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MECHANISM OF DIGESTION OF LEAVES OF GREEN PLANTS BY SOME LEPIDOPTEROUS CATERPILLARS

M. RYBICKI

Animal Physiology Department of the University of Warsaw Zoological Institute

(Received 15 May 1956)

Within the group of phytophagous insects the caterpillars of butterflies are — with few exceptions — closely connected with green plants. The problem of the utilization of various components of leaves, among which Hering (1951) puts protein to the fore, has been the object of much investigation.

A varying degree of the utilization of this component of the diet is implied by a number of works. Luciani et Lo Monaco (1897) found that *Bombyx mori* L. caterpillars assimilated 62.31% of azote. Evans (1939a) demonstrated that *Phalera bucephala* L. caterpillars fed on *Corylus avellana* leaves utilized 60% of proteins. On the other hand *Pieris brassicae* L. (Evans 1939b) caterpillars, fed on *Brassica oleracea capitata* utilize 83% of proteinous azote, *Malacosoma neustria* L., fed on *Salix viminalis* leaves 72%, and *Aglais urticae* L., fed on *Urtica dioica* leaves 63%. A comparative analysis of two species of caterpillars, carried out by Rybicki (1954), showed that as regards the ten species of plants the leaves of which were food for *Phalera bucephala* L. caterpillars those fed on *Carpinus betulus* leaves utilized 27% and those fed on *Quercus pedunculata* leaves 83% of azote. *Mimas tiliae* L. ceterpillars fed on leaves

of four species of plants utilized anything from 36% (the *Betula* series) to 71% (the *Tilia* series). No correlation between the azote content of leaves and the degree of its utilization by these two species of caterpillars was observed.

The varying degree of the utilization of plant protein by this group of caterpillars has not so far received any proper explanation, the latter being made difficult by the presence of cellulose membranes in cells and the absence of cellulase in these organisms.

The power of utilizing the protein of cells with undamaged cellulose membranes was the object of some earlier research (Verson 1881; Nazari 1899; Acqua 1916; Biedermann 1919) and its aim was to find an enzyme capable of digesting the plasmatic protein of plant cells.

Acqua and Biedermann claim that *Bombyx mori* and *Gastropacha rubi* caterpillars have no cellulase enzyme. According to the same authors in the guts of these caterpillars existed some protease (which was not however discovered), active in an alcaline environment. The difference between the points of view of these authors concerns the fundamental problem whether the content of cells with undamaged cellulose membranes may be utilized or not. The presence of fully digested cells in pieces of a leaf taken out of the gut, as observed by Biedermann, is a fact which has not so far been explained. Thus the necessity arises to examine the mechanism of the digestion by Lepidopterous caterpillars of the content of the plant cells whose membranes were not damaged during the biting of the leaf.

The organization of the experimental cultures and in vitro experiments made in this work is based on the present author's general observations of the Lepidopterous caterpillars which feed on leaves of green plants under natural conditions. Only few representatives of this group of organisms feed on cultivated plants, while their overwhelming majority feed on wild ones. These observations were associated with a mineral fertilization of soil and with the results of the research of Duchâteau, Florkin and Leclerque (1953) asserting that considerable quantities of potassium besides magnesium occurred in the hemolymph of phytophagous caterpillars. An intimation was made by Rybicki (1954) on the presumable mechanism of the utilization of the content of leaf cells whose cellulose membranes had not been damaged. In

this mechanism a great role was ascribed to potassium ions, which are known to be very mobile in the organism as they do not appear in permanent compounds.

METHODS

The experiments were made chiefly on *Phalera bucephala* L. and *Bombyx mori* L. caterpillars, and on some *Sphinx ligustri* L. and *Smerinthus ocellata* L. ones. *Bombyx mori* caterpillars were bred from eggs and the experiments on them were made during the fifth stage of development. The other caterpillar species were collected under natural conditions: *Phalera bucephala* ones during the second stage of development and the rest during the fifth stage.

The experimental culture was kept in crystallizers in an atmosphere saturated with water vapour at a temperature of 26°. *Phalera bucephala* caterpillars were fed on *Tilia platyphyllos* Scop. (Krüssman 1937; Schneider 1906, 1912) leaves of the tree they had fed on in natural conditions. *Sphinx ligustri* caterpillars were fed on *Spiraea Van Houttei* Zbl. leaves, those of *Smerinthus ocellata* on *Salix caprea* L. ones, and those of *Bombyx mori* on *Morus alba* ones.

The lime twigs whose leaves were eaten by *Phalera bucephala* during the fifth stage of development had been kept for twenty-four hours in KCl solutions of concentrations of 1%, 2% and 3% respectively, while the control caterpillars were fed on leaves from twigs kept in water.

The same experiment was carried out on the same species of caterpillars, applying a KH_2PO_4 solution of concentrations of 0.5M and 1M respectively.

Bombyx mori caterpillars were fed on mulberry leaves picked in the morning. Before they were given to the caterpillars the enzymes in the leaves were inactivated by slowly passing the latter twice through a stream of steam coming of a vessel with boiling water. The control caterpillars were fed on normal leaves. All through the fifth stage of development the caterpillars were weighed every day and their excreta collected daily, preserved in a 10% solution of formalin and stored for further cytological examination.

The gut was taken out of slightly narcotized caterpillars and cut into three sections: fore, mid and hind, in which the pH was measured. The gut content was taken from each section and fixed in Regaud's fixative (Langeron 1949; Romeis 1953).

The microtomic sections of the gut content were stained first with iron haematoxylin (the Regaud method) and then with Janus green.

In the in vitro experiments freshly picked leaves were cut with sharp scissors and put at once into 0.5M KH_2PO_4 and 1M KH_2PO_4 solutions, phosphate buffer of a pH of 9.1, and 10%, 15%, 20%, 25% and 30% $KHCO_3$ solutions. The vessels containing pieces of leaves in the solutions were thightly covered to preserve the concentration of the compound and kept a temperature of 26°C. for two, four and six hours.

The further procedure with the plant material and the excreta of mulberry silkworm was the same as that with the caterpillar gut content. The fixed pieces of leaves from the gut content and those subjected to the action of solutions of various salts were measured under the microscope and their area calculated.

EXPERIMENTS

I. The influence of the concentration of potassium compounds in leaves upon the development of caterpillars

This part of the work concerned *Phalera bucephala* caterpillars only. KCl in the first experiment and KH_2PO_4 in the other were respectively used as compounds containing potassium. The results of the experiments are shown in Table I.

Table I

Results of a culture of *Phalera bucephala* caterpillars fed on leaves kept in various potassium compounds

Date of experiment	Number of caterpillars	Compound and its concentration	Life of caterpillars in days	Results
		KCl		
4.VII	15	1%	9	died
4.VII	18	2%	7	died
4.VII	17	3%	5	died
4.VII	15	control leaves	10	pupae and butterflies
		KH_2PO_4		
28.VIII	20	0.5 M	7	died
28.VIII	20	1.0 M	7	died
28.VIII	20	control leaves	10	pupae and butterflies

The first part of the experimental culture was produced in July, the month of the most intense development of this species of caterpillars. Of the three cultures of caterpillars fed on leaves from twigs which had been kept in various concentrations of KCl for twenty-four hours the caterpillars from the first culture lived the longest without however reaching metamorphosis. The life-time of the caterpillars from the other cultures was shorter, and was very characteristically related to the concentration of potassium chloride in water solution. Since the control caterpillars passed their de-

velopment normally within ten days the result of this part of the culture warrants the suspicion that potassium chloride exert a toxical influence on caterpillars. This is borne out by the fact that the appearance of the leaves supplied was changed and their thicker vesicular bundles were red. Another potassium salt was applied (KH_2PO_4).

As a result of the second part of the experiment the caterpillars also died in seven days (Table I). In all the cultures the caterpillars, some in a lower degree and others in a somewhat higher degree, fed on the first day of the fifth stage. The behaviour of the experimental caterpillars was different from that of the control ones. Throughout the fifth stage of development the caterpillars of the control cultures were constant by feeding with short intervals for rest, while the caterpillars of the experimental cultures kept on being restless and moving about at the bottom of the vessel.

The way of feeding, behaviour and mortality of the caterpillars leave room for the supposition that these phenomena are due to an increased concentration of potassium in the leaves.

II. The histological examination of the gut content of caterpillars

The gut content of three caterpillar species: *Phalera bucephala* L., *Sphinx ligustri* L. and *Smerinthus ocellata* L. was histologically examined. When the gut content was fixed the size of the pieces of leaves consumed was measured (Table II).

Table II

Surface (in mm^2) of pieces of leaves found in caterpillar guts (average of ten measurements)

Name of caterpillar	Surface in mm^2	Name of plant
Phalera bucephala L.	0.09 ± 0.039	*Tilia platyphyllos* Scop.
Smerinthus ocellata L.	0.51 ± 0.039	*Salix caprea* L.
Sphinx ligustri L.	0.35 ± 0.006	*Spiraea Van Houttei* Zbl.

The caterpillars bite off pieces of leaves of varying surfaces and it is not known whether this should be ascribed to the anatomical structure of the leaf or to the morphological features of the mouth organs of the caterpillars or to the physiological condition of the plant cells. It ought to be added that the content of the

Phalera bucephala L. gut comes from a caterpillar killed on the fourth feeding day of the fifth stage of development while the *Smerinthus ocellata* L. caterpillar was killed on the eighth feeding day and the *Sphinx ligustri* L. caterpillar on the ninth day.

The stained microtomic sections of leaves were closely examined under the microscope in order to ascertain the changes undergone by the content of plant cells in various parts of the gut.

a) Fore-gut. pH-8.2*. In this part of the gut the cells are strongly plasmolyzed. Most strongly plasmolyzed are the cells of the lower epidermis and spongy tissue, those in the upper epidermis less so, and those of the palisade tissue the least. Closer observations of the lower epidermis allow quite contentless cells to be found there (Pl. I, Fig. 1, Pl. II, Fig. 1, Pl. III, Fig. 1).

b) Mid-gut. pH-9.1*. The picture of the content of the cells changes in the mid-gut more profoundly. Contentless cells are most often found in the lower epidermis (this tissue is not shown in Pl. I, Fig. 2), traces of plant plasm can very seldom be found in the cells of the upper epidermis.

The cells of the spongy tissue are more strongly plasmolyzed and in many cases clearer. A number of cells of this tissue have preserved cellulose membranes only and in some there are remainders of plasm (Pl. I, Fig. 2, Pl. II, Fig. 2, Pl. III, Fig. 2).

The cells of the palisade tissue undergo further plasmolysis. This can be seen clearly close to the upper epidermis, while in other sections — not visible in the figure — also close to the spongy tissue. The observation of a greater number of sections shows that the plasmolysis of the palisade tissue cells is stronger close to the upper epidermis and the spongy tissue and weaker close to the palisade tissue cells neighbouring with each other. It may be suspected that the access of the „digestive juices" to the leaf cells is considerably easier where they are looser. A difference in structure between the cellulose membranes may account for this. Besides plasmolyzed cells some others, whose plasm is clearer, may be found too. These changes most often occur close to the spongy tissue.

In some cells of the palisade tissue the plasm is shrunk. Closer observation of the contraction of the plasm due to plasmolysis shows that its margins are clear-cut while the plasm in the palisade tissue

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* The pH refers to the *Phalera bucephala* gut.

cells close to the spongy tissue gradually disappears. There is no clear boundary between the dark-stained plasm and the inside of the cell. The dark plasm in the middle of the cell becomes clearer and clearer till its stain disappears altogether, which should be considered the inchoation of the process of the digestion of the plasm. If we admitted that the observed disappearance of plasm were due to more advanced plasmolysis, the observed plasm, occupying less room, should be darker in colour. This picture may be observed in the fore-gut. An uneven margin and clear patches at the sites of the disappearance all show the changes taking place in the plasm.

c) Hind-gut. pH — 8,0 *. The pieces of leaves in the hind-gut are almost completely destroyed. The hind-gut is the place where faeces form. The remainders of vegetable food are crushed and ground by the muscles of the gut and a whole undestroyed piece of the leaf can hardly be found. Only fragments of tissues lend themselves to observation.

Cells of the lower and upper epidermis and of the spongy tissue containing remainders of weakly stained plasm can very seldom be found.

The palisade tissue cells present a highly varied picture. An inconsiderable part of them is devoid of plasm. Cells which have preserved plasm in a higher or lower degree prevail, but there are also many of those which have undergone distinct plasmolysis only close to the spongy tissue.

The picture of cells in this part of the gut is not uniform in all caterpillars and the differences may easily be seen in the figures (Pl. I, II, III, Fig. 3). A diagram showing the structure of the gut and the changes undergone by the plant cells throughout its length is presented in Pl. IV.

The comparison of the microscopic picture of the plasm of the plant cells allows us to observe strong plasmolysis already in the fore-gut and more intense plasmolysis in the mid-gut. The cells in a leaf to which the „digestive juices" find the easiest access undergo the strongest plasmolysis, although the varying degree of permeability of the cellulose membranes may not be devoid of influence here.

Microscopic observation does not show that the cellulose membranes undergo digestion. On the contrary, they are preserved in

* The pH refers to the *Phalera bucephala* gut.

Table III

The degree of the „digestion" of the content of plant cells in the guts of *Phalera bucephala* L., *Smerinthus ocellata* L. and *Sphinx ligustri* L. caterpillars

	Leaf tissue				Leaf epidermis				Total of cells found	
	palisade		spongy		upper		lower			
	Number of cells									
	with plasm	without plasm	with plasm	without plasm	with plasm	without plasm	with plasm	without plasm	with plasm	without plasm
					Phalera bucephala L.					
fore-gut	210	2 0.9 %	243	40 14.1%	79	12 13.2%	69	37 34.9 %	601	91 13.1 %
mid-gut	187	7 3.6 %	148	149 50.5%	63	17 21.3%	33	50 60.2 %	431	223 34.1 %
hind-gut	151	64 29.8%	100	357 78.1 %	6	80 93.0%	5	103 95.4 %	262	604 69.7 %
	548	73	491	546	148	109	107	190	1294	918

				Smerinthus ocellata L.						
fore-gut	266	9 3.3 %	305	32 9.5 %	82	8 8.9 %	96	27 22.0%	749	76 9.2 %
mid-gut	277	54 16.3 %	399	150 27.3 %	55	23 29.5 %	102	50 32.9%	833	277 25.0 %
hind-gut	159	122 43.4 %	52	169 76.5 %	11	35 76.1 %	7	71 91.0%	229	397 63.4%
	702	185	756	351	148	66	205	148	1811	750
				Sphinx ligustri L.						
fore-gut	422	9 2.1 %	209	54 20.5 %	83	19 18.6 %	104	43 29.3%	818	125 13.3%
mid-gut	416	76 15.4 %	253	80 24.6 %	97	30 23.5 %	142	121 46.0%	908	307 25.3%
hind-gut	572	167 22.6 %	92	235 71.9%	6	84 93.3 %	7	105 93.8%	677	591 87.3%
	1410	252	554	369	186	133	253	269	2403	1023

all the sections of the gut. The changes undergone by the plasm of the cells whose cellulose membranes have not been damaged indicate that plasmolysis is followed by a clarification of the plasm either uniform throughout the cell or partial, and a total disappearance of the plasm in many cells of the mid-gut. The movement of pieces of leaves in the caterpillar gut results in the „digestion" of the content of cells with undamaged cellulose membranes.

A quantitative picture of this phenomenon is presented in Table III, where the number of cells with plasm and those without plasm, and their percentage for each section of the gut is given.

In order to obtain the figures contained in Table III undestroyed transverse cuts of leaves were selected on which cells with and without plasm in every tissue were counted. Cells containing even some remainders of the dark-stained plasm were counted as ones having content and those unstained as contentless (digested). Columns showing the number of contentless cells present the percentage of cells digested in relation to the total number of cells in the given leaf tissue found in the given section of the gut.

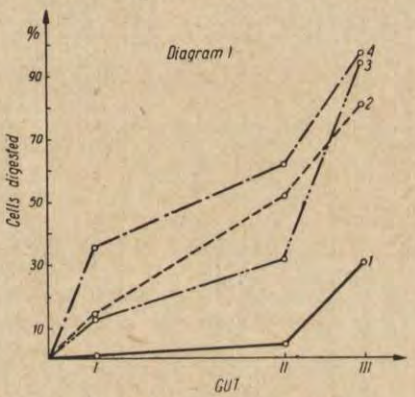
The figures of Table III give a picture of the processes going on in the three parts of the gut. Of the cells making up various tissues of a leaf those of the lower epidermis are the first whose plasm becomes digested in the fore-gut: 34.9% in *Phalera bucephala*, 22.0% in *Smerinthus ocellata* and 29.3% in *Sphinx ligustri*.

The plasm of the spongy tissue cells is digested in this part of the gut considerably less strongly (14.1%, 9.5%, 20.5%), even less so is the plasm of cells of the upper epidermis and that of palisade tissue cells least. It is very difficult to explain this fact. Some of these tissues, e. g., the spongy tissue, are located deeper inside the leaf and yet they are digested in a higher degree than e. g., the upper epidermis, which is exposed to the action of the „juices" in the gut immediately after it is consumed by the caterpillar. As to the upper epidermis, the lower number of digested cells should perhaps be linked with the presence of the cuticle on the outside or perhaps with a different structure of membranes in this part of the leaf.

In the mid-gut the number of digested cells in the lower epidermis continues to grow (this is not the case in *Smerinthus ocellata*). Compared with the number of cells digested in the other tissues their number in the spongy tissue is the greatest. In the mid-gut of *Smerinthus ocellata* caterpillars completely digested cells cannot

be seen in the number observed in the mid-gut of the other caterpillars, yet when more preparations are examined it may be found that in an overwhelming majority of tissue cells of the leaf the plasm is strongly clarified, which could prove that the process of digestion is advanced but not completed. This condition of the cells in this caterpillar species is clearly shown in Fig. 2 of Plate II. Leaves consumed by *Smerinthus ocellata* and *Sphinx ligustri* caterpillars differ in anatomical structure from the lime leaves consumed by *Phalera bucephala* caterpillars. (Compare Fig. 4 in Plates I, II, III). Lime leaves have fairly large air spaces while cells in other leaves particularly in *Salix caprea* are closely packed. It could be assumed that the presence of large air spaces in lime leaves makes the access of the „digestive juices" of the caterpillar easy, while in other plants access to cells is difficult. Hence, too, the rate of the digestion of spongy tissue cells in the mid-gut varies: in *Phalera bucephala* — 50.5%, in *Smerinthus ocellata* — 27.3% an in *Sphinx ligustri* — 24.6%. The age of the caterpillar, particularly the day of development in the fifth stage are not without importance here.

In the hind-gut the plasm of cells in the palisade tissue is digested in the smallest degree, but here, too, the caterpillar species examined differ widely from each other, the difference varying from 23% in *Sphinx ligustri* to 43% in *Smerinthus ocellata.*



Diagr. 1. The degree to which the plant cells are digested in *Phalera bucephala* L. caterpillars in the fore-gut (I), in the mid-gut (II), in the hind-gut (III). The diagram is based on the data put together in Table III.

1 — palisade tissue, 2 — spongy tissue, 3 — upper epidermis, 4 — lower epidermis

In the last vertical column (Table III) the total number of cells digested in various parts of the gut of the caterpillars under examination was given. This number grows from the fore to the hind parts of the gut. It follows hence that the process of the digestion of the plasm of cells with undamaged cellulose membranes varies and depends on the tissue of the leaf. Started in the fore-gut it continues all through the length of the gut (D i a g r a m 1).

III. The influence of the inactivation of plant enzymes in mulberry leaves on the development of *Bombyx mori* caterpillars

The material collected calls for making sure whether the proteases digesting the plasm of plant cells is of animal (Acqua, Biedermann) or of plant origin.

Table IV shows increases in body weight in silk-worm caterpillars fed on leaves with inactivated enzymes and those fed on normal leaves.

Table IV

Average body weight in caterpillars of *Bombyx mori* fed on normal leaves and those fed on leaves with inactivated enzymes.
(Average of 15 caterpillars)

t = 25°

Feeding time	Average body weight in (control) caterpillars fed on normal leaves	Average body weight in caterpillars fed on leaves with inactivated enzymes	Difference in body weight	
days	g	g	g	%
—	0.620	0.638	+0.018	+ 2.9
1	1.215	1.130	−0.085	− 6.9
2	2.719	1.315	−1.404	−51.6
3	3.235	1.520	−1.715	−53.0
4	4.582	1.320	−3.262	−71.2
5	5.730	1.115	−4.615	−80.1
6	5.020	0.976	−4.044	−80.1
7	4.270	0.850	−3.420	−80.1
8	3.364	0.820	−2.544	−75.6
	spinning of cocoon	death		

Observation made while the culture was kept showed no difference in the manner of feeding. Leaves with inactivated enzymes were just as readily consumed as normal leaves were by the control caterpillars. The results obtained from the culture might arouse the suspicion that the factor used for the inactivation of the enzymes might cause some deep changes in the plasm of the leaves so as to involve the mortality of the caterpillars. With this possibility in view the faeces of both series of caterpillars, collected on the first and the fifth days of feeding were subjected to cytological analysis.

The microscopic pictures if leaf sections from the faeces of the control caterpillars collected on the first day of feeding show an almost complete digestion of the plasm in spongy tissue cells and a very slight one of the plasm of palisade tissue cells. In the faeces collected on the fifth day of feeding the process of digestion is far more advanced (Pl. V, Fig. 1, 3).

In the leaf cells from the faeces of the caterpillars fed on leaves with inactivated enzymes the plasm is present everywhere on all feeding days (Pl. V, Fig. 2, 4).

The caterpillars fed on leaves with inactivated enzymes show smaller increases in body weight than those fed on normal leaves. These smaller increases must probably be due to the processes of the assimilation of the content of those cells which were damaged while the leaf was bitten. If we assume that this increase is apparent and due to the presence of food in the gut, we must assume that the caterpillars were in the condition of complete hunger for eight days. They could not have survived the temperature of 25° for so many days.

The result of this culture allows the conclusion to be drawn that the process of the digestion of the content of plant cells with undamaged cellulose membranes involves the enzymes contained in the plant cell, yet it takes place with the participation of the caterpillar.

Pieces of leaves in the faeces of the caterpillars fed on leaves with inactivated enzymes are not destroyed. This difference is clearly shown in the photographs (see Pl. V, Fig. 2, 4). The impression is obtained from the observation of more histological preparations that pieces of leaves with inactivated enzymes have preserved their elasticity, and owing to this their structure is not destroyed in spite of the action of the muscles of the alimentary tract on them while faeces are formed.

IV. Experimental in vitro digestion of pieces of leaves

In this series of experiments various KH_2PO_4 concentrations were made use of as warranted by the results of the experiments on the caterpillars (Table I).

a) Action of 0,5M KH_2PO_4 for two hours. Slight plasmolysis of the spongy tissue cells neighbouring directly with

the lower epidermis was observed in the preparations. The plasmolysis is weaker in the cells most distant from the lower epidermis and hardly noticeable in the cells of the palisade tissue adjoining the spongy tissue. The cells of the upper and lower epidermis undergo strong plasmolysis. There are swollen chloroplasts in the cells of the palisade, and spongy tissues. The changes in the cells are shown in Pl. VI, Fig. 1.

b) Action of 1M KH_2PO_4 for two hours. (Pl. VI, Fig. 2). The same changes as in experiment (a) except that the chloroplasts are darker in colour and more swollen.

c) Action of phosphate buffer with a pH of 9.1 for two hours (Pl. VI, Fig. 3). The picture of the cells in this experiment, too, shows slight changes in comparison with the picture obtained in experiments (a) and (b). The spongy tissue cells are more strongly plasmolyzed and so are the cells of the palisade tissue adjoining the spongy tissue. The upper epidermis cells are strongly plasmolyzed. Besides an advanced plasmolysis some single cells completely devoid of plasm may be found in this tissue. Chloroplasts, swollen.

In these three in vitro experiments were found no changes which would prove any considerable influence of the factors applied on the cellular plasm.

