Staehelin, M. (1958). *Biochim. biophys. Acta*, **29**, 43.
Suit, J. C. (1963). *Biochim. biophys. Acta*, **72**, 488.
Taki, M., Kondo, N. & Osawa, S. (1962). *Biochim. biophys. Acta*, **55**, 416.
Volkin, E. (1962). *Fed. Proc.* **21**, 112.
Volkin, E. (1963). In *Molecular Genetics*, vol. 1, p. 271. Ed. by Herbert Taylor, J. New York: Academic Press Inc.
Wade, H. E. (1961). *Biochem. J.* **78**, 457.
Wade, H. E. & Lovett, S. (1961). *Biochem. J.* **81**, 319.
Weiss, S. B. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 1020.
Williams, E. J., Shan-ching, S. & Laskowski, M. (1961). *J. biol. Chem.* **236**, 1130.
Ycas, M. & Vincent, W. S. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 804.

Biochem. J. (1964), **93**, 128

Pyruvate Oxidation and the Permeability of Housefly Sarcosomes

BY S. G. VAN DEN BERGH
Laboratory of Physiological Chemistry, University of Amsterdam, Amsterdam, The Netherlands

(*Received* 21 *November* 1963)

It has been shown by Van den Bergh & Slater (1962) that pyruvate is probably the most important physiological substrate for respiration and energy production in housefly flight-muscle mitochondria (sarcosomes). Isolated sarcosomes rapidly oxidize added pyruvate (Gregg, Heisler & Remmert, 1960), and this oxidation is accompanied by a rapid esterification of P_i and is under the control of the ADP and P_i concentrations. Evidence was presented that the low respiratory rates found with most substrates (e.g. the Krebs-cycle intermediates and glutamate) are caused by permeability barriers for these substrates in the sarcosomes.

In the present study the oxidation of pyruvate by isolated housefly sarcosomes was further investigated. During this work additional evidence was found in support of the suggested permeability barrier in the sarcosomes and information was obtained on the pathway of glutamate oxidation in housefly sarcosomes.

The work reported in the present paper was part of a Ph.D. Thesis, which was published in December 1962 (Van den Bergh, 1962).

METHODS AND MATERIALS

Isolation of sarcosomes. Sarcosomes were isolated from the thoracic muscle of the housefly, *Musca domestica* L., as described by Van den Bergh & Slater (1962). The medium used for the isolation was 0·154 M-KCl–1 mM-EDTA, pH 7·4.

Measurement of respiration and oxidative phosphorylation. The methods have been described by Van den Bergh & Slater (1962). The standard reaction medium used in all experiments contained a mixture of KCl (15 mM), EDTA (2 mM), $MgCl_2$ (5 mM) and tris (50 mM), adjusted to pH 7·5 by the addition of HCl, potassium phosphate buffer, pH 7·5 (30 mM), ADP (1 mM), glucose (30 mM) and hexokinase (150–180 Cori units). Unless otherwise stated, the respiratory substrate for the sarcosomes was 20 mM-pyruvate with or without 10 mM-L-malate. The gas phase in all experiments was air. In some experiments, the oxygen uptake was measured with an oxygen polarograph (Oxygraph, manufactured by Gilson Medical Electronics, Middleton, Wis., U.S.A.). The reaction medium in these polarographic measurements was the same as in the manometric experiments, but the reaction volume was 2·0 ml.

Qualitative demonstration of transaminase activity. The amino acid and the oxo acid (20 μmoles of each) were incubated in a medium containing 50 mM-potassium phosphate buffer, pH 7·5, 50 μg. of pyridoxal phosphate and 1 μg. of antimycin. After the addition of the enzyme preparation the volume was 1 ml. The reaction was run, with occasional shaking, for 45 min. at room temperature and was stopped by heating the reaction mixture for 2 min. at 100°. The protein was removed by centrifuging for 5 min. at 3000*g* and the deproteinized reaction mixture was analysed for its amino acid content by paper chromatography.

Analytical procedures. Protein determinations were made by the biuret method as described by Cleland & Slater (1953), with crystalline egg albumin as a standard.

Pyruvate was determined spectrophotometrically with NADH and lactate dehydrogenase by measuring the decrease of extinction at 340 mμ. For the determination of the sum of oxaloacetate and pyruvate, malate dehydrogenase was added to this system.

Succinate was determined manometrically as described by Borst (1962).

