CHAPTER 4

THE DISTRIBUTION OF PROTEIN, FAT, GLYCOGEN, DESOXYRIBONUCLEIC ACID, RIBONUCLEIC ACID, PHOSPHOLIPIDS AND URIC ACID IN THE LARVAL FAT BODY AND THEIR METABOLIC SIGNIFICANCE

The fat body of insects has long been looked upon as a static organ, a kind of store-house of reserve substances, predominantly fat and of excretory products such as uric acid. It is only in recent years that the significance of the fat body in the dynamic aspects of bodily functions has been recognized. This has come hand in hand with the realization that the adipose tissue of vertebrates is found to have a far more significant role in metabolism than has previously been believed. Experimental studies carried out in the last few years have changed the concept of adipose tissue from that of an inert storage site to that of an extremely active system primarily concerned with the synthesis, oxidation, storage and release of fats (Jeanrenaud, 1961). The insect fat body is something more than the adipose tissue of the vertebrates. The absence, in insects, of an organ comparable to the vertebrate liver is compensated by the fat body taking over some of the functions of liver. There is little doubt at present that the insect fat body is an important site of intermediary metabolism.

The deamination and transamination of aminoacids, which in vertebrates are carried out mainly in liver and kidney, were shown to take place in the fat body of Schistocerca (Kilby

and Neville, 1957) and Calliphora (Desai and Kilby, 1958). Synthesis of aminoacids from labelled acetate was also shown to occur in the fat body of this insect (Hines and Smith, 1963; Clements, 1959).

Fat body is also known to play a role in the synthesis of glycogen (Vardanis, 1963 in Periplaneta) and trehalose 1958; Clements, 1959; Candy and Kilby, 1961 in Schistocerca).

The existence of a system for synthesis of fatty acids was demonstrated in the fat body of Prodenia (Zebe and McShan, 1959) and Schistocerca (Tietz, 1961). Lipase activity was demonstrated histochemically in the fat body of Rhodnius (Wigglesworth, 1958), Schistocerca (George and Eapen, 1959) and Poecilocera (Hegdekar, 1963).

The above studies have unequivocally established the insect fat body as an important centre of intermediary metabolism comparable to the vertebrate liver. Attention must be drawn, however, to the fact that many of the above studies were carried out on a few laboratory favourites, more especially on the desert locust, Schistocerca gregaria. We need at present more information regarding the fat body of other insects if an integrated picture of the metabolic role of insect fat body is to evolve. Considering the wide variety of environmental conditions insects are known to cope with, with their consequent physiological problems, and the structural diversities noted in the fat body of different insects (Chapter 3), it seems possible that this tissue may exhibit different functional adaptations

in different insects. The present study on the localization and distribution of protein, fat, glycogen, DNA, RNA, phospholipids and uric acid in Anthrenus larval fat body was undertaken with a view to throw some light on the various metabolic processes that take place in the fat body.

MATERIAL AND METHODS

The insects used were obtained from a laboratory culture maintained at 32 ± 1°C, on a diet of dried and crushed pigeon breast muscle supplanted with 5% Brewer's yeast.

Histochemical methods

Fixation was done in the appropriate fixatives according to the procedure described in Chapter 3.

Lipids: Baker's improved Sudan Black technique (Baker, 1956) was employed for the demonstration of lipids. The larvae were fixed in formol-calcium, post-chromed in dichromate-calcium (Baker's acid-haematein fixation), embedded in gelatin, and sections cut on a freezing microtome. The sections were stained with Sudan Black B in 70% ethanol or ethylene glycol.

The tissues fixed in Ciaccio's fluid were also stained for lipids. Fixation in Ciaccio's fluid (Chou, 1957) was followed by washing and post-chromation first in 3% potassium dichromate for 24 hours and then in saturated potassium dichromate for a week at room temperature. The tissue was washed and embedded in gelatin. Sections were taken on a freezing microtome. This modification of Chou's method for the fixation of adipose fat (Chou, 1957) gave excellent preservation of lipids.

Neutral fat was demonstrated in the fresh fat body tissue by staining with Nile blue sulphate while Fettrot 7B was used (Pearse, 1960) for staining the tissue fixed as for the Sudan Black technique.

For the demonstration of phospholipids the acid-haematein method and the Copper phthalocyanin method were employed (Pearse, 1960). Weak Bouin fixed tissue after hot pyridine extraction was used as control.

