THE STORAGE OF PROTEIN, FAT, GLYCOGEN AND URIC ACID IN THE FAT BODY AND OTHER TISSUES OF MOSQUITO LARVAE

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(With Eleven Text-figures)

It is generally agreed that the main function of the fat body of insects is the storage of those reserve substances which require rapid mobilization during moulting and metamorphosis. But little attention has been given to the part taken by other organs and tissues in building up reserves and almost none to the influence of the composition of the food upon the type of reserve substance laid down.

These problems form the subject of the present paper. Mosquito larvae have been starved until all their reserves have been consumed. They have then been fed upon single substances and the storage of protein, fat and glycogen in the various tissues observed.

METHODS

Rearing and starving of larvae

All the experiments have been made at 28° C. on larvae of *Aedes aegypti* in their fourth or final instar. The eggs are immersed in a rich infusion of powdered dog biscuit. They hatch at once and the larvae reach the 4th stage in about 48 hr. If they are left in the infusion they pupate in a further 48 hr.

The larvae are starved by transferring them, within an hour of moulting to the 4th stage, to freshly prepared 0·01 *M* acid potassium phthalate in tap water adjusted to *p*H 4·0 with phosphoric acid. They are kept in this at 28° C. until their reserves are used up. This requires about 10–14 days. The fat body of the living larva can be observed by transmitted light under the microscope. By the time all fat droplets have disappeared the reserves are practically exhausted (p. 62). The larva then becomes sluggish and generally dies within 48 hr. or so.

Feeding of larvae

After this preliminary starvation the larvae are given pure food substances, twelve larvae being placed in 2·5–5 c.c. of the phthalate buffer at *p*H 4·0.

Protein is given in the form of casein (fat free and vitamin free). This is finely ground with kaolin and a few milligrams added on the point of a knife.

Carbohydrates. Starch is given in the same way in the form of soluble starch ground with kaolin and washed sand. *Sugars* are given in 2·5% solution with addition of kaolin. The larvae ingest the kaolin and so obtain the solution with

which it is moistened. Starved larvae immersed in such a mixture have the entire gut filled with kaolin within about 2 hr.

Fat is given in the form of olive oil. A few drops of olive oil containing a drop of oleic acid were emulsified with dilute sodium carbonate to form a stable emulsion of about the density of milk. Two or three drops of this are added to 5 c.c. of the phthalate buffer containing a large knife point of kaolin. The oil droplets are entangled by the kaolin and held at the bottom of the fluid where they are ingested by the larvae. Lecithin emulsified in water was given in the same way.

Exclusion of bacteria

This has been effected by keeping the larvae at the acid reaction of *p*H 4·0 and by transferring them to freshly prepared food mixture every 12 or 24 hr. It is not claimed that these cultures are sterile in the bacteriological sense. But in a number of tests in which casein mixtures (these are the most liable to contamination) were left unchanged after removal of the larvae, hanging drop preparations showed that micro-organisms were still very few at the end of 2 or 3 days. They are therefore unlikely to be of nutritional importance during the first 12 hr. of growth.

This belief is supported by the fact that although, as will be seen, casein is an adequate source of protein, fat and glycogen, larvae fed with casein under these conditions may still show no sign of growth beginning in the imaginal disks, etc., at the end of 7 days; whereas larvae fed with casein containing abundant micro-organisms (and so providing the accessory factors needed for growth; cf. Trager, 1935, 1937) will pupate within 4 days.

Nevertheless, yeasts which grow rather rapidly at *p*H 4·0 have occasionally appeared in the cultures. These have therefore been regularly examined in hanging drop preparations and the experiment rejected when such organisms were present.

Examination of the tissues

(i) Many observations on the fat body can be made in the living insect. Below the cuticle of the dorsal and ventral surfaces of the larva the flattened cells of the fat body form a thin single layer. In each cell the various inclusions lie for the most part in one plane and may be observed with the 2 mm. objective. It is thus possible to follow the inclusions in a given cell from day to day or from hour to hour, and at any desired stage to fix and test them. The distribution of glycogen in these cells can be seen by immersing the living larva in 0·05 % iodine solution under a supported cover-slip.

(ii) The general distribution of reserve substances is best studied in whole larvae dissected. After fixation the neck and the last abdominal segment are cut through. The larva is then slit with scissors along one side and the gut removed through the slit. The body may now be unrolled and mounted flat and all the tissues (epidermis with imaginal disks, fat body, tracheae, heart and pericardial cells, muscle fibres and sarcoplasm, oenocytes, reproductive and nervous systems) can be examined with the oil immersion in a single preparation (Fig. 1). The gut with caeca and Malpighian tubes is best cut longitudinally and mounted flat along-

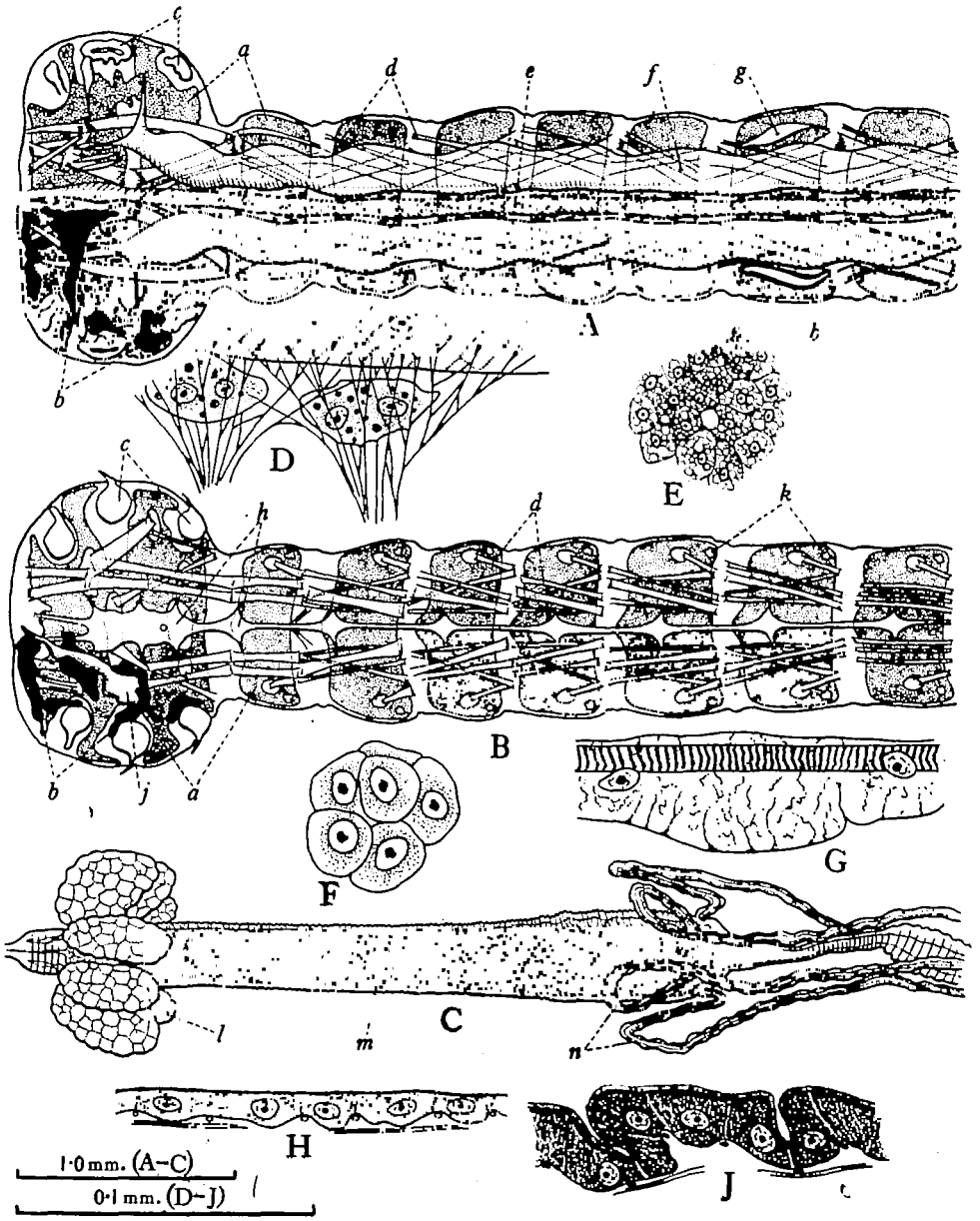

Fig. 1. Anatomy of mosquito larva showing the chief tissues used for storage. A, dorsal half of larva; B, ventral half of larva; C, alimentary canal; D, pericardial cells and margin of heart; E, fat body; F, oenocytes; G, muscle with its sarcoplasm; H, longitudinal section of anterior half of midgut; J, section of posterior half of midgut. *a*, parietal fat body; *b*, visceral fat body; *c*, imaginal disks; *d*, muscles; *e*, heart with pericardial cells; *f*, main tracheal trunk; *g*, gonad; *h*, ventral nerve cord; *j*, salivary gland; *k*, oenocytes; *l*, gastric caeca; *m*, midgut; *n*, Malpighian tubes.

side the remainder of the body after removal of the peritrophic membrane and its contents.