In the following experiments potassium salt of the weak acid occurring in the animal organism was used.

d) Action of 10% $KHCO_3$ for two hours. Some cells of the spongy tissue are slightly plasmolyzed and in many the plasm is strongly clarified. Chloroplasts, distinctly stained, most frequently placed on the margins of cells.

e) Action of 15% $KHCO_3$ for two hours. The picture of the plant cells is the same, except that the plasmolysis in the cells of all leaf tissues is more distinct.

f) Action of 20% $KHCO_3$ for two hours. Cells, more plasmolyzed. Plasm, homogenous. Chloroplasts, inconspicuous. Some cells of the spongy tissue are clearer and few cells in the upper epidermis contain no plasm.

g) Action of 25% $KHCO_3$ for two hours. Almost all cells of the spongy tissue are clarified and plasmolyzed.

h) Action of 30% $KHCO_3$ for two hours. (Pl. VII, Fig. 1). Most cells of the lower and upper epidermis preserved remains of the plasm. Digested cells are rare. The plasm of the

spongy tissue cells is clarified and digested cells are rare. The cells of the palisade tissue are distinctly plasmolyzed and their plasm clarified particularly close to the spongy tissue.

i) Action of 10% $KHCO_3$ for four hours. Plasmolysis, hardly distinct. Chloroplasts, observable in few cells. The plasm of the spongy tissue cells is strongly bleached and looks like a reticulum.

k) Action of 15% $KHCO_3$ for four hours. A distinct difference in the intensity of colour between the plasm of the spongy tissue cells and the palisade tissue cells. The plasm of the spongy tissue cells and of the lower epidermis is clear and chloroplasts on the margins of cells are rare. The plasm of the cells of the upper layer of the palisade tissue (under the upper epidermis) is stained dark and weakly plasmolyzed, its lower layer, above the spongy tissue, is clearer and their plasmolysis stronger.

l) Action of 20% $KHCO_3$ for four hours. Few of the cells of the lower epidermis are devoid of plasm (the inside of the cells is clear and transparent). The plasm of few cells of the spongy tissue forms a reticulum. Chloroplasts on the margins of cells can seldom be found. The plasm of the palisade tissue cells does not look like a uniform homogenous dark-stained mass, but is strongly clarified and slightly plasmolyzed; the outlines of the chloroplasts can very seldom be distinguished.

m) Action of 25% $KHCO_3$ for four hours. Only few of the cells of the upper epidermis contain remains of plasm. A considerable number of cells in the spongy tissue have preserved remains of plasm, the latter gathering next to the cellulose membrane. There are few chloroplasts in the remains of plasm. Cells of the palisade tissue are more strongly plasmolyzed close to the spongy tissue. In most cells the plasm is strongly clarified. Digested cells are very rare.

n) Action of 30% $KHCO_3$ for four hours. (Pl. VII, Fig. 2). Remains of plasm have preserved in single cells of the lower epidermis. A great many cells of the spongy tissue have no plasm. In the other cells of the tissue plasmolyzed cells, some of which have preserved remains of plasm, are very rare. Few of the cells of the palisade tissue are devoid of plasm. In the others the plasmolysis is far advanced close to the spongy tissue. Many of the palisade tissue cells have preserved changed plasm. It stains con-

siderably more weakly and shows many bleached patches. Chloroplasts may be found very seldom in some cells.

o) Action of 30% $KHCO_3$ for six hours. (Pl. VII, Fig. 3). The lime leaves used in this experiment were picked from the same tree as those used in the former experiments, yet they were picked a month later (Oct. 20). No digested cells. Plasm preserved even in the cells of the lower and upper epidermis. The cells of the spongy tissue show little plasmolysis. Chloroplasts, conspicuous. Cells showing slightly bleached plasm are very seldom found in this tissue. The cells of the palisade tissue are slightly plasmolyzed.

Table V

The degree of the disappearance of the plasm in leaf cells of *Tilia platyphyllos* under the action of 30% $KHCO_3$

t = 26°C

Time of action of solution in hours	Leaf tissue				Leaf epidermis				Total of cells found	
	palisade		spongy		upper		lower			
	Number of cells									
	with plasm	without plasm	with plasm	without plasm	with plasm	without plasm	with plasm	without plasm	with plasm	without plasm
2	201	6 3.0%	457	160 35.0%	96	49 51.0%	111	88 70.3%	865	303 35.0%
4	226	14 6.2%	403	274 68.0%	107	105 98.1%	108	107 99.1%	844	500 59.2%
6	232	0 0.0%	379	0 0.0%	89	0 0.0%	98	0 0.0%	798	0 0.0%

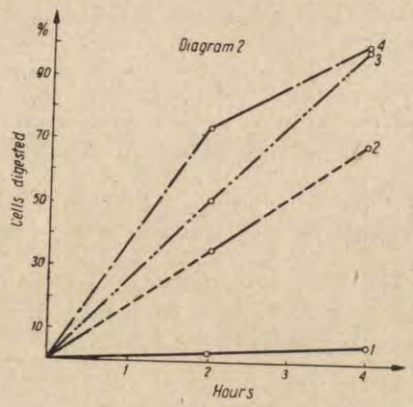
Table V shows the process of the disappearance of the plasm in quantitative terms under the influence of 30% $KHCO_3$ acting for various times.

It follows from the figures contained in the table that in proportion to the time and the concentration of the salt (but not the age of leaves) the order of the disappearance of the plasm in various tissues is the same as in the gut: lower epidermis < upper epidermis < spongy tissue < palisade tissue (Diagram 2).

The disappearance of the plasm in leaves under the action of potassium salt depends on time: in two hours 35%, in four 59%. The latter figure is only 10% smaller than the one found for *Phalera bucephala* fed on lime leaves (Table III). In order to ascertain the

reasons for the difference in the number of cells digested in vitro and in the gut, attention was paid to the size of pieces of leaves subjected to the action of salt in vitro and those found in the gut of the caterpillar. The area of pieces of leaves used in the in vitro experiments was 33 times as large in the two-hour experiments and 15 times as large in the four-hour experiments as the area of pieces of leaves found in the guts of the caterpillars.

The negative result of the action of 30% $KHCO_3$ for six hours leaves room for the supposition that as the leaf grows old the changes taking place in it make it difficult for the solution applied to penetrate inside the cells; therefore they must be changes undergone by the cellulose membranes.



Diagr. 2. The degree to which the plasm of various tissue cells of *Tilia platyphyllos* leaves disappears under the action of 30% $KHCO_3$. The diagram is based on the data put together in Table V.
1 — palisade tissue, 2 — spongy tissue 3 — upper epidermis, 4 — lower epidermis.

The application of a model approximately illustrating the possible process of the digestion of the content of plant cells in the gut of the caterpillars makes it possible to anticipate that electrolytes, of which potassium is the most important, participate in the process of the disappearance of the plasm. The action of potassium on various tissues of the leaf in vitro is about the same as in the gut and depends on the age of the leaf and the concentration of the electrolyte.

DISCUSSION OF RESULTS

The results obtained in these experiments seem to confirm the correctness of the hypothesis that potassium must be of essential importance in the mechanism of the digestion of the plasm contained in cells with undamaged cellulose membranes. What is meant by this is that there must probably be some difference in the concentration of potassium in the leaf and the lumen of the gut for a caterpillar to be able to utilize the plasm of the fragment of leaf it has

consumed. The correctness of this hypothesis is borne out by the results of the experiments as collected in Table I.

The cells of the leaf taken out of any of the three sections of the gut of the caterpillar are characterized by plasmolysis, the extent of the latter being dependent on the section of the gut and the tissue of the leaf. Hence two important conclusions:

1° The plasmolysis of plant cells indicates the presence of considerable concentrations of electrolytes in the lumen of the gut.

2° The degree to which cells are plasmolyzed depends on their physiological condition.

The fastest and most extensive plasmolysis occurs in the cells of the lower and upper epidermis, it is slower and less extensive in those of the spongy tissue, reaching a very low degree in the cells of the palisade tissue. This order and extent of plasmolysis is characteristically related to the order in which the plasm disappears in the cells of these tissues, as shown in Table III.

The observation of the cells in pieces of leaves from the hind-gut shows that the content of the cells of the palisade tissue was digested in the lowest degree (29.8%, 43.4%, 22.6%, Table III). This seems to imply that the reasons for the varying assimilation of protein by the caterpillars (Luciani et Lo Monaco 1897, Evans 1939a, 1939b, Rybicki 1954) should be ascribed to the physiological condition of the cells of the leaf consumed and probably to the structure of the cellulose membranes of these cells as well.

The degree to which the cytoplasm is digested also depends on the anatomical structure of the leaf, the arrangement of cells in the palisade and spongy tissues. This is shown in the photographs of pieces of a leaf from the mid and hind gut of *Smerinthus ocellata* caterpillar (Pl. II, Fig. 2, 3), where the cytoplasm of the palisade and spongy tissue cells is evenly clarified.

The access of electrolytes to loosely packed cells is easier, this being indicated by the degree to which they are plasmolyzed.

A high pH of the gut of *Phalera bucephala* caterpillar (9.1), the fast plasmolysis of the cells in particular which do not make up a compact tissue, and the results obtained by Verson (1881), showing that the alkaline character of the intestinal juice of mulberry silk-worm caterpillar is due to the presence of potassium carbonate, all allow us to assume that all the hitherto observed and described changes undergone by the cytoplasm of plant cells in the

alimentary tract of the caterpillar under examination are also brought about by potassium ions. This interpretation is also borne out by the fact that potassium occurs in very labile compounds in living organisms and this causes its great mobility.

Despite different results of their experiments both Acqua and Biedermann represent the view that the digestion of the cytoplasm of plant cells is due to a specific protease produced by the animal.

On the other hand the results of the experiments on *Bombyx mori* caterpillars in this study (Table IV) and the picture of the plasm of the cells of pieces of leaves from the excreta of those caterpillars both support the view that enzymes contained in the plant cell must participate in the mechanism of the digestion of cytoplasm.

The material collected hitherto allows us to lay stress not on the enzymes of the animal but on those of the plant, their action being contributed to by the animal.

The contribution of the animal to this process thus seems to consist in:

1° biting off and consuming a piece of a leaf of a suitable area determined by the anatomical structure of the leaf;

2° forming in the lumen of the gut a suitable alkaline environment which amounts to 9.1 in *Phalera bucephala* caterpillars;

3° throwing electrolytes — and potassium ions in all probability (Verson) — into the lumen of the gut, which results in the immediate plasmolysis of plant cells;

4° the rate of shifting the food consumed in the gut, which is related to the physiological condition of plant cells;

5° the rate of the digestion of the cytoplasm of the cells as a joint action of all factors prevalent in the gut, which is related to the structure of the cellulose membranes changing during the vegetative period of the leaf.

It may be anticipated on the basis of the experimental material collected hitherto that the digestion of the cytoplasm of plant cells of undamaged cellulose membranes results from the action of enzymes contained in the plant cell, the action of these enzymes in this direction being activated by potassium ions secreted by the intestinal epithelium of the caterpillar into the lumen of its gut.

The correctness of this interpretation is borne out by the results of the in vitro experiments. The picture of the cytoplasm of cells

of a lime leaf as obtained by the action of 30% $KHCO_3$ (Pl. VII, Fig. 1, 2) seems to indicate that the model applied reproduces to a great extent the processes occurring in the gut.

A number of objections might be made to the model applied.

The first of them concerns the potassium salts used in the experiments. These salts are characterized in Table VI.

Table VI

Characteristics of potassium compounds used in the in vitro experiments t = 26°

Date of experiment	Compound	Concentration	pH	Potassium content in g/l	Potassium content in m. equiv/l
4.IX	KH_2PO_4	0.5 M	4.1	19.549	500.0
4.IX	„	1.0 M	4.0	39.100	1000.0
4.IX	phosphate buffer		9.1		
30.VIII	$KHCO_3$	10%	8.7	39.054	998.8
30.VIII	„	15%	8.9	58.581	1498.2
30.VIII	„	20%	8.8	78.108	1997.6
30.VIII	„	25%	9.4	97.639	2497.2
30.VIII	„	30%	9.0	117.162	2996.5
24.IX	„	30%	9.0	117.162	2996.5
20.X	„	30%	9.0	117.162	2996.5

It follows from the comparison of figures contained in Tables VI and V that the degree to which cytoplasm is digested in vitro is related to the concentration of potassium, its pH, the duration of its action and the vegetative period of a leaf (the temperature being the same for all experiments).

As to the content of potassium in the caterpillars, the figures found in literature concern its concentration in haemolymph only and there are no figures concerning potassium content in the lumen of the gut. This leaves room for doubts whether the model applied in the experiment approximately corresponds to the conditions prevailing in the gut.

Histological research carried out by many investigators has proved that the epithelium of the gut of the caterpillar fulfils a secretory function. The secretion of the epithelium is however unknown and conclusions concerning its properties can be drawn from the pH of the gut, very high in phytophagous caterpillars (Jameson and Atkins 1921, Staudenmayer, Stellwaag 1939.

While relating the high pH of the gut to the secretory function of the epithelium and taking into account data from a paper by Verson which show that the alkaline character of intestinal juice is due to potassium carbonate, we have to assume that the secretion of the epithelium contains — among other possible components — a considerable quantity of potassium. The strong plasmolysis of plant cells, taking place already in the fore-gut seems to confirm this interpretation. The phenomenon of plasmolysis of the intensity observed in the gut may be brought about by electrolytes only, and since permanent compounds of potassium in organisms have been unknown, it may be assumed that the plasmolytic effect observed in the plant cells of pieces of leaves in the gut of the caterpillar is produced by large quantities of potassium thrown into the lumen of the gut.

The same, in proportion to the concentration of the solution used, may be observed in pieces of leaves applied in the model.

The high degree to which the plant cells are plasmolyzed in the gut and in vitro allows for a comparison to be drawn with the behaviour of leaf cells under the influence of potassium ions.

A closer analysis of histological preparations coming from the caterpillar gut and of in vitro experiments shows a gradual disappearance of the plasm in many cells, this being marked by all transition stages: the clarification of the plasm, the decrease in the quantity of the plasm, its remains, and finally, cells entirely devoid of the plasm. The similarity between the pictures of changes in the plasm of the plant cells in the gut and in the model applied is one more proof for the assumption that the decomposition of the protein of the cytoplasm in cells with undamaged cellulose membranes is effected under the participation of enzymes contained in the plant cell.

The high concentration of potassium used in the in vitro experiments may suggest another doubt: whether the changes observed in the cytoplasm are not due to its chemical decomposition without any plant enzyme contributing to them. The cytoplasm may be removed with the help of several factors and such techniques are often used in the cytology of plants. Eau de Javelle, chloral hydrate and other factors, all together referred to as bleaching agents are among those most frequently resorted to.

It seems that the disappearance of the plasm in the in vitro

experiments cannot be compared with its disappearance brought about by the action of Eau de Javelle or other bleaching agents.

Eau de Javelle dissolves the plasm of cells in all leaf tissues at the same rate, whereas a 30% $KHCO_3$ solution acts on the plasm of various tissues in an order almost analogous to that observed in the caterpillar gut.

The quantitative results of the action of 30% $KHCO_3$ on pieces of leaves as shown in Table V calls for an elucidation of two points:

1° the number of cells digested in vitro as compared to the number of cells digested in the gut;

2° the time of in vitro digestion as compared to the time the food remained in the caterpillar gut.

The number of cells digested in vitro in two hours amounts to 35% and this value roughly corresponds to the number of cells digested in the mid-gut of the *Phalera bucephala* caterpillar (34.1% — Table III).

The total number of cells digested in vitro under the action of 30% $KHCO_3$ for four hours is 10% lower that the number of digested cells found in the hind-gut of the same caterpillar.

If the number of cells digested in vitro under the action of a 30% solution of $KHCO_3$ for four hours is considered as a result of the action of plant enzymes, it must be admitted that the model applied reproduces the process going on in the gut in a fairly accurate manner. The high concentration of potassium in the solution used could be partly justified by a considerable difference between the area of pieces of leaves used in the in vitro experiments and that of the pieces found in the caterpillar gut. The ratio of the former to the latter is 33:1 in the first experiment (two hours), 15:1 in the second experiment (four hours) and 21:1 in the third experiment (six hours).

According to Acqua (1916) the time during which vegetable food remains in *Bombyx mori* caterpillar in the fifth stage of development amounts to anything between 1.2 and 2.5 hours, and to 1.6 hours on an average for the five caterpillars examined. Białaszewicz (1937) found that the time for which food remained in the gut of *Bombyx mori* caterpillar at a temperature of 22 to 25°C. since the cuticle was cast off amounted to 1.0 to 7.7, and to an average of 3.2 hours for three caterpillars and ten measurements, while in a caterpillar observed after a feeding period of 14 hours in the fifth development stage the time taken by the food

to shift in the gut amounted to anything from 0.9 to 1.9 hours, and to an average of 1.2 hours of seven measurements. The rate at which the food shifts in the gut is undoubtedly related to the feeding day as counted from the casting off of the cuticle and to the temperature, which some studies leave out of account. On the other hand according to Sakurai (quoted after Shinoda 1931) the average time taken by the food to shift in the gut of mulberry silk-worm caterpillar amounts to slightly over three hours in the fifth development stage. The time for which the food remains in the gut of silk-worm caterpillar varies widely but no mistake will be made if we assume that it amounts from two to three hours on the average.

The time of the in vitro action of a 30% $KHCO_3$ solution on pieces of leaves (in the second experiment) is one hour longer than the time for which the food remains in the caterpillar gut and the number of cells digested during the former is 10% lower than the number of cells digested in the caterpillar gut. These differences may be ascribed to:

1° the area of pieces of leaves used in the in vitro experiments, 15 times as large;

2° the age of the leaves (24.IX), which were one month older than those used in the first experiment (two hours, 30% $KHCO_3$ — 30.VIII) and those the caterpillars were fed on (28.VIII).

The conclusions based on the experimental material support the assumption that the disappearance of plant plasm observed by Biedermann (1919) in *Gastropacha rubi* and by the present author now in *Phalera bucephala, Sphinx ligustri, Smerinthus ocellata* and *Bombyx mori* caterpillars is due to the action of plant enzymes.

Some very cautious remarks on the subject are made by Biedermann when he writes: „Es ist im gegebenen Falle ausnahmslose Regel, dass die Stärkeeinschlüsse der Chloroplasten später gelöst werden als diese selbst, und man kann an verschiedenen Zellen alle Stadien des Vorganges beobachten" (p. 423).

Biedermann does not attempt to interpret the mechanism of the dissolution of the stroma of the chloroplasts but it follows from his view thus formulated that plant enzymes take part in this process, although he is of the opinion, „...dass im Verdauungssekret der Insekten entweder eine Protease enthalten ist, die spezifisch auf die Proteide des pflanzlichen Plasmas eingestellt ist, ..." (p. 424).

Acqua, on the other hand, sets much store by the disappearance of starch in mulberry leaves consumed by caterpillars. Although he assumes the presence in the gut of a specific protease produced by the epithelium of the caterpillar gut, his final conclusion is this: „L'amido primario o autoctono, contenuto nei cloroplasti, si trasforma incessantemente in glucosio, quando diminuisce o si arresta il processo fotosintetico, mentre, finchè dura tale processo la produzione del glucosio per sintesi diretta deve ritenersi continua. In realtà adunque il filugello è capace di utilizzare indirettamente l'amido contenuto nella foglia di gelso, ma tale utilizzazione, anzichè avvenire per opera di fermenti propri, si compie in virtù dei fermenti di natura vegetale che esistono nella foglia stessa, e nella quale esercitano continuamente la loro azione, prima dell'ingestione dell'alimento" (p. 43).

The very scrupulous histological observation made by Biedermann of pieces of leaves coming from the gut of *Gastropacha rubi* caterpillar and the less careful observation of the content of cells of mulberry leaves carried out by Acqua do not allow them wholly to question the active power of plant enzymes, particularly in reference to the stroma of the chloroplasts. The stroma of the chloroplasts is a proteinous substance; consequently, if this part of the cellular content may be digested under the participation of the enzymes contained in the plant cell, there is no ground to question the presence in the plant cell of an enzyme capable to decompose the protein of the cytoplasm under definite conditions.

Any historical analysis of the view on the problem of the mechanism of the digestion of the cytoplasm in the plant cell may not leave out of account another study the findings of which agree with what much later was confirmed by Biedermann. This study is mentioned by Acqua. As early as 1899 some interesting observations were made by Nazari on pieces of leaves taken out of the caterpillar gut. The following is Acqua's quotation from the study by Nazari: „in tutto l'intestino anteriore e nel primo terzo dell'intestino medio la struttura istologica della foglia si mantiene immutata, e si vedono gli elementi cellulari col loro protoplasma d'aspetto normale, e col piccolo nucleo distintissimo. Progredendo nell'intestino medio, si nota anzitutto la scomparsa del nucleo e un progressivo raggrinzamento del protoplasma. Nell'intestino posteriore gli elementi della foglia appaiono deformati e ridotti per la maggior parte alla semplice membrana di

cellulosi. Solo in alcuni pochi elementi notansi ancora residui di protoplasma fortemente coartati" (p. 9).

Nazari was not the only author to make these observations and the latter were quoted by many writers during his life-time. If however despite these proofs — of which many have been forgotten — it is assumed today that the disappearance of the plasm of plant cells in Lepidoptera (phytophagous) caterpillars is contributed to by an enzyme produced by the animal, it ought to be borne in mind on the basis of the data contained in literature that no evidence has been supplied for the presence in the lumen of the caterpillar gut of an enzyme capable to digest the cytoplasm of the plant cell.

In the light of the discussion carried on hitherto and of the results obtained in this research the question crops up whether in the conditions prevalent in the caterpillar gut plant enzymes may be active.

There may be no doubt that biting off a piece of a leaf, the caterpillar bites off a piece of a living plant which is transferred to different conditions at once, owing to which the whole metabolism of these cells, still living for some time, undergoes a rapid change.

In proportion to the area of piece of leaves, bitten off, various factors, the aggregate of which constitutes those different conditions, act more or less intensely each, and that is why not all leaf cells undergo the same changes.

All data seem to indicate that a piece of a leaf bitten off by caterpillars gets into an anaerobic environment in the gut. Taking into account the high pH (9.1) caused by the presence of potassium carbonate (Verson), we obtain an approximate picture of the conditions prevalent in the caterpillar gut and acting on the living cells of the leaf swallowed.

The action of all these factors prevalent in the caterpillar gut on various cells varies, because their physiological state — connected with the function they fulfil in the leaf — is not the same.

All these factors acting on pieces of leaves in the gut change the metabolism of cells in the direction of the decomposition of the compounds the synthesis of which was a dominant feature of the leaf in normal conditions. The process of decomposition, — the decomposition of the cytoplasm first of all — is probably

activated by potassium ions and lasts as long as leaf cells are living. Small wonder therefore that the degree in which the content of plant cells is utilized varies considerably, for in the condition of the bits of a leaf in the gut the lapse of time during which the cells may live is rather short and ought to be related to the rate of defecation in the caterpillar.

Not without significance in the mechanism of the digestion of the plasm of plant cells in the gut is lack of light, which is demonstrated by the results of a study by Yemm (1937). Although Yemm's study deals with plant physiology, its results bear upon the problem posed in the present paper inasmuch as they point out the influence of lack of light on the decomposition of proteinous compounds in the plant. If it is assumed that the time for which pieces of living leaves remain in the gut amounts from two to three hours, this seems to be of no essential importance for the processes discussed in this paper. Yet bearing in mind that the bits of a leaf found in the gut of the caterpillar examined have an area of 0.09 to 0.51 mm^2 (Table II) and considering that by the biting off of the piece of a leaf all mechanisms linking up physiological processes in the whole plant are destroyed and only the similar possibilities — limited in time — of the group of cells remain, lack of light may be regarded as one of the many factors accelerating decomposition in the cells of the cytoplasm, which no interpretation may leave out of account.

SUMMARY

1. It may be assumed in all probability that the capability of phytophagous caterpillars to feed on leaves of green plants depends on the difference in potassium content in leaves and in the lumen of the gut.

2. Cells of leaves consumed by caterpillars are characterized by strong plasmolysis due to a high concentration of electrolytes in the lumen of the caterpillar gut.

3. The process of the digestion of the plasm in leaf cells varies in various leaf tissues and is going on along the whole gut. The order of the rate of digestion for various tissues is as follows: lower epidermis < upper epidermis < spongy tissue < palisade tissue.

4. The process of the disappearance (digestion) of the cytoplasm

in plant cells with undamaged cellulose membranes is going on under the participation of plant enzymes activated by potassium ions thrown into the lumen of the gut by the intestinal epithelium.

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* Items inacessible in the original to the present author.

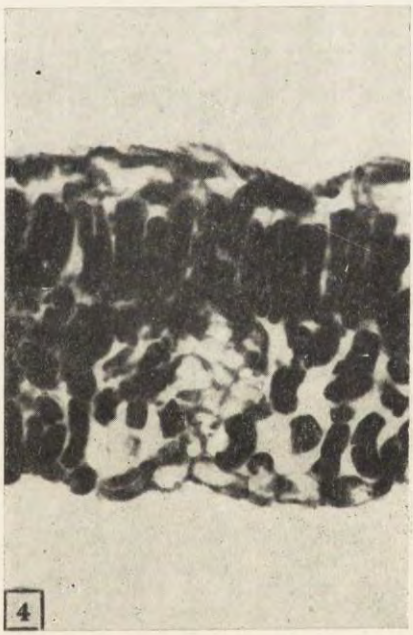


PLATE I

Phalera bucephala L.

Fig. 1. Fragment of a lime leaf from the fore-gut. Fig. 2. Fragment of lime leaf from the mid-gut. Fig. 3. Fragment of a lime leaf from the hind-gut. Fig. 4. Fragment of a control lime leaf.

All Photographs magnified about 370 times.

