Chapter 2

HISTOCHEMICAL DEMONSTRATION OF SUCCINIC DEHYDROGENASE IN THE FAT BODY OF THE DESERT LOCUST AND SOME GRASSHOPPERS

The fat body of insects has been shown to contain various metabolites and enzymes and is often compared with the mammalian liver. A number of enzymes including transaminases and glutamic dehydrogenase system has been shown in Schistocerca fat body (Kilby and Neville, 1957). In the same tissue George and Eapen (1958) demonstrated lipase and alkline phosphatase activity while Hearfield and Kilby (1958) who studied a number of enzymes associated with the tricarboxylic acid cycle, were able to show a triphosphopyridine nucleotide linked isocitric dehydrogenase, aconitase and fumarase, and a diphosphopyridine nucleotide linked malic dehydrogenase and cytochrome oxidase. However, their efforts to demonstrate the presence of enzymes such as condensing enzyme, succinic dehydrogenase and α-ketoglutarate oxidase were unsuccessful, and led them to infer that the tricarboxylic acid cycle might not play an important part in the intermediary metabolism of the fat body of the locust.

Remillard (1958) has reviewed the literature on the oxidative enzymes of the vertebrate adipose tissue. More recently George and Eapen studied the succinic dehydrogenase and lactic dehydrogenase activity in the visceral adipose tissue of the pigeon (1958) and the yellow and

brown adipose tissue of the bat (1959 c). β- Hydroxybutyric dehydrogenase has been demonstrated in the isolated mitochondria of the fat body of Locusta migratoria (Hess et al., 1958). Young (1959) found that the tissue fractions of the fat body of Periplaneta americana oxidized a variety of substances like sugar phosphates, reduced diphosphopyridine nucleotide, isocitrate, succinate, ketoglutarate and hydroxybutyrate. Succinic dehydrogenase has been demonstrated within isolated mitochondria of the fat body of the cockroach Periplaneta americana (Pearse and Scarpelli, 1958) but could not be shown in the isolated mitochondria of Locusta migratoria (Pearse, 1959). Bellamy (1958) obtained a low oxygen uptake for homogenates of Schistocerca fat body when incubated with α- ketoglutarate and succinate and suggested that α- ketoglutarate oxidase and succinic dehydrogenase were labile on homogenization. An in vitro study made by Clements (1959) of the incorporation of certain metabolites labelled with C^{14} into the fat body of Schistocerca gregaria revealed the presence of active succinic dehydrogenase. He incubated whole fat body with succinic acid-1,4-C^{14} which yielded highly radio active CO_2.

The high content of lipid in the form of fat droplets in the fat cells, particularly obscure the detection and localization of succinic dehydrogenase by the use of tetrazolium salts. Invariably, the fat droplets are stained

by formazan before any visible colour appears in cell cytoplasm. This is considered to be the result of either the diffusion into lipids of enzymatically reduced tetrazolium, or of the reduction by the lipid itself. However, the presence of colour in lipids did not enable one to believe that dehydrogenase activity was present in them (Shelton and Schneider, 1952).

Since the introduction of ditetrazolium chloride as an indicator dye for the histochemical demonstration of succinic dehydrogenase activity by Seligman and Rutenburg (1951), various modifications of the technique using different tetrazoles have been tried. Shelton and Schneider (1952) tried four tetrazolium salts and found neotetrazolium to be most efficacious. The use of activating ions, Al^{+++}, Ca^{++} and HCO_3^-, and the execution of the reaction under anaerobic conditions (Padykula, 1952;Rutenburg et al.,1953), further enhanced the detection of succinic dehydrogenase activity. Rosa and Velardo (1954) suggested the use of sodium cyanide in the buffer solution in which freshly cut sections were stored as well as in the incubation medium, and raised the pH of both from 7.6 to 8.2.