The following tests for the chief reserve substances have been used.

Protein. The larvae are fixed in Carnoy's or Bouin's mixtures and after dissection are warmed in 50% glycerol containing 1% of ninhydrin or in Millon's reagent freshly mixed with an equal volume of glycerol. The violet colour of the proteins in the ninhydrin and the rose red in Millon's reagent are permanent in preparations mounted in glycerol or in syrup.

Fat. The larvae are fixed in Bouin's fluid, stained with red or black Sudan B and mounted in glycerol. A few larvae have been fixed in the dilute Altmann's fluid (equal parts of 3% potassium dichromate, 2% osmic acid and water) recommended by Baker (1933). This gives excellent fixation of the cells, but the blackening of the inclusions is by no means specific for fats (Lison, 1936).

Glycogen. The larvae are fixed in Carnoy's fluid or in alcoholic Bouin. They are first stained with light green in 95% alcohol and then in a mixture of absolute alcohol saturated with iodine (65 parts) and 1% iodine in potassium iodide (35 parts), dehydrated in 95% alcohol saturated with iodine and mounted in euparal saturated with iodine. In these permanent preparations the glycogen deposits are conspicuously stained; but they are somewhat shrunken and distorted. Their natural form is better preserved if the larvae are mounted direct from the 65% alcoholic iodine into 'calsolene oil'.[1] Unfortunately these preparations are not permanent; the glycogen begins to fade after 24 hr.

In order to demonstrate fat, glycogen and protein in a single cell, the larva is fixed for 12 hr. in Bouin's mixture made up with 85% alcohol saturated with picric acid. It is then stained with B.Z.L. blue (Ciba) in 60% alcohol, mounted in syrup and the distribution of fat in the selected cell drawn. The fat stain is removed in 90% alcohol, the glycogen is stained in 65% alcoholic iodine, the larva mounted in 'calsolene oil' and the cell redrawn. Finally, the iodine is removed in tap water and protein is stained with ninhydrin. Figs. 6, 8 and 10 were obtained in this way.

(iii) Histological details have been confirmed in sections of larvae double embedded in celloidin and wax and stained with haematoxylin and eosin. The tests for protein and glycogen have also been applied to such sections.

RESULTS

Anatomy and histology of the larva

The general arrangement of the tissues and the histology of those used largely for storage are shown in Fig. 1. The *fat body* consists of a parietal layer composed of a single sheet of flattened cells applied to the surface of the body in each segment, and a visceral layer made up of a few small lobes in the prothorax, groups of cells applied to the imaginal disks and a thin membrane covering the gonads. The dorsal fat body is more deeply pigmented (it contains more uric acid) than the ventral; otherwise the structure is fairly uniform throughout.

The *gut* is shown in Fig. 1 C. Immediately behind the midgut caeca there are

[1] A wetting and clearing agent, miscible with alcohol and water, marketed by Imperial Chemical Industries, Ltd.

a few rather darkly staining cells, but beyond these the midgut falls into two sharply divided halves. In the anterior half (Fig. 1 H) the cells are clear, pale staining and homogeneous; in the posterior half (Fig. 1 J) they are granular and deeply staining with a conspicuous striated border. This division seems to be widespread in larvae of Nematocera. It is described among others by van Gehuchten (1890) in *Ptychoptera*, by Frederici (1922) in *Anopheles* and by Samtleben (1929) in *Culex*.

Reserve substances in the newly moulted 4th stage larva

In larvae newly moulted to the 4th stage *fat* droplets of varying size are plentiful throughout the fat body; there may be a small amount of fat in the cells of the anterior half of the midgut and there is a diffuse staining which cannot be resolved into droplets in the central fibrous region of the nerve ganglia, but no stainable fat elsewhere.

Protein in the fat body consists only of a condensed zone around the nucleus with filaments radiating outwards between the fat droplets and other vacuoles; there are no discrete deposits of protein. Elsewhere, also, protein exists only in the form of cytoplasm; it is most dense in the muscle fibres, oenocytes, pericardial cells and in the posterior half of the midgut.

Glycogen is widely dispersed. It is plentiful in the fat body (Fig. 2 A) and it occurs throughout the ganglia and nerve cord of the central nervous system in the form of granular deposits and small vacuoles (Fig. 2 C). But it is most abundant in the muscles, where the swollen masses of sarcoplasm which surround the fibres are practically solid with glycogen. There are also thread-like deposits between the muscle fibres (Fig. 2 B). Smaller amounts occur on the fibres of the gut wall and of the heart and there are small deposits in the intestinal epithelium, particularly in the caeca and the posterior half of the midgut. Glycogen is absent from the oenocytes and pericardial cells.

Fig. 4 A shows two typical fat body cells as seen in the living larva newly moulted. The cytoplasm appears as a coarse meshwork enclosing large vacuoles, and the cell boundaries are not visible. The vacuoles are of two types, one containing refractile droplets of fat, the other with non-refractile aqueous contents. The latter are mostly spherical but some are elongated or irregular in outline. Their interfacial tension is low, for these vacuoles are deformed and their contents move to and fro as the cells are pressed by the contracting heart. They range in size from minute vacuoles only a few μ in diameter to large spaces several times the diameter of the nucleus. They may be colourless, pale yellow or grey. They are responsible for the range in colour of the larva from white to dark grey.

After fixation (Fig. 2 A) some of these watery vacuoles, particularly around the periphery of the cells are seen to contain glycogen. But most of them contain none of the reserve substances.

If the larva is fixed in aqueous Bouin, dissected in 90% alcohol, and mounted in euparal (Fig. 4 A') it is seen that in many of the vacuoles small sheaves of needle-like crystals of uric acid[1] have separated out.

[1] These crystals are assumed to be uric acid because they are soluble in dilute alkalis, and in aqueous solutions of piperazine and hexamine; they give a positive murexide reaction and a blue colour with Folin's uric acid reagent (cf. de Boissezon, 1930).

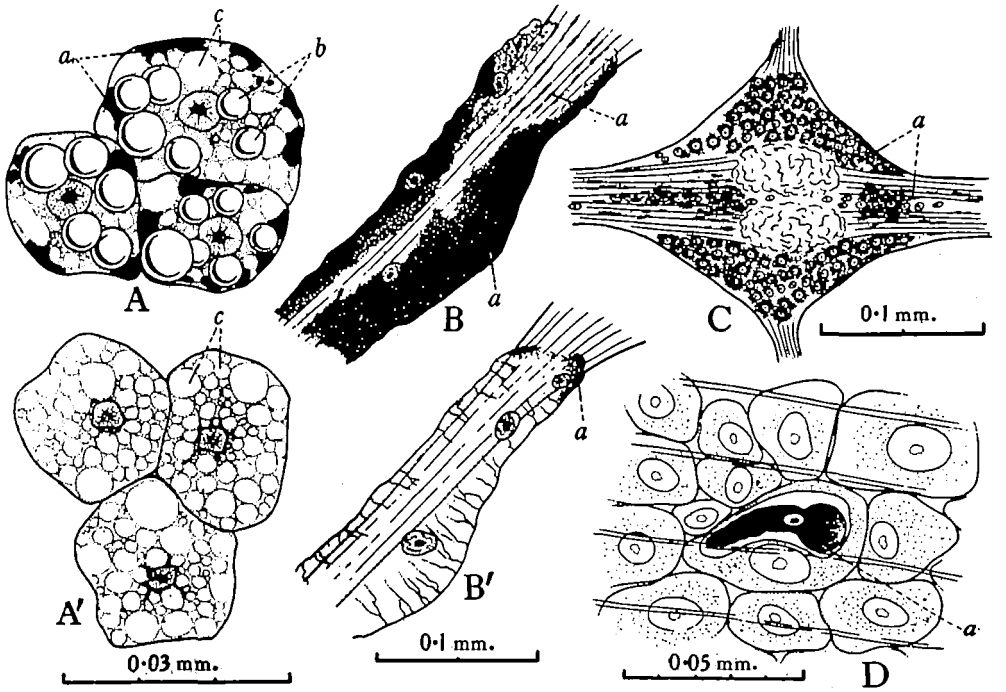

Fig. 2. A, fat body cells of newly moulted fourth stage larva; A′, the same in larva starved 12 days; B, part of muscle and sarcoplasm of newly moulted larva; B′, the same in larva starved 12 days; C, abdominal ganglion in newly moulted larva; D, surface view of midgut wall in completely starved larva, showing *Lankesteria culicis* in one of the cells. *a*, glycogen stained with iodine; *b*, fat droplets; *c*, watery vacuoles.