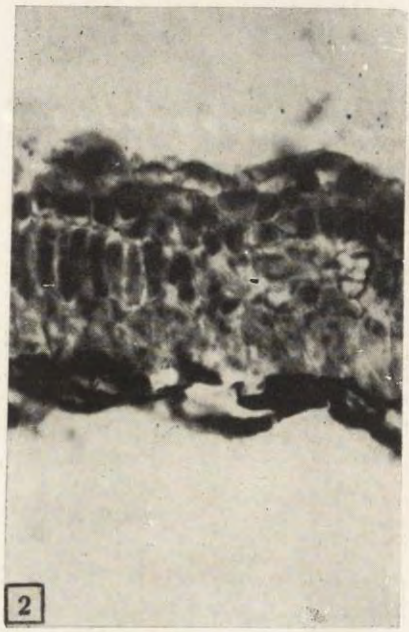


PLATE II

Smerinthus ocellata L.

Fig. 1. Fragment of a *Salix caprea* L. leaf from the fore-gut. Fig. 2. Fragment of a *Salix caprea* L. leaf from the mid-gut. Fig. 3. Fragment of a *Salix caprea* L. leaf from the hind-gut. Fig. 4. Fragment of a control *Salix caprea* L. leaf.

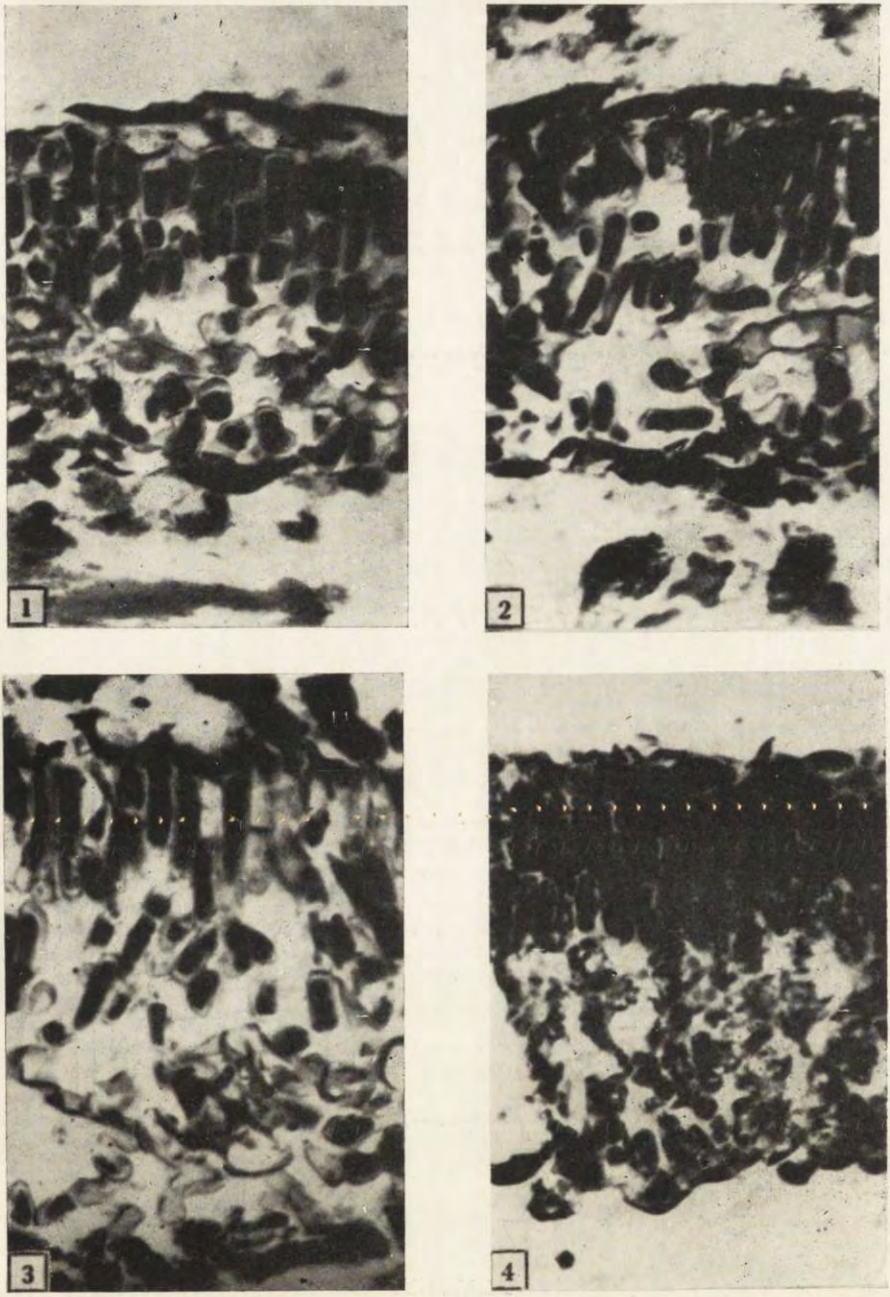


PLATE III

Sphinx ligustri L.

Fig. 1. Fragment of a *Spiraea* Van Houttei Zbl. leaf from the fore-gut.
Fig. 2. Fragment of a *Spiraea* Van Houttei Zbl. leaf from the mid-gut.
Fig. 3. Fragment of a *Spiraea* Van Houttei Zbl. leaf from the hind-gut.
Fig. 4. Fragment of a control *Spiraea* Van Houttei Zbl. leaf.

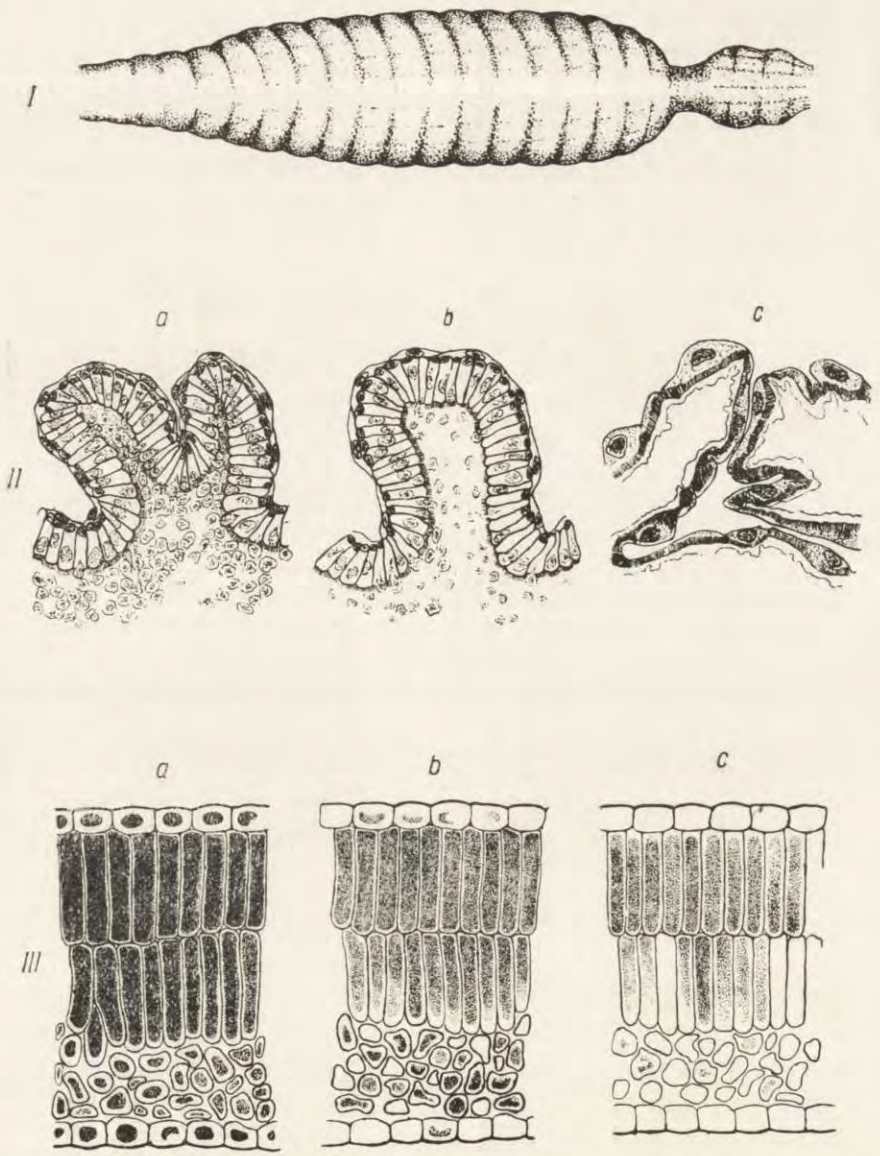
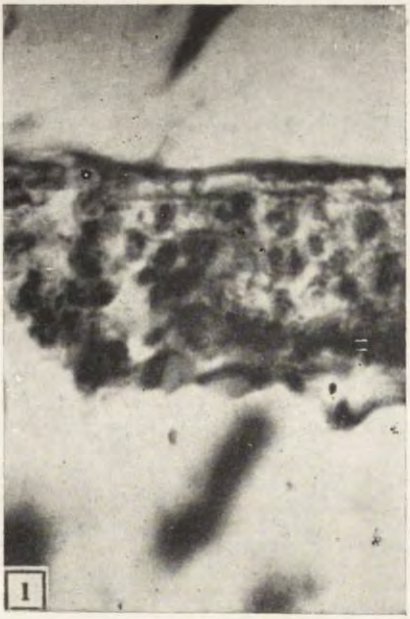


PLATE IV

Structure of the phytophagous caterpillar gut and changes undergone by the food in the gut.

I. Habitus of a mounted gut. II. Cross-cuts of the three parts of the gut: a) fore, b) mid, c) hind. III. Changes in leaf cells: a) in the fore-gut, b) in the mid-gut, c) in the hind-gut.



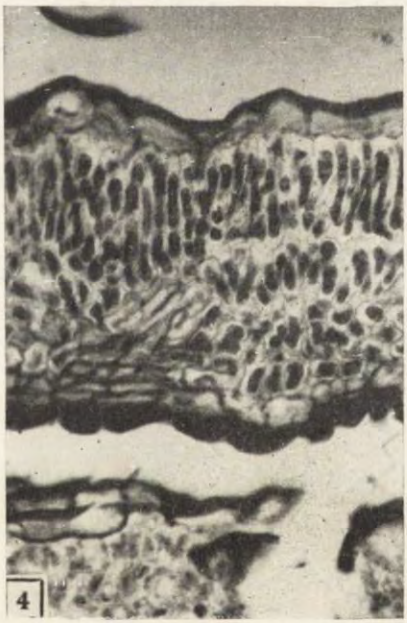
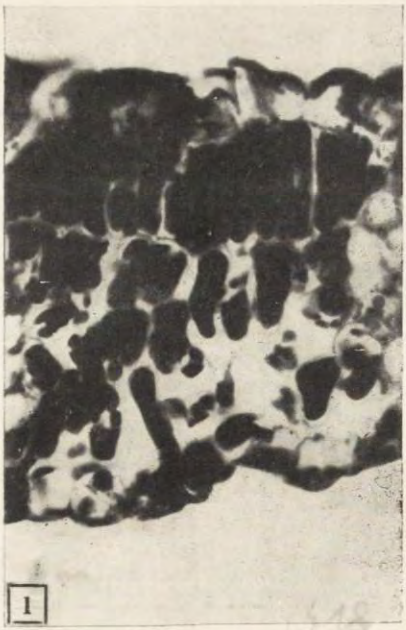


PLATE V

Microscopic picture of mulberry leaves from caterpillar excreta *Bombyx mori* L.

Fig. 1. Section of a normal (control) leaf from the excreta of the first feeding day of the fifth development stage. Fig. 2. Section of a leaf with inactivated enzymes from the excreta of the first feeding day of the fifth development stage. Fig. 3. Section of a normal (control) leaf from the excreta of the fifth feeding day of the fifth development stage. Fig. 4. Section of a leaf with inactivated enzymes from the excreta of the fifth feeding day of the fifth development stage.



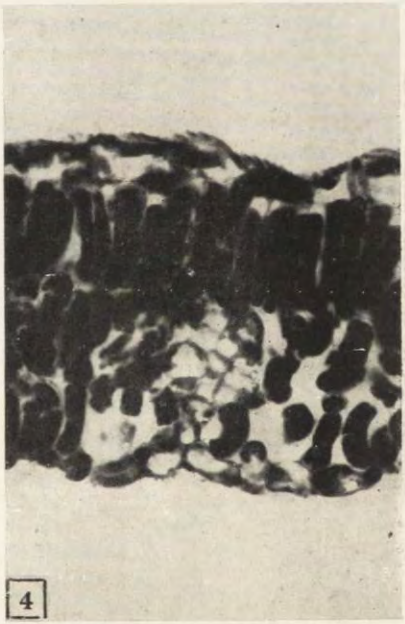
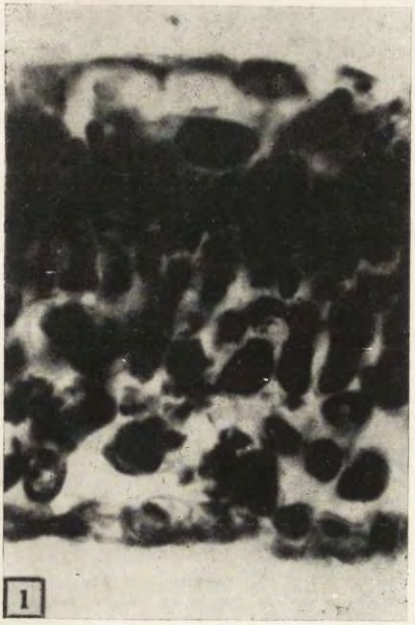
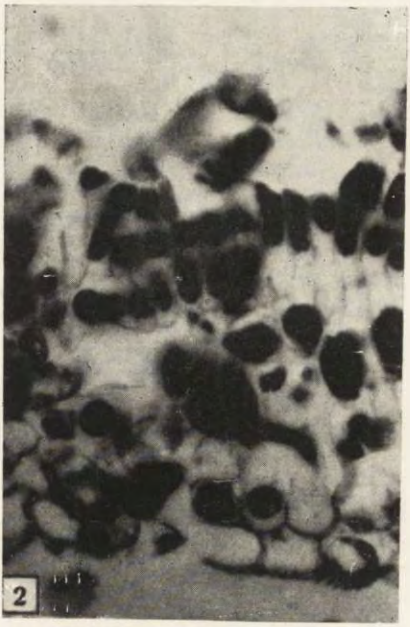


PLATE VI

Changes in *Tilia platyphyllos* Scop. leaves under the action of various potassium compounds.

Fig. 1. Changes in a leaf, caused by the action of 0.5M KH_2PO_4 for 2 hours (4.IX). Fig. 2. Changes in a leaf, caused by the action of 1.0M KH_2PO_4 for 2 hours (4.IX). Fig. 3. Changes in a leaf, caused by the action of buffer of a pH of 9.1 for 2 hours (4.IX). Fig. 4. Cross-cut of a control leaf.





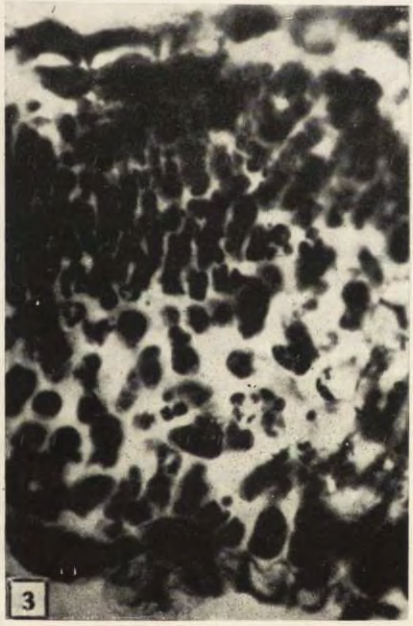


PLATE VII

Changes in *Tilia platyphyllos* Scop. leaves caused by the action of $KHCO_3$ Fig. 1. Changes in a leaf, caused by the action of 30% $KHCO_3$ for 2 hours (30.VIII). Fig. 2. Changes in a leaf, caused by the action of 30% $KHCO_3$ for 4 hours (24.IX). Fig. 3. Changes in a leaf, caused by the action of 30% $KHCO_3$ for 6 hours (20.X). Fig. 4. Cross-section of a control leaf (20.X).

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THE EFFECT OF THE PREFRONTAL LOBECTOMY ON THE VOCAL CONDITIONED REFLEXES IN DOGS

W. ŁAWICKA

Department of Neurophysiology, Nencki Institute of Experimental Biology, Warsaw

(Received 20 June 1956)

In the first paper of this series it was established that one of the most striking and constant symptoms of the ablation of prefrontal areas in dogs is the impairment of inhibitory conditioned reflexes. The instrumental effect of conditioned reflexes trained in those series of experiments consisted in the putting by the dog of his fore leg on the food tray in response to a conditioned stimulus, this movement being reinforced by food. Since it is generally accepted that the animals deprived of the frontal poles exhibit a strong locomotor hyperactivity (Kennard, Spencer, Fountain 1914, Langworthy and Richter 1939, Richter and Hines 1938, Richter and Hawkes 1939, Ruch and Shenkin 1943) it might be suspected that the above symptom is nothing more than a manifestation of this very disorder, i.e. of the tendency to move forward.

To establish whether this assumption is true or not it was necessary to choose, as an instrumental conditioned reaction, a movement which would have nothing to do with the locomotor hyperactivity, and which would not be exaggerated after prefrontal ablation. A reaction most suitable for this purpose seemed to be the vocal reaction of the dog. This reaction had been used by

several authors as an instrumental conditioned response (Frołow 1924, Konorski i Miller 1933, Szustin 1951) and had proved to be not less convenient and useful in this role than the motor reactions of the limbs. In addition our own observations of the prefrontal dogs have shown that they are rather more „silent", than normal dogs and never display an excessive vocal reaction.

METHODS AND EXPERIMENTAL PROCEDURE

The experiments were performed on 3 dogs. The preliminary training was usually conducted in the courtyard in the presence of other animals, where the dog was easily provoked to bark „emotionally". Since each bark was reinforced by food, the dogs soon learnt to bark instrumentally to the sight of the experimenter with a piece of bread in his hand. Then the experiments were transferred to a room in which the dog was allowed to move freely about, the experimenter sitting at a table. A new stimulus, the beating of a metronome, was introduced and only those barks were reinforced which were performed during the action of the metronome. It was applied about ten times in each experiment at intervals of 1—2 min. In the first few experiments the dog barked both to the sound of the metronome and in the intervals, but very soon barking in the intervals became inhibited and the vocal reaction appeared only to the metronome with a latent period of about 1 second. Immediately after the first bark the dog received food and the sound of the metronome was discontinued.

At this stage in the experiments the beating of a metronome with another frequency was also introduced and it was applied once or twice daily for 5 seconds without reinforcement. Owing to generalization this stimulus at first elicited a few barks during its action, but after several dozen trials this reaction disappeared and the dog stood quietly during the action of this metronome without any vocal or general alimentary reaction. As both metronomes, the positive and the negative one, differed not only in frequency but also in timbre, the differentiation was easy enough and after having been established was never disturbed.

In the same way a second inhibitory reflex was formed to the positive metronome combined with a new stimulus, a bell (conditioned inhibition). The bell rang for 5 seconds and was followed immediately by the metronome, lasting also 5 seconds, and this combination was not reinforced by food. The bell never produced any vocal reaction, but to the metronome following it barking was always manifest. After several dozen trials this reaction was inhibited and then we proceeded to prolong gradually the interval between the bell and the metronome. The length of the interval was increased up to the upper limit at which the animal was still able to solve the inhibitory task. Such an interval amounted to 5—10 seconds.

For the sake of illustration we give extracts from the protocols of several experiments with one dog in various stages of the conditioned-reflex training (Table I).

Table I

Extracts from some experiments in one of the dogs, representing the successive stages of the conditioned-reflex training before operation.

No of trial	Time	Conditioned stimulus	Its isolated period	Vocal reaction in secs of the cond. stim.	Reinforcement	Interval barking
		a) Experiment 2				
5	4 min. 30 sec.	$Metronome_1$	6 sec.	$4^1/_2$	+	3
6	5 min. 45 sec.	$Metronome_1$	3 sec.	$1^1/_2$	+	3
7	7 min. 50 sec.	$Metronome_1$	3 sec.	2	+	
		b) Experiment 13				
1	0 min. 30 sec.	$Metronome_1$	3 sec.	2	+	
2	1 min. 45 sec.	$Metronome_1$	2 sec.	1	+	
3	3 min.	$Metronome_2$	5 sec.	1; 3; 5;	—	
4	4 min.	$Metronome_1$	3 sec.	2	+	
5	5 min. 25 sec.	$Metronome_1$	2 sec.	1	+	
6	6 min. 30 sec.	$Metronome_2$	5 sec.	1; 3; 4; 5;	—	
7	7 min. 30 sec.	$Metronome_1$	2 sec.	2	+	
		c) Experiment 20				
3	3 min.	$Metronome_1$	$2^1/_2$ sec.	$1^1/_2$	+	
4	4 min. 15 sec.	$Metronome_2$	5 sec.	—	—	
5	5 min. 30 sec.	$Metronome_1$	$3^1/_2$ sec.	$2^1/_2$	+	
9	11 min. 15 sec.	$Metronome_1$	2	1	+	
10	12 min. 30 sec.	$Metronome_1$	$2^1/_2$ sec.	$1^1/_2$	+	
11	14 min.	Bell	5 sec.			
	14 min. 5 sec.	$Metronome_1$	5 sec.	2; 4;	—	
12	15 min.	$Metronome_1$	$2^1/_2$ sec.	$1^1/_2$	+	
		d) Experiment 103				
3	4 min. 15 sec.	$Metronome_1$	$2^1/_2$ sec.	$1^1/_2$	+	
4	5 min. 30 sec.	Bell	5 sec.			
	5 min. 40 sec.	$Metronome_1$	5 sec.	—	—	
5	6 min. 45 sec.	$Metronome_1$	$1^1/_2$ sec.	$^1/_2$	+	
6	8 min. 30 sec.	$Metronome_1$	$1^1/_2$ sec.	$^1/_2$	+	

a) First stage of elaboration of the vocal conditioned reflex to the Metronome. The dog barks both to the stimulus and in intervals.

b) The introduction of the differential inhibitory stimulus. Owing to generalization the dog barks to this stimulus as well as to the positive stimulus.

c) The inhibitory reflex to the differential stimulus is already established. The compound consisting of the conditioned inhibitor (Bell) followed immediately by the conditioned stimulus (Metronome) is introduced. The dog remains silent to the Bell , but barks to the Metronome.

d) The interval between the Bell and the Metronome in the inhibitory compound is prolonged to 5 sec. The inhibitory reflex to this compound is fully established.

Independently of the experiments performed in the room (situation I) with vocal conditioned reflexes, two of our dogs were also trained in an ordinary conditioned-reflex chamber on the stand (situation II), where the instrumental food reaction consisted in putting the fore leg on the food-tray. Here too, in addition to the positive conditioned reflexes, inhibitory conditioned reflexes, analogous to those in situation I, were established. The experiments in both situations were performed alternately in fairly long series. It must be emphasized that the two instrumental reactions established in two different situations are never intermixed and in no instance the animal would bark in situation II or lift his right fore leg in situation I.

When both excitatory and inhibitory conditioned reflexes in both situations were sufficiently firmly established, the operation was performed in which gyrus proreus and gyrus orbitalis just rostrally to sulcus praesylvicus were bilaterally ablated by suction. Olfactory tracts and bulbs as well as some portions of gyrus orbitalis were spared. Since all dogs are still alive the detailed description of the extent of lesion as well as its microscopical appearance will be given in future.

The dogs recovered a few days after operation and the conditioned-reflex experiments could be resumed. These experiments were conducted for many months till the conditioned-reflex activity of the animal seemed to achieve a stable state.

RESULTS

The effects of the prefrontal ablations on the conditioned-reflex activity in all our dogs were so similar that they can be presented jointly.

As far as conditioned positive reflexes are concerned the difference between the vocal reaction before and after operation consisted in that dogs barked now more profusely and loudly than in normal condition. While before operation the dogs barked to the conditioned stimulus only once and then stood quietly awaiting the food, now they managed to bark two or even several times in very quick succession, although the interval between the vocal reaction and presentation of food lasted, as before, not more than about 2 seconds.

But the most serious disturbances in animal's behaviour appeared in the intertrial intervals and in the response to inhibitory stimuli.

All our dogs, when brought to the experimental room after operation used to bark not only to conditioned stimuli but also in the intervals. In three dogs this intersignal barking was rather infrequent, no more than three dozen per experiment, and appeared

only during the first few days. In the fourth dog it was much more profuse, appearing in long series consisting of 20—30 barks.

As to the inhibitory stimuli both the differential metronome and the positive metronome preceded, even immediately, by the bell evoked a vigorous vocal reaction which consisted in repeated and frequent barking (up to 10 times) during and after the stimulus (Fig. 1 and 2). None of our dogs barked to the sound of the bell alone.