Glutamate was determined manometrically with glutamate decarboxylase (Gale, 1945). The reaction medium contained 150 mM-sodium acetate buffer, pH 5·0, and up to 5 μmoles of glutamate; 4 mg. of glutamate decarboxylase, dissolved in 0·1 ml. of 0·1 M-sodium acetate buffer, pH 5·0, was placed in the side arm. The final volume was 1·1 ml., the gas phase air and the temperature 25°. After temperature equilibration the enzyme was added to the main compartment of the manometer flask and readings were taken until a constant level was reached (10–20 min.).

α-Oxoglutarate was determined by the spectrophotometric method of Slater & Holton (1953).

Citrate and isocitrate were determined spectrophotometrically by conversion into α-oxoglutarate (Ochoa, 1948). To the reaction medium in the test cuvette (1·90 ml.) con-

taining 75 μmoles of tris–HCl buffer, pH 7·5, 5 μmoles of $MgCl_2$, 0·33 μmole of $NADP^+$ and up to 0·2 μmole of citrate + isocitrate, 0·05 ml. of a solution of isocitrate dehydrogenase was first added. When the reaction was finished, 0·05 ml. of a solution of aconitase was added. The amounts of NADPH formed in these reactions are equivalent to the amuntts of isocitrate and citrate (+*cis*-aconitate) respectively originally present.

Paper chromatography of amino acids was performed by the method described by Borst (1962).

Materials. Chemicals were obtained from the following sources: ADP, tris, NAD^+, $NADP^+$, pyridoxal phosphate, L-proline and α-oxoglutarate from Sigma Chemical Co.; succinate, pyruvate and oxaloacetate from C. F. Boehringer und Soehne G.m.b.H.; L-malate from Nutritional Biochemicals Corp.; egg albumin from Mann Research Laboratories; Amytal from Amsterdamsche Chinine Fabriek. Antimycin was kindly provided by the Kyowa Fermentation Co. and oligomycin by Upjohn Chemical Co. L-Isocitrate was prepared by the method of Pucher, Abrahams & Vickery (1948). NADH was prepared from NAD^+ by the procedure of Slater (1953). All other chemicals were supplied by British Drug Houses Ltd. Glass-redistilled water was used in preparing all solutions.

Lactate dehydrogenase, alcohol dehydrogenase, malate dehydrogenase and glutamate dehydrogenase were obtained from C. F. Boehringer und Soehne G.m.b.H.; glutamate decarboxylase was from Sigma Chemical Co. Hexokinase was prepared from yeast according to the procedure of Darrow & Colowick (1961), the final crystallization step being omitted. Isocitrate dehydrogenase and aconitase were prepared from pig heart by the methods of Grafflin & Ochoa (1950) and Buchanan & Anfinsen (1949) respectively.

RESULTS

Effect of 2,4-dinitrophenol on pyruvate oxidation. Remmert, Heisler & Gregg (1961) reported that dinitrophenol stimulated the respiration of housefly flight-muscle sarcosomes only in the presence of ATP (see also Gregg, Johnson, Heisler & Remmert, 1964). It was found, however, that 0·1 mM-dinitrophenol alone could fully release respiration when α-glycerophosphate was the respiratory substrate for housefly sarcosomes (Van den Bergh, 1962). The experiments reported in Table 1 show that the oxidation of pyruvate + malate, controlled by the absence of ADP or P_i, indeed could not be stimulated by the addition of dinitrophenol, in contrast with the respiration controlled by the absence of hexokinase, which was completely released by dinitrophenol. Two explanations for these surprising findings are possible. First, it is possible that dinitrophenol cannot abolish the need for ADP and P_i during pyruvate oxidation. Secondly, as concluded by Remmert *et al.* (1961), it may be assumed that ATP is certainly necessary for the oxidation of pyruvate in the presence of dinitrophenol, but possibly also in its absence. It should be noted from Table 2 that 0·1 mM-dinitrophenol, the optimum concentration for the stimulation of

Table 1. *Effect of 2,4-dinitrophenol on the oxidation of pyruvate + malate by isolated housefly sarcosomes*

The standard reaction medium contained KCl (15 mM), EDTA (2 mM), $MgCl_2$ (5 mM) and tris (50 mM), adjusted to pH 7·5 with HCl, potassium phosphate buffer, pH 7·5 (30 mM), ADP (1 mM), glucose (30 mM), pyruvate (20 mM), L-malate (10 mM) and hexokinase (150–180 Cori units).

