuli.

RESULTS

1) Alimentary conditioned reflexes.

The repeated extinction and restoration of alimentary conditioned reflexes was performed on two dogs, Bobik and Nepek. Since the experiments on Nepek were more exhaustive than those on Bobik, we begin with their presentation.

As an experimental stimulus we chose Lamp, an excitatory conditioned stimulus which had been firmly established and which hitherto had never been submitted to extinction. Each extinction series comprised 30 experimental sessions, while each restoration series consisted of 10 experimental sessions and was immediately followed by a succeeding extinction series. There were altogether 3 extinction and 3 restoration series. Throughout this whole series the experimental stimulus was applied once daily.

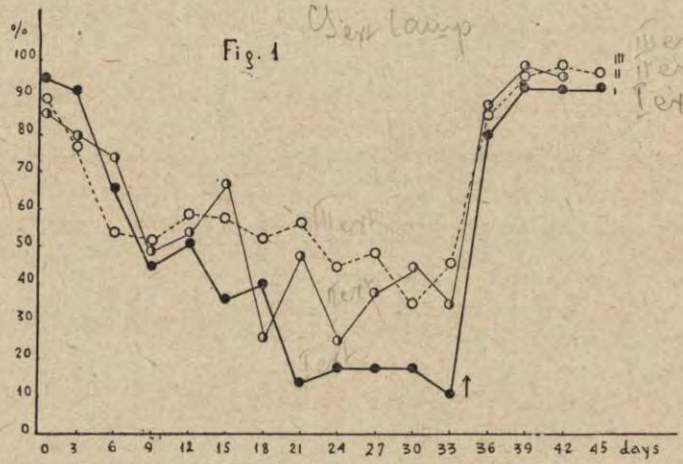

Fig. 1. Successive extinction and restoration series in Nepek. (Experimental stimulus, Lamp). Abscissae: experimental days. Ordinates: the magnitude of conditioned reflex in percentage of the magnitude of the reflex to the control stimulus (Bell). Dots, first extinction series, semi-circles, second extinction series, circles, third extinction series. ↑ beginning of restoration.

The results of these experiments are represented in Fig. 1. It may be seen that in spite of the fact that restoration series were much shorter than extinction series, each new extinction, contrary to occurring easier than the preceding one, was more protracted and less complete. And so, while after 30 sessions of the first extinction

series the conditioned reflex to Lamp fell to 11% of the control reflex, at the end of the second extinction series the respective percentage was 35, and at the end of the third 46.

If we sum up the total amounts of saliva secreted to Lamp during all three extinction series, the respective figures are: 1114 for the first series, 1282 for the second, and 1440 for the third. Thus, this computation shows the growing difficulty of the process of extinction with its repetition.

As far as the process of restoration is concerned it occurs very rapidly and more or less equally in all series.

Even more pronounced results pointing in the same direction were obtained in Bobik. In this dog the experimental stimulus was Metronome, a stimulus which had been hitherto trained for a period of 10 experiments (17 trials). In the first extinction series it was applied usually twice daily and after 13 experimental sessions (22 trials) its effect was reduced to 10%. After 3 sessions (10 trials) in which Metronome was applied with reinforcement the second extinction series was performed. Metronome was applied once daily during 22 experimental days and the reflex to Metronome dropped to 20%. After another short restoration series (9 days, 10 trials) the last extinction series followed. This time the process of extinction

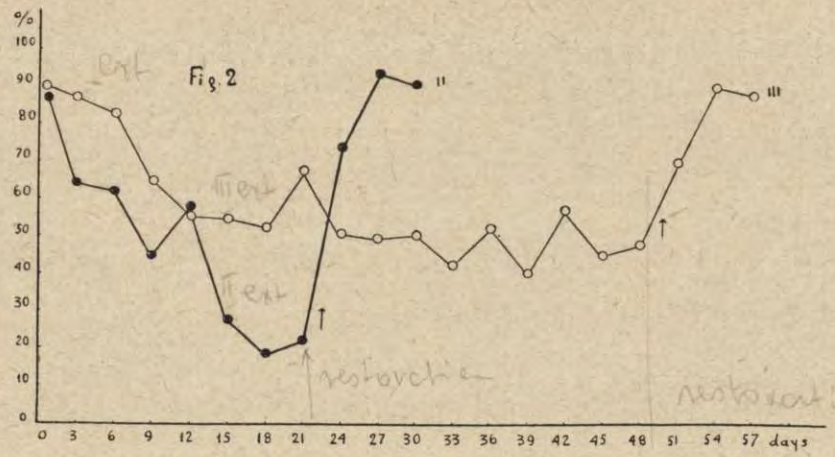

Fig. 2. Successive extinction and restoration series in Bobik. (Experimental stimulus, Metronome). Abscissae: experimental days. Ordinates: the magnitude of conditioned reflex in percentage of the magnitude of the reflex to the control stimulus (Bell). Dots, second extinction series, circles, third extinction series. → beginning of restoration.

was extraordinarily slow: in spite of 60 extinction sessions the reflex to Metronome did not fall lower than to 50% of the control reflex. The comparison of the second and third extinction series is represented in Fig. 2.

2) Defensive conditioned reflexes.

These experiments were performed on two dogs, Bobik and Cygan. On Bobik 4 extinction and restoration series were performed, on Cygan 3 such series. Extinction series were followed immediately by restoration series and vice versa. Figs. 3 and 4 represent

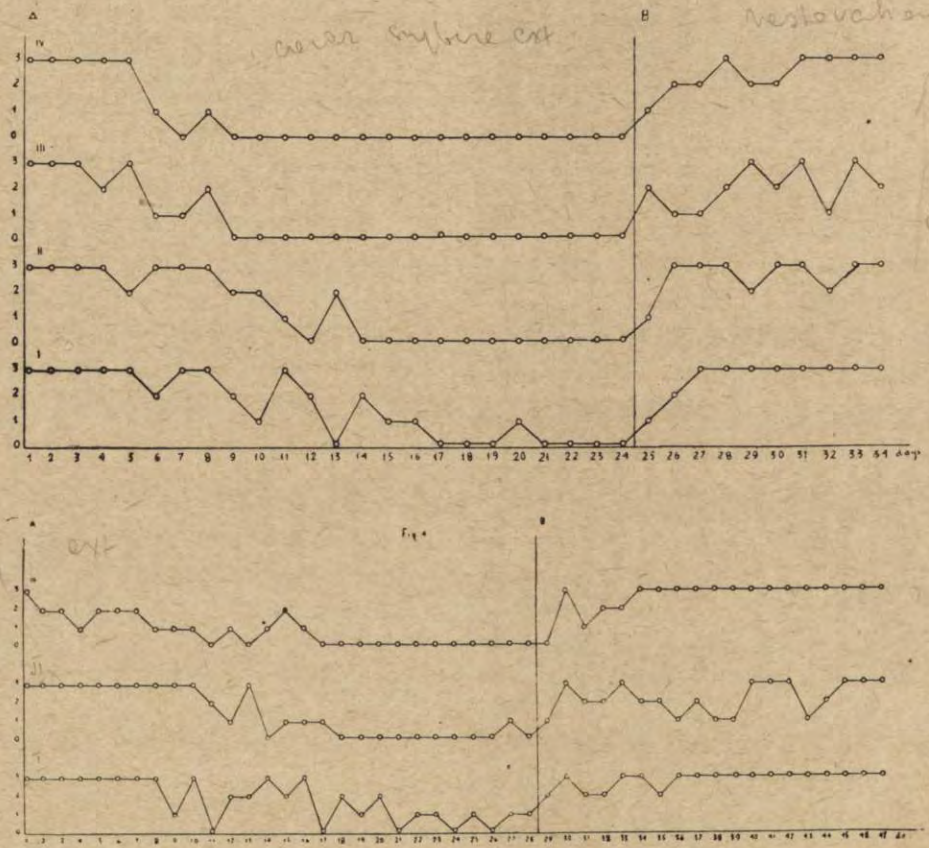

Fig. 3 and 4. Successive extinction and restoration series of defensive conditioned reflex in Bobik and Cygan. Abscissae: experimental days. Ordinates: the numbers of excitatory responses to the experimental stimulus in the course of extinction and restoration. I, first extinction and restoration series, II, second extinction and restoration series, III, third extinction and restoration series, IV, fourth extinction and restoration series. A — extinction, B — restoration.

the whole course of experiments performed on both dogs. Table I shows numbers of „faulty" reactions made by the dogs during every extinction and restoration series.

Table I.

The numbers of „wrong" responses in the course of successive extinctions and restorations in Bobik and Cygan.

No of series	Bobik		Cygan	
	Extinction	Restoration	Extinction	Restoration
I	36	3	50	4
II	30	4	40	15
III	18	10	20	4
IV	17	6		

It may be observed that in contradistinction to the alimentary conditioned reflexes the process of extinction, which is in the first series rather slow, in the successive series grows more and more rapid in both dogs. As to the process of restoration, no regular changes of its course can be observed, except that the first restoration is in both dogs the most rapid.

DISCUSSION

The growing speed of the chronic extinction of the defensive reflex can be easily explained in view of our previous results. It has been established (Konorski and Szwejkowska 1952) that the more inhibitory is the „composition" of a given stimulus, as determined by its previous „history", the more rapid is its chronic extinction and the more protracted is its restoration. Before the introduction of the first extinction the experimental stimulus was „purely" excitatory — it never had been submitted to an inhibitory training. But with each successive extinction its „composition" was being changed, viz. the stimulus was acquiring more and more inhibitory character, and hereby its transformation into an inhibitory stimulus became increasingly easier. If so, then the restoration of the extinguished reflex should become less and less rapid but this fact was not clearly exhibited (least clearly in Cygan), probably because the course of the restoration was generally very rapid and accidental fluctuations might have played a relatively larger part in total variation.

The situation is quite different in the case of alimentary conditioned reflexes. Here the course of extinction, instead of becoming

easier with each repetition of extinction series, becomes on the contrary, more and more difficult. In other words, the preceding extinctions instead of facilitating the succeeding ones, make the resistance to extinction increase.

What is the mechanism of this phenomenon?

It could be supposed that repeated transformations of a conditioned reflex into its counterpart present a difficult task to the dog, and consequently the process of inhibition becomes generally impaired. But this explanation is ruled out by the fact that the chronic extinction of other stimuli, which was conducted after the whole series with repeated transformations had been accomplished, turned out to be normal in both dogs. Because of this, we must accept that there is no general impairment of the inhibitory process, but only an invalidity of this process in respect to the stimulus subjected to such transformations.

Very similar, if not identical are the results obtained by Petrova (1937). In her dogs she carried out „conversions" of a pair of differentiated metronomes so that the positive metronome stopped to be reinforced and the negative one was reinforced. In some dogs the excitatory reflex to the previously excitatory metronome proved to be unremovable by the inhibitory process in spite of a lengthy inhibitory training. When this stimulus was submitted to the acute extinction, it appeared to be practically unextinguishable, although the acute extinction of other conditioned stimuli (sufficiently differing from the metronome) occurred quite normally. As it can be seen, in these experiments too there was an invalidity of the inhibitory process in respect to a particular stimulus. Pavlov has called this phenomenon „the inertia of the excitatory process" (Pavlovian Wednesdays 1949, I, p. 284, 328, 330; II, p. 9, 12, 539; III, p. 133, 147, 239, 269).

Not entering into the discussion of whether or not, and how far, this phenomenon of isolated maladaptation of the organism to external conditions found in Petrova's and our own experiments is pathological, we shall try to offer its tentative explanation.

In our experiments the stimulus that was subjected to the chronic extinction was from the very beginning applied as an excitatory conditioned stimulus, and therefore it can be classed as belonging to the cathegory of „purely" excitatory stimuli (Konorski and Szwejkowska 1952). Nevertheless it was possible, owing to an inhibitory training of some duration, to transform it partially into

an inhibitory stimulus. When now in the course of this training the stimulus was „unexpectedly" again reinforced, such a reinforcement possessed, so to say, an exceedingly great potency for the formation of excitatory connexions between the conditioned and the unconditioned centre. And so, the „strength" of the excitatory conditioned reflex to the stimulus in question was not to a very great degree weakened in a consequence of the inhibitory training as compared with the degree to which it was increased by the re-reinforcement following the inhibitory training. The varying relative potency of the reinforcement (or non-reinforcement) depending on the conditions of its application was emphasized in our previous paper of this series (Konorski and Szwejkowska 1952). It may be said that a great number of routine reinforcements of the established conditioned stimulus have in this respect a minor potency compared to a few reinforcements given after an inhibitory training of that stimulus.

It may be asked why is it that this mechanism did not work in our experiments with defensive conditioned reflexes, where the repeated extinction and restoration took quite a different course. Two suggestions may be offered. On the one hand, our defensive reinforcement* was much „weaker" than the alimentary one and this might be the cause that the re-reinforcement of the stimulus after an inhibitory training did not possess such a great potency for the farther fixation of the reflex as it had in the case of alimentary reflexes. If this is so, then it could be supposed that the course of the experiments with defensive reflexes would be different, if instead of one shock a strong faradic current were used. On the other hand, it may be supposed that the „positivity" of the alimentary reinforcement and the „negativity" of the shock reinforcement is the real cause of the above difference. The problem must await its elucidation until further experiments are performed.

SUMMARY

1) Some series of repeated chronic extinction and restoration of alimentary and defensive conditioned reflexes were performed in dogs with a view to studying the effect of the successive transformations of the stimulus.

2) As far as alimentary conditioned reflexes are concerned it was established that each successive extinction proceeded slower

* Only one, not very strong, condenser discharge was used as reinforcement.

and was more incomplete than the preceding one in spite of the fact that the restoration series were much shorter than the extinction series.

3) The results of the experiments with defensive conditioned reflexes were quite different: here each successive extinction proceeded more rapidly than the preceding one, while the process of restoration seemed to become increasingly protracted.

4) A tentative mechanism of these facts is discussed.

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STUDIES ON MOTOR CONDITIONED REFLEXES

4. THE EXTINCTION OF CONDITIONED REFLEXES OF THE MOTOR ANALYSER AGAINST THE EXCITATORY OR INHIBITORY BACKGROUND

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It is well known that in standard experiments with conditioned reflexes the „experimental situation" constitutes an important factor in determining both the character and magnitude of conditioned reflexes to sporadic stimuli, and its role in this respect cannot be neglected. There are however comparatively few works devoted directly to the analysis of the influence of the experimental situation on conditioned reflexes in various forms of experiments.

This paper represents an attempt to clarify the role played by the experimental situation in the course of the extinction of alimentary motor conditioned reflexes.

EXPERIMENTAL RESULTS

The present experiments were conducted on three dogs which had previously been used for a long time in experiments with the alimentary motor conditioned reflexes. The acquired movement consisted in lifting the right foreleg and putting it on the food-tray; this movement was performed to all stimuli and it was reinforced by food.

Both the motor and salivary reactions were registered. [Method has been given in detail in a former paper: Konorski and Wyrwicka, 1950].

Two series of experiments were performed.

I. THE EXTINCTION AGAINST THE EXCITATORY BACKGROUND

In this series only one conditioned stimulus — bubbling of water was used. It was applied several times in each experimental session at intervals of about 3—5 minutes and was not reinforced by food. Instead, food was given only in the intervals between the applications of bubbling without any correlation with the conditioned stimulus. We adduce the protocol of the first experimental session of this series.

Protocol No 1.

BIMBEK 20. IV. 1950

Time	Cond. stimulus	Motor reaction (latent period in sec)	Salivary reaction in manometer scales	Reinforcement	Salivation during eating (first 10 sec)
1′				Food	40
4′	Bubbling 10″	l* 4″5″ p* 6″	35	—	
8′				Food	43
10′	Bubbling 10″	p 7″	28	—	
13′				Food	44
15,5′	Bubbling 10″	l 3,5″ p 7″	27	—	
18′				Food	40
20′	Bubbling 10″	l 5″ p 7″	24	—	
22,5′	Bubbling 10″	l 6″ p 8″	21	—	
25′				Food	47
27′	Bubbling 10″	l 8″9″ p 12″	17	—	
29′				Food	46
31,5′				Food	40
34′	Bubbling 10″	l 12″	18	—	

* l = lifting the leg; p = putting the paw on the food-tray.

From this protocol it is seen that the conditioned reaction to bubbling gradually diminished and, at the end of this experiment, it was greatly reduced: the salivation decreased to half of its normal value and the motor reaction — putting the leg on the food-tray — disappeared altogether; instead, the dog performed only light movements of the leg. As to the unconditioned salivary reaction to food, it remained quite normal.

During the following experimental sessions both the motor and the salivary conditioned reaction gradually diminished and after about 6—8 experiments they disappeared while the unconditioned salivation to food continued to be normal.

When both the motor and salivary conditioned reaction were entirely extinguished, other conditioned stimuli [which had been not used during this period] were tested. It was established that they elicited a more or less normal conditioned reaction, both motor and salivary. Here are extracts of protocols of respective experiments.

Protocol No 2.

BIMBEK 5. V. 1950

Time	Cond. stimulus	Motor reaction (latent period in sec)	Salivary reaction in manometer scales	Reinforcement	Salivation during eating (first 10 seconds)
29′	Bubbling 10″	—	4	—	
32′	Tactile stimulus 10″	l 8″ p 10″	24	+	42
35′	Bubbling 10″	—	5	—	

Protocol No 3.

BIMBEK 6. V. 1950

24′	Bubbling 10″	—	3	—	
27′	Whistle 10″	p 4″	31	+	44
30,5′	Bubbling 10″	—	2	—	

After all conditioned stimuli had been tested in this way, bubbling was again applied with reinforcement with the result that the conditioned reflex to it [salivary and motor] was gradually restored [Wyrwicka 1952]. However it was observed that the process of this restoration was rather slow and it took several days [up to two weeks] before the conditioned reflex to this stimulus was totally re-established.

II. THE EXTINCTION AGAINST THE INHIBITORY BACKGROUND

After some months the second series of experiments was performed on the same dogs. As in the first series only one stimulus [whistle] was applied several times daily without reinforcement. But, in contradistinction to the first series, the food was not given during the intervals, and so no reinforcement at all was presented during the experiment.

When the reflex to whistle was entirely inhibited, other conditioned stimuli [which were not applied during the period of extinction] were tested. Here are extracts from protocols of two such experiments.

Protocol No 4.

BIMBEK 10. III. 1951

Time	Cond. stimulus	Motor reaction (latent period in sec)	Salivary reaction in manometer scales	Reinforcement
6,5′	Whistle 10″	—	0	—
10′	Tactile stimulus 10″	(p 15″)	4	—
15′	Whistle 10″	—	0	—

Protocol No 5.

BIMBEK 13. III. 1951

4,5′	Whistle 10″	—	0	—
9′	Bubbling 10″	(p 11,5″)	11	—
14,5′	Whistle 10″	—	0	—

As can be seen both the motor and salivary conditioned reaction to whistle is absent. As to the tactile stimulus the secretion of saliva is considerably less than in the analogous experiment of the first series [compare the protocol No 2]. The motor reaction is greatly delayed and appears only after the cessation of the conditioned stimulus. The value of conditioned reflexes to other stimuli is also considerably diminished [compare the magnitude of the conditioned reflex to bubbling in protocol No. 1 and 5]. The decrease of these reflexes was manifest both when they were tested after several applications of the extinguished stimulus and when the respective stimuli were applied at the beginning of the experiment.

In the following experiments all conditioned stimuli, including the extinguished one, were applied with reinforcement. Initially all reflexes were greatly reduced but gradually they increased and after several days became normal. The reflex to whistle behaved precisely in the same way as the reflexes to other strong stimuli and its restitution was even more rapid than the restitution of the reflexes to weak stimuli. This goes to show that the inhibition of the reflex to whistle itself was rather superficial.

DISCUSSION

In the following table the results of two series of extinction are compared.

Extinction				Effect of extinction	
Series	Extinguished stimulus	Other cond. stimuli	Food	Value of cond. reflexes to other stimuli	Reflex to the extinct stimulus
I	Bubbling	not applied	given in the exper. chamber	unchanged	strongly inhibited
II	Whistle	not applied	not given in the exper. chamber	much reduced	weakly inhibited
				the general fall of all conditioned reflexes	

There is no doubt that the difference in the effects of extinction between these two series is due to the fact that in the first series the food was given between the extinction trials and in the second series it was not.

It is well known that if a dog is submitted for a long time to an alimentary conditioned reflex training, then as soon as he is put into the experimental situation, his alimentary excitability (as well as a particular motor excitability, if motor conditioned reflexes are used] is greatly increased. Thus, the conditioned reflex to a sporadic stimulus applied in a given experimental situation consists of two components: 1^0 the subliminal conditioned reflex elicited by the experimental situation and operating throughout the experimental session and 2^0 the proper conditioned reflex to a sporadic conditioned stimulus itself.

Let us consider what is the fate of these two component reflexes in the course of the extinction of a sporadic stimulus conducted against two different background [excitatory and inhibitory].

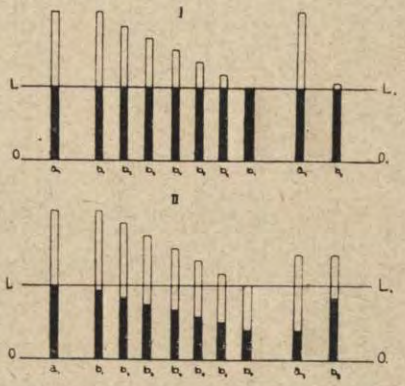

Fig. 1. The scheme of the course and the consequences of the chronic extinction of the conditioned reflex against the excitatory background (I) and against the inhibitory background (II). Columns represent the magnitude of conditioned reflexes in the course of experiments. LL_1 represents the level of liminal excitation. The black parts of the pillars denote the magnitude of the conditioned reflex to the experimental situation (for the sake of simplicity we assume that it is liminal). The white parts of the columns denote the magnitude of the conditioned reflex to a sporadic stimulus.

a_1 — the conditioned reflex to a control stimulus A before the extinction of the experimental stimulus B;

b_1 — b_7 — progressive decrease of conditioned reflex to B in the course of its extinction;

a_2 — the conditioned reflex to A after the extinction of the conditioned reflex to B;

b_8 — the conditioned reflex to B after its extinction, when it was applied with reinforcement among other conditioned stimuli.

I. The extinction against the excitatory background.

As may be seen, only the conditioned reflex to the sporadic stimulus is extinguished; the conditioned reflex to the experimental situation remains unchanged. In consequence, the conditioned reflex to stimulus A is not diminished ($a_2 = a_1$). The extinguished conditioned reflex to stimulus B is restored very slowly in spite of its reinforcement (it is deeply inhibited).

II. The extinction against the inhibitory background.

As may be seen, both the conditioned reflex to the sporadic stimulus and the conditioned reflex to the experimental situation are extinguished. In consequence, the conditioned reflex to stimulus A is much diminished ($a_2 < a_1$). The extinguished conditioned reflex to stimulus B is restored quickly, as soon as the dog again receives food in the experimental chamber (the conditioned reflex to stimulus B is superficially inhibited).

In our first series [Fig. 1, I] the subliminal reflex to experimental situation remained unchanged, since the food continued to be presented to the animal during the experimental session, while the reflex to the sporadic stimulus submitted to extinction was totally inhibited. Consequently conditioned reflexes to other sporadic stimuli which were not submitted to extinction, remained normal [$a_2 = a_1$]. In the second series [Fig. 1, II] the subliminal conditioned reflex, elicited by the experimental situation, was inhibited [as the food ceased to be given in the experimental chamber] paralelly with the reflex to the sporadic stimulus. As the subliminal reflex to the experimental situation is a component of reflexes to all sporadic stimuli, the general fall of all conditioned reflexes followed [$a_2 < a_1$].

As is seen in the scheme (Fig. 1) the rate of extinction of the reflex was in both series of experiments more or less equal. In spite of this, the conditioned reflex extinguished against the excitatory background was restored very slowly [i. e. it was deeply inhibited] while the conditioned reflex extinguished against the inhibitory background was restored rapidly [i. e. its inhibition was not very profound]. From this it may be concluded that the depth of inhibition depends not only on the number of the extinction trials but also on the conditions under which the extinction is performed.

SUMMARY

In two series of experiments the chronic extinction of the alimentary motor conditioned reflex to two different stimuli was performed in dogs. In the first series the food was given in the experimental chamber, but there was no correlation between stimulus and food; in the second series the food was not given at all in the experimental chamber during the period of extinction. It was found that in the first series, after the extinction was accomplished, other conditioned stimuli [which were not applied during the period of extinction] produced a quite normal effect, while in the second series their effect was greatly reduced.

These various results are easy to understand if we take into consideration the fact that the magnitude of a conditioned reflex represents the sum of the conditioned reflex to a sporadic stimulus and the conditioned reflex to the experimental situation. In the first series only the conditioned reflex to a sporadic stimulus was extin-

guished and the conditioned reflex to the experimental situation left unchanged; therefore conditioned reflexes to other sporadic stimuli remained normal. In the second series, both the reflex to a sporadic stimulus and the reflex to the experimental situation were extinguished; therefore the general fall of reflexes to all sporadic stimuli took place [see Fig. 1].

The author wishes to thank Prof. J. Konorski for his advice.

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Acta Biologiae Experimentalis Vol. XVI, Nr 10 1952

STUDIES ON MOTOR CONDITIONED REFLEXES

5. ON THE MECHANISM OF THE MOTOR CONDITIONED REACTION

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The problem of the mechanism of conditioned reflexes of the motor analyser was discussed by initial investigators of this type of reflexes [Konorski and Miller, 1933, 1936; Konorski, 1939, 1948] as well as by later experimenters [Hilgard and Marquis, 1940; Pietrowa, 1949; Skinner, 1938; Skipin, 1947; Woronin, 1948 and others]. Pavlov [1949] in his discussion on this type of reflexes expressed the view that the learnt movement of a dog [e. g. when he „gives his paw" to a corresponding command] is due to the connections established between the centre of this movement with both the alimentary and the auditory centre.

Facts reported in this paper, as well as in previous ones [Wyrwicka, 1950, 1952], present a strong experimental evidence supporting Pavlov's view concerning the cortical mechanism of the motor conditioned reaction.

METHOD

The experiments were performed on 6 dogs which were trained for a long time in alimentary motor conditioned reflexes in one and the same experimental situation. The motor conditioned reaction to all conditioned stimuli consisted in putting the right foreleg on the food-tray placed before the dog. Both the salivary and motor reaction were registered. The method has been given in detail in a previous paper [Konorski and Wyrwicka, 1950].

RESULTS

In 3 dogs the motor conditioned reflex to a conditioned stimulus [bubbling] was chronically extinguished and the problem arose as to whether or not it is possible to restore it without recourse to its elaboration from the very beginning [i. e. by combining the stimulus with passive movements of the leg].

For this purpose the action of the extinguished conditioned stimulus was repeatedly protracted, to as much as 60 seconds, but this measure proved to be unsuccessful: the dog stood quietly during the entire period of application of the stimulus without any tendency to lift his leg; his salivary reaction was also nil.

Then it was decided to change the experimental procedure in such a way that the stimulus in question was reinforced by food in spite of the lack of the learnt motor reaction. After a few reinforcements the alimentary reaction to the stimulus [turning of the dog's head towards the food-tray and salivation] reappeared and at the same time the dog again started to perform the learnt movement. Its latent period was rather long in the first trials, but it was very soon reduced and became quite normal [Fig. 1].

In the same dogs the differentiation between two conditioned stimuli was established in such a way that one of them [bubbling, low pitch] elicited the motor reaction and was reinforced by food, while the other [bubbling, high pitch] was not reinforced and consequently the motor reaction to it was totally inhibited. But as soon as we started to reinforce the inhibitory stimulus, it began immediately to evoke the learnt motor reaction [Fig. 2].

Similarly it was proved that in those dogs which in a given experimental situation performed only one and the same learnt movement to all conditioned stimuli, the application of a new stimulus with reinforcement led „automatically" to the formation of the motor conditioned reflex to that stimulus. This reflex was formed very quickly to acustic stimuli while to visual and olfactory stimuli its formation was more protracted. These results were obtained on 6 dogs.

DISCUSSION

The results obtained in this paper show that when in a dog one and the same motor conditioned response is trained to all conditioned stimuli applied in a given situation, then this response appears, sooner or later, to any new or inhibitory stimuli if they are rein-

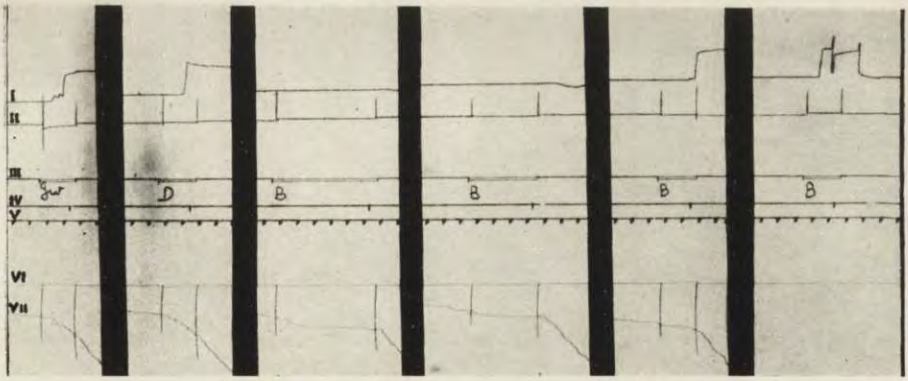

Fig. 1. The restoration of the chronically extinguished motor conditioned reaction [photocopy of the kymographic record].

I — motor reaction; II, VI — to show the isolated periods of conditioned stimuli in respect to motor and salivary reaction; III — conditioned stimuli: Gw — whistle, D — tactile stimulus, B — bubbling; IV — food reinforcement; V — time [5 sec]; VII — salivation [increases downwards]. Black thick vertical lines indicate the intervals between the successive trials.

As we see, initially bubbling [B] does not evoke any motor conditioned reaction; however after two reinforcements the conditioned motor reaction appears spontaneously. The fourth application of this stimulus evokes almost a normal motor reaction.

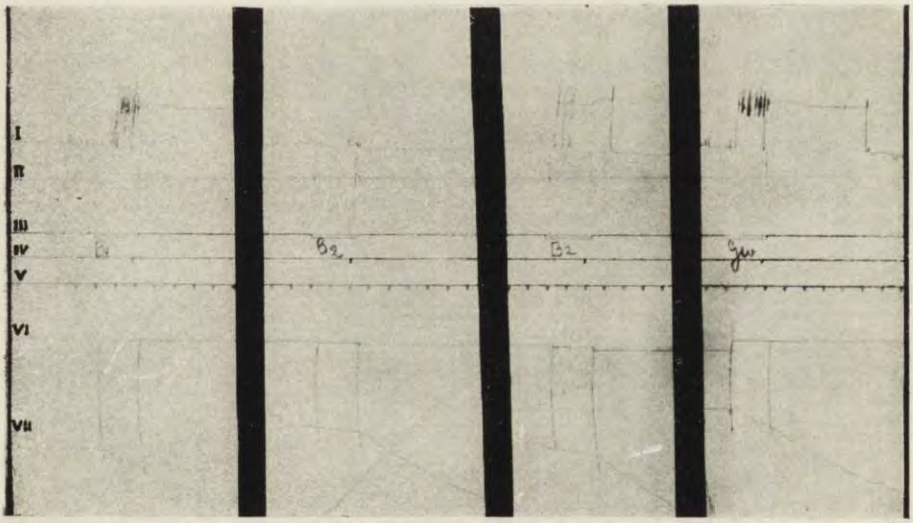

Fig. 2. The appearance of the motor conditioned reaction to differential inhibitor, bubbling II [photocopy of the kymographic record].

I — VII — as in fig. 1. B_1 — bubbling I, the positive stimulus; B_2 — bubbling II, the differential inhibitor; Gw — whistle.

Bubbling I evokes a normal motor conditioned reaction, bubbling II does not [only small movements of the leg are seen]. After 10 seconds action of this stimulus [B_2] the food is given. The succeding application of B_2 evokes the motor reaction though less intense than to bubbling I, and to whistle. The salivation to the second application of bubbling II is increased.

forced by food. In other words, in order to establish the motor conditioned reflex to a new [or inhibitory] stimulus, no special training is needed, since the reflex is formed spontaneously by mere reinforcement of this stimulus by food.

It seems to us that these facts [as well as facts reported in previous papers of this series (Wyrwicka, 1950, 1952)] throw some light on the mechanism of motor conditioned reflexes.

Let us imagine the most general, non-anatomic scheme of processes occurring in the cerebral cortex of a dog [for a long time trained in the same experimental situation and performing the same movement to all conditioned stimuli] during the action of a sporadic conditioned stimulus. This scheme [Fig. 3] represents the probable

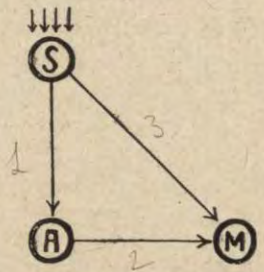

Fig. 3. The scheme of the probable mechanism of the motor conditioned reaction.
S — cortical parts of analysers [„centres of stimuli"]; A — cortical alimentary centre; M — learnt movement's centre.
Small arrows over S mean impulses from the experimental situation and the sporadic stimulus. Arrows between S, A and M indicate the direction of the excitatory impulses.

state of affairs which exists f r o m the moment of application of the sporadic conditioned stimulus t o the moment of performing of the learnt movement by the dog.

It is supposed that, when a motor alimentary conditioned reflex is established to a sporadic stimulus acting against a given experimental situation, the following connections are formed between the respective cortical centres:

1) the connections between the „centre of stimuli", S [including both the experimental situation and sporadic stimulus] and the cortical representation of the alimentary centre, A, [Fig. 3 S → A, path 1];

2) the connections between the alimentary centre, A, and the centre of the learnt movement, M, [A → M, path 2];

3) the connections between the „centre of stimuli", S, and the cortical centre of the learnt movement, M, [S → M, path 3].

