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FURTHER INVESTIGATIONS ON THE MECHANISM, DETERMINING BODY COLOUR IN DROSOPHILA MELANOGASTER.

BY SÁNDOR (ALEXANDER) WOLSKY, LAJOS CSIK AND GYULA FÁBIÁN.

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I. INTRODUCTION. EARLIER INVESTIGATIONS.

The experiments on which this paper is based were carried out in 1943 and 1944, i. e. during the most critical years of the war, when shortage of scientific material was increasing in Hungary and isolation from the scientific literature of the allied nations almost complete. At the final stage even the stock of „ebony" flies, on which the main bulk of the work was carried out, had been lost and there was no more possibility to replace it. Under such circumstances the work is by no means completed. But as after the war the authors had to depart company and there is little chance of their continuing the team work in the near future, they decided to publish this paper in its present, rather fragmentary form. The authors are well aware of the incompleteness of their exp eriments, and the many shortcomings of their paper, but they hope that even so it may contain some data which could be useful for further research.

Physiological genetics, i. e. the study of the mode of action of genes, has in recent years extended to the genes regulating theb ody colour in Drosophila melanogaster, such as ebony (e), black (b), yellow (y), etc., as well as their wild type allelomorphs. Research in this direction promised

to be extremely fruitful, as the mechanism of pigment formation is one of the fairly well known chapters in biochemistry and the process has long been known to be of a fermentative character. It could thus be hoped that the genes controlling the development of the body pigment in Drosophila could be shown to be in close connection with the enzymes responsible for pigment formation, and consequently the results would support the theory, according to which the genes produce their effects through enzymes, and are possibly of the nature of enzymes themselves (*Hagedoorn* 1911, *Goldschmidt* 1916, etc.). But the results so far accomplished in respect to the body colour genes do not fulfill these expectations.

Graubard (1933), the first who worked in this field, demonstrated that tyrosinase, which plays a decisive role in the formation of melanin, is not present in proportions corresponding the intensity of body colour in the different mutants. Thus in „yellow", which is lighter than the wild type, he found more tyrosinase than in the wild type, but in „black" and „ebony" (especially the later), which are darker than the wild type, he found distincly less enzyme than in the wild type. In respect to tyrosinase content *Graubard* otherwise found complicated relations, which cannot be reviewed here, but in any case, it was impossible to draw a conclusion as to the connection between the mode of action of the body-colour genes in question, and the enzymes responsible for pigment formation.

Recently *Danneel* (1943) made very detailed investigations in respect to the connection between body colour genes and pigment-forming enzymes, but his results, though they differ extensively from those of *Graubard*, and contain many new details (e. g., the demonstration of 3 different chromo-oxydases), are nevertheless negative in respect to the problem in question for they also do not show connection between the intensity of body colour and the quantity of pigment-producing enzymes (page 385: „Danach steht wohl fest, dass die Gene (+), (y), (e) und (b) mit der Entstehung der Chromo-Oxydasen nichts zu tun haben.").

Wolsky and *Csik* (1942) tried to approach the problem of the genes' mode of action by another way. As it is known that pigment-formation is in close relationship to biological oxidation, they investigated whether there was any difference in this respect between the wild type and one of the body-colour mutants, „ebony". Measuring the oxygen consumption by *Warburg's* manometric method, they found no difference. On the other hand, with *Thunberg's* vacuum tube methylene blue decoloration method, which in many respect seemed more suitable for the purpose (see *Wolsky* 1941), they found that at the beginning of metamorphosis

the ground contents of „ebony“ pupae decolorized methylene blue somewhat more rapidly than did the contents of wild type pupae. From this they concluded that the process of oxidation at the beginning of metamorphosis is more intensive in the ebony pupa (time of decoloration in wild type 28,9 ± 2,03 min., in „ebony“ 21,3 ± 0,94 min.). Thus it seemed that the different colour of the wild type and „ebony“ was due to this circumstance, though the difference in oxidation could not have derived directly from the chromo-oxydase activity, as it is known that the catalytic effect of chromo-oxydases is displayed only in the presence of oxygen. This cannot be substituted by other hydrogen-acceptors, as for example methylene blue. Thus the phenomena observed must have been due to other oxidizing (dehidrogenating) enzymes, which react with methylene blue, and which could only indirectly have influenced the process of pigment-formation, by way of the inner redox potential. But as will be pointed out later, another explanation is also possible.

Meanwhile *Pryor* (1940 a, b) has made most important contributions to the knowledge of the chemical processes, leading to the hardening and coloration of chitin (his observations on the hardening of the puparium of Calliphora are especially important in this connection), whereas *Waddington* (1941) showed mainly by phenogenetic observations, and simple experiments the role, which these processes may play in the production of body colour in Drosophila. The main point of these studies is that the body colour of Drosophila depends not only upon the amount of melanin formed, but also upon the intensity of the natural „tanning“, which takes place in the chitin of the integument and by which it becomes more resistant both physically and chemically and takes on a characteristic brownish „amber“ colour. (This process was called „sclerotization“ by *Pryor*.) In „ebony“ for example both melanin formation and sclerotization of the imaginal chitin takes place at a higher rate than in the wild type. These studies greatly contributed to a better understanding of the development of body-colour in Drosophila.

II. CHROMO-OXYDASES IN WILD TYPE AND „EBONY“.

Since the experiments with *Thunberg*'s method on the wild type and „ebony“ give greatly varying results in the later stages of metamorphosis, and the results with larvae are equally unsatisfactory (cf *Wolsky* and *Csik* 1942), we sought for other methods to demonstrate the mode of action of the „ebony“ gene. With methods similar to those employed by *Danneel* we investigated whether there was any difference in the chromo-oxydase content of wild type and „ebony“. As substrate we used tyrosine on

the one hand, and pyrocathechol and benzidin on the other. With the former we wanted to demonstrate the amount of tyrosinase, with the latter two the amount of phenol oxydase („dopa" oxydase), or that of the so-called benzidine-peroxidase. But the results obtained were similar to the findings of *Danneel*, as the difference of colour reactions of the extracts prepared from pupae and larvae was in some experiments in favour of ebony (as expected), in other experiments however the wild type extracts gave stronger colour. Thus, independent of *Danneel*, and contemporaneously with the publication of his paper (our experiments in this direction were carried out in 1943), we are in the position to confirm the statement that the „ebony" gene presents no difference from the wild type in respect to the amount of chromo-oxidase.

III. CHROMOGEN CONTENT IN WILD TYPE AND „EBONY".

From the results, reported above, we concluded that not the activity of the melanin forming enzymes but he amount of their substrate (tyrosine or other chromogen) must be different in the wild type and „ebony". This view is in good accordance with the findings of *De Luna* (1924), who showed by means of peroxidase tests the predetermination and prelocalization of chromogens in Drosophila. Earlier works of *Gessard*, *Durham*, *Weindl*, and especially of *Gortner* (1910, 1913) on the chromogen content of the colour varieties of the potato beetle — all before or around 1910 — seem to point in the same direction.

To establish a possible difference in the chromogen content, it seemed advisable to compare only those tissues in which the colour producing substances were deposited, i. e. the hypodermis, with perhaps the directly adjacent tissues. These we tried to obtain in the following way: we pinched off the anterior end of the larva or pupa and through the opening pressed out the inner organs with a blunt instrument (e. g. a flat dissecting needle). In this way superficial tissues dominated in what remained. In the finely ground pulp made from this, and without the addition of a substrate, the presence of chromogens could be shown, as the pulp in a short time turned lilac colour and finally took on a dark, dirty-gray appearance. The pulp prepared from the inner parts remained colourless or turned much less gray.

a) *Experiments with larvae.*

For these experiments we first used mature, IIIrd instar larvae, when they had already left the culture medium. The circumstance that

the pulp prepared from the superficial tissues of these specimens darkened, seemed to show that there was chromo-oxidase present in this stage. This stands in contradiction with *Danneel*'s statement (page 383): „verpuppungsreife Maden enthalten so gut wie keine Oxydasen". But it is possible that this contradiction is only apparent, for *Danneel* used dopa as substrate for his experiments, so they cannot throw light on the presence of tyrosinase in the strict sense (monophenolase), whereas it is known that the other reactions of the chromo-oxidation process are spontaneous, and take place in the presence of oxygen without catalysts. It is possible that in consequence of the mincing or grinding, the chromogens of the skin and the oxygen came into such direct and intensive contact that they could oxidize and produce pigment without the intervention of enzymes.