Omission from standard reaction medium	q_{O_2} Without dinitrophenol	q_{O_2} With dinitrophenol (0·1 mM)
None	625	613
ADP	36	39
P_i	33	40
Hexokinase	93	592
ADP + hexokinase	54	54
P_i + hexokinase	39	43
ADP + P_i	35	33

Table 2. *Effect of increasing concentrations of 2,4-dinitrophenol on the oxidation of pyruvate + malate by isolated housefly sarcosomes*

Experimental details are given in the text.

Concn. of dinitrophenol (μM)	q_{O_2} Without hexokinase	q_{O_2} With hexokinase	P:O ratio with hexokinase
0	91	732	2·53
5	122	745	2·28
10	159	732	2·12
20	223	—	—
50	395	715	1·79
80	668	—	—
100	714	708	0·97
120	704	—	—
140	511	—	—
150	—	509	0·52
170	364	—	—
200	247	312	0·12
250	123	—	—
300	78	61	0
400	—	75	0
500	72	35	0

respiration in the absence of hexokinase, did not completely abolish phosphorylation in the presence of P_i and phosphate acceptor. Higher concentrations of dinitrophenol stopped all phosphorylation, but at the same time greatly decreased respiration.

Indications that ADP and P_i play a role in pyruvate oxidation different from that in respiratory-chain phosphorylation were obtained when the relationship between the respiratory rate and the concentrations of these compounds was determined. The ADP concentration giving half-maximal respiration was about 0·1 mM (Fig. 1), where as 8 mM-phosphate gave half-maximal respiration (Fig. 2). These concentrations are very much higher than those obtained with mitochondrial pre-

parations from vertebrate sources (Slater & Holton, 1953; Chance & Williams, 1954, 1955; Chance & Connelly, 1957; Azzone & Ernster, 1961; Chance & Hagihara, 1961). Moreover, it was found that the concentrations of ADP and P_i required for optimum oxidation of pyruvate by housefly sarcosomes were not affected by the presence of 0·1 mM-dinitrophenol.

The presence of 20 mM-malonate inhibited the oxidation of pyruvate + malate by about 80 %. This showed how small the contribution of the added malate was in the supply of oxaloacetate necessary for the oxidation of pyruvate. Evidently by far the greatest part of the oxaloacetate is regenerated via the Krebs cycle from pyruvate. In fact, housefly sarcosomes respired equally rapidly with pyruvate in the presence and in the absence of added malate.

Some properties of the oxidation of pyruvate by housefly sarcosomes are summarized in Table 3. As expected, malonate inhibited pyruvate oxidation almost completely. The respiratory control, induced by the absence of ADP or P_i, was slightly greater than in the presence of malate (average control ratios 23 and 27, as against 19 and 24), and could again not be released by the addition of dinitrophenol. The inhibition by oligomycin (Lardy,

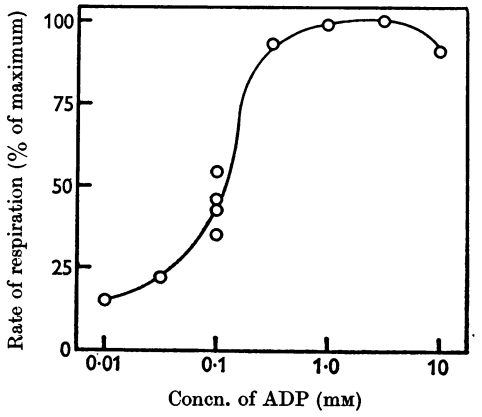

Fig. 1. ADP affinity for oxidation of pyruvate + malate by housefly sarcosomes. Experimental details are given in the text. The values for 0·1 mM-ADP were obtained in five different experiments.

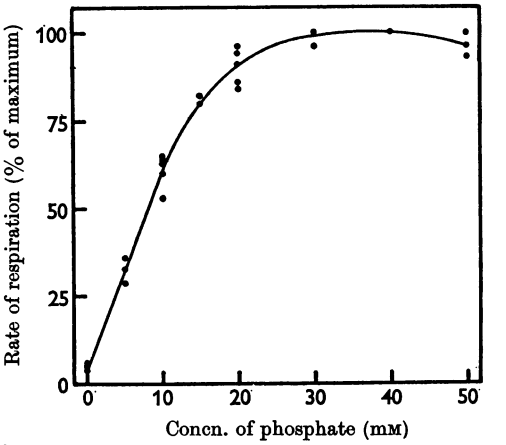

Fig. 2. Phosphate affinity for oxidation of pyruvate + malate by housefly sarcosomes. Experimental details are given in the text. The values were obtained in five different experiments.