Thus, when the dog is placed in the experimental situation and the sporadic stimulus operates, the excitatory process generated in respective receptors reaches the centre S and hence impinges on the centre M by two pathways: directly through the path S → M, and indirectly through the path S → A → M.

This supposition is based on the following considerations.

1⁰ If we assume for a moment that the centre M is excited only directly from the centre S [path 3], then the magnitude of the motor conditioned reflex should be quite independent of the alimentary centre. But the facts prove that this is not true. First, we have established that the motor conditioned reflex greatly depends on the alimentary excitability, being much diminished or even abolished when the dog is satiated [Wyrwicka, 1950]; secondly, it is well known that when the conditioned stimulus ceases to be reinforced by food, the learnt movement disappears. These facts prove that the performance of the motor conditioned response cannot be dependent solely on the connections between centre S and M [path 3].

2⁰ This leads us to the second assumption, namely that the motor conditioned reflex is accomplished exclusively through the way S → A → M [path 1 and 2]. But this assumption cannot be held in view of the following facts: 1) we have shown that even if the dog is quite satiated and takes no more food, he nevertheless continues to display the motor conditioned reaction to strong conditioned stimuli [Wyrwicka, 1950]; this shows that the alimentary excitability is not indispensable for the motor reaction to be performed; 2) if the only path to the centre M led from the centre A, then the animal, being hungry, would always perform a whole set of movements which in his previous practice led to the securing of food, independently of the situation in which it is placed; this is obviously not true: both in real life and under experimental conditions the animal is trained to perform v a r i o u s motor reactions, all leading to the reception of food, and the elicitation of this or that movement depends on the external stimuli to which the animal is submitted.

Thus we are compelled to admit that both the direct connections from S to M [path 3] and the indirect connections from S through A to M [path 1 and 2] play a role in the performance of the motor conditioned reaction; the connections S → A → M are chiefly concerned with the elicitation of movements, while the connections

S → M rather determine w h i c h of the trained movements is to be displayed at a given moment.

These considerations appear to explain all facts reported in this paper.

When a dog is placed on the stand in the experimental chamber, where he has been trained to perform a given movement, his centre M is subliminally excited both directly [path 3] and indirectly [path 1 and 2] [Fig. 4]. This is so because the experimental situation is

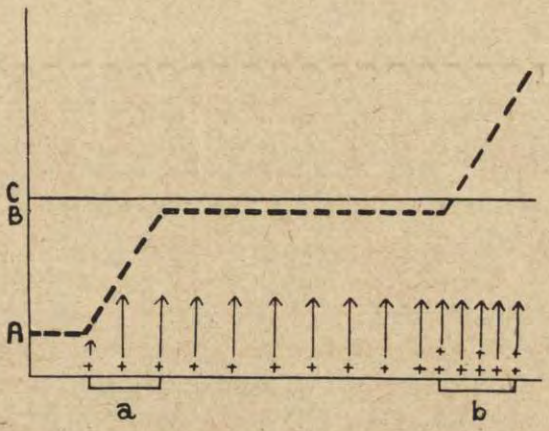

Fig. 4. The scheme to show probable changes occurring in the excitation of the cortical motor centre in the dog during the experiment.
Ordinate — degree of motor excitation: A — when the dog is going to the experimental chamber; B — after arriving in the experimental chamber; C — the liminal excitation. Abscissa — time; a — just after arrival of the dog in the experimental chamber; b — the action of a sporadic conditioned stimulus. Arrows with one cross refer to the excitatory impulses from the experimental situation; arrows with two crosses refer to the excitatory impulses from the sporadic stimulus.

joined both with the movement which was trained against its background, and with food repeatedly presented in this situation; therefore it has the power to evoke a subliminal conditioned excitation [see the previous paper of this series: Wyrwicka, 1952]. Now, if we repeatedly apply a new stimulus [or an inhibitory stimulus] and reinforce it by food, we transform it into an excitatory alimentary conditioned stimulus, i. e. this stimulus begins to produce an additional excitation of the alimentary centre which, for its part, sends additional impulses to the centre M. In consequence this centre becomes supraliminally excited and the learnt movement appears.

According to these considerations the hypothesis put forward by Konorski and Miller, [1933 and later] in their discussion of the mechanism of the motor conditioned reflexes, seems to be invalid. These authors claimed that the chief factor eliciting the trained motor response is the inhibition of the alimentary centre. The facts reported here lead us to the conclusion that this response is rather elicited by the e x c i t a t i o n of the alimentary centre.

SUMMARY

In the present paper the following problem was posed: if a dog has elaborated a number of alimentary motor conditioned reflexes consisting in the performance of one and the same movement to various stimuli, is it possible to form this reflex in response to a new stimulus or to restitute the reflex to an old stimulus [after it has been inhibited], without recurring to the special elaboration of the motor response, such as was applied at the beginning of the training?

It was found that the repeated application of the stimulus in question [i. e. either a new stimulus or an inhibitory conditioned stimulus] with food reinforcement is sufficient to the formation [or restitution] of the motor conditioned reflex to this stimulus without any specific training.

This fact seems to point out that the elicitation of the alimentary motor conditioned reflex is intimately bound up with the excitatory process arising in the alimentary centre in response to a stimulus signalling the presentation of food. The further implications of this fact, in respect to the mechanism of the motor conditioned reflexes, are discussed.

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VERÄNDERUNGEN DER INDIREKTEN ERREGBARKEIT DER PERIPHERISCHEN WARMBLÜTERNERVEN UND VERSUCH IHRER ERKLÄRUNG

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Die folgende Arbeit wurde unternommen, um die Veränderungen der indirekten Erregbarkeit der peripherischen, von ihren Zentren abgeschnittenen Warmblüternerven zu studieren und eine Erklärung für dieselben zu finden.

Zur Bestimmung der Nervenerregbarkeit wurde hier die Methode der mechanischen Reizung (Stossreizung) angewandt, die gegenüber derjenigen der elektrischen Reizung den Vorteil hat, dass ihre Wirkung — auch bei sehr hohen Schwellen — sich auf einen verhältnismässig geringen Abschnitt des Gewebes beschränkt. Bei dieser Verfahrungsweise lässt sich der Verlauf der Erregbarkeitsverhältnisse längs der ganzen, der Untersuchung unterworfenen Nervenstrecke verfolgen, wobei man die Gewissheit haben kann, dass durch den mechanischen Stoss tatsächlich die getroffene und nicht durch Stromschleifen gereizte Nervenstelle in Erregung gebracht wird.

VERSUCHSVERFAHREN

Die zu schildernden Versuche wurden an den *Nervi peronaei* und *tibiales* in der Schenkelgegend, wie auch an den *Nervi ulnares* und *mediani* in dem Arm und Unterarm der mit Somnifen (0,4—0,5 ml/kg) und Aether narkotisierten 31 Katzen und 3 Kaninchen durchgeführt.

Die Nerven wurden vollständing auspräpariert. *Nervi peronaei* und *tibiales*, die am häufigsten der Prüfung unterworten waren, wurden in der ganzen Schenkelgegend — von dem *Musculus pyriformis* bis an die Kniehöhle herab — von der Umgebung isoliert. Die *Arteria glutea inferior* blieb dabei unversehrt, während das der *Arteria profunda femoris* entspringende Gefäss, welches den mittleren Abschnitt der beiden Nerven mit Blut verseht, sowie die *Arteria saphena parva* und *Arteria poplitea* an der Kniehöhle, vollständig von den Nervenstämmen abgetrennt wurden. In den vorderen Extremitäten hat man den mittleren Abschnitt der Nerven blossgelegt, indem ihre Blutversorgung in der Peripherie und an der Achselhöhle erspart wurde.

Die Versuchstemperatur wurde in unmittelbarer Umgebung des Präparates gemessen und blieb während des Versuches ungefähr konstant, zwischen 30 und 35°C. Nach jedem Stossreiz wurde der Nerv und die anliegenden Muskeln mit einigen Tropfen der Ringer-Lösung befeucht.

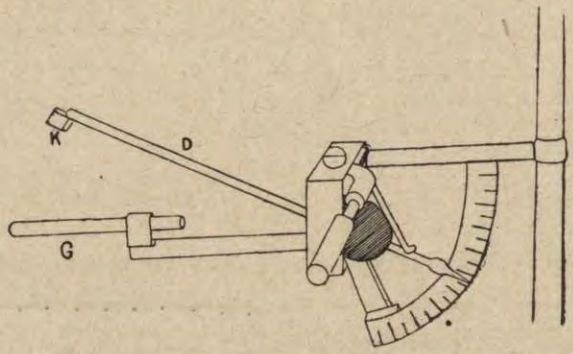

Abb. 1. Apparat zur mechanischen Nervenreizung.

Zur mechanischen Nervenreizung hat man eine ziemlich ähnliche Vorrichtung angewandt, wie sie seiner Zeit Hofmann und Blaas (1908) als Apparat zur Untersuchung der mechanischen Muskelerregbarkeit gebraucht hatten. Der hier benutzte Apparat besteht aus einem zweiarmigen Hebel D, an dessen längerem Arm ein etwas nach unten vorspringendes Korkklötzchen K befestigt ist. Das Klötzchen schlägt mit seiner abgerundeten Kante wie ein Hämmerchen quer auf den Nerven auf, welcher auf einem Glasstift liegt. Die Reizwirkung des stossenden Hämmerches gleicht der kinetischen Energie, die jenes im Moment des Auftreffens auf den Nerven ausübt (siehe Abb. 1).

Bei der Bestimmung der mechanischen Reizschwellen wurde die kleinste, immer gleich grosse und gleichartige Pfotenzuckung in Betracht gezogen. Manchmal, namentlich eine längere Zeit nach der Nervendurchtrennung fand man sich genötigt sich mit den überschwelligen Antworten zu begnügen, denn in solchen Fällen reagierte der Nerv auf einen abermaligen gleichwärtigen Reiz nicht mehr. Die Antwort wurde dann mit +, ++, oder +++ gekennzeichnet. Dieses Verfahren ermöglichtete wenigstens Ergebnisse zu bekommen, die den richtigen Reizschwellenbestimmungen annähernd entsprachen.

Im Laufe der Arbeit stellte es sich heraus, dass bei der mechanischen Reizung die Hauptsache ist, den freigelegten Nerven möglichst wenig umzulegen, weil davon die Gleichwertigkeit der Bestimmungen im hohen Grad abhängt. In der Tat ist das aber schwer zu vermeiden, und es gelingt nur selten einen und denselben Nervenpunkt wiederholt zu reizen. Aus diesem Grunde sollte man den Nerven — wenn's möglich ist — unbeweglich machen. Diesem Wunsche folgend, hat man letztens eine einfache Einrichtung angewandt; den betreffenden Nerven legt man nämlich nicht, wie vorher, auf einen einzigen Glasstift, sondern auf eine Reihe von denselben (in Paraffin eingeschmolzenen), die mit dem Reizapparat nicht verbunden ist. Bei der Reizung wird dann nur das Hämmerchen verschiebt, die Glasstiftenunterlage samt dem Nerven bleibt aber dabei unbeweglich. Dieses Verfahren ergibt genauere Werte und eignet sich dazu, eine grössere Zahl von Bestimmungen in kurzer Zeit zu erhalten.

Der Reizapparat wurde zu einer quantitativen Bestimmung der Reizschwellen benutzt. Wenn es aber im allgemeinen um die Frage ging, ob der gereizte Nerv eine Pfotenzuckung erlöst oder nicht, dann zerquetschte man die einzelnen Abschnitte des Nerven mit einer Pinzette.

ERGEBNISSE

Versuchsvariante:

1a) Der betreffende Nerv wurde freigelegt, gleich danach durchtrennt und auf eine 70—90 mm *(nn. peronaei et tibiales)* oder längere Strecke *(nn. ulnares et mediani)* von der Umgebung isoliert. Mit der Stossreizung fing man in der Regel binnen 2 Stunden nach der Durchschneidung an.

1b) Der Nerv wurde aseptisch freipräpariert und durchtrennt; die Wunde verschlossen. Mit der Reizung fing man mehrere Stunden (am häufigsten 24—48 und mehr) nach der Durchtrennung an.

2) Der Nerv wurde durchtrennt; von den umgebenden Geweben isoliert und dann gereizt wurde er aber erst mehrere Stunden (am häufigsten 24—48 und mehr, d. h. kurz vor dem Verschwinden der neuromuskulären Erregungsübertragung) nach der Durchtrennung.

3a) Der Nerv blieb entweder unverletzt oder die Durchtrennung galt ausschliesslich dessen hinteren Wurzeln. Mit der Stossreizung fing man unmittelbar nach der Nervenisolierung an.

3b) Nach sorgfältigem Freilegen vom zwei symmetrischen Nerven setzte man auf einen von den beiden, ohne ihn zu durchschneiden, eine dünne Gummihülle auf. Damit erhielt man eine gründliche Isolierung des betreffenden Nerven von der Umgebung.

1a. Die mechanische Erregbarkeit der freipräparierten und von den Zentren abgeschnittenen Nerven.

In den ersten Stunden nach der Nervendurchtrennung weist die von der Umgebung isolierte Nervenstrecke in ihrer g a n z e n L ä n g e einen hohen Erregbarkeitsgrad auf, auch wenn die Reizschwellen an den einzelnen Nervenpunkten nicht konstant gehalten werden. Die Schellenwerte sind in den meisten Präparaten ausserordentlich unstabil und zeigen spontane Variationen; sie schwanken von einigen zehnen bis zu einigen hunderten von Ergen, in der Regel werden aber 600 Erge nicht überschritten. Diese i n b e s t i m m t e n G r e n z e n s c h w a n k e n d e n R e i z-

Tabelle I

Katze Nr 67. Mechanische Erregbarkeit („Normalschwellen") des *Nervus peronaeus dex.* in der Schenkelgegend kurz nach seiner Durchschneidung geprüft. Die Länge der isolierten Nervenstrecke beträgt ca 90 mm. Zahlen in den einzelnen Spalten zeigen Reizschwellenwerte in Ergen angegeben.

Entfernung von der Durchschneidungsstelle in mm / Zeit nach der Durchschneidung	5	10	15	20	25	30	35	40	45	50	55	60	65
30 Min	330	65	110	460	570	460	460	270	100	100	15	40	
50 „	40	40	40	210	210	460	570	210	170	270	100	460	> 570
60 „	270		55		460		460		400		140		> 570
75 „		80		45		140		65		330		570	

s c h w e l l e n, die hier als „Normalschwellen" bezeichnet werden, treten immer nur zu Anfang des Versuches hervor. Tab. I gibt ein klares Bild über Schwellendifferenzen an dem Peronaeus-Nerven einer Katze.

Der distale Abschnitt des *Nervus peronaeus* in der Schenkelgegend, ca 20—30 mm vor seinem Eingang in die Tiefe des *Musculus peronaeus longus* zeigt jedesmal auffällig höhere Schwellen, als die übrigen Stellen des Nerven, auf. Die Ursache dieses eigentümlichen Verhaltens ist vielleicht auf die besondere Gestalt des *Nervus peronaeus* zurückzuführen. Die Faserbündel werden nämlich in diesen Nerven so angeordnet, dass ihr Querschnitt in der Mitte der Schenkelgegend einen ziemlich regelmässigen Kreis gegenüber einer Elipse an der Schnittfläche in der Nähe des *Musculus peronaeus* vorstellt. In beiden Fällen wird die Energie des stossenden Hämmerchens verschieden verteilt. Diese Verhältnisse illustriert Tabelle II.

Die folgenden Versuchsergebnisse, wenn sie mit den Peronaei-Nerven im Zusammenhang stehen, betreffen diejenigen Abschnitte, die einen kreisigen Querschnitt haben.

Hinsichtlich der Daten in der Tabelle I muss angedeutet werden, dass die hohe Erregbarkeit der durchtrennten Nerven nur bei den ersten nacheinander folgenden Schwellenbestimmugen festgestellt wird. Das Bild ändert sich aber in späterem Stadium nach der Nervendurchschneidung. In der Nähe der Durchschneidungsstelle steigen die Reizschwellen allmählich auf. Sehr oft, namentlich bei Fortdauer der Reizung, findet eine weitere Zunahme der Schwellenwerte in dieser Gegend statt. Die ausführliche Untersuchung der Nervenerregbar-

Tabelle II

Katze Nr 56. *Nervus peronaeus dex.* Abhängigkeit der mechanischen Erregbarkeit von der Gestalt des Nervenstammes. Die Länge der isolierten Nervenstrecke beträgt ca 85 mm. Weitere Ausführungen im Text.

Entfernung von dem Eingang des Nerven in den *M. peronaeus long.*	60	50	40	30	20	10
Reizschwellenwerte in Ergen	210	210	55	65	330	2400* 400++**

Vielmal mit immer demgleichen Ergebnis wiederholt

keit erbag aber auch, dass im Falle, wenn eine Pause von etwa 1—1½ Stunden zwischen je zwei Reizungen eingelegt wird, die Schwellen zum normalen Wert wieder zurückkehren. Es ist doch zu unterstreichen, dass — trotz einer gleichmässigen Reizung der ganzen, von der Umgebung isolierten Nervenstrecke — diese Änderungen nur in der Nähe der Schnittfläche aufzuweisen sind. Übrige Teile des Nerven sind dann immer noch „normal" erregbar.

Im Laufe der Zeit, besonders — wie oben schon erwänht — bei fortdauernder und intensiver Reizung, aber auch von der Reizung unabhängig, wandern die eben beschriebenen Änderungen nach der Peripherie hin, d. h. die Steigerung der Reizschwellen schreitet von der Nähe der Querschnittfläche aus immer mehr peripherwärts fort. Die Schwellen erreichen dann auch keine konstanten Werte. Wenn die Reizung des Nerven auf ein Paar Stunden gelassen wird, kommt es zu, dass die Schwellen wiederholt herabgesetzt werden, um schliesslich — in den nächsten Stunden — schon dauernhaft die „Normalwerte" vielfach zu überschreiten. Der Nerven-

* Normale Lage des Nerven, d. h. die längere Querschnittsachse ist der Reizwirkung senkrecht.

** Der Nerv wurde um 90° bezüglich der früheren Lage gedreht.

abschnitt, der von den vorstehenden Änderungen nicht beeinflusst wird, d. h. die Strecke, die weit von der Durchschneidungsstelle gelegen ist, weist noch vor dem Verschwinden der neuromuskulären Erregungsübertragung einen hohen Erregbarkeitsgrad auf, am häufigsten einen höheren, als bei Beginn des Versuches (Abb. 2).

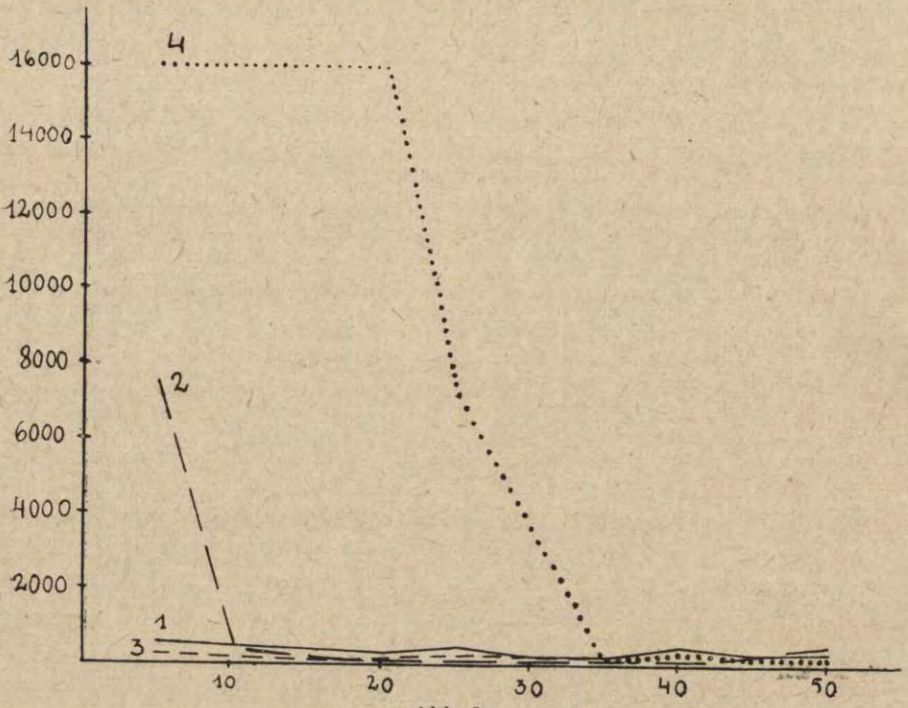

Abb. 2.

Katze Nr 65. Reizschwellenwerte des von seinen Zentren abgeschnittenen *Nervus peronaeus dex.*

Ordinate — Reizschwellenwerte in Ergen angegeben.

Abszisse — Entfernung von der Durchschneidungsstelle in mm.

Erläuterungen:

1. Der Mittelwert von 4 Messungen (2 Stunden, 2 Stunden und 30 Minuten, 3 Stunden sowie 4 Stunden und 15 Min. nach der Nervendurchschneidung) — „Normalschwellen".

2. Reizschwellenwerte 5 Stunden nach der Nervendurchschneidung bestimmt — „Ermüdungserscheinung" in der Nähe der Durchschneidungsstelle.

3. Reizschwellenwerte nach einer Pause, 6 Stunden und 30 Min. nach der Nervendurchschneidung bestimmt — die Schwellen kehren zu ihrer Ausgangslage zurück.

4. Der Mittelwert von 3 Messungen (24 Stunden, 24 Stunden und 15 Min. sowie 29 Stunden nach der Nervendurchtrennung) — dauernhafte Erregbarkeitsveränderung.

1b. Mechanische Erregbarkeit der isolierten und von den Zentren abgeschnittenen Nerven mehrere Stunden nach der Durchschneidung.

Die allmählich nach der Peripherie fortschreitende Steigerung der Schwellen wird nicht von der Reizung bewirkt, denn das Versuchsverfahren wurde so angeordnet, damit ihre destruktive Wirkung in gleichem Grad alle Abschnitte des isolierten Nerven beeinflüsse. Eine wichtige Stütze erhält jedoch diese Behauptung erst durch die Versuche, in denen der Nerv, vorhergehend ungereizt, mehrere Stunden (öfters 24—48 und mehr) nach der Durchtrennung ohne Weiteres mit stärksten zur Verfügung stehenden Reizen (bis zu 80 000 Ergen) gestossen oder peripherwärts Stück für Stück mit einer Pinzette zerquetscht wurde. Dabei stellte es sich heraus, dass der Nerv nur auf einer beschränkten Strecke, nämlich in der Nähe der Querschnittfläche seine Erregbarkeit verloren hat; von einem bestimmten Punkt an ist er aber immer noch „normal" erregbar (siehe Tab. III).

Tabelle III

Katze Nr 59. Mechanische Erregbarkeit des vorhergehend nicht gereizten *Nervus ulnaris sin.* zum ersten Mal 30 Stunden nach seinem Freilegen und Durchschneidung geprüft. Man hat Quetschung des Nerven und quantitative Bestimmung der Reizschwellen angewandt.

Entfernung von der Durchschneidungsstelle in mm	5 — 40	32	45	50	55	65	75
Reizschwellenwerte in Ergen	Keine Muskelzuckung bei der Nervenquetschung	+	7600	1000	65	65	85

+ die erste Muskelzuckung bei der Nervenquetschung gewonnen

Das völlige Verschwinden der Erregbarkeit in der Nähe der Durchschneidungsstelle des isolierten, aber nicht gereizten Nerven kommt oft schon in 24 Stunden zum Vorschein. Eine erhebliche Erhöhung der Schwellen im Vergleich zu ihren „Normalwerten" wird noch früher festgestellt.

Auch wenn die progressive, peripherwärts gerichtete Schwellensteigerung von der Reizung nicht bewirkt wird, wird sie doch von derselben recht beschleunigt. Wie oben schon erwähnt wurde, weisen die vorher nicht gereizten Nerven eine erhebliche Schwellenerhöhung am Querschnitt erst in 24 Stunden .Wenn sie aber in der Zwischenzeit oftmals gereizt werden, zeigen sie die cha-

rakteristische Steigerung der Schwellen auf einer viel grösseren Strecke auf. Leider, es ist nicht gelungen, die Schnelligkeit der nach der Peripherie fortschreitenden Vorgänge zu bestimmen. In zwei symmetrischen Nerven werden nämlich in einem bestimmten Zeitraum bei einem und demselben Tiere immer verschiedene Strecken gefunden, die auf mechanische Reize keine Pfotenzuckungen auslösen.

Die Reizung der Nerven dauerte mit Unterbrechungen bis die indirekte Erregbarkeit völlig verschwand. Dies geschah bei verschiedenen Individuen in 48—72 Stunden, am häufigsten aber in 50—60 Stunden nach der Durchtrennung.

Tabelle IV

Mechanische Erregbarkeit der isolierten und nicht isolierten Nerven mehrere Stunden nach ihrer Durchschneidung. Man hat die Nerven von der Durchschneidungsstelle aus peripherwärts Stück für Stück zerquetscht.

	Der durchschnittene und gleich danach isolierte Nerv	Der durchschnittene, aber erst unmittelbar vor der Prüfung seiner Erregbarkeit isolierte Nerv
Katze Nr 61	n. ulnaris dex.	n. ulnaris sin.
Erste Muskelzuckung bei der Nervenquetschung — 35 Stunden nach der Nervendurchschneidung	70 mm von der Durchschneidungsstelle	gleich an der Durchschneidungsstelle
Katze Nr 63	n. ulnaris sin.	n. ulnaris dex.
Erste Muskelzuckung bei der Nervenquetschung — 46 Stunden nach der Nervendurchschneidung	45 mm von der Durchschneidungsstelle	gleich an der Durchschneidungsstelle

Nun entsteht aber die Frage, wodurch die allmähliche Steigerung der Schwellen hervorgerufen wird. Überblickt man die bisher mitgeteilten Versuchsresultate, so kommt man zu der Vermutung, dass dies mit der Störung der normalen Blutzirkulation im Zusammenhang stehe. Wir werden sehen, dass diese Annahme in den folgenden Versuchen eine Bestätigung erfährt. Dieselben wurden nämlich an den durchtrennten, aber recht durchbluteten Nerven angestellt.

2. Mechanische Erregbarkeit der durchschnittenen Nerven, die erst kurz vor dem Verschwinden der neuromuskulären Erregungsübertragung isoliert wurden.

Wurde die Erregbarkeit eines durchschnittenen, aber erst mehrere Stunden nach seiner Durchtrennung (am häufigsten kurz vor dem Verschwinden seiner indirekten Erregbarkeit) isolierten Nerven mit mechanischen Reizen geprüft, so zeigte es sich, dass sie anfänglich in seiner ganzen Länge aufzuweisen ist. (Tab. IV) und, dass die Reizschwellen nicht von den „Normalwerten" abweichen (Tab. V). Sehr kurz nach seinem Freilegen kommt aber auch hier eine immer zunehmende, peripherwärts gerichtete Schwellensteigerung zum Vorschein, die in diesem Fall nie eine Erniedrigung — auch bei längeren Pausen zwischen je einzelnen Reizungsreihen — aufweist (Tab. V).

Tabelle V

Katze Nr 56. Mechanische Erregbarkeit des *Nervus peronaeus sin.* zum ersten Mal 46 Stunden nach seiner Durchschneidung geprüft. Der Nerv wurde erst ummittelbar vor der Reizung isoliert. Zahlen in der Tabelle zeigen Reizschwellenwerte in Ergen angegeben.

Entfernung von der Durchschneidungsstelle in mm / Zeit nach dem Freilegen des Nerven	5	15	20	25	35	45
unmittelbar	65	15		15		
1 Stunde	270 → 2000 →	570		210	210	
5 Stunden	Keine Muskelzuckung bei der Nervenquetschung		+	+	+	+

\+ eine geringe Muskelzuckung bei der Nervenquetschung gewonnen
→ die Reizschwelle wiederholt sich nicht; um eine Muskelantwort zu erzielen, müssen die Reize erhöht werden.

Diese Tatsachen zeigen, welch wichtigen Faktor die Blutversorgung des Warmblüternerven für die Erhaltung seiner Funktion vorstellt. Während ein durchschnittener und gleich danach sorgfältig von der Umgebung isolierter Nerv schon nach 24 Stunden seine Erregbarkeit in der Nähe der Schnittfläche verliert. bleibt der ebenfalls durchtrennte, aber erst kurz vor der Prüfung seiner Erregbarkeit isolierte, d. h. bis dahin recht durchblutete Nerv, noch vor dem Verschwinden der neuromuskulären Impulsübertragung, „normal" erregbar.

Es scheint also für richtig gehalten werden, dass die von der Schnittfläche aus nach der Peripherie hinlaufende Steigerung der Reizschwellen auf Störung der Blutzirkulation bei dem Freilegen des Nerven zurückgeführt werden kann.

Nachdem die Äbhängigkeit der mechanischen Erregbarkeit des Nerven von der Blutzirkulation erkannt war, entstand ferner die Frage, warum die allmählich zunehmende Erhöhung der Schwellen gerade peripherwärts gerichtet wird. Die bisherigen Versuchsergebnisse schienen dafür zu sprechen, dass diese Erscheinung durch eine Durchschneidung des Nerven bewirkt wird. Ob dies der Fall ist, konnte man sich leicht überzeugen, indem man die Prüfung der Erregbarkeit an den unverletzten Nerven vornahm.

3. Mechanische Erregbarkeit der isolierten, aber nicht durchtrennten Nerven.

Das Verhalten der freipräparierten, aber unverletzten Nerven ist mit dem der durchschnittenen zu vergleichen. In den ersten Versuchsstunden wird in der ganzen Länge der isolierten Nervenstrecke eine hohe Erregbarkeit nachgewiesen, und die Reizschwellen, auch wenn sie an den einzelnen Nervenpunkten nicht konstant gehalten werden, schwanken nur in bestimmten Grenzen, von einigen zehnen bis zu einigen hunderten von Ergen (in der Regel bis zu 600 Ergen). Aber auch hier wird die allmähliche Steigerung der Schwellen beobachtet und in diesem Fall beeinflusst dieser Prozess den m i t t l e r e n T e i l der isolierten Strecke. Der zeitliche Verlauf dieses Vorganges entwickelt sich jedoch an diesen Nerven langsamer und dauert 3—4 Tage.

Nachdem der betreffende Nerv auf eine bestimmte Strecke in seinem mittleren Abschnitt die Erregbarkeitsfähigkeit verloren hatte, wurde er in dieser Gegend durchtrennt. Hierauf prüfte man mit der mechanischen Reizung die Erregbarkeit des soeben abgeschnittenen peripherischen und zentralen Stumpfes. Dabei wurde festgestellt, dass aus der Nähe der Schnittfläche von den beiden Stümpfen aus — auch bei Anwendung stärkster mechanischen Reize (Stossreizung und Quetschung des Nerven) — keine Reflexe oder einfache Pfotenzuckkungen erzielt werden können. Dieselben Stümpfe weisen aber von bestimmten Pukten an einen ziemlich hohen Erregbarkeitsgrad auf

Die Schwellensteigerung des mittleren Abschnittes der undurchschnittenen, aber von den umgebenden Geweben isolierten Nerven, wird durch starke und frequente Reize bedeutend beschleunigt.

Werden die Nerven gar nicht gereizt, so zeigt es sich, dass sie in ihrer ganzen Länge ihr Erregbarkeitsvermögen einen beträchtlichen Zeitabschnitt hindurch (100 Stunden und mehr) bewahren.

Eine intensive Reizung ist ein Faktor, der anfänglich die Schwellensteigerung beschleunigt und danach die Funktionsfähigkeit des Nerven im allgemeinen verkürzt. Die Schwellen kehren aber zu ihrer Ausgangslage zurück, wenn das Intervall zwischen nacheinander folgenden Reizungen eine Stunde überschreitet.

Die durch eine Reizung bewirkte Schwellenerhöhung erfährt also immer eine Herabsetzung gegenüber der spontanen Erregbarkeitserniedrigung, die dauernhaft ist und nie zu ihrem „normalen" Wert zurückkehrt. Wie kommt es aber zu dem allmählichen Absinken und dann zu einem völligen Verschwinden der Erregbarkeit in der Nähe der Durchschneidungsstelle und später auch an den übrigen Stellen des Nerven? Wie schon oben erwähnt wurde, kann dieses Verhalten nur auf die ungenügende Blutversorgung des Nerven zurückgeführt werden. Aus den Versuchen geht es hervor, dass je schlimmere Bedingungen in der Umgebung des isolierten Nerven herrschen und vor allem je deutlicher die normale Blutversorgung gestört wird, desto schneller sinkt die mechanische Erregbarkeit der betreffenden Nervenstrecken herab. Dies kann recht schön in einem Versuch nachgewiesen werden, das hier als Beispiel angeführt wird:

Katze Nr 70. Nachdem *Nervus peronaeus dex.* und *N. medianus sin.* freipräpariert waren, setzte man auf die beiden dünne Gummihüllen auf, ohne die Nerven zu beschädigen. Hierauf wurden *N. peronaeus sin.* und *N. medianus dex.* von der Umgebung isoliert. Die Operation wurde aseptisch durchgeführt. Nach 48 Stunden machte man die Wunden auf und prüfte die mechanische Erregbarkeit aller vier Nerven. Dabei stellte sich folgendes heraus: der mittlere Teil des *N. peronaeus dex.*, mit sehr starken Schlägen gereizt, rief keine Muskelzuckung hervor. Eine in dieser Gegend vorgenommene Durchschneidung blieb ebenso ohne Antwort. Nach der Durchtrennung hat man den peripherischen und den zentralen Stumpf des Nerven mit einer Pinzette zerquetscht. Die erste Muskelzuckung von dem zentralen Stumpfe aus erhielt man 10 mm von der Schnittfläche entfernt. Der peripherische Nervenstumpf in der Schenkelgegend, d. h. dorthin, wo er isoliert wurde, war nicht im geringsten erregbar; nachdem er auch in der intakten Strecke in dem Unterschenkel isoliert wurde, erhielt man die erste Muskelzuckung bei

der Quetschung des Nerven in der Gegend, wo der *N. peronaeus* in den *M. peronaeus long.* eintritt.

