Aerobic Metabolism of the Muscle of *Locusta migratoria*

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During investigations of the aerobic metabolism of some invertebrates (Rees, 1952, 1953) the muscle of *Locusta migratoria* was examined. Barron & Tahmisian (1948) have shown that whole muscle fibres of the cockroach, *Periplaneta americana*, contain a glycolytic system and enzymes for the oxidation of citric acid cycle intermediates. Breis and acetone powders of *Drosophila* have also been found to contain enzymes for the oxidation of a number of citric acid cycle intermediates (Spirtes, 1951).

Watanabe & Williams (1951) described the isolation of sarcosomes from flight muscles of *Drosophila funebris* and of the blowfly, *Phormia regina*, and showed them to possess high respiratory activity. They concluded that these particles resembled mammalian mitochondria.

Sacktor (1953) was unable to demonstrate oxidative phosphorylation with insect sarcosome preparations. However, Lewis & Slater (1953) reported oxidative phosphorylation to occur with sarcosome preparations of the blowfly *Calliphora erythrocephala*.

In this present investigation, it has been shown that sarcosomes from *Locusta migratoria* possess citric acid cycle activity and the capacity for oxidative phosphorylation. The conditions necessary for such activity have been investigated, and it has been demonstrated that the addition of ethylenediaminetetraacetic acid is necessary for oxidative phosphorylation and that oxidation of citric acid is very slow unless either triphosphopyridine nucleotide or a mixture of diphosphopyridine nucleotide and adenosine triphosphate are added.

EXPERIMENTAL

All the experiments reported in this paper were carried out on adult male or female *L. migratoria*. The animals were kept at 32°.

Enzyme preparations

Cell-free tissue suspensions. By means of scissors, the head, abdomen and appendages were removed from the thoraces of the insects. The thorax was opened along the mid-dorsal line and the gut removed. The thoraces and appendages, without further dissection, were chopped with scissors in ice-cold 0.2M sucrose solution and then ground in an ice-cold Potter all-glass grinder. The suspension was diluted with 0.2M sucrose to give a final concentration of approximately 10% tissue and then centrifuged at 500 g for 5 min. to remove the chitin, cellular debris and nuclei.

For studying the synthesis of citric acid, KCl suspensions were prepared by the above method substituting 0.5% (w/v) KCl for 0.2M sucrose.

Sarcosome suspensions. A muscle suspension prepared as described above was centrifuged at 10 000 g for 10 min. at 2° in a Servall SS-1-a high-speed centrifuge. The reddish brown mass of sarcosomes was washed by suspending in 0.2M sucrose and again centrifuging. The sarcosomes from eight adult locusts were finally suspended in 6–7 ml. of 0.2M sucrose.

Microscopic examination of such preparations showed a practically pure preparation of sarcosomes, 3–8 μ . in diameter. The first supernatant from the high-speed centrifuging was found to be almost free of sarcosomes.

Myokinase was prepared by the method of Colowick & Kalckar (1943).

Hexokinase was obtained from yeast by the method of Berger, Slein, Colowick & Cori (1946), as described by Cross, Covo, Taggart & Green (1949). The preparation was stored as a frozen solution at –10°. The conditions for autolysis of baker's yeast were those described by Bailey & Webb (1948).

Analytical methods

Inorganic orthophosphate was determined by the method of Fiske & Subbarow (1925) and α -oxoglutarate and pyruvate by the method of Friedemann & Haugen (1943). Citrate was determined by the method of Weil-Malherbe & Bone (1949). Succinate was determined by the method of Krebs (1937). Oxygen uptakes were determined manometrically; the gas phase was air.

Materials

Triphosphopyridine nucleotide (TPN) was isolated by the method of LePage & Mueller (1949).

Flavin-adenine dinucleotide (FAD) was prepared by a modification of the method of Warburg & Christian (1938).

Yeast juice. Fresh baker's yeast was washed and centrifuged 3 times with 4 vol. of 1% NaCl. The washed yeast was added to an equal volume of water maintained at 90°. After the final addition of yeast, the temperature was maintained for 2 min. at 90°. The yeast suspension was cooled rapidly and centrifuged and the clear, bright yellow supernatant was stored at –10°.