But in respect to the amount of chromogen thus demonstrable in the skin of the larvae, again no difference could be shown between the wild type and „ebony". Sometimes the pulp prepared from the hypodermis of „ebony" larvae darkens more than that of the wild type, but at other times an equal degree of darkening, or even more could be seen in the pulp, made of wild type larvae.

b) *Experiments with pupae.*

The situation is different when pupae are compared, not immediately after puparium formation, but 24 to 48 hours later. In these stages the wild type and „ebony" pupae show differences in colour, as the puparium of the wild type turns brown soon after formation, while that of „ebony" remains colourless (cf. *Waddington* 1941). We found in experiments carried out with pupae of such age that the pulp made from the puparium and from the anlage of the imaginal skin of the wild type shows no change of colour, or at most gets a little browner, while the pulp similarly prepared from the same tissues of the ebony pupae darkens just like that made from the larval skin. This difference can be observed already, when pupae are grossly dissected. Where the puparium of ebony is injured, the borders of the wound turn black, which does not occur in the wild type.

Later investigations showed that even this difference is not always clear. It happened that the finely minced skin of „ebony" pupae showed no more colour change than that of the wild type, for which we could give at this time no reason. Until this question is cleared up, the above observations are communicated only with reservation.

IV. THE POSSIBLE ROLE OF REDUCING SUBSTANCES IN THE PRODUCTION OF COLOUR DIFFERENCES BETWEEN THE WILD TYPE AND „EBONY".

If further research would corroborate the observations, reported above, the conclusion should be drawn that „ebony" pupae in later stages of metamorphosis contain more chromogen than wild type pupae of the same age. In this case an interesting explanation offers itself of how the colour-difference between the pupae arises. The fact that the puparium of the wild type pupae turns brown at the beginning of metamorphosis, whereas that of „ebony" ones remains colourless, could be accounted for by the possibility that a certain reducing system in the ebony pupae inhibits the oxidation of the chromogens, while in the wild type this process is lacking or goes on more slowly and so a part of the chromogens, already in contact with air in the puparium becomes oxidized (and that is why the puparium turns brown), so less remains of it for the imaginal body colour development than in the „ebony" pupae. The situation may be similar to that which exists in the case of Addison's disease, where pigment formation and ascorbic acid content of the suprarenal are closely connected. *Szent-Györgyi* compared this case with the difference between a cut apple and a cut lemon. The former turns brown because it does not contain a sufficient amount of reducing substances (ascorbic acid), the latter remains colourless because a great amount of ascorbic acid is present. Whether this explanation holds good here, further investigations may establish. If a difference could be shown between the redox potentials of the two types of pupae, or in respect to the quantity of their reducing substances (more negative redox potential, or greater amount of reducing substances in the „ebony" pupae) the hypothesis in question would be confirmed. But even before this proof can be established numerous circumstances speak for the correctness of the hypothesis. Such are the earlier observations of *Wolsky* and *Csik*, above referred to, according to which the pulp made of „ebony" pupae at the beginning of metamorphosis reduces methylene blue more rapidly in *Thunberg* tube than does the pulp made from wild type pupae. Originally it was thought that this is a sign of stronger oxidation (dehydrogenation) in „ebony". But it may instead indicate the presence of a powerful reducing system in the „ebony" pupae, which is lacking in the wild type, or is present in a lower concentration. Already at the beginning of the experiments this reducing system could have reduced one part of the methylene blue in the tubes containing the „ebony" pupae, so that in the experiments carried out with wild type the decoloration process began with a certain handicap from the outset. Thus it is possible that the difference observed was not an indication of a difference in oxidation at

all. The fact that with manometric measurement of oxigen consumption *(Warburg)* no difference could be shown between wild type and „ebony" (which was explained then as due to inaccuracy of the method employed) can equally well be explained if we suppose that the difference obtained in the experiment with *Thunberg*'s method was not due to differences in the oxidative mechanism, but to the greater amount of reducing substances present in the „ebony" pupae. To-day, we must consider this later explanation as the more probable.

Also confirming this view is the circumstance that if we add a dilute ascorbic acid solution to the pulp prepared from larval hypodermis, the above described darkening does not take place. If we add the ascorbic acid to already darkened pulp, it fades again. All this therefore proves to a great extent that there must be a close connection between the formation and retention of chromogens, and the activity of the reducing systems present in the pupae.

V. EXPERIMENTS WITH TRANSPLANTATION OF IMAGINAL DISCS.

All that has been said above, indicates the conclusion that the differences between body colour mutants of Drosophila are based on specific differences (concerning chromogen content) of the individual cells of the hypodermis. These differences seem to be determined by the genotype of the cells in question and cannot be altered by changes of „internal environment". In order to obtain a definite proof for the correctness of this conclusion we repeated the experiments of *Medvedev* (1937) with transplantation of imaginal discs. (These experiments were carried out by *Gyula Fábián*.) We thought that a more detailed study of such grafts may reveal some minute influences of the host, for which there were some indications in an observation of *Sturtevant* (1932). He found that in individual cases of gynandromorph body colour mosaics, in which „yellow" type areas were wedged in between wild type hypodermal tissues, the „yellow" areas were somewhat darker than normal. This would make it probable that from the wild type cells some humoral factor can get into the „yellow" cells and makes them darker.

The details of our experiments were the same as described in *Medvedev*'s paper, i. e. imaginal discs of wing- and side-plates (dorsal-mesothoracic buds) were implanted from mature IIIrd instar larvae into hosts of same age. „Yellow" was used besides wild type, and „ebony" for the above mentioned reason. We let the hatched host imagos live for 24 hours with the foreign imaginal buds in their bodies, for the colour to ripen

completely. After that they were killed, the grafts removed and together with a small piece of the host skin, taken for comparision, examined in glycerine. The body colour of wild type and „yellow" could be clearly distinguished and compared on the basis of the macro-bristle colour. The characteristic dark-coloured macro-bristles of the wild type could be sharply distinguished from the quite light „yellow" bristles. Put in combination with „ebony", the colour differences were quite sharp and decisive.

The results fully confirmed *Medvedev*'s findings, as in all cases, without exception, the imaginal buds developed according to their original type in the foreign surroundings, and there were no signs of „external" influences. For example, the piece of „yellow" hypodermis transplanted in the heavily pigmented „ebony" host remained absolutely unchanged in colour. From these facts it follows that in the imaginal discs of mature larvae the cells, forming the future chitin, macro-bristles and body colour pattern, are already determined. Thus the „environment", which must have been furnished by the chromo-oxydase activity of the host does not influence the development of the body colour.

VI. DISCUSSION.

The cause and mechanism of body colour development thus depends upon internal factors of separate cells. But the experiments throw no light on what these factors consist of. It is possible that, in accordance with what was said above, there exist specific differences between the presumptive pigment cells of the different body colour mutants in respect to their capacity of chromogen production and retention. But there are also observations, according to which the differences consist not so much of the capacity, but of the *number* of chromogen producing cells. Thus in the light coloured „yellow" there are hardly any pigment-producing cells at all, in the wild type they are confined to those stripes, where the pattern will develop, whereas in „ebony" these cells are present abundantly beyond the borders of the pattern. It is of course possible that there is a causal connection between the number of the pigment cells, and their supposed capacity to accumulate chromogen. Perhaps the accumulation of chromogens necessarily results in the growth of these cells and the more intensive rhythm of their divisions.

