used in the present method, leaves this point uncertain.

The agreement between the titration curve estimation by the new criteria (Table 2, line 3) and the chromatographic estimation (Table 2, line 2) for the alkali-processed gelatin is so close as to be within the experimental error of the former. It is possible that there is a real discrepancy in the values for lysine, but this point is being further investigated. The correction of the imidazole figure by a reduction of approximately 50% automatically adjusts the arginine figure by the same absolute, but proportionately smaller, amount, and brings this latter value into good agreement.

SUMMARY

1. A rapid method of determining the titration curve of gelatins is described and the accuracy indicated.

2. Revised criteria for the interpretation of the titration curve are presented.

3. The anomalous values for the histidine content of gelatin by titration curve analysis are brought into agreement with those from other methods.

4. The whole of the analytical data obtainable by use of the titration curve and isoelectric point are shown to be in agreement with those from chromatographic analysis.

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Oxidative Phosphorylation in Insect Sarcosomes

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(Received 13 April 1954)

The unique structure of the indirect flight muscles of certain insects belonging to the natural orders Hymenoptera and Diptera has been known for many years. Aubert (1853) was the first to describe the unusually large and abundant granules, later named sarcosomes by Retzius (1890), which are a characteristic feature of these muscles.

Keilin (1925) stated that 'among all the organisms examined the highest concentration of cytochrome is found in the thoracic muscles of flying insects'. On the other hand, little cytochrome was found in the corresponding muscles of wingless insects. Keilin suggested that the high concentration in the thoracic muscle of flying insects had a connexion with their unusually high activity. The wing muscle of the housefly, for example, is capable of producing more than 300 separate contractions per second (Marey, 1874).

Recently, Williams and his colleagues have shown that the cytochrome in the thoracic muscles of *Drosophila funebris* and the blowfly *Phormia*

regina is contained in the sarcosomes (Watanabe & Williams, 1951, 1953; Levenbook, 1953). In this respect and in their chemical composition and enzymic content, the insect sarcosomes resemble mitochondria from vertebrate tissues. Nevertheless, until 1953, oxidative phosphorylation, which is a characteristic property of mitochondria and sarcosomes of vertebrate tissues, had not been demonstrated in insect sarcosomes. Sacktor (1953) reported that although sarcosomes isolated from the housefly *Musca domestica* L. oxidized α -ketoglutarate (α -oxoglutarate) rapidly, he was unable to show oxidative phosphorylation. He found that this was not due to the adenosinetriphosphatase (ATPase) activity of the sarcosomes, since the added hexokinase and glucose were able to compete successfully with the ATPase for added ATP. As already reported briefly (Lewis & Slater, 1953), we were able to demonstrate oxidative phosphorylation in sarcosomes isolated from the blowfly *Calliphora erythrocephala*. At about the same time, Watanabe & Williams (1953) quoted a personal communication from Sacktor stating that he had demonstrated

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oxidative phosphorylation, 'but only in the presence of proteinaceous media'.

The present paper describes our previously reported experiments in greater detail, and also includes additional experiments suggested by the work of Watanabe & Williams (1953). Since the completion of our experiments, Sacktor (1954) has published an account of his work referred to by Watanabe & Williams (1953). A comparison of his findings with ours will also be given in this paper.

METHODS

Isolation of sarcosomes

The flies (*C. erythrocephala*) were bred at 22° according to the technique described by Tate (1948). On emergence, the adults to be used in the experiments were maintained on a diet of glucose and water, and batches of mixed sexes were removed at intervals.

The method of isolating the sarcosomes was similar to that described by Watanabe & Williams (1951). The flies were immobilized by cooling at 0°, and placed on a cold glass plate. The head and abdomen were removed and the thoraces placed in an ice-cold mortar. When the required number of thoraces had been obtained they were crushed gently with a pestle (without sand) in about 1 ml. of suspension medium for each 20 thoraces. The suspension was then filtered through six layers of muslin, previously saturated with suspension medium, into a centrifuge tube immersed in an ice bath. The muscle pulp was washed with several portions of cold suspension medium using a total of 1 ml. for each thorax. The suspension was centrifuged at 0° for 4 min. at 200 g and the supernatant again centrifuged for 8 min. at 1650 g. The sedimented sarcosomes were resuspended in isolation medium to make a total volume of 0.8 ml. for each 20 thoraces used.

Several different isolation media were used in this study. The pH of all was 7.4, except the medium of pH 7.0 described by Watanabe & Williams (1953). Because of the high dilution of the sarcosomal suspension before centrifuging, the sedimented sarcosomes contained comparatively little sarcoplasm and further washing was not considered necessary. In trial experiments, the effect of washing the sarcosomes on the phosphorylation ratio was variable, decreasing it in one experiment, increasing it in another and having no effect in a third.

Measurement of activity of α -ketoglutaric oxidase system and of accompanying phosphorylation

The activity of the sarcosomes was measured by the procedure previously described (Slater, 1953; Slater & Holton, 1953). Except where stated otherwise, the reaction mixture used was that found optimum for heart-muscle sarcosomes and differed only slightly from that described as 'standard conditions' by Slater & Holton (1954). Usually 1–1.5 mg. sarcosomal protein and 3–4 μ moles α -ketoglutarate were used. At least 100 units of yeast hexokinase of specific activity (Berger, Slein, Colowick & Cori, 1946) 1550 were added for each mg. of sarcosomal protein. The reaction period was either 35 or 45 min. at 25°. Even though the reaction mixture contained 0.01M malonate to inhibit succinic dehydrogenase, the O_2 uptake (μ atoms) greatly exceeded the α -ketoglutarate (α -Kg) disappearance (μ moles).