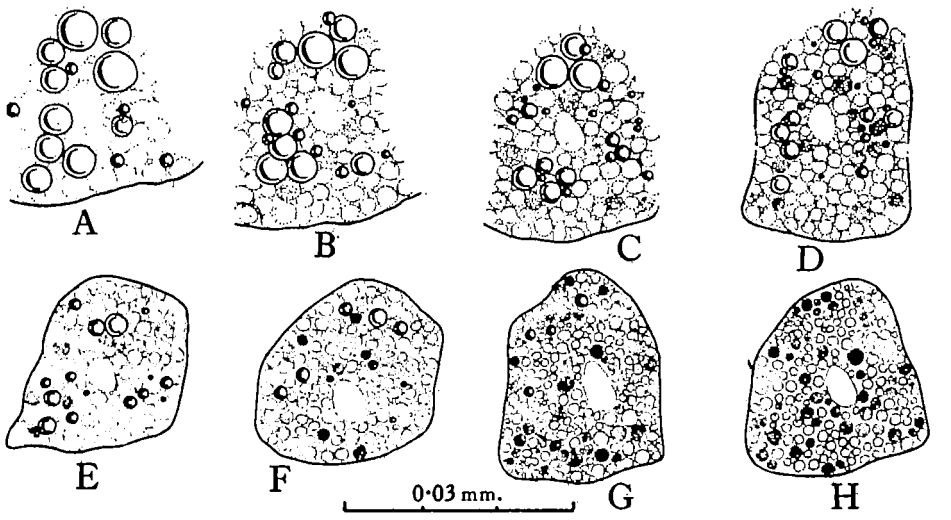

Fig. 3. A single fat body cell at the hind margin of the metathorax drawn from the living larva. A, immediately after moulting to the fourth stage; B–H, at 12 hr. intervals thereafter.

Utilization of reserve substances during starvation

Fat, protein and glycogen are used concurrently throughout starvation. There is no indication that glycogen is used up first. Fig. 3, for example, shows the fat droplets in a fat body cell drawn at intervals of 12 hr. during the first 3 days of starvation. The fat droplets move rather freely about the cell and are progressively reduced in size, minute droplets being apparently detached from the larger ones.

The fat droplets disappear first in the abdomen; they persist longest in the prothorax, where they finally disappear from 10 to 14 days after moulting. The larva dies about 48 hr. after this. Even in larvae completely starved, fat droplets remain at the centre of the disks of the pupal respiratory trumpets.

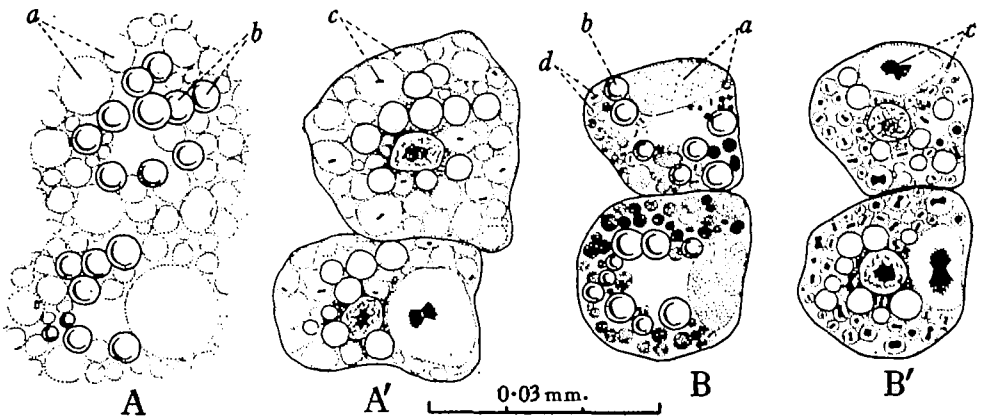

Fig. 4. A, fat body cells in thorax of newly moulted living larva; A′, the same cells after fixation in aqueous Bouin; B, fat body cells in thorax of living larva starved 7 days; B′, the same cells after fixation in aqueous Bouin. *a*, watery vacuoles; *b*, fat droplets; *c*, uric acid crystallized after fixation; *d*, uric acid separated in vacuoles of living cell.

The disappearance of glycogen from the fat body runs parallel with that of fat. The amount is much reduced at the end of 4 days; none remains after 6 days (Fig. 2 A′). At the same time the deposits in the gut have disappeared. In the sarcoplasm of the muscles the deposits are becoming less massive and solid by the end of 6 days; by 10 days they are very slender in most places; at 12 days, in larvae from which all fat had been used up, there were still faint traces of glycogen, particularly around the points of insertion of the muscles (Fig. 2 B′). It is of interest to note that when such larvae harboured gregarines (*Lankesteria culicis*) in the gut, these were still stuffed with glycogen (Fig. 2 D).

Alongside the utilization of fat and glycogen there is a progressive wastage of cytoplasm in all the tissues as proteins are used up, and the nuclei become much reduced in size. This is well seen in the muscle fibres and in the fat body cells. The oenocytes, pericardial cells, gut and central nervous system show a similar wastage.[1]

[1] Within the cells of the midgut, particularly in the caeca and just in front of the Malpighian tubes, there are refractile oval inclusions which colour faintly with ninhydrin and sometimes with fat stains. The nature of these is uncertain; they do not appear to consist solely of protein.

As starvation proceeds the aqueous vacuoles in the fat body become smaller and darker and probably increase in number (Fig. 3). Fig. 4 B shows two living cells in a larva starved 7 days. The contents of most of the vacuoles are homogeneous, but in some there are minute refractile particles, elongated crystals or clusters of needles all in Brownian movement. On fixation in aqueous Bouin masses of such crystals, carrying with them more or less grey or amber pigment, separate out in the form of spheres of radiating needles or double spheres or 'wheatsheaf' bundles of crystals (Fig. 4 B'). In the late stages of starvation, solid amber coloured deposits in the form of irregular spheres may separate during life. These crystalline deposits almost certainly consist of uric acid (see footnote, p. 60).

Thus during starvation, as the proteins are used up, the vacuoles of the fat body contain an increasing quantity of uric acid presumably in colloidal solution.

In the final stages of starvation the cells of the fat body may separate from one another and become rounded, many of the aqueous vacuoles shrinking or disappearing.

Formation of reserves after feeding with carbohydrates

Starch. Within 6 hr. of feeding these fully starved larvae on starch, deposits of *glycogen* appear in the cells of the posterior half of the midgut and there may be traces of glycogen in the cells of the fat body in the front of the prothorax.

At 12 hr. there are granules of glycogen in some of the cells of the caeca and in the ring of midgut cells immediately behind these. Then follows the region of clear cells; in these glycogen is absent or present only as a few sparse granules. Halfway along the midgut heavy deposits begin abruptly; they are very plentiful throughout the pyloric half. Glycogen is increasing in the prothorax but there is little elsewhere in the fat body. Scattered granules are beginning to appear in the sarcoplasm of some muscles and in the ganglia of the nerve cord.

Subsequently (24 hr. to 6 days) glycogen increases progressively in amount but retains the same distribution. That is, in the gut it is almost confined to the caeca and the cells just behind them, and to the pyloric half of the midgut where great masses of glycogen accumulate in the course of 2 or 3 days (Fig. 5 A). It occurs throughout the fat body and after several days it fills the greater part of each fat body cell (Fig. 6 A–D). The deposits in the muscles become gradually more dense and by the 3rd or 4th day the sarcoplasm is almost solid with glycogen (Fig. 6 E–H). Glycogen occurs throughout the central nervous system, both in the ganglia and in the connectives. But it is absent from the oenocytes, pericardial cells, epidermis and imaginal disks.

In some larvae glycogen is present in the form of fine granules in the cells of the Malpighian tubes, particularly in the proximal region; though sometimes there are extensive deposits throughout the tubes (Fig. 5 C). These deposits are not constant; but they may appear within 12 hr. of feeding, at a time when glycogen is still absent from the abdominal fat body. It seems therefore almost certain that they are derived from the gut contents which presumably may on occasion enter the Malpighian tubes and be there absorbed. Occasionally small deposits appear in the cells of the hindgut, suggesting some absorption here.