Coenzyme A (CoA), thiamine pyrophosphate (TPP) and glutathione (GSH) were commercial products. Other cofactors and reagents were gifts.

Standard medium. Final concentrations in the Warburg flasks were: KCl (0.025M), Mg^{2+} (0.0066M), adenosine 5'-phosphate (0.001M), cytochrome c (10^{-6} M), sodium phosphate buffer, pH 7.4 (0.014M), and substrate as indicated in the tables. Final volume, 3 ml. The enzyme preparation (0.5 ml.) was added in 0.2M sucrose solution. 0.1 ml. 20% KOH was placed in the centre well with filter paper. Temperature equilibration was carried out for 5 min.

RESULTS

Reactions of citric acid cycle

The effect of the addition of various glycolytic and citric acid cycle intermediates on the respiration of muscle suspensions in 0.2M sucrose was investigated (Table 1). All the substrates tested were found to increase the rate of respiration. The endogenous respiration of the suspension varied greatly from preparation to preparation. Dialysis of muscle suspensions against 0.2M sucrose resulted in a diminution of the endogenous respiration and of the ability of the preparation to oxidize citric acid cycle intermediates.

Table 1. *The effect of glycolytic and citric acid cycle intermediates on the respiration of muscle suspensions in 0.2M sucrose*

System: standard medium containing 30 μ moles of substrate (100 μ moles in case of succinate). Tissue suspension added equivalent to 1 mg. N. Duration 30 min.; 32° in air.

Expt.	Substrate	O ₂ uptake (μ l.)
1	Nil	20
	Fructose 6-phosphate	49
	Hexose diphosphate	47
	Phosphoglyceric acid	39
	L-Malate	54
	Succinate	160
2	Nil	51
	Oxaloacetate	74
	α -Oxoglutarate	105
	Fumarate	80
	cis-Aconitate	74
	Pyruvate	59

Table 2. *The effect of malonate and arsenite on the respiration of muscle suspensions in 0.2M sucrose*

System: standard medium with final concentrations of 0.033M malonate or 0.007M arsenite. Other additions as indicated. 60 min. respiration at 32° in air. Tissue suspension added equivalent to 1 mg. N.

Additions	O ₂ uptake (μ l.)	Keto acid accumulation
Nil	150	—
Malonate	7	—
Malonate + 5 μ moles L-malate	15	—
Malonate + 10 μ moles L-malate	33	α -Oxoglutarate (1.2 μ moles)
L-Malate (25 μ moles)	190	—
Arsenite	Nil	Pyruvate (0.5 μ mole)
Arsenite 2 μ moles L-malate	17	Pyruvate (2.0 μ moles)
Arsenite 5 μ moles L-malate	28	Pyruvate (3.0 μ moles)

Table 3. *Synthesis of citrate by muscle suspensions*

System: 0.0066M-Mg²⁺, 0.014M sodium phosphate buffer (pH 7.4), 0.0015M-ATP, 20 μ moles each of oxaloacetate and pyruvate. Reactions carried out anaerobically in Thunberg tubes. Tissue added as 1 ml. of muscle suspension in 0.5% KCl ($\equiv$ 1 mg. N). Duration of experiment, 30 min.

Substrate	Citrate synthesis (μ moles)	
	Expt. 1	Expt. 2
Nil	0	0.2
Pyruvate + oxaloacetate	2.0	3.1

Table 4. *The accumulation of succinate by muscle suspensions*

System: standard medium containing 0.033M malonate. 50 min. incubation at 32°. Tissue suspension in 0.2M sucrose equivalent to 1 mg. N. 10 μ moles α -oxoglutarate used.

Additions	O ₂ uptake (μ atoms)	α -Oxo-glutarate utilized (μ moles)	Succinate formed (μ moles)
Nil	1.0	—	—
α -Oxoglutarate	3.7	3.0	3.0
α -Oxoglutarate	4.1	3.8	3.4

The effects of malonate (0.033M) and arsenite (0.007M) on muscle suspensions were tested both in the presence and absence of added dicarboxylic acids (Table 2). The inhibition of the endogenous respiration by malonate was diminished by the addition of L-malate and α -oxoglutarate was found to have accumulated. The addition of arsenite resulted in complete inhibition of the endogenous respiration but under these conditions the system was capable of oxidizing L-malate to pyruvate, which accumulated.