The ratio ($\Delta O : \Delta \alpha$ -Kg) varied between 1.3 and 4.1, and was usually above 2. Although some of the excess O_2 uptake may be due to traces of residual substrates in the sarcosomal preparation or in the hexokinase preparation (the ratio of the rate of O_2 uptake in the absence of α -ketoglutarate to that in its presence was between 0.04 and 0.32, with a mean value 0.21, in five experiments), it is probably mainly due to further oxidation of the succinate formed from the α -ketoglutarate.

The activity of the α -ketoglutaric oxidase system is expressed as Q_{O_2} , but refers only to that fraction of the observed O_2 uptake concerned in the oxidation of α -ketoglutarate to succinate. It was calculated from the expression

$$Q_{O_2} = \frac{11.2 \times 60 \times \Delta \alpha\text{-Kg}}{wt} \mu\text{l. } O_2/\text{hr./mg., where } w \text{ (mg.) is the}$$

amount of sarcosomal protein and t (min.) the time of the experiment. The Q_{O_2} was found to vary with the α -ketoglutarate concentration (see p. 213). A correction curve was constructed to convert the observed Q_{O_2} obtained with between 0.0025 and 0.006M α -ketoglutarate into a standard concentration (0.005M). The correction factor varied between 1.16 with 0.0025M α -ketoglutarate and 0.94 with 0.006M α -ketoglutarate. The corrected activity is referred to as Q_{O_2} (corr.).

The amount of phosphate esterified (Δ esterified P) = Δ HMP (hexose monophosphate) + Δ ~P (reactive energy-rich phosphate groups of adenosine diphosphate (ADP) and adenosine triphosphate (ATP)). The myokinase activity, as measured by $-\Delta$ ~P, was about the same as in heart sarcosomes (Slater, 1953). The yield of phosphorylation is expressed either as P:O (atoms of phosphorus esterified per atom of oxygen consumed) or as P: α -Kg (atoms of phosphorus esterified per molecule of α -ketoglutarate disappearing). The true phosphorylation ratio for the oxidation of α -ketoglutarate to succinate will lie between these two values. When $\Delta O : \Delta \alpha$ -Kg is less than 2, P: α -Kg will be closer to the true value, since only slight phosphorylation accompanied the oxidation of succinate. The oxidation of endogenous substrate was also accompanied by only slight phosphorylation; the P:O ratio varied between 0.10 and 0.36, with a mean of 0.20, in four experiments. When the $\Delta O : \Delta \alpha$ -Kg ratio is much greater than 2, the P: α -Kg ratio becomes of little value.

RESULTS

Effect of age of blowfly

The phosphorylation ratios varied considerably from batch to batch of flies. Studies of individual batches at different ages (Table 1) showed that the

Table 1. *Effect of age of fly on phosphorylation ratios*

Isolation media: 0.36M sucrose containing 0.005M phosphate for batch A; 0.32M sucrose containing 0.01M ethylenediaminetetraacetic acid for batch B.

Batch	Age (days)	Q_{O_2} (corr.)	P:O	P: α -Kg
A	1-7	18.6	0.57	1.29
	6-12	12.6	0.72	1.56
	9-15	12.8	0.94	2.95
B	1-3	11.5	0.88	1.48
	8-10	9.4	1.79	4.81
	15-17	12.0	1.72	3.28

ratios were distinctly lower with young flies (less than 1 week from emergence). Watanabe & Williams (1953) have shown that the average diameter of the sarcosomes of *Drosophila funebris* and of the blowfly *Phormia regina* increases rapidly during the first week of life and then remains constant.

Effects of varying method of isolating sarcosomes

Time of standing of thoraces. In studies of oxidative phosphorylation, it is usually considered desirable to remove the tissue from the animal as soon as possible after death. In the case of blowflies, where 20–70 individuals are used in any one sarcosomal preparation, this is not convenient. In any case, the situation is not quite comparable, since the wing movements of the thorax are still obvious some time after dissection. Nevertheless, it was considered desirable to test whether the time of standing of the isolated thoraces, at or just below room temperature, had any effect on the phosphorylation ratios. Such an experiment is summarized in Table 2, which shows that prolonged standing (35 min.) caused definitely reduced ratios. In all the other experiments described in this paper, the time of standing of the thoraces did not exceed 5 min.

Degree of grinding of thoraces. A very mild crushing of the muscle with a pestle is sufficient to liberate the sarcosomes from the fibre. Even grinding for only 1 min. without sand caused a considerable decline in the phosphorylation ratios ($P:O = 0.58$, compared with 0.94; $P:\alpha\text{-Kg} = 1.69$, compared with 2.95).

Tonicity of the isolation medium. Mr T. D. Iles of the Department of Zoology, University of Cambridge, kindly measured the freezing-point depression of a sample of blowfly haemolymph for us. The value obtained was 0.72° , which is considerably greater than for rat serum (0.51° , Conway & McCormack, 1953). From data in the International Critical Tables (1928), it was calculated that 0.36M sucrose would be isotonic with haemolymph. Assuming that blowfly thoracic muscle is isotonic with haemolymph, it appeared logical to use this medium to isolate the sarcosomes. The effect of varying the tonicity of the isolation medium is shown in Fig. 1. It can be seen that the highest phosphorylation ratios are obtained in the region of the tonicity of the haemolymph. It is noteworthy that the preparation isolated under the most hypotonic conditions oxidized α -ketoglutarate most rapidly, but gave comparatively low phosphorylation ratios.

The low ratio obtained by the use of 0.56M sucrose containing 0.01M ethylenediaminetetraacetic acid as isolation medium is partly due to the high concentration of sucrose in the reaction mixture. A control experiment with another preparation showed that the inclusion of this additional

sucrose in the reaction mixture lowered the $P:O$ ratio of sarcosomes isolated with 0.36M sucrose from 1.03 to 0.79.