Protein: Protein was demonstrated employing the Millon reaction after Bensley and Gersh (Pearse, 1960) in alcohol-fixed tissue and the ninhydrin reaction (Wigglesworth, 1942) in fresh fat body.

Glycogen: Glycogen was demonstrated employing the periodic acid Schiff reaction (Pearse, 1960). Rossman's alcoholic picro-formalin was used as the fixative. Control sections were incubated with saliva at 37°C for half an hour. Tissues extracted with cold trichloracetic acid (TCA) for 8 hr before fixation were also used as control.

Nucleic acids: The Feulgen reaction was carried out in Zenker-fixed paraffin sections for the demonstration of DNA. The methyl green pyronin Y method (Kurnick) was used for the demonstration of both DNA and RNA simultaneously (Pearse, 1960). The stains were purified by repeated chloroform extractions. Tissues were fixed in Carnoy. Schneider extraction with 5% TCA at 90°C for 15 min was used as control for both DNA and RNA. Treatment with crystalline ribonuclease at 37°C (Pearse, 1960) and with saliva at 60°C were also employed as control for RNA.

Uric acid: The following histochemical methods were employed: (1) Hexamine silver method as given by Pearse(1960); (2) Hollande modification of Courmont-André method (Cowdry, 1952). The presence of uric acid was also tested with Benedict's uric acid reagent in homogenates of the fat body.

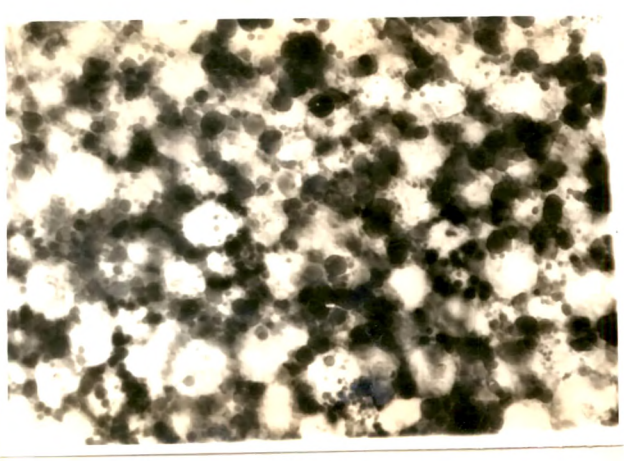
RESULTS

With Sudan Black B in both acid-haematein fixed and Ciaccio fixed gelatin sections the central globule took up a very dark colouration within a few minutes (Fig. 1). That the central globule comprises of neutral fat is evidenced by the following staining properties. With Nile blue sulphate in fresh fat body it showed a dark pink colouration while the peripheral globules stained blue. The pink colour due to the oxazone form of the dye is regarded as specific for neutral fat (Baker, 1960). With Fettrot 7 B, which is known to be specific for neutral fats (Pearse, 1960) it stained intense red. Except for Nile blue, the peripheral globules did not stain with the above dyes. A diffuse staining for fat was observed around the peripheral globules.

When Baker's acid-haematein test was carried out, the peripheral globules alone gave an intense dark blue colouration (Fig. 2). A similar result was obtained with Ciaccio fixed gelatin sections. Pyridine extraction, however, did not prevent them from maintaining an equally intense bluish tinge. With the Copper phthalocyanin method also the peripheral globules gave an intense staining both before and after pyridine extraction.

200 µ

Fig. 1 A transverse section of the larva stained for fat. (Sudan Black B. AC- Alimentary canal)



50 µ

Fig. 2 Section of the fat body subjected to acid-haematein staining. Note the tiny AH positive globules

With Millon's reaction the peripheral globules obtained a deep pink colour showing the presence of protein. With paraffin sections of the larva treated for the Millon reaction, however, these globules were not preserved as in life, but showed a tendency to become confluent. A positive ninhydrin reaction was also obtained in the peripheral globules.

The distribution of DNA was noted as large granules inside the nucleus (Fig. 3). The peripheral globules were intensely stained with pyronin. But the presence of RNA could not be confirmed as none of the controls employed viz. treatment with crystalline ribonuclease (1 mg. per ml in glass distilled water), saliva (both at 37°C and at 60°C) and trichloracetic acid (5% at 90°C for 15 minutes) could prevent the subsequent staining with pyronin.

Glycogen was found to be distributed diffusely around the peripheral globules. No granular concentrations of glycogen could be noticed in the fat body. In the prepupal stage, however, granular concentrations of glycogen appeared in the fat body (Fig. 4). This is in conformity with the finding (Chapter 5) that a quantitative increase in the glycogen content occurred in the prepupa.