Nach der gleichen Zeit reagierte der *N. peronaeus sin.* in seiner ganzen Länge auf Reize, die 600 Erge nicht überschritten. Danach setzte man auf ihn eine Gummihülle auf und prüfte seine Erregbarkeit abermals nach 7 Stunden nach. Man erhielt folgendes Ergebnis: der mittlere Nerventeil war vollkommen unerregbar gegenüber dem zentralen und peripherischen, die auf verhältnismässig kleine Reize antworteten.

Ähnliche Resultate hat man an den Mediani-Nerven gewonnen.

BESPRECHUNG DER VERSUCHSERGEBNISSE

Die mitgeteilten experimentellen Befunde haben ergeben, dass in jedem von der Umgbung isolierten — durchschnittenen oder gar nicht verletzten — Katzen-und Kaninchen-Nerven eine allmähliche Steigerung der Reizschwellen zum Vorschein kommt. In dem peripherischen und zentralen Stumpfe des durchtrennten Nerven wird dabei die Schwellenerhöhung in der Nähe der Durchschneidungsstelle am stärksten ausgedrückt gegenüber dem ebenfalls isolierten, doch an keiner Stelle durchschnittenen, d. h. mit seinen Zentren in Verbindung stehenden Nerven, wo der mittlere Teil eine allmähliche Schwellensteigerung aufweist. Bereits diese recht kurze Zusammenfassung zeigt, dass die spontane Schwellensteigerung nicht durch die Wallersche Degeneration hervorgerufen wird. Auch von der mechanischen Reizung wird sie nicht bewirkt (vergl. 1b). Aus den Versuchen geht es aber hervor, dass in den soeben abgeschnittenen peripherischen Nervenstümpfen eine progressive und peripherwärts gerichtete Steigerung der Schwellen v o r ü b e r g e h e n d mit einer intensiven und frequenten Reizung hervorgerufen werden kann. Wenn zu Anfang des Versuches eine Pause von etwa 1—1½ Stunden zwischen je zwei Reizungen eingelegt wird, kehren die Schwellen zum normalen Wert wieder zurück.

Die hier mitgeteilten Ergebnisse über die mechanische Erregbarkeit der Warmblüternerven stimmen mit den von J. Titeca (1935) an den elektrisch gereizten Froschnerven beachteten Ermüdungserscheinungen restlos überein. Titeca hat gezeigt, dass die vorübergehende Phase erhöhter und peripherwärts gerichteter Schwellen auf die Wirkung der fortdauernden Reizung zurückzuführen ist. Es ist zu unterstreichen, dass die durchtrennten, aber erst kurz vor dem Verschwinden ihrer indirekten Erregbarkeit (ca 50 Stunden)

isolierten und dann gereizten Nerven die grösste Ermüdung aufweisen. In diesem Fall kommt auch die Schwellenerniedrigung, die an den eben durchschnittenen Nerven so deutlich beobachtet werden kann, nicht mehr zum Vorschein.

Die Untersuchung der mechanischen Erregbarkeit der Warmblüternerven ergab aber auch, dass die Reizschwellen der isolierten Nerven nicht nur eine vorübergehende Erhöhung, die für die Ermüdungserscheinung charakteristisch ist, aufzeigen, aber auch eine dauernhafte Steigerung, die spontan nach der Peripherie fortgesetzt wird.

Die hier angeführten Versuchsresultate und das im vorstehenden Gesagte scheinen zu der Annahme zu führen, dass die Ursache der allmählichen und progressiven Erregbarkeitsherabsetzung, die an den isolierten Nervenstrecken zum Vorschein kommt, in der mangelhaften Blutversorgung des freipräparierten Nerven liege. Dies geht zunächst aus den Versuchen an den durchtrennten, aber erst mehrere (40—50 und mehr) Stunden nach der Durchtrennung der isolierten Nerven (vergl. 2) hervor, deren Erregbarkeit bis zum Verschwinden der neuro-muskulären Impulsübertragung keine Abstufung aufweist.

Fröhlich und Tait (1904), Koch (1925), Adams (1943) u. a. haben schon lange vorher nachgewiesen, dass die Blutversorgung des Warmblüternerven sehr reichlich ist. Dabei stellte es sich heraus, dass die Gefässversorgung eine streckenweise ist: es treten an die einzelnen Abschnitte des Warmblüternerven verschiedene Gefässe heran, die sich sowohl in peripherischer als auch in zentraler Richtung ausbreiten und mit analogen Gefässästen in Verbindung treten. Auf diese Weise entstehen längs des Nervenstammes mehrere Anastomosen.

Es ist allgemein bekannt, dass eine Nervenstrecke, welche ungenügend mit Blut versorgt ist, in kurzer Zeit ihre Erregbarkeit verliert, dieselbe aber wieder erhalten kann, wenn die Blutversorgung nach einer kleinen Unterbrechung günstiger wird (Fröhlich & Tait, 1904; Porter & Wharton, 1949). Für den Kaltblüternerven konnte schon Baeyer (1902) feststellen, dass der in eine sauerstofffreie Atmosphäre gebrachte Nerv allmählich unerregbar wird und seine Leitfähigkeit verliert, nach Sauerstoffzufuhr aber sich in verhältnismässig kurzer Zeit wieder erholt. Gerard (1930) zeigte, dass die Erstickung des Warmblüternerven (Hund) bedeutend schneller eintritt, als des Kaltblüternerven (Frosch). Bentley & Schlapp (1943)

bewiesen in den Tourniquet-Versuchen mit den Katzen-Nerven, dass der Aktionsstrom einer isolierten Nervenstrecke in 50 Min. vollständig verschwindet, während er in derselben Zeit bei andauernder O_2-Zufuhr von aussen nur um 15—20% herabgesetzt wird.

Auf eine grosse Abhängigkeit der Nervenerregbarkeit von der normalen Blutzirkulation und von dem damit zugeführten Sauerstoff weisen die Versuche hin, in denen die Nerven freipräpariert wurden, ohne durchschnitten zu werden. In solchen Fällen findet man die isolierten Nerven stunden - ja sogar tagelang in ihrer ganzen Länge völlig erregbar, weil das Freilegen des Nerven keine absolute Unterbrechung der Blutzufuhr bedeutet. Eine bedeutende Isolierung kann man aber künstlich erreichen, indem man z. B. auf den Nerven eine Gummihülle aufsetzt. Dies hat in wenigen Stunden eine merkliche Erregbarkeitsänderung zur Folge (Verg. Serie 3). Ungeachtet der sich in dem Nerven befindenden Arterienanastomosen erfährt die auf diese Weise isolierte Nervenstrecke eine bedeutende Erregbarkeitsherabsetzung. Daraus ergibt sich also, dass der von seinen Gefässen abgetrennte Nerv nur eine beschränkte Zeit überleben kann. Das Anastomosennetz genügt keineswegs, um die Funktionsfähigkeit des Nerven für längere Zeit zu sichern. Der vollkommen isolierte Nerv kann noch, wie es scheint, die für Erhaltung seiner Funktion notwendigen Substanzen (vor allem eine ausreichende Menge von Sauerstoff) von der Umgebung beziehen. Wie Bentley & Schlapp (1943) gezeigt hatten, d i f f u n d i e r t der Sauerstoff von den recht durchbluteten Muskeln in den Nerven hinein.

Obwohl nun die isolierte Nervenstrecke von ihren Blutgefässen sorgfältig abgetrennt wird, erhält der distal von der Schnittfläche gelegene Abschnitt eine genügende Sauerstoffmenge von den unverletzten Muskelgefässen. Diese Möglichkeit würde uns erklären, warum der Nerv in der Nähe des von ihm innervierten Muskels bis zum Verschwinden der neuromuskulären Impulsübertragung „normal" erregbar ist, während in der Nähe der Durchschneidungsstelle die Erregbarkeit bereits in 24 Stunden nach der Durchtrennung erloschen wird. Je ferner die entsprechenden Nervenstellen von dem Eingang des Nerven in den Muskel liegen, desto mangelhafter werden sie mit dem Sauerstoff versorgt. Dies würde ebenfalls mit der progressiven, von der Nähe des Querschnittes herauslaufenden peripherwärts gerichteten Steigerung der Reizschwellen übereinstimmen.

Auf diese Art und Weise sollte auch der zeitliche Verlauf der Erregbarkeitsverhältnisse an dem zentralen Stumpfe des durch-

trennten Nerven, sowie an dem unverletzten Nerven erläutert werden.

Frankenhäuser (1949) bewies, dass die isolierten Nervenstrecken auch beim Verschluss der Trachea nicht sauerstoffleer gemacht werden, weil in diesem Fall die L u f t w i r k u n g nicht ausgeschaltet wird. Diese Möglichkeit soll auch in den vorstehenden Erwägungen in Betracht gezogen werden — die Nerven wurden doch hier an einer langen Strecke isoliert und die Luft konnte leicht in dieselben eindiffundieren. Vermutlich dadurch kann man die ziemlich lange dauernde „normale" Erregbarkeit der freipräparierten Nerven erklären. Die späteren Schwellenänderungen zeigen aber, dass die Luftwirkung allein nicht ausreicht, um das Überleben des Nerven zu ermöglichen.

Wright (1946), der seine Untersuchungen über den Einfluss des O_2-Mangels aufs Überleben der Nerven an einer Anzahl von In-und Vertebraten durchgeführt hatte, zeigte, dass der grösste Sauerstoffverbrauch in der Nähe der Querschnittfläche ist und damit erklärt er das rascheste Absterben dieser Nervenstrecke. Wie hier mitgeteilt wurde, kommt die Steigerung der Reizschwellen bis zum völligen Verschwinden der indirekten Erregbarkeit am schnellsten gerade an der Durchschneidungsstelle vor.

Nach den obigen Ausführungen lässt sich nun ableiten, dass die „Ermüdungserscheinung", die dem dauernhaften Absturz der mechanischen Erregbarkeit vorangeht, als das erste Zeichen der mangelhaften Blutversorgung zu betrachten ist. Diese Erscheinung ist vorübergehend, weil der isolierte Nerv auch beim Ausbleiben der von ihm abgetrennten Gefässen, durch ein paar Stunden den Sauerstoff und andere Substanzen aus einigen ihm noch zur Verfügung stehenden Quellen aufnimmt: aus den reichlich durchbluteten Muskeln, von den sich in seinem Inneren befindenden Arterienanastomosen und schliesslich aus der Luft. Hervorzuheben ist es aber, dass der Nerv schon in diesem Stadium nicht mehr normal funktioniert, weil diese O_2-Quellen nur für einen streng bestimmten Zeitabschnitt ausreichen.

ZUSAMMENFASSUNG

1. Unter Anwendung einer mechanischen Reizmethode (Stossreizung) wurde die Erregbarkeit der *nn. peronaei* und *tibiales*, sowie der *nn. ulnares* und *mediani* bei Katzen und Kaninchen untersucht.

2. Die isolierten Nerven weisen in den ersten Stunden nach ihrer Durchtrennung einen hohen Erregbarkeitsgrad in ihrer ganzen

Länge auf. Ihre Schwellenwerte sind dabei ausserordentlich unstabil; sie schwanken von einigen zehnen bis zu einigen hunderten von Ergen, in der Regel werden aber 600 Erge nicht überschritten („Normalschwellen"). In späterem Stadium steigen die Reizschwellen in der Nähe der Durchschneidungsstelle auf, so dass die Erregbarkeit in dieser Gegend in 24 Stunden vollkommen erloschen wird. Im Laufe der Zeit schreitet die Steigerung der Schwellen von der Nähe der Querschnittfläche aus immer mehr peripherwärts fort.

3. Die allmählich nach der Peripherie fortschreitende Steigerung der Schwellen wird nicht von der Reizung hervorgerufen, doch beschleunigt.

4. Es ist nicht gelungen, die Schnelligkeit der peripherwärts gerichteten Schwellenveränderungen zu bestimmen, denn sie zeigt bedeutende Variationen auf.

5. Die neuromuskuläre Erregungsübertragung verschwindet in 48—72, am häufigsten aber in ca. 50 Stunden nach der Durchtrennung der Nerven.

6. Die durchschnittenen, aber durchbluteten (d. h. nicht isolierten) Nerven zeigen bis zum Verschwinden ihrer indirekten Erregbarkeit keine Schwellensteigerung.

7. In den isolierten, aber undurchschnittenen Nerven tritt die Steigerung der Schwellen in dem mittleren Teil der freipräparierten Strecke hervor. Der zeitliche Verlauf dieses Vorganges dauert hier 3—4 Tage, kann aber durch eine gründliche Isolierung des Nerven (wenn z. B. darauf eine Gummihülle aufgesetzt wird) recht beschleunigt werden.

8. Die Ursache der progressiven Steigerung der Reizschwellen liegt wahrscheinlich in einer mangelhaften Sauerstoffversorgung der isolierten Nervenstrecke.

Frau Dr L. Lubińska gestatte ich mir für gewährte Unterstützung und Erteilung wohlgemeinter Ratschläge bei der Durchführung vorliegender Arbeit auch auf diesem Wege meinen verbindlichsten Dank auszusprechen.

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

7. THE DIGESTION OF WAX AND UTILIZATION OF UNSAPONIFIABLE SUBSTANCES BY LARVAE

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As is well known, waxmoth larvae *(Galleria mellonella)* feed on the honey comb. Our earlier investigations have shown that the larvae utilized about 50% of the ingested wax, the remaining portion being excreted. The chemical composition of the wax from the comb and from the excreta has been found to be very similar. Each contains 35—40% of fatty acids and 60—65% of unsaponifiable matter. Balance experiments have enabled us to show that the amount of unsaponifiable substances utilized by the larvae is nearly twice as great as the amount of fatty acids. To explain this rather unexpected result it has been assumed that the unsaponifiable substances of the wax are transformed in the larval body into fatty acids. The fatty acids of the wax are utilized, but fresh amounts of fatty acids are constantly being formed from the unsaponifiable matter. (W. Niemierko and Włodawer, 1950 a).

A more complete study of utilization of wax by *Galleria mellonella* has been made in the present investigation and the problem of transformations of unsaponifiable substances has been more thoroughly studied. A preliminary account of these experiments has already appeared (W. Niemierko and Włodawer, 1950 b).

METHODS

The experiments were performed on full grown waxmoth larvae *(Galleria mellonella)* weighing 150—250 mg. The insects were raised in thermostates at 30°C as described by W. Niemierko and Cepelewicz (1950). The investigations were concerned with determination of lipids in the larval bodies, in the gut and in the excreta of the larvae, and with the determination of wax constituents in the honey comb. The analytical procedure were varied according to the character of the material analysed.

1. LARVAE. Five larvae were taken for each analysis. The dry substance was determined after drying the material to a constant weight in a vacuum dessicator over conc. H_2SO_4. The lipids were then extracted with hot chloroform in a Kumagawa extraction apparatus modified by W. Niemierko (1952). After evaporation of chloroform the residue was weighed and redissolwed in a known amount of chloroform. Samples of this solution served for the separation of lipids into fatty acids and unsaponifiable substances.

The procedure was as follows. A sample of the solution was measured into a micro-Kjeldahl flask and, after evaporation of the solvent, 4 ml of 0.5 N alcoholic alkali were added to the residue and the solution was boiled under reflux for one hour. After hydrolysis, 2 ml of 20% HCl were added and the released fatty acids, together with the unsaponifiable substances, were extracted with ether in a separating funnel. The ether solution was washed with water and, following evaporation, the residue was dried in a vacuum dessicator and weighed. The residue containing the ether soluble products of lipid hydrolysis was redissolved in 10 ml of petroleum ether and 4 ml of 0.5 N alcoholic alkali, and 4 ml of alcohol was then added to the solution. After addition of 10 ml water the mixture was shaken in a separating funnel and the fatty acid salts which had been formed transferred into the water phase. The extraction of fatty acid salts was repeated twice and, following addition of 2 ml of 20% HCl to the aqueous solution, the liberated fatty acids were extracted with petroleum ether. The quantities of fatty acids and of unsaponifiable substances were estimated after evaporation of the corresponding petroleum ether solution to dryness and weighing the residues.

2. WAX from the honey comb and from the excreta of the larvae was analysed in the following way. The material was extracted with hot chloroform (W. Niemierko, 1952) and a sample of chloroform solution containing about 50 mg of wax was boiled under reflux for 2 hours with 4 ml of 0.5 N alcoholic alkali; 10 ml of water, 15 ml of benzene and 2 ml of 20% HCl were added and the mixture was again boiled under reflux for 10 min. The mixture was then shaken in a separating funnel and the products of hydrolysis of wax transferred quantitatively into benzene solution.

The solution was washed with dilute alcohol (20%), the benzene evaporated and the residue, which contained the products of hydrolysis of wax, was dried to constant weight. The residue was redissolved in 5 ml of benzene and boiled under reflux for 1 hour with 5 ml of 0.5 N alcoholic alkali. After addition of 5 ml of water the boiling was continued for another half-hour; 10 ml of benzene and 20 ml of 50% alcohol were then added and the benzene fraction separated from the alcohol-water solution of fatty acids salts in

a separating funnel. The solution was acidified with 20% HCl and the liberated fatty acids extracted with benzene in a separating funnel. The amount of fatty acids and of unsaponifiable substances from the wax were finally estimated after evaporation of the corresponding benzene solutions to dryness and weighing of the residues.

3. THE LIPIDS FROM THE ISOLATED GUTS of the larvae were analysed by the method of Kumagawa-Suto using the micromodification of W. Niemierko (1947).

EXPERIMENTAL RESULTS

1. Lipid metabolism during starvation.

The first series of experiments was performed upon 50 larvae and the period of starvation was varied up to a maximum of 20 days. All the larvae were ligated in order to prevent metamorphosis (W. Niemierko and Wojtczak, 1950). The larvae were maintained at a temperature of 30°C and were taken for analyses at different periods of starvation.

Table I.

Dry substance and lipids in starving waxmoth larvae.
(Calculated as mg per 100 mg of initial fresh weight)

Day of starvation	Fresh substance	Dry substance	Lipids	Fatty acids	Unsaponifiable subst.
0	100,0	45,1	25,0	20,7	2,4
1	97,0	42,6	23,6	20,8	1,3
2	95,6	40,0	21,1	18,1	1,3
3	91,1	37,2	19,1	15,9	1,1
4	85,2	38,4	20,4	17,6	1,4
10	64,3	30,0	14,5	12,7	0,7
15	60,4	25,8	10,9	8,9	1,0
20	47,9	21,3	9,9	8,4	0,7

The analytical results are presented in Table I where the chemical body constituents are calculated as mg per 100 mg of initial fresh body weight. This method of calculation makes it easier to follow the variation in the amount of body constituents during starvation. From the figures of Table I, each of which represents the mean for five larvae, it can be seen that the total lipid content of

the larval body gradually decreases during the 20-day starvation period, from 25.0 to 9.9 mg per 100 mg of fresh initial body weight.

The behaviour of the fatty acids and unsaponifiable substances during the initial period of starvation is, however, quite different. During the first 24 hours starvation the quantity of fatty acids remains unchanged; by contrast, the quantity of unsaponifiable substances drops sharply. Prolongation of the starvation period results in a gradual diminution of fatty acids and a slight decrease in the content of unsaponifiable matter.

During the first 24 hours of starvation of a larva with a weight of 150—250 mg, the excretion amounts to about 1—2 mg. During the following days of starvation this drops to 0.5 mg per day. As compared to the excreta of feeding larvae (which have a lipid content of 50—60%), that of starving ones contains only small quantities of lipids: about 20% during the first day of starvation and only about 4% on subsequent days. Analyses of the excreta have enabled us to show that the disappearance of large quantities of unsaponifiable substances from the larval body, during the first day of starvation, is not due to their elimination in the excreta. The unsaponifiable matter found in the excreta forms not more than 15% of the total which disappears from the body.

The above experiment therefore shows that there was no change in the content of fatty acids during the first 24 hours starvation; while about one-half of the unsaponifiable matter really disappeared.

In order to verify these findings we undertook further experiments involving short starvation periods. In order to eliminate the effect of individual variations in lipid content (W. Niemierko and Cepelewicz) the experiments were performed on a large number of larvae. Three series of experiments were performed, in each of which 60 or 100 larvae were used, analyses being performed on groups of five. In each series of experiments one half of the larvae were analysed immediately after cessation of feeding, and the other half after 8, 18, or 24 hours starvation. One series, with a starvation period of only 8 hours, was performed without ligation of the larvae. The results, together with those mentioned above, are given in Table II which contains the mean values for each series. We see that, in all cases, without exception, the quantity of unsaponifiable substances which disappeared during the short starvation period was significant and amounted to 35—50% of the initial value. The ana-

lyses of excreta performed at the same time showed that, in all the experiments, the amount of unsaponifiable substances found formed only a small fraction of that which disappeared from the body. The amount of fatty acids in the starving larvae underwent, at the same time, only a small change, if at all. The last series of experiments S-v shows that the non ligated larvae losed more lipids during 8 hours starvation than the ligated ones did during 24 hours. This is in accord with the result of W. Niemierko and Wojtczak who showed that the rate of metabolism falls rapidly following ligation.

Table II.

Lipids in waxmoth larvae after short starvation period. Mean values for each series of experiments as mg per 100 mg of initial fresh weight.

Series	Duration of starvation. Hours	Amount of larvae	Lipids			Fatty acids			Unsaponifiable matter		
			Feeding larvae mg	Starved larvae mg	Difference %	Feeding larvae mg	Starved larvae mg	Difference %	Feeding larvae mg	Starved larvae mg	Difference %
SII	24	20	25,0	23,6	—5,6	20,7	20,8	+0,5	2,4	1,3	—45,5
SIII	25	60	24,9	23,2	—6,8	20,3	20,2	—0,5	2,6	1,4	—46,2
SIV	18	100	28,2	25,9	—8,1	23,7	22,8	—3,8	2,0	1,3	—35,0
SV	8	100	24,6	22,2	—9,8	20,7	19,2	—7,2	1,8	0,9	—50,0

It does not appear likely that, during the initial period of starvation, the larvae oxidize exclusively the unsaponifiable substances of the lipids and not the fatty acids. It may be supposed that, in the larval body, a transformation of unsaponifiable substances into fatty acids takes place, the extent of which obscures the oxidation of fatty acids. In order to clear up this problem we undertook several experiments the aim of which was to follow the processes occuring in the intestinal tract of the larvae.

2. Investigations on the intestinal tract of larvae.

The analyses performed on the intestinal tract of the larvae involved the determination of total lipid content, the degree of hydrolysis of lipids, the amount of fatty acids and the amount of unsaponifiable substances.

The experimental procedure was as follows: the intestinal tracts were removed from 10—20 large larvae, weighed and hydrolysed with conc. KOH solution. The results are presented in Table III

and show that the mean weight of a single, well filled, intestinal tract is about 37 mg, of which 4—7% is composed of lipid, with a mean value of 5.2%. The mean value of the relative proportion of the unsaponifiable substances in the gut lipids is 34%, and that of the fatty acids 63%. The composition of the gut lipids is thus seen to be quite different from that of the consumed wax in which the content of unsaponifiable substances is 60% and that of the fatty acids 40%. It is important to note that the contents of the intestinal tracts are in a state of emulsification and that the total amount of lipids is small.

Table III.

Lipids in the intestinal tract of feeding waxmoth larvae.

Experiment No.	Amount of guts in experiment	Mean weight of 1 gut. mg	Lipids %	Unsaponifiable substances %	Fatty acids %	Relative composition of lipids	
						Unsaponif. subst. %	Fatty acids %
1	9	44,2	5,15	2,01	3,01	39,0	58,5
2	10	40,0	5,50	2,00	3,37	36,4	61,3
3	12	46,5	5,45	1,61	3,76	29,5	69,0
4	8	37,5	6,82	2,50	4,07	36,7	59,9
5	20	32,0	5,31	1,74	3,33	32,7	62,8
6	15	27,6	4,15	1,45	2,65	34,9	63,9
7	18	30,0	4,90	1,66	3,14	33,9	64,1
8	20	36,4	4,54	1,30	3,02	28,6	66,5
Mean		36,8	5,23	1,78	3,29	34,0	63,3

A determination of the degree of hydrolysis of the lipids showed that 22—26% of the fatty acids is in the free state. The lipids in the intestinal tract are therefore hydrolysed at a very rapid rate, since the free fatty acids content of the wax is quite small.

In order to investigate the changes which occur in gut lipids during a short starvation period, analyses were made on intestinal tracts of larvae starved for 24 hours. Five such experiments were made in each of which 15—25 larvae were maintained at 30°C. The intestinal tracts were then isolated and analysed with the results shown in Table IV.

The gut of the starving larva is noticeably less filled than that of the feeding larva, and its mean weight is 26 mg. The lipid content

of the gut of the starving larva is 3.17% of the fresh weight in comparison to 5.23% for the gut of feeding larvae. The fatty acids content falls from 3.3% to 2.5% i. e. a drop of 24%; simultaneously the content of unsaponifiable substances drops, after 24 hours starvation, from 1.78% to 0.56%, a decrease of 69%.

It was possible, from these results, to calculate that the composition of the gut lipids undergoes an appreciable change. The proportion of fatty acids rises from 63.3% to 78.5%, in contrast to the decrease of the proportion of unsaponifiable substances from 34.0% to 17.6%. The analyses of the excreta showed that the amount of unsaponifiable substances eliminated from the organism during the course of the experiment forms, as in the previous experiments, only 15% of the total amount which disappears during the experiment.

Table IV

Lipids in the intestinal tract of larvae starved for 24 hours.

Experiment No.	Amount of guts in experiment	Mean weight of 1 gut. mg	Lipids %	Unsaponif. substances %	Fatty acids %	Relative composition of lipids	
						Unsaponif. subst. %	Fatty acids %
1	11	33,0	2,76	0,41	2.34	15,0	84,8
2	17	25,9	3,16	0,39	2.36	12,4	82,9
3	19	28,9	2,15	0,28	1,78	13,0	82,8
4	22	18,2	3,38	0,75	2,38	22,1	70,6
5	25	24,0	4,40	0,96	3,27	21,8	74,1
Mean		26,0	3,17	0,56	2,49	17,6	78,5

It seemed of interest to investigate the transformation of the gut lipid in vitro. Seven experiments of this kind were performed in which the guts of 15—25 larvae were kept in 2 ml of solutions such as Ringers, 1% NaCl, etc. at 30°C for 24 hours. No change in lipid content or composition could be found, but the degree of hydrolysis was somewhat greater. The guts maintained in Ringers solution for 24 hours contained 45% of fatty acids in the free state in comparison with 25% found in freshly isolated guts.

3. Feeding of larvae on paraffin.

The present study, as well as a previous one (Niemierko W. and Włodawer, 1950) showed that the waxmoth larva utilizes the unsaponifiable substances present in wax. The question then arose as to

whether the larvae are able to utilize only the alcohol fraction of the wax, as some authors assume, or whether they are also able to utilize hydrocarbons. To clear up this point we attempted to feed the larvae exclusively on hydrocarbons, namely on pure paraffin. However the larvae were unable to grow under these conditions and, within a short while, died. Therefore in subsequent experiments the paraffin was mixed with honey comb.

Portions of honey comb were immersed in melted paraffin, removed and weighed to determine the amount of paraffin, which was usually about equal to the weight of honeycomb. Ten larvae, weighing about 20 mg each, were fed on this mixture at 30°C. The larvae grew, consuming both the paraffin and honeycomb. After a period of several days the larvae and the excreta were removed and analyses were performed on the larval bodies, the excreta and the remainder of the food medium. The determination of the composition of the medium was made in a special control analysis. On the basis of the initial and final compositions of the medium, and the larval bodies and the excreta, a balance was established of the utilization by the growing larvae of the fatty acids and the unsaponifiable substances. The results (Table V) show that utilization of hydrocarbons must be very important. From experiment B_1, it is seen that the larvae utilized 0.62 g of unsaponifiable substances and only 0.03 g of fatty acids. The corresponding figures in experiment B_2 are 0.74 and 0.08. Taking account of the fact that the amounts of unsaponifiable substances utilized by the larvae (0.62 g and 0.74 g) are larger than the total content of unsaponifiable substances of the wax (0.57 and 0.53 g) it must be assumed that the larvae utilized not only the hydrocarbons present in the wax, but also a certain amount of the paraffin.

DISCUSSION

On the basis of numerous studies there is no doubt that the waxmoth larva utilizes the consumed wax. However, published results (Sieber & Metalnikov, 1904; Metalnikov, 1908; Dickman, 1935; Manunta, 1935) do not provide an answer as to what transformations the wax constituents undergo in the larval body. From our previous investigation (W. Niemierko and Włodawer, 1950 a) it appeared that the disappearance of the unsaponifiable substances from the wax consumed by the growing larvae was nearly twice as great as the disappearance of fatty acids. The role played by the unsaponifiable substances in the metabolic processes is, therefore, undoubt-

Table V
Utilization of wax and paraffin by waxmoth larvae.

Experiment No.	Material under investigation	Amount of lipid and paraffin			Amount of unsaponifiable substances (including paraffin)			Amount of fatty acids		
		Initial g	Final g	Difference g	Initial g	Final g	Difference g	Initial g	Final g	Difference g
B_1	Paraffin (2,34 g)	2,34			2,34			0		
	Honey comb (2,40 g)	0,95	3,29	1,84	0,57	2,91	1.58	0,38	0,38	0,26
	10 larvae (0,12 g)	0,02		0,07	0,001		0,01	0,02		0,06
	Excreta	—		0,75	—		0,70	—		0,05
		Sum: 3,31		2,66	2,91		2,29	0,40		0,37
B_2	Paraffin (2,16 g)	2,16			2,16			0		
	Honey comb (2,19 g)	0,88	3,04	0,20	0,53	2,69	0,17	0,35	0,35	0,03
	10 larvae (0,24 g)	0,05		0,17	0,002		0,03	0,05		0,14
	Excreta	—		1,90	—		1,75	—		0,15
		Sum: 3,09		2,27	2,69		1,95	0,40		0,32

Difference g
−1,45
+0,05
+0,75
−0,65
−2,84
+0,12
+1,90
−0,82
−1,33
+0,01
+0,70
−0,62
−2,52
+0,03
+1,75
−0,74
−0,12
+0,04
+0,05
−0,03
−0,32
+0,09
+0,15
−0,08

edly of considerable importance. It was assumed that the waxmoth larvae are able to form fatty acids from the unsaponifiable fraction of the wax. As far as we are aware, processes of this kind have not heretofore been noted in animal organisms.

From our present experiments on short period starvation of the waxmoth larvae it may be seen that, during the initial period of starvation, a rapid diminution of the unsaponifiable substances occurs while the fatty acid content remains substantially unchanged. It should be emphasized that these results were obtained, without exception, in all the experiments which were performed on a total of more than 300 larvae.

As the excreta of the starving larvae contained only 15% of the amount of unsaponifiable matter which disappeared during the course of the experiment, it must be assumed that, during the first few hours of starvation, processes take place leading to some transformations of the unsaponifiable substances.

To explain these unusual results it is suggested that the rapid diminution of the amount of unsaponifiable substances, and the constant level of the quantity of fatty acids in the larval body during the initial period of starvation, is due to the transformation of unsaponifiable matter into fatty acids. The situation is such that the fatty acids are constantly metabolized, but are continously replaced by fatty acids formed from unsaponifiable matter.

Our experiments performed on intestinal tracts of the larvae appear to support these assumptions. It is known that the waxmoth larva consumes very large quantities of honeycomb which is poor in water content and includes constituents which are chemically very stable. It could therefore be supposed that the gut of the feeding larva must be filled with wax which only slowly undergoes digestion, whereas in fact the contents appear to be in the nature of an emulsion. The dry substance of the entire intestinal tract, together with its contents, amounts to 15% of the total weight while the total content of lipid is only 5.2%. Not more than 1.5—2.5 mg of wax can be found in the well-filled gut of a feeding larva, whereas we know from our observations that the larva is able to consume during a 24-hour period 70—80 mg of honeycomb. It seems, therefore, that the larva ingests the wax in small portions, grinds it by means of the „chytin teeth" of the foregut and acts upon it with a large quantity of digestive juices which emulsify it and produce the different chemical transformations.

The analysis of the lipids found in the intestinal tract shows that their composition is appreciably different from that of the wax. The content of the unsaponifiable substances in the wax is much higher than the content of fatty acids, whereas in the gut lipids this relation is reversed (Table III). Our results, in all of which an increase in the relative content of the fatty acids was observed along with a diminution of the relative content of the unsaponifiable substances in the gut lipids, suggests that a transformation of the alcohol and hydrocarbon fractions into fatty acids occurs precisely in the intestinal tract.

Our analysis further showed that the degree of hydrolysis of the wax is considerable, as the amount of free fatty acids, in the gut of a feeding larva, may be as high as 25%.

The fate of the consumed wax is therefore clear, to a certain extent. The consumed wax undergoes emulsification in the gut, is then hydrolyzed and a considerable portion of the unsaponifiable substances transformed into fatty acids. The quantity of food which moves through the gut is very large and only about one-half of the wax consumed is excreted. The fact that the actual amount of the lipids found in the gut is small indicates that the digestion of wax and resorption of the products of transformation occurs very rapidly.

The investigations performed on the intestines of the larvae starved during 24 hours only, confirm this view. It appeared that, after 24 hours, only 30% of the initial content of unsaponifiable matter, and 80% of the fatty acids, may be found. It is probable that, during this period of starvation, the fatty acids passed through the intestinal wall but were partially replaced by fresh amounts formed from the unsaponifiable matter.