In most of the experiments reported in this paper the tonicity of the isolation medium was 0.36 osmolar; in a few it was 0.20 osmolar, which would be expected from Fig. 1 to give a $P:O$ ratio about 10% lower than that obtained with the optimum isolation medium.

Table 2. *Effect of time of standing of isolated thoraces on phosphorylation ratios*

Isolation medium: 0.20M sucrose containing 0.002M ethylenediaminetetraacetic acid. The time of standing refers to the time between removing the head and abdomen of the fly and commencing the isolation procedure. The preparations were made simultaneously from the same batch of flies. Concn. of α -ketoglutarate, 0.003M.

Time of standing (min.)	Q_{O_2}	$P:O$	$P:\alpha\text{-Kg}$
0	9.5	0.83	1.88
5	11.9	0.86	1.68
35	11.4	0.65	1.39

Fig. 1. Effect of varying the tonicity of the isolation medium on the activity of the α -ketoglutaric oxidase system and on the phosphorylation ratios. The isolation media contained 0.01M ethylenediaminetetraacetic acid and 0.06, 0.16, 0.28 or 0.56M sucrose, respectively. The four preparations were made simultaneously from the same batch of blowflies, using 20 blowflies to each preparation. All preparations were tested in the same reaction mixture, except that the sucrose concentration varied between 0.009 and 0.084M. The arrow gives the osmolarity of sucrose with the same freezing-point depression as blowfly haemolymph. (The osmolarity is defined as the total concentration of all species of solute in the isolation medium (g. moles or g. ions/l.), i.e. osmolarity = [sucrose] + 4[ethylenediaminetetraacetate], since at pH 7.4, the chelating agent is present largely as the tribasic anion.) Concn. of α -ketoglutarate, 0.003M.

Composition of isolation media. (1) *Ethylene-diaminetetraacetic acid.* The effect of including ethylenediaminetetraacetic acid in the isolation medium is shown in Table 3. It caused a statistically significant ($P=0.05$) decrease in the activity of the α -ketoglutaric oxidase system. This is in contrast to the behaviour of rat-heart sarcosomes where the inclusion of ethylenediaminetetraacetic acid in the isolation medium considerably increased the activity of the α -ketoglutaric oxidase system (Slater & Cleland, 1953*b*). However, in agreement with the experience with rat heart, the presence of ethylenediaminetetraacetic acid in the suspension medium stabilized the α -ketoglutaric oxidase system in blowfly sarcosomes (Table 4). In contrast with the behaviour of heart sarcosomes, the phosphorylation ratios were more affected than the rate of oxidation, by incubation of the preparation in the absence of the chelating agent. It should be noted that except in some experiments described in Tables 5 and 7, ethylenediaminetetraacetic acid was always present in the reaction mixture.

The other differences in Table 3 are not statistically significant ($P>0.05$).

(2) *Saline.* Only one preparation was made with saline (0.173M-KCl containing 0.01M ethylenediaminetetraacetic acid). This had a very high Q_{O_2} (50, compared with 18.7 given by sarcosomes isolated in 0.36M sucrose containing 0.005M phosphate using the same batch of flies on the same day) and a low phosphorylation ratio ($P:O=0.61$, $P:\alpha\text{-Kg}=0.73$, compared with 0.90 and 1.70, respectively, for the sucrose preparation). Sacktor (1954) has also found lower phosphorylation ratios using saline isolation media.

(3) *Albumin.* Watanabe & Williams (1953) have found that a solution of 2.5% bovine-plasma albumin in 0.16M phosphate, pH 7.0, was the optimum medium for the preservation of the microscopic appearance of isolated sarcosomes. Accordingly, this medium was compared with sucrose in the experiment summarized in Table 5. Since the addition to the reaction mixture of the sarcosomes suspended in this medium introduced a considerable concentration of albumin (0.37%) and additional phosphate (0.024M) and lowered the pH somewhat, the comparison was made by adding the same concentration of albumin and phosphate to the reaction mixture used for measuring the activity of the sarcosomes isolated in sucrose. As will be shown later, the addition of albumin to the reaction mixture markedly stimulated the oxidation of α -ketoglutarate. However, it is clear from Table 5 that the isolation medium of Watanabe & Williams is not as suitable as sucrose for obtaining maximum phosphorylation ratios. In fact, the former preparation resembles that obtained with potassium chloride.

The addition of 2.5% albumin to the usual sucrose medium was also tried in the experiment shown in Table 6. The addition increased the phosphorylation ratios slightly, but the increase was well within the range of variation between individual preparations and was no more than that often obtained by adding ethylenediaminetetraacetic acid to the sucrose.

Table 3. *Comparison of preparations made with and without ethylenediaminetetraacetic acid*

The values given are mean $\pm$ standard error of the mean. Four preparations with abnormally low phosphorylation ratios ($P:\alpha\text{-Kg}$ less than 1) have been omitted from this series.

	Isolation medium	
	0.36M Sucrose + 0.005M phosphate	0.32M Sucrose + 0.01M ethylene- diaminetetraacetic acid
No. of preparations	7	9
Age of flies (days)	10.2	8.6
Q_{O_2} (corr.)	17.35 ± 1.95	12.9 ± 0.76
$P:O$	1.00 ± 0.13	1.19 ± 0.15
$\Delta O:\Delta\alpha\text{-Kg}$	2.64 ± 0.28	2.04 ± 0.13

Table 4. *Effect of incubating sarcosomes*

The incubation procedure was the same as that described by Slater & Cleland (1953*b*).