Urate or uric acid crystals were seen inside the peripheral globules, which could be demonstrated by both the hexamine-silver and the Courmont-André methods. Small amounts of these deposits were seen in most of the peripheral globules but in some regions heavy deposits were noticed (Fig. 5). Uric acid deposits were also noticed during the routine staining in Bouin and Carnoy

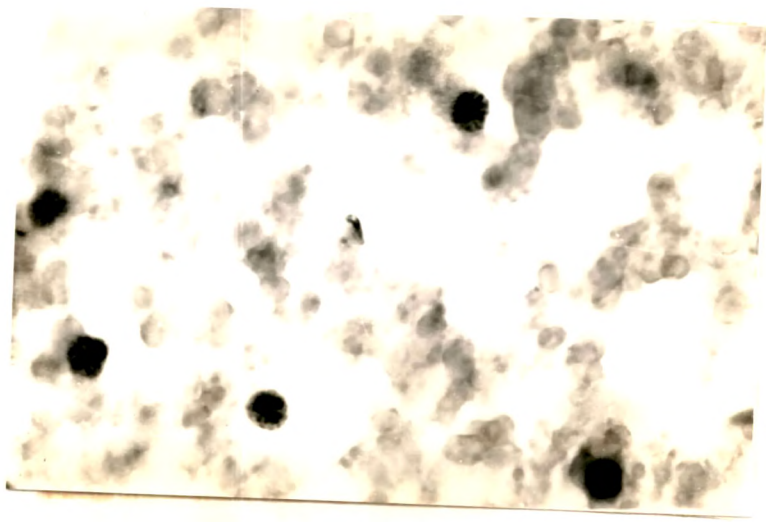


Fig. 3 Showing the localization of DNA in the nucleus (Feulgen reaction, counterstained with light green)

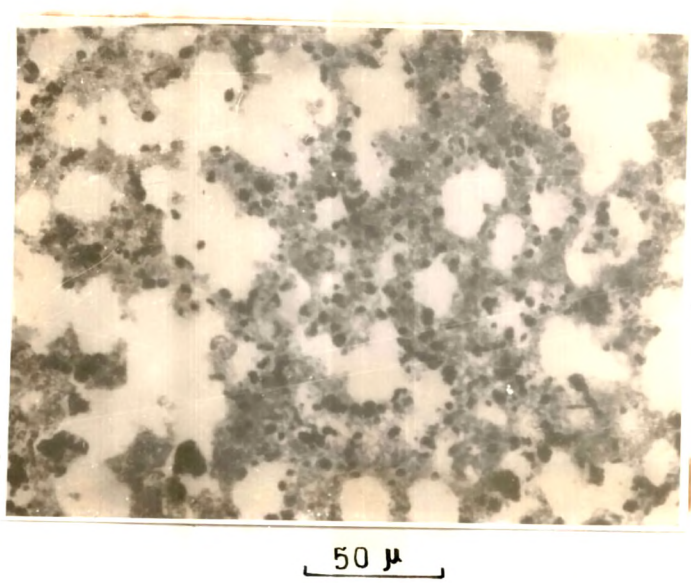


Fig. 4 Glycogen in the prepupal fat body (PAS). Note the granular concentrations

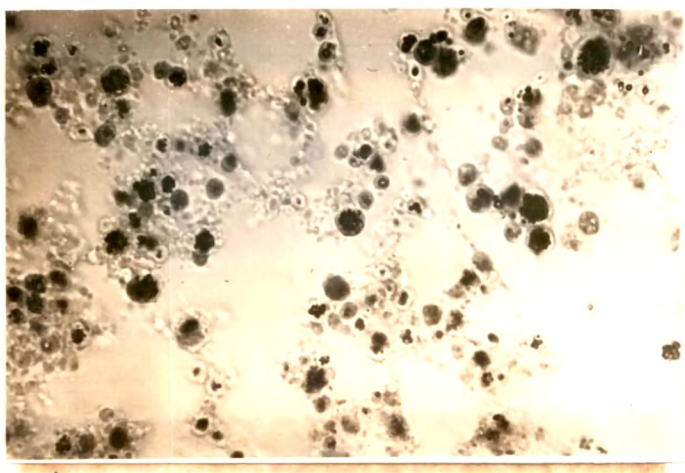


Fig. 5 Showing the concentrations of uric acid in the peripheral globules
(Hexamine silver)

