The Structure and Metabolic Properties of Tissue Preparations from *Schistocerca gregaria* (Desert Locust)

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This work is part of a survey of the sedimentation characteristics and metabolic properties of mitochondria isolated from a wide variety of animal species. The desert locust (*Schistocerca gregaria*) was chosen as a representative of the class Insecta because of its large size, and also because many individuals could be obtained at one time from standard populations.

The only comparable work on the metabolic processes of isolated locust tissues is that of Rees (1954), who used homogenates and a particulate preparation from the combined thorax and appendages of *Locusta migratoria* (the Moroccan locust). These preparations oxidized tricarboxylic acid-cycle intermediates and carried out oxidative phosphorylation under suitable conditions.

In the present work three tissues were selected for study: the nervous tissue of the head, the muscle from the thorax and legs, and the fat body. The latter organ was used because it is in some respects similar to the liver of vertebrates (Wigglesworth, 1950; Kilby & Neville, 1957).

This paper records methods of preparing mitochondrial suspensions and gives a comparison of the rates of oxidation of α-oxoglutarate and succinate by different tissue preparations in a simple standard medium without the addition of cofactors. The concentrations of glutamic acid and acetylcholine were measured in particles from the head, and some observations were made on the choline acetylase activity of these particles. Some properties of isolated pigment granules from the integument have also been investigated.

MATERIALS AND METHODS

Care of insects. Immature adult male and female insects were obtained from the Anti-Locust Research Centre, London, about 10 days after the final moult, and were either used at once or fed on fresh grass and kept 1 or 2 days at 28°. Occasionally insects were obtained from the Department of Entomology, Oxford.

Preparation of homogenates. All dissections were carried out on cracked ice and subsequent operations were performed as close to 0° as possible.

Homogenizing media. These were 0·25M-sucrose, 0·25M-sucrose containing 2·5% of dried human serum as supplied for transfusion purposes and 0·15M-KCl. The media were adjusted to pH 7·6 with 0·1M-2-amino-2-hydroxymethylpropane-1:3-diol before use.

Thorax and leg homogenates. The head, appendages and abdomen were removed from the thorax of each insect with scissors. The thorax was cut open along the mid-dorsal line and the gut removed. For the preparation of the thorax and appendage homogenates, each tissue was cut up finely with scissors and homogenized with 4 vol. of ice-cold medium in a tightly fitting stainless-steel homogenizer of the Potter–Elvehjem type (working volume, 40 ml.). The pestle was moved up and down seven times, revolving at a speed of 2000 rev./min.

Muscle and integument homogenates. For the preparation of muscle homogenates and the suspensions of integument, the muscle was removed from the thorax integument as completely as possible with small scissors and forceps. The muscle was homogenized as above without further treatment, and the integument was treated in the same manner as the whole thorax. To increase the amounts of material, the abdominal integument was often combined with that from the thorax. For this the abdomen was cut open longitudinally along the mid-dorsal line, and about 3 mm. of the posterior end, containing the developing reproductive organs, was cut off. After removal of the underlying tissues, the cleaned integument was treated as the thorax integument above. For the preparation of pigment granules in 0·15M-KCl the integument was disintegrated in an ice-jacketed blendor (MSE Nelco-10 homogenizer) for 1 min. with 10 vol. of medium.

Head homogenates. After removal of the heads the mouthparts were cut off and the remainder was chopped up finely with scissors and treated as the thorax above.

Fat-body homogenates. The severed abdomen was cut open as described above and the yellow amorphous material comprising the fat body was removed from either side of the gut with a pair of forceps. The tissue was homogenized as above without further treatment.

Preparation of particles

In general, the procedure adopted for each preparation was as follows. The homogenate was diluted with 5 vol. of medium and the cell debris and nuclei were sedimented by two or three successive centrifugings of the homogenate at the same speed, the residue being discarded each time. The supernatant material was examined microscopically for contaminating tissue fragments in every preparation. After the sedimentation of the particles the pellet was washed three times with 10 vol. of fresh medium, the last centrifuging being done at 25 000 *g* for 10 min. A final

resuspension was carried out to give 100 mg. wet wt./ml. The homogeneity of the fractions and the structure of the particles were assessed by examination of sections through the sedimented pellets under the electron microscope. The sections showed the presence of an external limiting membrane and internal reticular structure (Witter, Watson & Cottone, 1955).

Thorax and integument. Cell debris and nuclei were sedimented at 650 *g* for 10 min. A dark-red supernatant fraction was obtained, consisting of particles from 1 to 3 μ in diameter. These were sedimented by centrifuging at 10 200 *g* for 10 min. Occasionally further subfractionation was carried out, intermediate centrifugal forces being used for the same length of time. The final, slightly turbid, yellow supernatant was discarded. The wet-weight yield was approx. 4% for the thorax and about 7% for the integument.

Muscle. After centrifuging at 650 *g* for 10 min. the supernatant fraction from the muscle homogenate was only slightly cloudy and contained particles from 1 to 3 μ in diameter. These could be sedimented when centrifuged at 10 200 *g* for 10 min. The final clear supernatant fluid was discarded. The wet-weight yield was approx. 1%.