	Isolation medium	
	0.36M Sucrose + 0.005M phosphate	0.32M Sucrose + 0.01M ethylene- diaminetetraacetic acid
Not incubated		
Q_{O_2}	18.7	7.4
$P:O$	0.90	0.88
$P:\alpha\text{-Kg}$	1.70	1.48
Incubated 15 min. at 25°		
Q_{O_2}	14.6	7.2
$P:O$	0.57	0.83
$P:\alpha\text{-Kg}$	0.69	1.34

Table 5. *Comparison of aqueous solutions of bovine-plasma albumin and of sucrose as isolation media*

The reaction mixture included 0.37% albumin and 0.024M phosphate buffer, pH 7.0, in addition to 'standard conditions'.

	Isolation medium	
	0.16M Phosphate, pH 7.0 + 2.5% albumin	0.36M Sucrose + 0.005M phosphate, pH 7.4
Q_{O_2}	46*, 47	25*, 27
$P:O$	0.82*, 0.78	1.00*, 0.95
$P:\alpha\text{-Kg}$	0.99*, 0.93	1.49*, 1.39

* Measured without ethylenediaminetetraacetic acid in the reaction mixture.

Table 6. *Effect of adding albumin to isotonic sucrose isolation medium*

The isolation medium was 0.36M sucrose + 0.005M phosphate, pH 7.4, with or without 2.5% albumin. The reaction mixture included 0.37% albumin in addition to 'standard conditions'.

	Isolation medium	
	With albumin	Without albumin
Q_{O_2}	33	30
P:O	1.39	1.24
P: α -Kg	3.14	2.55

Fig. 2 (a). Effect of varying the amount of hexokinase on the P:O ratio and on $\Delta\sim P$. Sarcosomes isolated in 0.20M sucrose containing 0.002M ethylenediaminetetraacetic acid.

Fig. 2 (b). Fig. 2 (a) plotted according to procedure of Lineweaver & Burk (1934).

Effects of varying reaction mixture

Concentration of hexokinase. With any preparation containing ATPase, it is important that sufficient hexokinase should be added so that virtually all the ATP formed by oxidative phosphorylation reacts with this enzyme and none with the ATPase. The effect of varying the amount of hexokinase on the P:O ratio is shown in Fig. 2 (a). It can be shown (see the Appendix) that in a system in which there is competition between hexokinase and ATPase (or

other ATP-consuming systems) for available ATP, the relationship between the concentration of hexokinase and the amount of ATP which reacts with it may be represented by a rectangular hyperbola. Fig. 2 (b) shows the same results plotted by the Lineweaver & Burk (1934) method, which converts a rectangular hyperbola into a straight line. From this line, it can be calculated that 50, 90, 95 and 99% of the ATP will react with the glucose in the presence of 2.5, 22, 47 and 246 units of hexokinase/mg. sarcosomal protein respectively, with this particular sarcosomal preparation. Provided that more than 100 units of hexokinase/mg. sarcosomal protein are used, the error due to loss of ATP by hydrolysis will be insignificant. It is interesting that the amount of hexokinase required for 50% of the ATP to react is identical with that calculated by the same method from the results of a similar experiment with rat-heart sarcosomes described by Slater & Holton (1953). Fig. 2 (a) shows also that the decrease of $\sim P$ is maximum with about 70 units of hexokinase/mg. sarcosomal protein. With less hexokinase, the loss of $\sim P$ caused by the combined action of myokinase and hexokinase (or ATPase) is partly compensated for by the building up of a steady-state concentration of ATP (cf. Slater & Holton, 1953).

Another possible source of error in measuring phosphorylation is the presence in the reaction mixture of phosphatases hydrolysing hexose monophosphate, the final product of the reaction. Direct test by the method described previously (Slater, 1953) showed, however, that no phosphatase (phosphomonoesterase) was detectable.

Ethylenediaminetetraacetic acid and albumin. Table 7 shows the effects of adding ethylenediaminetetraacetic acid and bovine plasma albumin, either separately or together, to the reaction mixture. Ethylenediaminetetraacetic acid had an appreciable effect on the activity of the α -ketoglutaric oxidase system and a considerable effect on the phosphorylation ratios when added in the absence of albumin, but little effect in the reaction mixture containing 3.7 mg. albumin/ml. (Expt. 2). Expt. 3 shows that 3×10^{-4} M ethylenediaminetetraacetic acid was insufficient for maximum phosphorylation ratios, either in the absence or in the presence of 1.2 mg. albumin/ml. Albumin itself very markedly increased the activity of the α -ketoglutaric oxidase system, without usually appreciably affecting the phosphorylation ratios. In Expt. 4, the P:O ratio was increased somewhat, while the P: α -Kg ratio was decreased. This can be explained by the decreased $\Delta O:\Delta\alpha$ -Kg ratio associated with the addition of the albumin. In Expt. 5, the phosphorylation ratios were slightly decreased by the addition of albumin. In Expt. 6, they were appreciably increased.

Table 7. *Effect of including bovine plasma albumin or ethylenediaminetetraacetic acid or both in reaction mixture*

The isolation media were: 0.36M sucrose + 0.005M phosphate in Expts. 1, 2, 4 and 5; 0.20M sucrose + 0.002M ethylenediaminetetraacetic acid in Expt. 3; and 0.36M sucrose + 0.01M ethylenediaminetetraacetic acid in Expt. 6. The concentrations of ethylenediaminetetraacetic acid, albumin and cytochrome *c* refer to the final reaction mixture.