Many authors such as Metalnikov (1908), Dickman (1933), Duspiva (1935), Florkin (1949) are in agreement that the unsaponifiable substances play some not clearly defined role in the metabolism of wax by *Galleria mellonella*. Our observations make it possible to assume that the wax metabolism occurs through the transformation of the alcohol, and perhaps also the hydrocarbon, fraction into fatty acids.

Many authors are of the opinion that the waxmoth larva does not utilize the hydrocarbons of the wax. Duspiva (1935) supposes that they utilize only the fatty acids and the alcohols and that they eliminate the unchanged hydrocarbons in the excreta. Florkin (1949)

thinks that the larvae, with the aid of bacteria, utilize only the fatty acids and their esters with higher alcohols.

On the basis of our present investigation, it would appear that no doubt exists as to the possibility of the utilization of the hydrocarbons by the larvae. This is confirmed by our experiments involving the feeding of paraffin. In both experiments performed the utilization of the unsaponifiable substances was larger than the total amount of alcohols and hydrocarbons in the wax consumed by the larvae. We can, therefore, suppose that the larvae utilized not only the hydrocarbons present in the beeswax, but also a part of the added paraffin. We have reason to believe that, under normal circumstances as well, the larvae utilize the honeycomb hydrocarbons.

Many authors (Metalnikov, 1908; Duspiva, 1935) attempted to isolate from the waxmoth larvae enzymes which are capable of hydrolyzing wax. These attempts gave no positive results. Only recently Mankiewicz (1949) was able to isolate from the larval body an enzyme which split not only beeswax but also the wax of *Mycobacterium tuberculosis.*

Some authors (Dickman, 1935; Rybicki, 1949; Florkin 1949) are of the opinion that the digestion and utilization of wax by the waxmoth larva is connected with the activity of specific bacteria. They were able to isolate several kinds of bacteria from the intestines, which could be cultured on a wax medium and which could hydrolyze the wax (Dickman 1933) and even oxidize the fatty acids and esters present in the wax (Florkin 1949). The decomposition of wax by bacteria, in the experiments of Dickman and Florkin, proceeded at a very slow rate. These processes are quite different from the rapid changes which we observe in the intestinal tract of the larva. On the other hand the chemical transformations occuring in the isolated tract of the larva, as we have shown, are insignificant. We may suppose that, if the metabolism of wax by *Galleria mellonella* were due exclusively, or even mainly, to bacterial activity, the changes in the intestinal tract isolated from the body would be more pronounced than was actually found. We do not exclude the possibility of an indirect participation of floral bacteria, but we are of the opinion that wax metabolism is due chiefly to enzymes present in the waxmoth gut. On the basis of our results we can assume that the waxmoth larvae are able to utilize all the principal constituents of wax, acting upon them with highly specific enzymes and transforming unsaponifiable substances into fatty acids.

SUMMARY

Experiments on the waxmoth larvae *(Galleria mellonella)* show that, after a short period of starvation, a considerable portion of unsaponifiable substances disappears from the body. At the same time the amount of fatty acids remains nearly constant.

The wax from the honey comb, on which the larvae feed, consists usually of about 60% of unsaponifiable matter and of 40% of fatty acids. On the contrary the lipids found in the gut of feeding larvae contain 30—40% of unsaponifiable substances and 60—70% of fatty acids, 25% of the latter being in the free state. After a day of starvation the proportion of the fatty acids in the gut lipids increases to 78% and the proportion of the unsaponifiable matter decreases to 17%.

It seems that the larvae are able to utilize not only fatty acids but also alcohols and hydrocarbons of the wax. They can utilize, to a certain degree, even paraffin. During the metabolic processes unsaponifiable substances are probably transformed into fatty acids.

A possible participation of microorganisms in wax utilization can not be excluded. It seems however that the chief role in these processes is played by the enzyme systems of the larvae.

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STUDIES ON THE BIOCHEMISTRY OF THE WAXMOTH
(Galleria mellonella)

8. NITROGEN METABOLISM OF THE LARVAE

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The present work is concerned with the nitrogen metabolism of waxmoth larvae *(Galleria mellonella)* chiefly during starvation. In insects, as in other animals, generally the first substance to be metabolised during starvation is glycogen, followed by the lipids, while the extent of protein metabolism shows, depending on the species, a wide range of fluctuations (Wigglesworth, 1950).

In the body of the waxmoth larva lipids constitute 25% of the fresh substance, that is more than one half of the dry matter. The investigations of W. Niemierko and Włodawer (1952) have shown that, in the first days of starvation, a distinct loss of lipids may be observed, and its content after three weeks is down to 40% of the initial quantity.

Glycogen appears in the body of the larva in small quantities, about 0.2% of fresh substance (W. Niemierko and Cepelewicz, 1950), and therefore can play no important role in metabolism during starvation.

The aim of our work was to become acquainted with the influence of long starvation on the nitrogen metabolism. In the body of the larvae the following components were determined: total nitrogen,

protein nitrogen, nonprotein nitrogen, uric acid, amino acids and ammonia, and, in some of the experiments, lipid nitrogen as well. The investigations on the starvation metabolism were supplemented by analyses of the excreta and of the beecomb, which constitutes the sole food supply of the larvae.

MATERIAL AND METHODS

Waxmoth larvae were taken from a breed kept in a thermostat at a temperature of 30°C (W. Niemierko and Cepelewicz, 1950). For some particular series of experiments about 100 grown specimens weighing 163 ± 18 mg each were taken. Larvae *Galleria mellonella* in the last period of growth when deprived of food, undergo a metamorphosis independently of the size they attain. In order to prevent this a ligature was laid just behind the head (W. Niemierko and Wojtczak, 1950). In general the starving larvae were kept in separate vessels and in some of the series — in groups. In view of the great diversity between individuals it was advisable to select them at random for the analyses.

Total nitrogen was determined by the Kjeldahl method in single larvae which were previously dried in the vacuum over sulphuric acid. The preliminary analyses showed that the quantities of nonprotein nitrogen, when determined on material dried in a dessicator are in general higher than after fresh material has been extracted. It may be supposed this is due to some decomposition processes during drying, so the investigation of nitrogen fractions were performed exclusively on fresh material. For each analysis 5—10 specimens were taken. The separation of nitrogen into nonprotein nitrogen, and insoluble nitrogen (protein nitrogen, chitin nitrogen, and lipid nitrogen) was done according to Haden (1923). Nitrogen of each of these fractions was determined by means of micro-Kjeldahl method. Ammonia nitrogen was determined directly by distillation of alkalinised filtrate. In all cases ammonia was distilled in a Parnas-Wagner or Markham (1942) apparatus into a solution of boric acid according to Conway (1947). Uric acid was determined by a modified method of Fearon (1944) or by Brown's method (1945). When the Brown method was applied the measurements were performed with a Hilger Spekker absorptiometer, using a red filter OR 2 instead of a green one, recommended by the author. Amino acid nitrogen was determined according to Frame, Russel and Wilhelmi (1943).

The application of this method to biological material containing certain quantities of uric acid and ammonia was verified (Zielińska, 1952). Urea nitrogen was determined after treating the filtrate with urease by the microdiffusion method according to Conway (1947).

The collection of excreta was started from the moment when the larvae under investigation were weighed. The excreta were collected either during the whole period of starvation, or taken from successive sections of the starvation period. In the majority of experiments nitrogen compounds of the excreta were fractioned into soluble (in a lithium carbonate solution) and into insoluble. In elaborating the method of extraction of nitrogen substances from the excreta, extractions by water, by tungstic acid and by a 0,5% solution of lithium carbonate were tried. The most appropriate proved to be a hot solution of lithium carbonate. Beside the total soluble nitrogen, the uric acid nitrogen, ammonia nitrogen and, in some experiments, urea nitrogen also were determined in the excreta.

To determine the fractions of nitrogen compounds of the beecomb the same methods were applied as in the investigation of nitrogen fraction of the larvae. The beecomb was freed of lipids with chloroform and was extracted by means of tungstic acid. Total nitrogen, lipid nitrogen, nitrogen of soluble in tungstic acid compounds, and nitrogen in lipid-free insoluble residue were determined. In the soluble fraction ammonia nitrogen and uric acid nitrogen were also measured.

TOTAL NITROGEN

In order to study the extent of nitrogen metabolism in waxmoth larva the determination of total nitrogen in feeding and in starving larvae was undertaken. Analyses were perfomed on single specimens and the figures present average values taken of a several larvae.

The average content of nitrogen in feeding larvae amounts to 2.4% of the fresh weight. The digestive tract of normally-fed larvae is filled to a large extent with food pulp. The value 2.4% means therefore the average content of nitrogen in the body of the larva and in the food pulp. During the first twenty-four hours after ligation a great part of the intestinal content is either absorbed or excreted. The weight of the larvae twenty-four hours after ligation is in this work considered as initial and the data of nitrogen determination are based on this weight (Białaszewicz, 1936). The total nitrogen amounts to 2.7% of fresh substance.

During starvation period (Table I) which lasted four weeks, a reduction of the body-weight of up to 65% of the initial state could be observed. At the same time, the larvae lose 76% of their water and 47% of their dry substance in consequence of which the actual content of dry substance is raised to 65%, an indication of rather intense drying. The losses of total nitrogen reach 42%, and are therefore somewhat lower than the losses of the total dry substance; this can be explained by the large consumption of lipids

Table I.

Some chemical constituents of waxmoth *(Galleria mellonella)* larvae during starvation.

Starvation period days	Body weight mg	Water %	Dry substance %	Total nitrogen %	Lipids %
1	163 ± 18	55 ± 2	45 ± 2	2.7 ± 0,3	25
Losses during starvation in percentage of initial value.					
5	16 ± 9	18 ± 10	12 ± 6	11 ± 4	17
10	20 ± 6	20 ± 7	21 ± 8	20 ± 6	41
17	35 ± 8	34 ± 11	25 ± 10	28 ± 6	56
21	52 ± 1	56 ± 12	45 ± 8	36 ± 11	60
27	65 ± 6	76 ± 9	47 ± 5	42 ± 2	

which, after three weeks time, reaches 60% (Niemierko and Włodawer, 1952). The rapid disappearence of lipids during the first days of starvation (Niemierko and Włodawer, 1952) and the intensive drying up of larvae are the cause of the increase in the relative content of total nitrogen up to 4.4% in spite of losses of nitrogen which exceed 40% of the initial content.

THE NONPROTEIN NITROGEN

The average content of nonprotein nitrogen in the body of larvae which were examined after one day of starvation amounts to 333 ± 21 mg %. About 50% of this value is amino acids nitrogen, a few percent uric acid nitrogen (this quantity is liable to large variations) and 10—20% ammonia nitrogen. Other nitrogen compounds were not identified and nitrogen which they contain amounts to about 25% of the total nonprotein nitrogen.

During starvation period which lasts from three to four weeks the content of uric acid in the body of the larvae increases considerably and in some cases reaches a tenfold quantity of the initial content. The quantity of the other nitrogen compounds is gradually decreasing and at this stage falls down to one half of the initial value. It concerns the amino acids and ammonia as well as other compounds which are not clearly identified.

(The amount of amino acid nitrogen determined by the method used in this work is always somewhat greater than the true value; as the results include the ammonia nitrogen and part of uric acid nitrogen. It can be supposed that the decrease of the amino acids content in the larval body during starvation is in reality even pronounced).

Table II gives average values from a few series of analyses and illustrates the content of the particular fractions of nonprotein-nitrogen and their changes in the starvation period.

Table II

Nonprotein nitrogen in the waxmoth larvae *(Galleria mellonella)* during starvation in mg% of initial body-weight.

Period of starvation days	Loss of body-weight %	Nonprotein nitrogen	Amino acid nitrogen	Ammonia nitrogen	Uric acid nitrogen
0 — 1	—	333 ± 21	178 ± 2	44 ± 15	20 ± 10
5 — 10	19 ± 6	272 ± 47	150 ± 10	39 ± 5	18 ± 8
11 — 15	39 ± 5	248 ± 48		31 ± 8	72 ± 34
19 — 29	55 ± 8	230 ± 46	95 ± 4	25 ± 3	126 ± 47

As stated above the larvae, during a long period of starvation, lose large quantities of water and the loss reaches up to 76% of the initial content. At the same time the excretion capacity diminishes. When such larvae are dissected a strong filling of the Malpighi tubules and of the final sections of the digestive tract with the products of metabolism may be observed. In normally-fed larvae the content of narrow tubules is almost colourless. With the progress of starvation they are swelled by a yellow semi-liquid matter, which after it has passed to the intestine presents a material which is, to a large degree, dried out. The water resorption, which undoubtedly occurs here, is one of the numerous manifestations of the struggle of the insect's organism for water (Wigglesworth, 1950).

We supposed too that, in connection with the general drying up of the larvae, this explains, at least partly, an occasional almost complete obstruction in the elimination of excreta.

With the loss of weight of the body amounting to about 44—60% the quantity of nitrogen in the Malpighi tubules and in the hind sections of the digestive tract, after a period of one month of starvation, may, in particular cases raise to 9—12% of the total nitrogen contained in the larva. As the weight of walls of the tubules and the digestive tract is small in relation to the weight of their content, one may, with but a small error, consider the total nitrogen found there to be a product of starvation metabolism and in the first place as uric acid nitrogen, especially since the quantities of uric acid nitrogen contained in the body of the larvae, starved for three to four weeks, found directly in experiments, are of the same order of value. They amount on the average to 7% of the total nitrogen which is actually contained in the organism.

NITROGEN INSOLUBLE IN DEPROTEINIZING REAGENTS

Analyses performed on the larval body have shown that protein nitrogen amounts to over 90% of the total insoluble nitrogen, lipid nitrogen to 1—2%, chitin nitrogen to 5% of insoluble nitrogen. The part played by chitin and lipid nitrogen in the metabolism as a whole does not therefore influence the picture of the protein metabolism. In our experiments therefore the total nitrogen insoluble in tungstic acid was determined and the changes in its content, which characterise the protein metabolism, were analysed as a whole. In the larvae examined one day after ligation the insoluble nitrogen amounted on the average to 2.1% (Table III), with the progress of starvation the quantities of insoluble nitrogen found in starving larvae diminished. With the loss in body weight amounting to 60% during a 3—4 weeks experiment, a loss of insoluble nitrogen amounting to almost 50% was observed. It indicates the consumption of about one half of the protein contained in the larvae during the period of starvation. It is easily understood that as the result of a large drop in the content of the lipids and of an extensive drying out of larvae, is an increase in the actual content of the protein despite their large share in the metabolism. This is proved by an increase in the value of insoluble nitrogen as expressed in percentages of the actual fresh substance.

Table III

Insoluble nitrogen in the waxmoth larvae *(Galleria mellonella)* during starvation.

Number of experiments	Period of starvation days	Loss of body-weight %	Insoluble nitrogen		
			in % of actual body-weight	in % of initial body-weight	Loss in % of initial value
6	0 — 1	—	2,1 ± 0,1		—
4	5 — 7	16 ± 2	2,1 ± 0,0	1,7 ± 0,2	16 ± 5
4	8 — 10	26 ± 3	1,9 ± 0,1	1,5 ± 0,0	28 ± 2
5	11 — 15	40 ± 3	2,4 ± 0,3	1,4 ± 0,1	31 ± 7
7	19 — 29	60 ± 4	2,7 ± 0,2	1.1 ± 0,1	48 ± 1

NITROGEN EXCRETED AND NITROGEN METABOLISED

The excreta of insects present, as we known, a mixture of metabolites and of unused remains of food, together with the products of their decomposition. Feeding larvae *Galleria mellonella* retain about 40% of the consumed food. The content of nitrogen in the excreta of feeding larvae amounts on the average to 2.9 ± 0.3% In the Table IV the results of one of series of experiments which illu-

Table IV

Nitrogen of the excreta of the waxmoth larvae *(Galleria mellonella)* during starvation. (8-th Experiment).

Period of starvation days from — to	Total-N %	Soluble-N %	Uric acid-N %	Soluble-N in % of total-N	Uric acid-N in % of soluble-N
0	2,7	2,4	0,8	82,8	33,3
0. — 1.	3,2	2,6	0,7	82,5	26,9
2. — 5.	11,5	9,5	7,1	82,6	74,7
6. — 18.	17,4	16,7		96,3	
19. — 27.	17,4	16,8	12,4	96,8	73,8
starved for a long-term	18,3	17,8	15,0	97,3	84,3

strate the content of the nitrogen fractions in the excreta of feeding and of starving larvae are presented. With the progress of starvation the percentage content of nitrogen in the excreta increases rapidly and after 3—4 weeks reaches about 20%. The nitrogen of soluble compounds which in the excreta of feeding larvae reaches above 80% of total nitrogen, after a few days of starvation amounts to 96—

97%. In time of starvation distinct displacements of the fractions of soluble nitrogen appear. Uric acid nitrogen shows an increase after a few days of from 30% even to 75—80% of the total soluble nitrogen, reaching 12—15% of the weight of excreta. Beside of uric acid, which is, as we see, the chief product of nitrogen metabolism of waxmoth larvae the presence of ammonia has been proved in the excreta. The quantities of ammonia nitrogen found in particular experiments fluctuate from 0.4 to 1.2% of the weight of excreta, independently of the degree of starvation of larvae. Uric acid nitrogen and ammonia nitrogen comprise about 80% of soluble nitrogen. The remainder is nitrogen in compounds which were not identified by us. In a special series of analyses it has been proved that the urea nitrogen constitutes only about 1% of the excreted nitrogen. The production of urea by larvae *Galleria mellonella* plays therefore no role of importance in the nitrogen metabolism.

The quantities of excreta produced by larvae depend on their size and on the state of nutrition. In the last period of growth larvae weighing on the average 120 mg consume about 60 mg of beecomb in 24 hours, 60% of it is excreted. In the first 24 hours after ligation the quantity of excreta amounts only to 2.5—3 mg, in the subsequent days it falls even further, reaching 0.5 mg for every 24 hours.

It has been told above that in the subsequent periods of starvation of *Galleria mellonella* a high fall in the excreting capacity could be observed. This is connected with accumulation of large quantities of products of metabolism in the Malpighi tubules and in the final sections of the digestive tract. With this in mind one cannot judge the rate of nitrogen metabolism during long-term starvation of larvae by the quantities of nitrogen in the excreta. Beside nitrogen which is removed one must take in consideration also the nitrogen of metabolites which are accumulated in the larvae.

As uric acid constitutes a prevailing product of nitrogen metabolism by the term metabolised nitrogen we shall mean the sum of excreted nitrogen and uric acid nitrogen found in the larval body. In part of the experiments the metabolised nitrogen was calculated as the sum of excreted nitrogen and total nitrogen found in the Malpighi tubules and in the hind sections of the digestive tract. The results obtained by this method of calculation gave approximately the same value for the extent of nitrogen metabolism as the previous method.

The amount of nitrogen metabolised during the experiment expressed as percent of initial nitrogen content in the larvae was calculated for different periods of starvation in relation to the losses of the weight of the body. The results are shown in Fig. 1 and point to a rather considerable regularity in the rate of nitrogen metabolism during starvation.

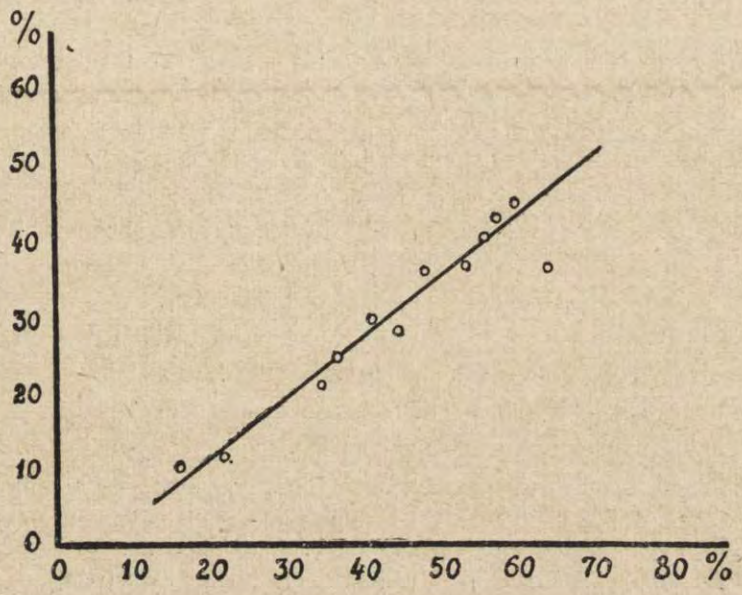

Fig. 1. Amount of nitrogen metabolised during starvation as a function of the loss of body-weight. Abscissae — Loss of body weight. Ordinates — Metabolised nitrogen.

THE BEECOMB

The composition of the beecomb, which is the sole food source of larvae *Galleria mellonella*, varies and is dependent upon many factors. In our breeds a natural beecomb was applied, avoiding combs with an artificial comb foundation because such comb, made of ceresine, differ in composition from beecomb produced by the honey bees. For our experiments we chose beecomb which served during a few years for bees for the storing of honey or pollen and for breeding worker bees larvae. The queenbees cells and drone comb were not used.

A beecomb contains only about 2% water. It may be supposed therefore that waxmoth larvae satisfy most of their demand for water from oxidising processes.

The chief fraction of beecomb is wax. The wax content varies considerably, mean value is about 50%.

In our present work we were interested in nitrogen components of the beecomb. In the first place the total nitrogen, the lipid nitrogen and the nitrogen contained in a lipid-free residue were determined. Next the compounds soluble in tungstic acid were extracted from the lipid-free residue, and the total soluble nitrogen, ammonia nitrogen and uric nitrogen were determined in the extract. The insoluble residue was also examined for their nitrogen content. The content of total nitrogen reaches about 2—3%. In the samples taken from different pieces of beecomb fluctuation observed are rather high.

In the lipids of beecomb only about 0.15% of nitrogen was found. It constitutes only about a few percent of the total nitrogen in the beecomb, while nitrogen which remained in the lipid-free residue reaches over 90%.

Further analyses have proved soluble nitrogen to constitute 20—25% of the total nitrogen of the beecomb; the ammonia nitrogen about 10% of the soluble nitrogen, the uric acid nitrogen appears in negligible quantities only (0.4% of the soluble nitrogen). The remaining 90% of soluble nitrogen is represented by nitrogen of unidentified substances.

The chief nitrogen fraction of the beecomb, reaching up to 80% of the total nitrogen, constitutes nitrogen of non-lipid insoluble compounds.

A hot KOH hydrolysis lasting a few hours, of beecomb freed of lipids, and a later determination of nitrogen in the hydrolyzate and in unhydrolysed residue was made with a view to obtaining some idea as to whether the beecomb contains much of remains of bees' chitin. The answer was negative, the results indicating that nitrogen of the unhydrolysed residue does not exceed 5%, while nitrogen in the hydrolyzate reaches over 70%. The sum of these fractions reaches about 80%, so that the losses connected with the above procedure are of the order of 20% of the total nitrogen.

We have up to-day no answer to the question as to what are the components of this chief fraction of the beecomb, which embraces

nitrogen compounds, lipid-free, insoluble, but yielding to an alkaline hydrolysis. This fraction, as it seems, is the most important source of nitrogen to waxmoth larvae since in the excreta of feeding larvae the content of insoluble nitrogen does not reach 20% of the total nitrogen of the excreta.

DISCUSSION OF RESULTS

The extent of nitrogen metabolism of insects during starvation is widely spread. In some species the part played by nitrogen compounds in the starvation metabolism is very considerable and may reach 50%. So for example *Deilephila* (Heller, 1926), *Melolontha* and *Geotrupes* (Slovtzoff, 1909) consume, during a long-term starvation, from 20—40% of proteins contained in their body. In other insects however, such as *Chorthophaga viridifasciata* (Ludwig, 1950), the losses of nitrogen by inanition do not exceed 11%. Finaly such cases are known as that of *Popilio Japonica* larvae (Ludwig, 1949) which after a few days of starvation do not show a decrease of proteins content. The consumption of proteins in this insect is slight also during metamorphosis (Anderson, 1948).

Refering to our investigation on nitrogen metabolism of starved larvae *Galleria mellonella* we must lay emphasis on its extent. During the 27 days of starvation with losses of body weight amounting to 65% and losses in dry substance equal to 47%, the fall in total nitrogen content reaches 42% of the initial quantity, and a simultanous decrease in the content of protein nitrogen even 50%. The consumption of lipids in starvation metabolism is also very high and may reach 60% of the initial quantity. Glycogen, because of its low content, can play no role of importance in the metabolism as a whole.

The starvation metabolism of *Galleria mellonella* larvae is therefore characterized by its large consumption of lipids and proteins. In his research on the products of nitrogen metabolism of oak silkworm larvae, *Antheraea pernyi*, Leifert (1935) found 327 mg% of nonprotein-nitrogen in grown larvae of this butterfly, that is the same value which we established for waxmoth larvae.

The quantities of uric acid which were discovered by Leifert in the oak silkworm after its last moult exceed many times the quantities of this compound in waxmoth larvae. So in *Antheraea pernyi* the amount of uric acid nitrogen is 140 mg%, while in *Galleria mello-*

nella it amounts on the average to 20 mg%. For the mulberry silkworm, *Bombyx mori*, 30—60 mg% (our own data, not yet published).

The ammonia nitrogen of *Galleria mellonella* reaches 50 mg% of fresh substances. In some series of our experiments, however, lower values were also found amounting to about 20 mg%. For *Antheraea pernyi* ammonia nitrogen amounted to about 25 mg% (Leifert, 1935).

In the body of waxmoth larvae we found 175 mg% of amino acid nitrogen. As was shown above the presence of uric acid and of ammonia causes of some increase in obtained quantities of amino acid nitrogen over the true values. It may be supposed that the true quantities of amino acid nitrogen are lower than those found by us and probably do not exceed the value of 120—150 mg% of fresh substance.

Urea nitrogen appears only in very small quantities in the waxmoth larvae (about 3 mg%, while in oak silkworm larvae it may attain up to 20 mg% (Leifert, 1935).

The fraction of nitrogen which remains after the quantities of measured uric acid nitrogen, ammonia nitrogen, and urea nitrogen were substracted from the values of nonprotein nitrogen, is regarded by Leifert as amino acid nitrogen. These conclusions of Leifert do not seem to be quite certain. In the protein-free filtrate from waxmoth larvae do occur some nitrogen compounds which have not been identified in our investigation and which form about 20% of the amount of nonprotein nitrogen compounds.

The comparison of *Galleria mellonella* and *Antheraea pernyi* shows, however, that in both insects the total amount of nonprotein nitrogen is nearly the same and that the chief constituent of this fraction is amino acid nitrogen.

The very high content of uric acid in the body of *Antheraea* larvae is probably due to the synthesis of this compound in the fat body, which will be discussed later in detail. The amino acid nitrogen consitutes the chief fraction of nonprotein nitrogen in the haemolymph of insects. In the bee worker the average content of amino acid nitrogen in haemolymph amounts to 290 mg%, that is to 85% of nonprotein nitrogen (Bishop, 1925), in *Gastrophilus intestinalis* larvae — 94 mg%, that is 71% of nonprotein nitrogen (Levenbook, 1950). In *Antheraea* (Leifert, 1935) nitrogen corresponding to amino acids reaches in haemolymph 91 mg%, that is 83% of

nonprotein nitrogen. A direct determination of amino acids in haemolymph of the oak silkworm was reported recently by Demianowskij and Filipovič (1950). They found, in the larvae examined, a few days before the spinning of cocoon was started, about 125 mg% of amino acid nitrogen and this value is later liable to decrease. According to our analyses (unpublished data) the content of amino-acid nitrogen in haemolymph of silkworm larvae amounts before the spinning has begun to about 250 mg%. The same amount is found in heamolymph of full-grown *Galleria mellonella* larvae, which constitutes about 50—60% of the non-protein nitrogen. Uric acid is found in the haemolymph of insects in larger quantities than in the blood of other animals. Emphasis should be laid however upon the fact that in the haemolymph of *Gastrophilus intestinalis* larvae, which lives in the ventricule mucose of the horse, the content of uric acid is only 2.2 mg% with quite large quantities of urea — 20 mg%, just as in plasma of mammalian blood. This fact illustrates the influence of the environment on the character of the metabolism.

The chief product of nitrogen metabolism of insects is in most cases uric acid. For example in the full grown silkworm larvae, *Bombyx mori*, uric acid nitrogen constitutes one half of the excreted nitrogen (Kawase, 1917), in the meconium of *Deilephila euphorbiae*— 38—58% (Heller and Aremówna, 1934), in the excreta of *Antheraea pernyi* larvae as much as 77% (Leifert, 1935). According to our data the chief nitrogen component in the excreta of feeding *Galleria mellonella* larvae is also uric acid. During starvation of the wax-moth larvae most of the metabolised nitrogen is excreted in the form of uric acid.

The sole food source of *Galleria mellonella* larvae, beecomb, contains only about 2% of water. The main supply of the organism for water therefore must be satisfied by water formed in oxidative processes. The losses of water by evaporation through the cuticle are very large as it was found that the larvae dry up considerably during starvation (S. Niemierko, 1950). The features of all insects render them very prone to lose water (Wigglesworth, 1934); *Galleria mellonella* larvae must struggle for water more than many other insects. It is therefore a need for the existence in the organism of the larvae of some biochemical and physiological adaptation which act against loss of water. The production of uric acid in the processes of nitrogen metabolism and the of almost dry excreta are probably two of such numerous adaptations.

Recent investigations show (Sonne, Buchanan and Delluva, 1948) that the synthesis of uric acid in animal body is an extremely complicated process. The steps in the formation of the purine ring must be the result of precisely coordinated action of many enzyme systems. It seems, therefore, to be quite remarcable that the formation of uric acid in the starving waxmoth larva, even just before the moment of death of the exhausted insects, is normal and uric acid is always the chief product of nitrogen metabolism.

In our study of the composition of the beecomb we established that the chief nitrogen fraction contains lipid-free, insoluble compounds, which are susceptible to an alkaline hydrolysis. It may be supposed that this fraction is the chief source of nitrogen for *Galleria mellonella* larvae. Microscopic examinations have confirmed the existence of large quantities of pollen in the beecomb. It is probable moreover that other insoluble nitrogen substances (which hydrolyse under action of alkali) are also present in the becomb and that they are produced by bees or their larvae. These substances, beside pollen, may constitute a source of nitrogen for waxmoth larvae.

The study on the respiratory metabolism of waxmoth larvae (Niemierko and Wojtczak, 1950) has proved that the curve of oxygen consumption during starvation is U — shaped as a result of the primary fall and later rise in the oxidative processes in the organism. Our study which established a rather regular course of nitrogen metabolism indicates that the changes in the intensity of respiratory metabolism during starvation cannot be due to the oxidative processes of the proteins.

SUMMARY

At the beginning of the starvation period the *Galleria mellonella* larvae contain about 2.5% of protein nitrogen, 330 mg% of nonprotein nitrogen of which one half is amino acid, a further 20% ammonia and uric acid and a remainder which has not been identified.

The participation of nitrogen substances in the starvation metabolism is quite considerable as within three to four weeks about one half of the protein contained in the organism is metabolised, and the intensity of metabolism is quite regular. The chief product of nitrogen metabolism of waxmoth larvae is uric acid, the quantity of which in the excreta may reach 45%.

During starvation the losses of water exceed the total loss of body-weight and this leads to a drying up of the larvae, to a decrease

in the excretion capacity and to an accumulation of large quantities of uric acid in the organism.

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STUDIES ON THE BIOCHEMISTRY OF THE WAXMOTH

(Galleria mellonella)

9. VARIATIONS IN INSOLUBLE PHOSPHORUS COMPOUNDS DURING THE GROWTH OF THE LARVAE

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Biochemical processes occuring during the growth of larvae *Galleria mellonella* were investigated by Teissier (1931) and by W. Niemierko and Cepelewicz (1950). These authors show that, from a biochemical point of view the period of growth of larvae can be divided into three subperiods, which differ in the character of the chemical processes taking place: the first period corresponds to larvae weighing up to 50 mg; the second, to larvae from 50 to 100 mg, and the third, to larvae weighing over 100 mg and up to the period of spinning. In the first and third periods, i. e. for larvae up to 50 mg and those above 100 mg, the increase in body weight is connected with a gradual correlative increase of its various constituents. In the second period, however, rapid changes occur in the accumulation of lipids and therefore in the weight of dry substance as well. According to W. Niemierko and Cepelewicz the third period corresponds to the final stage of growth after the last moult. Tessier determined the content of the dry substance, the total lipids and the content of total phosphorus. W. Niemierko and Cepelewicz investi-

gated mainly metabolism of lipids, determining at the same time the content of saturated and unsaturated fatty acids and of unsaponifiable substances.

Our previous investigations (S. Niemierko, 1950, S. Niemierko and W. Niemierko, 1950) point to a considerable, though partially unclear, importance of phosphorus compounds in the overall biochemical processes occuring in *Galleria mellonella*. These studies were concerned only with the final stage of growth of larvae (prior to spinning) in which the content of total phosphorus and of fractions of acid-soluble phosphorus compounds were determined. These investigations did not, however, include the determination of fractions of lipid phosphorus and of nucleic acid phosphorus, fractions that are of great importance and activity in biochemical metabolism.

The aim of our present work, therefore, was to investigate the chemical changes occuring during the growth of *Galleria mellonella*, with special attention to the content of the particular insoluble phosphorus compounds.