Head. After centrifuging the homogenate at 600 *g* for 10 min. three distinct zones appeared. At the bottom of the tube there was a small pellet of cell debris, nuclei and chitin. Above this was a lightly packed dark-red particulate fraction and a light-brown turbid supernatant fluid. The supernatant was poured off together with the red particulate layer. A heavy-particle fraction was sedimented from this mixture by centrifuging it at 1000 *g* for 10 min. The supernatant fraction was decanted and a light-particle fraction was isolated from it by centrifuging at 10 200 *g* for 10 min. The wet-weight yield of the light-particle fraction was 3% and of the heavy-particle fraction 10%.

Fat body. The homogenate was centrifuged at 850 *g* for 10 min. and the greenish residue discarded. The particles were sedimented as a yellow pellet by centrifuging at 10 200 *g* for 10 min. A cloudy supernatant fraction from this centrifuging was discarded. The wet-weight yield was 4%.

In all preparations, except those from the head, difficulty was encountered in removing the large quantities of yellow fat associated with locust tissues. It was removed as completely as possible from the surface of the supernatant fractions with filter-paper strips before pouring off from the sedimented pellet.

Preparation of electron micrographs of particulate suspensions

Fixation and dehydration. The fixing medium contained 1% of osmic acid adjusted to the osmolarity of the isolation medium with the major osmotically active component of the latter. A small drop (approx. 0·02 ml.) of the particle suspension was added to 0·5 ml. of the fixing mixture in a glass centrifuge tube. When centrifuged at 650 *g* for 5 min., this was just sufficient to give a thin smear 1 mm. in diameter at the bottom of the tube. The drop of tissue suspension was stirred up with a nylon rod for 1 min. in the fixing medium before centrifuging. The supernatant fluid was discarded and the particles were dehydrated with successive samples (2 ml.) of 40, 60, 80 and 90% ethanol, azeotropic ethanol and absolute ethanol. The particles were stirred up for 5 min. with each fluid and then centrifuged for 5 min. at 650 *g*. The complete dehydration took approx. 65 min.

Embedding. All but approx. 1 ml. of absolute ethanol was removed from the centrifuge tube and 1 ml. of 50% *n*-butyl methacrylate in absolute ethanol was added. The pellet was resuspended and left for 30 min. After centrifuging at 650 *g* for 10 min., the supernatant fluid was discarded and the procedure was repeated with 1 ml. of butyl methacrylate soln. and then twice with 1 ml. of butyl methacrylate soln. with added catalyst (1%, w/v, benzoyl peroxide). Most of the supernatant fluid from the final operation was discarded. The particles were then resuspended in butyl methacrylate and catalyst and transferred to gelatin capsules. After mild centrifuging of the capsules to sediment the particles the resin was polymerized overnight at 45°.

The particles were sectioned and electron micrographs prepared by Dr G. Meek of the Department of Anatomy, University of Oxford, who also supervised the embedding procedure.

Analytical methods

Measurement of oxygen uptake. The incubation mixture contained 10 μmoles of substrate, 0·01 M-potassium phosphate buffer, pH 7·4, and 2 μmoles of $MgCl_2$. The tissue suspension (0·5 ml. containing about 10 mg. dry wt. of particles) was added to give a total fluid volume of 1 ml. The solute content was adjusted to 0·25 osmolar with sucrose solution. The centre well contained 0·05 ml. of 2 N-NaOH with filter paper. All additions were made with the vessels standing in cracked ice. Incubations were carried out in Warburg flasks of approx. 5·5 ml. total volume for 40 min. at 30° with air as the gas phase, and the rate of uptake of O_2 was calculated from the initial constant rate after an equilibration period of 10 min. The results are expressed as Q_{O_2} (μl. of O_2/mg. dry wt./hr.).

Determinations of dry weight. The dry weight of homogenates and particle suspensions was determined as 'acid-insoluble dry matter', according to the method of Werkheiser & Bartley (1957).

Measurement of optical-density changes in pigment granules. Granules containing insectorubin, a redox pigment (Goodwin & Srisukh, 1950; see also Butenandt, 1957), could be obtained from homogenates of integument by differential centrifuging. These particles changed from yellow to pink on reduction. Complete reduction of insectorubin by added substrate could not be demonstrated in an aerobic suspension of integument particles, because of the high rate of reoxidation of the pigment.

In preliminary studies of the reduction of insectorubin in the spectrophotometer, N_2 was bubbled through the suspension in a cuvette, but this resulted in frothing and sedimentation of the aggregated particles. To overcome this a 1 cm. glass cuvette was waxed on to the side of a cylindrical glass chamber (volume 23 ml.) with two gassing stoppers. By emptying the contents of the cuvette into the chamber and passing a stream of N_2 through, adequate anaerobiosis could be achieved within 1 min.

Incubations were carried out at 20° with 0·01 M-potassium phosphate buffer, pH 7·4, 15 μmoles of $MgCl_2$, 30 μmoles of substrate, 0·33 m-mole of KCl and 0·2 ml. of a particle suspension (100 mg. wet wt./ml.). The total volume was 3 ml. and the mixture had an optical density of approx. 1·5 at 440 mμ compared with water. The vessel was gassed

with N_2 and agitated constantly for 1 min. Substrate and other additions were made by adding them in a small volume, and regassing with N_2 each time. Optical-density measurements were carried out with a Beckman (model DU) spectrophotometer adjusted to be most sensitive to small changes in optical density according to the method of Holton (1955). The linear decrease in optical density of the particle suspension, as the insectorubin was reduced, was measured every 30 sec. for 5 min. at 440 mμ, the wavelength of maximum change. The rate of reoxidation was investigated by flushing the vessel rapidly with O_2 after measurements in N_2 had been completed, and following the subsequent increase in optical density at 440 mμ.