Investigations of *Pryor* (1940) and *Waddington* (1941) have shown that the body colour of Drosophila depends not only upon the amount of melanin formed, but also upon the intensity of the process of „tanning", by which the chitin becomes harder and takes on a characteristic „amber"

colour. This „tanning" was put down by *Pryor* (1940 a) to be a reaction between water-soluble proteins and oxidation products of a dihydroxyphenol, probably dihydroxyphenyl-acetic acid (cf *Schmalfuss, Heider* and *Winkelmann* 1933). It was also shown that if the oxidation of the dihydroxyphenol is prevented (either by M/200 sodium cyanate or by simple exclusion of oxygen) the tanning is also prevented. Thus oxidation is just as important factor in the process of tanning as it is in melanin formation. It seems that what has been said above about the possible role of reducing substances in the production of colour difference between the wild type and „ebony" concerns in the first place the oxidation of the dihydroxyphenol, which is the „chromogen" primarily responsible for the coloration of the puparium.

The independence of the body colour from the „internal environmental" factors (in the first place from the humoral factors present in the surrounding haemolymph) is not surprising in view of *Sturtevant*'s (1932) earlier observation, referred to above. His observation, if it is not based on some optical illusion, shows equally well the great independence of the colour forming mechanism from the surrounding influences. If the effect of the continued foreign influence, starting immediately at the outset of the embryonic development is capable of altering only to a very slight extent the process of colour development, so that the „yellow" patches of the skin can always be distinguished in the wild type surroundings, then it is obvious that transplantations, which take place at the end of larval life are not capable of even so much as slightly modifying the colour forming activity of the cells, and they keep their hereditary characteristics entirely.

VII. SUMMARY.

1. The genes influencing body colour do not exert their effects through differences in the amount or activity of pigment producing chromo-oxydases. In this respect the observations confirm *Danneel* (1943) and other authors.

2. In several cases a difference was observed in respect to the chromogen content of wild type and „ebony" pupae. Ground hypodermic tissues of „ebony" pupae, aged 24 hours or more (at 25^0 C) take on a darker colour on standing than similarly prepared tissues of the wild type. In earlier stages no such differences were observed.

3. The difference may be explained by differences in the activity of intracellular reducing substances, which would be more effective in „ebony" and would prevent the early oxidation of the chromogens in the

puparium of these mutants. The quicker decoloration of methylene blue by „ebony" pupae in comparision to wild type, as observed earlier (*Wolsky* and *Csik* 1942) can now also be explained thereby.

4. In respect to the amount of chromogen the above differences are already very early determined in some of the cells of the epidermis and cannot be influenced by humoral factors (internal environment). This was proved by transplanting imaginal buds reciprocally between wild type and body colour mutants (repetition and confirmation of the results of *Medvedev* 1937).

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THE LEYDIG INTERSTITIAL CELLS IN TESTES OF GUINEA PIGS

BY DR. GÁBOR STOHL (BUDAPEST)

To this day it has not been possible to determine whether the germinative epithelial, the *Sertoli*, or the *Leydig* interstitial cells of the testes produce the male sexual hormone which in all probability is testosteron. *Steinach* (in 1912, cited by *Harms*, 1926) considers that the sexual hormones are produced in the *Leydig* interstitial cells, whereas *Harms* & *Stieve* (see *Harms*, 1926) in the germinative epithelium itself. Both hypotheses have had many adherents, but of late researchers occupied with this question have inclined rather to *Steinach*'s view. The works proving hormone production in the interstitial cells have the great drawback that it has not been possible to bring the morphological changes observable in the interstitial cells into any cause-effect relation with the hormone-producing process; that is, with the appearance of the hormones in the interstitial cell. We can already rightly suppose that, with the formation of the testosteron molecule, that is, with the accumulation of testosteron created in the cell, parallel changes would occur morphologically in the interstitial cytoplasm and cell nucleus too. As the appearance of the sexual hormones in the organism cause definite changes showing increased activity in the accessory sexual organs (vas deferens, vesicula seminalis, prostate) the possibility exists of determining the finer structure of the interstitial cells during increased and decreased activity of the accessory sexual organs. In this way the connections might be analysed without disturbing the equilibrium of the organism. In the following I give an account of my investigations in this direction.

MATERIAL AND METHOD OF INVESTIGATION

As material for the investigation 28 (350—700 g) mature and 3 young (200—280 g) male guinea pigs were employed. Besides these, for comparison, the testes of some other mammals (musk rats, rabbits and bats) were examined. The seasonal rhythm of the other organs of internal secretion of 19 of the 28 mature male animals was also examined; of 5

ones the daily rhythm. The animals were killed with overdose of ether and the immediately dissected testes, while still bodywarm, were fixed in 4% formaldehyde, in "Susa", and in *Wiesel* potassium-bichromat-formaldehyde mixture as modified by me on the basis of physico-chemical considerations. This modification of *Wiesel's* fixation consists in putting the substance first in 12 ml 2,5% pure potassium-bichromat solution, to which only 2½ hours later is added a mixture of 8 ml distilled water and 2 ml 40% formaldehyde. The substance is left in this mixture for 2 days, then for 1 day put into pure 3,5% potassium-bichromat solution, afterwards washed with running water, etc. (Compare *Stohl*, 1943.) The material which was fixed in Susa and in bichromat-formaldehyde is made water-free in an alcohol series, then imbedded with the *Péterfi* methylbenzoat-celloidin-paraffine method. From the material imbedded in paraffine I prepared 4—6-μ slices and stained them with *Heidenhain*'s ferri-haematoxylin-eosin, *Ehrlich's* alum-haematoxylin-eosin, and *Heidenhain's* azan stain. From the material fixed in formaldehyde I prepared frozen slices and used them by staining with Scarlet-*R* to show the fats, that is, the lipoids.

RESULTS OF THE INVESTIGATION

Compared with other rodents, the interstitium in the guinea pig's testes is very scanty. The interstitial cells, as their name implies, are placed in the spaces and gaps between the testicular ducts, more particularly round the capillaries. Their relation to the capillaries is constant; if here and there we find a smaller or greater interstitial cell group which no capillary penetrates, the condition is only apparent, as in the next slices we invariably come upon a capillary situated on the border or in the center of the cell group. A net of very fine connective fibres surrounds the interstitial cell groups, but of that tissue only 1 or 2 weak fibres penetrate among the individual cells; only in a few animals was there a well-developed connective network among the interstitial cells. In many cases it could be observed that the fibres on the surface of the interstitial cell group went through into the capillary fibres without interruption, so that the interstitial cells seemed to be imbedded in the capillary connective tissue integument, almost making a sheath around the capillary. This arrangement of the interstitial cells corresponds — essentially, at least — remarkably to the so-called myoid-epithelial cells found in the capillary wall of the thymus of different vertebrates (*Bargmann*, 1943). A very interesting development takes place in the interstitium in the testes of guinea pigs kept for 2 weeks in complete darkness (in litteris

Kokas & Stohl): though the animals' testes are not in the least smaller than those of the control animals, the typical interstitium is almost entirely lacking in them. The spaces between the ducts are filled with an extraordinarily strong connective tissue and with an interstitial substance which, stained with azan, develops a bright red, or blue, colour; and in it we come across only sporadically a few cells of connective type having a fuchsinophil cytoplasm and nucleus. Though no far-reaching conclusions can as yet be drawn from these investigations, so much seems in any case probable, that — as *Tonutti* (1943) emphasised — the interstitium is not some permanent formation, but can in certain circumstances transform itself into connective tissue.

In what follows, with the aim of analysing the relations of cytological conditions and the hormone-producing processes, we compare the finer structure of the interstitium in increased or diminished activity of the accessory sexual organs.