Table 3 shows the synthesis of citrate from oxaloacetate and pyruvate. The reaction was investigated in Thunberg tubes under anaerobic conditions to prevent oxidation of the citrate formed.

The reaction α -oxoglutarate $\rightarrow$ succinate was studied in the presence of malonate to prevent further oxidation of succinate. Table 4 shows the results of two experiments.

These experiments indicate that the muscle of *L. migratoria* contains enzymes which will oxidize citric acid cycle intermediates. The inhibition of the endogenous respiration in the presence of malonate and arsenite further suggests that the citric acid cycle plays a part in the endogenous respiration.

The oxidation of citric acid cycle intermediates by sarcosomes from whole muscle suspensions was investigated. The rates of oxidation are low (Table 5).

The addition of ATP, ADP, FAD, GSH, TPP or CoA, singly or in combination, had no effect on the rates of oxidation. DPN was found however to increase the rate of oxidation (Table 5).

Table 5. *The effect of various cofactors and protein-containing solutions on the respiration of sarcosomes*

System: standard medium, with further additions as shown. CoA (5 Lipmann units), ADP and DPN (3 μ moles) and TPP (100 μ g.) when added. 30 min. incubation at 32° in air. 0.5 ml. of supernatant or yeast juice added. Sarcosomes added equivalent to 0.3 mg. N and muscle suspension equivalent to 1.5 mg. N (0.3 mg. of sarcosome N derived from 1.5 mg. of cell-free suspension N). Succinate (100 μ moles) and L-glutamate, α -oxoglutarate or L-malate (30 μ moles), where added.

Expt.	Substrate	Enzyme preparation	Additions	O ₂ uptake (μ atoms)
1	Succinate	Cell-free muscle suspension	Nil	6.5
		Sarcosomes	Nil	14.7
		Sarcosomes	Supernatant	20.2
		Sarcosomes	ADP	15.3
		Sarcosomes	CoA + TPP	15.3
2	L-Glutamate	Cell-free muscle suspension	Nil	13.3
		Sarcosomes	Nil	1.7
		Sarcosomes	Supernatant	7.0
	L-Malate	Sarcosomes	Nil	3.8
		Sarcosomes	Supernatant	14.0
		Sarcosomes	Boiled supernatant	6.1
		Sarcosomes	Yeast juice	9.8
		Sarcosomes	DPN	5.6
		Sarcosomes	CoA + TPP	4.4
		Sarcosomes		
3	L-Glutamate	Cell-free muscle suspension	Nil	13.0
		Sarcosomes	Nil	3.3
		Sarcosomes	Supernatant	14.0
		Sarcosomes	Boiled supernatant	6.8
		Sarcosomes	Yeast juice	6.9
		Sarcosomes	DPN	4.7
		Sarcosomes	CoA + TPP	3.1
	α -Oxoglutarate	Sarcosomes	Nil	4.8
		Sarcosomes	Supernatant	9.5
		Sarcosomes	Boiled supernatant	7.7
		Sarcosomes	Yeast juice	8.0
		Sarcosomes	DPN	5.5
		Sarcosomes	CoA + TPP	4.4
		Sarcosomes		

Table 6. *The effect of various protein-containing solutions on the oxidation of α -oxoglutarate by sarcosome preparations*

System: standard medium with or without 0.01M α -oxoglutarate; final volume, 3 ml., including 0.5 ml. sarcosome suspension ($\approx$ 0.6 mg. N) with or without 0.5 ml. yeast juice. 20 min. incubation at 32° in air.

Additions	O ₂ uptake (μ atoms)	α -Oxo-glutarate utilized (μ moles)
α -Oxoglutarate	8.5	8.0
α -Oxoglutarate + yeast juice	16.0	14.3
Yeast juice	6.5	—
Nil	1.0	—

It was found that the addition to such sarcosome preparations of some of the supernatant fluid from which they had been isolated resulted in a large increase in the respiration (Table 5). This supernatant fluid still retained some activity after being kept at 100° for 2 min. The supernatant fluid in the absence of added sarcosomes possessed no residual

respiration and was found not to show any L-glutamic dehydrogenase activity as determined manometrically in the presence of DPN and with methylene blue as carrier. The supernatant fluid effect was not lost after dialysis for 12 hr. at 0° against 0.2M sucrose. Yeast juice was observed to increase the rate of oxidation of citric acid cycle intermediates by sarcosomes. The results are also included in Table 5. The rates of oxidation were now of the same order as whole muscle suspensions despite the fact that the yeast juice gave no oxygen uptake in the absence of added substrate. This point was confirmed by following the oxidation of α -oxoglutarate in the presence of sarcosomes and yeast juice. It was found that oxidation of α -oxoglutarate was in fact accelerated by the addition of yeast juice (Table 6).