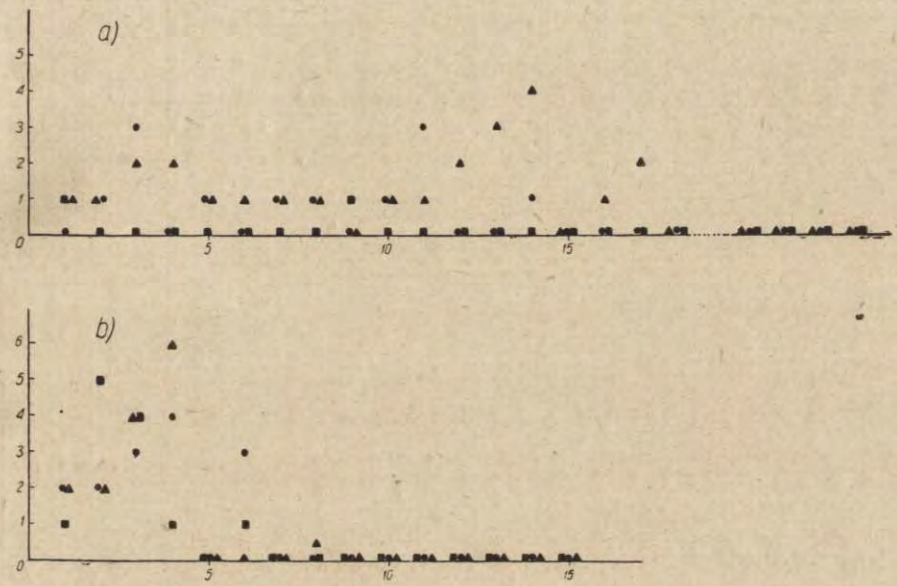

Fig. 1. The course of differentiation before *a* and after *b* prefrontal ablation in all our dogs. Abscissae, successive trials in which a differentiated stimulus was applied. Ordinates, the number of barks in a given trial. Circles, triangles and squares denote different dogs used in these experiments. In *a* the beginning of training and a few trials just before the operation. In *b* the postoperational training till the full restitution of differentiation. After operation the differentiation is strongly disinhibited but very soon it returns to normal.

In the subsequent experiments the inhibitory reflexes gradually improved in that the barks to the inhibitory stimuli became less frequent and eventually disappeared completely. The inhibitory reflex to the differential metronome was restored sooner than that to the positive metronome preceded by the bell, and when the inhibition to these stimuli applied in immediate sequence had been

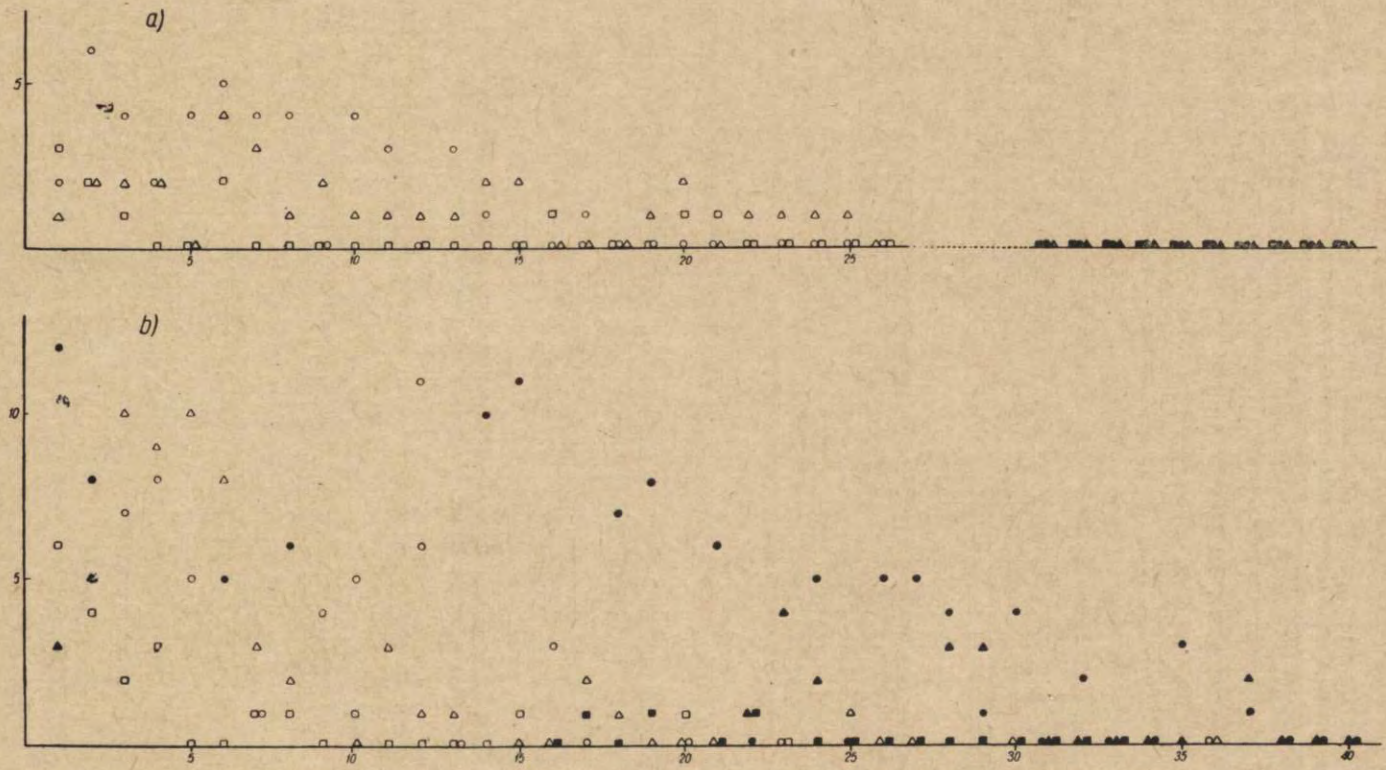

Fig. 2. The course of conditioned inhibition before *a* and after *b* prefrontal ablation in all our dogs. Denotations as in Fig. 1. Hollow signs denote trials with immediate sequence of conditioned stimulus; heavy signs denote trials in which interval between them lasted 2—5 sec. Elaboration of conditioned inhibition before operation was more difficult than that of differentiation (compare with Fig. 1). Accordingly the disturbance of this inhibition after operation was more severe and long-lasting.

restored, the prolongation of the interval between them caused disinhibition.

In general the retraining of inhibitory reflexes after operation resembled the corresponding training before operation. Thus the more difficult for a given dog was the given sort of inhibition, the more disinhibited it became after operation. The dog which used to bark rather infrequently in intervals at the beginning of the training did so also after operation. Another dog continued barking in the intervals for a long period, even when inhibitory conditioned reflexes were already established. The same appeared after operation and the dog stopped barking sooner to inhibitory stimuli, than in the intervals.

When the dogs were brought into situation II they were disinhibited in just the same degree as in situation I but there the reaction performed consisted exclusively in putting the fore leg on the foodtray. They performed this movement both in the intervals and to inhibitory stimuli, just as did the dogs described in the previous paper. But even in the period when the disinhibition was at its highest, none of our dogs barked in situation II or lifted his fore leg in situation I.

DISCUSSION

The experiments presented here seem to show that the disturbances of inhibitory reflexes after prefrontal lobectomies in dogs described in the previous paper are not the result of the locomotor hyperactivity displayed sometimes by such animals, but are a true manifestation of impairment of cortical inhibitory processes. This view is substantiated by the following facts:

1. The vocal conditioned reaction is disinhibited in a similar manner and degree to the motor conditioned reaction of the limb.

2. The animals do not show any exaggerated vocal reaction outside the experimental situation in which it has been trained, and, in particular, this reaction is not transferred to the second experimental situation where another instrumental reflex has been established.

3. The course and the degree of disinhibition after prefrontal ablation resembles the state of the corresponding reflex before and during inhibitory training.

In one of our dogs (unpublished experiments), in which the

instrumental conditioned response consisted in standing on the hindlegs, the inhibitory reflexes after prefrontal lobectomy were disinhibited in just the same way, as in experiments reported here. The same effect was obtained in Brutkowski's experiments (1956) concerning classical salivary conditioned reflexes. We are therefore compelled to conclude that the disinhibition of inhibitory reflexes after frontal lobectomy is, at least in the domain of alimentary reflexes, a general phenomenon, and may prove to be the chief symptom of this injury.

It remains to comment upon the relation of this symptom to the general locomotor hyperactivity often described in animals after prefrontal ablations. According to our observations this symptom appears in dogs only after more extensive prefrontal ablations encroaching on gyrus sigmoideus anterior. This ablation is followed not only by persistent circling but also by perseverative performance of the learnt instrumental movement, whether it be putting the fore leg on the food tray or barking.

In this connection we should like to draw attention to a feature of the vocal behaviour of our prefrontal dogs observed both in response to positive and to inhibitory stimuli. We have pointed out above that the dogs after prefrontal ablations bark to the positive conditioned stimuli not once, as before operation, but several times in very quick succession. As to the inhibitory stimuli, they also bark to them more persistently than they did at the beginning of the corresponding preoperative inhibitory training. Whether such a behaviour may be considered simply as a symptom of profound disinhibition of inhibitory reflexes, or as a comparatively mild form of perseveration, or both, remains to be elucidated.

SUMMARY

1. Vocal instrumental alimentary conditioned reflexes, both excitatory and inhibitory, were established in dogs in order to examine changes produced in them after prefrontal ablations.

2. Excitatory vocal conditioned reflexes are slightly changed after operation in that the dog barks to the conditioned stimulus repeatedly in very quick succession.

3. Inhibitory conditioned reflexes both to the experimental situation and to inhibitory stimuli are disinhibited.

4. All these changes gradually disappear in the course of weeks or months.

The author is greatly indebted to professor J. Konorski and professor L. Stępień for their valuable criticism and advice, and for performance of operations.

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THE EFFECT OF PREFRONTAL LOBECTOMIES ON SALIVARY CONDITIONED REFLEXES IN DOGS

S. BRUTKOWSKI

Department of Neurophysiology, Nencki Institute of Experimental Biology, Warsaw

(Received 20 June 1956)

In the previous papers of this series (Brutkowski, Konorski, Ławicka, I. Stępień and L. Stępień 1956, Ławicka 1956) it was established that damage to the prefrontal areas in dogs produces a more or less pronounced disinhibition of the motor inhibitory conditioned reflexes. These results raise the question as to whether the disturbance of the inhibitory processes affects only the motor behaviour of the animal, or whether it also involves the autonomous reactions. In order to solve this problem a new series of experiments was undertaken in which the effect of prefrontal ablations upon the classical salivary conditioned reflexes was investigated. The results of these experiments are reported in the present paper.

METHODS AND COURSE OF EXPERIMENTS

Experiments were performed on 6 mongrels in an ordinary semi-soundproof conditioned-reflex chamber. All the dogs had a parotid gland fistula performed by the Glinski method. The salivary secretion was recorded by Kozak's method (Kozak, 1950).

In all our dogs the training began with the elaboration of excitatory

alimentary conditioned reflexes to a number of stimuli. At the beginning of training the conditioned stimuli were immediately reinforced by food, and then the intervals between stimulus and reinforcement were protracted to 20 sec. and had a determined length. The intervals between stimuli lasted from 4 to 8 min. in a given series of experiments.

After the positive conditioned reflexes were firmly established the following inhibitory reflexes were elaborated:

1. Differentiation. A stimulus similar to that used as a positive conditioned stimulus was introduced and applied for 20 sec. without reinforcement.

2. Conditioned inhibition. A new stimulus was applied for 10 sec. and followed immediately by a conditioned stimulus lasting for 20 sec. and in this combination not reinforced by food. When the inhibitory reflex to the conditioned stimulus preceded by the extra stimulus was established the interval between both stimuli (which we shall call the inhibitory interval) was gradually lengthened up to 6—10 seconds.

3. Alternation. In a given series of experiments one and the same stimulus was applied throughout each session and was alternatively either reinforced or not reinforced by food. It has been proved in our laboratory that alternation may be considered as a special case of conditioned inhibition in which every presentation of food plays the role of a conditioned inhibitor in respect to the subsequent trial.

Inhibitory stimuli were applied usually only once in an experimental session accompanied by 5—8 positive stimuli. Since some inhibitory tasks (as e. g. conditioned inhibition with a lengthy inhibitory interval) were rather difficult for some of our dogs and they caused time and again functional disturbances of the conditioned-reflex activity, it was necessary to apply the inhibitory stimuli rather cautiously, and even to cease from time to time their application altogether till the condition of the animal returned to normal. The administration of bromides was also very helpful in these cases. Usually salivary reactions to the inhibitory stimuli did not drop to zero but amounted to 10—30% of reactions to positive stimuli. This was due in the first place to the preponderance of excitatory stimuli used in all our experiments. It must also be added that as Kozak's method of registration of salivary secretion is very precise, it detects even small salivary reactions which would be completely undetectable in the old Ganike-Kupalov method.

When the full experimental training was completed and conditioned reflexes, both excitatory and inhibitory, were stable (which usually took about one year), one of two operations was performed: either prefrontal areas including gyrus proreus and orbitalis rostrally to sulcus presylvius were bilaterally amputated, or, for the sake of control, medial parts of parietal lobes, including gyrus suprasplenialis, gyrus entolateralis and gyrus ectolateralis, were bilaterally removed. The surgical procedures are described elsewhere (Brutkowski, Konorski, Ławicka, J. Stępień and L. Stępień 1956).

The schedule of training and operations performed on each of our dogs is given below:

Dogs	Preoperational training	Its duration (months)	First operation	Duration of post-operative training (months)	Second operation	Further fate
No 1	Differentiation Conditioned inhibition	12	prefront.	14	—	still alive
No 2	Differentiation Conditioned inhibition	$15^1/_2$	prefront.	$2^1/_2$	—	strong rheumatism, killed
No 3	Conditioned inhibition	9	prefront.	$4^1/_2$	parietal	still alive
No 4	Differentiation Conditioned inhibition	10	prefront.	$8^1/_2$	parietal	died after epilep. attack
No 5	Differentiation Conditioned inhibition	$12^1/_2$	prefront.	5	parietal	still alive
No 6	Alternation	9	parietal	$1^1/_2$	prefront.	killed 14 months after prefront. operation

Convalescence after all operations was uneventful and usually after 3—5 days conditioned-reflex experiments could be renewed. They were conducted in few cases for about one year, so that the conditioned-reflex status in the end of the series was quite stable.

RESULTS

In the first few experiments after prefrontal ablation the whole conditioned-reflex activity was greatly impaired. This fact must be considered as the immediate sequel of the surgical intervention, since it was equally observed after parietal operation. Then positive conditioned reflexes returned to normal, or even a little exceeded their preoperative level. On the other hand inhibitory reflexes were strongly and more or less lastingly disturbed.

Fig. 1, 2, 3, 4 and 5 give the whole pattern of disturbances of differentiation and of conditioned inhibition in each of the five dogs,

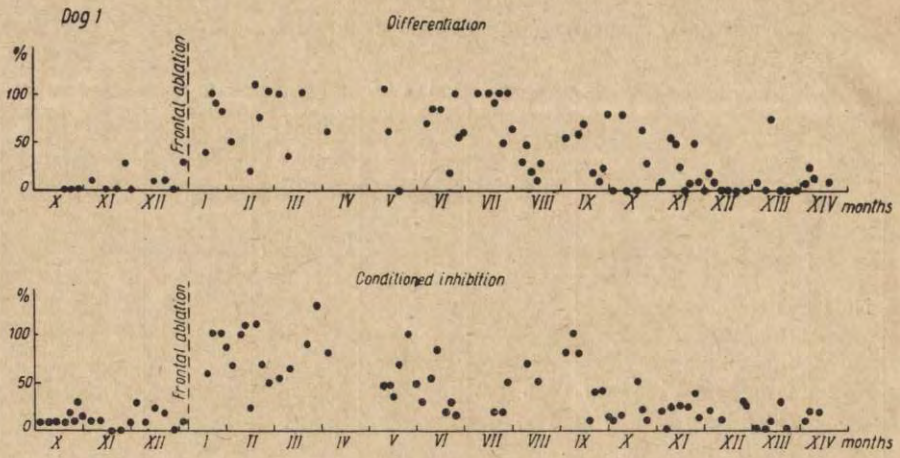


Fig. 1. The course of differentiation and conditioned inhibition in the last period of preoperative training and after prefrontal lobectomy in dog 1.
Abscissae: consecutive months before and after operation. The dots indicate days on which a determined inhibitory stimulus was applied. Ordinates: salivary reactions to the inhibitory stimulus in per cent of the magnitude of reaction to the immediately preceding excitatory stimulus.

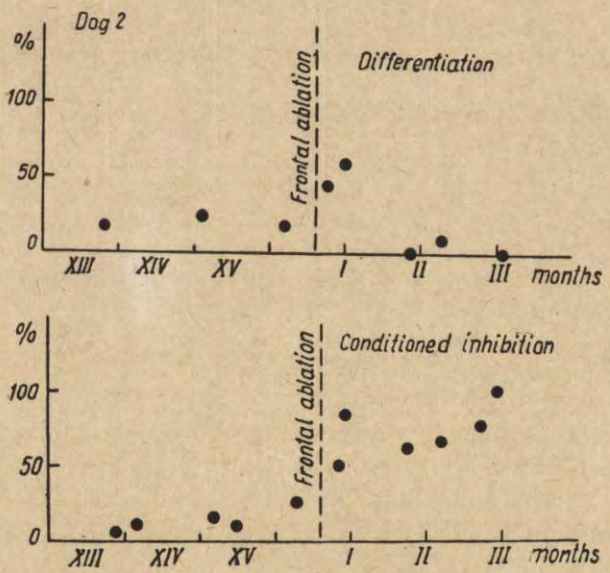


Fig. 2. The course of differentiation and conditioned inhibition in the last period of preoperative training and after prefrontal lobectomy in dog 2.
Abscissae: consecutive months before and after operations. The dots indicate days on which a determined inhibitory stimulus was applied. Ordinates: salivary reaction to the inhibitory stimulus in per cent of the magnitude of reaction to the immediately preceding excitatory stimulus.

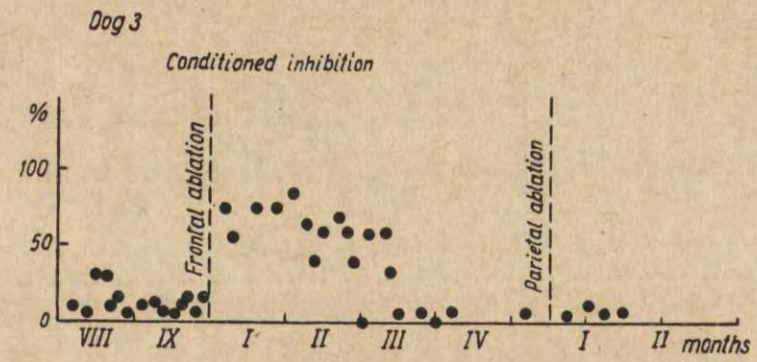


Fig. 3. The course of conditioned inhibition in the last period of preoperative training and after prefrontal and parietal lobectomies in dog 3.

Abscissae: consecutive months before and after operations. The dots indicate days on which a determined inhibitory stimulus was applied. Ordinates: salivary reaction to the inhibitory stimulus in per cent of the magnitude of reaction to the immediately preceding excitatory stimulus.

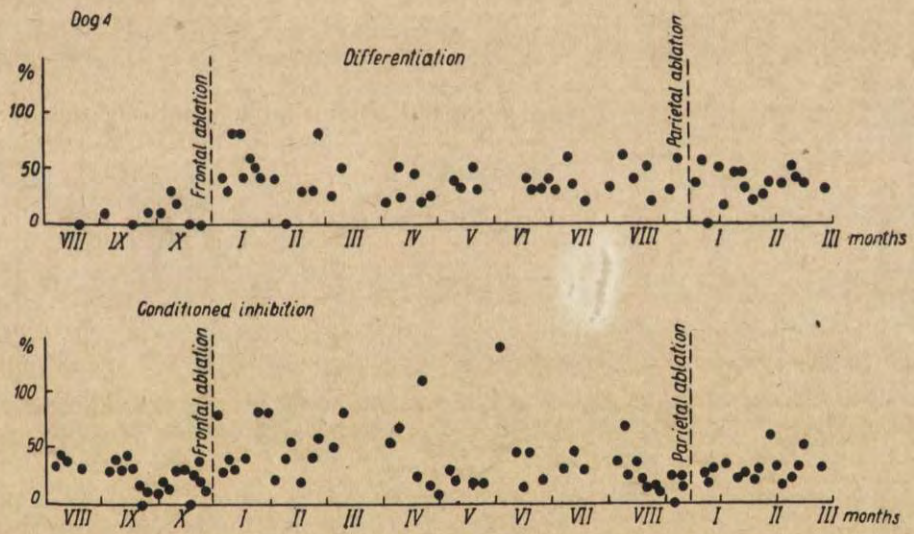


Fig. 4. The course of differentiation and conditioned inhibition in the last period of preoperative training and after prefrontal and parietal lobectomies in dog 4.

Abscissae: consecutive months before and after operations. The dots indicate days on which a determined inhibitory stimulus was applied. Ordinates: salivary reaction to the inhibitory stimulus in per cent of the magnitude of reaction to the immediately preceding excitatory stimulus.

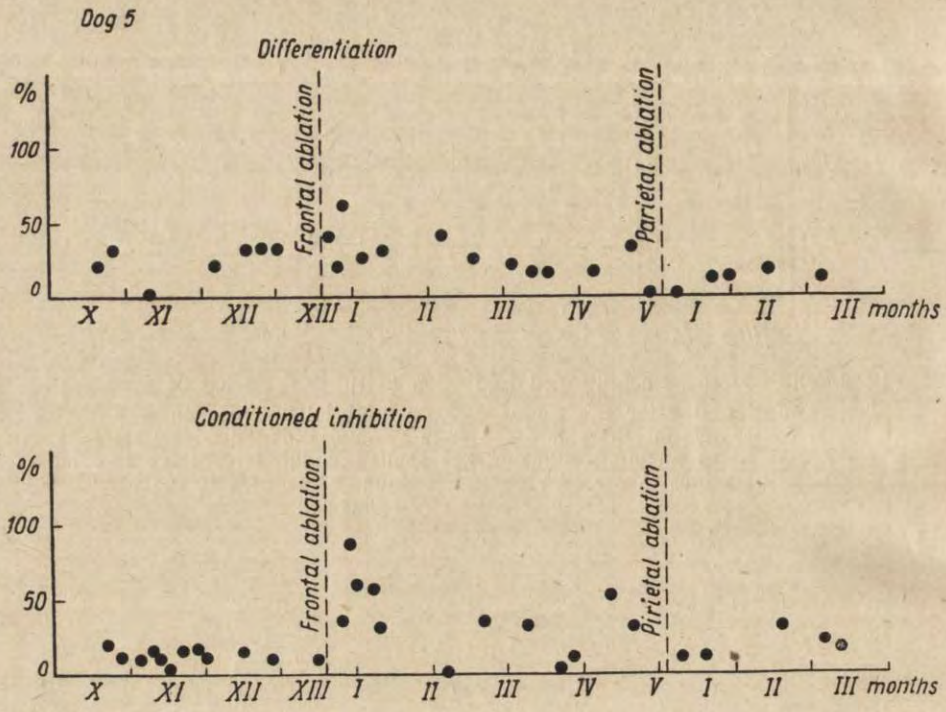


Fig. 5. The course of differentiation and conditioned inhibition in the last period of preoperative training and after prefrontal and parietal lobectomies in dog 5.