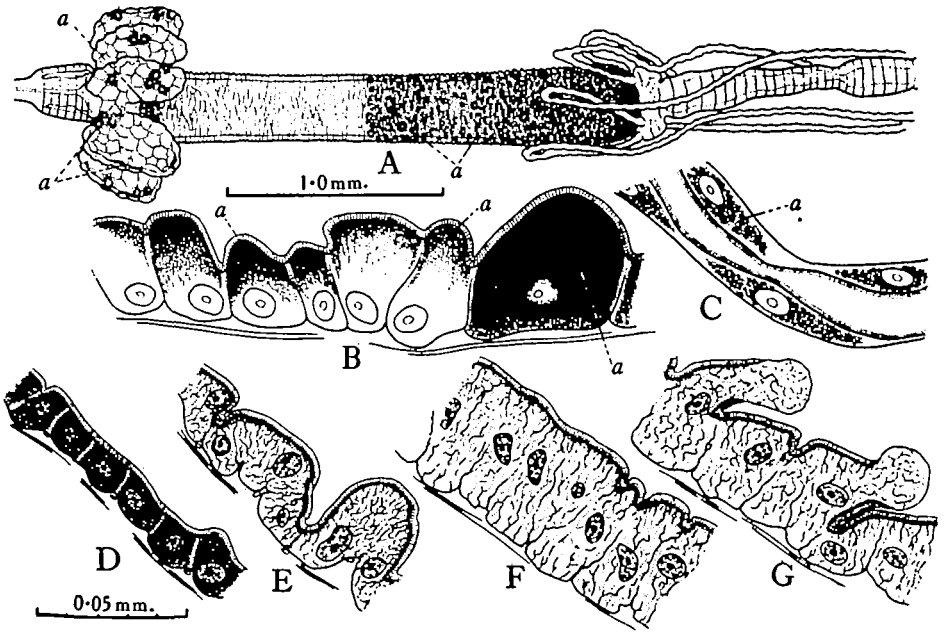

Fig. 5. A, gut of larva fed 2 days on starch; B, optical section of pyloric segment of midgut in this larva showing distribution of glycogen in the cells; C, portion of Malpighian tube of larva fed 24 hr. on sucrose showing glycogen in the cells; D–G, longitudinal sections of pyloric half of midgut (fixed in Carnoy, stained haematoxylin and eosin); D, in starved larva; E, larva fed 12 hr. on starch; F, larva fed 3 days on starch; G, the same showing droplets filled with glycogen protruding through the striated border. *a*, glycogen stained with iodine.

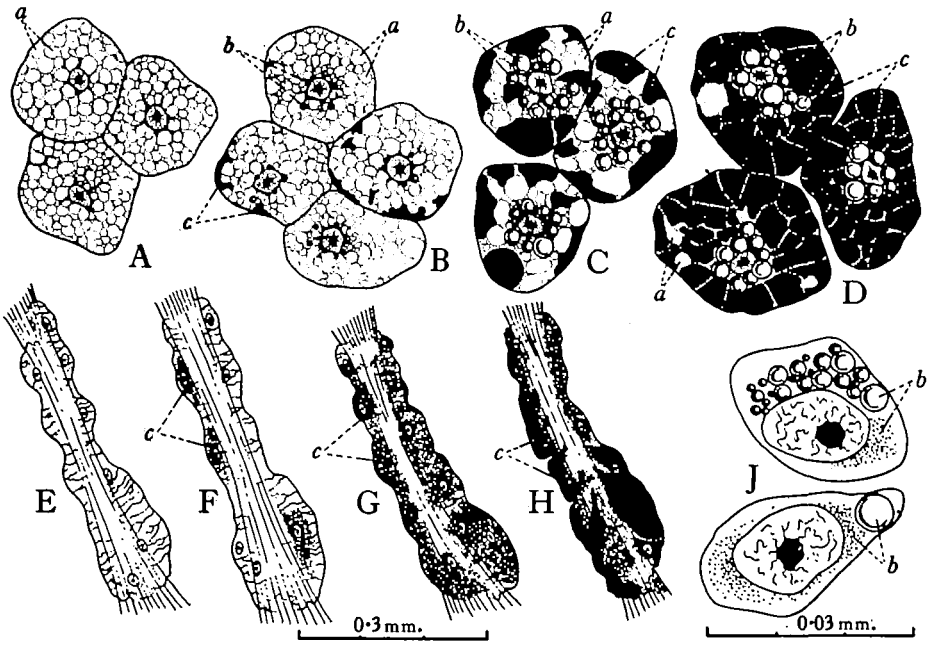

Fig. 6. A, fat body cells in fully starved larva; B, after feeding on starch for 24 hr.; C, after 2 days; D, after 4 days; E, abdominal muscle in fully starved larva; F, after feeding on starch for 24 hr.; G, after 2 days; H, after 4 days; J, oenocytes after feeding on starch for 2 days. *a*, watery vacuoles; *b*, fat droplets; *c*, glycogen stained with iodine.

As to the location of the glycogen in the cells; in the pyloric half of the midgut glycogen occurs in diffuse form in the cytoplasm. It first appears in a layer immediately below the striated border (Fig. 5 B); but later, and in many of the cells, it may extend and accumulate until the swollen cell is practically solid with glycogen (Fig. 5 B). Sections of such cells show a delicate meshwork of cytoplasm with the interstices filled with glycogen (Fig. 5 F). Very often there are large droplets of cytoplasm filled with glycogen protruding through the striated border (Fig. 5 G). It is probable, however, that these are artefacts of fixation: I have been unable to see them in optical sections of the living larva.[1]

Similarly, in the cells of the fat body, glycogen is first seen in vacuoles (and perhaps in the cytoplasm) at the periphery of the cells (Fig. 6 B). Later these vacuoles, with delicate strands of cytoplasm lying between them, almost fill the entire cell (Fig. 6 D). Meanwhile the uric acid vacuoles are displaced, chiefly to the outer surface (Fig. 7 B), and the total quantity of uric acid present appears to be greatly reduced.

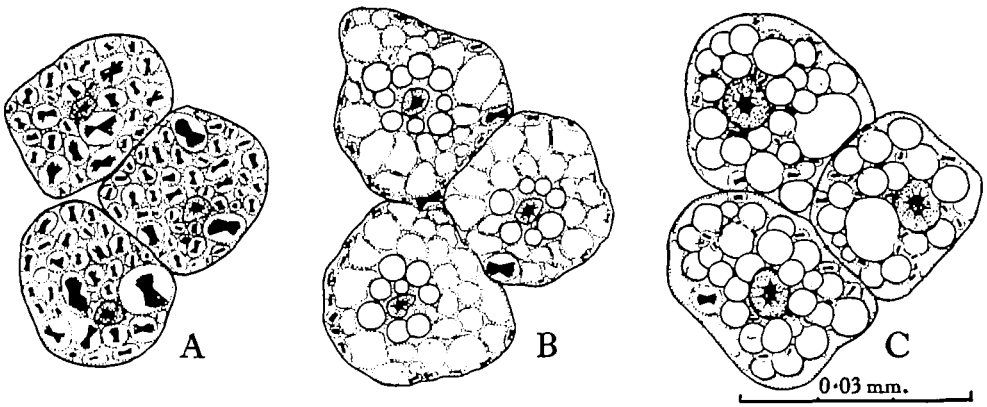

Fig. 7. A, uric acid crystals in fat body cells of fully starved larva (fixed in aqueous Bouin); B, in a similar larva fed for 3 days on starch; C, in a similar larva fed for 3 days on casein.

After feeding on starch, *fat* first makes its appearance in the oenocytes. It is seen here between 24 and 48 hr. from the start as a diffuse staining in which no particles can be resolved, as a fine 'dust' or as small scattered droplets (Fig. 6 J). At about the same time fat droplets appear in the fat body at the front of the prothorax and later in the fat body elsewhere. They may occur in cells from which glycogen is still absent. They arise first as minute droplets close to the nuclear membrane (Fig. 6 B) and even in the later stages when these fat droplets are enlarged and the cell is filled with glycogen they generally retain this distribution (Fig. 6 D). Occasional droplets of fat occur in the cells of the caeca and the anterior half of the midgut.

There is of course no accumulation of *protein* during starch feeding. The cytoplasm remains very deficient in protein and the nuclei remain small.