Extraction and estimation of insectorubin. The method of Goodwin & Srisukh (1950) was used. Insectorubin was extracted from the tissues at 0° with methanol containing 1% (v/v) of conc. HCl. All extracts were diluted to give an optical density of 0·25 at 495 mμ, the isosbestic point of the oxidized and reduced forms. The resultant solution contained 2·5 units of insectorubin/ml.

Extraction and estimation of acetylcholine. Acetylcholine was extracted from the homogenates and particle suspensions by a modification of the method of Lewis & Smallman (1956). After incubation of the tissue samples, the contents of the flasks (3 ml.) were transferred to 5 ml. glass centrifuge tubes, which were at once subjected to 10 000 *g* in a refrigerated centrifuge for 10 min. The clear-yellow supernatant liquid was poured off into test tubes and the residue was suspended in 3 ml. of water. All tubes were heated in a boiling-water bath for 90 sec., and, after cooling to room temperature, 0·3 ml. of 10% trichloroacetic acid was added to each sample. The tubes were allowed to remain at room temperature for 30 min., with occasional stirring, and were then centrifuged at 6000 *g* for 10 min. Reference dry weights of the tissue samples were determined on the tubes containing the residues from the initial centrifuging.

Ion-exchange resins were used in order to remove a substance present in the incubation mixture, which behaved as acetylcholine, but decreased the sensitivity of the assay system. The supernatant fluids containing acetylcholine were put on to a column (0·5 cm. × 6·0 cm.) of well-washed Dowex 50, Na^+ form, followed with 15 ml. of water. Acetylcholine was eluted from the column with 9 ml. of 0·15% NaCl soln., and the eluent was immediately freeze-dried. The samples obtained could be conveniently stored, and when required for assay the solid was dissolved in 2·5 ml. of mM-sodium acetate. Complete recovery of acetylcholine chloride added to the test solutions was achieved by eluting with twice the test volume of 0·15% NaCl soln. Acetylcholine was measured by the method of Chang & Gaddum (1933), with the controls suggested by Feldberg (1942–3).

Determination of glutamic acid and ammonia. The sum of glutamic acid and glutamine was determined by the manometric measurement of the CO_2 liberated by the glutamic decarboxylase of *Clostridium welchii* (Krebs, 1948). Glutamine was calculated from the ammonia liberated from the amide group of glutamine in the same sample by the glutaminase of *C. welchii*. Ammonia was determined colorimetrically, after microdiffusion (Conway, 1947), with Nessler's reagent and by reading the optical density in a spectrophotometer at 475 mμ.

Lactate. This was determined by the method of Barker & Summerson (1941).

RESULTS

Comparison of the total respiration of Schistocerca gregaria *with that of isolated tissues.* It was thought desirable to obtain values of the total respiration of the live insect and the endogenous respiration of its component tissues. These results are of use in evaluating the data obtained from respiration studies on cell-free homogenates and isolated particle suspensions. Results are recorded in Table 1. The respiration of the whole insect was measured in a Warburg vessel of about 60 ml. total volume where flying was impossible and movement restricted. Any movements during the experiment were noted (Table 1) and related to the oxygen uptake over 5-min. periods. Variability in the respiration was found only during movement and a comparatively steady rate was obtained when the insect was immobile. After the experiment the insect was dissected into its major component tissues, which were suspended, whole, in 0·25M-sucrose. Owing to the thinness of the tissues and the presence of tracheae it was thought unnecessary to slice or chop the tissues. A linear rate of oxygen uptake was measured over 50 min., for each tissue.

The sum of the respiration of the individual tissues approximately equals the respiration of the whole resting insect (Table 1). This suggests that the rates obtained from isolated tissues represent the resting rates in the intact animal. In a similar

Table 1. *Comparison of the total respiration of* Schistocerca gregaria *with that of isolated tissues*

The experiment was carried out on one insect. Respiration of the live insect was recorded every 5 min. during three 30 min. periods. The animal was then killed and the respiration of the component tissues, placed in 0·25M-sucrose, was followed for 1 hr. All measurements were carried out in conventional Warburg vessels at 30° with air as the gas phase.

	Wet wt. (mg.)	Total uptake of O_2 (μl.)	Q_{O_2} at 30°
Live insect			
Active (no wing movements)	1252	3800	15·8
Resting	—	880	3·6
Isolated tissues			
Head	126	100	2·9
Legs	236	102	1·9
Wings	109	42	1·2
Gut	184	96	4·6
Thoracic muscle	177	126	6·9
Integument	397	230	3·1
Cuticle	159	0	—
Fat body	23	44	7·8
Total uptake of O_2 by isolated tissues	—	740	—

study on the snail *Helix pomatia* Kergut & Laverack (1957) obtained somewhat different results. The resting respiration of the whole animal was 40–70% of the sum of the rates of the isolated tissues.

The greatest contribution to the total oxygen uptake of the isolated tissues was made by the integument. The highest rates of respiration were shown by the fat body and the thoracic muscle. However, the integument included the cell-free cuticle which, on a wet-weight basis, made up 30% of the integument and contained 52% of dry matter. The cuticle incubated alone had no oxygen uptake and, after making a correction for the dry weight of the cuticle, the Q_{O_2} of the integument can be recalculated as 5·2. On this basis the cells of the hypodermis are of the same order of activity as the fat body and thoracic muscle.