MATERIAL AND METHODS

The breeding of larvae was performed at temperature of 30°C in the manner described by W. Niemierko and Cepelewicz. Difficulties related with the exact determination of the stage of growth of the larvae are fully described in the same work. In the present investigations, therefore, the determination of the exact age of larvae was of necessity neglected and larvae were selected from the general breed and graded according to weight. It has been assumed that larvae of approximately equal weight are in the same stage of growth. For these experiments we used groups of larvae with an initial weight of 0.55 mg and ending with full grown ones weighing 140—180 mg each, which are in the finale stage of growth before spinning of the cocoon. The number of specimens used in an experiment was depended on the weight of the larvae and amounted to from 8 to 850 (the latter figure for the smallest ones). Larvae were taken directly from the breeding, without previous starvation. The undigested food contained in the digestive tract undoubtedly obscures somewhat the results of analyses of the larvae bodies. But the fasting of larvae, even through a short duration, must influence the metabolism and may have a different effect on a large larva and on a small one. It is difficult to define exactly the time necessary for the removal of the remains of undigested food from larvae of different size and this would require special study by itself. The normal physiological state of larvae is related to a continuous ingestion, of large quantities of food, which as was proved by the experiments of W. Niemierko and Włodawer (1950) is liable in a short time to far reaching transformations. Taking this into consideration, it was decided to use normal feeding larvae

for the experiments. Because of the large number of larvae, especially of the smaller ones, needed for experiments, it occasionally requires considerable time to select them from the colony. In order to reduce the rate of the physiological processes occurring during the preparation of material needed for analyses, the selected larvae were maintained at a temperature of about 0°.

The analyses included the determination of dry substance, of the lipid content and of the particular fractions of phosphorus compounds. Water and lipids were eliminated by means of an acetone-chloroform mixture and acid-soluble phosphorus compounds removed from the residue by means of 5% TCA at a temperature of 0°. In the remainder containing proteins, nucleoproteins and chitin the determination and separation of nucleic acids was effected by the method of Schmidt and Thannhauser, 1945, and Schmidt, Hecht and Thannhauser, 1948.

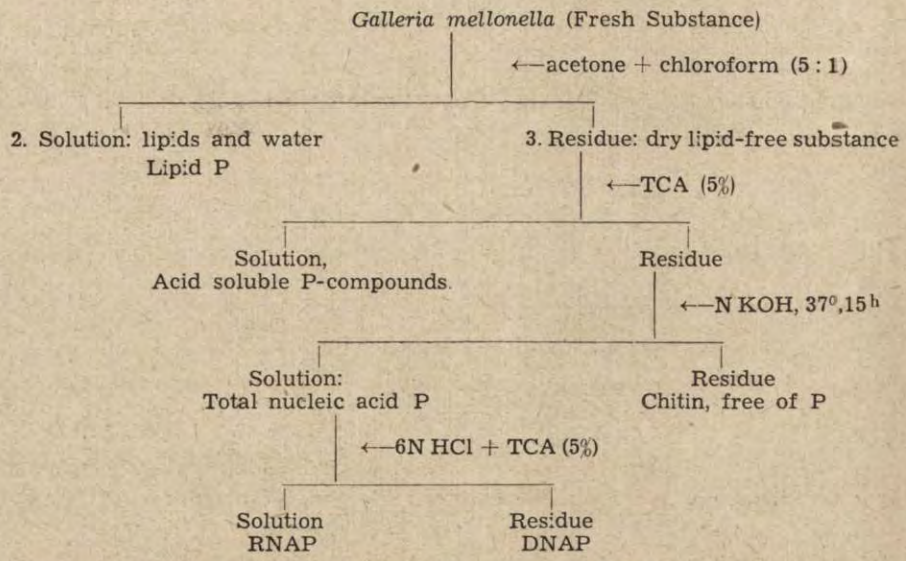
When the applicability of the method of Schmidt and Thannhauser to our biological material was tested it was found out that with incubation periods of 15 hours at 37°C the alkali attacked the glass. In blank analyses, when reagents for phosphorus estimation were used, a distinct blue colour, characteristic of silicium compounds, appeared. Under the action of alkali Si was detected in test tubes made of different kinds of glass: Jena glass, as well as pyrex and Italian glass. In order to avoid this difficulty when the investigated material was hydrolysed with alkali, the glass test tubes were replaced with tubes made of a plastic material. Special analyses performed by us proved that the plastic material used in test tubes withstood the infuence of alkali and did not yield substances which give a blueish color with the reagents used for P determination.

The body of the larva contains a large amount of chitin which, following exposure to alkali for 15 hours at a temperature of 37°C, does not undergo hydrolysis and remains in the form of thin skins. Before attempting to define the nucleic acid P, chitin was removed by filtration of the alkaline hydrolysate. Chitin which remained on the filter was washed out with alkali. In some of the preliminary determinations chitin was digested by means of HNO_3 and H_2SO_4 and shown to contain no phosphorus compounds. Consequently in regular experiments the residue which contained chitin was removed.

In order to determine the total nucleic acid P, the RNA phosphorus (RNAP) and the DNA phosphorus (DNAP), samples were taken from a volumetric flask containing the alkali hydrolysate of nucleoprotein compounds. To avoid dissolution of glass by alkali the analyses on an alkaline hydrolysate was performed as rapidly as possible.

The amount of DNAP was determined, not from a difference between the total nucleic acid P and RNAP, as is usually done according to the original method of Schmidt and Thannhauser, but directly by digesting the residue of DNA according to a modification by Schmidt, Hecht and Thannhauser. As the preliminary determinations showed no presence of protein phosphorus, these analyses were discontinued in subsequent experiments.

The schematic fractionation procedure for the separation of phosphorus compounds is as follows:



Lipids (2) + lipid-free dry substance (3) = dry substance.
Phosphorus determinations were made according to the method of Fiske and Subba Row applying amidol instead of euconogen.

EXPERIMENTAL AND DISCUSSION OF RESULTS

Sixteen series of analyses were performed on larval bodies with a weight of from 0.6 mg to 190 mg. Results obtained are presented in Table I. This table includes the content of dry substance, of lipids, of lipid phosphorus and of nucleic acid phosphorus in %% of fresh weight.

From the initial period of growth up to the moment the larvae have reached a weight of about 60 mg the percentage content of dry substance is more or less constant and fluctuates from 23% to 27%. In the subsequent period of growth there is a sudden increase in the content of dry substance and for large larvae with a weight of one hundred and more mg it reaches a value of about 40%. Finally for larvae above 112 mg up to the time they attain the final size the % content of dry substance amounts to 40—44% and as for small larvae (up to 60 mg) remains almost constant. The % increase in the content of dry substance is due to an accumulation of lipids in the larval body. It may be seen from Table I that the % content of lipids increases several times during period of growth (from 6%

Table I.

Dry substance, lipids, and insoluble phosphorus compounds in waxmoth larvae during growth (in % of fresh weight).

Experiment No.	Weight of larva mg.	Number of specimens used in analysis	Dry substance %	Lipids %	Lipid P mg %	Nucleic acid P mg %	RNAP mg %	DNAP mg %
1	0,55	810	23,1	5,5	59,0	90	70	15
2	4,3	200	23,3	6,0	61,8	86	74	14
3	10,7	100	24,6	6,0	62,5	85	75	11
4	21,0	70	25,0	5,8	61,0	65	—	—
5	29,2	20	25,2	8,0	53,0	56	47	11
6	36,9	30	24,9	6,8	55,6	63	—	—
7	58,0	20	23,5	7,4	53,2	79	70	9
8	70,8	11	31,0	14,4	48,0	51	46	5*
9	72,0	17	32,5	15,4	52,3	58	51	8
10	93,0	10	34,2	16,8	50,0	49	43	7
11	112	10	39,0	22,0	41,6	48	38	10*
12	145	7	42,8	24,3	54.9	49	—	—
13	158	7	41,9	23,6	42,9	48	40	6
14	174	6	42.9	25,6	48,5	45	40	5
15	182	8	42,0	23,0	44,5	66	57	9*
16	191	9	40,3	20,5	44,6	57	53	4*

for small larvae to 26% for large larvae). These results agree with those of Teissier and W. Niemierko and Cepelewicz. It should be stated here that the above authors investigated larvae beginning with a weight above 10 mg. The results of our analyses, which were performed on larvae with a weight of 0.6 mg and of 4.0 mg show that the percentage content of the dry substance, as well as that of lipids, is the same for the smallest larvae examined by us as for the larger ones with a weight of up to 60 mg.

It may be seen from Table II that for larvae with a body weight of from 0.6 mg up to 20—30 mg, lipids constitute about 25% of the dry weight, and for large larvae, above 100 mg, the fat-content amounts to over one half of all the solid constituents of the larval body.

The figures on the content of lipid phosphorus (Table I) enabled us to estimate the proportion of phospholipids in the total fat content during the growth of larvae.

* Calculated from difference between total nucleic acid P and RNAP.

It may be assumed that lipids, with the exception of phospholipids and of a small quantity of unsaponifiable substances which in our analyses were left without consideration, are in most cases composed of neutral fats. In our calculations it was assumed that the content of phosphorus in phospholipids amounts to 4%.

The results presented in Table II show that the percentage content of phospholipids in the total quantity of fats diminishes during the period of growth. For small larvae up to 60 mg phospholipids amount on the average to 22% of all fats — for larger larvae, up to 145 mg, there is a rapid drop to 8%. For the largest larvae, just before spinning begins, the relative content of phospholipids diminishes even further and amounts to only 5% of all the lipids, so that it is only about one fifth of that at the initial period of growth.

Table II.

Changes in lipid composition in waxmoth larvae during growth.

Experiment No.	Weight of larva mg	Lipids % in dry substance	Neutral fats	Phospholipids
			% in lipids	
1	0,55	23,7	74,8	25,2
2	4,3	25,7	74,9	25,1
3	10,7	24,3	74,9	25,1
4	21,0	23,2	74,8	25,2
5	29,2	31,9	83,5	16,5
6	36,9	27,2	79,5	20,5
7	58,0	31,4	82,0	18,0
8	70,8	46,3	91,7	8,3
9	72,0	47,5	91,5	8,5
10	93,0	49,5	92,5	7,5
11	112	56,5	92.7	7,3
12	145	57,0	94,5	5,5
13	158	56,6	92,5	4,5
14	174	59,3	95,2	4,8
15	182	54,7	95,2	4,8
16	191	50,9	94,5	5,5

In the present investigations the contents of lipid-free substance, of lipids and of phospholipids, were determined directly. Although in analyses no constituents of the body other than lipids and phosphorus compounds were estimated it was nevertheless possible to calculate the changes in the percentage of lipids and lipid-free

substances in the larval body during growth. The results are presented in Table III and plotted in Fig. 1. It may be seen from this diagram that, in contrast to neutral fats, the content of phospholipids and of lipid-free substances of the body undergo no appreciable changes during the growth of the larvae. The percentage of lipid-free constituents of the body is a quantity more or less constant and amounts to 17 ± 0.9%. It was mentioned above that the content of

Table III.

Dry lipid-free substance and nucleic acid phosphorus in waxmoth larvae during growth.

Experiment No.	Weight of larva mg	Dry neutral fat-free substance %	Dry lipid-free substance %	Nucleic acid P mg % in dry neutral fat-free substance
1	0,55	17,6	19,0	474
2	4,3	17,4	18,8	458
3	10,7	18,5	19,9	423
4	21,0	19,2	20,6	314
5	29,2	17,1	18,5	302
6	36,9	18,1	19,5	296
7	58,0	16,6	17,9	296
8	70,8	16,7	17,9	283
9	72,0	15,2	16,5	313
10	93,0	17,2	18,5	266
11	112	16,7	18,3	266
12	145	18,7	20,0	248
13	158	18,1	19,2	248
14	174	17,6	18,7	239
15	182	18,9	20,0	328
16	191	19,7	20,8	272
Mean		17,7±1,12	18,8±0,9	

phospholipids also show only small variations during the period of growth, and it can therefore be said that the percentage content of the solid constituents of the larval body, with exception of neutral fats, does not change during the period of growth and amounts to 19% of the body weight. The chief lipid-free component of the larval body is protein. Calculations of the data of W. Niemierko and Cepelewicz show that the % content of proteins increases but slightly during the period of growth and, during the initial stage of growth, amounts to 14.2% — in the second to 14.5% and in the third to

15.5%. As the content of the solid components of the body, fats excluded, amounts to about 17%, it follows that the lipid-free, non-protein compounds (i. e. nucleoproteins, carbohydrates, chitin and ash) appear in very small quantities and amounts to only 2—3% of the fresh weight.

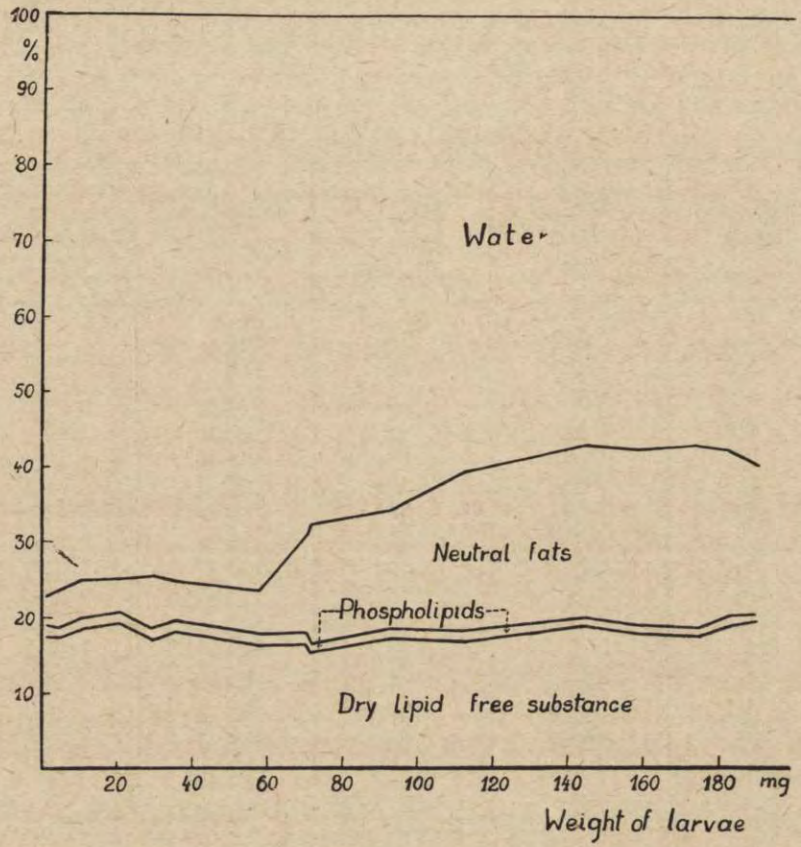


Fig. 1. Chemical composition of the growing waxmoth larvae.

Changes in the contents of other components of the larval body during growth are to a certain degree obscured by the accumulation of neutral fats. For example, according to W. Niemierko and Cepelewicz, the content of proteins in percentages of the dry weight amounts in the first period to 54.6%, in the second period to 48.5%, and in the third to 37.8%. The protein content of the dry substance free of neutral fats is a more or less constant quantity. The same is

true for phospholipids, the percentage of which, in relation to the solid components of the body free from neutral fats, is also more or less constant and decreases only just prior to spinning. (Table III).

In order to stress the changes which occur in the content of nucleic acid phosphorus during growth these data were calculated in relation to the dry substance free from neutral fats.

The figures presented in Table III show that the relative content of nucleic acid P is highest for the smallest larvae with a weight of about 10 mg. It than drops rapidly, reaching a more or less steady level for larvae with a weight of about 30 mg. In subsequent periods of growth there is a further tendency to decrease. The percentage content of nucleic acid P of large larvae is about one half of that found in the smallest larvae. It is difficult to explain the physiological significance of this phenomenon. The establishment of a higher percentage of nucleic acid P in younger individuals than in older ones is by no means an isolated fact, and has been referred to by Davidson (1950).

In the course of the present experiments desoxyribonucleic acid phosphorus (Table I) was in some cases determined. The amount of ribonucleic acid phosphorus is several times greater than that of desoxyribonucleic acid phosphorus. For large larvae (about 150 mg) it was established that there is a certain tendency for the ratio RNAP: DNAP to increase in comparison with values for small larvae. The number of analyses is, however, to small and, taken together with the low absolute values of the determined DNAP do not permit the drawing of wellfounded conclusions. It seems probably however that the greater value of the ratio RNA to DNA for large larvae is related to the production of silk. From the works of Denucé (1952) it is known that the silk glands of *Bombyx mori* are very rich in ribonucleic acid. According to the opinion of Brachet (1947) and of Caspersson (1947) the high content of RNA is characteristic for cells capable of intensive protein synthesis. The silk glands are not, however, the chief factor in the high content of ribonucleic acid, as it was shown above that young larvae, whose silk glands are but slightly developed, also have high ribonucleic acid content. There can be no doubt therefore that the functions of ribonucleic acid are more variable and of a more important and general significance.

Taking into consideration the results of the present analyses and the data of our previous studies (S. Niemierko 1950) we can

set the content of the various phosphorus compounds in the body of full grown waxmoth larvae (in mg% of fresh substance) as follows:

Total P	Ac. sol. P	Lipid P	Nucleic P
228 ± 33	107 ± 8	47 ± 2,3	50 ± 2,8

The insoluble phosphorus constitutes therefore about one half of the total phosphorus content of the larval body. The proportion of lipid phosphorus and of nucleic acid phosphorus (NAP) are approximately equal. Ribonucleic acid P constitutes about 90% of the total nucleic acid P.

A number of phosphorus compounds occuring during the metamorphosis of *Calliphora erytrocephala* were determined by Levenbook (1951). He established only small changes during metamorphosis and gives the following data: acid soluble P 75-78 mg%, lipid P 36-46 mg%, NAP 60-72 mg%. Khouvine and Gregoire (1944) give for the same *Calliphora* equal values for NAP and for lipid P, using a different method of determination.

By comparing our results with those of Levenbook we see that the content of total P of *Galleria mellonella* is somewhat higher than that of *Calliphora*. For *Calliphora*, as for *Galleria*, the lipid P constitutes about 22% of the total P, whereas the acid-soluble P compounds are less (42%) and NAP is higher (65%) than for *Galleria*. Ribonucleic acid phosphorus accounted also, as for *Galleria*, for 90% of the total nucleic acid P.

It is generally known that the growth of larvae is characterised by alternate periods of feeding and moulting. In time of feeding there occurs an increase in the size of the body, a period of muscular activity, and intensive ingestion and assimilation of food. The period of moult, proceeded by a more or less (depending on the species) distinct period of dormancy, is a phase of apparent quiescence. It would seem that the metabolic processes which take place in the period of moult and in the period of feeding should differ greatly from each other and that these differences would be reflected in the chemical composition of the larval body. In our experiments we did not analyse separately larvae in periods of moult and in periods of feeding. It was impossible to do so for technical reasons indicated above in the section on methods.

Analyses of single individuals were impossible because of their small size. A factor which can also obscure the results of our analyses is that in the present experiments larvae were not dif-

ferentiated with regard to sex. It was impossible to establish the external sexual characteristics even with full grown larvae. A quick preparation and identification of sex according to gonads, especially with small individuals, presented great difficulties and could not be done. Taking the above into consideration attention should be called to the fact that the results of the various analyses are the resultants for a large number of individuals which were not necessarly in the same physiological state. Several of the characteristic features for some particular periods of growth may have therefore been obscured and no striking differences in the chemical composition of the body could be observed. It appears to us that experiments performed on other species of insects, in which the particular stages of growth and the identification of sex are easier to establish, will make it possible to distinguish more clearly the eventual differences appearing at particular periods of growth.

SUMMARY

During the growth of the larvae *Galleria mellonella* an increase in the percentage content of dry substance, due to an accumulation of neutral fats, takes place. The proportion of phospholipids to total fats decreases.

The percentage of the solid components of the body free from neutral fats is constant during the period of growth.

The percentage of nucleic acid P is highest for larvae with a weight up to 10 mg, then it drops suddenly to a level which, in a subsequent period of growth, decreases further slightly. The amount of ribonucleic acid phosphorus is several times greater than the amount of desoxyribonucleic acid P. In the body of fully grown larvae in which the total amount of phosphorus is equal to 228 ± 33 mg%, the proportion of lipid phosphorus is 47 ± 2.3 mg% and that of nucleic acid P — 50 ± 2.8 mg%.

ADDENDUM IN PROOF.

W. Niemierko et al. (1952) have shown recently that by treatment of the tissue with acetone-chloroform mixture, lipoproteins are not split entirely and hence a part of lipid phosphorus remains in the residue and may be found in the nucleic acid fraction. A complete removal of lipids may be accomplished by subsequent action of alcohol-ether upon residue. As the last

operation has not been performed in the present experiments, it is likely that the amount of lipid phosphorus in the larval body is somewhat larger and the amount of nucleic phosphorus correspondingly smaller than in the figures presented above.

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STUDIES ON THE BIOCHEMISTRY OF THE WAXMOTH
(Galleria mellonella)

10. RESPIRATORY ENZYMES OF THE LARVA

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In the course of a study on the biochemistry of the waxmoth *Galleria mellonella* L. the necessity arose of acquiring some knowledge of the respiratory enzyme system of this insect. Despite the numerous reports on work in this field our knowlegde of the respiratory enzyme systems of insects is rather limited and is in any case much less precise than our knowledge of analogous systems of Vertebrates. And yet the problem of the oxidoreductive enzymes of insects contains many interesting and but little cleared-up aspects, and is of considerable importance from theoretical as well as practical respects. Of much interest also is the net difference between the respiratory enzyme systems of insects and of Vertebrates, which is confirmed by many workers. One of those differences is the presence of very active phenoloxidase in insect tissues (Bhagvat and Richter 1938, Bodine and Allen 1941, Heller 1947, Dennell 1947, Sussman 1949 and others). This enzyme was found in Mammalia not long ago in small quantities only (Fitzpatrick et al. 1950, Lerner and Fitzpatrick 1950, Foster 1951). Of much interest also is the fact that it was found in large quantities in some kind of tumours of

Vertebrates i. e. in melanoma (Hogeboom and Adams 1942, Fitzpatrick, Lerner et al. 1950). It is maintained (Fitzpatrick et al. 1950, Foster 1951) that phenoloxidase which is to be found in the skin tissue of Mammalia participates in the production of skin pigment. The part played by this enzyme in insects is not as yet cleared up. Dennell (1947) points to the role it plays in the construction of insect cuticle. Other authors put forth the supposition that phenoloxidase in insects may play the role of a terminal oxidase, like cytochrome oxidase does in Verterbrates (Heller 1947). Boswell (1945) ascribes an analogous importance to phenoloxidase present in vegetable tissues. But this problem has not been definitely cleared up to date (Sussman 1949). There are results which indicate that phenoloxidase may participate in vitro in the oxidative desamination of amino acids (Jasckson and Kendal 1949, Trautner and Roberts 1950). The investigations of Boswell (1945) on tissue of potato tuber seem to indicate the participation of phenoloxidase in the oxidation of a series of amino acids.

Another characteristic, which distinguishes the respiratory enzyme system of insects from that of Vertebrates, is a considerable immunity of certain developmental stages of a number of insect species to some enzyme inhibitors e. g. to KCN (Bodine 1934, Sacktor 1951). This aspect is of some practical importance in the fight against harmfull insects.

We consider the present work to be an introduction to further investigations which are intended to deal with respiratory enzymes of insects. Its purpose is to determine the optimal conditions of research on the respiratory enzymes of waxmoth, to investigate the general character of the respiratory metabolism of the tissue homogenate of the larva and to identify some respiratory enzymes or some enzyme systems which are active in the matter under investigation.

Side by side with the research on the respiratory enzymes of waxmoth the present work contains some comparative measurements on some other insects, namely: *Achroea grisella* (Lepidoptera), *Bombyx mori* (Lepidoptera), and *Carausius morosus* (Orthoptera). Only some data, which were obtained while working on this material, are presented in this paper. A more detailed report of these investigations, especially on the research on silkworm, will be published later.

MATERIAL AND METHODS

Experiments were performed on full-grown larvae of waxmoth in the last instar, mostly with a weight of 150—250 mg. These insects were descendents from a common breed on beecomb at a temperature of 30° and 75% relative humidity.

The measurements of oxygen consumption were performed on homogenates of whole larvae or of isolated organs in a Warburg manometric apparatus at a shaking frequency of about 100 oscillations per minute. CO_2 was absorbed by means of 0.1 ml of 10% KOH. In the majority of measurements which were performed on fresh substance a tenfold dilution was applied, which means that a weighed quantity of larvae was ground with a ninefold quantity of diluting medium (water, salt solutions or buffer solutions). As the specific gravity of the examined insects is close to unity, 1 ml of homogenate so prepared contained 100 mg of biological substance. Grinding was performed in a glass homogenizer according to Potter and Elvehjem (1936). The larvae, as well as the diluting liquid, were, before grinding, chilled to a temperature of about 0°. Grinding itself was performed at the same temperature. The homogenate being prepared was usually subjected to a very light centrifugation (in a manual centrifuge at about 600 cycles per minute for about 1/4 of a minute) in order to eliminate some chitin particles which could interfere in the pipetting of homogenate. The measured quantities of homogenate were transferred to Warburg flasks which were placed in the bath. Shaking was then started. Measurements began 5 minutes after the flasks had been at a temperature of 30°. Numerous trials have shown that a period of 5 minutes was sufficient to equalize the temperature of the homogenate with that of the bath and to stabilize the vapour pressure of water inside the flasks. The flasks contained 2—4 ml of the tissue homogenate. In the event that the enzymatic oxidation of different substrates was to be examined they were added from a side vessel in the course of the experiment. Solutions of the substrates were added in quantities of 1.0 to 1.5 ml, but the amount of substrate poured over from the side vessel to the main compartment was not exactly known as it was impossible to pour the entire contents of the side vessel owing to the dampness of its walls.

The oxygen consumption was usually expressed in ml O_2 at N. T. P. per gram of fresh substance of larvae per hour, and it was marked with the symbol Q'_{O_2}; the results of some experiments were calculated in terms of one gram of dry substance (Q_{O_2}) or of one gram of lipid-free dry substance (Q''_{O_2}).

Further details of the methods are submitted in the experimental part.

EXPERIMENTAL AND RESULTS

The diluting medium. In searching for the most appropriate diluting medium to prepare the homogenate the following were tried: 1) distilled water, 2) 0.90% NaCl solution, 3) mammalian Ringer solution (carbonates free), 4) phosphate buffer M/15, after Sörensen, with a pH close to that of the haemolymph of the wax-

moth larva. The pH of haemolymph of larva in the last instar, as determined by means of indicator papers, was close to 6.6.

The respiratory metabolism of the homogenates did not appear to depend in a distinct way on the solutions tested. In consequence phosphate buffer was used exclusively as a diluting medium in further work to ensure a constant pH, which may be subject to some changes especially when substrates are added.

Table I.

Oxygen consumption of homogenates of waxmoth larvae at different pH of the diluting phosphate buffer solution. 10-fold dilution.

pH	Q'_{O_2} in consecutive 15 minute intervals			
5,3	0,65	0,38	0,26	0,22
6,0	0,96	0,59	0,38	0,29
6,6	1,28	0,76	0,50	0,36
7,2	1,17	0,64	0,42	0,35
7,8	0,88	0,54	0,37	0,27
8,1	0,68	0,46	0,32	0,23

The influence of the pH. The oxygen consumption of homogenate, as a function of the pH buffer used, was next examined. The measurements were made within the pH range from 5.3 to 8.1 at intervals of ca. 0.6 pH. They show (Table I) that the highest rate of O_2 consumption was attained in the neighbourhood of pH = 6.6. As such a hydrogen ion concentration corresponds at the same time to that of the haemolymph of the insect, a phosphate buffer with pH = 6.6 was exclusively used in further experiments.

The „diluting effect". In investigating the influence of conditions, under which the homogenate was being prepared, on its respiratory metabolism, attention was drawn to the influence of the degree of dilution of the biological substance. The level of oxygen consumption by homogenates of the following dilutions was analysed: 1) a twentyfold dilution (1 g of larvae and 19 ml of the buffer), 2) a tenfold dilution, and 3) a threefold dilution. The oxygen consumption was calculated per unit weight of the undiluted biological material. It turned out that the intensity of oxygen consumption measured in this way decreases with an increase in dilution of the homogenate. The intensity of the oxidative processes in a homogenate diluted twentyfold is about two times lower than with a tenfold dilution. Homogenate diluted threefold shows a consump-

tion 50% higher in relation to one diluted tenfold (Table II). This phenomenon of a decrease in the intensity of oxidative processe in the homogenate with an increase in its dilution has been observed and described under the name of „diluting effect" (Krebs 1935, Potter and Elvehjem 1936, Potter and Schneider 1942). In order to eliminate the influence of this „diluting effect" on the comparative value of further measurements a constant dilution of homogenate with buffer solution, namely a tenfold one, was adapted in subsequent experiments.

Endogenous respiration of the homogenate. The next experiments were devoted to the examination of oxygen uptake by homo-

Table II.

Oxygen consumption of homogenates of waxmoth larvae at different dilutions (phosphate buffer, pH = 6,6).

Dilution	Q'_{O_2} in consecutive 15 min. intervals				in per cent of the 10-fold diluted homogemate			
20 - fold	0,34	0,25	0,21	0,16	47	57	67	64
10 - fold	0,72	0,44	0,31	0,25	100	100	100	100
3 - fold	1,07	0,71	0,53	0,41	149	161	171	164

genate with no addition of substrate (endogenous respiration). It was remarked that the intensity of the respiratory processes undergoes rapid changes during the course of a single experiment, namely the level of oxygen uptake falls steadily during the one hour the experiment lasts. This phenomenon was observed in all experiments. Table III presents the results of several such experiments and the average values from 57 experiments. The average oxygen consumption for the first 15 minutes amounts to 0.72 ml/g/h., while in the next 15 minutes it is only 0.44 ml/g/h., i. e. it decreases about 40%. Subsequently we can observe a steady but slower decrease in the intensity of respiratory processes. The average uptake of O_2 in the course of one hour amounts to about 0.43 ml/g/h. The process of oxygen consumption of homogenate is illustrated in Fig. 1.

The comparative measurements which were performed on homogenates of other insects, namely of *Carausius morosus*, larvae of *Bombyx mori*, larvae of *Achroea grisella* and larvae of *Tenebrio molitor* showed a steady decrease of respiratory activity of homogenates of these insects as well.

Table III.

Endogenous oxygen uptake of homogenates of waxmoth larvae in last instar and oxidation of phenol and succinate. 10-fold dilution with phosphate buffer, pH = 6.6. Phenol 2% solution; sodium succinate 0,2 M. Substrates added after 60 minutes.

Experiment No.	Endogenous Q'_{O_2}				Mean for the whole 60 min. period	Q'_{O_2} phenol added			Q'_{O_2} succinate added		
	time 0—15′	15′—30′	30′—45′	45′—60′		60′—75′	75′—90′	90′—105′	60′—75′	75′—90′	90′—105′
28	0,72	0,34	0,29	0,26	0,40	1,54	2,29	2,30	0,28	0,30	0,30
29	0,81	0,51	0,38	0,26	0,49	1,10	1,60	1,79	0,52	0,48	0,50
30	0,79	0,36	0,27	0,23	0,41	1,90	2,74	2,52	0,24	0,22	0,19
31	0,78	0,43	0,34	0,23	0,45	0,59	1,19	1,34	0,45	0,56	0,51
32	0,60	0,46	0,32	0,31	0,42	0,72	1,15	1,04	0,60	0,82	0,72
33	0,80	0,53	0,42	0,28	0,51	0,55	0,75	0,84	0,69	0,77	0,77
34	0,45	0,25	0,20			1,10	1,94	1,56	0,27	0,23	0,18
35	1,00	0,48	0,37	0,30	0,54	1,19	1,78	1,61	0,42	0,51	0,47
36	0,99	0,52	0,36	0,26	0,53	1,21	1,62	1,19	0,64	0,76	0,62
44	0,62	0,49	0,37	0,28	0,44	1,28	2,11	1,94	0,69	0,68	0,57
	Mean 0,72	0,44	0,31	0,25	0,43	0,95	1,43	1,36	0,49	0,55	0,51

Considering the rapid decrease in intensity of the oxidative processes in the homogenates of waxmoth a more precise investigation of the course of these changes seemed to be wanted. Several trials were therefore undertaken and readings taken at shorter intervals, namely every 5 minutes. Table IV presents the results of

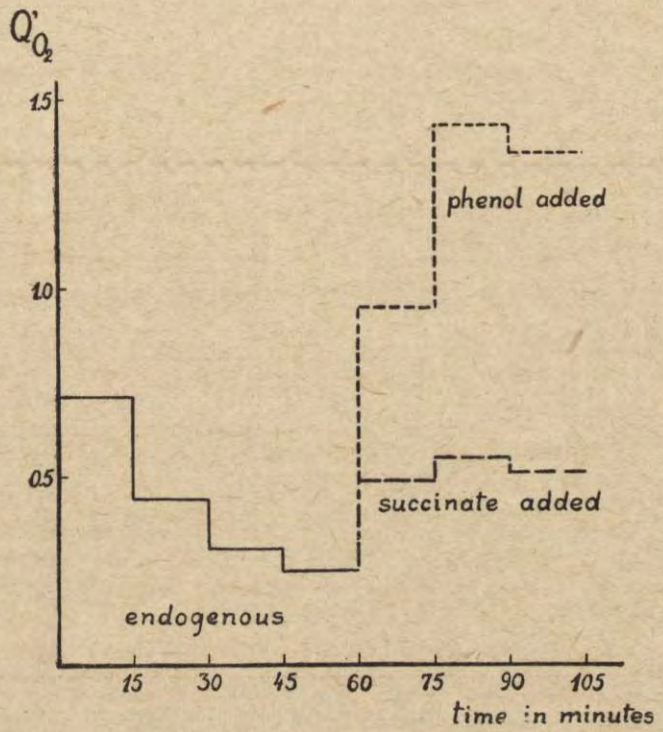

Fig. 1. Course of oxygen consumption of homogenate of full-grown larvae of *Galleria mellonella*.

Substrates added after 60 minutes: 1) phenol 2% solution, 2) sodium succinate 0.2 M. O_2 uptake in ml per gram per hour (Q'_{O_2}).

several such measurements for the first 15 minutes of the experiment. It may be seen from these data that in the majority of cases a decrease of oxygen consumption has taken place in the initial stage also.