[1] Droplets of this type were described by van Gehuchten (1890) in *Ptychoptera* as secretory vesicles. They are regarded by Vignon (1899) in *Chironomus*, by Steudel (1913) in various insects, and by de Boissezon (1930) in *Culex* as artefacts.

Sugars. The question arises whether the deposition of glycogen in the cells of the pyloric half of the midgut is a specific function of the cells in this region, or whether it is merely the result of digestion of starch being delayed until it reaches this segment of the gut.

That there is a real difference in the absorptive or storage function of the cells is suggested by the fact that some glycogen is deposited also in the cells of the caeca and in the ring of cells just behind them. A further proof has been obtained by feeding the larvae on glucose in 2·5 % solution. The deposition of glycogen in the gut has exactly the same distribution as after feeding with starch: after 6 hr. feeding there is a sharp boundary between the pyloric half of the midgut which contains plenty of glycogen and the anterior half without.

This rapid deposition of glycogen in the gut wall after feeding with carbohydrates provides a means of comparing the ability of the mosquito larva to deal with different sugars and sugar alcohols. These have been given in 2·5 % solution to batches of twelve larvae which have been fixed and dissected after 6 and 12 hr.

The results prove clearly enough that even among sugars which can serve as a source of glycogen some are more efficient than others; they seem to form a graded series in this respect. There are other sugars which do not yield any glycogen at all. The individual variation is such that accurate grading is impossible; but the sugars tested have been roughly sorted into five groups.

(*a*) *Glucose* is definitely the most efficient. At the end of 6 hr., besides the deposits in the gut, there is plenty of glycogen in the fat body in the prothorax and elsewhere and it is beginning to accumulate in the sarcoplasm of the muscles. There are massive accumulations at the end of 12 hr.

(*b*) *Fructose* does not lead to quite such a rapid deposition as glucose, but plenty of glycogen appears in the prothoracic fat body within 12 hr. The disaccharides *sucrose* and *maltose* fall into this group, being only slightly less efficient than glucose.

(*c*) *Galactose* produces fairly large deposits in the pyloric half of the midgut within 12 hr., but no glycogen has appeared in the fat body. Other substances falling into this group are: *mannose*, *trehalose*, *α-methyl-d-glucoside*, *mannitol*, *sorbitol* and the disaccharide *lactose*.

(*d*) Sugars which lead to the formation of only very small traces of glycogen in the gut in 12 hr. are the pentose *xylose*, the sugar alcohol *dulcitol* and the disaccharide *cellobiose*.

(*e*) No glycogen is formed from the hexose *sorbose*, the pentoses *arabinose* and *rhamnose* or the trisaccharide *raffinose*.

Formation of reserves after feeding with fats

Within 24 hr. of feeding with olive oil minute droplets of fat are usually present throughout the fat body, lying chiefly, though not exclusively, close to the nuclear membrane (Fig. 8 B). Exceedingly fine particles of fat are present also in the oenocytes and may appear in these cells at a time when none is laid down in the fat body (Fig. 8 F). In later days there is a progressive increase in the fat droplets in these tissues.

In the gut, fat is confined to scattered cells in the caeca and to the clear cells of the anterior half of the midgut (Fig. 9 A). 24 hr. after feeding with olive oil the cells of this region contain abundant fat: some are filled with fairly large droplets,

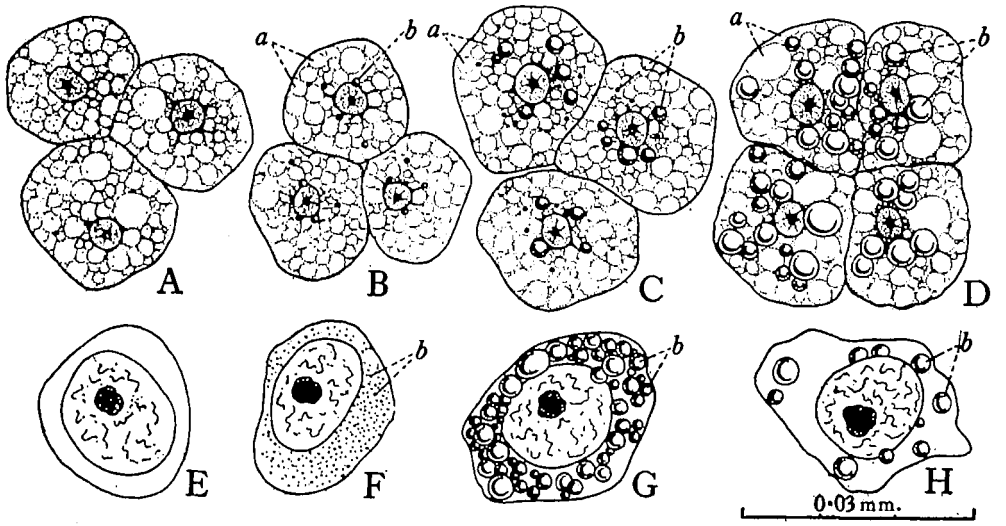

Fig. 8. A, fat body cells in fully starved larva; B, after feeding on olive oil for 24 hr.; C, after 2 days; D, after 3 days; E, oenocyte in fully starved larva; F, after feeding on olive oil for 24 hr.; G, after 2 days (in this larva the oenocytes contained an exceptionally large amount of fat); H, after 3 days. *a*, watery vacuoles; *b*, fat droplets.

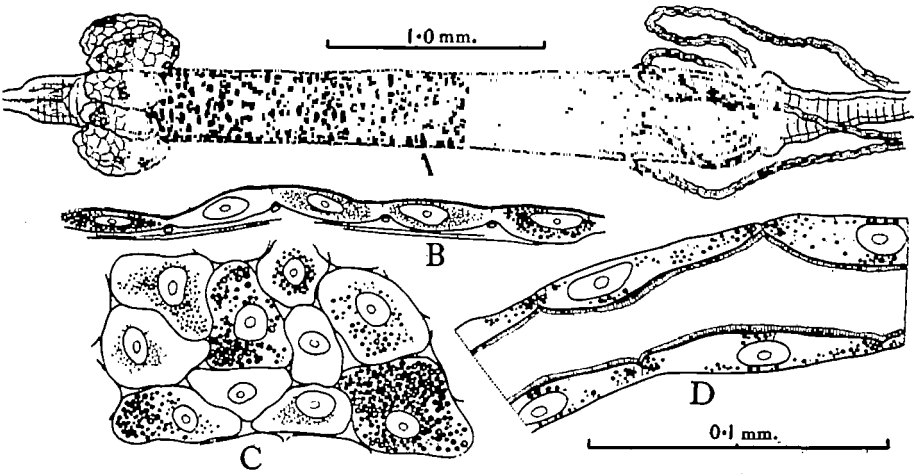

Fig. 9. A, gut of larva after feeding on olive oil for 24 hr., showing fat in caecal cells and anterior half of midgut; B, optical section of the midgut epithelium showing fat in the cells; C, the same in surface view; D, portion of Malpighian tube showing fat droplets in the cells.

others show only a fine 'dust' and yet other cells contain no fat at all (Fig. 9 B, C). In some larvae, on the other hand, there may be fat in the cells of the fat body but none deposited in stainable form in the gut wall. In a few larvae minute fat droplets appear in the cells at the lower ends of the Malpighian tubes (Fig. 9 D).

At no stage does there seem to be any accumulation of glycogen in the tissues after feeding with olive oil.

Similar results are obtained after feeding with egg 'lecithin', but absorption perhaps occurs more readily. In some larvae droplets of fat, besides filling the anterior half of the midgut, were plentiful in the caecal cells and in the cells of the cardiac region which secrete the peritrophic membrane. A barely resolvable fat 'dust' could sometimes be seen in the blood within the heart.

Formation of reserves after feeding with protein and amino acids

Casein. In larvae starved until all fat and glycogen have been used up, the proteins in all the tissues are largely wasted. Everywhere the nuclei are very small, the nucleoli deficient in protein. In the fat body there is a little protein in the form of strands radiating from the nucleus, but elsewhere the protein meshwork between the vacuoles in these cells is almost invisible (Fig. 10 A). The oenocytes are similarly wasted (Fig. 10 F), protein in the muscles is reduced and the epithelium of the midgut is greatly attenuated (Fig. 11 A, A').