The supernatant fluid and yeast juice (which contains protein) could exert their effects by the removal of inhibitory metals. In view of the findings of Slater & Cleland (1953), the effect of ethylenediaminetetraacetic acid (EDA) (Versene)

was studied and was found to increase the rate of respiration to the same extent as the yeast juice and supernatant fluid. Histidine, however, would not substitute for EDA in these sarcosome preparations (Table 7).

Table 7. *The effect of ethylenediaminetetraacetic acid and L-histidine on the oxidation of L-malate by sarcosome preparations*

System: standard medium with 30 μ moles L-malate. Sarcosomes added equivalent to 0.5 mg. N, and incubation at 32°.

Additions	Concn. ($M \times 10^{-4}$)	O ₂ uptake (μ l.)	
		1st 60 min.	2nd 60 min.
Nil	—	94	25
Ethylenediaminetetraacetic acid	3.3	111	53
Ethylenediaminetetraacetic acid	1.0	115	49
Histidine	3.3	86	19
Histidine	1.0	102	19

Table 8. *The oxidation of citrate, acetate and octanoate by sarcosome preparations*

System: standard medium. Substrates: citrate (30 μ moles), pyruvate and acetate (20 μ moles), L-malate and octanoate (3 μ moles). Other additions: ATP (3 μ moles), DPN and TPN (1 μ mole); final concn. of ethylenediaminetetraacetic acid in all cases $3.3 \times 10^{-4}M$. Sarcosomes added equivalent to 0.5 mg. N. Incubated for 40 min.

Expt.	Substrate	Other additions	O ₂ uptake (μ l.)
1	Citrate	Nil	30
	Citrate	ATP + DPN	327
	Citrate	TPN	404
2	L-Malate	Nil	37
	L-Malate + pyruvate	Nil	147
	L-Malate + acetate	Nil	74
	L-Malate + octanoate	Nil	32

Table 10. *The effect of fluoride on oxidative phosphorylation of 0.2M sucrose muscle suspensions*

System: standard medium; substrates, 30 μ moles, excepting succinate (100 μ moles); 0.05 ml. hexokinase solution; 100 μ moles glucose. Final concentration of sodium fluoride, 0.014M. Duration of experiment 20 min. with 5 min. equilibration, at 32°. Tissue suspension added equivalent to 2 mg. N.

Expt.	Substrate	NaF	O ₂ uptake (μ atoms)	P esterified (μ moles)	P/O ratio
1	Nil	—	7.5	13.5	1.8
	Nil	+	3.0	Nil	0
	Succinate	—	44.0	24.5	0.56
	Succinate	+	21.0	16.5	0.79
	L-Malate	—	12.0	20.0	1.66
	L-Malate	+	5.1	10.0	1.95
	cis-Aconitate	—	8.0	10.0	1.25
	cis-Aconitate	+	4.0	3.0	0.75
2	Nil	—	1.5	Nil	0
	Nil	+	1.5	Nil	0
	L-Glutamate	—	9.5	20.0	2.1
	L-Glutamate	+	6.0	14.0	2.3
	α -Oxoglutarate	—	6.5	13.5	2.0
	α -Oxoglutarate	+	4.5	12.0	2.7

Table 9. *Component requirement for oxidative phosphorylation by a 0.2M sucrose muscle suspension*

Complete system: standard medium, 0.03M glucose, 0.01M L-glutamate, 0.014M-NaF, 0.05 ml. hexokinase solution. Final volume, 3 ml. 30 min. incubation in air at 32°; equilibration 5 min. (allowed for in results). Tissue suspension added equivalent to 2 mg. N.