The influence of KCN. Before starting the experiment, KCN solution was added to the homogenate so that the final concentration of cyanide amounted to 10^{-4} or 10^{-3} M. At the same time instead of potassium hydroxide a mixture of KOH and KCN, accord-

Table IV

Oxygen consumption of homogenates of waxmoth larvae during the initial 15 minutes. Readings taken every 5 minutes. 10-fold dilution with phosphate buffer, pH = 6.6.

Experiment No.	Q'_{O_2}			
	time: 0 — 5′	5′ — 10′	10′ — 15′	Mean for 0 — 15′
48	1,06	0,77	0,77	0,87
49	1,02	0,81	0,83	0,89
50	1,65	1,55	1,42	1,54
51	0,82	1,11	0,72	0,85
53	0,94	0,73	0,78	0,82
55	1,05	1,18	0,80	1,01
Mean	1,09	1,03	0,89	1,00

ing to Krebs (1935), was used to absorb CO_2, thus preventing the diffusion of HCN from the homogenate to the hydroxide solution. When the respiratory enzyme system was blocked with cyanide the intensity of oxygen consumption fell to about one half of the values obtained without KCN (Table V). Values obtained for KCN 10^{-3}

Table V

Oxygen consumption of KCN blocked homogenate. Final KCN concentration 10^{-4} to 10^{-3} M.

Experiment No.	Q'_{O_2} in consecutive 15 min. intervals			
1	0,41	0,19	0,11	
2	0,44	0,18	0,14	0,10
3	0,33	0,23	0,13	0,07
Mean	0,39	0,20	0,13	0,09

and 10^{-4} M are close to each other. The gradual drop in the intensity of respiratory processes for one hour of the duration of the experiment takes place also in case the homogenate is blocked by KCN and to an even greater degree than in non poisoned homogenate. The average consumption of O_2 which in the first 15 minutes amounts to 0.39 ml/g/h. drops during the next 15 minutes to 0.20 ml/g/h., that is by 49%.

Oxydation of substrates. In order to examine further, and at least to identify partly, the enzyme systems active in the homogenate of the larva of *Galleria mellonella*, different substances which are known to be oxidized by living tissue were added to the homogenate. Those compounds which, after being added to the homogenate, caused an increase in oxygen consumption were proved to be: 1) tyrosine and other derivatives of phenol and phenol itself, 2) succinic acid, 3) ascorbic acid, and 4) some aldehydes (e. g. benzaldehyde). The following did not lead to an increase in O_2 uptake: glucose, lactic acid (racemate), citric acid, glycocol, glutamic acid, pyruvic acid.

In the present work we payed no further attention to systems which oxidize ascorbic acid and aldehydes but we made a more precise investigation of the oxidation of phenol derivatives and of succinic acid.

Phenoloxidase (tyrosinase). Oxidation of the derivatives of phenol points to the presence of enzymes of the phenoloxidase group in the tissue homogenate. Among the phenols which were examined the following are subject to enzymatic oxidation in the homogenate: a) phenols with one —OH group (so called monophenols): phenol C_6H_5OH, cresol, and tyrosine, b) phenols with several hydroxide groups (so called polyphenols) or their derivatives: hydroquinone, catechol, and guaicol, c) aminophenols: 2,4-diaminophenol (amidol) and p-methylaminophenol (methol). Resorcinol and phloroglucinol did not lead to an increase in oxygen uptake.

Oxidation of the above-named phenols was accompanied by the appearance of a pigment from red through different shades of brown to a black colour, depending on the substrate. Tyrosine for example dyes black; phenol, reddish-brown. These coloured substances are in all probability products of oxidation of phenols of the melanin type (Lerner and Fitzpatrick 1950). During an examination of the endogenous respiration of homogenate it was noted that it gradually turned dark. This is probably due to melanin formation by the oxidation of natural derivatives of phenols, which are present in the tissue of larva, tyrosine or „dopa" maybe.

Investigating further the activity of phenoloxidase of waxmoth a water solution of phenol at a concentration of 2% was used exclusively. Preliminary measurements indicated that the increase of oxygen consumption under the influence of phenol reached approximately the same level independently of whether a substrate

was added at the beginning of the experiment or after the homogenate was shaken for one hour at a temperature of 30° without substrate. Relying on this observation we applied a procedure in subsequent experiments consisting in the addition of phenol after the homogenate had been shaken for one hour in a temperature of 30°. It enabled us to make a measurement of the endogenous oxygen consumption on the same portion of homogenate. About 1 ml of phenol solution was added to the homogenate (the quantity of homogenate being 3—4 ml) from a side vessel. The quantity of phenol used in this experiment was excessive in relation to the enzyme concentration as it has been proved that the intensity of phenol oxidation (the increase in oxygen uptake) was the same whether a phenol solution of 2% or of 1% was added. Table III and Fig. 1 show the increase in oxygen consumption after phenol has been added, as exemplified on several experiments. The numbers indicate the consumption of O_2 in ml/g/h. in consecutive 15 minute periods before and after the substrate has been added. Before phenol has been added the Q'_{O_2} amounted on the average to 0.25 for a period of 45 to 60 minutes from the beginning of the experiment. In the first 15 minutes after the addition of the substrate the oxygen uptake increases to 0.95 ml/g/h. and then reaches usually a maximum intensity of 1.43 ml/g/h. on the average and is later usually maintained on the same level or undergoes a slight reduction. After a short period (15—30 minutes) appears an intensive reddish-brown colouring. In further experiments the intensity of phenol oxidation was adopted as a measure of phenoloxidase activity.

In relation to the activity of phenoloxidase a very marked effect of dilution could be seen. In a homogenate diluted 20-fold the oxidation of phenol was several times weaker than with a ten-fold dilution.

The presence of phenoloxidase was ascertained also in *Carausius morosus* and in full grown larvae *Achroea grisella*. Comparing the activity of phenoloxidase of waxmoth with the activity of phenoloxidase of silkworm larvae determined by us in the same way (unpublished) we find that, in the waxmoth, this enzyme is several times more active.

The poisoning of the respiratory enzymes by means of KCN at a concentration of 10^{-4} M and higher blocks entirely the oxidation of phenol: The addition of phenol causes no increase in O_2 uptake. There is also no appearance of the characteristic colouring. No dark-

ning of homogenate without phenol when poisoned with cyanide was observed as well. KCN in a concentration of 10^{-5} M only partly lessens the oxidation of phenol and the formation of a reddish-brown coloured substance.

The succinoxidase system. Following measurement of the endogenous oxygen consumption for one hour, 1 ml of solution of sodium succinate at a concentration of 0.2 M and pH made up to 6.6, was added to the homogenate. An increase in O_2 consumption was in evidence, (Table III and Fig. 1.), which indicates an oxidation of this substance through the enzyme systems of the homogenate. This increase was however much less than after addition of phenol. KCN in concentration of 10^{-4} and 10^{-3} M blocked entirely the oxidation of succinate, while in a concentration of 10^{-5} M abrogated only partly the oxidation of this substrate.

The influence of drying and of delipidation. In order to examine the stability of respiratory enzymes of waxmoth a number of experiments on the activity of homogenate from dried larvae was undertaken. The insects were dried *in vacuo* over concentrated sulphuric acid according to W. Niemierko (1947) at room temperature. The drying usually lasted 3—4 days, following which the insects, in chilled buffer solution, were crushed with a mortar. Dilutions of 10-fold and 20-fold, in relation to the weight of dry material, were made. As the full grown larvae of waxmoth contained on the average 56% water a 20-fold dilution of dry substance corresponds approximately to a tenfold dilution of fresh substance. Tests have proved that dry substance after being crushed and diluted preserves its ability to catalyze oxygen consumption (Table VI). In order to compare the rate of oxygen uptake of homogenate from dry larvae with that from fresh larvae we must base the former on the fresh substance too. (The dry substance of full grown waxmoth larvae, as determined in many measurements, amounts on the average to 44% of the fresh weight). By comparing these data we can deduce that drying reduces the respiratory activity to about 60%.

Experiments performed with the addition of phenol showed that the activity of phenoloxidase was not weakened. No succinate was however oxidized in homogenate prepared from dry larvae. An addition of this substrate have absolutely no effect on the increase of oxygen consumption (Table VI).

The homogenate made from dried larvae was completely inactivated when warmed up to 100°, thus proving the enzymatic character of the process.

Table VI

Oxygen consumption of homogenate of vacuum dried larvae. 10-fold dilution with phosphate buffer. O_2 uptake related to dry substance (Q_{0_2}). Q'_{0_2} calculated on the basis of 44% of dry substance in fresh material.

Experiment No.	Q_{0_2} in consecutive 15 min. intervals				Q_{0_2} for the whole 60 min. period	Q_{0_2} phenol added		
1	0,74	0.36	0,25	0,21	0,39			
2	0,92	0.39	0,32	0,30	0,48			
3	1,07	0,65	0,54	0,45	0,68			
4	1,00	0,55	0,48	0,44	0,62			
5	1,13	0,66	0,47	·	·			
6	1,05	0,63	0,41	0,41	0,63			
7	1,03	0,61	0,46	0,44	0,63	4,11	4,25	3,38
Mean Q_{0_2}	0,99	0,55	0,42	0,38	0,57			
Mean Q'_{0_2}	0,44	0,24	0,18	0,17	0,25	1,81	1,87	1,49

The dried substance preserves its enzymatic activity for several days with no visible changes when stored at room temperature. Some two weeks after it has been dried, however, a distinct lowering of its activity could be observed with time. The respiratory activity of the dried substance, after it has been kept for several months, has fallen to zero, although in one case a slight but perfectly distinct respiratory activity was ascertained after the lapse of one and a half years. If the substance is kept *in vacuo* over sulphuric acid it is more stable than when kept in the open air, although even in the former case the activity continues to decrease.

Dried substance was much more resistant to short term action of higher temperatures and of some chemicals. The heating of larvae in dried state for several minutes at a temperature of 100° lessens only to a certain degree the respiratory activity of homogenate prepared from them, but does not suppress it entirely, as is the case when fresh insects are heated to the same temperature. Ethyl alcohol when added to the homogenate suppresses the respiratory action, while absolute alcohol acting for some time on dried substance only partly inactivates the respiratory enzymes. (Alcohol

was eliminated prior to the grinding and its residue evaporated at room temperature).

The application of dried substance to our experiments presented the inconvenience that such a material, because of large contents of lipids, about 50% of dry weight, was difficult to grind and to be mixed with aqueouse buffer solution. There was the possibility of some fragments remaining uncrushed and unmoistened by water. Because of this some experiments were made with a delipidated substance.

Dry larvae were either extracted several times for a couple of hours with benzene or chloroform at room temperature or the delipidation was performed in an extraction apparatus by means of boiling ethyl ether (35°). The fat-free substance obtained in this way was ground together with a buffer solution as formerly. 20-fold and 40-fold dilutions were applied. This latter one corresponds to a 10-fold dilution of fresh substance with regard to the content of lipid-free substance, as this amounts to about 1/4 (average of several determinations, 22% of the fresh weight).

Table VII presents results of these experiments. The respiratory activity after drying and delipidation underwent a reduction of about 30% in relation to the fresh material on the average. In relation to a substance which is dried only, the dried and delipidated preparation possesses a something higher values of oxygen consumption. This is probably due to the fact that in the homogenate of insects which were only dried a part of the substance was not moistened by water because of large contents of lipids, and took no part in the enzymatic processes. Delipidation removed these obstacles.

Measurements of the activity of phenoloxidase in delipidated material (Table VII) show no inactivation of this enzyme. However, as in previous cases, no oxidation of succinate could be established.

Studies on the localization of respiratory enzymes in the organs of waxmoth larva. Besides the investigations concerning the activity of respiratory enzymes of whole waxmoth larvae, measurements were also made on the separate organs and fragments of the insect body. These measurements are thus far only of preliminary character. It is hoped to extend these investigations in the future.

Larvae were chilled on ice and the following parts were isolated: 1) the silk glands, 2) the alimentary tract together with its

contents, and 3) the fat body. The remainder included integument with muscles and the remains of the fat body. The close anatomical connection of the various organs of the insect prevented often an exact isolation of the particular organs.

Table VII

Oxygen consumption of homogenates of dried and delipidated larvae. O_2 uptake related to fat free substance (Q''_{O_2}). Q'_{O_2} calculated on the basis of 22% of fat free substance in fresh material.

No. Experiment	Dilution	Endogenous Q''_{O_2}				Q''_{O_2} for whole hour	Q''_{O_2} phenol added		
1	20 - fold	2,17	0,98	0,90	0,65	1,17	·	·	·
2	20 - fold	2,69	1,75	1,33	1,06	1,71	·	·	·
3	20 - fold	2,39	1,53	1,02	0,92	1,47	·	·	·
4	20 - fold	2,69	1,74	1,33	1,06	1,71	·	·	·
5	40 - fold	2,89	1,50	1,17	0,73	1,57	5,58	7,49	6,31
6	40 - fold	2,61	1,05	0,92	0,65	1,31	5,36	6,36	5,51
7	40 - fold	1.45	0,92	0.58	0,63	0,90	4,42	5,93	·
Mean Q''_{O_2}		2,41	1,35	1,04	0,81	1,40	5,12	6,59	5,91
Mean Q'_{O_2}		0,53	0,30	0,23	0,18	0,31	1,13	1,45	1,30

The first tests showed an almost complete lack of oxygen consumption of the homogenate of silk glands so that, in subsequent experiments, these glands were discarded. Table VIII presents the results of some of the experiments performed on homogenates of: 1) the alimentary tract, 2) the fat body, 3) the integument with muscles (and with remains of the fat body). The average level of O_2 consumption is, for all three examined parts, approximately equal. Attention is drawn to the large oxygen uptake of homogenate of alimentary tracts. This is of considerable interest as the alimentary tract of waxmothe larva contains only 20% of dry substance (W. Niemierko and Włodawer 1952), that is one half less than the whole larva and, moreover, a large part of it consists of the food substance of the intestine. The level of oxygen consumption calculated on the basis of dry substances is therefore considerably higher for homogenate of alimentary tracts than for homogenates of the remaining parts of the insect organism. Despite such a high endogenous oxygen consumption no presence of phenoloxidase could be

Table VIII

Oxygen consumption of homogenates of organs of waxmoth larvae. Tenfold dilution with phosphate buffer, pH = 6.6.

A) Alimentary tracts:

Experiment No.	Endogenous Q'_{O_2}				Q'_{O_2} phenol added		Q'_{O_2} succinate added		
1	0,52	0,39	0,32	0,32	0,13	0,12	·	·	·
2	0,65	0,46	0,31	0,25	0,17	0,19	·	·	·
3	1,28	0,59	0,40	0,37	·	·	0,30	0,36	0,26
4	0,59	0,35	0,22	0,22	0,10	0,14	0,28	0,26	0,20
Mean	0,76	0,45	0,31	0,29	0,13	0,15	0,29	0,31	0,23

B) Fat body:

Experiment No.	Endogenous Q'_{O_2}				Q'_{O_2} phenol added			Q'_{O_2} succinate added		
1	0,62	0,47	0,45	0,40	1,15	1,45	1,32	0,73	0,66	0,45
2	0,45	0,35	0,29	0,27	1,69	2,54	1,84	0,58	0,65	0,50
Mean	0,54	0,41	0,37	0,34	1,42	2,00	1,58	0,66	0,66	0,48

C) Integument and muscles:

Experiment No.	Endogenous Q'_{O_2}				Q'_{O_2} phenol added			Q'_{O_2} succinate added		
1	0,67	0,39	0,29	0,23	1,58	2,07	1,66	·	·	·
2	0,51	0,30	0,25	0,22	·	·	·	0,62	0,82	0,68
3	0,78	0,48	0,37	0,32	2,91	2,82	2,44	0,95	1,25	1,04
Mean	0,66	0,39	0,30	0,26	2,25	2,45	2,05	0,79	1,04	0,85

detected in the homogenate of alimentary tracts: The addition of phenol causes no increase in oxygen consumption. Oxidation of succinate in the homogenate of alimentary tracts is very slight. Phenol, as well as succinate, are energetically oxidized by the remaining parts, that is, by the fat body and by integument with muscles.

DISCUSSION

We shall analyse the obtained results and experimental data from the point of view of the respiratory enzyme systems which are active in the tissues of the investigated insect. While interpreting the results it should be born in mind that they are obtained in experiments performed not on a living tissue, but on a material whose biological structure has been destroyed to a considerable degree.

Our study on the respiratory enzyme systems of waxmoth larva have proved the presence of phenoloxidase and of succinoxidase system. Phenoloxidase has previously been found in various insect species, e. g. in *Bombyx mori* (Yamafuji 1934), *Celerio euphorbiae* (Heller 1947), *Sarcophaga falculata* (Dennell 1947), *Melanoplus differentialis* and *Tenebrio molitor* (as proenzyme, Bodine and Allen 1941) and other (Bhagvat and Richter 1938). Our comparative investigations have confirmed the presence of this enzyme in *Carausius morosus* and in full grown larvae of *Achroea grisella*. Phenoloxidase is therefore widely distributed among insects. This enzyme was also found in Crustacea (Bhagvat and Richter 1938, Bodine and Allen 1941, Krishnan 1949 and others). From the point of view of comparative biochemistry it would be of interest to investigate whether phenoloxidase exists also in the remaining classes of Arthropoda, that is in Arachnoidea and Myriapoda. We know no work on this subject, however.

The high activity of phenoloxidase in insects and its universal presence in this group leads to the supposition that it plays an important part in the respiratory system of insects. Suppositions are advanced that phenoloxidase in insects may play a role of terminal oxidase, similar to that played by cytochrome oxidase in Vertebrates (Heller 1947). Other authors, however, do not support this view (Sussman 1949). In the light of the present investigations the phenoloxidase of high activity which was established by us in the waxmoth larva may be a further contribution to the knowledge of the part played by this enzyme in insects. We intend to pursue further work in this direction.

In literature dealing with phenoloxidase we find a division of these enzymes into monophenoloxidases and polyphenoloxidases, catalyzing the oxidation of phenols with one hydroxyl group (so called monophenols), and with two or a greater number of —OH

groups (polyphenols), respectively. Later investigations, however, undermined the validity of such a division and supported the hypothesis of the existence of only one phenoloxidase, namely polyphenoloxidase (Californo and Kertész 1939, Kertész 1947). Oxidation of monophenols would be a more complicated phenomenon, where in addition to enzymes there participate also slight quantities of o-diphenols, eventually of o-quinons. A confirmation of this hypothesis is the appearance of an induction period in the oxidation of monophenols. This matter is however not yet cleared up. Kendal (1949) put forth the supposition that the enzyme possesses independent groups which are active as mono and polyphenoloxidases. In our experiments diphenols as well as phenols with one — OH group were energetically oxidized. During the oxidation of these latter an induction phase could be observed. The intensity of O_2 uptake reached its maximum only after a lapse of 15 minutes from the moment phenol was added. This is an additional proof that oxidation of monophenols is a complicated process.

Measurements have shown that the increase in oxygen consumption, after phenol has been added, reaches approximately the same level, independently of whether substrate was added at the beginning of the experiment or after an hour of endogenous respiration (with no substrate). It indicates the relative stability of phenoloxidase of waxmoth.

The enzymatic oxidation of phenols leads to the appearance of pigment which is probably akin to natural melanins (Lerner and Fitzpatrick 1950).

The oxidizing system of succinate is, as we know, more complicated. It is probably composed of several enzymatic elements, namely of the cytochromic system and of dehydrogenase of succinic acid, which are active with Ca ions participating (Straub 1942, Potter and Schneider 1942). According to this supposition oxidation of succinate in the homogenate from waxmoth larva is a resultant of the activity of the separate elements of this enzymatic system.

In our present work we established, moreover, oxidation of ascorbic acid in the homogenate, which is indicated by increase in O_2 consumption after this substrate has been added. This phenomenon has an enzymatic character, proved by the almost entire lack of oxidation of ascorbic acid in a homogenate warmed to 100^0. It is possible that phenoloxidase participates in oxidation of ascorbic acid (Keilin and Mann 1938); or that a specific oxidase of ascorbic

acid, analogous to the enzyme which was discovered in vegetable tissues (Diemar and Zerban 1943, Dumm and Davson 1951), is here active.

Oxidation of certain aldehydes in homogenate, which was proved by us, seems to point to the presence of an aldehyde oxidase.

Considerable difficulties arise when interpreting values of the so called endogonous respiration of homogenate. Oxygen consumption of homogenate alone, without the addition of a substrate, is a resultant of a number of oxidative processes in which participate, on the one hand various enzymatic systems and, on the other, natural substrates present in the tissues of larva. The intensity of endogenous O_2 consumption depends therefore on the activity of separate respiratory enzymes: oxidases and dehydrogenases, and on the quantities of natural substrates as well. A phenomenon which is very much in evidence is a rather rapid drop in oxygen consumption during the one hour duration of the experiment. This drop of the respiratory activity of homogenate was observed by us in relation to other species of insects as well, namely *Achroea grisella*, *Bombyx mori* and *Carausius morosus*. This phenomenon may be due to the exhaustion of natural substrates or to a gradual inactivation of enzymes, or both. We are inclined to reject the first interpretation on the following grounds: 1) An addition of a portion of an inactivated homogenate at 100^0 to the active homogenate in the course of the experiment does not results in an increase in oxygen consumption. The inactivated homogenate should contain a certain quantity of natural substrates. Despite such an addition a further drop in oxygen uptake was observed. 2) The intensity of respiration is considerably lower when the homogenate is poisoned with KCN, the wearing off of natural substrates should be therefore slower. In spite of this a decrease in O_2 consumption was in this case also observed in the course of experiment, and to a similar degree as in the case of unpoisoned homogenate. It is therefore likely that the decrease of oxygen consumption is due to a gradual but rather rapid inactivation of respiratory enzymes in the homogenate. Experiments have proved that the inactivation of phenoloxidase does not proceed so rapidly. A fall in oxygen consumption was observed also in the case of homogenate poisoned with cyanide in conditions where phenoloxidase, as well as other heavy metal enzymes eventually present, were inactivated. From this we may drow the conclusion that the fall in oxygen consumption is due to a gradual inactivation

of some non-metalic oxidases or of dehydrogenases. The darkning of homogenate may point to a decrease in activity of dehydrogenases. As is supposed by Heller (1947) the presence of active dehydrogenases leads immediately to the reduction of the oxidized derivatives of natural phenols and no compound of the melanin type can be formed.

Experiments with homogenate poisoned with KCN, show an interesting property of the respiratory enzyme system of the insect under examination. Cyanide in concentrations of 10^{-4} and 10^{-3} M blocks the oxygen consumption of homogenate by only 50% or a little more. We know however (Baumann and Myrbäck 1941) that cyanide, in concentrations used by us, blocks almost entirely the activity of cytochrome oxidase. It blocks also, as was seen from our experiments, the activity of phenoloxidase. The rather high level of homogenate respiration, observed by us, after it has been poisoned with cyanide indicates the presence of some cyanide resistant oxidases, flavoproteins maybe.

In investigating the respiratory metabolism in relation to the degree of dilution, we observed a fall in oxygen consumption following the dilution of homogenate. This phenomenon has already been observed by a number of authors on different enzymatic systems (Krebs 1935, Potter and Elvehjem 1936, Potter and Schneider 1942) and was named the „dilution effect". The above named authors explain this effect by a dissociation of enzymes and by the presence of higher degree reaction. „Dilution effect" was observed not only in relation to the endogenous oxygen consumption in homogenate of waxmoth larvae but in relation to the phenoloxidase activity of the examined insect as well.

Some interesting data are furnished by tests with homogenate of dried insects. The dried matter, after being ground with water or with a buffer solution, preserves a large portion of its respiratory activity. Drying has in effect a decrease on endogenous respiration to about 60%, in relation to the fresh substance. Delipidation of the dry material at room temperature does not cause any further decrease in respiratory activity of homogenate. Experiments with dried insects and with insects dried and delipidated prove that part of the respiratory enzymes active in the examined material is not strictly connected with the biological structure of the living substance and preserves its activity even after such a structure is destroyed through drying and delipidation.

Phenoloxidase has proved to be the enzyme which stood up well to the operations of drying and delipidation herein described. Its activity is approximately the same in fresh material as in dried one, and also in dried and delipidated substance (in relation to the fresh substance).

The succinoxidase system was, however, inactive in dried material. We may suppose that one or more of the enzymatic components of this system has been destroyed, which would suggest the more intimate connection with the structure of living matter.

Some resistance of the dried substance to the action of high temperature and of alcohol may be explained by the fact, that proteins in general, and enzymatic proteins too, in anhydrous state are more resistant to denaturation under the influence of these factors.

After it had been ascertained that homogenate from dried, and also from dried and delipidated insects, preserves respiratory activity, the question arose as to whether would it be possible to use a dried and eventually delipidated material to examine the respiratory enzymes of waxmoth. The use of such a material would have some advantages, since it would make it possible the preservation of the material for some days for further examination. Some further experiments however, performed with the use of dried material, induced us to reject this idea, as it turned out that such a material as regards the respiratory enzymes does not correspond to fresh material since: 1) in consequence of drying the endogenous respiration of the homogenate is liable to decrease, 2) drying causes an inactivation of the succinoxidase system, so that dried material does not correspond to fresh material in relation to quality also. It seems, nevertheless, that for certain experiments, such as examination of phenoloxidase, dried insects or dried and delipidated ones can be advantageously used. However, because of gradual inactivation, such material should not be kept longer than 10 days.

In experiments on the respiratory activity of the various fragments of the body of larva of *Galleria mellonella* attention is, in the first place, drawn to the high level of oxygen consumption by homogenate of alimentary tracts. It equals and sometimes even surpasses the O_2 consumption of homogenates of other parts of larva's body, and also of the insect as a whole. As the contents of dry substance in alimentary tracts of waxmoth larva amount to only 20% (W. Niemierko and Włodawer 1952), compared to 44% for the whole body of larva, then the oxygen consumption per unit

of dry substances is considerably higher for homogenate of alimentary tracts than for homogenate of the larva as a whole. It should also be taken into consideration that feeding matter composed a large part of the alimentary tracts which were examined and that only the remainder consisted of the living elements of the intestine walls. We are of the opinion that the high respiratory metabolism of alimentary tracts of the waxmoth larvae may be related to the specific capacity of this insect to digest and to metabolize the wax constituents (W. Niemierko and Włodawer 1950). On the other hand, taking into consideration the existence of rich intestinal flora in waxmoth larva (Florkin et al. 1949, Rybicki 1950) we may suppose that a part of oxygen consumption of homogenate of alimentary tracts may be ascribed to the microorganisms which are present. Interest may be aroused by the lack of phenoloxidase in the homogenate of alimentary tracts and by the lack of succinate oxidation. This points to a certain difference in the respiratory enzymes which are active there.

A number of problems which appeared in the course of the present work require a broader investigation and further work is being devoted to them.

SUMMARY

Experiments were performed on oxygen consumption of homogenates prepared from larvae of *Galleria mellonella* in the last instar and the respiratory enzymes active in the homogenate were examined. The Warburg manometric method was used for measurements.

A method of preparation and examination of tissue homogenate was worked out and optimal conditions of activity defined. Optimal pH was 6.6. „Dilution effect" in relation to the endogenous oxygen consumption and to oxidation of phenol was ascertained. The endogenous oxygen consumption diminishes quickly within one hour after homogenate has been prepared. At a temperature of 30^0 and with a tenfold dilution, Q'_{O_2} within the first 15 minutes amounts on the average to 0.72; after a lapse of one hour it falls to 0.25. The average Q'_{O_2} for one hour is 0.43. Homogenate from waxmoth larva contains highly active phenoloxidase and succinoxidase. Moreover an enzymatic oxidation of ascorbic acid and of some aldehydes were ascertained. An induction period, when phenol was being oxidized, was observed. The oxidation of phenol is accompa-

nied by the appearance of coloured bodies. KCN in concentrations 10^{-4} to 10^{-3} M diminishes the endogenous oxygen consumption by about 50%, while it checks entirely the oxidation of phenol and of succinate. Homogenates from dried and from dried and delipidated material preserve the respiratory activity. The activity of phenoloxidase undergoes no decrease as a result of drying and delipidation. The activity of succinoxidase disappears entirely. The level of endogenous oxygen consumption falls to about 60%. Preparations that are dried, or dried and delipidated, are liable to inactivation when preserved for a long time.

A high level of endogenous oxygen uptake of homogenate of alimentary tracts of larvae, and the absence of phenoloxidase and succinoxidase in alimentary tract, were noted.

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STUDIES ON THE BIOCHEMISTRY OF THE WAXMOTH

(Galleria mellonella)

11. RESPIRATORY ENZYMES IN DEVELOPMENT

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The respiratory metabolism of insects during their development, especially during metamorphosis, has been the object of many investigations. In the course of these studies a characteristic shape of the curve of oxygen consumption by pupae, as a function of their age, was established (Needham 1929, Dobzhansky and Poulson 1935, Heller 1947, Olifan 1949, Tarvit 1951 and others). The respiratory metabolism of the pupa diminishes beginning with the moment of pupation, it then remains for some time at a low level, and finally increases. Just before the imago emergence a recurring fall in oxygen consumption of short duration was usually observed. The course of oxygen consumption just described was observed in many representatives of various orders of Holometabola and seems to be a general phenomenon in the life of pupae. Similar changes in O_2 consumption were ascertained also for the pupae of *Galleria mellonella* (Taylor and Steinbach 1931). In connection with these investigations the question arose as to whether changes in the intensity of respiratory metabolism of pupa have corresponding parallel changes in the activity of respiratory enzymes. Studies on this

subject, small in number, gave on the whole a positive answer. Heller (1947) established that homogenates of pupae of *Celerio euphorbiae* show a dependence of the level of oxygen consumption upon the age of the pupa similar to living individuals. Sacktor (1951) found for pupae of *Musca domestica* a change in activity of cytochrome oxidase of a similar character with regard to the intensity of the pupal respiration: first a fall of the activity, later an increase. Agrell (1949) points to a like course in the change of dehydrogenase activity for pupae of *Calliphora erythrocephala*.

Our knowledge concerning the respiratory enzymes of other developmental stages of insects are much more scarse than these concerning the pupal period. We can mention here the researches of Dennell (1947) on the changes in phenoloxidase activity in the larva of *Sarcophaga falculata* during the prepupal period and a number of works of Bodine and co-workers on the respiratory enzymes of the embryonic stage of *Melanoplus differentialis* (Bodine 1934, 1950, Bodine and Lu 1950, 1951 and other).

The aim of the present work was to gain some comparative data concerning the activity of respiratory enzyme systems in various developmental stages of waxmoth, namely in larval stages and in the pupal period. Here we undertook the task of investigating the younger larval stages and the pupal stage, while experiments on the last instar were taken from our previous work (Wojtczak 1952).

METHODS

Experiments were performed on larvae, pupae and imagines of waxmoth *(Galleria mellonella)* descending from a collective breed, raised at a temperature of 30°. Larvae which were used for experiments were taken out of collective breeds. For research on the pupal stage single full-grown larvae were put in separate vessels and supervised several times a day. Thanks to such a method the moment of pupation could be determined with an accuracy of a few hours. During their pupation larvae were watched through well illuminated cocoons. In doubtful cases a window was cut in the cocoon. Respiration of living pupae was investigated in Warburg apparatus without cocoons.

The activity of respiratory enzymes was examined by measuring the oxygen uptake of homogenates of insects (endogenous oxygen uptake) and the oxidation of substrates which were added to the homogenate. Homogenates were prepared as described previously (Wojtczak 1952). A tenfold dilution with phosphate buffer of pH = 6.6 was used. The experiment was performed in Warburg apparatus at a temperature of 30° and shaking frequency of about 100 oscillations per minute. Following substrates were added in quan-

tities of 1.0 to 1.5 ml after one hour of measurement of the endogenous respiration: 1) phenol, 2% water solution, and 2) sodium succinate 0.2 M. Oxygen consumption was expressed in ml per gram of fresh substance per hour and was marked with the symbol Q'_{0_2}. More details of this method are to be found in our previous work (Wojtczak 1952).

EXPERIMENTAL AND RESULTS

Investigations are started beginning with the younger larval stages of *Galleria mellonella*. To define approximately the age of larvae their weight was taken as base, because a direct definition of insect age was blocked by many difficulties. Larvae which were used for experiments were divided in two groups according to their

Table I

Oxygen consumption of homogenates of waxmoth larvae of the body weight below 40 mg.
10-fold dilution with phosphate buffer, pH = 6.6.

Experiment No.	Endogonous Q'_{0_2} in consecutive 15 min. intervals				Q'_{0_2} phenol added		
3	0,43	0,42	0,34	0,28	0,28	0,29	0,26
5	0,38	0,20	0,24	0,20	0,12	0,06	0,05
6	0,50	0,34	0,41	0,32	0,16	0,11	0,11
9	0,36	0,26	0,25	0,25	0,17	0,17	.
10	0,44	0,38	0,32	0,33	0,23	0,20	0,13
12	0,24	0,25	0,23	0,19	0,09	0,14	.
13	0,45	0,19	0,18	0,15	0,09	0,05	.
15	0,58	0,47	0,33	0,31	0,31	0,31	0,28
16	0,53	0,42	0,35	0,28	0,28	0,29	0,26
Mean from 18 experiments	0,43	0,35	0,29	0,23	0,20	0,19	0,18
Mean for the whole 60 minute period = 0,33							

body weight. The first group included individuals below 40 mg and the second group, individuals from 40 to 80 mg. The body weight of this latter group corresponds to the period of the last larval moult (W. Niemierko and Cepelewicz 1950).