Effect of the homogenizing medium on the respiration of homogenates of tissues of Schistocerca gregaria. When the flight muscle and the integument of the thorax were homogenized separately in 0·25M-sucrose the endogenous respiration of each tissue was lower than that of the whole-thorax homogenate (62 and 27·5% respectively of that given by a mixed preparation). The fat-body homogenate showed a very low endogenous respiration and lower response to succinate than thorax muscle and integument (Table 2).

The addition of human serum to the homogenizing medium used above did not affect the endogenous respiration or the oxidation of α-oxoglutarate in the whole-thorax preparations. Homogenates made from the legs showed about one-sixth of the oxidative activity of the whole-thorax homogenate. The percentage dry weight of both the whole thorax and legs was the same.

Homogenates of both muscle and integument had a higher rate of succinate oxidation in 0·15M-KCl than in 0·25M-sucrose. The endogenous rate was not altered.

Effect of the medium on the respiration of particle suspensions from tissues of Schistocerca gregaria. When 0·25M-sucrose was used for the isolation and suspension of particles from the whole thorax or legs there was no endogenous respiration but α-oxoglutarate was oxidized. Particles from the fat body had an endogenous Q_{O_2} of 3, which was increased on adding α-oxoglutarate and succinate, reaching a maximum of 7. The Q_{O_2} of α-oxoglutarate oxidation by particles from the legs was twice that of α-oxoglutarate oxidation by particles from the thorax (Table 3). The addition of dried human serum at a concentration of 2·5% in the isolation medium (and therefore 1·25% in the incubation medium) caused the appearance of a high rate of respiration in particles from the whole thorax and legs. It was expected that lactate would be the oxidizible substrate added in greatest quantity to the medium in the serum. The amount of lactate was measured (Barker & Summerson, 1941) and was present in the incubation medium at a concentration of 0·6 mM. This quantity of lactate would not be expected to give rise to the increased respiration since the oxygen uptake when α-oxoglutarate and succinate were at a concentration of mM could not be measured under the experimental conditions.

Because of the above stimulating effect, isolation of particles in subsequent experiments was carried out including serum in the media. The oxidation of α-oxoglutarate by integument particles was 60% faster than the oxidation of this substrate by particles from the thorax muscle: and the oxidation of α-oxoglutarate by the integument particles was about twice as fast as succinate oxidation. The latter result contrasts with most findings on mitochondria from the majority of tissues, where the

Table 2. *Oxidation of α-oxoglutarate and succinate by tissue homogenates from* Schistocerca gregaria

Incubations were in Warburg manometers of approx. 5·5 ml. total volume. Contents: 10 μmoles of substrate, 0·01M-potassium phosphate buffer, pH 7·4, 2 μmoles of $MgCl_2$, 0·5 ml. of tissue suspension (10–20 mg. dry wt.), NaOH in the centre well. Total fluid volume, 1 ml. Flasks were incubated for 40 min. at 30° with air as gas phase, and Q_{O_2} values calculated from the initial constant rate after an equilibration period of 10 min.

Tissue	Homogenizing medium	Q_{O_2} No substrate	Q_{O_2} α-Oxoglutarate	Q_{O_2} Succinate
Whole thorax	0·25M-Sucrose + 2·5% dried serum	14·3	28·5	—
	0·25M-Sucrose	14·9	28·0	—
Legs	0·25M-Sucrose + 2·5% dried serum	2·4	4·9	—
Thoracic muscle	0·25M-Sucrose	9·2	—	14·0
	0·15M-KCl	8·9	—	27·0
Integument	0·25M-Sucrose	4·1	—	13·5
	0·15M-KCl	4·8	—	18·8
Fat body	0·25M-Sucrose	1·7	2·4	4·1

Table 3. *Oxidation of α-oxoglutarate and succinate by particle suspension from* Schistocerca gregaria

Standard incubations were as given in Table 2.

Particles isolated from	Homogenizing and isolating medium	Q_{O_2} No substrate	Q_{O_2} α-Oxoglutarate	Q_{O_2} Succinate
Whole thorax	0·25 M-Sucrose + 2·5% dried serum	6·2	20·5	—
	0·25 M-Sucrose	0	7·3	—
Legs	0·25 M-Sucrose + 2·5% dried serum	5·1	12·1	—
	0·25 M-Sucrose	0	13·4	—
Thorax muscle	0·25 M-Sucrose + 2·5% dried serum	4·0	7·1	8·9
Integument	0·25 M-Sucrose + 2·5% dried serum	3·8	11·4	6·5
	0·15 M-KCl	4·9	—	5·8 *6·5
Fat body	0·25 M-Sucrose	3·0	5·5	7·0

* Adenosine triphosphate (2 μmoles) was added.

ratio of succinate to α-oxoglutarate oxidation is usually greater than 1.

A high rate of endogenous respiration was found when the integument particles were isolated in 0·15 M-KCl. This was increased by only 16% on the addition of succinate, which compares with the doubling of succinate oxidation in the whole homogenate when 0·15 M-KCl was the suspending medium.

Comparison of the endogenous respiration of whole tissues with the endogenous respiration of tissue preparations. Comparing the results of Table 1 with those of Table 2 it can be seen that the Q_{O_2} of the whole-thorax homogenate in 0·25 M-sucrose was two to five times as high as that of the intact component tissues. The respiration of the thorax-muscle homogenate was 30% higher than that of the intact tissues. The Q_{O_2} values of homogenates of both legs and integument were only slightly higher than those of the original tissues, and the fat-body homogenate showed only one-quarter of the activity of the intact organ.