System	O ₂ uptake (μ atoms)	Phosphate esterified (μ moles)
Complete system	9.2	19.0
No glucose	9.2	19.0
No magnesium	9.0	19.0
No NaF	14.5	29.0
No hexokinase	7.3	14.0
No AMP	8.6	19.0
No L-glutamate	3.7	1.0

Citric acid was oxidized by sarcosome preparations only in the presence of TPN or of ATP and DPN. In the latter case presumably a synthesis of TPN had occurred. The preparations oxidized added pyruvate and acetate in the presence of a primer such as L-malic acid. It was not possible to demonstrate the oxidation of octanoic acid even in the presence of added cofactors (Table 8).

Oxidative phosphorylation in muscle

The endogenous respiration of whole muscle suspensions was found to be accompanied by the esterification of inorganic phosphate as was the oxidation of citric acid cycle intermediates. The components of the oxidative phosphorylation systems were investigated (Table 9). There is a small but significant effect of added hexokinase. It is presumed that there is present in the locust preparations a large amount of endogenous hexose and a powerful hexokinase (Leaback, 1953, private communication). This contrasts with the large

Table 11. *The effect of protein-containing solutions on oxidative phosphorylation by sarcosome preparations*

System and conditions as described in Table 10. 0.5 ml. of the supernatant from which the sarcosomes were isolated or 0.5 ml. of a yeast juice extract used. Sarcosomes added equivalent to 1.0 mg. N (NaF not added).

Expt.	Substrate	Additions	O ₂ uptake (μ atoms)	P esterified (μ moles)
1	Nil	Nil	0.5	Nil
	Nil	Supernatant	0.5	Nil
	Succinate	Nil	10.0	Nil
	Succinate	Supernatant	22.0	16.0
	Succinate	Boiled supernatant	15.5	6.1
	Succinate	Yeast juice	25.0	17.0
	Nil	Yeast juice	6.3	9.0
2	Nil	Nil	0.5	Nil
	L-Glutamate	Nil	3.3	Nil
	L-Glutamate	Supernatant	14.0	21.0
	L-Glutamate	Boiled supernatant	6.8	5.3
	L-Glutamate	Yeast juice	6.9	7.5
	Nil	Yeast juice	3.6	0.7
	α -Oxoglutarate	Nil	4.8	Nil
	α -Oxoglutarate	Supernatant	9.5	6.7
	α -Oxoglutarate	Boiled supernatant	7.7	7.1
	α -Oxoglutarate	Yeast juice	8.0	9.3

Table 12. *The effect of ethylenediaminetetraacetic acid and 2:4-dinitrophenol on oxidative phosphorylation by sarcosome preparations*

System and conditions as described in Table 10. Ethylenediaminetetraacetic acid (EDA) added to a final concentration of 0.001M; 0.5 ml. yeast juice and 2:4-dinitrophenol (DNP) final concn. 10^{-4} M used. Sarcosomes added equivalent to 1.0 mg. N (NaF not added).

Expt.	Substrate	Additions	O ₂ uptake (μ atoms)	P esterified (μ moles)
1	Nil	Nil	0.5	Nil
	α -Oxoglutarate	Nil	11.4	10.0
	α -Oxoglutarate	EDA	15.8	32.0
	α -Oxoglutarate	Yeast juice	16.5	37.0
	α -Oxoglutarate	DNP + EDA	11.0	0.2
2	Nil	Nil	0.5	Nil
	L-Glutamate	Nil	6.7	4.0
	L-Glutamate	EDA	10.0	29.0
	L-Malate	Nil	7.3	Nil
	L-Malate	EDA	16.5	38.0
	L-Malate	DNP + EDA	16.0	3.0

effects seen with rat-liver suspensions (Lardy & Wellman, 1952). The addition of sodium fluoride is found to inhibit the respiration of muscle suspensions both in the presence and absence of added citric acid cycle intermediates; there is, however, sometimes an increase in the P/O ratio, although it was found with *cis*-aconitate that the addition of fluoride gave the opposite effect. It will be seen from Table 10 that the P/O ratios obtained were of the same order as those obtained in mammalian tissue (Judah & Williams-Ashman, 1951).