1. In the case of *incraesed* activity of the accessory sexual organs, the epithelium of the vas deferens and the vesicula seminalis is strongly pleated, the gland epithelium of the prostate is high. In the testes of such animals the interstitium is well developed, consists of compact masses of cells. After fixation in Susa solution (Figure 1) the fine, comb-like, foamy structure of the cytoplasm of the interstitial cell is more strongly eosinophil than is that of the germinative cells. In the round, possibly a little elongated, nucleus, its framework of fine chromatic granules is clearly to be seen, together with 3—4, possibly more, round corpuscles which are normally situated under the nuclear membrane, or at least in its neighbourhood. We can find ferrihaemotoxylin-positive particles in

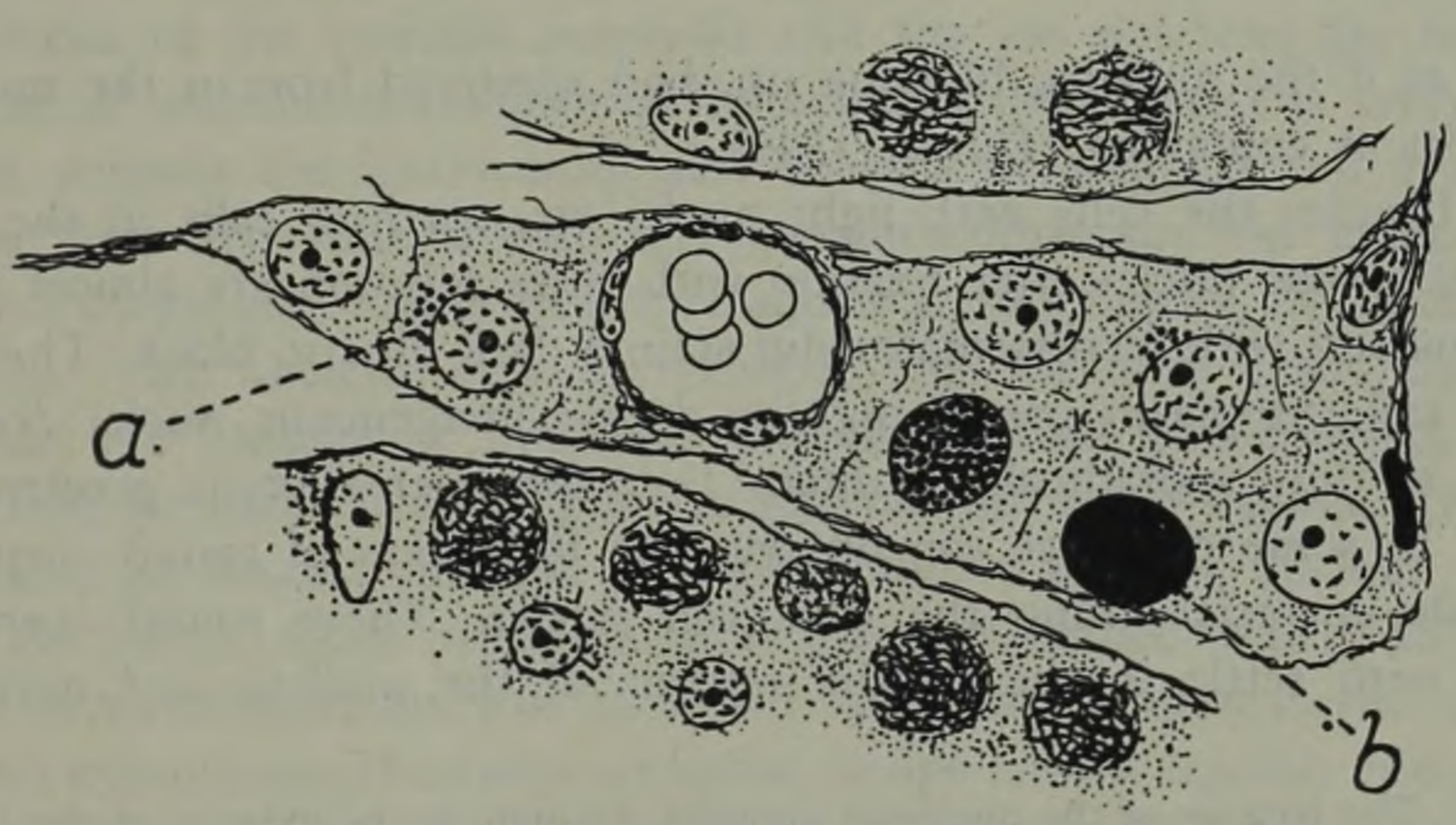

Figure 2. Guinea pig male. Interstitial group. Susa, ferrihaematoxylineosin. Magnified 950. *a*) ferrihaematoxylin-positive granules. *b*) Dark-staining interstitial nucleus.

the cytoplasm of interstitial cells having such nuclei. In many cases these ferrihaemotoxylin-positive particles appear directly in the neighbourhood of the cell nucleus, as if lying on it, and, as in such nuclei similar round ferrihaematoxylin-positive granules occur in the neighbourhood of the inner surface of the nuclear membrane, it is highly probable that nucleolar granules pass out of the nucleus into the cytoplasm. The exodus of the nucleolar granules sometimes assumes such proportions that it causes the destruction of the nucleus.* The nuclear membrane splits at one place and through this gap a great number of ferrihaematoxylin-positive granules escape into the cytoplasm. So the histological picture

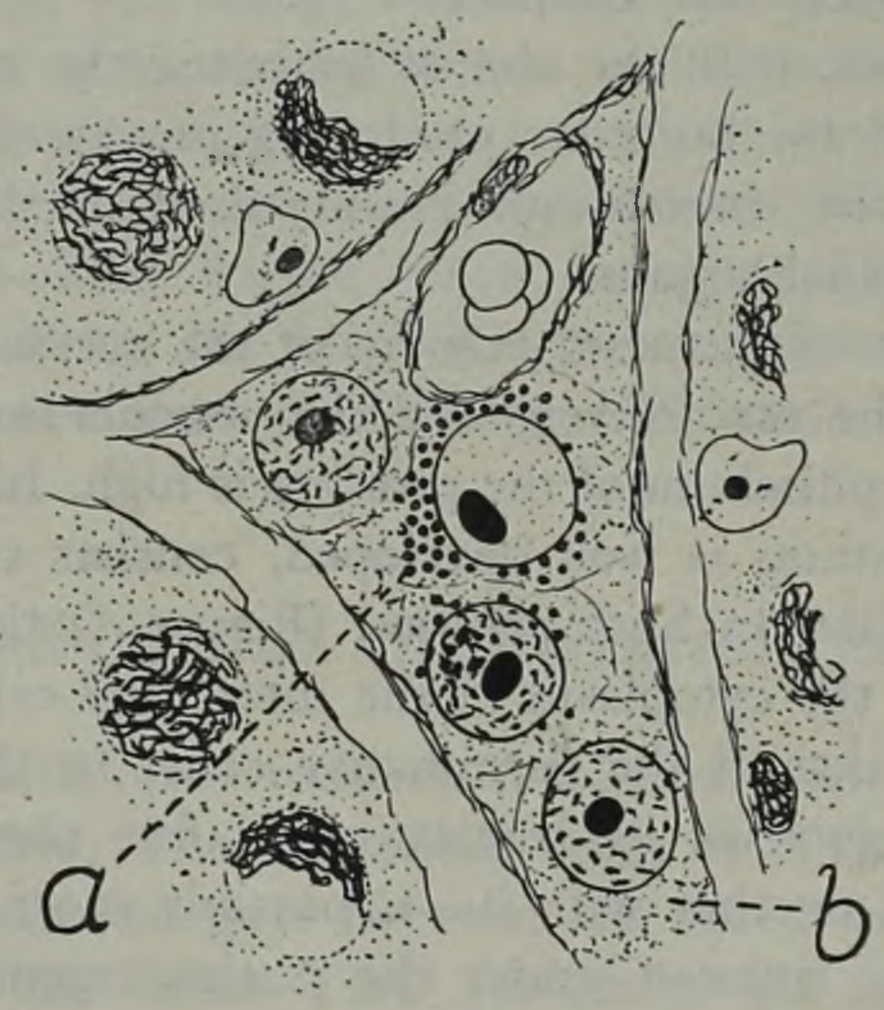

Figure 2. Guinea pig male. Interstitial group. Potassiumbichromat-formaldehyde, azan. Magnified 950. *a*) Fuchsinophil granules. *b*) lipoid vacuoles.

looks as if the nucleus, blowing up, had scattered from it the nucleolar granules it contained (*Dittus*, 1940).