Within 24 hr. of feeding on casein the nuclei in the fat body are enlarged, the protein meshwork, particularly around the nucleus, is more evident, and everywhere the tint given with ninhydrin is much darker. Minute droplets of fat may have appeared, chiefly in the neighbourhood of the nuclear membrane, but little glycogen is present as a rule except in the prothorax (Fig. 10 B). The oenocytes may show a very fine fat 'dust' or scattered droplets or they may still be free from fat (Fig. 10 G). The sarcoplasm of the muscles may show a little glycogen finely dispersed (Fig. 10 M) and traces appear in the central nervous system. The cells of the gut epithelium are becoming enlarged but they contain neither fat nor glycogen (Fig. 11 B, B').

After 2 days, protein in the cytoplasm of the fat body cells is further increased and in the thorax minute spheres of protein are making their appearance on the nuclear membrane. There are filaments in the nucleus radiating from the plasmosome to the nuclear membrane and in many places the protein droplets have the appearance of being attached to these filaments. Fat droplets are larger and more plentiful and are often scattered throughout the cells (Fig. 10 C). Glycogen occurs in many of the fat cells, chiefly at the periphery, but not in all; it is most abundant in the prothorax. The oenocytes are further enlarged and contain small fat droplets (Fig. 10 H). Glycogen is increasing in the sarcoplasm of the muscles (Fig. 10 N) and in the nervous system, and traces may occur throughout the midgut.

After 3 days the droplets of protein in the fat body cells are enlarged and plentiful everywhere. Although many of them still rest upon the nuclear membrane, others are dispersed throughout the cell among the enlarged fat droplets (Fig. 10 D). Glycogen is plentiful throughout the fat body; it occurs chiefly in the outer parts of the cell in deposits distorted by other cellular inclusions and sometimes in vacuoles with sharply defined boundaries. Fat droplets in the oenocytes (Fig. 10 J) and glycogen in the sarcoplasm and in the other tissues mentioned continue to increase. Glycogen may occur also in the cells of the Malpighian tubes.

From sections stained with haematoxylin and eosin there seems little doubt that the protein spheres in the fat body arise from the nucleus. They appear first as minute basophil droplets on the nuclear membrane and are often seemingly connected by radiating filaments with the nuclear plasmosome. As they enlarge and pass outwards into the cytoplasm they become eosinophil.

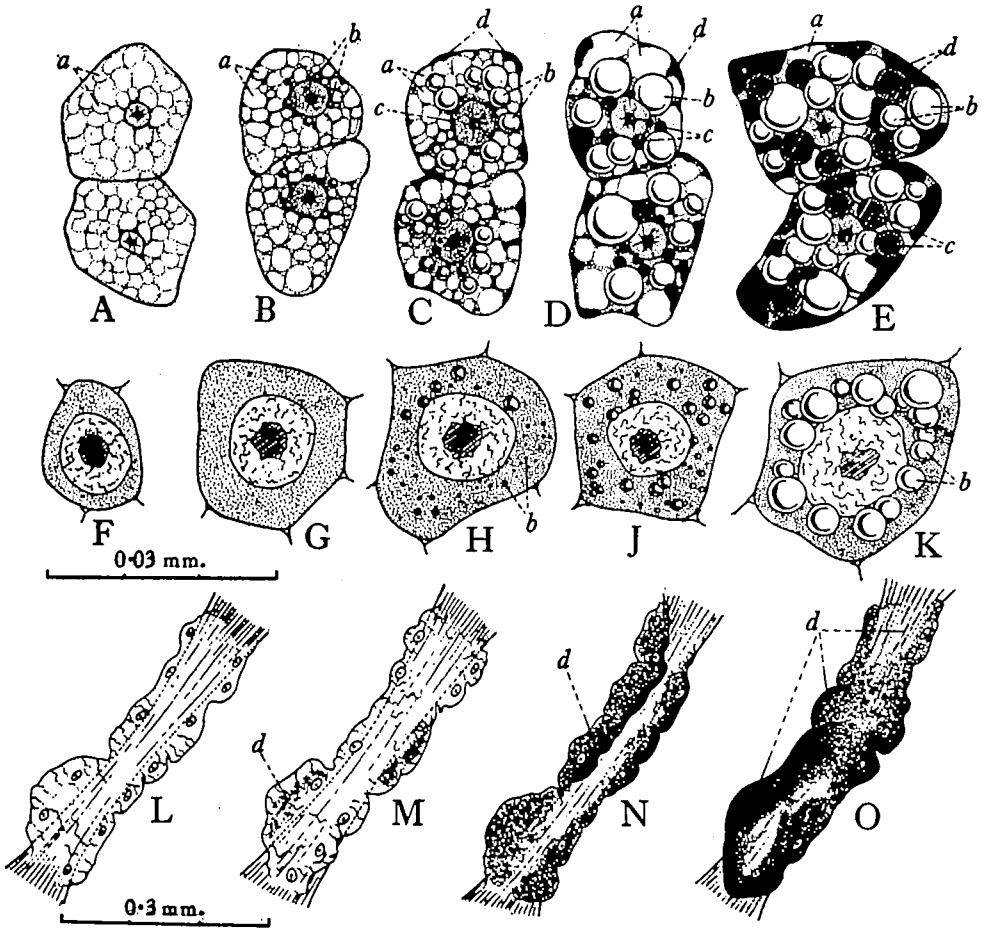

Fig. 10. A, fat body cells in fully starved larva; B, the same in larva after feeding on casein for 24 hr.; C, after 2 days; D, after 3 days; E, after 7 days; F, oenocyte in fully starved larva; G, after feeding on casein for 24 hr.; H, after 2 days; J, after 3 days; K, after 7 days; L, abdominal muscle in fully starved larva; M, after feeding on casein for 24 hr.; N, after 2 days; O, after 5 days. *a*, watery vacuoles; *b*, fat droplets; *c*, protein droplets; *d*, glycogen stained with iodine.

After 5 or 6 days the cells of the fat body are almost filled with refractile spherical inclusions (Fig. 10 E). In the living cell these cannot be differentiated into fat and protein and both sorts blacken with osmic acid. But when stained with fat stains and with ninhydrin there is a sharp division into protein and fat droplets. Between these are a few distorted aqueous vacuoles; and also interspersed among them, but

chiefly around the periphery of the cells are numerous deposits and ill-defined vacuoles of glycogen. It is probable that glycogen occurs also in the cytoplasmic framework of the cell. The oenocytes contain plenty of fat, embedded in the dense protein rich cytoplasm, but no glycogen (Fig. 10 K). The sarcoplasm is in many places practically solid with glycogen and there are elongated deposits between the muscle fibrils (Fig. 10 O). The midgut epithelium is much thickened and, particularly in the posterior half, very rich in protein (Fig. 11 C, C′). All parts of the gut wall may contain small amounts of glycogen, but the largest deposits occur at the hind end. There is some glycogen in the epidermal cells and in the cells of the oesophageal invagination. The pericardial cells have by now increased to nearly three times their diameter in the starved larva; they contain much protein but no fat or glycogen.

Larvae fed for several days with casein and then fixed in aqueous Bouin have the uric acid vacuoles dispersed among the various other inclusions (Fig. 7 C). The

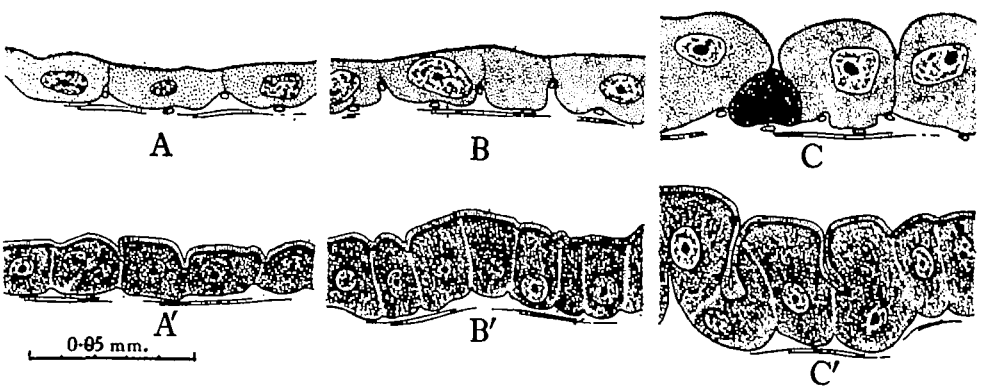

Fig. 11. Longitudinal sections of midgut of larvae; A, B, C, anterior segment; A′, B′, C′, posterior segment. A, A′, in fully starved larva; B, B′, after feeding on casein for 24 hr.; C, C′, after 3 days.

uric acid is probably reduced in quantity, but this has not been established with certainty.