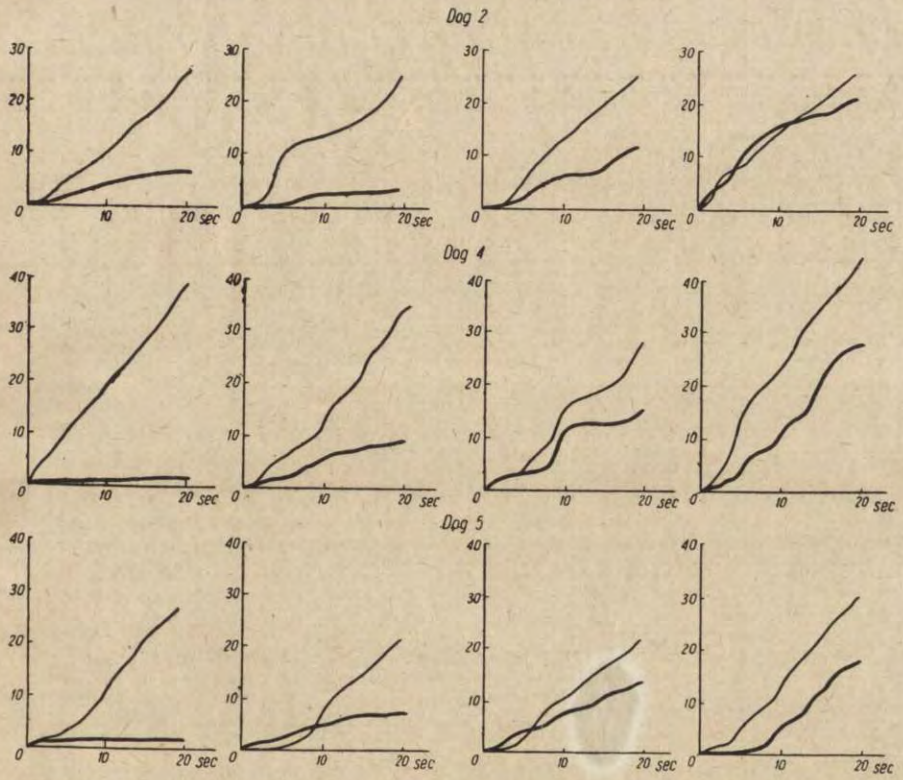
Abscissae: consecutive months before and after operations. The dots indicate days on which a determined inhibitory stimulus was applied. Ordinates: salivary reaction to the inhibitory stimulus in per cent of the magnitude of reaction to the immediately preceding excitatory stimulus.

in which these kinds of inhibitory reflexes were established. Here are the main features of these disturbances:

1. The degree of disinhibition of inhibitory reflexes is not equal in all the dogs. It was much more severe and of longer duration in dogs 1 and 4 than in dogs 2, 3 and 5. It is worth mentioning that in the first two dogs a strong preponderance of excitatory over inhibitory processes has been observed: the elaboration of inhibitory reflexes took more time in them than in the other dogs and these reflexes during the preoperative training were easily disinhibited by trivial occurences in the experiments.

2. The disinhibited inhibitory reflex rarely attains or exceeds its excitatory counterpart. Usually in the first period after opera-

tion it amounts to 50—80% of the control excitatory reflex. But even in this period there are inhibitory trials in which the salivary reaction is just the same as before operation. This shows that the prefrontal dog does in principle differentiate between the positive and inhibitory stimulus or the combination of them, but is generally not able to develop a sufficient inhibitory process.



Several days before prefrontal lobectomy. Several days after prefrontal lobectomy.

Fig. 6. Salivary secretion to the inhibitory stimulus (heavy line) and to the excitatory stimulus which preceded it immediately (light line).
Abscissae: duration of the conditioned stimulus (in seconds) Ordinates: amount of secreted saliva.

3. The records of salivary reactions to inhibitory stimuli show after operation spectacular irregularities almost never seen in control records (Fig. 6). This indicates that even in a state of strong disinhibition there is a struggle between excitation and inhibition, one of these processes alternately taking the upper hand.

4. After a lapse of time the inhibitory ability of a dog gradually improves but not always does it return to its preoperational level.

5. The effect of the conditioned inhibitor itself (which is what we have called a primary inhibitor (Konorski and Szwejkowska 1952) is never disturbed after prefrontal ablation.

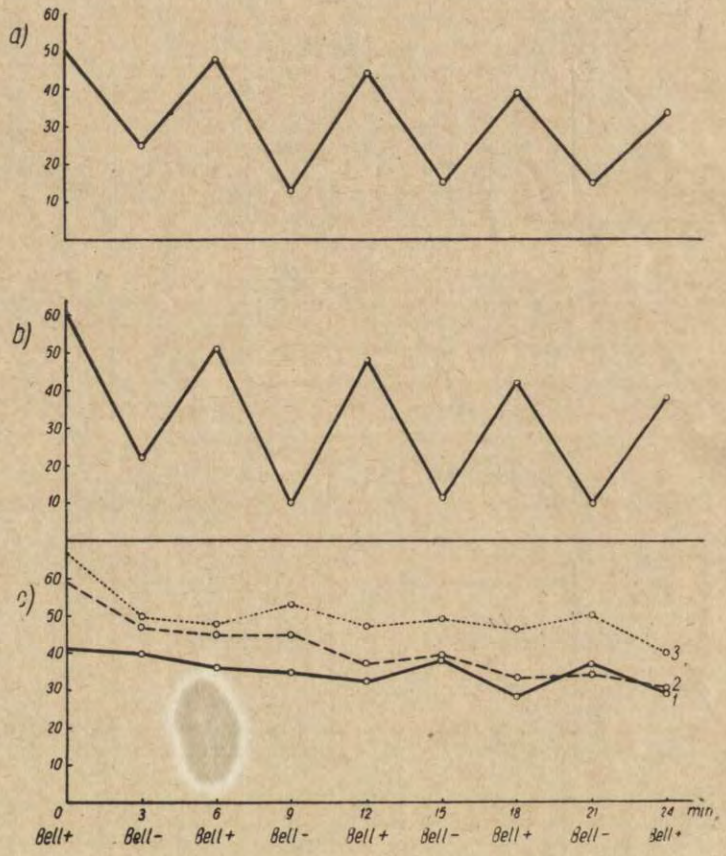


Fig. 7. Alternation in dog 6

a. in the last month before parietal operation; b. during one month after parietal operation; c. 1. during first month after prefrontal operation; 2. during second month after prefrontal operation; 3. during third month after prefrontal operation. Abscissae: times in minutes. Dots correspond to consecutive applications of the conditioned stimulus (Bell) alternatively reinforced (+) and not reinforced (—) by food. Ordinates: mean values of salivary reactions obtained in successive experiments.

As seen in Fig. 3, 4 and 5 the parietal operation performed in three dogs several months after prefrontal operation did not change

in any way the status of inhibitory reflexes. Excitatory reflexes were also left unimpaired after this operation.

In dog 6 alternation was established before operation, i. e. the dog was taught to display inhibitory reaction to every second application of the conditioned stimulus. The elaboration of this kind of inhibition was exceedingly difficult for the dog. It lasted about 9 months and from time to time the dog became neurotic. The administration of bromides in such periods greatly improved the condition of the dog, and in the end enabled it to master the task. The state of excitatory and inhibitory reflexes in the last month before the first operation is given on Fig. 7a.

The dog was at first submitted to the parietal operation, and, as it is seen in Fig. 7b, this operation did not change the performance of the dog. The inhibitory reflexes were even better than before operation.

Two months after the first operation the second operation was performed, in which prefrontal poles were removed. As is seen in Fig. 7c this operation totally and permanently destroyed the elaborated conditioned-reflex pattern. Experiments with this dog were carried out for nearly one year, but alternation could not be reestablished.

Fig. 7c shows that salivation in inhibitory trials is not only less than in excitatory trials, but on the contrary slightly exceeds it. This is easily understood, if we take into account the fact that every non-reinforcement of the stimulus makes its subsequent application less effective, while every reinforced trial facilitates salivation to the next stimulus.

It is worth mentioning that while before operation every disturbance in the performing by the dog of the task to which it had been trained had been immediately cured by the administration of bromides, after the operation this drug turned out to be absolutely useless and neither small nor very large doses of it led to any improvement.

In order to test the inhibitory ability of the dog by other methods, the training with alternation was abandoned and we proceeded to the elaboration of a simple differentiation. This task proved to be quite soluble, while, on the other hand, elaboration of conditioned inhibition was impossible.

DISCUSSION

The present series of experiments proves that after prefrontal lobectomies inhibitory salivary reflexes are as much disturbed as inhibitory motor reflexes, i. e. that the disturbance in question is not limited to the motor behaviour only.

Comparing the extent of disturbances of inhibitory reflexes in all our dogs, we see that there are great differences between them. In those dogs in which conditioned inhibition and differentiation was established, the inhibitory reflexes turned out to be more or less impaired, but not totally destroyed: the salivation to the inhibitory stimuli was nearly always smaller than that to the control stimuli, and sometimes it was even as small as before the operation. On the other hand alternation in dog 6 was completely destroyed and in spite of a long postoperative training there has been no slightest sign of improvement.

It seems that the following factors may play a role in determining the influence of operation on salivary conditioned reflexes:

1. The difficulty of the inhibitory task. As a good example of the significance of this factor may serve the dog 6 in which the alternation could not be restored, whereas the simple differentiation was easily established after operation.

2. The type of the nervous system of the dog. It was emphasized above that in dogs which belonged to the „unbalanced", excitatory type the impairment of inhibitory processes after operation was more pronounced and of longer duration than in normal well-balanced dogs.

3. The extent of lesion. Although in this series we have no direct proof of the importance of this factor, our earlier experiments (cf Brutkowski et al. 1956) seem to indicate that the degree of disinhibition depends on extent of cortical ablation.

The effect of prefrontal ablations on the salivary conditioned reflexes was investigated earlier by Shumilina of Anochin's laboratory (Shumilina 1949) and quite recently by Shustin (1953).

In Shumilina's experiments, among other things, differentiation was established, and she claimed that it was not disturbed after frontal ablation. This seems to be due to the easiness of the inhibitory task and also to the not very perfect recording of salivary reactions in these experiments. On the other hand in Shustin's experiments trace conditioned reflexes were dealt with and they

were abolished after prefrontal ablations. As trace reflexes are based on inhibition, this is another example of the impairment of this process after prefrontal lobectomies.

SUMMARY

1. In dogs, in which conditioned salivary alimentary reflexes, both excitatory and inhibitory, are established prefrontal lobectomies produce a pronounced impairment of inhibitory reflexes, whereas the excitatory reflexes are left unchanged.

2. The degree and duration of impairment of inhibitory reflexes depends on the difficulty of the inhibitory task, the type of the nervous system of the dog, and the extent of lesion.

3. The rate of restitution of inhibitory reflexes depends on factors enumerated in 2. In some cases the disturbance of inhibition seems to be irreversible.

4. The control ablations of similar extent in the parietal region do not produce this disorder.

The author wishes to thank Prof. J. Konorski, who suggested the subject of these experiments, for his interest and advice throughout this work and Prof. L. Stępień who performed the operations on dogs.

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THE CHRONIC FISTULA OF SHORTENED STENSEN'S DUCT IN DOGS

STEFAN SOŁTYSIK AND ANDRZEJ ZBROŻYNA

Laboratory of Physiology of The National Psychoneurological Institute, Pruszków, and Department of Neurophysiology, Nencki Institute of Experimental Biology, Warsaw

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Continuous graphic registration of salivary secretion in dogs, proposed by W. Kozak (1950), has enabled a much more accurate recording of this process than was possible by the hitherto applied

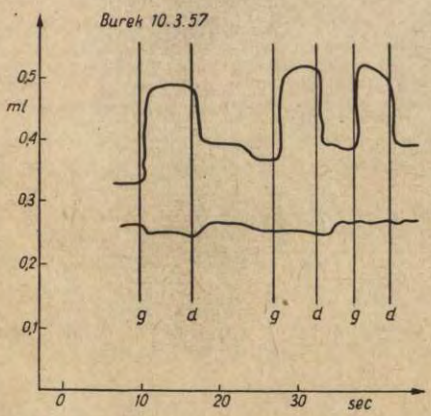

Fig. 1. The influence of movements of the head on the recording of salivary outflow from the Glinski fistula on the right side (upper record), and from the shortened one on the left (lower record). g — upward movement. d — downward movement. Ordinates: 1 division of the scale corresponds to 0,1 ml. Abscissae: time.

methods. Kozak's method has enabled, among other things, the detection of many irregularities in the seemingly uniform flow of saliva. It has been suspected that these irregularities result not so

much from the uneven secretion of saliva from the parotid gland, but rather from the changes of the lumen of the salivary duct, caused by movements of the dog's muzzle or head (Fig. 1). To avoid these

Fig. 2. Parotid glands with Glinski's (lower) and our (upper) fistulae.

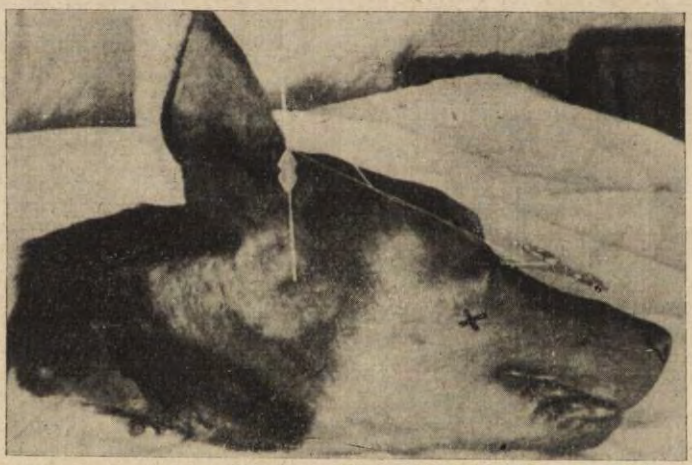

Fig. 3. The location of the orifice of Glinski fistula (×) and of the shortened fistula (with sound inserted).

artefacts it was thought useful to produce a fistula of the parotid gland in which the greater part of the duct would be excluded (Fig. 2), i.e. to sever the duct as near as possible to the gland, and to suture the distal end of its proximal portion directly to the skin

(Fig. 3). Attempts to produce such a fistula were made time and again by various authors, but they usually resulted in obliteration of the orifice of the duct (cf. Abuladze K. S. 1953, Kvasnicki A. W. 1955). Kozak however succeded in obtaining the parotid fistula with a shortened duct which remained open permanently for several years (pers. comm.) .

SURGICAL TECHNIQUE

The set of surgical instruments used in the Glinski operation should be supplemented by two silver sounds about 0,3 mm diameter. Both sounds are inserted into the duct as deeply as possible without overcoming resistance. The tips of the sounds should be palpable in the region of the parotid gland. The skin is cut perpendicularly to the duct about 3 cm rostrally to the tips. The portion of the duct between the incision of the skin and the tips is then carefully cleaned of the connective tissue. Care must be taken not to injure the glandular tissue and the ramifications of the duct, otherwise the saliva will spread and gather under the skin. The duct with sounds inserted is then severed at a distance of at least 2 cm from the last branchings or from the gland.

The skin in the region directly over the proximal portion of the duct is then scarified and incised with the Graefe cataract knife. The duct will be threaded through this incision. The site must be carefully chosen in such a way as to accomodate the duct without folds and traction. The size of the incision must correspond to the diameter of the duct. To thread the stump of the duct through the incision two ligatures are stitched on the end to facilitate manipulation, and then the terminal portion of the stump, with sounds inserted, is drawn through the incision. The protruding portion of the stump is then cut lengthwise after the sounds are pushed slightly aside. The bleeding is stopped by hot saline. The duct is left intact over 2—3 mm after traversing the skin. The split portion of the duct is reduced to some 5—7 mm and sutured to the scarified surface of the skin, the epithelium lining the lumen of the duct upwards.

The length of the duct from the gland to the surface of the skin is about 1,5 cm (Fig. 2).

In order to prevent excessive displacements of the skin, which might cause the tearing off of the duct, the skin near the fistula was

stitched to the underlying tissue with one or two catgut sutures. The closing of the skin and the removal of sounds end the operation.

For 10—15 days postoperative care is necessary. The dressing should be changed at least once in 24 hours, the scab softened and removed, the outflow of saliva facilitated by the cleaning of the fistular region, and the duct made patent. Only then the animal can be fed. A rapid and uncomplicated healing is necessary to obtain a good fistula.

The animal is ready in 2—3 weeks for the recording of the salivary secretion.

RESULTS

In order to compare the outflow of saliva from the Glinski fistula and from fistula with a shortened duct we made a Glinski fistula on one side and a fistula with shortened duct on the other in 3 dogs.

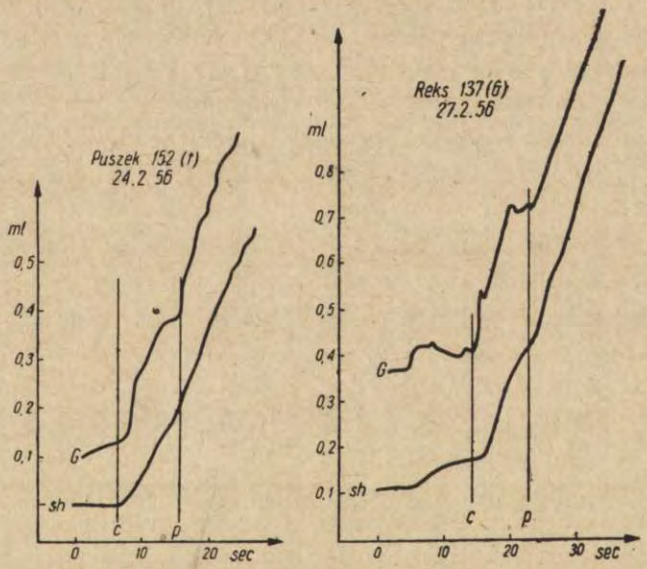

Fig. 4. Kymographic records of the outflow of saliva from the Glinski fistulas (G) and from fistulas with a shortened duct (sh). Each pair of curves is obtained by simultaneous registration of salivation from two fistulas in a conditioned-reflex experiment. c — the beginning of the conditioned stimulus, p — presentation of food. Ordinates: 1 division of the scale corresponds to 0,1 ml.
Abscissae: time.

The recording of the salivary outflow shows many irregularities in the Glinski fistula, whereas the shortened duct fistula gives a smooth

course of outflow (Fig. 4). Although the secretion from the two glands may be asymmetrical, the regularity obtained from shortened duct fistula suggests strongly that the kinks in the curve from Glinski fistula are artefacts, due to extensibility and varying volume of the Stensen's duct.

The record shown in Fig. 5 is especially instructive in this respect. Here an entirely new stimulus — the lightening of a lamp — was applied. In the record from the Glinski fistula a stepped rise of the curve is seen, which would indicate a strong and rapid outflow

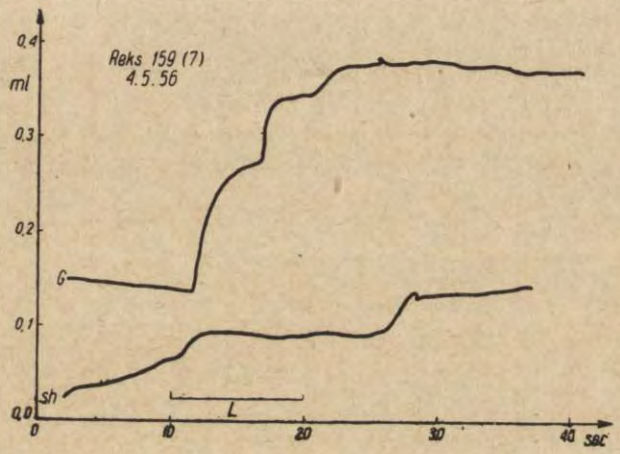

Fig. 5. A pair of curves obtained from salivation through the Glinski fistula (G) and the fistula with a shortened duct (sh) during the action of a new stimulus — lamp (L). The rise of the curve G is due only to the lifting of the dog's head. Ordinates: 1 division of the scale corresponds to 0,1 ml. Abscissae: time.

of saliva. As seen on the other curve this rise is probably a pure artefact connected with the rapid lifting of the dog's head. We are convinced that many such artefacts were observed in conditioned-reflex experiments and led sometimes to quite erroneous conclusions.

SUMMARY

1. A new method of producing salivary fistula of the parotid gland in dogs is described. In this method a large part of Stensen's duct is excluded and the distance between the gland and fistula is thereby greatly reduced.

2. The comparison of the outflow of saliva from the Glinski fistula and from the fistula described here shows that many irregularities observed in the course of the outflow from the first of these fistulae are artefacts, and that almost all of these artefacts are eliminated by the present method.

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STUDIES ON THE BIOCHEMISTRY OF THE WAXMOTH (*GALLERIA MELLONELLA*). 16. WEIGHT OF TISSUES AND ORGANS DURING STARVATION OF THE LARVAE

Z. M. ZIELIŃSKA AND A. WRONISZEWSKA

Department of Biochemistry, Nencki Institute of Experimental Biology, Warsaw

(Received 12 January 1956)

In connection with the studies made in this laboratory on the biochemistry of starvation of the waxmoth, *Galleria mellonella*, it was necessary to determine the weight of separate tissues and organs of the larvae.

The following tissues and organs were investigated: the blood, the silk glands, the alimentary canal together with the Malpighian tubes, the fat body, the muscles, and the integument. The tracheal and the nervous systems were examined together with the muscular system.

During the starvation period the larvae were kept separately in conditions described previously (Niemierko W. and Cepelewicz 1950, Niemierko W. and Wojtczak L. 1950, Zielińska 1952).

Before dissection the larvae were weighed and cooled to about 0° C. The amount of haemolymph was determined either by subtracting the weight of the larvae after bleeding from the weight of the intact larva, or by subtracting the weight of all separated tissues from the body weight as a whole.

The body weight of the larvae after twenty four hours of starvation was considered as initial (Zielińska 1952). Individual larvae, weighed after the same period of starvation, show different losses in body weight. It must be noted that specimens which suffer smaller losses in body weight live longer and changes produced in the course of starvation (desiccation and clogging of the intestine by dry excreta) set in later (Zielińska 1952). This is the reason why we consider the loss in body weight as more symptomatic for the degree of starvation of the larvae than the time length of starvation. In this study only organs of larvae with about 60 per cent loss in initial body weight were examined. The period of starvation lasted from three to four weeks.

As shown by the accompanying table the weights of all the organs diminished during the starvation period. Losses in the weight of particular organs, measured in percentage of their initial weight (Table I, column 7), appear not to differ much except for the integument. While the loss in weight of the latter amounted to 30 per cent of its initial weight, other organs lost over 60 per cent, and the silk glands even more than 70 per cent of their initial weight. This is the reason why the weights of organs expressed in percentages of the actual body weight (Table I, column 4 and 5) decreased only slightly, the silk glands lost more, whereas the weight of the integument increased considerably in relation to the weight of the whole body.

The analysis of the losses in weight of the particular organs in relation to the general loss of body weight in the starving larvae (Table I, column 8) shows that 3/4 of the loss of general body weight during starvation is made up by a loss of the blood weight, the fat body and the muscles. The integument shows the smallest decrease in the percentage of the body weight.

In his studies on *Celerio euphorbiae* Heller and Świechowska (1947) determined the percentages of weights in relation to the body weight for the blood, the alimentary canal, the fat body and the rest of the organs called „muscles". In the alimentary canal of full grown caterpillars of the hawk moth, *Celerio euphorbiae,* the relation in question was from 25 per cent to 64 per cent, according to the degree to which the intestine was filled with food pulp. As a result, the weight of the other organs investigated, expressed in percentages of the body weight, varied strongly. On the average the corresponding relation for the alimentary canal

Table I

Weights of the tissues and organs of waxmoth larvae during starvation

Organ	Mean values of the organ weights					Loss of weight after prolonged starvation	
	in mg		in % of the body weight		in % of initial body weight		
	starvation		starvation			in % of initial organ weight	in % of whole body weight loss
	beginning	prolonged	beginning	prolonged	prolonged starvation		
1	2	3	4	5	6	7	8
Silk glands	16.9	4.7	9.4±0.8	6.5±1.1	2.7±0.7	71.2	11.4
Alimentary canal and the Malpighian tubes	18.0	6.6	10.0±1.5	9.1±1.6	3.8±1.1	62.0	10.5
Blood	51.0	20.3	28.3±2.3	28.1±1.1	10.6±1.6	62.6	26.9
Visceral fat body	38.7	13.7	21.5±1.7	19.0±2.6	7.9±2.1	63.2	23.1
Muscles	33.8	11.9	18.8±1.5	16.5±1.1	6.8±1.4	63.8	20.4
Integument	21.6	15.1	12.0±0.5	20.8±3.3	8.4±0.6	30.0	7.7
The whole larva	180.0	72.3	100.0	100.0	40.2	59.8	100.0

was 37,9 per cent, for the blood — 18,6 per cent, and the fat body 11,2 per cent. During a starvation period of 2 to 50 hours Heller and Świechowska noticed reductions up to a few percent in the relative weight of the alimentary canal owing to which the percentage for the blood increased.

In the body of the feeding waxmoth larvae, *Galleria mellonella*, the quantity of haemolymph (21 per cent of its body weight) is higher than in the body of the feeding caterpillars of the hawk moth. The result was quite unexpected. It was due to the fact that the total water content in the body of *Galleria mellonella* larvae amounts only to about 55 per cent (Niemierko S. 1950) while the water content in the body of *Celerio euphorbiae* larvae is considerably higher (Heller 1938).

The weight of the alimentary canal of the waxmoth larvae drops after the first twenty four hours of starvation to such a degree, that its percentage relation to the body weight falls by a half (namely from 22 per cent in the feeding larvae to 10 per cent in the larva starved for one day). In *Celerio euphorbiae* this was noticed after 48 hours of starvation (Heller and Świechowska 1947).

The decrease in weight of all body organs of the starving waxmoth larvae points to the fact that all of them participate in the metabolism of starvation.

The silk glands of *Galleria mellonella* larva were those which during prolonged starvation lost most of their weight.

SUMMARY

The weight of particular tissues and organs of the waxmoth larvae was investigated. The results show that the weight of each of the organs diminishes during prolonged starvation. The participation of the particular organs in the general loss of body weight was calculated.