Expt.	Ethylene-diaminetetraacetic acid (m-moles/l.)	Albumin (mg./ml.)	Cytochrome <i>c</i> (mg./ml.)	-ΔO (μatoms)	-Δα-Kg (μmoles)	Δ Esterified P (μmoles)	P:O	P:α-Kg
1	0	0	0.6	1.29	0.45	0.57	0.44	1.26
	1.5	0	0.6	1.38	0.73	1.24	0.90	1.70
2	0	0	0.6	1.33	0.50	0.78	0.59	1.56
	1.5	0	0.6	1.61	—	1.15	0.72	—
	0	3.7	0.6	2.16	1.44	2.15	1.00	1.49
	1.5	3.7	0.6	2.37	1.62	2.24	0.95	1.39
3	0.3	0	0.6	1.77	0.76	2.36	1.33	3.10
	1.5	0	0.6	1.73	0.73	2.91	1.68	4.00
	0.3	1.2	0.6	2.67	1.31	4.31	1.62	3.30
	1.5	1.2	0.6	2.64	1.26	4.52	1.71	3.61
4	1.5	0	0.6	2.86	0.91	2.68	0.94	2.95
	1.5	3.7	0.6	3.84	2.18	4.62	1.20	2.12
	1.5	10.0	0.6	3.78	2.23	4.12	1.09	1.85
5	1.5	0	0.6	4.07	1.74	5.17	1.27	2.98
	1.5	1.2	0.6	5.00	2.53	6.46	1.29	2.56
	1.5	2.4	0.6	5.30	2.69	6.20	1.17	2.30
	1.5	3.7	0.6	5.16	2.51	6.40	1.24	2.55
	1.5	0	0	4.09	1.61	5.35	1.31	3.32
	1.5	0	3.7	3.90	1.91	4.42	1.14	2.32
	1.5	3.7	0	5.34	2.60	6.35	1.19	2.44
6	1.5	0	0.3	0.89	0.53	1.30	1.46	2.45
	1.5	1.2	0.3	1.60	0.90	2.85	1.78	3.16
	1.5	5.0	0.3	1.805	0.98	3.44	1.91	3.51
	1.5	10.0	0.3	2.06	0.89	4.10	1.99	4.60

* Calculated as cytochrome *c* containing 0.45% iron.

Fig. 3. Effect of bovine plasma albumin on the oxidation of succinate by blowfly sarcosomes and on the P:O ratio. Reaction mixture same as for α-ketoglutarate, except 0.01M succinate replaced α-ketoglutarate, and fluoride and malonate were omitted. Sarcosomal protein, 0.77 mg./ml.; hexokinase, 165 units/mg. sarcosomal protein. The reaction commenced at zero time, the first manometric readings were taken at the times corresponding to zero oxygen uptake on the curves.

In Expt. 5, the same degree of stimulation was obtained with all the concentrations of albumin tested (between 1.2 and 3.7 mg./ml.). In Expt. 4,

10 mg. albumin/ml. gave the same result as 3.7 mg./ml. In Expt. 6, however, higher concentrations of albumin were appreciably more effective than 1.2 mg./ml.

Expt. 5 shows that omitting the cytochrome *c* had no effect on any of the measurements, either in the presence or in the absence of albumin. Apparently, the sarcosomes contain sufficient cytochrome *c* for optimum respiration (cf. Sacktor, 1954). A high concentration of cytochrome *c* could not substitute for the albumin; in fact, it was appreciably inhibitory to the phosphorylation (contrast the effect on rat-heart sarcosomes, Slater & Cleland, 1953a).

The action of albumin was also tested with succinate as substrate. Fig. 3 shows that albumin had little effect on the initial rate of oxidation of succinate, but prevented the marked fall off in the rate of oxygen uptake. It is possible that albumin promotes the oxidation of the inhibitory oxaloacetate, thereby preventing its accumulation in the system. The P:O ratio was also markedly increased by the addition of albumin.

α-Ketoglutarate concentration. It was thought possible that the activating effect of albumin was due to its ability to increase the permeability of the

sarcosomes to α -ketoglutarate. This was tested by measuring the activity, both in the absence and in the presence of albumin, at different concentrations of α -ketoglutarate (see Table 8). If the rate of penetration of α -ketoglutarate to its dehydrogenase in or on the sarcosomes were a rate-limiting step in the oxidation, it would be expected that the rate of oxidation would be increased by increasing the concentration of α -ketoglutarate. This was found to be the case. It should be noted that the rate of oxidation of α -ketoglutarate by heart sarcosomes is scarcely affected by variation of the α -ketoglutarate concentration within the range shown in Table 8 (see Slater & Holton, 1954). Further, as would be expected if there were a permeability barrier to α -ketoglutarate, the relationship between α -ketoglutarate concentration and rate of oxidation shown in Table 8 does not follow that predicted from the Michaelis & Menten (1913) theory.

Table 8. *Effect of varying the α -ketoglutarate concentration, both in the presence and absence of albumin*

Isolation medium: 0.32M sucrose + 0.01M ethylenediaminetetraacetic acid. Ratio = value in presence of albumin/value in absence of albumin.

	Albumin (mg./ml.)	α -Ketoglutarate (m-moles/l.)		
		2.0	4.5	9.8
- ΔO (μ atoms)	0	1.02	1.33	1.87
	1.2	1.86	2.51	3.21
Ratio		1.82	1.89	1.72
- $\Delta\alpha$ -Kg (μ moles)	0	0.48	0.70	—
	1.2	1.03	1.36	—
Ratio		2.15	1.95	—
Δ Esterified P (μ moles)	0	1.77	2.18	3.27
	1.2	2.76	4.45	5.78
Ratio		1.56	2.04	1.76
P:O	0	1.73	1.64	1.75
	1.2	1.49	1.77	1.80

It seems possible then that the slow rate of oxidation of α -ketoglutarate is partly due to the slow penetration of α -ketoglutarate to its dehydrogenase. But even if this is so, the activating effect of albumin cannot be explained on this basis, since the relative effect of increasing the concentration of α -ketoglutarate was independent of the presence of albumin.