From Tables 1 and 3 it can be seen that the Q_{O_2} of particles isolated from the legs in 0·25 M-sucrose was twice that of the whole tissue from which they were derived. The Q_{O_2} of particles from the thorax muscle and fat body was about 50% of that of the whole tissues. In the integument, particles and whole tissue gave about the same Q_{O_2} value.

Pigment granules of the integument

On removal of the manometers from the bath after incubations of thorax and integument preparations, a pink zone developed at the bottom of the vessels containing tissue and substrate on standing. In vessels without added substrate the suspension remained a uniform yellow colour. There was no microscopic difference between the particles in either zone, and on shaking the vessels the suspension resumed its original colour. If left for prolonged periods the entire contents of the vessels turned red, and on shaking in air the yellow colour returned. This indicated the presence of a pigment that can undergo reversible oxidation and reduction. Extracted by the methods of Goodwin & Srisukh (1950), the coloured material, both in the reduced and oxidized forms, had the same absorption spectra and the same chromatographic behaviour as insectorubin, an insect pigment that changes colour from yellow to pink on reduction (see Goodwin & Srisukh, 1950).

The pigment was concentrated in granular bodies in the hypodermis of the intact integument. These particles have been described before by Chauvin (1941) and Nickerson (1956). They were seen microscopically in the teased-out integument and in particulate preparations from the homogenized tissue.

The particles of the integument consisted of spherical bodies, ranging in size from 0·5 to 3·5 μ in diameter, of which only the larger ones were noticeably coloured. On adding dithionite or ascorbic acid to a suspension under a cover slip these particles changed from yellow to red.

Isolation of granules containing insectorubin. Results of attempts made to isolate the pigment-containing particles by differential centrifuging are tabulated in Table 4. When the insects were reared at 28°, a granular fraction in which the content of insectorubin was about six times as high as in the whole homogenate sedimented between 650 and 1000 *g* for 10 min. Particles with a high pigment content were also present in the fraction which sedimented between 1000 and 10 200 *g* for 10 min., the sedimentation range of most animal mitochondria.

All particulate matter visible under the light microscope was removed by centrifuging at 25 000 *g* for 40 min. The material which sedimented between 10 200 and 25 000 *g* gave an intense-yellow fatty-looking pellet and consisted of particles with a wide size range, 3 μ to less than 1 μ in diameter. This fraction contained no insectorubin. The final supernatant fraction was usually pink coloured and contained soluble insectorubin.

Insectorubin was removed from the particulate fractions by washing at 0° in the medium used for isolation. For example, two-thirds of the insectorubin was lost from freshly prepared appendage- or thorax-granules by three washes in 0·25M-sucrose containing 2·5% of dried human serum. Because of this the particulate suspensions always contained the soluble pigment.

Redox behaviour of insectorubin in particle suspensions. Insectorubin in the particle suspensions from the integument prepared in 0·15M-KCl was reduced anaerobically by the several substrates tested (Table 5). The reduction by malate was stimulated twofold on adding diphosphopyridine nucleotide (DPN) and the rate of reduction by succinate was doubled on adding adenosine triphosphate (ATP). In the particles sedimenting between 650 and 1000 *g* for 10 min., succinate increased the rate of reduction of insectorubin only

Table 4. *Fractionation of insectorubin in particles from the integument, thorax and appendages of* Schistocerca gregaria

Particles were isolated in 0·25M-sucrose + 2·5% of dried human serum, or in 0·15M-KCl. Homogenate was fractionated at the gravitational forces shown, as described in Materials and Methods. One unit of insectorubin in 1 ml. of methanol containing 1% (v/v) of conc. HCl gave an optical density of 0·1 at 495 mμ.

	Insectorubin content [units/mg. dry wt. extracted with methanol containing 1% (v/v) of conc. HCl]					
	Preparation in 0·25M-sucrose + 2·5% of dried serum			Preparation in 0·15M-KCl		
				Integument from insects reared at 28°		Integument from insects reared at 35°
Expt. no.	Appendages 1	Thorax 1	Integument 2	3	4	5
Tissue preparation						
Homogenate	—	—	1·0	1·1	1·0	0·7
Sedimenting force (*g*, for 10 min.)						
0–650	—	—	—	—	0·7	0·5
650–1000	—	—	—	—	5·6	1·4
650–2200	—	—	4·1	—	—	—
650–10 200	—	—	—	3·0	—	—
1000–1400	2·7	2·3	—	—	—	—
1000–2200	—	—	—	—	2·4	—
1000–10 200	—	—	—	—	—	0·03
1400–10 200	1·0	1·1	—	—	—	—
2200–10 200	—	—	—	—	0·9	—

Table 5. *Reduction of insectorubin in isolated pigment granules from the integument of* Schistocerca gregaria

Incubation mixture: 30 μmoles of substrate, 0·01M-potassium phosphate buffer, pH 7·4, 15 μmoles of $MgCl_2$, 0·2 ml. of particle suspension (100 mg. wet wt./ml. in 0·15M-KCl) and 0·33 m-mole of KCl. Total vol. 3 ml.; optical density (*E*), approx. 1·5. Gas phase, nitrogen. Decrease in optical density was measured by the method described earlier. Particles used were sedimented for 10 min. between the gravitational forces shown.