Besides the cells with light nuclei we can find cells in the interstitium whose nuclei after fixation with Susa solution are almost homogeneous and with ferrihaematoxylin stain a dark blue to black. The interstitial cell cytoplasm containing the dark homogeneous nuclei does not at all, or only sporadically, contain 1—2 ferrihaematoxylin-positive granules. In some cases we can observe the formation of round corpuscles from the material filling the dark cell nuclei. These round corpuscles begin with settle in the middle regions of the nucleus and only later

* The passage of the nucleolar granules through the boundaries of the nucleus can be observed, in every direction, thus we do not need to qualify their presence as an artificial product brought about by the pull of the microtome knife.

withdraw under the surface of the nuclear membrane, from where they afterwards emerge as nucleolar granules in the cytoplasm. From these premises it seems very probable that the cells with dark nuclei can change into cells with light nuclei.

We could also observe the passage of the nucleolar granules indicating internal secretion production in the material fixed in the bichromat-formaldehyde mixture and stained with azan (Figure 2). The blue-stained cytoplasm in many of the interstitial cells contains smaller or larger round lipoid vacuoles. One part of the vacuoles, arrangend in a wreath-like form around the nucleus, is in contact with it, and, what is the most remarkable, these vacuoles are filled with a redstainable substance, or at least covered with a membrane of such material. The appearance of these fuchsinophil particles is extraordinarily important

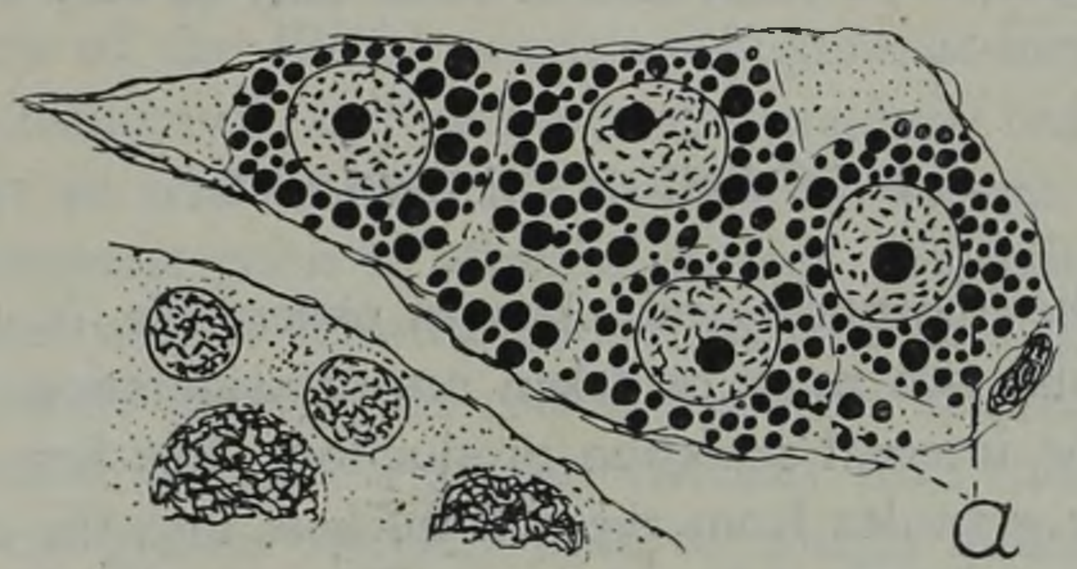

Figure 3. Guinea pig male. Interstitial group. 4% formaldehyde, Scarlet-R-haema-alum. Magnified 950. *a*) Lipoid drops.

from the point of view of elucidating internal secretion production.

2. In the case of *diminished* activity of the accessory sexual organs the epithelia of the vesicula seminalis and the vas deferens are hardly pleated at all and the epithelium in the acina of the prostate is very low. In these animals the interstitium also consists of compact cell groups. Fixed in Susa and stained with ferrihaematoxylin-eosin, the cytoplasm has a fine, comb-like, foamy structure and is at times more, at times less eosinophil. The nucleus is bubble-shaped, its structure easily discernible, but the passage of nucleolar granules cannot be observed, nor can we find ferrihaematoxylin-positive granules in the cytoplasm. In the interstitium of August animals a larger number of dark-nucleus cells occurs. In frozen slices prepared from material stained with Scarlet-*R* fixed in formaldehyde, fat, that is, lipoid drops can be seen in the interstitial cell cytoplasm. The fatty, or lipoid, drops have a regular, spherical form and, evenly distributed, fill the entire cytoplasm (Figure 3). Though a good lot of fatty drops crowd one another in the individual interstitial

cells, they are always conspicuously distinct: the minuscule drops never run together.

The extraordinarily fine, foamy, almost homogeneous cytoplasm of the interstitial cells in the material fixed in the bichromat-formaldehyde mixture and stained with azan takes a blue colour under this staining and contains many sharply defined, round lipoid vacuoles. With this preparation it can very well be seen that the lipoid vacuoles, no matter how densely they fill the interstitial cell cytoplasm, are always separated from one another by a thin cytoplasmic border. The cell nucleus also stains blue with azan but slightly violet in comparison with the cytoplasm. The structure of the nucleus is extremely indistinct, only in a few cases can 2—3 orange-yellow coloured "nuclear corpuscles" be distinguished. We could scarcely find granules stained red with azan ("fuchsinophil"), in 1—2 cells, and in these only in very small numbers.

DISCUSSION OF RESULTS.

Among the morphological changes observable in the interstitial cells, in particular two must be taken into consideration from the view of estimating the internal secretion production: The first is the passage of the nucleolar granules from the cell nucleus into the cytoplasm; the other is the accumulation of lipoid drops. The question is which of these two processes antccous for testosteron production (and possibly other sexual hormones similar in structure). To decide the question we must compare the interstitial tissue structure of the animals with accessory sexual organs of increased and of diminished activity. As we have seen from the foregoing, the accessory sexual organs increased in activity in those animals in whose interstitial cells we could observe the passage of the ferrihaematoxylin-positive nucleolar granules in Susa fixative, while the material in the bichromat-formaldehyde fixative showed "fuchsinophil" granules (saitning red with azan). On the other hand, in those animals where the interstitial cells were filled with many small globules — that is, with definitely dispersed lipoid drops, and whose cytoplasm, fixed in bichromat-formaldehyde turned blue after azan-staining, the action of the accessory sexual organs was decidedly diminished. In these animals' interstitial cells (the substance being fixed with Susa) the passage of the nucleolar granules could be observed only very rarely.

From the foregoing we can conclude that testosteron is produced in the *Leydig* interstitial cells of the testes in guinea pigs, and that the production of this sterol hormone is connected with the passage of nucle-

olar granules from the cell nucleus into the cytoplasm, as well as the appearance of "fuchsinophil" granules.

Parenthetically I should remark that the state of activity of the accessory sexual organs is independent of the germinative epithelium, furthermore that in the germinative cells, fixed with bichromat-formaldehyde and stained with azan, I could not show red-stained granules. Only the spermiogonium of testes of animals kept in the dark stained red with azan, but as the degeneration of the germinative epithelium was evident in these animals it is likely that the spermiogonia's "fuchsinophil" change is in relation to the degeneration and not to the production of sexual hormones.