Amino acids. Of the readily available amino acids cystine and tyrosin are so insoluble that they are probably not absorbed rapidly enough to supply the energy requirements of the larva. For when starved larvae are fed on these substances they die, apparently of starvation, in about 48 hr. Glycine, on the other hand, while freely soluble, has such a small molecular weight that solutions of adequate strength are hypertonic and cause shrinkage of the larva. Alanine in 1·5% solution with kaolin and glutaminic acid 2·5% are satisfactory foods. The latter is more than saturated so that there is plenty of solid amino acid present. Both mixtures are adjusted to *p*H 4·0, the alanine with phosphoric acid, the glutaminic acid with sodium bicarbonate, and they are made up fresh every 12 hr.

After feeding on alanine for 48 or 60 hr. there may be minute quantities of visible fat in the fat body of a few larvae but most show no fat. Glycogen on the other hand is plentiful. It is present within 24 hr. in some of the cells at the pyloric

end of the midgut and there are traces in the ganglia. By 60 hr. there is abundant glycogen in the pyloric half of the gut, it is present in some cells of the gastric caeca and occasionally in traces in the Malpighian tubes. It is plentiful also in the ganglia and connectives of the central nervous system, in the sarcoplasm of the muscles and throughout the fat body.

Glutaminic acid gives identical results, though perhaps rather more larvae develop visible fat in the fat body cells, and glycogen in the gut epithelium and in the fat body may not be so conspicuous.

Storage of uric acid in the fat body

Under the conditions in which larvae have been reared in these experiments, where they have developed without interruption in a rich mixture of foods, there is, as we have seen, very little uric acid in the fat body at the time of moulting to the fourth stage (Fig. 4 A, A′). After fasting for 24 hr. there is a great increase, and this continues throughout starvation (Figs. 4 B′, 7 A).

This accumulation of uric acid has been regarded as evidence that the consumption of protein for energy purposes takes place from the outset during starvation (p. 62). This belief is supported by the fact that the accumulation of uric acid is greatly reduced and sometimes seems to be entirely prevented if the larva is fed continuously from the time of moulting. And this is so whether the larvae are fed on the mixed infusion, on casein alone or on starch alone.

It is obvious from preparations such as that shown in Figs. 4 B′ and 7 A that the total amount of uric acid collecting within the fat body cell during starvation is greater than can have been produced from the protein originally present in the cell. Uric acid or precursors of uric acid must be taken up from the blood. When larvae are fed with solid uric acid during starvation, the quantity of uric acid in the fat body cells is not obviously greater than that accumulating during simple starvation; and if newly moulted larvae are fed on a mixture of casein and uric acid, the uric acid appearing in the fat body is much less than that in unfed larvae.

Although this evidence cannot be regarded as conclusive, it thus seems likely that the uric acid which collects in the vacuoles of the fat body is the product of deamination within the cell of amino acids or proteins brought from elsewhere.

During starvation, when the reserve substances are diminishing, conditions in the cells of the fat body are presumably ideal for the accumulation and persistence of uric acid within the vacuoles. When the starved larva is fed on starch or protein the quantity of uric acid is reduced (Fig. 7). Presumably the physico-chemical or mechanical state of the cell now favours the discharge of uric acid into the blood and its consequent elimination by the Malpighian tubes.

DISCUSSION

Storage in different tissues

The results described in this paper emphasize the important part played by other tissues besides the fat body in storing reserves. The muscles, the oenocytes, the gastric caeca and the posterior half of the midgut are particularly important as

reserves of protein. The masses of glycogen laid down in the sarcoplasm of the muscles are at least as great as those in the fat body; while smaller amounts are stored in the nervous system, epidermis, gut and Malpighian tubes.

De Boissezon (1932) has stated that in larvae of *Culex* glycogen is confined to the fat body; but the widespread distribution observed in *Aedes* agrees with that described by Bogojawlensky (1935) and Paillot (1938) in the silkworm. In the silkworm the amount of glycogen in the muscles is quite small; a larger amount occurs in the muscles in *Ephestia* (Zeller, 1938); but in no insect have massive accumulations, such as are seen in *Aedes*, been recorded.

In the oenocytes of *Aedes* glycogen is consistently absent; but it has been recorded as occasionally present in the oenocytes of *Bombyx* (Bogojawlensky, 1935); while it is plentiful in those of aquatic Hemiptera (Poisson, 1924), in *Polistes* and *Melasoma* (Pardi, 1939*b*) and in several other insects (Hollande, 1914*a*).

As in all insects, fat is stored in *Aedes* chiefly in the fat body. Some is laid down in the oenocytes—as has been noted also by Poisson (1924) in aquatic Hemiptera. And when the food contains much fat this may collect in large quantities in the epithelium of the gastric caeca and the cardiac half of the midgut. Hobson (1931) observed a storage of fat in both anterior and posterior segments of the midgut of *Lucilia* larvae.

Influence of the nature of the food on the reserves

When the larva is fed on casein alone, the reserves in the fat body and elsewhere do not differ from those formed in larvae feeding on the normal diet of micro-organisms: abundant protein, fat and glycogen are laid down. This confirms the evidence of other authors that protein in insects may serve as a source of both fat and glycogen. Thus Kulz (1881) showed that *Calliphora* larvae can form glycogen from egg albumen and Weinland (1907) supported this conclusion; while Pardi (1938 *a*, *b*) noted that at the end of larval life in *Polistes* and *Melasoma* glycogen first makes its appearance in the form of granules actually inside the protein globules. Weinland (1909) and Mishikata (1922) have shown that *Calliphora* larvae will lay down fat on a diet of fat-free fibrin or egg albumen.

Starch and other carbohydrates lead to very large accumulations of glycogen and, to a lesser extent, of fat. Fat, on the other hand, although it can obviously serve as a source of muscular energy, does not lead to the deposition of visible glycogen.

We have seen that the sugars differ much in the readiness with which they are transformed into glycogen. Comparative results of this kind have been obtained by Vogel (1931) on bees, Haslinger (1936) and Fraenkel (1940) on blowflies (*Calliphora*) and Hinman (1933) on newly hatched mosquito larvae (*Aedes*) by noting the time of survival of insects fed solely upon different sugars. The results for bees and flies have been brought together by Fraenkel. When compared with the present results on *Aedes* it may be noted that in all these insects glucose, fructose, sucrose and maltose are the most readily utilized sugars; in none is sorbose used; and for all of them the most available of the pentoses is xylose. Apart from this there are

striking differences which need not be reviewed here. *Aedes* larvae, like *Calliphora* adults show no sign of the 'toxic' effect exerted by mannose on bees, an effect which is ascribed by Staudenmayer (1939) to 'competitive inhibition' of enzyme action. Nor is there any sign of sorbitol having exceptional value as it seems to have for both bee and blowfly.

During starvation in *Aedes* protein, fat and glycogen are used up concurrently; uric acid begins to collect in the fat body at once and larvae starved until all their fat has been consumed may still have a little glycogen in their muscles. Other insects show differences in these respects. In the cockroach glycogen is used first, then fat and finally protein; only during this final stage do the uric acid concretions in the fat body increase (Philiptschenko, 1907). In *Dytiscus*, protein is used in large amounts from the outset (Pilewiczówna, 1926). Other examples are reviewed elsewhere (Wigglesworth, 1939).

Fat body inclusions

(i) *Fat droplets*. These are the most obvious of the inclusions. They usually appear first around the nucleus, at least after feeding on starch or protein. Quite often when the fat first becomes visible it is in the form of a slender crescent applied to a spherical non-fatty inclusion in this position. Large crescentic fat drops, which are sometimes seen, are certainly artefacts occurring in badly fixed material; some doubt therefore exists about the genuineness of the small crescents. They have not been detected in living larvae.

It may be noted that Popoff (1910) (quoted by Schreiner, 1915) has stated that in the fat body of muscids the fat droplets are derived from 'chromidia' discharged through the nuclear membrane and Schreiner (1915) holds the view that fat droplets in cells (in *Myxine*) are produced by mitochondria which are themselves produced by discharge from the nucleolus. The mitochondrial origin of fat droplets in *Culex* is supported by de Boissezon (1930).