Recently George and Talesara (1961), with certain modifications of the incubation medium, succeeded in demonstrating the localization of succinic dehydrogenase activity in the broad fibres of the pigeon breast muscle

which had defied earlier attempts to demonstrate the enzyme histochemically(George and Scaria, 1958). This modified incubation medium has been used in the present attempt to demonstrate succinic dehydrogenase activity in the fat body of insects.

MATERIALS AND METHODS

Fat bodies of the following insects were used for the study (insects identified according to Kirby(1914) and Lefroy (1909)).

Schistocerca gregaria (F)
Acrida exaltata (Wlk.)
Aeolopus affinis (Bol.)
Epacromia dorsalis (L.)

Specimens of Schistocerca gregaria were obtained from the locust research centre, Government of India Bikaner through the kindness of Dr. D.R.Bhatia, locust entomologist. The grasshoppers were collected locally.

The insects were decapitated and placed in ice cold 0.85% saline. Sheets of perivisceral fat body, which contains fewer oenocytes than the peripheral fat body (Coupland,1957), were dissected out carefully so as to exclude air sacs and most of the tracheal tissue. They were then immediately transferred to ice cold phosphate buffer at pH 7.6 containing 0.1% sodium cyanide (Rosa and Velardo, 1954).

The incubation medium used was the same as that used by George and Talesara (1961), which consisted of the following ingradients;

1. PO_4 buffer (0.1 M, pH 7.6).....2.5 ml
2. Sodium succinate (0.5 M, neutralized to pH 7.6) ..... 0.6 ml
3. $CaCl_2$ (0.004 M) ..... 0.5 ml
4. $AlCl_3$ (0.004 M) ..... 0.5 ml
5. $NaHCO_3$ (0.6 M, freshly prepared) ..... 0.3 ml
6. NaCN (0.03 M, neutralized to pH 7.6) ..... 0.5 ml
7. Neotetrazolium (3 mg/ml, freshly prepared) 1.0 ml
8. $MgSO_4$ (0.005 M) ..... 0.05 ml
9. Methylene blue (2 mg/ml) ..... 0.05 ml

In order to remove any trace of dissolved oxygen from the medium, it was boiled, cooled and kept in full, tightly covered glass cuvettes. Sheets of whole fat body from the phosphate buffer were then transferred to the incubation medium and incubated for two hours at 37°C. After incubation they were rinsed with phosphate buffer, fixed in neutral 10% formalin for 30 minutes, washed in distilled water and mounted in glycerine jelly.

In addition to sheets of whole fat body, sections of frozen fat body and thin sheets of fat body spread on

clean coverslips and air dried at room temperature were also used. Some of the material was subjected to simultaneous metal chelation (Pearse, 1957) along with neotetrazolium. In another set of experiments, sheets of whole fat body were treated seperately at 4°C with ethyl acetate, ether, 3:1 ether-acetone mixture, and acetone alone, for 20 to 40 minutes. Considerable amount of fat was found to be removed from the tissues treated with ethyl acetate, and with the other solvents almost complete removal of fat was achieved.

RESULTS

Neotetrazolium chloride , on reduction, is known to produce a deep purple diformazan and a red monoformazan. Within ten minutes of beginning of incubation the sheets of fat body turned red, the fat globules pink. Only after the continued incubation did deep purple diformazan granules appear in the cells of the fat body, the granules being distributed at the periphery of the cells and around the fat globules (Figs. 1, 2, 3). This was possible only with sheets of whole fat body. Sections cut from frozen material and air dried whole fat body, did not show this reaction.