A further question is, to what chemical processes the morphological changes observed in the interstitial cells point. In other works I have discussed in detail (*Stohl.* 1943, 1944) how, in material fixed with my modification of the *Wiesel* potassium-bichromat-formaldehyde mixture, the "fuchsinophil" cell-constituents (staining red with azan) in general indicate an acid reaction and oxidative processes, while the blue-staining cell-elements point to alkaline reaction and reduction processes. The speculation was as follows. Model experiments of *Florentin* and myself have clearly shown that cell components stained red with azan (in the fixed material) have a greater viscosity than those, stained blue. The cause of the greater viscosity must be sought in the fact that those components which were stained red were coagulated by potassium-bichromate whereas the blue ones were fixed by formalin, which was added later. Potassium-bichromat coagulated those components, the hydratation of which was less at the time of fixation. The smaller degree of hydratation may be caused — among else — by the acid reaction of the medium, as the proteins of protoplasm, the isoelectric point of which is less than *pH* 7, were in this case nearer to their isoelectric point. The elements on the other hand, which are stained blue, were not coagulated by potassium-bichromat because in basic medium they were far from their isoelectric point. It is known from the electrochemistry of the redox systems that there is a cause-effect connection between the acid reaction of the medium and the incresae in oxidative potential and conversely between basic reaction and increased reducing capacity. This view is confirmed by the researches of *Brachet* (1942), *Brachet & Jeener* (1944) and *Chantrenne* (1944) according to which the cytoplasmic granules, containing — like the nucleoli — pentose nucleinic acids, are linked with more oxidizing enzymes. In these works I have pointed out that in the cells of the suprarenal cortex of mammals the production of cortico- and desoxy-corticosteron is related to the accumulation of

fine, scattered lipoid drops and the cell's vigorous reducing capacity, while the formation of andrenosteron, having a male sexual hormone effect, is connected with the appearance of fuchsinophil granules indicative of oxidative processes. Considering that the chemical structure of testosteron and andrenosteron is extremely similar the part relating to andrenosteron production in the above findings can be applied without further question to the formation of the testosteron molecule.

Mutatis mutandis we can say that testosteron is produced by the reciprocal effect of the lipoids accumulating in the interstitial cell and the nucleolar substance leaving the cell nucleus. We have seen when discussing the histological structure that the fuchsinophil granules always appear in the immediate neighbourhood of the nucleus, while the nucleolar granules fixed with sublimate already develop in the interior of the nucleus in the form of round corpuscles. The ferrihaematoxylin-positive nucleolar granules, fixed with sublimate, thus represent the substance which forms in the nucleus and which leaves it for the cytoplasm; it there sets going chemical reactions which lead to the formation of the testosteron molecule. As the granules appearing in the neighbourhood of the nucleus stain red with azan, which indicates an acid reaction and oxidative processes we have every right to suppose that the nucleolar granules leaving the nucleus for the cytoplasm set oxidative processes at work. In the present case this means that the testosteron is formed from some larger molecule by oxidative processes. As far as testosteron is concerned, we have to abandon the supposition that it is formed directly from the breakdown products of glucose possessing 3 *C* atoms, that is from glycerinealdehyde and dioxyacetone through condensation and following reduction. The circumstance that while the cortico- and desoxy-corticosteron molecules contain 21 (i. e., 7×3) *C*-atoms, testosteron contains only 19, which is not divisible by 3, speaks of itself against the possibility of a direct condensation.

From this the question arises of itself as to what that larger molecule can be from which by oxidation testosteron is formed. Physiological investigations show that probably the suprarenal cortex hormone, cortico or desoxy-corticosteron, is the basic material of the testicular hormone (*R. Abderhalden,* 1943, p. 198). But the findings of physiological researchers in this direction can also be brought into relation with the morphological changes observable in the interstitial cells. The production of suprarenal cortical hormones, as I mentioned before, is connected with the cell's reduction capacity and the accumulation of the widely dispersed lipoid drops. But we can observe the same appearances in the interstitial cells: the interstitial cytoplasm containing the fine, scat-

tered, round lipoid drops, after bichromat-formaldehyde fixation and azan staining, turns blue; and the blue colour again points to reduction processes. Thus under certain circumstances we find in the interstitial cells exactly the same cytological conditions as in the suprarenal cortical cells showing increased activity, from which we can draw the conclusion — which may at first glance seem daring — that, in the *Leydig* interstitial cells of the testes of guinea pigs, suprarenal cortical hormone can also be formed.

In support of this hypothesis I can bring forward the following:

1. That the male sexual hormone is not formed in the fine, scattered, separate round and widely dispersed lipoid drops is proved by the fact that in those animals whose interstitial consist predominantly of such cells the action of the accessory sexual organs shows a decrease. *Ito & Oinuma* (1939) in a series of investigations carried out on human material likewise found that the endocrine activity of those testes whose interstitial cells contained much fat was much diminished.

2. *Wolf—Heidegger* (1942), using the *Giraud—Leblond* reaction showed that in the interstitial cells of testes of young rats after extirpation of the suprarenal a great quantity of strongly reducing material accumulated. We have already seen that the appearance of reducing substances has a close relation to the formation just of cortico- and desoxycorticosteron, or similar molecules. *Wolf—Heidegger's* experimental results can thus be explained, in that after extirpation of the suprarenal the interstitium of the testes takes over its work and thus, in consequence of the absence of the suprarenal cortex, the normally only small cortical hormone production assumes greater dimensions.

3. The embryological findings likewise strengthen our hypothesis as to the interstitial cell's cortical-hormone producing capacity. The investigation of *Goormaghtigh* (mentioned by *Harms*, 1926) of the development of the testicular interstitium in guinea pigs is very extensive. It shows that the suprarenal cortex and sexual glands are of common origin; both organs develop from the thickening of the coeloma epithelium which streches on both sides the length of the body in the form of small, bud-like sprouts. The cranial buds later joining form the suprarenal cortex, the caudal sprouts, in which the elementary sexual cells are also imbedded, form the sexual glands. *Goormaghtigh* emphasises that the morphological and physiological similarity existing between the interstitial cells and the suprarenal cortex cells can be explained from the tissue development.

On the basis of all this and with a physico-chemical explanation of the cytological conditions, we can form the following picture of the

activity of the interstitial cells in the testes of guinea pigs: The interstitial cells, through oxidative processes from some great molecule, probably cortical hormone (or some other compound having a similar sterol structure) form the hormone of the testes, testosteron. The initial product of testosteron can also be formed by the reducing processes of the interstitial cells themselves, which, however, does not in the least preclude the possibility that the interstitial cells can transform into testosteron not only the cortical hormone formed and accumulated there but also that formed in the suprarenal cortex and conveyed to them through the circulation.

The results of the present investigation cannot be considered in the least conclusive; the aim of this communication is merely to point out the relation between the suprarenal cortex and the *Leydig* interstitial cells of the testes, as well as those connections existing between the morphological changes of the interstitial cells and the activity of the accessory sexual glands. These morphological changes can also be brought into a relationship with the possibilities of sterol compound formation.

SUMMARY

1. After fixation with potassium-bichromat-formaldehyde mixture *red* particles can be shown with azan stain in the interstitial cells of the testes of the guinea pig at the time of *increased* activity of the accessory sexual organs. The particles, or granules, are probably formed in the cytoplasm as effect of nucleolar substance passing out of the nucleus.

2. *Decreased* activity of the accessory sexual organs was linked with *blue* azan colouring of interstitial cytoplasm containing a large quantity of fine, scattered lipoid drops after the above fixation.