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STUDIES ON THE BIOCHEMISTRY OF THE WAXMOTH (*GALLERIA MELLONELLA*). 17. NITROGEN METABOLISM IN THE TISSUES AND ORGANS OF THE LARVAE

Z. M. ZIELIŃSKA

Department of Biochemistry, Nencki Institute of Experimental Biology, Warsaw

(Received 12 January 1956)

The extensive literature on the metabolism of insects includes chiefly studies on the chemical composition of whole insects. The results thus obtained could not afford to give more than a general outline of the metabolism.

There are comparatively few studies concerning the biochemical transformations occurring in the separate tissues and organs of the insect body. Alone the composition of the haemolymph has been investigated by a number of authors (Heller 1930, 1932, Beadle and Shaw 1950, Ludwig 1953, 1954 and others). But changes in blood composition afford only indirect evidence of the processes occurring in other tissues.

A certain number of studies on the integument of insects has been published lately (e. g. Pryor 1940, Fraenkel and Rudall 1940, 1947, Lafon 1942, Dennel 1947, Wigglesworth 1948, 1949, Hackman 1953, 1954, 1955). The main problem studied was that of the chemical composition of the cuticle; however, the participation of the insect integument in the general metabolism of the organism has not been sufficiently taken into consideration.

The biochemical study by Wigglesworth (1942) deserves attention. The author observed the accumulation of lipid, glycogen and protein during feeding, and the disappearance of these storage substances in the fat body and in several other organs of the mosquito *Aedes aegypti* larvae during starvation. The studies by Demjanowskij, Wasiljewa and Konikowa (1950) in which methionine labelled with S^{35} was applied, concern protein metabolism during the growth and metamorphosis of the oak silkworm, *Antheraea pernyi*. In these investigations, the changes in the intensity of nitrogen transformations in each separate organ were examined.

The previous investigations of the present author (1952) have shown that the participation of nitrogen substances in the starvation metabolism of the waxmoth larvae is rather considerable. About one half of the protein contained in the organism was metabolised while the general loss of body weight amounted to 60 per cent.

In the present study, the nitrogen metabolism in the separate tissues and organs of the waxmoth larvae has been investigated.

MATERIAL AND METHODS

Full grown waxmoth (*Galleria mellonella*) larvae were examined. They were kept separately and starved for a long time under conditions previously described (Zielińska 1952, Zielińska and Wroniszewska 1956). The blood, the silk glands, the visceral part of the fat body, the alimentary canal with the Malpighian tubes, the muscles together with the parietal part of the fat body and the integument were now investigated. The tracheal and the nervous systems were analysed together with the muscle system.

Before dissection the larvae were weighed and cooled. For the dissection and separation of the larval organs two different methods were used alternatively.

Following the first one, the larva was dissected, the tissues and organs were separated as rapidly as possible with almost no loss at all and they were immediately submitted to chemical analysis, without being weighed. This procedure afforded the possibility to determine the quantities of the constituents investigated contained in the tissues, and to estimate them in relation to the larval weight as a whole. Thus the distribution of the particular nitrogen compounds in the body of the waxmoth larvae could be estimated.

Following the second method, each tissue or organ was examined separately. After the dissection of the body, the organ investigated was separated, weighed and analysed.

The total nitrogen was determined in a special series of experiments. In the other series, the non-protein nitrogen, the amino acids and the uric

acid were determined in the tungstic acid protein-free filtrate. Total nitrogen and non-protein nitrogen were estimated by the Kjeldahl method, amino acids according to the method previously applied (Zielińska 1952 a and b) and uric acid according to the modified Fearon (1944) method (Zielińska, non published). Chitin nitrogen was determined by the Kjeldahl method in the hydrolysate after hot KOH hydrolysis of the material investigated. The protein nitrogen content in the integument was estimated by subtracting the chitin nitrogen and the non-protein nitrogen content from that of the total nitrogen. In other tissues and organs chitin is absent or else its content is rather insignificant. In these cases the protein nitrogen content was estimated by subtracting the non-protein nitrogen content merely from that of the total nitrogen.

RESULTS

Feeding larvae, larvae starved for one day and larvae after prolonged starvation with about 60 per cent loss of initial weight were examined. The starvation period was 3 to 4 weeks.

Table I

Total nitrogen content in mg for 100 mg of the initial weight of the larva

Organ	At the beginning of starvation mg	After prolonged starvation mg	Differences	
			mg	in % of the initial content in the tissue
Silk glands	0.60	0.10	−0.50	−83
Alimentary canal and Malpighian tubes	0.60	0.24	−0.36	−60
Blood	0.52	0.10	−0.42	−81
Visceral fat body	0.43	0.11	−0.32	−75
Muscles with parietal fat body	0.40	0.20	−0.20	−50
Integument	0.95	0.75	−0.20	−21
The whole larva	3.50	1.50	−2.00	−57

The changes in total nitrogen contents gave a rough picture of the nitrogen metabolism in the material investigated. At the onset of the starvation period* the total content of nitrogen in the body of the waxmoth larvae amounted to 3.5 mg for 100 mg

* After one day of starvation.

of larval body weight. After prolonged starvation this value was merely 1.5 mg for 100 mg of the initial body weight.

The general loss of nitrogen was 2.0 mg for 100 mg of the initial body weight of the larvae. It represents 57 per cent of the protein initially present in the body (Table I). The loss of body weight reached simultaneously 60 per cent. The loss of nitrogen compounds was different in each separate tissue and organ. Most considerable was decrease of the total nitrogen content in the silk glands and the blood (Table I). About 80 per cent of the initial content of nitrogen compounds disappeared from these tissues during starvation. The fat body lost 75 per cent, the muscular system — 50 per cent, the alimentary canal — 60 per cent and the integument — 20 per cent of the initial content of all nitrogen compounds.

Table II

Non-protein nitrogen content in μg for 100 mg of the initial weight of the larva

Organ	At the beginning of starvation μg	After prolonged starvation μg	Differences	
			μg	in % of the initial content in the tissue
Silk glands	20	20	0	0
Alimentary canal and Malpighian tubes	50	80	+30	+60
Blood	75	45	−30	−40
Muscles and fat body	115	90	−25	−22
Integument	100	70	−30	−30
The whole larva	360	305	−55	−16

The decrease of the total nitrogen in each of the tissues and organs of the waxmoth larvae under investigation shows that all these tissues and organs participate in the nitrogen metabolism during starvation.

Chitin, the characteristic constituent of the Arthropods integument, is also present in the tracheal trunks as well as in the fore and hind gut of insects. According to data obtained in this laboratory the chitin content in *Galleria mellonella* larvae shows considerable deviations in specimens derived from different cultures. In several series of analyses the chitin nitrogen content varied

from 50 to 75 μg for 100 mg of larval body weight. However, deviations from the mean value were slight.

The content of chitin nitrogen in a one-day-starved waxmoth larvae amounted to 53.9∓5.2 μg for 100 mg of body weight. During prolonged starvation of the waxmoth larvae, the chitin nitrogen content remained unchanged, its mean value being 52.2 ∓ 5.2 μg for 100 mg of the initial body-weight of the larvae. The data obtained in these investigations seem to suggest that chitin does not participate in the nitrogen metabolism of the waxmoth larvae during starvation.

The data concerning the non-protein nitrogen fraction of the nitrogen compounds are presented in Table II.

At the onset of the experimental period the non-protein nitrogen amounted to 360 μg for 100 mg of body weight and at the end of the starvation experiments it amounted to 305 μg for 100 mg of the initial body weight of the larvae.

Table III

Free amino acid nitrogen content in μg for 100 mg of the initial weight of the larva

Organ	At the beginning of starvation μg	After prolonged starvation μg	Differences	
			μg	in % of the initial content in the tissue
Silk glands	10	7	− 3	−30
Alimentary canal and Malpighian tubes	15	22	+ 7	+47
Blood	40	21	−19	−48
Muscles and fat body	50	27	−13	−46
Integument	45	37	− 8	−18
The whole larva	160	114	−46	−29

The quantity of non-protein nitrogen in the alimentary canal and in the Malpighian tubes rose during starvation. The increase reached 60 per cent of the initial value. The quantity of non-protein nitrogen in the silk glands remained constant. In other tissues and organs it decreased. The loss in nitrogen of this fraction in each separate organ was 20 to 40 per cent of the initial values.

In insects the main part of the non-protein nitrogen components

are free amino acids. The content of amino acids is expressed in this paper as the alpha-amino nitrogen (Zielińska 1952 a and b). At the beginning of starvation, the amino acids nitrogen content was 160 μg for 100 mg of body weight; at the end of the experimental period, this value was 114 μg for 100 mg of initial body weight. These data compared with those in Table II show that amino acid nitrogen constitutes at the beginning about 45 per cent of the total non-protein nitrogen and 37 per cent at the end of the experimental period.

In nearly all the organs the amino acid nitrogen constitutes 45 to 55 per cent of the non-protein nitrogen. In the alimentary canal with the Malpighian tubes this relation amounts only to 30 per cent.

Table IV

Uric acid nitrogen content in μg for 100 mg of the initial weight of the larva

Organ	At the beginning of starvation μg	After prolonged starvation μg	Differences	
			μg	in % of the initial content in the tissue
Silk glands	0	0	—	—
Alimentary canal and Malpighian tubes	5.5	44.3	+38.8	+705
Blood	1.6	3.8	+ 2.2	+135
Muscles and fat body	4.3	29.0	+24.7	+575
Integument	5.5	17.3	+11.8	+215
The whole larva	16.9	94.4	+77.5	+460

After prolonged starvation, the amount of free amino acids represents, in many organs, a smaller fraction of the total quantity of non-protein nitrogen compounds than at the beginning; in the blood and in the integument this ratio remains unchanged.

The content of free amino acids decreases in nearly all the organs (Table III). In the alimentary canal and in the Malpighian tubes, somewhat larger amount of amino acids was found after prolonged starvation. We may as well add that in the presence of large amount of uric acid, the amino acid nitrogen values obtained according to the applied method, were higher than the real ones (Zielińska 1952 b). During starvation, large quantities

Table V

Protein nitrogen content of the larvae

Organ	At the beginning of starvation	After prolonged starvation	Differences				
			in mg for 100 mg of the initial body weight	in % of the initial content in the tissue	in % of the total loss of the protein	in % of the total protein nitrogen content of the larvae	
	in mg for 100 mg of the body weight	in mg for 100 mg of the initial body weight				At the beginning of starvation	After prolonged starvation
Silk glands	580	80	−500	−86	25.7	18.8	7.0
Alimentary canal and Malpighian tubes	550	160	−390	−71	20.0	17.9	14.1
Blood	450	55	−395	−88	20.3	14.5	4.8
Muscles and fat body	710	220	−490	−69	25.2	23.2	19.4
Integument	790	620	−170	−22	8.8	25.6	54.7
The whole larva	3080	1135	−1945	−63	100.0	100.0	100.0

of uric acid accumulate in the hind gut and in the Malpighian tubes. It is possible that the presence of uric acid in these organs may have introduced some inaccuracy in the determination of amino acids.

Uric acid, the chief end product of nitrogen metabolism of the waxmoth larvae was found in practically all the organs except the silk glands (Table IV). At the beginning of the experiment, the uric acid nitrogen content was 17 μg for 100 mg of larval weight; after prolonged starvation it reached 95 μg for 100 mg of the initial body weight. The content of uric acid increased in all the organs, except the silk glands alone. The quantities of uric acid in the intestine and the Malpighian tubes, as well as in the fat body and the muscles increased about eight times.

The protein nitrogen was estimated merely as the difference between the total nitrogen content and the chitin and non-protein nitrogen. At the onset of the experimental period, the protein nitrogen reached 3 mg for 100 mg of larval weight; after starvation it was no more than 1.1 mg for 100 mg of the initial body weight (Table V). Hence, the loss of the protein nitrogen amounts to nearly 2 mg for 100 mg of the initial body weight i. e. the loss of proteins amounts to over 60 per cent of the initial proteins content of the organism. The quantities of proteins dropped in all the tissues and organs (Table V). The consumption of protein was different in each separate organ. The silk glands and the blood are those which during prolonged starvation lose most of their proteins. The consumption of proteins in these organs reaches 90 per cent of the proteins initially present. About 70 per cent disappears from the fat body and the muscles as well as from the alimentary canal. Even 20 per cent of the initial quantity of protein from the integument is utilized.

As a result of the unequal consumption of proteins from each separate organ, the proteins content of these organs in relation to the total proteins content of the organism differed at the beginning and at the end of the starvation period. The proteins of the integument which constitutes one fourth of the total proteins (Table V) formed after starvation to about a half of them. The protein of the silk glands and of the blood, which at the beginning constituted 15—20 per cent of the body protein formed at the end of the starvation period only a small fraction of the total amount of body proteins.

Table VI

Different nitrogen compounds in μg N for 100 mg of the initial weight of the whole larvae

Organ	At the beginning of starvation						After prolonged starvation					
	total N	protein N	chitin N	non--protein N	amino acid N	uric acid N	total N	protein N	chitin N	non--protein N	amino acid N	uric acid N
Silk glands	600	580	—	20	10	0	100	80	—	20	7	0
Alimentary canal and Malpioghian tubes	600	550	—	50	15	5.5	240	160	—	80	22	44.3
Blood	520	450	—	75	40	1.6	100	55	—	45	21	3.8
Visceral fat body	430	710	—	115	50	4.3	110	220	—	90	27	29.0
Muscles with parietal fat body	400						200					
Integument	950	790	60	100	45	5.5	750	620	60	70	37	17.3
The whole larva	3.500	3 080	60	360	160	16.9	1500	1135	60	305	114	94.4

Table VII

Changes in nitrogen content of the tissues after prolonged starvation
I. In μg N for 100 mg of the initial weight of the whole larva
II. As % of the initial content in the tissues

Organ	I						II					
	Total N	Protein N	Chitin N	Non-protein N	Amino acid N	Uric acid N	Total N	Protein N	Chitin N	Non-protein N	Amino acid N	Uric acid N
	μg	μg	μg	μg	μg	μg	%	%	%	%	%	%
Silk glands	−500	−500	—	0	− 3	—	−83	−86	—	0	−30	—
Alimentary canal and Malpighian tubes	−360	−390	—	+30	+ 7	+38.8	−60	−71	—	+60	+47	+705
Blood	−420	−395	—	−30	−19	+ 2.2	−81	−88	—	−40	−48	+135
Visceral fat body	−320	−490	—	−25	−13	+24.7	−75	−69	—	−22	−46	+575
Muscles with parietal fat body	−200		—				−50		—			
Integument	−200	−170	0	−30	− 8	+11.8	−21	−22	0	−30	−18	+215
The whole larva	−2000	−1945	0	−55	−46	+77.5	−57	−63	0	−16	−29	+460

The fact that in all the tissues and organs of the waxmoth larvae the quantity of protein decreased suggests that during starvation the proteins of all the tissues and organs are consumed. Most considerable is the lability of the silk glands proteins, whereas the proteins of the integument show the most considerable stability.

Table VI illustrates the content of all the nitrogen compounds investigated. Table VII presents the changes in nitrogen content after prolonged starvation.

Most interesting are the processes occurring in the silk glands of the starved waxmoth larvae. In full grown waxmoth larvae, the proteins content of the silk glands constitutes about 35 per cent of their wet weight. During prolonged starvation the loss of silk gland weight amounted to 70 per cent of its initial value (Zielińska and Wroniszewska). At the same time the consumption of proteins used up reached almost 90 per cent of the initial protein content (Tables V, VI and VII). The percentage of protein in the silk glands dropped to a half. The quantities of free amino acids present in these organs diminished insignificantly during starvation, but simultaneously, their concentration rose. No uric acid was detected in the silk glands of starved larvae.

The proteins content of the alimentary canal (analysed together with the Malpighian tubes) may amount to 35 per cent of its wet weight. During prolonged starvation, the loss in weight of these organs amounts to about 60 per cent of the initial value (Zielińska and Wroniszewska). The decrease of proteins reached at the same time 70 per cent of the initial proteins content (Tables V, VI and VII). Simultaneously, large amounts of uric acid accumulated in the Malpighian tubes and in the hindgut. The absolute quantity of uric acid increased here eight times; the concentration rose twenty times, namely from 55 μg to 1150 μg uric acid for 100 mg of the organs wet weight. A certain increase in the quantity of free amino acids was also observed.

The integument of the waxmoth larvae contains about 50 per cent of water. The determination of water content in the insect skins is rather difficult owing to the fact of their rapid drying up during dissection. This fact was already reported by Lafon (1942), Tsao and Richards (1952). For this reason, the composition of the integument is expressed in per cent of the dry weight.

The chitin content of the integument of the waxmoth larvae

varies from 22 per cent, to 33 per cent of the dry weight*. The proteins content amounts to about 65 per cent of the dry integument. Chitin and protein constitute together over 90 per cent of the dry skin substance. Amounts of non-protein nitrogen compounds do not exceed several per cent of the dry weight of the integument.

During starvation, the weight of the integument of the waxmoth larvae decreases to a lesser degree than the weight of the other organs (Zielińska and Wroniszewska), the loss of integument weight being not over 30 per cent of its initial value. The amount of chitin in the skin of the waxmoth larvae remained unchanged during prolonged starvation. The total non-protein nitrogen decreased in the integument, whereas the content of uric acid rose (Tables IV, VI and VII).

In the body of the waxmoth larvae, like in that of many other insects one can distinguish the central or visceral and the peripheral or parietal part of the fat body. The visceral layer of the fat body in the waxmoth larvae forms compact, folding, whitish lobes which can be easily dissected. The parietal layer of the fat body is composed of loose, small, yellowish elements that are not separable from the muscles by dissection. That is why the parietal layer of the fat body was investigated in the present studies together with the muscle system. The content of lipids in the visceral part of the fat body reaches 60 per cent of the wet weight, thus constituting about 90 per cent of the dry substance of the fat body (Włodawer private communication). The parietal part of the fat body and the muscles contain jointly a smaller quantity of lipids than the visceral part of the fat body. The proteins content in the visceral part of the fat body is 7 to 8 per cent, in the parietal layer of the fat body together with the muscles — about 10 per cent of the wet organ weight. The content of free amino acids in the muscles and the parietal fat body was also higher than in the visceral fat body. In the former, the amino acid nitrogen reached 220 μg, whereas in the latter it did not exceed 140 μg for 100 mg of organ weight.

During prolonged starvation the weight of the visceral fat body dropped considerably. The loss amounted to 60 per cent of the

* The contents of the chitin were estimated by multiplying the empirically obtained data concerning the chitin nitrogen by 15.4 (Lafon 1942, Fraenkel and Rudall 1940).

initial weight (Zielińska and Wroniszewska). The general decrease of total nitrogen in the visceral fat body was 75 per cent, in the parietal fat body and the muscles — 50 per cent of the initial quantity. During prolonged starvation the visceral fat body of the waxmoth larvae disappeared almost entirely. Consequently the nitrogen fraction in starved larvae were estimated in visceral fat body together with the parietal fat body and the muscles.

About 70 per cent of the proteins and about 50 per cent of the free amino acids initially present were used up in these organs during prolonged starvation. At the same time the quantity of uric acid present in the fat body and in the muscles rose eight times in comparison to the initial quantity while its concentration rose as much as twenty times.

The blood of the waxmoth larvae contains about 14.5 per cent of dry substance, the lipids being 4,5 per cent (Włodawer, private communication) proteins — 8.1 per cent and the amino acids about 1 per cent. Hence, all the other organic compounds and mineral constituents amount to no more than 1 per cent of the fresh blood weight.

During prolonged starvation, the quantity of blood dropped, the loss reaching 60 per cent of the initial value (Zielińska and Wroniszewska). At the same time, about 90 per cent of the proteins initially present, disappeared from the haemolymph (Tables V, VI and VII). Simultaneously, the concentration of the blood proteins dropped to a half of its initial level. The total amount of free amino acids decreased in the blood but their concentration rose a little. The concentration of uric acid in the blood of full grown waxmoth larvae amounts to several microgramms for 100 mg of blood. After prolonged starvation, the uric acid concentration was several times higher, and the total quantity of uric acid was also larger.

Finally, we must point out that during prolonged starvation of the waxmoth larvae the proteins of all the tissues and organs are used up: 25 per cent of proteins that has been consumed is derived from the silk glands, 25 per cent from the fat body and the muscles, 20 per cent from the blood, 20 per cent from the alimentary canal and about 10 per cent from the integument (Table V).

DISCUSSION

The present results indicate that about 90 per cent of the proteins contained in the silk glands of the *Galleria mellonella* larvae disappears during prolonged starvation. This is evidence of a great biochemical plasticity of the organism. As it is known, intensive processes of proteins synthesis occur foremost in the silk glands.

According to Demjanowskij and Filipowicz (1950), the blood amino acids seem to be the only, directly utilised material in the silk proteins synthesis of the oak silkworm *Antheraea pernyi* larvae. The pseudo globulin fraction of the blood proteins may participate in the silk synthesis as an intermediary link between the tissues proteins and the blood amino acids. The protein synthesis, according to these authors, takes place within the cells of the silk gland walls. The usage of methionine labbeled with S^{35}, directly indicating the intensive incorporation of S^{35} into the proteins of the silk gland walls in *Antheraea pernyi* larvae, whereas the proteins content of the silk glands does not exhibit any radioactivity (Demjanowskij, Wasiljewa and Konikowa 1952). Bheemeswar (1955) moreover has stated that extracts from the silk glands of the silkworm *Bombyx mori* show transaminase activity. Amino acids active in these transamination reactions besides aspartate were alanine, phenylalanine, and asparagine. Aspartate is the most active substrate. The author pointed: „The discovery of an enzyme system in the glands of the silkworm capable of converting aspartate to alpha-alanine is of particular significance from the point of view of the synthesis of precursors of silk fibroin. This mechanism securs for the organism an additional source of endogenous alpha-alanine, which constitutes about 20.0 per cent on the weight of silk".

The main question bears on the pattern according to which the starving organism of the waxmoth larvae utilized the protein accumulated in the silk glands. It does not seem likely that the high molecular silk proteins can penetrate from the silk glands through their walls into the blood. It seems more probable that during starvation the silk proteins are split and the products of decomposition are gradually removed by the haemolymph.

Histochemical studies of the separate tissues of the mosquito larvae *Aedes aegypti* (Wigglesworth 1942) go to prove that

during starvation, the intestinal walls become thinner and the epithelial cells smaller. Similar changes indicative of atrophia of the intestinal epithelium were observed in the waxmoth larvae (Przełęcka, private communication). These data suggest that during starvation, the walls of the alimentary canal participate in the general metabolism of the organism.

The previous investigations (Zielińska and Wroniszewska) have established that the loss in weight of the alimentary canal amounts to 60 per cent of the initial value, whereas the utilization of proteins is of 70 per cent. At the same time, there is a marked accumulation of large quantities of uric acid in the Malpighian tubes and in the hindgut. The amount of amino acids present in the alimentary canal and in the Malpighian tubes increases to a certain extent. In larval urine of the silkworm glutamic acid, serine, glycine, alanine, leucine, valine, histidine and glutamine were founded; glutamic acid in pupal urine (*maeconium*) is quite abundant (Fukuda, 1951). Courtois (1929) found also a certain amount of amino acids in the maeconium of several species of Lepidoptera. It is known that maeconium contains the end products of the metabolism from the whole period of metamorphosis.

The main function of the fat body of insects is the storage of lipids, glycogen and proteins. But the fat body is also a tissue where many different metabolic processes occur owing to which the fat body may be considered as an organ comparable to the liver of other animals.

The occurrence of uric acid concretions in the fat body of starved cockroaches was observed by Filipczenko (1907) who attributed the fact to the disappearance of other nitrogen compounds. Anderson and Patton (1955) have determined uric acid in the fat body of both feeding and starving cockroaches, *Periplaneta americana*. They established that during prolonged starvation the content of uric acid in the cockroach fat body rises considerably. During starvation, the fat, glycogen and proteins of the mosquito larvae *Aedes aegypti* (Wigglesworth, 1942) are entirely used up. The nuclei of the intestinal cells become smaller, the cytoplasm undergoes changes which Wigglesworth attributes to the utilization of protein, at the same time a progressive accumulation of uric acid in the aqueous vacuoles of the fat body was observed. Leifert (1935) studied the synthesis

of uric acid in the oak silkworm *Antheraea pernyi*. She regards the fat body as a tissue where uric acid may be synthetized. Anderson and Patton (1955) while studying the homogenate of the fat body of *Prodaenia eridana* larvae and of *Tenebrio molitor* larvae established that such purine derivates as xanthine, hypoxanthine, guanine, adenine and inosine as well as monoethyl oxalacetate are uricogenically active. „The activity of oxalacetate and the inhibition by malonate may indicate that the Krebs citric acid cycle has a role in uric acid production" (Anderson and Patton). Inactivity of urea in extracts of *Prodenia* and *Tenebrio* larvae seem to contradict the supposition that urea is a precursor of uric acid. Hence, the authors statement that „the process of uric acid synthesis in insects follows the same pattern as has been demonstrated from other uricotelic forms" seems quite convincing.