(ii) *Protein droplets* certainly arise in *Aedes* in contact with the nuclear membrane. Berlese (1901) and Pérez (1910) have described this in many insects. They often appear in *Aedes* as though connected to the nucleolus by filaments. But their origin is a subject of much controversy. Schreiner (1916, 1918) describes filaments connecting granules in the cytoplasm (which he regards as precursors of the mitochondria) to the nucleolus; and Ludford (1925) has described the apparent discharge of nucleolar substance into the cytoplasm in living cells in tissue culture. De Boissezon (1930) and Hosselet (1931) in the fat body of *Culex* and Paillot & Noel (1926, 1928) in *Pieris* and *Bombyx* hold that the mitochondria produce the protein inclusions. Bishop (1922, 1923) describes the nuclear membrane in *Apis* as breaking down and the basophil nucleoli as being set free into the cytoplasm to give rise to the protein droplets. But no such rupture could be seen by Schnelle (1923) and Pardi (1935) has shown that the basophil cytoplasmic inclusions around the nucleus do not stain by the Feulgen method. In reviewing the subject Pardi (1939*a*) considers that it is not proved that the nucleus gives rise to either fat or protein inclusions.

The nature of the protein droplets has been equally disputed. Possibly this varies in different insects. Kreuscher (1922) describes the protein droplets in *Dytiscus* as replacing the fat droplets almost entirely before pupation; and Pérez (1920), Schnelle (1923) and Schmieder (1928) all take the view that the 'albuminoid granules' are formed largely by transference of material from the fat droplets. Zakolska (1928) refers to these granules in *Tenebrio* as 'albumino-fatty granules' because they give reactions for both fatty acids and proteins.[1] Bishop (1922, 1923) likewise believes that in *Apis* the fat contents of the cell are incorporated with the protein to form a common raw material for tissue use. His evidence is the blackening of the protein droplets with osmic acid. Pardi (1939*a*) finds some support for this belief in young larvae of *Melasoma* where the protein globules stain with Ciaccio's stain. But there is no sign of this in older larvae, nor in *Polistes*, *Pieris* or *Aporia*. Zeller (1938) describes the highly refractile protein inclusions in *Ephestia* as staining yellowish brown with osmic acid. In *Aedes* also, as we have seen, they darken in osmic acid, although they fail to stain with fat stains. Perhaps this merely indicates that a certain amount of lecithin is incorporated with the protein. There is certainly no obvious reduction in the fat droplets as the protein droplets enlarge.

(iii) *Glycogen* is much less sharply differentiated than the other inclusions. Sometimes it seems to be diffused in the cytoplasmic framework of the cell. But usually it is confined to vacuoles. These, however, appear to have a low interfacial tension and are so readily deformed by the fatty and protein droplets that their boundaries are not always easy to make out. As described by Bogojawlensky (1935) in the silkworm and by Zeller (1938) in *Ephestia* these irregular deposits occur chiefly at the periphery of the fat body lobes.

(iv) *Watery vacuoles* in the fat body have been described by Voinov (1927) in *Chironomus*. She likewise often observed a 'dense secretory granule' in Brownian movement in the vacuolar fluid. She states that neighbouring vacuoles may fuse until a single enormous vesicle fills the cell. Hosselet (1931) supposes that, in *Culex*, enzymes from the nucleus are concerned in the formation of these vacuoles which are described as later migrating to the periphery of the cell, fusing to larger vacuoles, becoming smoky in tint and finally 'approximating to urates' (cf. Hollande, 1914*b*).

In *Aedes*, as we have seen, uric acid collects in the vacuoles chiefly during starvation; and this was attributed to the endogenous production of uric acid from proteins in course of deamination. Hollande (1914*b*, 1925) came to the same conclusion when he found, as Philiptschenko (1907) had already found in the cockroach, that injection of urates into the blood of Orthoptera or larvae of *Vanessa* does not lead to any increase in uric acid in the urate cells of the fat body. In *Polistes* uric concretions become very numerous in the urate cells at the time when glycogenic activity in the fat body at the expense of the reserve proteins is at its maximum (Pardi, 1939*a*). Even if the vacuoles have a nuclear origin there is no reason to suppose that the uric acid collecting in them is derived solely from purines in the nucleus as de Boissezon (1930) suggests.

[1] Fatty acids were demonstrated with Nile blue and by Fischler's method, both of which are regarded by many writers as non-specific.

Importance of gut and Malpighian tubes in intermediary metabolism

Van Gehuchten (1890) regarded the clear cells of the anterior half of the midgut of *Ptychoptera* as absorbing, the granular cells of the posterior half as secreting. De Boissezon (1930) showed that absorption of iron saccharate is limited to the posterior half of the midgut in *Culex* larvae. If the deposition of absorbed substances can be taken as an index of the site of absorption, it would seem from the present experiments that, in *Aedes*, fat, sugar and amino acids are absorbed in the caeca, that the clear cells of the cardiac half of the midgut absorb fat, and the granular cells of the pyloric end sugar and amino acids, while all three may be absorbed by the Malpighian tubes if the gut contents happen to enter them.

But more important is the light these results throw on the part played by the gut in intermediary metabolism. It is claimed by Bogojawlensky (1935) that on feeding starved silkworm larvae, glycogen appears first in the fat body and later in the other organs beginning with the midgut epithelium. He concludes that sugars are absorbed from the gut and first synthesized to glycogen in the fat body. But in *Aedes* larvae it is quite certain that sugars of many sorts are converted into glycogen in the epithelium of the pyloric half of the midgut before any appears in the fat body, and its deposition in the fat body may synchronize with its appearance in the ganglia and muscles.

This same sequence is observed after feeding with alanine and glutaminic acid, which can evidently be deaminated and converted into glycogen in the midgut epithelium. This recalls that Brown & Farber (1936) and Brown (1938) produced evidence of a deaminase for peptones in the posterior part of the midgut of blowfly larvae and this enzyme has recently been shown by Dr H. Hurst[1] to act also on free amino acids. On the other hand, after feeding with casein, fat and glycogen are usually laid down in the fat body before they appear in the gut wall.

Glycogen formation both from the various sugars and from amino acids is possible also in the Malpighian tubes; though only in a few larvae apparently do the gut contents pass into them.

SUMMARY

In the newly moulted fourth stage larva of *Aedes aegypti* the cells of the fat body contain fat droplets and numerous watery vacuoles. Protein is present only in the form of cytoplasmic strands between these inclusions. Some of the watery vacuoles contain glycogen; in others there is uric acid in solution. Glycogen is present also in the ganglia and connectives of the central nervous system and in great masses in the sarcoplasm enveloping the muscles and between the muscle fibrils.

During starvation, fat, glycogen and protein are used up concurrently. In 10–15 days (at 28° C.) all the stainable fat has disappeared and glycogen is absent or present in minute traces in the sarcoplasm only. Nuclei and cytoplasm in all

[1] Unpublished work to which I am kindly permitted to refer.

tissues are much wasted. The utilization of protein is marked by a progressive accumulation of uric acid in the aqueous vacuoles of the fat body. In some of these uric acid may crystallize out during life. This accumulation of uric acid can be largely prevented by feeding on casein or on starch.

When starved larvae are fed on starch there is a massive deposition of glycogen in the epithelium of the *posterior half* of the midgut. A little is deposited in the cells of the gastric caeca and occasionally in the cells of the Malpighian tubes. Subsequently glycogen collects in the cells of the fat body until these are enormously distended with it and around the muscles until the sarcoplasm is practically solid with glycogen. Some appears also in the central nervous system. Rather small fat droplets collect round the nuclei of the fat body and in the oenocytes.

After feeding on sugars, glycogen and fat have the same distribution as above. Different sugars vary in their efficiency as precursors of glycogen; from some (raffinose, sorbose, rhamnose, arabinose) no glycogen seems to be formed.

After feeding on olive oil, droplets of fat are limited to the cells of the *anterior half* of the midgut and to some cells in the gastric caeca. Occasionally droplets appear in the cells of the Malpighian tubes. Much fat collects in the oenocytes and in the fat body, but no glycogen is laid down.

After feeding on casein there is a rapid increase in protein in the cytoplasm of all the tissues and the nuclei enlarge. Minute droplets of fat appear in the fat body, chiefly around the nuclei, and later pass outwards and enlarge. Fat accumulates also in the oenocytes. Glycogen is deposited in large amounts in the cytoplasm and in indefinite vacuoles chiefly at the periphery of the fat body cells. It is laid down in great quantity in the sarcoplasm of the muscles, to a less extent in the central nervous system and later in the epidermis, Malpighian tubes and gut wall. Minute droplets rich in protein appear on the nuclear membrane of the fat body cells; they have the appearance of being discharged from the nuclei; gradually they enlarge and pass outward among the fat droplets. These protein droplets blacken with osmic acid but they do not stain with fat stains. They are basophil when first formed, becoming acidophil as they enlarge.

After feeding on alanine or glutaminic acid glycogen is laid down, as after carbohydrates, in the caecal cells, the pyloric half of the midgut, the central nervous system, sarcoplasm and fat body. Small amounts of fat become visible in the fat body.

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