Malonate and fluoride. The effect of omitting malonate and fluoride from the reaction mixture is shown in Table 9. In Expts. 1 and 2, the omission of the malonate caused a greatly increased rate of respiration (especially in the first 15 min.), a decreased disappearance of α -ketoglutarate and a considerably increased esterification of inorganic phosphate. The stimulation of the disappearance of α -ketoglutarate by the addition of malonate is interesting. It is also shown, but to a smaller degree, in similar experiments with rat-heart sarcosomes (Table 5 of Slater & Holton, 1954). It is probably an indirect effect; the rapid oxidation of succinate and its oxidation products which occurs in the absence of malonate might suppress the oxidation of α -ketoglutarate by competition for a common intermediate, involved either in the hydrogen-transfer to oxygen or in the accompanying phosphorylation. By slowing down these reactions, malonate will promote the oxidation of α -ketoglutarate. This effect of malonate was not shown in the presence of albumin (Expts. 3 and 4 in Table 9).

The markedly different $\Delta O:\Delta\alpha$ -Kg ratios obtained in the presence and absence of malonate in Expts. 1 and 2 makes it difficult to compare the respective P:O ratios. Expt. 3 shows, however, that 0.01M malonate has little if any effect on the phosphorylation associated with the oxidation of α -ketoglutarate to succinate. Doubling this concentration of malonate also had little effect (Expt. 4).

Omission of fluoride had no marked effect on the phosphorylation ratios. In Expt. 1, Table 9, the

Table 9. *Effect of malonate and fluoride*

Isolation medium: 0.36M sucrose + 0.005M phosphate in Expts. 1, 2 and 3; same medium containing 2.5% albumin in Expt. 4.

Expt.	Malonate (M)	NaF (M)	- ΔO (μ atoms)	- $\Delta\alpha$ -Kg (μ moles)	$\Delta O:\Delta\alpha$ -Kg	Δ		
						Esterified P (μ moles)	P:O	P: α -Kg
1	0.01	0.04	1.61	—	—	1.15	0.72	—
	0	0.04	2.76	0.66	4.19	2.48	0.90	3.76
	0.01	0	1.52	0.60	2.54	1.40	0.92	2.33
	0	0	1.69	0.48	3.52	1.23	0.73	2.56
2	0.01	0.04	2.86	0.91	3.14	2.68	0.94	2.95
	0	0.04	4.80	0.66	7.29	4.57	0.95	6.94
	0.01	0	2.34	0.68	3.44	1.94	0.83	2.85
3*	0.01	0.04	5.16	2.51	2.06	6.40	1.24	2.55
	0	0.04	7.60	2.57	2.96	6.87	0.91	2.68
4*	0.01	0.04	5.45	2.42	2.25	7.59	1.39	3.14
	0.02	0.04	4.01	2.30	1.75	5.26	1.31	2.30

* Reaction mixture contained 3.7 mg. albumin/ml.

initial rate of oxygen uptake was considerably increased by omitting the fluoride, but it rapidly declined. This may be associated with the very low concentration of ADP which is obtained in the absence of fluoride, owing to action of myokinase and hexokinase. Fluoride is a strong inhibitor of myokinase (Kotelnikova, 1950; Barkulis & Lehninger, 1951).

Effect of time of experiment

Fig. 4 shows the results of an experiment in which one of five replicate flasks was removed and the contents deproteinized at various time intervals. The first point to be noted is that, as might be expected, the $\Delta O:\Delta\alpha\text{-Kg}$ ratio increased with time, particularly during the first 10 min. The ratio after 35 min. was unusually high in this experiment. Secondly, after a rapid phase in the first 5 min., the rate of esterification of phosphate remained constant between 5 and 35 min. Probably as a consequence of the change of $\Delta O:\Delta\alpha\text{-Kg}$ ratio in the first 10 min., the P:O ratio declined and the P: $\alpha\text{-Kg}$ ratio increased during this period. However, both phosphorylation ratios remained almost constant between 10 and 35 min. It seems probable, then, that no inactivation of the phosphorylating enzymes occurred in 35 min. under the conditions of this experiment. The marked fall in both the P:O and the P: $\alpha\text{-Kg}$ ratios between 35 and 60 min. shows, however, that a considerable inactivation occurred after 35 min.

P:O ratios

Fig. 5 summarizes our results with 27 blowfly sarcosomal preparations, isolated with media containing 0.20–0.36M sucrose, with or without ethylenediaminetetraacetic acid, and measured under 'standard conditions'. The results with albumin in the reaction mixture are excluded.

The P:O ratios of the blowfly sarcosomes are much lower and show much greater variation than those of heart sarcosomes (cf. Fig. 5 of this paper with Fig. 3 of Slater & Holton, 1954).

One might expect somewhat lower P:O ratios with the blowfly sarcosomes, since a greater proportion of the oxygen uptake is associated with the oxidation of succinate to fumarate, a reaction with a low phosphorylation ratio. Nevertheless, the difference in P:O ratios is much greater than can be accounted for by the different $\Delta O:\Delta\alpha\text{-Kg}$ ratios and must reflect a true difference between the phosphorylative abilities of the two preparations.

The following factors probably contribute to the variability of the P:O ratios of blowfly sarcosomes: (i) age of fly, (ii) a real difference between different batches of flies, (iii) variation of degree of crushing of thoracic muscle during the preparation of the sarcosomal fraction. It is likely that the second

factor is the most important. Variation between individual blowflies is not likely to be important, since at least 20 flies were used in each preparation. It might be significant that most of the very low ratios were obtained within a few weeks during the summer. The temperature during the development of these blowflies sometimes exceeded 22°.