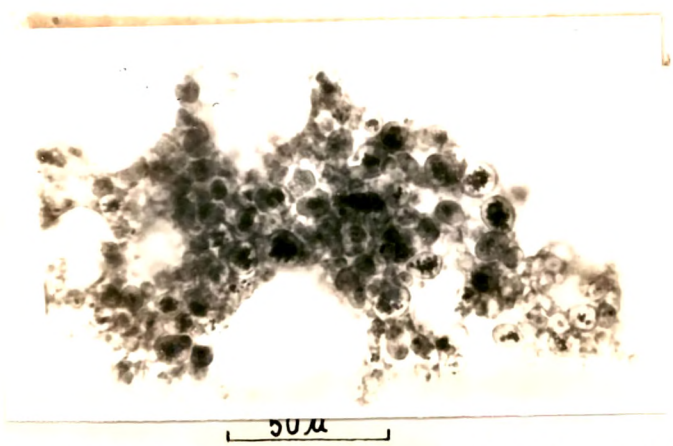


Fig. 6 Showing the precipitation of uric acid in Bouin-fixed fat body
(Mallory's triple stain)

fixed fat body (Fig. 6). Homogenates of the isolated fat body gave a positive reaction with Benedict's uric acid reagent.

In the fresh fat body, the peripheral globules were noticed to have a tendency to take up colour from dilute solutions of vital dyes such as Janus green and Methylene blue. In paraffin sections these globules were stained with both acidic and basic dyes.

DISCUSSION

It is obvious from these studies that the central globules consist of neutral fats. The fat body occupies a major part of the hemocoelic space and enormous amounts of fat could be stored in the larval body. It is shown in Chapter 5 that in Anthrenus vorax fat is utilized during metamorphosis and that it is also mobilized for the formation of the reproductive elements in the imago. The consummation of reproductive function soon after the emergence of the imago necessitates the raw-materials for egg production to be made available from larval reserves. Consequently, the task of building up and the storage of reserve materials leans heavily on the larval stage. The larval diet consists mainly of protein, but the large deposits of fat noticed here suggest that a major part of it is converted to fat since fat is a convenient form in which the energy could be stored . The site of such conversion could probably be the fat body.

The exact nature of the peripheral globules - their structure, chemical composition and the metabolic significance are not clearly understood at present. Vacuoles of different

kinds are known to occur in the fat body of different insects. Vacuoles which contain fat, glycogen or protein have been described in the mosquito larva (Wigglesworth, 1942). Some vacuoles which have been generally designated as 'watery vacuoles' were reported in the larvae of Chironomus (Voinov, 1927) and Aedes (Wigglesworth, 1942). It may seem that the peripheral globules of Anthrenus fat body have an apparent similarity to these vacuoles. But their preservation in dissociated fat body cells around the central globules and in properly fixed histological sections (Chapter 3), suggests that they are not to be looked upon as vacuoles. The tendency to become confluent in paraffin sections after fixation in ordinary fixatives would indicate the presence of a delicate limiting membrane rather than its absence. Their excellent fixation with Ciaccio's fixative and other lipid fixatives are indicative of the importance of lipids in the structural frame-work of these bodies.

Certain albuminoid granules have been described in the fat body of many insects. It should be conceded that 'albuminoids' is a vague term which has been ascribed to granules which stain deeply with cytoplasmic stains. They are supposed to be stored protein nutrients that may be utilized during starvation or metamorphosis. A more detailed study of these so-called albuminoids is required for a clearer understanding of their composition and metabolic significance. Though the peripheral globules described in the present study would correspond to the albuminoid granules of other authors, it is difficult to

conceive that they are merely protein inclusions. The positive histochemical reaction of these bodies for phospholipids by the acid-haematein method merits some consideration. The inability of hot pyridine to extract the stainable material suggests that they are not to be considered as free phospholipids. Pearse (1960) points out that certain bound lipines are resistant to extraction by pyridine. The presence of protein in the peripheral globules has already been mentioned. It may appear that these globules are composed of a complex of phospho-lipo-protein. The positive reaction obtained with the Copper phthalocyanin method before and after pyridine extraction lends further support to this contention. The characteristic affinity of the peripheral globules for vital dyes could also be attributed to the presence of phospholipids (Baker, 1960). Byrne (1962) has shown that basic dyes, without exception, are taken up by isolated phospholipids. Such a property of the peripheral globules has already been mentioned.