3. The production of testosteron is in all probability connected with the appearance of fuchsinophil particles. On the basis of the physico-chemical explanation of the histological picture, one must suppose that the fuchsinophil granules are indicative of oxidative processes and the testosteron is formed through such processes from some bigger molecular compound.

4. On histophysiological grounds we have to count with the possibility that in certain circumstances the interstitial cells can produce suprarenal cortical hormone, or a substance similar to it.

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NITROGEN-BINDING BY KALOTERMES FLAVICOLLIS (ISOPTERA) AND ITS SYMBIONTS.

BY LÁSZLÓ TÓTH.

FIXATION OF ATMOSPHERIC NITROGEN BY INSECTS.
(MADE AT THE HUNGARIAN BIOLOGICAL RESEARCH INSTITUTE.)

INTRODUCTION.

The supposition that the symbionts of insects play the same biological role as do the well-known root-bacteria of Leguminosa is not a novelty. *Cleveland* (1925) attempted to prove the question experimentally with termites. He stated: "if these insects can maintain themselves in a perfectly normal manner indefinitely on a cellulose diet, they must be able in some way to fix atmospheric nitrogen, which they use in manufacturing proteins; or else, contrary to the current opinion, they must be able to transform carbohydrates into proteins." However reasonable and natural this conclusion, with his experiments he by no means succeeded in proving it satisfactorily. "when termites (Termopsis) are confined in air with barometric changes being noted and temperature being kept constant, a negative pressure is very soon developed. This indicates that nitrogen is being fixed, but analyses of air samples taken from tubes where the negative pressures have developed have shown very little, if any, change in the nitrogen percentage."

The new methods employed in recent years by *Virtanen* and his colleagues in research in Leguminosa have proved suitable for insect symbiosis too. The investigations of *Tóth, Wolsky* and *Bátory* (1942) on surviving systems in plant lice have shown that the binding of free atmospheric nitrogen really does take place in plant lice, and from the very high values thus obtained they deduced the remarkable degree of action of the nitrogen-binding system. It goes without saying that it is a question, not of single exceptional phenomena, but of a so far unknown, apparently extensive category of nitrogen supply. This hypothesis they have since in fullest measure confirmed by new experiments *(Tóth, Wolsky & Bátyka,* 1944). The cases which are first to be considered are of course those in which the insect's food is highly deficient in protein. It is clear that here too the organism obtains the protein materials indispensable for life by a similar route.

THE SYMBIOTIC SYSTEM OF KALOTERMES FLAVICOLLIS.

All species of termites have symbiont micro-organisms. In Kalotermes flavicollis these micro-organisms settle in a sack-like extension of the postintestine. Their mass is very considerable, from the systematic standpoint most varied, containing approximately at least 6 Flagellatae, 3 Spirochaetae and 6—8 species of Bacteria. But besides these free-living species, bacteria are also regularly to be found in the body of the large Polymastigina Flagellatae (Joenia annectena).

In the young termite larva emerging from the egg there are none of these intestine dwelling micro-organisms, but it acquires them quickly in the first days of its life. In the first larval stadium if feeds exclusively on the liquid intestinal excrement deposited by its elder nest-fellows. This is nothing other than the contents of the distended part of the post-intestine which contains all the sorts of intestine-inhabiting micro-organisms in enormous quantities. It is by this route that they enter the intestine of the new-born, though most of them will fall victim on the way to the proteolytic enzyme of the digesting fluid. But such as arrive in the post-intestine will multiply hugely in course of time, so that by the IIIrd larval stage they crowd the now fully developed ampulla.

They continue to propagate, however, and this leads to unbearable overcrowding. There are two ways of ending this. The one we already know, the secretion of the liquid excrement. The other leads through the pylorus valve into the mesenteron where the micro-organisms are soon digested by the action of the proteolytic enzyme. Thus in both cases they serve as food, in the first for their nest-fellows, in the second for themselves. At any rate, it can be established without possibility of doubt that the mass of symbiont micro-organisms is an important factor in the protein supply of the termites.

The activities of the micro-organism in the house-hold of the host animal can, of course, be brought to light only by experiment. In Kalotermites the sterilization hoped for by means of heat and hunger unfortunately did not succeed, as the micro-organisms are more resistant to these influences than the host animal itself. Dietetic experiments were more successful and from them several important facts can be deduced. In the first place, any variation in the composition of the food is likely to produce a corresponding modification in the composition of the intestine-inhabiting micro-organisms.

On a diet of water-soluble carbohydrates the great flagellates (Joenia annectens) disappear. From this the negative conclusion can be drawn that on such a diet these micro-organisms play no part in the

house-hold of the host animal. On the other hand, on a pure cellulose diet the same kind of ampulladwelling micro-organism will, after a temporary relapse, increase in size as well as in numbers. But if termites containing no large flagellates are put on an exclusive cellulose diet they will inevitably starve. This all indicates that the Joenia flagellates play an indispensable part in the decomposition of cellulose. All that remains problematical is whether the flagellata itself, or the bacteria living in it, are responsible.

From the fact that on a pure carbohydrate diet termites can live and propagate for a long time without presenting any deficiency phenomena it follows of necessity that they must somehow be able to produce out of their incomplete diet the other two important groups of nutritvie substances, fat and protein. Their supply of fat might more or less be explained by the general capacity of animal organisms to build up fats from carbohydrates. But this is not true of proteins.

It can be established that the mass of ampulla-inhabiting micro-organisms is an important factor in the provision of termites with proteins, yet for the micro-organisms confined in the host animal certainly no other source of proteins is available either, than those available to the host animal itself. In the diet experiments all that was available was carbohydrate. But in order to convert these into proteins or amino acids, nitrogen is also required, and this can be got only from atmospheric air.

NITROGEN-BINDING EXPERIMENTS WITH KALOTERMES FLAVICOLLIS.

The method employed in the experiments which follow is sketched only in general outlines, the reader being referred for wider discussion to older investigations of the subject (principally *Tóth, Wolsky* and *Bátyka,* 1944). In this case also it is a question of so-called "model experimets," in which the essential is to create a surviving system in as high a degree as possible containing all the factors of a natural system. These factors are the following: The crushed Kalotermite body with the surviving cells and the living symbionts (with all optimu factors possible as to physiological medium, *pH,* osmotic pressure, temperature etc.); the air serving as source of nitrogen; and finally, as nitrogen acceptor, a suitable carbohydrate, in this case oxalic acid or succinic acid is necessary. From this experimental material samples are taken at definite intervals, their total nitrogen content determined by the Parnas-Wagner-micro-Kejdahl method, and the resulting values expressed in %.

1st series of experiments: 10 specially prepared hind-guts are crushed in a solution, containing no carbonic acids, of 0,5% *NaCl* + 0,5% glucose, which solution possesses a hydrogen-ion concentration of 7,5. Keeping the solution at a temperature of 26 C°, it is impossible, after 24 hours, to show any gain in nitrogen.

2nd series: To the preceding solution is added: n/75 oxalic acetic acid, under the same experimental conditions as before. The gain in nitrogen after 24 hours is 5%.

3rd series: To the preceding solution is added 0,1% K_2HPO_4 + 0,1% $MgSO_4$ + 0,1% Na_2CO_3 + 0,2% $CaCO_3$ + one small drop of cupreine for each 3 ml. After 24 hours the gain in nitrogen is 50%.

4th series: In a solution with a hydrogen-ion concentration of 7,5, 0,5% *NaCl* + 0,5% glucose + n/75 oxalic acetic acid, 2 whole specimens of Kalotermes per ml are crushed and the whole kept at a temperature of 26 C°. After 24 hours the gain is 6%.

5th series: With the same experimental conditions as before, but adding to the solution a further 0,1% K_2HPO_4 + 0,1% $MgSO_4$ + 0,1% Na_2CO_3 + 0,2% $CaCO_3$ + per 3 ml one drop of cupreine. The gain in nitrogen after 24 hours is 67%.