Measurements of oxygen uptake of homogenate were taken. Results obtained in a number of experiments on the endogenous

oxygen consumption of homogenate of the first group of larvae (below 40 mg) are given in Table I. The average values of Q'_{O_2} in consecutive intervals of 15 minutes duration are:

0.43 0.35 0.29 0.23

The average value of Q'_{O_2} for the whole hour is equal to 0.33. The corresponding values for homogenate of larvae of body weight 40—80 mg are:

Q'_{O_2} = 0.47 0.43 0.32 0.23

The average for one hour Q'_{O_2} is 0.36.

Results of the particular measurements are given in Table II.

The average values which are here presented and the results of the particular experiments (Tables I and II) show that the fall in intensity of the respiratory metabolism of homogenate during the 60 minutes of the experiment is less strongly marked in the case

Table II.

Oxygen consumption of homogenates of waxmoth larvae of the body weight of 40—80 mg.

Experiment No.	Endogenous Q'_{O_2} in consecutive 15 min. intervals				Q'_{O_2} phenol added		
1	0,34	0,42	0,32	0,25	0,28	0,31	0,28
2	0,74	0,48	0,31	·	0,45	0,56	·
3	0,25	0,31	0,25	0,20	0,18	0,24	· ·
4	0,42	0,35	0,23	0,18	0,45	0,86	1,00
5	0,52	0,54	0,40	0,29	0,27	0,24	0,23
6	0,52	0,50	0,42	·	0,23	0,17	0,13
Mean	0,47	0,43	0,32	0,23	0,31	0,40	
Mean for the whole 60 minute period = 0,36							

of homogenates, which were here examined, than in the case of full-grown larvae, previously examined (Wojtczak 1952, Table III). In particular experiments (Tables I and II) we observed more than once even a transitory increase in the level of O_2 uptake in the period 15—30 minutes following the beginning of the experiment.

We established next that homogenate of larvae of a weight below 40 mg and also of a weight 40—80 mg oxidizes succinate just as was done by homogenate of full-grown larvae (Wojtczak 1952). More detailed experiments in this direction were not performed.

Homogenate of larvae weighing below 40 mg moreover did not catalyze at all or catalyzed to a very slight degree only the oxidation of phenol. An increase in oxygen consumption was not observed after phenol had been added. In most cases a further fall in Q'_{O_2} took place. In a small number of experiments the level of O_2 consupmtion stays on a steady but low level after phenol has been added, which fact points to a very weak oxidation of this substrate (Table I).

Somewhat different is the case of homogenate of the larger larvae, of a body-weight 40—80 mg. It is true some measurements here also do not show phenol to be oxidized. In some other experiments however homogenates did oxidize phenol to a lesser or a greater degree, as proved by an increase in oxygen consumption after this substrate has been added (Table II). The increase in oxygen uptake after phenol has been added was always accompanied by the appearance of the reddish-brown pigment which is, we suppose, a product of phenol oxidation. In a homogenate which did not show an increase of Q'_{O_2} after phenol has been added, the appearance of a coloured body was not observed.

From the works of Bodine et al. (1939) and Bodine and Allen (1941) we know that phenoloxidase (tyrosinase) may appear in certain insect species *(Tenebrio molitor, Melanoplus differentialis)* in the form of an inactive proenzyme (protyrosinase). This proenzyme goes over to an active form under the influence of sodium oleate. These results induced us to investigate whether in the younger larval stages of waxmoth, in which an active phenoloxidase was not found by us, an enzyme in an inactive form may be present. In this connection we applied a device, made known by the above mentioned authors, of protyrosinase activation by means of adding Na oleate to the homogenate.

0.5 ml of 1% solution of sodium oleate was added to 3 ml of homogenate of larvae *Galleria mellonella* weighing below 40 mg and after 1 hour of incubation with the oleate in temperature 30° phenol solution was added. No oxidation of phenol could, however, be observed. The same procedure was applied to homogenate of larvae of *Tenebrio molitor,* repeating the experiment of Bodine and Allen (1941). The homogenate was prepared exactly in the same way as in the case of waxmoth. The homogenate of *Tenebrio molitor* larvae did not oxidize phenol without oleate, but an intensive oxidation of phenol followed after 0.5 ml of 1% sodium oleate solu-

tion was added to each 3 ml of homogenate. This experiment convinced us that the procedure applied by us of protyrosinase activation was performed properly although in the case of the waxmoth the result obtained was negative.

Experiments were performed on homogenates blocked with KCN at concentrations 10^{-4} and 10^{-3}M. They have shown the oxygen uptake by homogenate of larvae weighing below 40 mg and by those weighing 40—80 mg to be checked by cyanide to a considerable degree.

Table III.

Oxygen consumption of living waxmoth pupae. Mean values.

Pupal age days (*d*) and hours (*h*)	Number of pupae	O_2 uptake ml/g/h.
before pupation	12	1,24
6^h	2	1,28
8^h	15	0,97
10^h	5	0,83
21^h	9	0,77
$1^d\,10^h$	6	0,66
$1^d\,22^h$	4	0,52
$2^d\,21^h$	10	0,51
$3^d\,9^h$	11	0,63
$3^d\,21^h$	8	0,64
$4^d\,9^h$	11	0,73
$4^d\,20^h$	5	0,86
$5^d\,10^h$	10	0,87
$6^d\,10^h$	16	1,25
$6^d\,22^h$	2	1,49
$7^d\,15^h$	7	1,62
$8^d\,3^h$	4	1,54
imago		3—8

Researche on the final larval stage is presented in our previous work (Wojtczak, 1952). We have now taken up the prepupal period. We measured the respiration of homogenates of larvae removed from cocoons just prior to pupation. The level of endogenous oxygen consumption in this case is near to the O_2 uptake of homogenate of feeding larvae in the last instar and on the average amounts to 0.42 ml/g/h. for the duration of a one hour experiment.

We started our investigations on the pupal period by measuring oxygen consumption of living pupae as a function of their age. This part of our work was a repetition of the research of Taylor and Steinbach (1931). The results obtained by us were the same as these obtained by the above mentioned authors and are presented in Table III and in Fig. 1. (curve A). It was established that the level

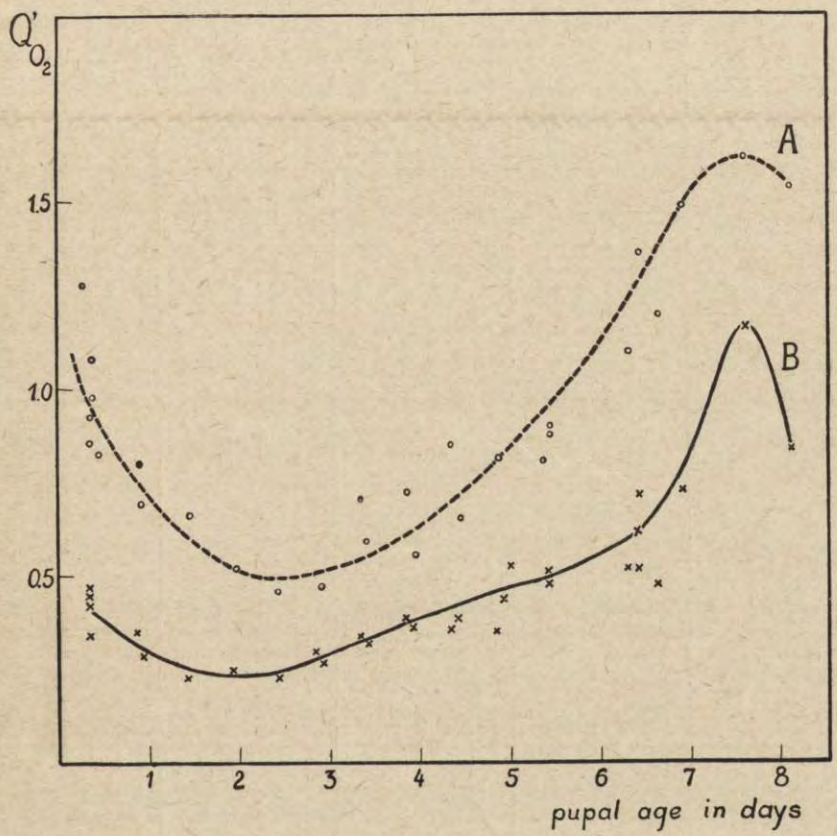

Fig. 1. Oxygen consumption of pupae of *Galleria mellonella* (curve A) and of their homogenates (curve B) in ml per gram per hour (Q'_{O_2}).

of oxygen consumption decreases gradually during the first few days of pupal life, reaching its minimum about the 2nd — 3rd day. From this moment on an increase in O_2 uptake begins, reaching a value above 1.5 ml/g/h. shortly before the emergence of imago. Immediately before the emergence a repeated fall in oxygen uptake, of short duration, was observed.

Next a homogenate of pupae, whose age was known, was prepared and its respiratory activity examined. It has been proved that oxygen uptake of homogenates, measured for a period of 60 minutes, shows changes depending on the age of the pupa, similar

Table IV

Oxygen consumption of homogenates of waxmoth pupae. Mean O_2 uptake in the whole 60 minute period.
10-fold dilution with phosphate buffer, pH = 6.6.

Pupal age days (*d*) and hours (*h*)	Endogenous Q'_{O_2}	Q'_{O_2} phenol added	Succinate oxidation Q'_{O_2}	
			succinate added before	after
Before pupation	0,42	1,13	0,19	0,32
8^h	0,42	0,59	0,26	0,23
21^h	0,32	1,97	.	.
$1^d\,10^h$	0,23	2,10	0,09	0,09
$1^d\,22^h$	0,29	2,16	.	.
$2^d\,10^h$	0,23	1,94	0,07	0,08
$2^d\,21^h$	0,28	2,14	0,08	0,09
$3^d\,9^h$	0,34	1,42	0,06	0,11
$3^d\,21^h$	0,33	2,00	0,08	0,13
$4^d\,9^h$	0,38	1,20	0,12	0,20
$4^d\,22^h$	0,44	.	0,19	0,28
$5^d\,10^h$	0,50	2,37	.	+
$6^d\,10^h$	0,57	2,00	0,25	0,85
$6^d\,22^h$	0,73	1,45	.	.
$7^d\,15^h$	1,17	1,47	0,41	1,68
$8^d\,3^h$	0,84	1,02	.	.
imago	~ 0,8	2,11	0,12	0,82

to changes in the respiration of living pupae. Within two days from the moment of pupation a fall in respiratory activity of the homogenate from 0.42 ml/g/h. shortly after pupation, to 0.23 ml/g/h. for pupae 2 days 10 hours old was observed. This was followed by an increase of Q'_{O_2} until a value of 1.17 for a homogenate of pupae of an age 7 days 15 hours was reached. Immediately before the

emergence a decrease in oxygen consumption of homogenate was also observed (Table IV). Fig. 1. presents the curves of oxygen consumption by living pupae (curve A), and of homogenate (curve B) as a function of age. The course of both curves is alike.

The intensity of O_2 uptake of homogenates of pupae undergoes some changes during the 60 minutes of the duration of the experiment as do those observed by us in relation to homogenates of larvae. Values given in Table IV and illustrated in Fig. 1. (curve B) express the average consumption of O_2 for the entire period of 60 minutes. A more exact analysis of the respiration of homogenates has proved however that curves representing the inactivation of enzymatic systems in homogenates of pupae at various ages are not similar. A homogenate of pupae 8 hours old shows in general a slower inactivation in relation to homogenates of older pupae. Table V presents examples of the results of some characteristic experiments on homogenates of pupae of various ages. Oxygen consumption of homogenate of pupae 8 hours old falls from a value of 0.80 ml/g/h. in the first 15 minutes to 0.28 ml/g/h. at the end of the hour, that is it shows a decrease to 35% of the initial value. Meanwhile oxygen consumption of homogenate of pupae 2 days 10 hours old falls to 15% of the initial value after a lapse of as little as 15 minutes. With the aging of the pupa oxygen consumption of the homogenate increases and at the same time the inactivation of homogenate again appears weaker. For example in the case of homogenate of pupae 6 days 22 hours old the activity after 15 minutes falls to 42% and after one hour, to 15% (Table V). Attention is drawn to the case of homogenates of older pupae the initial oxygen consumption (in the first 15 minutes) of which is, as a rule, somewhat higher than the oxygen consumption by living pupae. For example in the case of pupae 6 days 22 hours old Q'_{O_2} of the homogenate for the first 15 minutes amounts to 1.64, while the oxygen uptake by pupae from which the homogenate has been prepared, amounted on the average to only 1.49 ml/g/h.

After the experiment on the endogenous respiration of the homogenate had proceeded for one hour the following substrates were added: 1) phenol, and 2) sodium succinate. Oxidation of phenol took place during the entire pupal period (Table IV presents the maximal values of oxygen uptake after phenol has been added). At the very beginning only, namely for pupae 8 hours old, the activity of phenoloxidase is rather weak (after an addition of phenol

Q'_{O_2} = 0.59). Later however, beginning with the 24th hour of the pupal life, we observe a rather considerable intensity of phenol oxidation of the homogenate, which corresponds to an oxygen consumption from 1.2 to 2.4 ml/g/h. on the average to about 1.9 ml/g/h. At the end of the pupal period a quite distinct fall in the activity of phenoloxidase takes place and continues until it reaches the value of 1.0 ml/g/h.

Table V

Course of inactivation of respiratory enzymes in homogenates of waxmoth pupae.

Pupal age days (*d*) and hours (*h*).	Oxygen uptake of homogenate in consecutive 15 minute intervals				
		0 — 15′	15′ — 30′	30′ — 45′	45′ — 60′
8^h	in ml/g/h.	0,80	0,50	0,33	0,28
	in per cent of the initial uptake	100	63	41	35
$2^d\,10^h$	in ml/g/h.	0,67	0,10	0,06	0,07
	in per cent of the initial uptake	100	15	9	10
$6^d\,22^h$	in ml/g/h.	1,64	0,69	0,32	0,25
	in per cent of the initial uptake	100	42	20	15

In the case of phenol oxidation we observe an induction period and the appearance of a reddish-brown colouring.

Oxidation of succinate in the homogenate of pupae is subject to interesting changes. In the homogenate of young pupae, until the third day of pupal life, after the addition of succinate no growth of oxygen uptake can be observed. Beginning only from the age of 4 days the homogenate of pupae oxidizes succinate distinctly and with an intensity which increases with age. A maximal consumption of O_2 after succinate has been added amounts to 1.68 ml/g/h. for homogenate of pupae 7 days 15 hours old (before the addition of substrate, 0.41 ml/g/h.), (Table IV).

Finally measurements of respiration of adult insects were undertaken. The O_2 uptake by living butterflies amounted to from 3 to 8 ml/g/h. The spread of the values depends, we suppose, on

the varying mobility of insects. The oxygen uptake of homogenate of young adults amounts on the average during one hour of the experiment to about 0.8 ml/g/h. The homogenate catalyzes to a large degree the oxidation of phenol as well as of succinate (Table IV).

DISCUSSION

Our experiments on the respiratory metabolism of homogenates of waxmoth larvae of various ages indicate that with the development and growth of larvae the respiratory enzyme systems in their tissues are subject to changes. The results of experiments of our present work concerning the younger larval periods will be discussed here together with the results of our previous publication (Wojtczak 1952), in which we were concerned only with the last instar. Such a setting unveils the differences in the activity of the respiratory enzymes of the younger larvae below 40 mg on the one hand and of full-grown larvae, weighing above 100 mg, on the other. Larvae of medium body weight occupied a transitory place with regard to the respiratory enzymes in relation to the two above groups. The differences remarked by us can be brought down to the following points: Homogenates of larvae weighing below 40 mg show in relation to homogenates from full-grown larvae: 1) a lower level of endogenous oxygen consumption, 2) a weaker fall in O_2 uptake during the one hour of the experiment duration, 3) a lack of phenol oxidation.

The lack of phenol oxidation in homogenates of larvae below 40 mg indicates the absence of phenoloxidase, or more strictly monophenoloxidase, in the body of the younger larvae. We have proved at the same time the presence of a highly active phenoloxidase in the homogenates of full-grown larvae (Wojtczak 1952). Taking in consideration the possibility of appearance of an enzyme in an inactive form in the younger larvae we applied the method, made known by Bodine and Allen (1941) to activate protyrosinase by means of sodium oleate. The negative result obtained by us leads to the conclusion that in the younger larvae phenoloxidase-proenzyme also is absent. Phenoloxidase appears therefore in older larvae only and probably at once in an active form.

The studies of W. Niemierko and Cepelewicz (1950) made known to us that the larvae of *Galleria mellonella* are passing through the last larval moult when their weight amounts to from 43 to 87 mg,

63 mg on the average. It leads to the supposition that phenoloxidase appears in the tissues of larvae just in the period of their last moult. Such supposition is confirmed by experiments performed on the other group of larvae weighing from 40 to 80 mg, the homogenate of which usually already catalyzes, to a lesser or geater degree, the oxidation of phenol.

For the time being we are unable to explain the importance of the appearance of phenoloxidase in the tissues of waxmoth during or after the period of the last moult. Moreover the part played by phenoloxidase in insects in general is not sufficiently cleared as yet. In connection with our investigations we may mention here the work of Dennell (1947) who watched the appearance of an active phenoloxidase in the blood and tissues of larva of *Sarcophaga falculata* shortly before its pupation. This enzyme participates in the construction of the pupal cuticle by impregnating it with products of phenol oxidation. The phenomenon observed by us bears some analogy to the one discovered by Dennell, with this difference, that the enzyme in *Galleria mellonella* appears much earlier than in *Sarcophaga*. We suppose that the part played by phenoloxidase in waxmoth is not limited to the impregnation of cuticle. It seems probable that this enzyme, appearing in the last larval period, is strictly connected with the metabolism of this stage as a whole. We know from other experiments (Niemierko et al. in preparation) that the last larval stage of waxmoth, with respect to metabolism, shows a number of differences in relation to the younger periods. The diversity of the respiratory enzyme systems emphasize these differences still more. In the last instar processes which prepare the organism for the pupal period are taking place. It is possible that phenoloxidase also plays an important role in these processes. Dennell (1947) also points to a possible connection between the enzymatic oxidation of phenols and a hormonal control of metamorphosis.

The diversity of respiratory enzyme systems in the younger larval stages is disclosed further by the different course of oxygen consumption of homogenates. The values of Q'_{0_2} fall much slower during the one hour duration of the experiment than was observed in the case of homogenate of full-grown larvae (Wojtczak 1952).

Finally homogenate of younger larvae shows a lower intensity of oxygen consumption (Q'_{0_2} for one hour is 0.33) than the homogenate of larvae in their last instar (Q'_{0_2} is 0.43). This may be a consequence of the lack of phenoloxidase in the younger larval periods.

Supposition have been advanced that phenoloxidase plays the role of a terminal oxidase in respiratory systems of insects (Heller 1947, Sussman 1949). If this supposition could be applied to larvae of waxmoth in the last larval stage where phenoloxidase exists, then in relation to the younger stages, where this enzyme is not found, we must presume the existence of some other oxygen activators. Perhaps the cytochrome system with cytochrome oxidase as final oxygen activator, is present. The presence of the cytochrome system was already established in insect tissues. Among others Sacktor (1951) found cytochrome oxidase in *Musca domestica* and Keilin (1933), cytochrome c in *Galleria mellonella*. In our work we did not investigate directly the presence of cytochrome oxidase. However the existence of succinoxidase in full grown larvae as well as in larvae in younger periods may indirectly point to the presence of the cytochrome system, because, as we know (Straub 1942), the cytochrome system is supposed to be one of the links of the succinoxidase system. Moreover, as we have ascertained, the endogenous oxygen uptake of homogenate is considerably inhibited by KCN, which is a further proof that some oxidative enzymes containing metals are here active. And to such systems, beside phenoloxidase, belongs the cytochrome system.

The studies on the respiration of living pupae and on the intensity of oxygen consumption of homogenates of these pupae have proved a far reaching parallelism of these processes. A similar phenomenon was observed by Heller (1947) in *Celerio euphorbiae* and by Sacktor (1951) in *Musca domestica*. In our work we have not succeeded in defining more precisely the enzymes responsible for such a course of respiration of homogenates. In any case phenoloxidase does not enter the picture, as its activity is kept at a high level during the pupal period and not only does not decrease at the moment af a lowering of the Q_{O_2} of the homogenate and of living pupae but, on the contrary, shows at this moment maximal activity. It seems probable however that the increase in intensity of respiratory metabolism in the second part of the pupal period is conditioned, among other things, by an increase in activity of the succinoxidase system. We are not in possession of data which would indicate which of the components of the succinoxidase system is the cause of the increased oxidation of succinate by homogenate of older pupae. Sacktor (1951) discovered in *Musca domestica* a growth of cytochrome oxidase in a later pupal period. But Agrell

(1949) ascertained in *Calliphora erythrocephala,* a change in the activity of dehydrogenases which run according to the „U" curve in the development of pupa. The increase in dehydrogenase activity in the second half of the pupal life is explained by the author on the basis of a histogenesis of the imaginal organs, especially of muscles.

The results of our research on the pupal stage of waxmoth are therefore a further confirmation of the supposition that a characteristic course of the intensity of respiratory processes in pupae of insects (curve „U") finds its reflection in changes of activity of respiratory enzymes. Our supposition that during metamorphosis not only quantitative, but also qualitative changes, occur in the composition of respiratory enzymes is confirmed by the different course of inactivation established by us during the 60 minutes of experiment duration.

SUMMARY

Oxygen consumption of homogenates from various development stages of *Galleria mellonella* and oxidation of phenol and of succinate were investigated.

In homogenates of larvae, prior to the last moult, no appearance of enzymatic phenol oxidation could be established. Phenoloxidase appears only after the last moult and discloses a high activity in full-grown larvae.

The succinoxidase system is present in the younger larval stages as well as in the last instar.

Oxygen consumption of living pupae of waxmoth runs according to the U-shaped curve depending on the age. The diagram of oxygen consumption of homogenates of pupae at various ages has a similar shape. Phenoloxidase is present during the entire pupal period. Phenoloxidase activity reaches its maximum in the middle of the pupal period. The succinoxidase system shows but a very low activity during the first three days of the pupal life. The activity of the succinoxidase system increases beginning with the fourth day and reaches a high level before the emergence of imago.

A highly active phenoloxidase and succinoxidase were found in the homogenates of adult insects.

The author wishes to express his thanks to Prof. W. Niemierko for his interest and advice during this investigation.

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STUDIES ON THE BIOCHEMISTRY OF THE WAXMOTH
(Galleria mellonella)

12. RESPIRATORY ENZYMES OF THE LARVA DURING STARVATION

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During an investigation of the respiration of starving waxmoth larvae in last instar W. Niemierko and Wojtczak (1950) observed the following phenomenon: the intensity of oxygen consumption of the larvae is quickly diminishing in the first few days of starvation, reaching a level of 0.2 to 0.8 ml/g/h. (oxygen uptake by feeding larvae amounts to 3—8 ml/g/h.) usually between the 5th — 10th day. The degree of respiratory metabolism of starving larvae then remains for some time on a rather low level and in the end, usually between the 6th and the 15th day of starvation, quickly rises to a value of 1.5 to 4 ml/g/h. or even higher, and then remains at this level almost to the death of the animal, that is on the average to the 20th day of starvation or even later. The experimental starvation of larvae was performed in such a way that a ligature was made on the larva just behind its head, preventing pupation and enabling it maintained the larval stage in spite of lack of food. The ligature caused at the same time a complete immobilisation of the animal. It is only at the end of this starvation period that a spon-

tanous increased excitation and mobility of the insect appeared. The period of increased excitation however usually did not appear simultanously with the increase of oxygen uptake. We had therefore to look elsewhere for an explanation of this increase in oxygen consumption. Among other things we directed our attention to a possible connection between the increase in oxygen consumption and the dehydration of the larval body, which takes place in the late period of starvation (S. Niemierko 1950). The character of the respiratory metabolism of the starving larvae of waxmoth bore some superficial resemblance to the changes in the intensity of oxygen consumption by *Galleria mellonella* in its pupal period (Taylor and Steinbach 1931) and by other insects, although in ligated larvae no signs of metamorphosis were ever noticed.

The present study was made with a view to clarifying the question as to whether the changes in the level of O_2 uptake, by living larvae in a state of starvation, show corresponding parallel changes in the activity of respiratory enzymes, as was ascertained for the pupal period of *Galleria mellonella* (Wojtczak 1952a). With this in view we analysed the respiration of homogenates of starving ligated larvae of waxmoth, comparing it with oxygen uptake by living larvae before grinding.

METHODS

Full-grown larvae, weighing 150—250 mg, were used for the experiments. A ligature of strong thread was placed between the head and the first thoracal segment (W. Niemierko and Wojtczak 1950). Such ligated larvae were kept at a temperature of 30° and 75% relative humidity. Oxygen consumption by living larvae and by homogenate was measured in Warburg apparatus at 30°. Preparation and examination of the homogenate were performed in a manner described previously (Wojtczak 1952). Larvae were ground with a ninefold quantity of phosphate buffer of pH = 6.6. Measurements of the homogenate respiration were conducted during one hour and manometer readings were taken every 15 minutes. After one hour the following substrates were added: 1) phenol 2% water solution, 2) sodium succinate 0.2 M. Oxygen consumption was expressed in ml per gram of fresh substance per hour (Q'_{O_2}).

EXPERIMENTAL AND RESULTS

As we have already established the increase in oxygen consumption of starved larvae occured at various times, some on the 6th day of starvation, and for other only after the lapse of 15 days. It was therefore absolutely necessary to examine the individual

intensities of respiratory metabolism of each separate larva prior to the preparation of the homogenate. With this in mind each separate individual at a definite period of starvation, was subjected to an examination of its oxygen consumption in Warburg apparatus. The insects were then separated into two groups, one of which included larvae with a low respiratory metabolism (oxygen consumption below 1 ml/g/h.), the other, individuals whose O_2 uptake showed a rather high level (above 1 ml/g/h.). In accordance with previous observations (W. Niemierko and Wojtczak 1950) larvae that were starved for a short period showed mainly a low level of oxygen consumption, whereas groups of animals which were starved for a long time contained a majority of individuals with a high rate of respiratory metabolism. Homogenates were prepared of both groups and the level of oxygen uptake of the homogenates and oxidation of the substrates were examined.

The results of these experiments are contained in Table I, where the following data can be found: 1) the level of oxygen consumption of living larvae at a specific period of starvation, 2) oxygen consumption of homogenates of these larvae during the first 15 minutes of measurement, 3) oxygen consumption of the homogenates during the entire hour of the experiment, 4) oxidation of phenol, 5) oxidation of succinate. The table contains separately values for lots of larvae with a low oxygen consumption, and for lots of insects with an increased respiratory metabolism.

From the above it may be seen that the average oxygen consumption by living larvae, which are ranked in the second group is 3—4 times higher than in the case of individuals of the first group, whereas Q'_{O_2} of the homogenate of larvae from the second group is only 10%—20% higher. The intensity of phenol oxidation shows considerable fluctuations within both groups, and the average value of oxygen consumption after phenol has been added is higher for larvae with a low level of respiratory metabolism. Oxidation of succinate was very slight in all cases when homogenates of starving larvae were examined.

These measurements concern later stages of starvation. It seemed of interest however to proceed with investigations relating to respiratory enzymes in the initial stage of starvation. We know (W. Niemierko and Wojtczak 1950) that in this period there is initially a rapid, and later a slower, drop in the intensity of oxygen consumption for every individual. We examined here the respiratory

Table I

Oxygen consumption of starving waxmoth larvae and of their homogenates.

Days of starvation	Low oxygen uptake					High oxygen uptake				
	living larvae Q'_{O_2}	homogenate Q'_{O_2}				living larvae Q'_{O_2}	homogenate Q'_{O_2}			
		for the first 15 minutes	for the whole hour	phenol added	increase in Q'_{O_2} after succinate added		for the first 15 minutes	for the whole hour	phenol added	increase in Q'_{O_2} after succinate added
4	0,38	0,47	0,27	2,22	0,03	·	·	·	·	·
13	0,61	0,60	0,42	2,18	·	1,77	0,73	0,42	1,42	0,10
14	0,41	0,52	0,24	3,40	0,06	1,88	0,75	0,36	2,02	0,06
16	0,63	0,65	0,40	1,33	·	1,35	0,82	0,48	1,49	0,10
16	0,52	0,55	0,32	0,83	0,10	1,44	0,55	0,30	0,54	·
17	0,40	0,51	0,29	2,34	·	1,78	0,49	0,25	1,97	·
18	0,46	0,56	0,32	2,38	0,12	1,76	0,59	0,42	1,39	0,18
14	0,40	0,56	0,33	1,42	·	·	·	·	·	·
20 — 22	·	·	·	·	·	1,68	0,61	0,27	0,79	0,06
Mean	0,48	0,55	0,32	2,01	0,08	1,67	0,65	0,36	1,37	0,10

metabolism of homogenates of starving larvae on consecutive days following the beginning of starvation. It was established by us that the level of oxygen consumption of homogenates falls in the first two days of starvation to a level amounting to about 70% of oxygen uptake by homogenate of feeding insects and is sustained on this level. The activity of phenoloxidase is steadily maintained on a rather high level, whereas the capacity to oxidize succinate by homogenate falls rapidly. A homogenate of larvae after two days of starvation oxidizes succinate at almost one-half the rate of homogenate of feeding larvae. After four days of starvation the oxidation of succinate diminishes almost to zero (following an addition of substrate no increase in oxygen consumption can be seen), and stays at this level until the death of the starved larva. The results of these measurements are shown in Table II.

Table II.

Oxygen consumption of homogenates of starving waxmoth larvae in the initial period of starvation.

Days of starvation	Endogenous Q'_{O_2}				Q'_{O_2} succinate added	
	0—15′	15′—30′	30′—45′	45′—60′	60′—75′	75′—90′
Normal larvae (before ligation)*	0,72	0,44	0,31	0,25	0,49	0,55
1	0,79	0,55	0,42	0,38	0,79	0,70
2	0,48	0,33	0,28	0,20	0,43	0,32
3	0,55	0,27	0,19	0,16	0,23	0,21
4	0,43	0,21	0,20	0,16	0,14	0,15

DISCUSSION

The results of the experiments herein described may be summarized as follows:

1) The respiratory metabolism of waxmoth larvae which were starved when ligated shows in the first few days of starvation a very considerable decrease, falling from a value of 3—8 ml/g/h. for feeding larvae, through 2—3 ml/g/h. immediately after ligation, to 0.2—0.8 ml/g/h. after few days of starvation (W. Niemierko and Wojtczak 1950). At the same time the oxygen consumption by homogenates diminishes only by about 30% of the oxygen uptake of the feeding larvae.

* Wojtczak, 1952.

2) In the first four days of starvation the rate of succinate oxidation diminishes considerably.

3) In a later period (about the 6th — 15th day of starvation) the level of oxygen uptake of living larvae is very much increased: 5—10 times, in relation to the minimal oxygen uptake in a but little advanced stage of starvation (W. Niemierko and Wojtczak 1950). The oxygen consumption by homogenate of the same larvae shows an uptake which is only about 10%—20% higher.

4) Homogenates of larvae with a low respiratory metabolism in general oxidize phenol more intensively than homogenates of larvae with an increased respiratory metabolism.

The fall in activity of the respiratory enzymes, which happens in the first days of starvation, concerns largely the activity of the succinoxidase system. The fall of the activity of enzymes on which the endogenous respiration of the homogenate depends is, however, much weaker than the decrease in the intensity of respiration of living larvae. Similarly the increase in oxygen consumption of starving larvae is distinctly accentuated, whereas the increase in O_2 uptake of homogenate is very weak.

In our work on the respiration of waxmoth larvae during starvation (W. Niemierko and Wojtczak 1950) we have put forth a supposition that the increase in oxygen consumption in the later period of starvation may be connected with the increasing dehydration of the insect body, which happens at this stage. It is then possible that a small increase in oxygen consumption of 10%—20% of homogenates of larvae with an increased respiratory metabolism can be explained by an increase in the amount of dry substance and that values calculated on a dry substance would show no difference. Such a supposition requires independant verification.

At present, as previously, an increase in excitation was established in larvae at an advanced stage of starvation. They reacted with violent contraction of the body even to a slight touch. The increased excitation usually succeeded the increase in oxygen consumption. It seems, then, that increased respiration is not dependant on the mobility of the insect and that some other explanation must be sought .The present work showed no parallelism in the changes of activity of the respiratory enzymes of the homogenate and of respiration of the starving larvae, such as were established in the case of the pupal stage of *Galleria mellonella* (Wojtczak 1952a). This does not enable us, however, to assert that the changes in the inten-

sity of respiratory processes of starving larvae could not be related to changes in the activities of enzymes. In order to examine more thouroughly this problem one would have to make a study of the particular enzymes and enzyme systems and of their changes during period of starvation. A suggestion comes to mind that in the starvation period some other mechanisms may be involved other than enzymatic, which regulate respiratory metabolsm. Such may be: 1) nervous influences, and 2) hormonal influences. These possibilities deserve attention, because the ligature which is laid between the head of the larva and the rest of the body destroys in this place the nervous trunk, and at the same time isolates the endocrine glands, which are located in the head. It is very probable that the factors mentioned above react on the respiration of the larva through the respiratory enzyme system, but such a reaction is disclosed in a living organism only, and it vanishes after the homogenate has been prepared.

SUMMARY

Research on the oxygen consumption of homogenates of starving larvae of *Galleria mellonella* was carried out. The intensity of O_2 uptake diminishes during the first few days of starvation to about 60% of oxygen consumption of homogenate of feeding larvae and stays at this level for some time. In the period of increased respiratory metabolism of larvae at an advanced starvation period the intensity of oxygen consumption of homogenate increases by about 10%—20%.

A comparison of the intensity of the respiratory metabolism of starving larvae with one of homogenates prepared from them proves the consumption of oxygen by the homogenate to show no changes in the run of the „U" curve during starvation to the same degree as observed in living larvae.