	100 × (ΔE at 420 mμ/5 min./20 mg. wet wt.)				
		650–1000 *g*		1000–10 200 *g*	
Additions	650–10 200 *g*	Fresh	Aged	Fresh	Aged
Nil	—	2·4	2·5	3·0	3·2
DPN (2·7 μmoles)	1·2	—	—	—	—
ATP (10 μmoles)	1·3	—	—	—	—
Sodium malate (0·01M)	5·9	—	—	—	—
Sodium malate + 2·7 μmoles of DPN	11·5	—	—	—	—
Sodium succinate (0·01M)	5·7	2·4	5·6	6·3	6·5
Sodium succinate + 2·7 μmoles of DPN	7·0	—	—	—	—
Sodium succinate + 10 μmoles of ATP	9·9	—	—	—	—
α-Oxoglutarate	8·9	—	—	—	—

after the particle suspension had been left at 0° for about 16 hr. (aged particles). Although particles prepared in 0·15M-KCl had a high endogenous respiration compared with the increase on adding succinate the substrates responsible for this did not seem capable of reducing insectorubin. The rate of endogenous reduction was slow and soon came to an end. Reoxidation of insectorubin by oxygen was 10 times as fast as the rate of reduction by succinate and the oxidation reaction was completely inhibited by mM-cyanide, suggesting that a heavy metal is involved. This concentration of cyanide had no effect on the reduction of insectorubin. Ethylenediaminetetra-acetate (5 mM) completely inhibited the reduction of insectorubin in the particles and decreased the oxidation of α-oxoglutarate by 38%.

Effect of temperature on insectorubin production. It has been found (for example, Goodwin, 1950) that the insectorubin concentration (as measured by the amount extracted by the standard techniques) in the locust integument is decreased by raising the environmental temperature of the insects. Increasing the temperature from 28° to 35° caused a 30% fall in the insectorubin content of the homogenate (Table 4). Concomitant with this there was a drop in the concentration of pigment in each fraction of the integument homogenate. This amounted to 97% in the particles sedimenting between 1000 and 10 000 *g*.

The distribution of total pigment and dry material in the various tissue fractions is altered when the insects are maintained at different temperatures (Table 6). The decrease in insectorubin at higher temperatures is most striking in the fraction sedimenting between 1000 and 10 200 *g* in 10 min. Less than 0·01% of the total is found in this fraction at 35° compared with 13% at 28°. When the environmental temperature of the insect was increased from 28° to 35° there was a 98% decrease in the dry weight of this fraction of the integument homogenate. The production of insectorubin may thus be linked with the appearance of particles sedimenting at a high centrifugal force, and these particles provide an indicator of the environmental temperature of the insect.

Fine structure of isolated particles from the integument. Electron micrographs of isolated integument particles show granules with an internal membrane system. The isolated particles are spherical with an average diameter of 3 μ compared with 1 μ for the diameter of the short axis of the rod-like forms that are found in the intact integument. Often the membranes are confined to a small area on the edge of the particle membrane.

No particles other than mitochondria were found to be associated with the fraction of the homogenate having the highest pigment concentration. There was no obvious structural differences between particles sedimented at a lower centrifugal force and those sedimented at a higher one. The difference in size between fractions was not appreciable either, suggesting that the sedimentation difference was one of density only.

Particles isolated from the integument of insects reared at 35° were smaller than those from insects reared at 28°. The maximum diameter was 2 μ, but a large proportion of them were less than 1 μ. This difference in size did not alter the sedimentation pattern of the pigment and the fraction with the highest content still sedimented between 650 and 1000 *g*.

Occasionally, strongly osmiophilic granules were observed in all fractions. These had no organized structure and could not be correlated with the presence of a high concentration of insectorubin in any one fraction. They were few in number, probably less than 1 in 100 mitochondria, and the conclusion is that insectorubin is contained in mitochondria.

Isolation of particles derived from nervous tissue of head homogenates

To avoid the difficulties of dissection it was thought possible that a particle fraction derived from the cerebral ganglion could be isolated from a

Table 6. *Distribution of insectorubin and dry matter in homogenates of locust integument*

The integument was homogenized in 0·15M-KCl and centrifuged successively for 10 min. at the gravitational forces given below. One unit of insectorubin in 1 ml. of methanol containing 1% (v/v) of conc. HCl gave an optical density of 0·1 at 495 mμ. Results are expressed in terms of the percentage of total insectorubin and dry material recovered from the homogenate.

	Insectorubin content (% of total recovered)		Dry wt. (% of total recovered)	
	Insects reared at 28°	Insects reared at 35°	Insects reared at 28°	Insects reared at 35°
Expt. no.	4	5	4	5
Sedimenting force (*g*)				
0–650	53·2	46·4	42·0	70·3
650–1000	33·4	53·6	10·7	29·0
1000–2200	6·0	<0·01%	10·0	0·60
2200–10 200	7·3		37·2	

head homogenate by differential centrifuging. In order to identify the particles, acetylcholine content and choline acetylase activity were taken as characteristics of the mitochondrial fraction from nerve cells (Hebb & Smallman, 1956; Whittaker, 1958; D. Bellamy, unpublished work).