Materials treated with cold ethyl acetate, ether, 3:1 ether-acetone mixture and acetone alone did not show any succinic dehydrogenase activity. Sheets of whole fat body subjected to simultaneous metal chelation with cobalt chloride showed a black deposition of the metal formazan

FIGS. 1 and 2 Fat body of *Schistocerca gregaria* and *Epacromia dorsalis* respectively, stained for succinic dehydrogenase, showing the distribution of formazan granules at the periphery of the fat cells

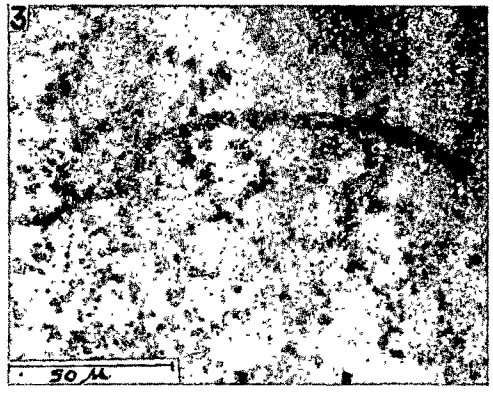

FIG. 3 Portion of Fig. 1 enlarged

OF SUCCINIC DEHYDROGENASE IN LOCUST AND SOME GRASSHOPPERS

D B. M. HEGDEKAR

nd Entomology, Depaıtment of Zoology,
ty, Baroda, Indıa

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The high content of lıpıd in the form of fat droplets in the fat cells, particularly obscures the detection and localization of succınic dehydrogenase by the use of tetrazolıum salts. Invariably, the fat droplets are stained by formazan before any visıble color appears in cell cytoplasm. This is consıdered to be the result of either the dıffusion into lipıds of enzymatically reduced tetrazolıum, or of the reduction of tetrazolium by the lipid itself However, the presence of color in lipıds did not enable one to belıeve that dehydrogenase activity was present ın them (26).

Since the ıntroduction of ditetrazolium chlorıde as an ındicator dye for the histochemical demonstration of succınic dehydrogenase activity by Selıgman and Rutenburg (24), various modıfications of the technique using different tetrazoles have been trıed Shelton and Schneider (26) tried four tetrazolium salts and found neotetrazolıum to be most efficacıous. The use of actıvating ions, Al^{+++}, Ca^{++}, and HCO_3^-, and execution of the reactıon under anaerobıc condıtıons (17, 23), further enhanced the detection of succinic dehydrogenase activıty. Rosa and Velardo (22) suggested the use of sodıum cyanide in the buffer solution ın whıch freshly cut sections were stored as well as ın the ıncubatıon medıum, and raısed the pH of both from 7.6 to 8.2.

FIG.1.

Photomicrograph of the fat body of Schistocerca gregaria, stained for succinic dehydrogenase, showing the distribution of formazan granules at the periphery of the fat cells

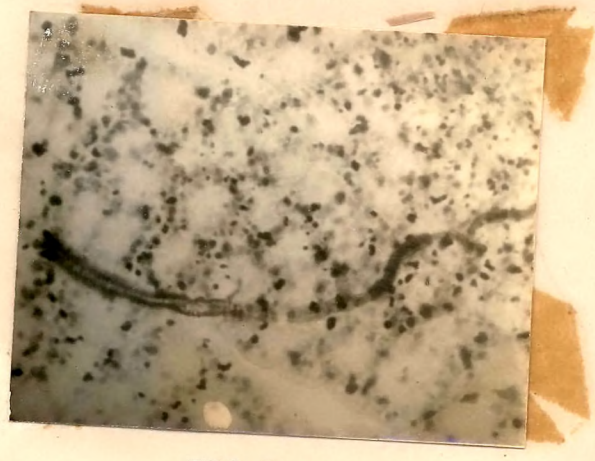

FIG.2.