Sacktor's (1954) figures for the P:O ratios of housefly sarcosomes are within the range covered by Fig. 5.

Fig. 4. Oxygen uptake, α -ketoglutarate disappearance, esterification of phosphate and the various ratios as a function of time. Sarcosomes (isolated with 0.36M sucrose + 0.005M phosphate), 1.33 mg. sarcosomal protein/ml.; α -ketoglutarate, 0.0045M.

Fig. 5. Frequency distribution of P:O ratios obtained with 27 blowfly sarcosomal preparations.

DISCUSSION

Activity of the α -ketoglutaric oxidase system

The oxidation of α -ketoglutarate by the blowfly sarcosomes is slow, the average Q_{O_2} of preparations isolated in sucrose-ethylenediaminetetraacetic acid being only 13. This is to be compared with 80, obtained under identical conditions with rat-heart sarcosomes. The activity of the blowfly sarcosomes can be increased by adding either bovine plasma albumin or 2:4-dinitrophenol (Slater & Lewis, 1953), neither of which has any effect on the rat-heart sarcosomes.

The experiments with albumin were prompted by the paper of Watanabe & Williams (1953) which showed that a solution of 2.5% bovine plasma albumin in 0.16M potassium phosphate buffer, pH 7.0, was the optimum medium for the preservation of the microscopic appearance of isolated sarcosomes. Although we did not find this medium as suitable as sucrose for isolating sarcosomes with a high P:O ratio, we did find that the addition of albumin to the reaction mixture had a remarkable effect on the oxidation of α -ketoglutarate. The full effect was usually obtained with the lowest concentration tested (0.12%), which was considerably below the concentration in the isolation medium of Watanabe & Williams. The oxygen uptake proceeded at practically a uniform rate (in the presence of ethylenediaminetetraacetic acid) whether or not albumin was added. This shows clearly that albumin acts not just by stabilizing the system, but as a true activator.

Sacktor (1954) has also reported that albumin increased the rate of oxygen uptake with housefly sarcosomes oxidizing α -ketoglutarate. However, the chief effect of albumin in his experiments was on the phosphorylation. No uptake of phosphate occurred in the absence of albumin, while 2% or more of albumin was necessary for maximum phosphorylation. In our experiments, because ethylenediaminetetraacetic acid was in the reaction mixture, albumin had only a slight effect (or none) on the phosphorylation ratios with α -ketoglutarate as substrate. However, it considerably increased the P:O ratio when succinate was the substrate. For this reason and because of the marked stimulation of the rate of oxygen uptake, we would agree with Watanabe & Williams (1953) and Sacktor (1954) that it is advantageous to include albumin in the reaction mixture.

The mechanism of the activation by albumin is obscure. It is unlikely to be the same as that suggested for 2:4-dinitrophenol (Slater & Lewis, 1953) for two reasons: (1) the α -ketoglutaric oxidase systems of some preparations were only slightly stimulated by 2:4-dinitrophenol, but markedly accelerated by albumin; (2) albumin did not de-

crease the phosphorylation ratios like 2:4-dinitrophenol.

Some other treatments which caused decreased phosphorylation ratios also increased the activity of the α -ketoglutaric oxidase system, for example isolation with hypotonic sucrose or with isotonic saline. The explanation in these cases might be the same as that given for 2:4-dinitrophenol. In any case, these results show that the activity of the α -ketoglutaric oxidase system is by itself of little value in assessing the effect of a particular treatment of the blowfly sarcosomes. This is different from the behaviour of rat-heart sarcosomes in which case the activity of the α -ketoglutaric oxidase system is a more sensitive index than the phosphorylation ratios (Slater & Cleland, 1953a).

Effect of ethylenediaminetetraacetic acid

Although the effect of adding ethylenediaminetetraacetic acid to the reaction mixture was less striking than in similar experiments with rat-heart sarcosomes (Slater & Cleland, 1953b), it was still quite appreciable, in the absence of albumin. A high concentration of albumin appears to be able to substitute for the chelating agent, as well as having a marked effect on its own, since the addition of ethylenediaminetetraacetic acid to a medium already containing 0.37% albumin had little effect on either the rate of oxidation of α -ketoglutarate or on the phosphorylation ratios. This may be because albumin, in the high concentrations used, can also effectively bind calcium (Greenberg, 1944; Katz & Klotz, 1953). Our results are in complete opposition to the surprising finding of Sacktor (quoted by Watanabe & Williams, 1953) that the addition of 0.001M ethylenediaminetetraacetic acid abolished the oxidation of α -ketoglutarate by isolated insect sarcosomes. Sacktor (1954) does not refer to this experiment, but states that the inclusion of 0.01M ethylenediaminetetraacetic acid in the isolation medium (0.25M sucrose) completely inhibited both respiration and phosphate uptake. We are also in complete disagreement with this result (see Table 3).

The inclusion of ethylenediaminetetraacetic acid in the isolation medium had no appreciable effect on the phosphorylative activity of sarcosomes tested immediately after preparation in a reaction mixture containing the chelating agent. However, such preparations, like the corresponding preparations from heart, could be incubated at 25° for 15 min. without inactivation of oxidative phosphorylation, in contrast to the considerable inactivation in sarcosomes isolated in sucrose alone.

P:O ratio

It is always a difficult problem to assess the activity of enzymes *in vivo* from studies with cell-free preparations. In the case of the P:O ratio of

rat-heart sarcosomes, we were unusually well-placed, since the ratio was found to be almost independent of considerable variations in the isolation medium, the reaction mixture and the treatment of the sarcosomes (Slater & Holton, 1954). It was possible then to extrapolate from the situation in the isolated sarcosome to that in the intact cell with some degree of confidence.