Acetylcholine and choline acetylase in the head tissues. No inhibitor of choline esterase was added during the homogenization and fractionation procedures. In spite of this, the head homogenate contained 60 μg. of acetylcholine/g. dry wt. (Table 7). Two particle fractions were isolated from the homogenate. The first sedimented between 600 and 1000 *g* in 10 min. and the second between 1000 10 200 *g* in 10 min.; they were called the 'heavy'- and 'light'-particle fractions respectively. Only the 'light'-particle fraction contained bound acetylcholine (130 μg./g. dry wt.) and about 56% of the bound acetylcholine in the homogenate was recovered in this fraction. Choline acetylase has been found to be localized in the mitochondrial fraction of rabbit brain (Hebb & Smallman, 1956) and in the mitochondrial fraction of rat brain (D. Bellamy, unpublished work). In head homogenates a considerable synthesis of acetylcholine occurred with no added choline (Table 7). This resulted in a 2·5-fold increase of the ester in 1 hr. at 38°.

After fractionation the 'light'-particle fraction was the only fraction in which choline acetylase activity could be detected. No synthesis of acetylcholine occurred unless choline was added. After the incubation without choline about 50% of the acetylcholine that was originally particle-bound appeared in the surrounding medium in a soluble form. Adding choline increased the concentration of acetylcholine in the particle suspension by 50%. Most of the ester was found to be soluble but the particle-bound acetylcholine was higher than in the control incubation although this never reached the original level.

Non-peptide glutamic acid and glutamine in locust tissues

All the locust tissues examined contained glutamic acid and glutamine (Table 8). The head contained the highest level of glutamic acid (15·8 μ-moles/g. wet wt.) with a ratio of glutamine to glutamic acid of 0·1. The head differed in this respect from other tissues, which contained less glutamic acid and glutamine, with higher ratios of glutamine to glutamic acid, ranging from 2·2 in the fat body to 11 in the thoracic muscle. On fractionation of the head homogenate over 60% of the glutamic acid and glutamine was found in the supernatant fraction (Table 9). This was not due to the inclusion of blood, which contained only about 1 μmole of glutamic acid/g. and 9 μmoles of glutamine/g. (B. A. Kilby, personal communication).

Table 7. *Effect of choline chloride on acetylcholine synthesis by head tissues of* Schistocerca gregaria

Incubations were carried out with 0·017 M-potassium phosphate buffer, pH 7·4, 40 μmoles of $MgCl_2$, 10 μmoles of ATP, 100 μM-sodium acetate, 150 μM-sodium citrate and 0·1 mg. of eserine sulphate. The tissue suspension (2 ml. containing 100 mg. wet wt./ml.) was added to give a final fluid volume of 3 ml. and the mixture incubated at 38° in air for 1 hr. in a Dubnoff shaking incubator. The acetylcholine content of the homogenate was 60 μg./g. dry wt., and of the 'light'-particle fraction 130 μg./g. dry wt. After incubation the mixtures were centrifuged at 10 000 *g* for 10 min. The acetylcholine contents of the supernatant fluid (supernatant) and the sedimented tissue (residue and particles) were determined separately.

	Acetylcholine (μg./g. dry wt. of sedimentable tissue)			
	Homogenate		'Light'-particle fraction	
	Residue	Supernatant	Particles	Supernatant
Without choline	63·5	95·5	47·3	89·4
With choline (6·16 mg.)	81·9	109	89·0	156
Increase in acetylcholine (%)	34	14	88	74

Table 8. *Content of glutamic acid and glutamine in tissues from* Schistocerca gregaria

	Content in tissue (μmoles/g. wet wt.)			
	Thoracic muscle	Abdominal integument	Fat body	Head
Glutamic acid + glutamine	9·6	5·3	4·8	17·3
Free ammonia + amide nitrogen	10·2	5·0	4·6	1·5
Free ammonia	1·4	1·8	1·3	0
Glutamic acid	0·8	1·8	1·5	15·8
Glutamine	8·8	3·5	3·3	1·5
$\frac{\text{Glutamine}}{\text{Glutamic acid}}$	11	1·9	2·2	0·1

Table 9. *Glutamic acid and glutamine distribution in head tissue from* Schistocerca gregaria

Head tissue (8 g. wet wt.) was homogenized in 0·25 M-sucrose (80 ml.) and fractionated at the gravitational forces shown.

Tissue fraction	Gravitational force (*g*, 10 min.)	Total glutamic acid and glutamine in fraction (μmoles)	Total glutamine in each fraction (μmoles)	Glutamic acid (μmoles/g. dry wt.)	Glutamine (μmoles/g. dry wt.)
Homogenate	—	138	12·3	51·3	5·0
Cell debris, nuclei and chitin	0–650	8·4	0·6	11·7	<0·1
'Heavy' particles	650–1000	49·4	7·4	143	21·4
'Light' particles	1000–10 200	2·6	1·0	30·8	19·2
Supernatant	—	80·1	2·3	—	—
Recovery in all fractions	—	140	11·3	—	—

The 'heavy'-particle fraction contained 36% of the glutamic acid and glutamine in the homogenate and the content (165 μmoles/g. dry wt.) was about three times that in the whole homogenate. This fraction had an intense-red colour and contained a pigment with the spectral characteristics of insectorubin and thus it was probably derived from the integument. Glutamine was found in both the 'heavy'- and 'light'-particle fractions at a concentration about four times that in the whole homogenate. The ratio of glutamine to glutamic acid in these fractions was 0·15 and 0·63 respectively.