Portion of Fig. 1 enlarged

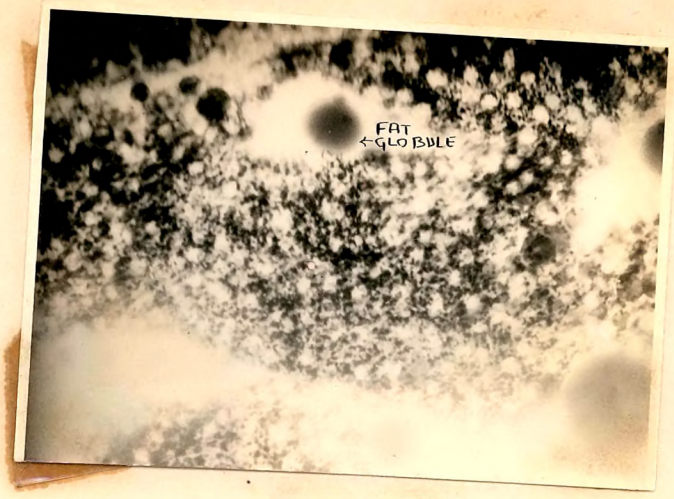

FIG. 3.

Photomicrograph of the fat body of Epacromia dorsalis, stained for succinic dehydrogenase, showing the distribution of formazan granules at the periphery of the fat cells.

in addition to the blue granules of diformazan. In this case the pink staining of the fat globules was scarcely visible.

DISCUSSION

The demonstration in the whole fat body of succinic dehydrogenase activity, one of the key enzymic reactions in the oxidative processes in animal tissues, clearly suggests the normal functioning of the tricarboxylic acid cycle in this tissue, contrary to the suggestion of Bellamy (1958). It is, therefore, reasonable to expect that the tricarboxylic acid cycle plays an important part in the intermediary metabolism of the insect fat body.

Bellamy (1958), who obtained a very low oxygen uptake on incubating homogenates of Schistocerca fat body with succinate, suggested that succinic dehydrogenase was labile on homogenization. Rat intestinal mucosa has been shown to contain a factor identified as fatty acid which inhibits succinoxidase activity on homogenization (Nakamura et al., 1959). Clements (1959) observed that homogenization of Schistocerca fat body reduced the production of $C^{14}O_2$ by about 95% on incubation with succinic acid-1,4-C^{14} and attributed this to the release of such an inhibitor on homogenization. However, he found that the fat body homogenate had no effect on the sheep heart muscle succinoxidase system. It is possible that the inhibition of succinic dehydrogenase activity in sections of frozen material might well be due to the release of such an inhibitor as result of cell injury during cutting and processing of the sections.

Oxaloacetic acid formed in the tissues during processing might, by virtue of its similar chemical structure act as an inhibitor of succinic dehydrogenase, but this difficulty was overcome in our studies by the introduction of sodium cyanide in the incubation medium as well as in the preincubation buffer (Rosa and Velardo, 1954). Sodium cyanide, in addition to acting as an effective blocking agent of cytochrome oxidase, by the formation of cyanhydrin helped to trap any oxaloacetic acid formed.

The fat solvents tried in the present investgations definitely acted as inhibitory agents of succinic dehydrogenase activity.

Seligman and coworkers (Rutenburg et al., 1953; Seligman and Rutenburg, 1951) have suggested that the areas of deposition of blue diformazan of blue tetrazolium represents sites of high activity and red or pink areas represent sites of low activity. This observation however, must be interpreted with caution as pointed out by Farber et al (1956). If, at a certain locus, the degree of enzyme activity alone determines the intensity of staining, namely, red or blue, at that particular region, regions which ultimately stain blue should stain first and should be expected to become red before turning blue. The pink colour in the fat globules of fat body appears long before any colour in the fat cells is discernible. These observations may be compared to those obtained by Shearer et al (1952) in frozen sections of cat spinal cord. They observed that the cytoplasm of nerve cells stained blue and the surrounding grey matter stained red, and yet, when sections were observed periodically during the staining process, it was seen that the grey matter began to stain red before the nerve cells had any staining whatsoever. They had no explanation to offer for these observations.

The distribution of much of the deep purple granules of diformazan at the periphery of the larger fat globules might well support the suggestion of Novikoff (1953), that we are dealing with an enzyme which is free to diffuse and be adsorbed to the fat, as xanthin oxidase does in milk.