In the first few days of starvation the activity of succinoxidase is considerably diminished. The activity of phenoloxidase shows, at the same time, no distinct changes. In a later period of starvation, however, it is higher in larvae with a low respiratory metabolism and lower in individuals with an increased level of oxygen consumption.

The author wishes to express his thanks to Prof. W. Niemierko for his interest and advice during this investigation.

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THE EXTRACTION AND FRACTIONATION OF PHOSPHORUS COMPOUNDS IN ANIMAL TISSUES. (Part I)

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The methods in current use for the fractionation of phosphorus compounds in animal tissues (Schmidt and Thannhauser 1945, Schneider 1945, 1946) do not allow a simultaneous determination of water content. The procedure of these methods begins with cold TCA extraction of acid-soluble P-compounds, followed by extraction of lipids with alcohol, hot ether-alcohol (1 : 3) and sometimes hot chloroform. Protein phosphorus and nucleic acids are then determined in the residue. The estimation of both nucleic acid contents is based upon pentose and desoxypentose or phosphorus determination.

Recently Ogur and Rosen (1950) have worked out a new method for nucleic acid determination in plant tissues. They have modified the extraction and fractionation of nucleic acids by using perchloric acid instead of TCA. Their procedure has been as follows: 1) cold extraction with 70% alcohol, 2) hot extraction of lipids with ether-alcohol, 3) removing of perchloric acid-soluble compounds and 4) extraction and estimation in the residue of nucleic acids and of protein phosphorus. The authors checked the method also in rabbit liver and found good agreement with the results obtained according to the procedure of Schneider. The acid soluble phosphorus fraction

has not been examined. In addition the fraction of compounds soluble in 70% alcohol is not clear.

The aim of the present investigations was to work out a method of fractionation of phosphorus compounds in animal tissues which would permit a simultaneous determination of water and lipids, of all the acid soluble phosphorus compounds, and of lipid, nucleic acid and protein phosphorus.

Special attention was paid to obtain reliable results with material particularly rich in lipids such as wax moth larvae which form one of the principal objects of our investigations (W. Niemierko et al. 1950).

The first problem was to find a solvent which would remove all water, dissolve the lipids and in which the other substances, contained in the sample under the investigation, were insoluble. In addition the procedure of dehydration and delipidation must avoid the possibility of splitting of the labile P compounds. As a result of numerous trials the most suitable solvent was found to be a mixture of acetone and chloroform, in the ratio of 5 : 1. The particular advantages of acetone for our purposes are on the one hand, its property of dehydration and, on the other, its ability to dissolve easily all lipids with the exclusion of phospholipids. The addition of a small proportion of chloroform makes it possible to dissolve phospholipids as well. Since, as we have found, an acetone-chloroform mixture does not split all the lipoproteins the final removal of lipids, esp. phospholipids, from these compounds, must be accomplished by means of alcohol-ether*.

PROCEDURE

1. *Removal of water and lipids.* The substance under investigation is chilled in a mortar at a temperature of several degrees below 0°C. It is then extracted with acetone-chloroform mixture (5 : 1), also at a temperature below 0°. This extraction is repeated three times, followed by one or two extractions with chloroform alone and finally two or three extractions with hot alcohol-ether (3 : 1). The total extract is filtered into a volumetric flask. For 1 g fresh weight of tissue a total of about 50 ml of acetone-chloroform mixture, about 10 ml of chloroform, and about 25 ml of alcohol-ether are required.

For estimation of the lipid content of Fraction 1 (containing the dissolved lipids) aliquots are tranferred to small, tared Erlenmeyer flasks. The sol-

*Details of the behaviour of the lipoproteins in connection with the present method will be published later.

vent is evaporated on a water bath and the lipids dried in a dessicator, first over concentrated H_2SO_4, and then over paraffin, to constant weight.

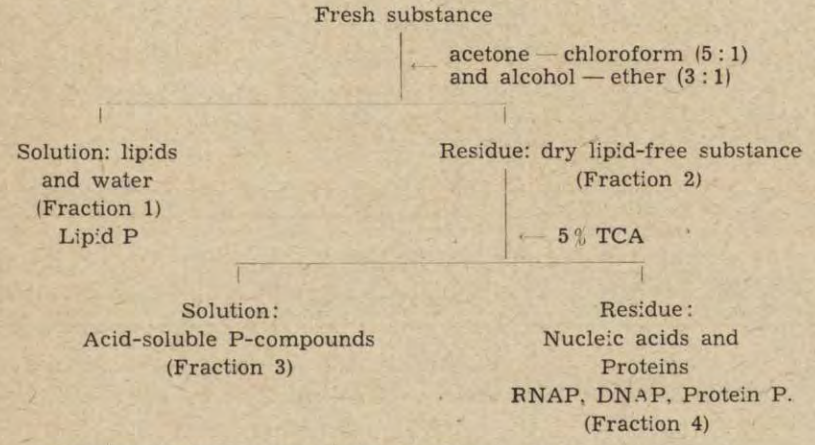
For estimation of lipid phosphorus aliquots of Fraction 1 are evaporated to dryness and digested with conc. H_2SO_4 and HNO_3 (Umbreit, 1945).

2. *Estimation of dry matter.* Following removal of lipids and water, the residue is dried, first freely in air and then under vacuum, over paraffin, to constant weight. (The dry, lipid-free substance is designated as Fraction 2). The total dry matter is the sum of Fractions 1 and 2, and the water content is equal to the Fresh Weight minus the sum of Fractions 1 and 2.

3. *Estimation of acid-soluble phosphorus compounds.* The dry lipid-free substance is transferred to a chilled mortar and treated with cold 5% TCA (10 ml per g fresh tissue weight), following which it is filtered on a chilled funnel into a volumetric flask kept in ice. The TCA extraction is repeated 3 times, according to Umbreit (1945). The different phosphorus compounds are determined in this extract. (Fraction 3).

4. *Determination of nucleic acids and protein phosphorus.* Nucleic acids and protein phosphorus are determined in the residue resulting from stage 3 (Fraction 4). The total phosphorus content of Fraction 4 is determined, following digestion with conc. H_2SO_4 and HNO_3. The separation of nucleic acids and phosphoproteins is achieved by the method of Schmidt and Thannhauser (1945) or Schneider (1946) or Ogur and Rosen (1950).

The entire procedure is schematically as follows:



P determinations were made according to Fiske and SubbaRow.

TEST OF VALIDITY OF METHOD

The reliability of our procedure was tested by analyses on wax-moth larvae, *Galleria mellonella*. First, following lipid extraction with cold acetone-chloroform and hot alcohol-ether, the residue was further extracted with hot chloroform in extraction apparatus. The lipid content of the hot chloroform extract was found to be not

more than 1 or 2% of the total lipids, thus showing that the solvents used quantitatively extract the lipids, even from a substance as rich in lipids as *Galleria mellonella* larvae.

To verify that the solvents used extract no substances other than lipids, samples of fraction 1 were evaporated to dryness, and the residue treated with petroleum ether. It was seen that the entire residue dissolved to give a clear solution. Because of the known specificity of petroleum ether as a solvent for lipids we thus see that, by means of our procedure, the lipids are extracted selectively and quantitatively.

The mean value of lipid phosphorus, found by this method, for one series of experiments, was 57 ± 2 mg%. Using the method of Schmidt and Thannhauser we obtained values from 50 to 60 mg%, and the individual variations were appreciably greater.

Table I

Acid-soluble P compounds in waxmoth larvae.

Method used	P inorganic mg %	P arg. mg %	P_7, mg %	P tot. ac. sol. mg %
TCA extraction from fresh material (Schmidt and Thannhauser)	$14,6 \pm 1,1$	$5,7 \pm 1,6$	$23,1 \pm 2,8$	$106,5 \pm 8$
TCA extraction from lipid-free substance (present method)	$13,5 \pm 0,8$	$6,8 \pm 1,4$	$22,5 \pm 2,6$	$105,0 \pm 5$

The following problems were encountered in connection with the extraction of acid-soluble phosphorus: 1) it must be shown that, following extraction of the lipids, all acid-soluble compounds can be extracted with TCA. Schmidt and Thannhauser assumed that it is more difficult to extract acid-soluble phosphorus compounds from dry lipid-free matter, 2) it must be shown that labile P-containing fractions are not hydrolysed by our procedure.

A comparison was therefore made between analyses of acid-soluble P-compounds obtained a) by TCA extraction of the fresh substance according to the method of Schmidt and Thannhauser or Schneider, and b) extraction of the lipid-free, dry substance according to our procedure. From the results shown in Table I it can be seen that the content of total acid-soluble P, inorganic P, and labile P-fractions, is the same for both procedures.

In order to verify that cold acetone-chloroform does not extract inorganic P, in addition to the lipid P, the following experiments were made. To about 1 g of larvae was added a quantity of KH_2PO_4 containing 0.86 mg P, and the mixture was ground with acetone-chloroform. Subsequent operations were as outlined above. The P content of the deproteinized filtrate was then determined.

From the results shown in Table II it may be seen that the total phosphate added was completely recovered as inorganic P in the fraction of acid-soluble P-compounds. Analyses of the acetone-chloroform fraction showed no increase in the content of lipid P as compared to the result obtained by extraction without prior addition of KH_2PO_4.

Table II.

Weight of larvae taken for analyses g	Inorganic phosphorus			
	Amount present in larval body* mg	Amount added to the sample mg	Total amount in the sample mg	Amount found mg
0,972	0,142	0,860	1,002	1,010
0,926	0,135	0,860	0,995	1,020

It is of some importance to know whether the dry, lipid-free substance may be preserved for some time, since this would be of considerable value when analyses cannot be made immediately.

The lipid-free, dry substance of two groups of ten larvae was analysed immediately, and that of two other groups was kept in a vacuum dessicator for ten weeks. The content of inorganic P in all four samples was found to be nearly identical. This result proves that the labile phosphorus compounds present in the dry lipid-free substance do not undergo decomposition during preservation for at least ten weeks (Table III).

In connection with this it is worth noting that one of us has previously shown (S. Niemierko, 1950) that drying of waxmoth larvae in vacuum over sulphuric acid leads to an immediate decomposition of labile phosphorus compounds and to a considerable increase of the amount of inorganic phosphorus. Contrary to this the present method not only allows the drying but also the preservation of the material, which may be of great practical importance in serial analyses.

* calculated from mean usual content.

Table III.

Inorganic P content in waxmoth larvae calculated as mg% of fresh substance

Samples analysed immediately	Samples analysed after preservation during 10 weeks
12,9	12,7
13,4	14,4

SUMMARY

A new procedure for fractionation of phosphorus compounds in animal tissues is described.

Lipids and water are first removed from the chilled fresh material by means of cold acetone-chloroform (5 : 1); folloved by alcohol-ether (3 : 1). The dry lipid-free residue is afterwards extracted with trichloracetic acid. The amount of particular acid-soluble phosphorus compounds in this TCA extract is identical with that found in the TCA protein-free solution prepared from fresh tissue.

The new method makes possible the determination of the lipid and water content of the material. The dry lipid-free substance can be preserved for a long period of time without decomposition of labile phosphorus compounds.

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ON THE „REDUCING VALUE" OF BIOLOGICAL MATERIAL AND A MICRO-METHOD FOR GLUCOSE AND FRUCTOSE

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Most of the methods for sugar determination in animal tissue are based on their reducing power. The methods are not specific and, depending on the method of deproteinization used, the reducing values obtained may be different. Non sugar reducing substances are chiefly eliminated by deproteinization, but the protein-free filtrate is never completely free from them. A comparison of the reducing value with the value for the „true sugar" obtained by biological methods (using yeast fermentation or an enzymic reaction) shows that the true sugar value may often be not more than one half, or even less, than that obtained from the reducing value. The methods based on reducing value possess many advantages because of their simplicity and the ease of conducting serial determinations, which explains the reason for their widespread use especially in clinics.

During an attempt to use the well-known methods of Hagedorn & Jensen (1923) and Fujita & Iwatake (1931) in analyses upon waxmoth larvae *(Galleria mellonella)* we were confronted with phenomena which made the use of these methods inapplicable. Different volumes of the same protein-free filtrate gave different reducing values. The smaller the volume of filtrate used for ana-

lysis, the higher its relative reducing value. The differences obtained were as high as 50—60%. The results of one series of determinations are illustrated in Table I.

The use of the method of Fujita & Iwatake on solutions of pure glucose gave results strictly proportional to the amount of glucose present. It might therefore be supposed that the irregularities observed in the reducing power of the protein-free filtrate from waxmoth larvae are related to some processes of non-sugar reduction.

Table I

Determination of glucose by the method of Fujita and Iwatake in waxmoth larvae.

Volume of protein-free filtrate ml	Amount of glucose found	
	in the sample µg	in the whole material mg %
2.0	118	330
3.0	140	269
4.0	162	236
5.0	179	207

Heller & Swiechowska (1939) proposed a method for the determination of „non-sugar reduction" by means of the method of Hagedorn & Jensen at room temperature and subtracting the result thus obtained from that found according to usual procedure. Experiments made in the course of the present investigation show that „cold reduction" really does take place in the material under analysis. But, as already pointed out above, the less the volume of protein-free filtrate used as a sample, the higher its relative reducing value. Similar results are obtained whether the analyses are performed at 37° or 50°C, or whether the time of heating is prolonged to 30, 60 or even 180 minutes. The reducing values increase with increase in temperature or time of heating but in no case is a proportionality obtained between the relative volume of the same sample and the relative amount of ferricyanide reduced.

From the above it follows that the methods of Hagedorn & Jensen, Fujita & Iwatake, and perhaps other analogous methods, are occasionally incapable of providing reliable results. In particular cases the methods not only fail to give the „true sugar value", they may not even give the „true reducing value", a fact which does not appear to have been given consideration.

In our search for a more specific and, at the same time, simple method of glucose determination, our attention was drawn to the well-known fact that glucose heated in alkaline medium undergoes enolization and is partially transformed into fructose. (Lobry de Bruyn & Ekenstein, 1910). Since fairly specific colour reactions for fructose are known (e. g. the reaction of Seliwanow) it was decided to attempt the development of a new method for glucose based on its transformation to fructose, the latter being then determined colorimetrically. The method will give reliable results only, of course, if the material under investigation does not contain fructose or contains it in relatively small amounts.

Simultaneously a micro-modification of the method of Roe (1934) has been developed which makes possible the determination of 1—5 μg fructose.

EXPERIMENTAL

The following conditions for the enolization of glucose, most suitable for our purposes, were elaborated as a result of numerous trials. The solution containing the glucose in a phosphate buffer at pH 9.5 is heated on a water bath, at 100°C. Under these conditions a state of equilibrium is reached after 10—15 minutes in which 31—32% of the sugar is fructose. If the pH is less than 9 only small amounts of fructose are formed, while a pH greater than 11.5 causes considerable decomposition of the sugar. A 0.1 M mixture of di- and tri- basic sodium phosphates, in the ratio 19 to 1, was used as a buffer medium. One-half ml buffer solution is added to 1 ml of a standard solution of glucose. Following enolization of the glucose, the fructose formed is determined by the method of Roe (or by our micro-modification) using a Hilger Spekker absorptiometer.

Procedure for samples containing 80—200 μg of glucose („Macro-method")

1—2 g of the material is ground in a mortar with 2 ml 5% $ZnSO_4$ and 2 ml 0.25 N NaOH. It is filtered and washed with small amounts of distilled water and the filtrate brought to 10 ml with water. (The material may be also homogenized in a Potter homogenizer and the insoluble material removed by centrifugation). From 0.5 to 1.5 ml of the protein-free solution, containing 80—200 μg glucose, is transferred to a 10 ml volumetric flask and 0.5 ml of the phosphate buffer mixture added. Sufficient water is then added to bring the volume to approximately 2 ml. At the same time a standard solution of

glucose, containing also 80—200 μg per ml, is treated in the same way. The solutions are heated on a boiling water bath for 15 minutes. They are then cooled and 2 ml of alcoholic resorcinol solution and 6 ml of 25% HCl are added, following which the contents of the flasks are thoroughly mixed, and then heated for a further 10 minutes at a temperature of 80—85°C. After cooling water is added to the mark and the colour determined in the Spekker absorptiometer, using a violet filter.

Procedure for sampls containing 4—15 μg of glucose („Micro-method")

100—300 mg of material are homogenized in the homogenizer of Potter and Elvehjem (1936) with 1 ml of acetone-chloroform mixture (5 : 1) (Niemierko et al. 1952) for elimination of most of the water and lipids. Following centrifugation the acetone-chloroform filtrate is discarded and the precipitate is allowed to dry at room temperature for a few minutes. One then adds 0.4 ml of 5% $ZnSO_4$ and 0.4 ml of NaOH, the mixture is homogenized and centrifuged or filtered. The precipitate is washed several times with a few drops of water. The final filtrate must be nearly neutral, and is diluted to exactly 1.0 or 1.5 ml.

Exactly 0.1 ml of deproteinized solution, and of the standard glucose solution, are transferred to 30 × 7 mm test tubes, and 0.05 ml of the buffer mixture is added. The solutions are then heated at 100°C for 15 minutes, cooled, and 0.1 ml alcoholic resorcinol and 0.3 ml 25% hydrochloric acid added, following which the contents of the tubes are well mixed and again heated for 10 minutes at 80—85°C. After cooling water is added to bring the volume to exactly 0.6 ml and the colour determined in the 0.5 ml microcells of the Spekker absorptiometer, using a violet filter.

The procedure of mixing, dilution and measurement, following deproteinization, are performed according to the technique of Niemierko.

Determination of fructose in amounts of 1—5 μg.

Preparation of the material, deproteinization and the subsequent procedure are identical with the micro procedure described above, except that the enolization step (addition of the phosphate buffer mixture and heating at 100°C) is omitted. The standard solution consists of 0.1 ml containing 1—5 μg of fructose.

Reagents

1. Buffer mixture: 0.1 M Na_2HPO_4 and 0.1 M Na_3PO_4 (19 : 1).
2. 5% solution of $ZnSO_4$.
3. 0.25 n solution of NaOH. Solutions (2) and (3) are prepared according to Roe (1934).
4. 25% hydrochloric acid.
5. 0.1% solution of resorcinol in 96% alcohol.
6. Freshly prepared standard solution of glucose containing in 1 ml — 100 μg of glucose.

Calculation of the amount of glucose in the presence of fructose-containing material

The fructose content of the material under investigation may be directly determined by means of the procedure described above. The enolization of a solution containing either glucose or fructose produces a state of equilibrium in which the relative proportion of fructose is 31—32%, that of glucose 65—67%, and small proportions of other sugars. Therefore, when fructose is present in the material under investigation, the quantity of glucose determined must be corrected by substraction of about 33% of the fructose present. As has been mentioned above, considerable amounts of fructose present in the material under investigation makes, however, the determination of glucose by the present method impossible.

VERIFICATION OF THE METHOD

The accuracy of the above-described methods of fructose and glucose determinations was checked by trials on pure glucose and fructose solutions, and by analyses on waxmoth larvae. The results of these analyses are presented in Tables II, III, IV and V.

Table II.

Determination of glucose in a pure glucose solution.

Amount present µg	Amount found µg	Difference µg	Difference %
„Macro-method"			
106	106	0	0
106	107	1	0,9
109	108	1	0,9
136	136	0	0
136	138	2	1,5
160	163	3	1,9
160	164	4	2,5
254	255	1	0,4
254	257	3	1,2
„Micro-method"			
4,5	4,4	0,1	2,2
4,5	4,6	0,1	2,2
6,8	6,9	0,1	1,5
7,2	7,4	0,2	2,8
7,2	7,2	0	0
9,0	8,9	0,1	1,1
9,0	9,1	0,1	1,1
10,8	10,5	0,3	3,0
10,8	10,8	0	0
13,5	13,7	0,2	1,5

Table III.

Determination of glucose in waxmoth larvae.

Number of larvae	Weight of larvae mg	Glucose content		
		in a single determination µg	calculated for the whole material	
			mg	mg%
„Macro-method"				
3	548	118	0,590	107,5
		116	0,580	105,8
		117	0,585	106,7
3	525	114	0,574	108,5
		112	0,560	106,6
7	1345	118	1,19	88,5
		180	1,20	89,2
7	1397	117	1,17	83,7
		177	1,18	84,4
„Micro-method"				
1	215	12,3	0,191	88,5
		12,0	0,187	86,6
		12,0	0,187	86,6
1	214	15,0	0,225	105,1
		15,2	0,228	106,3
		15,0	0,225	105,1
1	198	13,0	0,207	104,5
		13,1	0,208	105,0
		13,2	0,209	105,5

Table IV.

Determination of fructose in a pure fructose solution.

Amount present µg	Amount found µg	Difference	
		µg	%
1,63	1,60	0,03	1,8
1,63	1,61	0,02	1,2
1,63	1,63	0	0
1,63	1,64	0,01	0,8
3,27	3,22	0,05	1,6
3,27	3,30	0,03	0,9
3,27	3,36	0,09	2,4
4,90	4,86	0,04	0,9
4,90	4,92	0,02	0,4
4,90	4,98	0,08	1,6

Table V.

Determination of fructose in waxmoth larvae.

Weight of larva mg	Fructose content		
	in a single determination µg	in the whole material	
		µg	mg%
270	1,89	43,0	15,9
	1,88	42,7	15,8
269	1,74	39,4	14,7
	1,75	39,6	14,7
266	2,05	46,5	17,5
	2,08	47,0	17,7
251	1,53	34,5	13,7
	1,56	35,2	14,0
296	1,76	40,5	13,7
	1,78	40,7	13,8

It is readily seen that the errors of determination of glucose and fructose in pure solutions do not exceed 3%. Repeated determinations of glucose and fructose in the waxmoth *Galleria mellonella* gave concordant results, irrespective of the concentrations used for analyses. Differences between duplicate determinations did not usually exceed 3%.

SUMMARY

Experiments have been made which show that methods for sugar determination in animal tissue based on their reducing power are occasionaly incapable even to give the „true reducing value".

A new method for glucose is described based on its transformation to fructose, the latter being then determined colorimetrically. The method permits to determine glucose in quantities from 4 to 200 µg with an error of about 3%.

A micro-modification of the method for fructose of Roe is developed which makes possible to estimate 1—5µg of fructose with an error of about 3%.

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OXYGEN CONSUMPTION OF TISSUE HOMOGENATE IMMEDIATELY FOLLOWING HOMOGENIZATION

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In our previous work (Wojtczak 1952) it was established that a homogenate of larvae of *Galleria mellonella* shows a rapid fall in respiratory activity: Oxygen consumption of the homogenate diminishes considerably during the 60 minutes of the experiment duration. The method that was applied by us made it possible to begin measurements only 5 minutes after the cooled homogenate was placed in the bath at a temperature of 30^0. Yet in view of the rapid changes in the intensity of the oxidizing processes that were observed it seemed to be of interest to examine the level of oxygen consumption immediately after the homogenate has been prepared, that is in a period when an examination by the Warburg method is not feasible. An attempt was therefore made to solve this problem in the following way: Larvae were to be ground in a buffer solution of known content of dissolved atmospheric oxygen. When the exchange of gases with the atmosphere is prevented then the processes of enzymatic oxidation will for some time run at the expense of the dissolved oxygen. It may be assumed that for a short enough period, so long as the concentration of dissolved oxygen will be high enough, these processes will proceed at the same rate as would be the case with a free diffusion of atmospheric oxygen to the homogenate.

If the enzymatic reactions in the homogenate were now stopped and the remaining free oxygen determined, it would be possible to count over the intensity of respiratory processes within a short period of time immediately after the homogenate has been prepared. The difficulty consists in finding a device to estimate the free oxygen in the tissue homogenate. While the estimation of O_2 dissolved in pure buffer solutions could be done without special difficulties by the Winkler method (Abderhalden 1931), in relation to the homogenate this method was not applicable because of large contents of organic substances. A number of tests which were performed by us to measure the free oxygen in a biological material by chemical methods gave no positive results.

While making inquiries the method of polarographic estimation of dissolved oxygen, published by Davies and Brink (1942) was noted. The basis of this method is as follows: To the solution under examination are introduced: a platinum electrode as cathode and a calomel (or an AgCl-covered silver) electrode as anode, between which is applied a potential difference of 0.2—0.8 V. With such a system the current flowing through the solution is, under certain conditions, proportional to the amount of free oxygen dissolved in the liquid. The current may be measured with special sensitive measuring devices, and often there is a necessity to use high-gain amplifiers. This method finds its application in industry (Marsh 1951), and above all, in biological studies (Davis et al. 1944, Roseman et al. 1946, Entina and Jakovlev 1951).

We tried to apply this method to measurements of free oxygen in homogenate. Because of technical difficulties connected with the lack of proper equipment the results obtained by us by this method have, for the time being, a tentative character only. Larvae were ground with a ninefold amount of phosphate buffer of pH = 6.6 with an addition of NaCl solution, at temperature of 30°. Previously the content of oxygen, dissolved in the solution, was measured. The enzymatic processes were blocked after 20, 30 or 60 seconds by an addition of trichloracetic acid and the remaining O_2 was measured. In order to prevent a possibility of a diffusion of O_2 from the atmosphere the homogenate was covered with a layer of liquid paraffin. The quantity of oxygen consumed per gram of fresh biological material per hour amounted on the average to 2.2 ml/g/h. (Table I). To date the measurements are not accurate enough and

to small in number to give an idea whether in the first minute after grinding a fall in intensity of respiration has already taken place.

Table I.

Oxygen consumption by homogenate of waxmoth in the initial period after homogenization.
Tenfold dilution with phosphate buffer — NaCl solution, pH = 6.6.
Temperature 30°.
Values obtained by polarographic method.

Period after homogenization in seconds	O_2 uptake in µl per gram	Q'_{O_2} (ml/g/h.)
0 — 20	12	2,2
0 — 20	9,5	1,7
0 — 30	15	1,8
0 — 30	17,5	2,1
0 — 30	33	4,0
0 — 60	45	2,7
30 — 60	11,5	1,4
30 — 60	14,5	1,7
30 — 60	18,5	2,2
60 — 90	17	2,0
		Mean 2,2

The polarographic measurements of oxygen contents in homogenate have shown moreover the following phenomenon: If the homogenate was inactivated after the lapse of 90 seconds or later from the moment of grinding, it showed, as a rule, no presence of oxygen. This proves that the respiratory enzyme system of waxmoth larva has used up, within the limits of accuracy of the measurements, the whole or almost the whole supply of the dissolved oxygen, which means that this system may work at a very low partial pressure of O_2. This is substantiated also by numerous observations on the representatives of various insect species which were able to live at a very low oxygen content in the atmosphere (Wigglesworth 1947).

The polarographic method may be very valuable in researches on respiratory metabolism. We intend therefore to improve the devices used by us with a view to their extended application.

The author wishes to express his thanks to Prof. W. Niemierko for his interest and advice during this investigation.

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THE DETERMINATION OF AMINO ACIDS IN THE PRESENCE OF AMMONIA AND URIC ACID

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The colorimetric method for the determination of amino acids introduced by Folin (1922) utilizes the reaction of alpha-amino groups of amino acids in alkaline medium with beta-naphtoquinone-4-sulphonate. Subsequently this method was modified (Danielson 1933, Frame, Russel and Wilhelmi 1943, Sahyun 1944). The colour reaction is not specific, however. The colour products of condensation are produced also by ammonia, uric acid and some other nitrogen compounds. In our experiments on the nitrogen metabolism of insects (Zielińska, 1952) we had to deal with biological material containing small quantities of ammonia and often large quantities of uric acid. In verifying the reliability of the Folin method to our material we had to show what is the influence of ammonia and uric acid, which are present in the material, on the determination af amino acids.

The first series of analyses was performed to check the amounts of nitrogen found by the above method in a pure uric acid solution. With a variation in uric acid content from a few micrograms to 1.5 mg, the amount found as nitrogen varied from a few to 20 micrograms.

The results obtained made it necessary of performing analyses on standard solutions of amino acids with the addition of known quantities of uric acid. The results are assembled in Table I. Values which were found for amino acid nitrogen often exceed the quantities of amino acids present in the sample. In addition the excess was not proportional to the quantity of uric acid.

Table I.

Determination of amino acids in a pure solution and in the presence of uric acid.

Amount of amino acid nitrogen in the sample µg	Amount of uric acid nitrogen added to the sample µg	Amount of amino acid nitrogen found in the sample µg	$\frac{C}{A} \cdot 100$ %
A	B	C	
15,0	—	15,0	100,0
20,0	—	19,7	98,5
25,0	—	25,4	101,5
30,0	—	30,0	100,0
40,0	—	39,7	99,3
45,0	—	44,9	99,8
50,0	—	50,2	100,3
15,0	12,1	16,6	111,5
15,0	24,2	18,4	122,8
15,0	36,3	21,1	140,5
30,0	12,1	32,2	107,5
30,0	24,2	32,8	109,5
45,0	12,1	44,5	99,0
45,0	24,2	44,9	99,8

It was therefore necessary to find out to what degree the presence of uric acid will influence the determination of amino acids in biological material. For this purpose known amounts of uric acid were added to samples of homogenates prepared from wax moth larvae. After deproteinization, according to Haden (1923), amino acids were determined in the protein-free filtrate. From the results shown in Table II it can be seen that small quantities of uric acid have no influence on the determination of amino acids. But if, in a sample containing 15 to 30 µg amino acid nitrogen, the quantity of uric acid nitrogen is over 10 µg the values obtained for the amino acid N are increased considerably.

Table II.

Determination of amino acids in the homogenate of waxmoth larvae and in the homogenate treated with known quantity of uric acid solution.

Amount of uric acid nitrogen added to the sample µg	Amount of amino acid nitrogen found in the sample µg
—	18,2
1,25	17,2
12,5	20,8
—	17,2
10,0	19,2
30,0	23,8

Table III.

Determination of amino acids in the protein-free filtrate from the waxmoth larvae *(Galleria mellonella)* in presence of different amounts of uric acid.

Material	Volume of protein-free filtrate taken for analysis ml	Amount of uric acid nitrogen in the sample µg	Amount of amino acid nitrogen found in the sample µg/ml	Deviation from the mean value %
Feeding larvae	0,5	2,2	55,8	— 2,4
	1,0	4,4	57,7	0,9
	1,0	4,4	57,9	1,0
	1,0	4,4	59,0	3,2
	2,0	8,8	55,0	— 3,0
			mean value 57,2	
Starving larvae	0,5	35	29,6	4,2
	1,0	70	29,4	3,5
	1,0	70	28,0	— 1,4
	1,5	105	26,6	— 6,3
			mean value 28,4	

Besides, experiments were performed on normally feeding waxmoth larvae, which contain only small amounts of uric acid (18 mg% uric acid N), and on starving larvae rich in uric acid (170 mg% uric acid N; Zielińska, 1952). The figures in Table III indicate that despite the great differences in the uric acid content of the analysed samples, and despite the diverse mutual relation

of amino acid N to uric acid N the deviations from the average values of amino nitrogen fluctuate in the range of a few percent in the given instances. The standard deviation for 60 analysis is ± 4.6%. In a next experiment known amounts of amino acids were added to the protein — free filtrate of larvae. The amino acid added were recovered in about 100% (Table IV).

Table IV.

Determination of amino acids in the protein-free filtrate of waxmoth larvae treated with known amino acids solutions.

Amount of amino acid nitrogen in the sample of protein-free filtrate	Amount of amino acid nitrogen added to the sample	Total amount of amino acid nitrogen present in the sample	Amount of amino acid nitrogen found in the sample	$\frac{C}{A+B} \cdot 100$
µg	µg	µg	µg	%
A	B	A + B	C	
25,1	15,0	40,1	40,8	101,7
25,1	15,0	40,1	41,2	102,7
33,4	20,0	53,4	52,7	98,7
41,8	25,0	66,8	66,0	98,8
50,1	30,0	80,1	77,8	97,1
50,1	30,0	80,1	83,0	104,6
50,1	30,0	80,1	81,8	102,1

The result discussed above indicate that in the biological material, examined by us in the presence of various quantities of uric acid, it was possible to determine amino acid nitrogen with an error about 5%. Only in cases when the amount of uric acid present in the material under investigation is very large the amount of amino acid nitrogen found may be considerably increased.

The influence of ammonia on the amino acid nitrogen determination were investigated in a special series of experiments. To the protein-free filtrate of larvae with a known content of ammonia nitrogen and of a determined content of amino acids, a solution of ammonia chloride was added. The results assembled in Table V show that the total ammonia nitrogen added to the sample is recovered in the range of amino acid nitrogen. The values of amino acids which were obtained by this method include therefore also

ammonia nitrogen and this should be taken in consideration when the exiperimental data are interpreted. In case bigger quantities of ammonia are present in the examined samples a part only of ammonia nitrogen is detected in the range of amino acid.

Table V.

Determination of amino acids in the protein-free filtrate of the waxmoth larvae in presence of known amounts of ammonia.

Amount of amino acid nitrogen in the sample of protein-free filtrate μg	Amount of ammonia nitrogen added to the sample μg	A + B μg	Amount of amino acid nitrogen found in the sample μg	$\frac{C}{A+B} \cdot 100$
A	B		C	
16,0	2,6	18,6	18,4	98,9
16,0	2,6	18,6	18,4	98,9
16,0	1,3	17,3	17,1	98,8
16,0	1,3	17,3	17,1	98,8
32,0	2,6	34,6	33,7	97,4
32,0	1,3	33,3	32,4	97,4
32,0	1,3	33,3	33,0	99,1

SUMMARY

The reliability of the method introduced by Folin to determine the alpha-amino nitrogen of amino acids was verified on biological material containing varying quantities of uric acid and ammonia.

Amino acids added to the protein filtrate can be recovered with an error not exceeding a few percent.

Small quantities of uric acid have no influence on the estimation of amino acids. Quantities over 10 μg of uric acid nitrogen in a sample may considerably increase the amount of the determined amino acid nitrogen.

Ammonia nitrogen in quantities not exceeding a few micrograms in the sample is detected totally as amino acid nitrogen, and this should be taken into consideration in their determination.

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