DISCUSSION

Evaluation of the results on mixed-tissue preparations. When working with insects the necessary dissection of different tissues for individual investigation is time-consuming and tedious, and there has been a tendency to use mixed-tissue preparations (Sacktor, 1953; Chefurka, 1953; Rees, 1954) or even whole insects (Gonda, Traub & Avi-Dor, 1957). When an insect or part of an insect contains more than one highly active tissue, as in the desert locust, this approach could be misleading. The Q_{O_2} of tissue preparations from the separated thorax muscle and thorax integument was lower than those prepared from the whole thorax. The integument made up 50% of the whole thorax and from the Q_{O_2} values it was calculated that the hypodermis was the most active tissue present. The oxidative activity of particles derived from the integument was higher than particles from the muscle and when each tissue was treated and centrifuged in the same way 7% of the wet weight of the integument was recovered from the homogenate in a particulate form but only 1% from the muscle. Thus unless the activities and proportions of the different tissues comprising the insect are known it is impossible to obtain a clear picture of metabolic activities from mixed homogenates.

Insectorubin as a respiratory pigment. Insectorubin (for review of similar pigments see Butenandt, 1957) in the particles from the integument was reduced anaerobically by many substrates and reoxidized rapidly on admitting oxygen. Assuming a maximum extinction coefficient of 1000 for the particle-bound pigment it has been calculated that the rates of insectorubin reduction by α-oxoglutarate and succinate are of the same order as the rates of oxidation of these substrates by particle suspensions. Ethylenediaminetetra-acetate (5mM) inhibited both the reduction of insectorubin and the oxidation of α-oxoglutarate in the particles. These results suggest that a substantial oxidative pathway could operate through insectorubin *in vitro*. The involvement of insectorubin in the oxidation of substrate could be an artifact arising from the methods of preparation. Insectorubin does not seem to be indispensable to the locust, because an albino strain is known (Hunter-Jones, 1957) in which the insectorubin of the integument is decreased by 85% (D. Bellamy, unpublished work) but the insects appear normal in most other respects.

Comparison of particles isolated from the locust with mitochondria from rat tissues. This work is only part of an as yet incomplete survey of particles isolated from animal tissues, but already certain similarities have been shown to exist between particles from locust tissues and mitochondria from, for example, rat liver and brain. Rees (1954) has already established the similarity of particles from the thorax of the Moroccan locust to rat-liver and brain mitochondria in terms of oxidation of citric acid-cycle intermediates and associated phosphorylation of adenosine diphosphate. In the present work α-oxoglutarate and succinate were oxidized by particles from all the tissues studied, although certain quantitative differences from rat tissues were apparent. The higher rate of α-oxoglutarate oxidation by the integument particles compared with succinate has already been emphasized. The endogenous Q_{O_2} values of particles from the thorax tissues were higher than those found for mitochondria from rat liver and brain, although the maximum Q_{O_2} values obtained on

adding substrate were lower than the maximum Q_{O_2} values of the latter particles under the same conditions. In the fat body there are indications of a lability of these oxidative enzymes. There was a decreased rate of endogenous respiration on homogenizing this tissue and the maximum Q_{O_2} on adding succinate was lower than the endogenous Q_{O_2} of the intact tissue. Hearfield & Kilby (1958), using homogenates of fat body, could not detect succinate or α-oxoglutarate oxidation and this is in agreement with the above interpretation.

The binding of glutamic acid, glutamine, acetylcholine and insectorubin in mitochondria-like particles from the locust has similarities to the binding of citrate (Schneider, Striebich & Hogeboom, 1956), acetylcholine (Whittaker, 1958), adrenaline and noradrenaline (Blaschko, Hagen & Welch, 1955) and glutamic acid and glutamine (D. Bellamy, unpublished work) in particulate fractions isolated from mammalian tissues.

SUMMARY

1. Mitochondria were prepared by differential centrifuging from tissues of *Schistocerca gregaria* (desert locust). The preparations, which were essentially free from nuclei and cell debris, were examined under the electron microscope.

2. The Q_{O_2} values of α-oxoglutarate and succinate oxidation by homogenates and particle suspensions from whole thorax, legs, integument, thoracic muscle and fat body were compared with the respiration of the live insect and the intact tissues. The Q_{O_2} values obtained at 30° for the whole tissues ranged from about 1 for the wings to about 7 for the fat body and thoracic muscle. Values for the homogenates were on the whole higher than those of the intact tissue and the increased respiration on the addition of substrate was four to six times as high as in the intact tissue.

3. Mitochondria-like particles containing the pigment insectorubin, which is partially responsible for the external colour of the locust, were isolated from the integument and characterized.

4. Acetylcholine (about 60 μg./g. dry wt.) was found in 0·25 M-sucrose homogenates of head tissue. After centrifuging, acetylcholine (130 μg./g. dry wt.) was found in the particulate fraction which sedimented between 1000 and 10 200 *g* for 10 min. A choline acetylase system was demonstrated in head homogenates and in the particles described above.

5. Values for the sum of glutamic acid and glutamine (μmoles/g. wet wt.) in tissues from *Schistocerca gregaria* are: fat body 4·8, integument 5·3, thoracic muscle 9·6 and head 17·8. The proportion of glutamine to total glutamic acid and glutamine ranged from 0·9 in the thoracic muscle to 0·1 in the head. On fractionation the particulate fraction of the head homogenate (containing 55 μmoles of glutamic acid and glutamine/g. dry wt.) sedimenting between 650 and 1000 *g* in 10 min. contained 165 μmoles of glutamic acid and glutamine/g. dry wt., and that sedimenting between 650 and 10 200 *g* in 10 min. contained 50 μmoles of glutamic acid and glutamine/g. dry wt.

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