The oxidative phosphorylation in sarcosomes was investigated. There was wide variation in the ability of preparations to esterify inorganic phosphate. The addition of the pyridine nucleotides, adenine nucleotides and myokinase singly or in combination failed to elicit oxidative phosphorylation.

As in the case of respiration, both supernatant fluid and yeast juice stimulated oxidative phosphorylation (Table 11) and again EDA substituted

satisfactorily for the crude extracts (Table 12). Slater & Lewis (1953) observed that 2:4-dinitrophenol (DNP) stimulated the respiration and increased the overall phosphate esterification of sarcosomes from *Calliphora erythrocephala* in the presence of hexokinase and glucose. This is in contrast to observations on mammalian mitochondria (Lardy & Wellman, 1952). In *Locusta migratoria* sarcosome preparations studied in the presence of hexokinase and glucose, DNP was without effect on the respiration but lowered the efficiency of oxidative phosphorylation (Table 12).

DISCUSSION

Suspensions of locust muscle in 0.2M sucrose show high rates of endogenous respiration. The addition of glycolytic or citric acid cycle intermediates either increases the rate of respiration or stabilizes the initial rate. The reactions α -oxoglutarate $\rightarrow$ succi-

nate and the synthesis of citric acid from pyruvate and oxaloacetate occur in muscle suspensions. The results of studies with malonate and arsenite indicate that the major part of the endogenous respiration passes through the citric acid cycle and the activity/mg. N values for citric acid cycle substrates give figures of the same order as those obtained for cell-free sucrose suspensions of rat liver.

The muscle suspensions were found to esterify inorganic phosphate and the level of esterification was increased by the addition of citric acid cycle intermediates. The suspensions contained enough endogenous hexose to accept phosphate groups from ATP. Enough hexokinase was present in the suspension and addition of yeast hexokinase only increased the esterification of inorganic phosphate by 25%.

Different sarcosome preparations showed great variation in their ability to oxidize citric acid cycle intermediates. Variation was also found to occur in oxidative phosphorylation. The rate of respiration of a preparation in the presence of a citric acid cycle intermediate proved to be no guide to its ability to phosphorylate. High rates of respiration often did not result in phosphate esterification whereas, in contrast, a low rate of respiration might well be associated with oxidative phosphorylation. It was also found that with a given sarcosome preparation there was substrate variation, e.g. with L-malate, oxidative phosphorylation might occur, whereas with the oxidation of succinate it did not. Sacktor (1953) has shown that the inability to demonstrate oxidative phosphorylation in insect preparations was not due to a rapid breakdown of ATP.

The addition of the sucrose supernatant fluid to the isolated sarcosomes was found to confer on the preparation the power of oxidative phosphorylation. Treatment of this supernatant at 100° for 2 min. followed by centrifuging caused some loss of the activity. This effect was also displayed by yeast juice. It was considered that these protein solutions were abstracting an inhibitor from the sarcosome preparations.

Calcium is a well known inhibitor of oxidative phosphorylation (Potter, 1947; Lehninger, 1949). Slater & Cleland (1953) had found large quantities of calcium present in mammalian heart preparations and used EDA to remove it. The addition of 0.001 M EDA to the locust sarcosomes gave preparations capable of oxidative phosphorylation. There are small variations in the effect of EDA. The maximum effective final concentration varied from one preparation to another, depending on whether the sarcosomal preparation was or was not capable of oxidative phosphorylation in the absence of EDA. However, in the range 3.3×10^{-4} – 10^{-3} M EDA not more than 10% inhibition of respiration was found and in the majority of cases increases of respiration

and of oxidative phosphorylation from 2 to 4 times were observed.

With sarcosome preparations in the presence of EDA the oxidation of citric acid cycle intermediates and of pyruvate and acetate was found to take place but we were unable to demonstrate manometrically the oxidation of octanoate. 2:4-Dinitrophenol was found to inhibit oxidative phosphorylation, in contrast to the effect reported by Slater & Lewis (1953) with insect sarcosomes, where increased phosphate esterification occurred in the presence of DNP.

Watanabe & Williams (1953) investigated the effects of various media on the size and organization of isolated sarcosomes of *Drosophila* and *Phormia*. Protein-containing solutions were extremely effective in preserving the sarcosomes but EDA was ineffective. These authors quote unpublished results of Sacktor which show that oxidative phosphorylation occurs with isolated sarcosomes only in the presence of proteins whereas 0.001 M EDA abolished the oxidation of α -oxoglutarate. Watanabe & Williams considered this to be in good agreement with their results.