The present investigation showed that during prolonged starvation about 70 per cent of the proteins initially present and about 50 per cent of free amino acids disappear from the fat body and the muscles of the waxmoth larvae. Simultaneously, large amounts of uric acid accumulate and their concentration rises almost twenty times. These data seem to provide indirect evidence that the fat body constitutes that tissue of the waxmoth larvae where the biosynthesis of uric acid takes place.

The uric acid concentration in the parietal fat body analysed together with the muscles was, in starved waxmoth larvae, somewhat higher than in the visceral fat body. It would be interesting to separate the parietal fat body from the muscles and to investigate each of them separately.

During the last fifteen years there has been a great advancement in the knowledge of the insect cuticle, the outer part of the integument.

According to Lafon (1942) chitin amounts to about 1/3, insoluble proteins likewise to 1/3, mineral salts to 3—4 per cent and lipids to 2—3 per cent of the dry substance of the insect integument. There is also in the insect skin a certain quantity of pigments and substances extractable with water and alcohol (including phenols).

The chitin content in the cuticle of waxmoth larvae amounts according to Tsao and Richards (1952) to somewhat more than 1/3 of the dry cuticle weight. This value is thus almost the same as the one indicated by Lafon for the waxmoth larvae

integument. In our experiments the chitin contents in the *Galleria mellonella* larvae varied considerably, the mean value being 27 per cent of the dry skin weight.

According to Lafon (1942) the protein content of the integument of waxmoth larvae constitutes not less than 36.7 per cent and not more than 61.3 per cent of the dry weight. Both these values Lafon obtained by different methods. The first one is based upon the formol estimation of amino acids in the hydrolysate after acid hydrolysis of the integument. The second one on the estimation of the protein nitrogen content by subtracting the chitin nitrogen from total nitrogen. Lafon supposes the first (minimum) value to be more reliable than the second (maximum) value which may include the pigments and extractable substances.

Our own data concerning the protein content in the integument of the waxmoth larvae correspond with the maximum values of Lafon: about 65 per cent of the dry weight of the integument consisted of proteins. As pointed out above, the protein nitrogen was estimated, in our studies by subtracting the chitin and non protein nitrogen from the total nitrogen.

Chitin content in blow-fly *Sarcophaga falculata* (Fraenkel and Rudall 1940) is considerably higher than in *Galleria mellonella*. In the larval cuticle, chitin amounts to more than half of the dry weight, proteins being the chief constituents of the rest. During pupation, the *S. falculata* larvae do not throw of their cuticle and at the same time the chitin contents of the integument remains unchanged. The fraction of the insoluble proteins increases partly at the expense of the soluble proteins content. As known, the formation of insoluble complexes of proteins and phenols is accompanied by the hardening and darkening of the insects cuticle. According to Fraenkel and Rudall (1947) tyrosine of the blood may be a source of phenols. At the time of hardening and darkening of the cuticle, the level of tyrosine in the blood decreases. It seems probable that during these processes there is a desamination of tyrosine, since the nitrogen quantity in the puparium of *S. falculata* does not increase (Fraenkel and Rudall 1947).

The histochemical studies (Wigglesworth 1942, 1948) indicate that insect integument does not constitute a stable material, as considered earlier, but participates in the general metabolism. Our investigations confirm this statement on a biochemical basis: during prolonged starvation of the waxmoth larvae about 20 per

cent of protein disappears from the skin. But proteins of the integument seem to be somewhat less labile than the proteins of other organs.

The skin of waxmoth larvae contains substances which yield with a chlorimide reagent coloured products of condensation. These substances are considered in the present paper as uric acid. During the last years, the presence of pterinlike pigments has been found in the insect integument. Bodine and Fitzgerald (1948) found pterin pigments not only in the skin of Orthoptera larvae but also in the eggs. From the larval skin of the lemon type of silkworm *Bombyx mori* Hirata, Nakanishi and Kikawa have isolated, besides uric acid also xanthopterin and a certain amount of some other, unidentified pterin. It is possible that in the integument of the waxmoth larvae are contained some amounts of the pterinlike pigments. We have founo that vanthopterin gives no eoloured reaction with chlorimid reagent. Our next study will be concerned with questions whether other pterins react to chlorimide reagent and whether in the skin of the waxmoth larvae there are pterin pigments at all*.

Present investigations show that about 90 per cent of the protein initially contained in the blood disappeared in the course of metabolism during starvation. The quantity of free amino acids decreased at the same time to half of its initial value.

In his studies on the biochemistry of the hawk moth *Celerio euphorbiae*, Heller (1930, 1932) has found that after a few days of starvation the level of proteins in the haemolymph of the larvae decreases by half while the level of the non-protein compounds fraction remains stable.

In starved aquatic larvae of *Sialis lutaria* (Beadle and Shaw 1950) the proteins concentration decreased twenty times, but the fraction of non protein nitrogen remained at the level that assures the osmotic balance.

The studies of Ludwig and Wugmeister (1953) on the chemical composition of the blood of the japanese beetle *Popilla*

Addendum in proof.

* Our recent experiments (Zielińska and Klita 1957, Acta Physiol. Polon. in press) have shown the presence of leucopterin, as well as of another fluorescent substance, in skin of the waxmoth larvae. Leucopterin reacts with chlorimid giving a colour reaction product. It is clear therefore that the values for uric acid reported in this paper include leucopterin too.

japonica larvae present another picture. During four weeks of starvation no significant changes occurred in the proteins concentration of the blood. The amino nitrogen and non-protein nitrogen rose steadily during the entire experimental period; the level of lipids and of reducing compounds varied a little. The water content and the osmotic pressure of the blood remained constant. Therefore, the authors suggest that while certain materials are taken from the haemolymph during starvation, others are being added to it as a result of tissues metabolism*.

The facts described above afford indirect evidence that all the tissues and organs of insects play a part in the metabolism during starvation. The present studies furnish the additional and more direct argument to this statement.

SUMMARY

The part played by each particular tissue and organ in the nitrogen metabolism of the waxmoth larvae during starvation was investigated. The total nitrogen content, non-protein nitrogen, amino acids and uric acid were tested in all the organs.

It was established that during three to four weeks of starvation, about 60 per cent of the total proteins content and about 30 per cent of the total amino acids content of the organism were used up. During the entire starvation period, the content of chitin remained unchanged whereas the content of uric acid increased considerably.

On the whole, 25 per cent of the proteins consumed during starvation was derived from the silk glands, 25 per cent from the fat body and the muscles, 20 per cent from the blood, 20 per cent from the alimentary canal and 10 per cent from the integument. The results obtained show that all the tissues and organs participate in the metabolism of the waxmoth larvae during starvation.

The percentage of uric acid increased almost twenty times in the alimentary canal and the Malpighian tubes as well as in the fat body.

* Addendum in proof.

In april 1956 Ludwig and Cullen published (Physiol. Zool. *29*, 153) their work on the effects of starvation on insects blood. They stated that increase in non-protein nitrogen in blood of the Japanese beetle larvae during starvation can be only in part explained by the increases in amino nitrogen and urea nitrogen. It may also result from increases in other nitrogenous compounds, such as glucosamine, various purines and so on.

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INVESTIGATION OF FREE AND PROTEIN-BOUND NUCLEOTIDES IN ACETONE-CHLOROFORM-DRIED MUSCLE POWDER

W. NIEMIERKO, M. DYDYŃSKA, W. DRABIKOWSKI, I. KĄKOL and H. ZAŁUSKA

Department of Biochemistry, Nencki Institute of Experimental Biology, Warsaw

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Since the discovery of ATP'ase activity of myosin by Engelhardt and Ljubimowa (1939) the nucleotides (esp. nucleoside polyphosphates) are still in the centre of interest in muscle biochemistry. Until recently it has been almost generally accepted (cf. Weber 1952, 1955) that dephosphorylation of ATP is probably the first known exergonic reaction in muscle contraction. Although the latest experiments of Fleckenstein *et al.* (1954, 1955) and Mommaerts (1954, 1955) may modify this hypothesis, which is based chiefly on model experiments, there seems to be no doubt that the breakdown of ATP is essential for the contraction of the isolated myofibril (Perry 1954). Recently Perry (1952) and Chappel and Perry (1953, 1954) were able to show that not only the myofibrils but also the sarcoplasm possess the ATP'ase activity. Consequently it appears that the functions of ATP in muscle are manifold and are not restricted to the contractile process.

During the last several years many authors which have been working with muscle proteins have found a certain quantity of ATP, ADP as well as of some other phosphate-containing compounds in their preparations of actin, myosin and actomyosin

(Buchthal *et al.* 1949, Straub and Feuer 1950, Laki *et al.* 1950, Laki and Clark 1951, Dubuisson and Mathieu 1950, Mommaerts 1951, Szent - Györgyi 1951, Perry 1952, Biró and Nagy 1955, and others). The amount of the P present in the isolated proteins was small and variable.

So far as we know, there are no systematic investigations to show, what proportion of the nucleotides in the muscle is bound to the proteins and what part appears in free form. There exist only occasional data concerning this problem (Biró and Nagy 1955; Lange 1955).

This point merits however more detailed investigation. Moreover it seems especially interesting to find out with which proteins are combined the particular P compounds in the muscle, what is the character of the combinations, and whether and how does the situation change in various physiological states of the muscle. The elucidation of these questions may throw more light upon the various roles of the phosphate-containing compounds and may help to approach the problem of interrelations between structure and metabolism in muscle.

Perry (1956) assumes that about 10% of the nucleoside polyphosphates are bound to the myofibrillar structure, quite a small quantity is associated with the mitochondria and the remainder is in free solution in the sarcoplasm.

On the other hand, some of our preliminary experiments, (Niemierko 1954, Niemierko *et al.* 1954), seemed to indicate that probably about one half of the nucleotides of the resting frog muscle exist in a protein-bound form.

The usual procedure for determination of P fractions in animal tissues involves TCA (trichloracetic acid) for deproteinization. The total amount of so called acid soluble P compounds can be found afterwards in the protein free filtrate. Consequently, such filtrate contains not only the free P compounds but also the originally protein-bound fractions liberated by TCA. Taking this into consideration and trying to estimate in the muscle the total amount of compounds containing protein-bound phosphate, it was necessary to elaborate a procedure which would prevent the breakdown of the P-protein linkage whatever character it may possess.

The present paper deals with investigation of the free and bound nucleotides in the acetone-chloroform-dried muscle powder („muscle powder") prepared from resting frog muscles.

MATERIAL AND METHODS

The experiments were performed on leg muscles of the frog *Rana esculenta*. In order to obtain the muscles in a state of complete relaxation the following procedure was chosen. The living frogs were cooled gradually to about −10°C and the muscles were quickly removed without decapitation or destruction of the spinal cord. The cooled animals did not react during this operation. Control experiments showed that if the cooled frogs were allowed to warm they were, in the majority of cases, still alive.

The muscles were quickly weighed and immediately afterwards ground in a mortar with a chilled (−10°C) acetone-chloroform mixture (5:1). About 10—20 ml of the mixture were used for 1 g of muscle. The solution was filtered and the remainder repeatedly extracted with fresh portions of the chilled mixture. The muscle powder obtained after the last extraction was free of water and of most of the lipids. After evaporation at room temperature of the traces of the organic solvents some larger particles (fragments of tendons?) which were always present in very small quantity and which could not be ground in the mortar* were separated from the powder by sifting. The fine powder obtained after sifting was preserved in vacuum at a temperature of 0°C.

The results of our previous investigations have shown (Niemierko *et. al.* 1952; Dydyńska, unpublished results) that the TCA extract of such a powder contains nucleotides in quantities nearly identical with those which contains the TCA protein free filtrate from the corresponding amounts of the fresh tissue.

Samples of the different muscle powder preparations were analysed for control purposes by a routine technique in which TCA was applied for deproteinization. The results of the analyses furnished data concerning the total quantity of protein and of particular acid soluble phosphorus compounds in the investigated muscle powder.

The following analytical methods were used. The protein nitrogen was estimated by micro-Kjeldahl in the residue after precipitation with TCA. The destillation of ammonia was made in the apparatus of Markham (1942). Inorganic phosphorus together with creatine phosphate phosphorus (P-1) were estimated after one minute heating (100°C) of the TCA filtrate which was previously neutralized and subsequently acidified to about 0.1—0.2 N. The nucleoside polyphosphate labile phosphorus (P-15) was determined by means of estimating the surplus (i. e. over P-1) ortophosphate released after 15 minutes hydrolysis of the TCA filtrate at 100°C in 1N sulphuric acid. For estimation of the orthophosphate the method of Fiske and Subbarow (1925) was employed. Ribose was estimated by the method of Mejbaum (1939).

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* The analyses have shown that the total quantity of phosphorus in this material was quite unimportant.

EXPERIMENTS AND RESULTS

The experiments which were to supply information about the amount of protein-bound nucleotides in muscle powder were based on following considerations.

It is well known that the solubility of ATP in water solutions is very great. Even in the presence of 50% alcohol it is quite important (cf. Berger 1956). Consequently ATP, in amounts corresponding to physiological conditions, will by easily soluble either if it is in a free form or if it is in combination with soluble proteins. On the other hand ATP will remain after extraction in the residue or will be precipitated from the solution only then when the conditions are such that the proteins with which it is bound are or become insoluble.

Accordingly, the following general scheme of experiments was elaborated. The muscle powder was extracted either with water or with buffer mixtures. Both, the solution obtained and the residue, were analysed. If the solution contained proteins they were afterwards precipitated by various methods. The phosphate containing substances present in the residue, or in the precipitate, which were presumably in a combination with proteins were „liberated" by means of TCA. The different P fractions were estimated afterwards in the protein free filtrate.

It was necessary, however, in performing such experiments 1° to take into consideration the possibility of an adsorption of phosphate containing compounds on the precipitated proteins, 2° to be aware of the possibility of splitting off the protein-P linkage by the applied procedure.

The results obtained by Straub and Feuer (1950), Biró and Nagy (1955), as well as by several other authors indicate that the protein bound nucleotides of the muscle are present chiefly in combination with actin. As a starting point of the present investigation it has been assumed that this view is correct. For this reason two types of experiments were performed in which the good solubility of actin in destilled water and the minimal solubility of actin, and of most other muscle proteins, at pH-4.6 were made use of.

In the first type of experiments the muscle powder was extracted with destilled water and it was expected that the solution would contain actin, actin bound nucleotides, as well as free nucleotides.

In the second type of experiments the muscle powder was ex-

tracted with an acetate buffer mixture at pH-4.6. It was thought that in such experiments most of the muscle proteins (in particular actin) and the nucleotides bound to insoluble proteins would remain in the residue, whereas the solution would contain the free nucleotides and possibly the nucleotides connected with some soluble proteins.

I. Aqueous extract of muscle powder

Aqueous extracts were prepared by grinding samples of muscle powders with destilled water (0°C) in a mortar taking about 20 ml water for 1 g of powder. After 20 minutes the extract was filtered into a volumetric flask, the residue reextracted 2 or 3 times with small amounts of cold water and the combined filtrates brought to the mark. The pH of the solutions was usually about 6.5.

When the residues and samples of the aqueous extracts were analysed it was found that the aqueous extracts contained about 1/10 of the proteins present originally in the powder and contained most of P-1 and P-15. Table I gives some of the results which were obtained after treating the residues and the aqueous extracts with TCA.

Table I

Protein, P-1 and P-15 in aqueous extracts of muscle powders. Extracts prepared at 0°C. All figures are expressed as mg or micromoles per g muscle powder and as percentage of the total quantity of the substances in material

Exp. No	Protein		P — 1		P — 15		Ribose	
	mg/g	%	μM/g	%	μM/g	%	μM/g	%
5	78.6	9.8	58.7	92.1	34.2	90.0	—	—
8	99.3	12.4	76.1	92.6	66.7	97.4	—	—
12	73.8	9.2	65.5	93.2	54.7	90.0	—	—
12a	70.9	8.8	65.7	98.0	56.0	95.5	—	—
13	101.5	12.7	69.6	94.2	48.9	98.5	31.1	94.7
14	102.0	12.7	68.7	94.6	45.5	87.3	27.3	76.0
15	99.3	12.4	76.1	92.6	71.0	97.8	—	—

The proteins present in the aqueous extract were precipitated with various methods and the precipitate was investigated on the content of P-1 and P-15 fractions which could be liberated by means of TCA.

The following methods of protein precipitation were used

1° Heat coagulation (t = 100°C).

2° Addition of 0.2 M acetate buffer (pH-4.6) in cold (t = 0°C).

3° Addition of a mixture of 0.2 M acetate buffer (pH-4.6) and alcohol (1:1) in cold (0°C).

4° Addition of hot 0.2 M acetate buffer (pH-4.6) followed by heating the mixture in boiling water bath during 5 minutes.

Table II

P-1, P-15 and ribose in the filtrate obtained after heating (100°C) the aqueous extract. All figures are expressed as micromoles per g muscle powder and as percentage of the total quantity of the substances in extract

Exp. No	P — 1		P — 15		Ribose	
	μM/g	%	μM/g	%	μM/g	%
10	75.4	99.6	35.8	99.4	—	—
13	68.6	98.5	48.6	99.4	31.6	100

The results which were obtained with aqueous extracts prepared from different muscle powder preparations are presented in Tables II, III, IV and V.

One can see, that the quantity of proteins precipitated from the aqueous extracts by different methods, as well as the amounts of phosphate containing compounds which were precipitated with the proteins, varied greatly.

Table III

Protein, P-1 and P-15 in the precipitate obtained from aqueous extract after addition of buffer solution (pH-4.6) at 0°C. All figures expressed as mg or micromoles per g muscle powder and as percentage of the total quantity of the substances in extract

Exp. No	Protein		P — 1		P — 15	
	mg/g	%	μM/g	%	μM/g	%
1	11.2	14.7	3.6	6.7	8.2	13.7
2	37.2	49.0	4.7	9.1	5.5	9.0
4	15.3	18.3	1.9	2.7	1.9	4.7
5	3.7	4.8	0.4	0.7	4.1	12.6
6	—	—	0.4	0.5	2.2	4.5

In the case of heat coagulation (Table II) the precipitate was free of phosphate fractions which remained almost completely in the solution (over 99%).

After addition of a cold acetate buffer solution (pH-4.6) to the aqueous extract (Table III) 4.5 to 13.7% of the total amount of P-15 fraction and various amounts of proteins were precipitated. The precipitate contained also a small quantity of P-1 fraction.

Table IV

Protein, P-1 and P-15 in the precipitate obtained from the aqueous extract after addition of buffer solution (pH-4.6) and alcohol at 0° C.
All figures expressed as in Table III

Exp. No	Protein		P — 1		P — 15	
	mg/g	%	μM/g	%	μM/g	%
1	76.2	100	2.9	5.4	11.6	19.4
4	39.2	47	2.4	3.5	6.4	10.4
5	33.7	42.7	0.7	1.2	3.4	10.0
6	—	—	0.4	0.5	5.6	11.4
13	62.9	62.0	0.6	0.9	10.1	20.6

The addition of a cold buffer solution (pH-4.6) + alcohol (Table IV) caused the precipitation of a considerable amount of proteins (43—100%) and of a somewhat greater proportion (10—20%) of P-15 than was found in the previous experiments.

Table V

Protein, P-1 and P-15 in the precipitate obtained from the aqueous extract after addition of buffer solution (pH-4.6) at 100°C.
All figures expressed as in Table III

Exp. No	Protein		P—1		P — 15	
	mg/g	%	μM/g	%	μM/g	%
2	65.2	86.3	1.8	3.3	12.4	20.2
3	—	—	0.2	0.2	11.4	30.6
4	57.2	68.5	2.1	3.0	6.5	16.1
5	77.3	98.0	1.7	2.9	4.1	12.0
6	—	—	0.3	0.4	6.1	12.4
10	—	—	1.8	2.5	4.9	11.0
13	100.8	99.5	0.7	1.0	11.6	23.7
18	—	—	0.4	1.0	8.4	19.0
19	—	—	2.6	3.0	8.8	26.2
20	—	—	1.0	1.8	6.0	12.5

The next table (Table V) shows that the greatest amounts of proteins (68—100%) as well as of P-15 (11—30%) were precipitated by addition of a hot buffer solution (pH-4.6) to the extract.

In all the experiments the amount of the precipitated P-1 fraction was always much lesser than the P-15 fraction.

Table VI

Content of P-15 fraction in the precipitate obtained from the aqueous extract to which ATP was added

Precipitation of protein by	ATP added to the aqueous extract μg P — 15	P — 15 found in the protein precipitate μg
Buffer solution (pH-4.6) + alcohol 0°C.	0 330	188 195
Buffer solution (pH-4.6) 100°C.	0 267	143 168
,,	0 267	120 120
,,	0 443	136 141

In a few cases the amount of ribose was also estimated. The tentative results obtained seem to indicate that the nucleoside polyphosphates precipitated together with the proteins were either ATP or a mixture of ATP, ADP and AMP.

Table VII

Protein, P-1, P-15 and ribose in hot aqueous extract of muscle powder (100°C). All figures are expressed as in Table I

Exp. No	Protein		P — 1		P — 15		Ribose	
	mg/g	%	μM/g	%	μM/g	%	μM/g	%
11	31.7	3.9	104.0	99.8	33.0	87.4	33.4	89.5
11a	35.5	4.4	97.5	94.7	34.0	93.8	29.1	94.4
14	46.5	5.8	96.7	94.7	38.9	94.2	30.1	95.0

To decide whether the nucleotides found in the precipitate were present in a protein bound form or were merely adsorbed by the precipitating proteins, the following experiments were performed. To samples of the aqueous extract considerable amounts of ATP were added and the proteins were precipitated afterwards by one of the methods described above. As can be seen from Table VI in all cases the added ATP remained free in solution

and the amount of P-15 fraction found in the protein precipitate was practically unchanged.

In one series of experiments the aqueous extract was prepared by treating the muscle powder with boiling water. The whole P-1 fraction (95—100%) and most of the P-15 fraction (87—94%) were found in the filtrate (Table VII). Only a small quantity of proteins was present in the solution and these proteins were found to be free of phosphate. It could be supposed therefore that the nucleotide-protein linkages were split by boiling and that the phosphate containing compounds were present in the solution in free form.

II. Acetate buffer extracts of muscle powder

In the second type of the experiments the muscle powder was extracted by means of an acetate buffer solution at pH — 4.6. As mentioned above, it was expected that at this pH a considerable part of the proteins, esp. actin, would remain in the residue and if some of the phosphate containing compounds were combined with the proteins these P compounds should be undissolved.

The extractions were made by a procedure similar to that used in the case of the aqueous extraction. The solutions and the residues were analysed after treating them with TCA.

Table VIII shows the results obtained by a hot extraction. One can see that 4 to 10% of the P-1 fraction and 20 to 40% of the nucleotides (P-15) remain in the residue.

When a cold acetate buffer extraction of the muscle powder was made the amount of the P-15 fraction found in the residue was even greater. Table IX shows that the residue contained 43 to 67% of the nucleotides present in the muscle powder.

DISCUSSION

The results of the present investigation show that, apparently, a considerable part of the nucleotides which are present in the acetone-chloroform-dried muscle powder prepared from resting frog muscles may form a combination with proteins. The amount of these protein bound P-compounds found in different types of experiments is very variable.

The largest amount of undissolved nucleotides was found when

Table VIII

Protein, P-1, P-15 and ribose in the residue and the extract after extraction of the muscle powder with buffer (pH-4.6) at 100°C. All figures are expressed as in Table I.

Exp. No	Residue						Extract							
	P − 1		P − 15		Ribose		Protein		P − 1		P − 15		Ribose	
	μM/g	%	μM/g	%	μM/g	%	mg/g	%	μM/g	%	μM/g	%	μM/g	%
7	6.6	8.6	13.0	36.3	—	—	—	—	73.7	91.5	23.0	64.2	—	—
9	6.5	5.9	11.4	31.4	—	—	—	—	107.0	94.0	25.0	69.1	—	—
9a	8.8	7.2	17.0	38.8	—	—	—	—	111.4	93.0	26.1	60.8	—	—
11	10.0	10.0	11.8	28.5	8.3	22.3	2.5	0.3	92.2	90.0	29.7	71.4	28.9	77.6
11a	3.6	3.8	7.1	20.8	5.5	15.9	3.8	0.5	90.1	96.2	27.1	79.1	28.5	84.1
14	4.2	4.0	9.6	22.0	5.4	16.1	5.3	0.7	99.5	95.9	34.6	77.7	28.1	83.9

Table IX

Protein, P-1, P-15 and ribose in the residue and the extract after extraction of the muscle powder with buffer (pH-4.6) at 0°C. All figures are expressed as in Table I.