The nature of the proteinous inclusions in the fat body of insects has been much disputed (Wigglesworth, 1942). Zakolska (1928) referred to the albuminoid granules in Tenebrio as 'albumino-fatty granules' because they gave reactions for both fatty acids and proteins. The protein inclusions of Ephestia was noted to stain yellowish brown with osmic acid (Zeller, 1938). In the fat body of Aedes, Wigglesworth (1942) noted that the protein inclusions darkened with osmic acid though they failed to stain with fat stains. He attributed this to the possible

incorporation of a certain amount of lecithin with the protein.

The complex nature of the peripheral globules is also emphasized by its staining with pyronin even after treatment with RNA-ase, saliva or TCA. The stainability of nucleic acids with pyronin is due to the binding of the basic stain by the phosphate group which are originally free or are liberated by the displacement of protein (Kurnick, 1955). The presence of pyroninophilia even after the removal of RNA suggests the existence of other phosphate groups. These were resistant to extraction with 5% TCA at 90°C which should be suffieient to remove the free organic esters of phosphoric acid. This fact suggests the complex nature of phosphate binding in these globules.

In view of the established role of the fat body in the deamination and transamination processes of amino acids (Kilby and Neville, 1957; Desai and Kilby, 1958) and the synthesis of lipids (Zebe and McShan, 1959; Tietz, 1961), it may be inferred that in Anthrenus larval fat body a major part of the dietary aminoacids are deaminated and the resulting keto-acids are converted to fat. Part of it may also be converted to glycogen. The conversion of protein to fat and glycogen are known to occur in insect fat body. On a carbohydrate and fat free diet, the mosquito larva has been shown to synthesize both glycogen and fat and these were laid down in the fat body before they appeared in the gut wall (Wigglesworth, 1942). The peripheral globules have been shown to contain protein. A diffuse distribution of fat and glycogen was noticed around these globules. It was not possible, however, to demonstrate

the presence of fat and glycogen inside these bodies. The intense reaction obtained with the acid-haematein and Copper phthalocyanin methods, though could not be held as sufficient evidence to conclude the presence of phospholipids, as already indicated, it may be maintained that phospholipids are present in some bound form as part of a phospho-lipo-protein complex. If the peripheral globules described here really constitute a phospho-lipo-protein complex they may well have some implications in the elaboration of the specific type of fat. Complexes of a lipoprotein nature are thought to provide a sequential arrangement in space for the multi-enzyme systems. The characteristic arrangement of the peripheral globules around the central fat globule and a diffuse staining of fat around them would indicate that specific fats or fatty acids are produced inside these bodies and are being constantly passed on to the central fat globule for storage. The uric acid which appears inside these globules may be the product of deamination of the aminoacids from which fat is synthesized. It is logical to assume then that the peripheral globules are the lipopoietic centres of the fat body rather than being a mere protein reserve. It should, however, be admitted that this is a possible hypothesis which should be put to the test of more intensive and elaborate studies.

The peripheral globules may be looked upon as definite organelles which have become specialized to perform certain specific functions rather than as stored protein nutrients.

The size of the peripheral globules was found to vary, globules of minute size could be clearly seen by the acid-haematein method of staining (see Fig. 2). It may be mentioned here that on staining for mitochondria with aniline oil-fuschin (Cowdry, 1952) in Regaud fixed fat body, all the peripheral globules of various sizes took up a deep red colour and it was not possible to distinguish any mitochondrial structures. The presence of uric acid or urate could not be seen in all the peripheral globules, but at the same time such of those which did contain uric acid could not be clearly distinguished from those that did not. It is possible that the deamination of aminoacids and the synthesis of fat or at least fatty acids occur in these globules, they being sent to the central globules for storage and the amino part of the deaminated aminoacids being converted to uric acids at these sites and stored there. When a large amount of uric acid has thus been formed, these globules might enlarge in size and may appear as the uric acid containing ones. The concentration of uric acid was more in the fat body lobes found in the sub-cuticular region of the body. Excretion of uric acid through the alimentary canal in Anthrenus larva was confirmed by testing the excreta with Benedict's uric acid reagent, but it is not known how the uric acid finds its way into the alimentary canal.

The present observations call for a more detailed study of the so-called albuminoids of other insects. The peripheral globules of Anthrenus fat body are certainly homologous to the

albuminoids of other insects. However, the views expressed here are in striking contrast to the views of Perez (1920), Schnelle (1923) and Schnieder (1928) who consider that the albuminoid granules are formed largely by transference of material from the fat droplets.