According to the results reported in this paper, EDA substitutes for the proteins. These results with EDA confirm some of the findings of Lewis & Slater (1953) with *Calliphora erythrocephala* sarcosomes who reported oxidative phosphorylation with α -oxoglutarate as substrate in the presence of EDA.

SUMMARY

1. The oxidation of a number of citric acid cycle intermediates takes place in muscle suspensions and sarcosomes of *Locusta migratoria*.
2. Oxidation of citric acid by sarcosomes has been shown to require added triphosphopyridine nucleotide.
3. Oxidative phosphorylation has also been demonstrated in muscle suspensions and sarcosomes.

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The Titration Curve of Gelatin

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Titration curves have played a considerable part in both the determination of the amino acid composition of proteins and in studying their behaviour as acids and bases (Cohn & Edsall, 1943). A serious discrepancy was known to exist for many years between the analytically determined dicarboxylic acid content of gelatin (or collagen) and the values derived from titration. The discrepancy persisted even after it became clear that some of the dicarboxylic acid residues were present, in collagen and in certain types of gelatin, as amides. Improved determinations of the dicarboxylic acids showed that there had been errors in previous analyses. There remained, however, a discrepancy (Ames, 1952) in the content of histidine, the titration curve giving too high a value. In spite of recent improvements of methods of amino acid analysis, titration remains a valuable tool for studying the peculiarities of dissociating groups, especially when these are chemically labile; for example, where ionizing groups are blocked or modified by treatment with reagents, or where carboxylic groups are present in part as amides.

The present paper describes the experimental method and its application to two types of gelatin: this has enabled four of the five most important reactive groups to be estimated, namely carboxyl, imidazole, α -amino, guanidino.

EXPERIMENTAL

Materials. Alkali-processed ox-hide gelatin (1), isoelectric point $pI=4.92$. The gelatin was prepared commercially from ox hide, which had been soaked for several months in

a saturated solution of $\text{Ca}(\text{OH})_2$ at about 15° . After neutralization, gelatin was extracted from the hide using warm water ($ca. 60^\circ$).

Acid-processed pig skin gelatin (2), $pI=9.1$. The gelatin was prepared commercially from pig skin by extraction at $pH\ 3.5-4.0$, with warm water. No alkaline pre-treatment was used.

De-ionization. 4-6% (w/v) solutions of each of the two gelatins were de-ionized by passage through a mixed bed of Amberlite resins IR-120 and IRA-400 (Rohm and Haas Co., Philadelphia 5, Pa.) as described by Janus, Kenchington & Ward (1951). The pH of the solution leaving the resin bed gave the pI of the gelatin. The de-ionized solutions were set, dried and ground to give a coarse powder. The pH of the solution of the acid-processed gelatin was brought, after de-ionization, from 9.1 to $ca. 5.0$ by the addition of HCl, before drying and grinding. The ash content of the gelatins was reduced to less than 0.1% (w/w) from 2.5% for gelatin (1) and 0.5% for gelatin (2).

Preparation of solutions for titration. A weighed quantity of the de-ionized gelatin, the water content of which had been determined by drying for 18 hr. at 105° in the form of a thin film, was allowed to swell and then dissolved by gentle warming to 40° . Concentrations of 4.5% (w/v) were used. The concentration was checked by drying a weighed quantity of solution at 105° for 18 hr.

pH standardization. All pH measurements were made with Doran 'Alkacid' glass electrodes (L.S.B. Components Ltd., Stroud, Glos.) with a modified Cambridge pH meter (Kenchington & Ward, 1954), which enabled several electrodes to be used in succession, without re-standardization, for the duration of a titration-curve determination. The following solutions were used for standardization, all pH values being for 40° . (I) 0.1 N-HCl, pH 1.08 (Hitchcock, 1931); (II) 0.05 N potassium hydrogen phthalate, pH 4.03 (Bates, 1948); (III) 0.1 N-KOH, pH 12.38.

The value for 0.1 N-KOH is the mean of measurements obtained in this laboratory.