The blowfly sarcosomes present a more difficult problem. The P:O ratio was found to depend in a critical manner on the isolation procedure, which in comparison with that used for isolating heart sarcosomes was very mild, and on variations of the composition of the reaction mixture. Moreover, there were large unexplained variations in the P:O ratio from experiment to experiment, using apparently the same procedure with blowflies bred in the same way.

There are three possible explanations for the finding that the phosphorylation ratios obtained with blowfly sarcosomes are considerably below those obtained with rat-heart sarcosomes under identical conditions. (1) The yield of phosphorylation in blowfly sarcosomes, *in vivo*, may be lower than in mammalian heart. (2) The phosphorylation enzymes of the blowfly sarcosomes may be more labile than the corresponding enzymes in the rat-heart sarcosomes. (3) The P:O ratio of blowfly and rat-heart sarcosomes are identical *in vivo*, there is no difference in the stability of the respective enzymes, but the activity of the phosphorylating enzymes is not the rate-limiting step in the rat-heart sarcosome, but is limiting in the blowfly sarcosome. Thus the same degree of inactivation of a phosphorylating enzyme could conceivably have no effect on the P:O ratio in the rat-heart sarcosomes, but appreciably depress the ratio in the blowfly sarcosome.

The available evidence does not allow us to choose between these three alternatives.

SUMMARY

1. Oxidative phosphorylation has been demonstrated with sarcosomes isolated from the thoracic muscle of the blowfly *Calliphora erythrocephala*.

2. The phosphorus: oxygen ratio varied considerably from preparation to preparation, but never exceeded 2.0. The ratio was lower with sarcosomes isolated from young flies.

3. Factors causing low ratios were (i) isolation in media hypotonic or hypertonic to the blowfly haemolymph (tonicity equal to that of 0.36M sucrose); (ii) isolation in potassium chloride or phosphate; (iii) allowing the dissected thoraces to stand at room temperature for 35 min.; (iv) subjecting the thoraces to more than gentle crushing to liberate the sarcosomes; (v) omission of ethylene-

diaminetetraacetic acid from the reaction mixture; (vi) prolonging the experiment beyond 35 min. (at 25°). The treatments referred to in (i) and (ii) gave preparations with higher oxidative activity.

4. The inclusion of fluoride or malonate in the reaction mixture or variation of the α -ketoglutarate concentration between 0.002 and 0.01M had no effect on the phosphorylation ratios.

5. Sarcosomes isolated in sucrose containing ethylenediaminetetraacetic acid oxidized α -ketoglutarate more slowly than those isolated in sucrose alone. The P:O ratio was little affected.

6. The addition of small amounts of albumin (1.2 mg./ml.) to the reaction mixture markedly stimulated the rate of oxidation of α -ketoglutarate, without usually affecting the phosphorylation ratio. Albumin considerably increased the P:O ratio when succinate was the substrate.

7. Albumin is a true activator of the oxidase system, in contrast with ethylenediaminetetraacetic acid which stabilizes the system.

8. Apart from the activation caused by the introduction of albumin into the reaction mixture, the inclusion of albumin in the isolation medium had little effect.

This work was carried out while S. E. L. was on leave from D.S.I.R. Pest Infestation Laboratory, London Road, Slough. E. C. S. is working on behalf of the Agricultural Research Council. We wish to thank Professor D. Keilin, F.R.S., and Dr P. Tate for their interest and advice.

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APPENDIX

Relationship Between Amount of Hexokinase and Amount of Phosphate Esterified

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(Received 13 April 1954)

The reactions concerned may be expressed in a simplified form as follows

Reaction (1) represents the rate of synthesis of ATP by the oxidative phosphorylation reaction. Except at very low concentrations of ADP, this is independent of the concentration of ADP (Slater & Holton, 1953). Reaction (2) is the hexokinase reaction; it is assumed that the concentration of ATP is low, so that with saturating concentrations of glucose, the rate of this reaction will be proportional to the concentrations of ATP and to the concentration of hexokinase (hK). Reaction (3) is the ATPase reaction; again it is assumed that the ATP concentration is so low that the rate of reaction (3) is proportional to it. Since the enzymes catalysing reactions (1) and (3) are in the sarcosomal

preparation, the amount of which is not varied, the concentrations of these enzymes are included in the rate constants k_1 and k_3 .

In the steady state,

$$d[\text{ATP}]/dt = k_1 - [\text{ATP}] (k_2[\text{hK}] + k_3) = 0, \quad (4)$$

$$d[\text{HMP}]/dt = v = k_2[\text{hK}] [\text{ATP}]. \quad (5)$$

Adding (4) and (5),

$$v = k_1 - k_3[\text{ATP}]. \quad (6)$$

From (4),

$$[\text{ATP}] = \frac{k_1}{k_2[\text{hK}] + k_3}. \quad (7)$$

Combining (6) and (7)

$$v = k_1 \left[1 - \frac{k_3}{k_2[\text{hK}] + k_3} \right],$$

i.e.

$$v = \frac{k_1 k_2 [\text{hK}]}{k_2[\text{hK}] + k_3},$$

and

$$1/v = \frac{k_2 + k_3[\text{hK}]}{k_1 k_2 [\text{hK}]}.$$

Thus

$$\frac{1}{v} = \frac{1}{k_1} + \frac{k_3}{k_1 k_2} \cdot \frac{1}{[\text{hK}]}. \quad (8)$$

That is, $1/v$ is a linear function of $1/[\text{hK}]$.

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