6th series: The hind-gut is previously removed from the experimental animals; in other respects the conditions of the preceding series are maintained. The gain in nitrogen in 24 hours is 69%.

7th series: If, under the same experimental conditions 10 Kalotermes heads per ml are crushed into the solution, no gain in nitrogen can be detected after 24 hours.

8th series: Under the same experimental conditions 5 Kalotermes thoraxes are crushed per ml into the solution; the gain in nitrogen is 65% after 24 hours.

9th series: With the same experimental conditions, but crushing 4 Kalotermes abdomina per ml into the solution, the gain in nitrogen after 24 hours is 88%.

All these figures represent averages of tests repeated several times. From the results the following conclusions can be drawn: In a simple kitchensalt-glucose solution no gain in nitrogen takes place. If carbonic acids only are added to the solution the gain in nitrogen will still be slight. But if certain further salts are added in weak concentration, nitrogen fixation will increase intensively. Cupreine also acts favourably, though the extent of its effect is much more limited. Among the various carbonic acids, in addition to oxalic acetic acid, succinic acid, and tartaric acid can similarly be employed, though with not quite as good results.

It is surprising that it is not only the hind-gut, that is, the mass of micro-organisms, which can fix atmospheric nitrogen, but also the body of Kalotermes without the hind-gut. What degree of nitrogen-fixing capacity exists in the various parts of the Kalotermes body is shown by the experiments detailed below.

10th series of experiments : 10 Kalotermes larvae of medium size are divided into 4 parts, and the 10 hind-guts, heads, thoraxes and abdomens examined separately in optimal physiological solutions (kitchen salt + + glucose + salts cupreine + oxalic acetic acid). The amounts of nitrogen fixation are shown in the following Table:

Per 10 units of material examined	Quantity of N observed (mg)	Quantity of nitrogen fixed in 24 hours		
	At beginning of test	After 24 hours	Hours mg	%
Hind-gut	0,100	0,198	0,098	9
Head	0,112	0,113	0.001	0
Thorax	0,431	0,713	0,282	25
Abdomen	0,490	0,866	0.376	33
Total	1,133	1,890	0,737	67

I regret to say that for lack of the necessary experimental animals (Kalotermes) I was unable to repeat these experiments. The data are probably correct, however, because the total gain in nitrogen in 24 hours (from 1133 to 1890 mg) is 67%, exactly the figure we know as the mean figure of numerous experiments. It is only the nitrogen fixation of the hind-gut that seems a little too high; it could hardly attain 9%.

It is difficult to explain how the Kalotermes body deprived of its intestine, and therefore according to our present knowledge without any micro-organisms, is capable of so substantial an amount of nitrogen fixation. According to such experience as we have to date only nitrogen-fixing micro-organisms possess this capacity. So far similar observations are known only of some Heteroptera species. *(Tóth, Wolsky & Bátyka,* 1944.) Here too perhaps the same explanation can be given as there; that is, that, in spite of negative results so far, they contain symbionts not yet discovered. If we consider that the most diverse lurking places are at the disposition of the symbionts in the insect's body, moreover that their demonstration is often subject to very great micro-technical difficulties, it can be supposed that in such cases too, where today a total conception cannot be established, the future will bring proof, of the connection between the symbionts and the fixation of nitrogen. In any case,

it may be of some instruction that in the termites the head has no capacity for nitrogen-binding.

Research so far has not been able to reveal the presence of micro-organisms elsewhere in the termite's body than in the distended part of the hind-gut. The only exception is the germs of primeval termites, Mastotermes, represented by a single species, in the adipose body of which a mass of intracellular symbiont bacteria live. In Kalotermes such bacteria cannot be found. There are extremely small granules in the fat body, however, just visible under light microscope, which absorb colour to a very slight degree. It would not be surprising if, given a suitable technique, we might identify them as bacteria. To attribute nitrogen-assimilating activity to them would, of course, be too hazardous an assertion. On the other hand, it is certain that the known intestine-inhabiting micro-organisms play at most an insignificant part in nitrogen fixation.

With the establishment of the termites' nitrogen-fixing capacity we have got the answer to the question of the source from which the nitrogen necessary for building up the proteins is obtained. But at the same time a host of new problems arise. The most important of these is the question of the mechanism of nitrogen assimilation. The great similarity the termites show in this respect, partly to other insects, partly to the Leguminosa, permits the supposition that we have to deal with a general biochemical phenomenon. On the basis of research thus far, unfortunately, we cannot create a clear picture of this mechanism; it can only be sketched in large outline, according to the following schema:

N-acceptor Carbohydrate		N-donator Air
Carbonic acid	+ ?	Gaseous nitrogen
	Amino acid	
Biological factors		Nitrogen assimilating activity
Host animal		Micro-organisms (symbionts).

The carbohydrate food supplies the nitrogen acceptor substratum for the nitrogen binding, in our case in the form of a carbonic acid. Into this the nitrogen furnished by the air is built up (as nitrogen donator) resulting in an amino acid as end-product. The active work of nitrogen assimilation is performed by the micro-organisms (the symbionts). The micro-organisms moreover probably produce still another factor

("biological factor") necessary for the production of amino acids, which however was probably supplied originally by the host animal.

The experiments carried out in nitrogen assimilation of Kalotermes resulted in the following average values: A medium-sized Kalotermes larva (IV. stadium) has a total nitrogen content of about 0,12 mg. This in 24 hours in an optimum surviving system mounts to 0,20 mg. The increase in total nitrogen in 24 hours is therefore 0,08 mg, which amounts to 67%. Of this at least half belongs to the abdomen without intestines, 40% to the thorax, 10% to the post-intestine, while the head is not capable of nitrogen assimilation.

Recapitulating briefly, the nutrition biology of the termites can be characterized as follows: In respect to nourishment they are extremely unpretentious, inasmuch as they are content with an exclusively carbohydrate diet. Nourishment taken per os fulfills all their own and all their symbionts' carbohydrate requirements — and through them their fat requirements. On the other hand, it can be demonstrated experimentally that they are capable of assimilating the nitrogen of the air in a great measure. In this way, then, the amino acids necessary for binding up proteins are also supplied, not only for themselves but for the intestine-dwellers also. According to this, the latter find excellent life conditions in the termites' bodies where, propagating in dense masses, they supply by their bodies ready-made, valuable protein materials for the metabolism of the host animal.

COMPARISON OF NITROGEN FIXATION IN KALOTERMES AND IN OTHER INSECTS

If we compare the data obtained as to the nitrogen binding of Kalotermes with what is thus far known of that of other insect species, complete harmony can be established. *Tóth, Wolsky & Bátyka* (1944), on the basis of experiments carried out on 22 different insects of the Rhynchota order, showed a direct connection to exist between the development of the symbiont system and the degree of activity of the nitrogen binding. According to this, the insects with powerful symbiont systems, that is, which possess well-developed symbiont-containing organs (mycetoma), have a very great degree of nitrogen binding capacity too; plant lice, for example. In the case of less powerful symbiont systems on the other hand, the nitrogen binding capacity is also less, as in the Heteroptera group, which have no mycetoma. The nitrogen binding capacity of termites can also be ranged in this category, that is, where the capacity corresponds to the degree of development of the symbiont-

system, the category of animals possessing no independent symbiont-containing organ (mycetoma) but where the micro-organisms find place in the intestinal system.

From the standpoint of the mechanism of nitrogen binding, it may further be mentioned that, according to newer, as yet unpublished experiments, not only oxalic acetate acid but apparently many of the carbonic acids, indeed perhaps other carbohydrates, will prove suitable as nitrogen acceptors. It is probable that the different sorts of nitrogen binding micro-organisms would behave differently in this respect, but it is also certain that the same micro-organism can use different kind of carbohydrates as nitrogen binders, if to a different degree. Concerning this, as in general the other questions bearing on the mechanism of nitrogen binding, only wide experiments can give an answer.

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