Exp. No	Residue						Extract							
	P − 1		P − 15		Ribose		Protein		P − 1		P − 15		Ribose	
	μM/g	%	μM/g	%	μM/g	%	mg/g	%	μM/g	%	μM/g	%	μM/g	%
1a	6.1	5.9	12.1	67.0	—	—	—	—	98.6	94.0	6.4	33.0	—	—
14	10.7	10.8	22.7	44.8	16.2	51.2	65.8	8.2	88.7	88.4	30.3	54.9	15.4	48.7
15a	5.6	6.2	26.8	43.0	20.0	57.0	—	—	85.1	93.8	35.9	57.0	15.4	42.8

the muscle powder was extracted with cold acetate buffer at pH-4,6 (Table IX).

When a hot acetate buffer extraction is made (Table VIII) the amount of nucleotides which can be established as protein bound is somewhat lesser. A still smaller amount of the bound nucleotides is found in a cold aqueous extract (Tables III, IV and V) although it contains most of the nucleotides present in the muscle powder. If the aqueous solution is heated (Table II), or when a hot aqueous extract (Table VII) is prepared from the muscle powder, the quantity of protein bound nucleotides found in the material is negligible. All these findings indicate that the stability of the protein bound nucleotides is essentially different in different conditions.

It has been observed that when muscle powder, or the protein precipitated from the aqueous extract by addition of acetate buffer at pH-4.6, are washed repeatedly with cold buffer solution, the

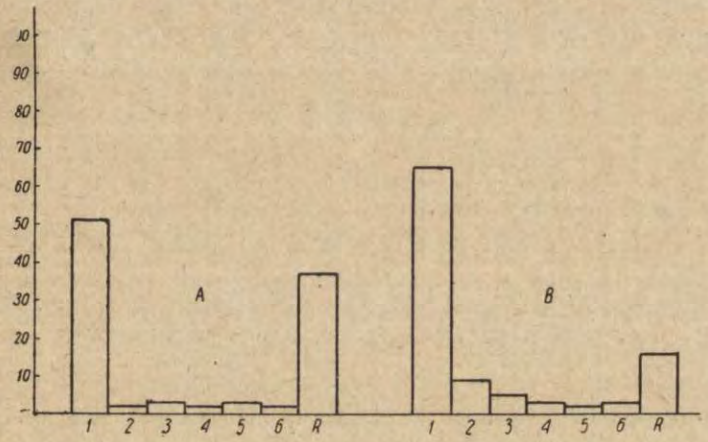

Fig. 1. Effect of repeated washing of muscle powder with cold (A) and hot (B) buffer (pH-4.6) solution. Columns denote the P-15 content expressed as percentage of its total quantity in the material. 1 — first extraction, 2—6 successive washings, R — residue

amount of protein-bound nucleotides found in the residue is always considerable. After repeated washing of the powder with hot buffer (pH-4.6) nucleotides are still found in the residue although in somewhat smaller quantities (Fig. 1). Consequently the association seems to be rather stable at this pH, especially at low temperature. The combination of the nucleotides with proteins appears to be less stable in distilled water, even at 0°C.

The amount of nucleotides found in purified muscle protein preparations or in isolated myofibrils by the different authors mentioned above is much lower than it might be expected from the results of the present experiments.

Biró and Nagy (1955) report the presence of protein bound nucleotides in muscles of different species in amounts corresponding to 5—16 μg P/g fresh muscle. This forms probably not more than 5—10% of the total quantity of nucleotides in muscle. Other authors mentioned above give figures which are approximately of the same order. In all the experiments the isolated proteins were prepared either after a previous extraction of myosin from the minced muscle by KCl solution or by using prolonged dialysis or repeated washing with water or with neutral or alkaline buffer solutions. It is to be expected that all such manipulations may affect the integrity of the nucleotide-protein complexes and may break down certain linkages. Straub and Feuer (1950) mention that „some data suggest the possibility that a fraction of the actin-ATP may have been lost during the process of isolation of actin". Nevertheless, it follows from the results of Biró and Nagy (1955) that even after a very prolonged dialysis of muscle proteins with boric acid buffer (pH-7.2) a nearly constant amount of nucleotides is found as protein-bound. It is just this small amount of the nucleotides that Biró and Nagy accept as the bound nucleotides.

All these findings give impression that different protein-nucleotide complexes with differing properties may exist in muscle. The forces binding the molecules may be stronger or weaker depending upon the character of the P-protein linkage.

The only author who obtained high figures for the amount of bound nucleotides was Lange (1955). Lange showed that the nucleotides of the *rectus abdominis* of the frog are distributed equally (1:1) between the „sarcoplasm" and „the structural proteins". He has made moreover a very interesting discovery that only the latter fraction takes part in the dephosphorylation processes during muscular contraction. Lange is however of opinion that his experiments do not allow to decide whether and how the nucleotides are bound with the structural proteins in muscle.

As mentioned above, we found in the muscle powder also a protein-bound P-1 fraction. Its amount was much smaller than the amount of the protein-bound P-15 fraction. The P-1 fraction comprised inorganic phosphate and phosphocreatine which were not

estimated separately. The presence of small quantities of inorganic phosphate in actomyosin threads was shown by Buchthal et al. (1949). The existence of protein-bound phosphocreatine in muscle was foreseen by Eggleton and Eggleton. Already in 1929 these authors supposed that the whole creatine phosphoric acid is present in muscle „in combination with some colloidal substance".

All the results obtained so far seem to indicate that all the mentioned P compounds may occur in the muscle in a combination with proteins. The amount of protein-bound nucleotides found in the muscle powder in the present investigation was especially large and the stability of the complexes at pH 4.6 was quite considerable. Further examination of the nature of these compounds is still in progress.

SUMMARY

1. Free and protein-bound phosphate containing compounds were investigated in acetone-chloroform-dried muscle powder prepared from resting frog muscle.

2. During extraction of the muscle powder with a buffer solution at pH 4.6 about 50% of nucleotides and a far smaller percentage of orthophosphate and phosphocreatine remain in the residue presumably in a protein-bound form.

3. The nucleotide-protein complexes appear to be relatively stable at pH-4.6 and less stable in distilled water.

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INVESTIGATION OF SOME PROTEIN-BOUND PHOSPHORUS COMPOUNDS IN FRESH MUSCLE

W. NIEMIERKO, M. DYDYŃSKA and I. KĄKOL

Department of Biochemistry, Nencki Institute of Experimental Biology, Warsaw

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Previous experiments (Niemierko *et al.* 1954, 1957) had demonstrated that after extraction of the acetone-chloroform-dried muscle powder with an acetate buffer solution at pH 4.6 50% of polyphosphate nucleosides remained undissolved and could be found in the residue presumably in a protein-bound form. The complexes appeared to be fairly stable at pH-4.6 but were much less stable in distilled water. Hot water and trichloroacetic acid (TCA) split off the complexes easily. It had been accepted tentatively (Niemierko, 1954; Niemierko *et al.* 1954) that the amount of the P-protein complexes found in the above experiments corresponds to that present in the intact muscle. It could be supposed however that the manner of preparation of the powder had an influence on the nucleotide-protein complexes. Dehydratation and delipidation of muscle tissue obviously causes a partial denaturation of certain muscle proteins and probably also a destruction of some of the lipoproteins. Such processes might cause a liberation of a part, at least, of the nucleotides originally associated with protein (lipoprotein?) in the intact muscle. It seemed therefore necessary to perform the experiments on fresh frog muscle also.

MATERIAL AND METHODS

The experiments were performed on *mm. gastrocnemii* of the summer and autumn frogs *Rana esculenta*. The animals were cooled gradually to —10°C and treated as described in the previous paper (Niemierko *et al.*, 1957). The removed muscles were quickly weighed and immersed into liquid air. The frozen muscles were ground afterwards in a chilled mortar and extracted with a cold (0°C) mixture (1:1) of 0.2 M acetate buffer (pH-4.6) and alcohol. The final pH of the buffer-alcohol mixture increased usually to 5.6. About 10 ml of the mixture were used for one *gastrocnemius* each muscle being analized separately.

The solution obtained after the extraction was quickly filtered through a chilled Buchner funnel (dense filter paper being used) into a chilled flask and the residue (R-1) quickly washed three times with the cold (0°C) buffer-alcohol mixture. The collected filtrates (F-1), which were slightly opalescent, usually contained 2—3 mg of protein per g muscle. The filtrates were again filtered, now through a chilled Seitz filter disc under suction. A small quantity of a residue (R-2) was thus separated from the filtrate and the latter was collected into a chilled volumetric flask. This filtrate (F-2) was practically free of protein.

In this way three fractions (R-1, R-2 and F-2) were obtained according to the scheme shown in Table I. In all three fractions the inorganic orthophosphate + phosphocreatine (P-1) and the labile nucleotide phosphate (P-15) were determined in a manner similar to that described in the previous paper (Niemierko *et. al.* 1957) i. e. after treating the fractions with TCA.

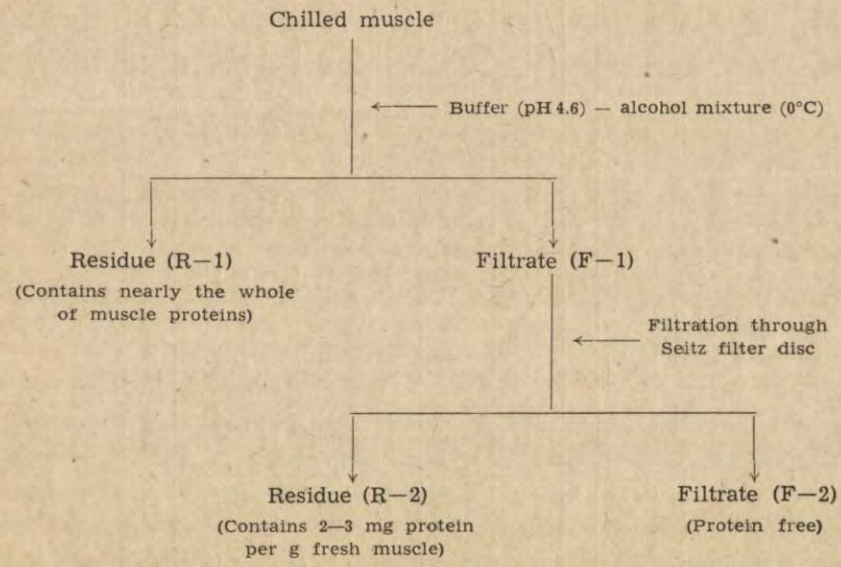
EXPERIMENTAL AND RESULTS

The determination of the amount of free and of protein-bound nucleotides (P-15), as well as of the P-1 fraction, in fresh muscle was based upon considerations described in the preceding paper (Niemierko *et al.* 1957). The muscles were extracted with a buffer (pH-4.6) - alcohol mixture (see the previous section) in conditions when most of the muscle proteins remain undissolved. We considered as protein-bound: 1° P-1 and P-15 fractions which were found in the residue (R-1) and 2° that part of the soluble P-fractions which could be separated from the solution (F-1) together with proteins by filtration through Seitz filter disk (R-2). The rest of the P-1 and P-15 fractions was assumed to be free (F-2). It was shown in the preceding paper that the above considerations seemed to be correct because the amount of the added ATP could always be recovered in the solution in free form and did not precipitate with protein.

The results of experiments in which the muscles were extracted with the buffer (pH-4.6)-alcohol mixture are presented in Tables II, III and IV. One can see that although the content of the estimated P compounds varies considerably in muscles of different frogs, the quantities of particular fractions present in the symmetrical muscles are very similar. The mean values show that somewhat more than one half of the polyphosphate nucleosides (P-15) can be found in the R-1 fraction and 17% on the average is present in the R-2 fraction (Table IV). This means that under these experimental conditions about 70% of the nucleotides were probably protein-bound and that only 30% appeared to be free.

Table I

Scheme of fractionation of the fresh frog gastrocnemii.



The results summarized in Table IV show that most of P-1 fraction (83%), composed of inorganic orthophosphate and phosphocreatine, appear in free form. The quantities of protein-bound compounds in the P-1 fraction were nevertheless large. This becomes evident if we take into consideration the absolute amounts of the compounds expressed as micrograms or micromoles per one gram of fresh muscle (Tables II and III). One can see that the

Table II

Fractionation of phosphorus compounds of fresh frog *gastrocnemii*. All figures expressed as μg P per g muscle. P-1 orthophosphate + phosphocreatine phosphorus; P-15 nucleotide labile phosphorus. R-1, R-2 and F-2 fractions as in Table I.

Frog No	Residue												Filtrate			
	R − 1				R − 2				R − 1 + R − 2				F − 2			
	P − 1		P − 15		P − 1		P − 15		P − 1		P − 15		P − 1		P − 15	
	muscle		muscle		muscle		muscle		muscle		muscle		muscle		muscle	
	left	right	left	right	left	right	left	right	left	right	left	right	left	right	left	right
32	86	84	107	103	33	37	24	31	119	121	131	134	583	596	62	60
38	79	67	93	94	44	45	49	52	123	112	142	146	584	660	82	86
38a	73	75	126	126	50	51	56	53	123	126	182	179	604	604	68	71
39	105	102	88	83	—	—	22	24	—	—	110	107	676	660	46	44
44	151	156	150	159	29	25	27	31	180	181	177	190	593	593	36	37
45	91	94	70	70	20	21	26	24	111	116	96	94	596	589	36	36
46	103	116	83	78	32	27	20	16	135	143	103	94	620	607	50	55
Mean value	98	99	102	102	35	34	32	33	132	133	134	133	608	616	54	56

Table III

Fractionation of phosphorus compounds of fresh frog *gastrocnemii*, All figures expressed as micromoles per g muscle. Symbols as in Table II.

Frog No	Residue												Filtrate			
	R − 1				R − 2				R − 1 + R − 2				F − 2			
	P − 1		P − 15		P − 1		P − 15		P − 1		P − 15		P − 1		P − 15	
	muscle		muscle		muscle		muscle		muscle		muscle		muscle		muscle	
	left	right	left	right	left	right	left	right	left	right	left	right	left	right	left	right
32	2.8	2.7	3.4	3.3	1.1	1.2	0.8	1.0	3.9	2.9	4.2	4.3	18.8	19.2	2.0	1.9
38	2.5	2.2	3.0	3.0	1.4	1.4	1.6	1.7	3.9	3.6	4.6	4.7	18.8	21.3	2.6	2.8
38a	2.3	2.4	4.1	4.1	1.6	1.6	1.8	1.7	3.9	4.0	5.9	5.8	19.5	19.5	2.2	2.3
39	3.4	3.3	2.8	2.7	—	—	0.7	0.8	—	—	3.5	3.5	21.8	21.3	1.5	1.4
44	4.9	5.0	4.8	5.1	0.9	0.8	0.9	1.0	5.8	5.8	5.7	6.1	19.2	19.0	1.2	1.2
45	2.9	3.0	2.3	2.3	0.6	0.7	0.8	0.7	3.5	3.7	3.1	3.0	19.2	19,0	1.2	1.2
46	3.3	3.7	2.7	2.5	1.0	0.9	0.6	0.5	4.3	4.6	3.3	3.0	20.0	19.6	1.6	1.8
Mean value	3.2	3.2	3.3	3.3	1.1	1.1	1.0	1.0	4.3	4.3	4.3	4.3	19.6	19.9	1.8	1.8

Table IV

Fractionation of phosphorus compounds of fresh frog *gastrocnemii*.
All figures expressed as percentage of the total amount of the substance in the muscle.
Symbols as in Table II.

Frog No	Residue												Filtrate			
	R − 1				R − 2				R − 1 + R − 2				F − 2			
	P − 1		P − 15		P − 1		P − 15		P − 1		P − 15		P − 1		P − 15	
	muscle		muscle		muscle		muscle		muscle		muscle		muscle		muscle	
	left	right	left	right	left	right	left	right	left	right	left	right	left	right	left	right
32	12.3	11.7	55.5	53.2	4.8	5.2	12.4	15.9	17.1	16.9	67.9	69 1	82.8	83.0	32.1	30.9
38	11.0	8.8	41.6	40.0	6.2	5.6	22.2	22.7	17.2	14.4	63.8	62.7	82.8	85.5	36.1	30.3
38a	9.8	10.2	50.6	50.6	6.8	6.8	22.2	21.0	16.6	17.0	72.8	71.6	83.3	82.9	27.2	28.4
39	13.5	13.4	56.0	55.0	—	—	14.0	14.3	—	—	70.0	69.3	86.5	86.5	30.0	28.6
44	19.6	20.0	69.5	69.9	3.6	3.2	13.0	13.7	23.2	23.2	82.5	83.6	76.8	76.8	17.4	16.4
45	12.8	13.2	53.5	54.7	2.6	3.1	18.6	16.7	15.4	16.3	72.1	71.4	84.5	83.7	27.9	28.6
46	13.4	15.3	55.1	52.1	4.1	3.7	12.2	10.4	17.5	19.0	67.3	62.5	82.2	81.0	32.7	37.6
Mean value	13.2	13.2	54.5	53.7	4.7	4.6	16.4	16.4	17.9	18.0	70.9	70.1	82.7	82.8	29.0	29.7

quantities of the P-1 and P-15 fractions found as protein-bound are of the same order.

In a series of experiments one of the symmetrical muscles was extracted as previously with buffer (pH 4.6)-alcohol mixture, where-

Table V

Comparison of extraction of the muscles with the buffer (pH-4.6)-alcohol mixture and with 60% alcohol.
All figures expressed as percentage of the total amount of the substance in the muscle. Symbols as in Table II.

Frog No	Right muscle:extraction with buffer (pH 4.6) — alcohol mixture				Left muscle: extraction with 60% alcohol			
	R − 1 + R − 2		F − 2		R − 1 + R − 2		F − 2	
	P − 1 %	P − 15 %	P − 1 %	P − 15 %	P − 1 %	P − 15 %	P − 1 %	P − 15 %
28	22	66	78	34	6	9	94	91
28a	17	80	83	20	2	7	98	93

as the other muscle was treated with 60% alcohol. The results presented in Table V show that after treatment with alcohol the undissolved residue (R-1 + R-2) contains only a small part of the

Table VI

Effect of repeated extractions of the muscles with buffer (pH-4.6) solution. Figures and symbols as in the previous Tables.

Extractions	P − 1		P − 15	
	µg/g	%	µg/g	%
1 st extraction: buffer (pH-4.6) — alcohol mixture	592	79	90	38
2 nd extraction: buffer (pH-4.6)	26	3	28	11
3 rd „ „ „	15	2	6	2
4 th „ „ „	13	2	2	0.8
5 „ „ „ „	9	1	2	0.8
6 „ „ „ „	7	1	0	0
7 „ „ „ „	16	2	0	0
8 „ „ „ „	4	0.5	2	0.8
9 „ „ „ „	8	1	0	0
10 „ „ „ „	12	2	2	0.8
11 „ „ „ „	7	1	0	0
12 „ „ „ „	11	1	3	1.3
Residue	9	1	85	36

investigated phosphorus compounds, most of them being present in the solution (F-2), presumably, in free form.

Analogously to our previous experiments (Niemierko *et al.*, 1957) the influence of repeated extraction (washing) of the residue was also examined. Four *gastrocnemii* of two frogs were extracted in the usual manner with the buffer(pH-4.6)-alcohol mixture. Subsequently 11 successive extractions with the buffer solution (pH-4.6) without alcohol were performed. The figures summarized in Table VI indicate that after all the extractions the amount of nucleotides (P-15) found in the residue was still considerable, whereas the quantity of P-1 fraction was very small.

Table VII

Effect of addition of ATP to the muscle during extraction with buffer (pH-4.6)-alcohol mixture.

Frog No	M. gastrocnemius	Weight of muscle g	ATP added to muscle (as μg P—15)	P—15 found in the residues (R—1 + R—2) μg
33	right	0.68	0	146
	left	0.67	150	149
34	right	1.30	0	288
	left	1.27	150	294
34a	right	1.06	0	172
	left	1.07	150	188

Finally, in a last series of experiments considerable amounts of ATP were added to the frozen muscle immediately before extraction with the buffer (pH-4.6) - alcohol mixture. Table VII shows that the quantity of the bound nucleotides found in the residue was practically equal to that in the control muscle.

DISCUSSION

The experiments presented in this investigation give a further support to the view, expressed in the previous paper (Niemierko et al., 1957), that most of the nucleotides present in the muscle are not extractable at pH 4.6 and remain in the residue presumably in a protein-bound form. The results of the experiments performed on fresh muscles are even more marked than our previous data

obtained with acetone-chloroform-dried muscle powder. It seems therefore to be quite possible, as was already mentioned above, that the delipidation procedure used during preparation of the muscle powder splits off certain lipoproteins which may form an association with nucleotides.

One can see from Table IV that the percentage of the protein bound P-1 fraction (mean value 17%) is much smaller than the percentage of the bound nucleotides (mean value 70%). Nevertheless, the absolute amount of each of the bound P fractions is large and the figures for each of them are very similar (Tables II and III). A further characterization of the P-1 fraction is however needed.

Biró and Nagy (1955) as well as other authors quoted in our preceding paper give much lower values for the amount of protein-bound nucleotides. The experiments presented in this investigation show that a large part of the nucleotides associated with protein is easily liberated under certain conditions (comp. Table V). In fact, we were able to show that the nucleotide-protein complexes are fairly stable at pH-4.6—5.5 but that they are split in distilled water and in 60% alcohol. Most of the previous authors determined probably only the more firmly bound nucleotides whereas by the method used in the present investigation some weaker linkages could also be revealed. Further experiments are necessary to determine the stability of the association at other pH as well as to elucidate the influence of different ions and of different salt concentrations upon the complexes. Such experiments are in progress in our laboratory.

The highest figures (about 50%) for the nucleotides found in the „structural residue" („Strukturrückstand"), which can be accepted as protein bound, are given by Lange (1955). But even these figures are somewhat lower than ours. It is not to be excluded that the fraction called by Lange „Sarcoplasm" might also contain a certain quantity of protein-bound nucleotides. Lange deproteinised this fraction by treating it with 60% alcohol and estimated the nucleotides in the protein free filtrate. Such procedure, as can be concluded from our results, could easily split the nucleotide-protein complexes, if they were present in the „sarcoplasm".

A few additional remarks are needed concerning the protein containing muscle fractions (Table I) designated in the present investigation as R-1 and R-2. As was mentioned above the residue which remained after the extraction of the muscle with the buffer

(pH-4.6)-alcohol mixture contained nearly the whole of the muscle proteins. In the extract (F-1) only 2—3 mg of proteins per g fresh muscle were usually present. If the extract was filtered through a Seitz filter disc all the protein could be separated (Residue 2) so that the new Filtrate (F-2) was practically free of protein. Quite considerable amounts of P-1 and P-15 fractions were always present in the Residue 2 (Table II, III, IV). It is difficult so far to decide what is the nature of the Residue 2. May be that it consists merely of minute particles of denaturated proteins formed under the influence of alcohol during extraction. The figures in Table II, III and IV show that the amounts of P-1 and P-15 fractions in Residue 2 in muscles of different frogs are variable. On the other hand, however, the content of the P-fractions in the symmetrical muscles of the same animal is very similar. These last findings indicate that it is not to be excluded that Residue 2 may consist of some structural elements of the muscle tissue (mitochondria?). To elucidate this point further experiments are under way.

SUMMARY

1. After extraction of frozen frog muscles (*mm. gastrocnemii*) with acetate buffer (pH-4.6) - alcohol mixture about 70% of the nucleotides are found as protein-bound in the residue, and only 30% are free in solution. The percentage of the protein-bound P-1 fraction (orthophosphate + phosphocreatine) found in the residue is much lower (17%).

2. The absolute amounts of nucleotides and P-1 fractions found at pH-4.6 as protein-bound are, however, very similar making up to about 4.3 micromoles of each per g fresh muscle. The mean values for the free nucleotides and free P-1 fractions are 1.8 micromoles and 20 micromoles respectively.

3. The protein-bound nucleotides are fairly stable at pH-4.6 whereas P-1 fractions are much less stable. All the investigated P-protein complexes split off easily under influence of 60% alcohol in distilled water.

Addendum in proof.

On the grounds of the experiments discussed in the present paper it seemed justifiable to suppose that the nucleotides found in the residue after extraction of the muscle at pH 4.6 are protein-bound

also in the intact muscle. Such a view was expressed in our earlier preliminary communication (Niemierko 1954, Niemierko *et al.* 1954). Our recent experiments show however that the situation is much more complicated (Dydyńska *et al.* 1957). We have demonstrated that the amount of protein-bound nucleotides depends on the extraction medium used (buffer solution + alcohol), especially on its pH. At a lower pH (3.6) up to 90% of the nucleotides could be found in the protein residue; whereas in an alcaline medium nearly all of the nucleotides were soluble. When pH was changed during the process of extraction it could be observed that acidification led to a formation of nucleotide-protein combinations whereas alcalization led to their break up. Moreover, Drabikowski (1957) was able to show that different proteins, not only muscular, could form combinations with ATP, ADP and AMP. The amount of protein--bound nucleotides was strictly dependent on pH. Thus, so far it is difficult to decide what is the proportion of protein-bound nucleoside polyphospates in the intact living tissue. The observed lability of the compounds makes it, however, possible that also in the organism such combinations can be formed and split easily.

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