Table 3. *Oxidation of pyruvate by isolated housefly sarcosomes*

Experimental details are given in the text. The values are given as means and ranges, with the numbers of experiments in parentheses.

Omission from standard reaction medium	Addition to standard reaction medium	Dinitrophenol (0·1 mM)	q_{O_2} Mean	q_{O_2} Range	P:O ratio Mean	P:O ratio Range
None	None	−	678	500–919 (28)	2·38	2·07–2·70 (17)
		+	631	428–927 (15)	0·77	0·57–1·17 (5)
P	None	−	24	14–31 (8)	—	—
		+	27	23–34 (7)	—	—
ADP	None	−	28	16–44 (6)	0·68	0·63–0·73 (2)
		+	30	15–48 (4)	—	—
Hexokinase	None	−	87	75–96 (7)	—	—
		+	477	408–580 (9)	—	—
None	Oligomycin*	−	18	11–27 (5)	0·17	0·15–0·21 (3)
		+	291	240–347 (5)	0·18	0·15–0·20 (3)
None	Malonate (20 mM)	−	27	21–29 (4)	2·45	2·26–2·60 (4)
None	Amytal (1·75 mM)	−	7	6–9 (3)	—	—

* 2–3 μg. of oligomycin was added/mg. of sarcosome protein.

Table 4. *Inability of added Krebs-cycle intermediates to restore inhibited pyruvate oxidation*

Experimental details are given in the text. The omissions from and additions to the standard reaction mixture are given. The following concentrations were used: succinate (60 mM), fumarate (20 mM), L-malate (10 mM), oxaloacetate (20 mM), malonate (20 mM) and dinitrophenol (0·1 mM).

	Omission from standard reaction medium	Additions to standard reaction medium	q_{O_2}
Expt. A	None	None	788
	None	Malonate	28
	None	Malonate + fumarate	143
	None	Malonate + L-malate	177
	None	Malonate + oxaloacetate	137
Expt. B	None	Dinitrophenol	927
	P_i	Dinitrophenol	31
	P_i	Dinitrophenol + succinate	155
	P_i	Dinitrophenol + fumarate	86
	P_i	Dinitrophenol + L-malate	70
	P_i	Dinitrophenol + oxaloacetate	63
Expt. C	None	Dinitrophenol	610
	ADP	Dinitrophenol	22
	ADP	Dinitrophenol + succinate	80
	ADP	Dinitrophenol + fumarate	57
	ADP	Dinitrophenol + L-malate	51
	ADP	Dinitrophenol + oxaloacetate	34

Johnson & McMurray, 1958; Huijing & Slater, 1961) was also higher than in the presence of malate (average inhibition 97 %, as against 91 %).

Since added malate cannot penetrate into the sarcosomes (Van den Bergh & Slater, 1962), it cannot support pyruvate oxidation if the operation of the Krebs cycle is inhibited by malonate.

It is also clear now why ADP and P_i are required for the oxidation of pyruvate + malate in the presence of dinitrophenol. For, in the presence of dinitrophenol, the omission of ADP or P_i leads to inhibition of the overall operation of the Krebs cycle. Under these conditions the step from α-oxoglutarate to succinate is selectively inhibited, since the substrate-linked phosphorylation involved in this step (Hunter, 1949) is not uncoupled by dinitrophenol (Hunter, 1951; Judah, 1951).

Endogenous substrates of sarcosomes. In experiments with the oxygen polarograph it was found that housefly sarcosomes oxidized added pyruvate without a demonstrable lag period. Therefore an initial amount of oxaloacetate must either be present in the sarcosomes or it must rapidly be formed from some precursor in the sarcosomes.

To investigate this point, we extracted the sarcosomes essentially by the technique described by Bellamy (1962). No detectable amounts of succinate, α-oxoglutarate, glutamate, oxaloacetate or pyruvate could be found in the extracts. However, in agreement with Bellamy's (1962) findings on locust sarcosomes, a large amount of endogenous citrate could be demonstrated. In two extracts we found 5·07 and 6·78 μmoles of citrate/g. of protein respectively. In addition, 0·34 and 0·57 μmole of isocitrate were found.

Low permeability of the sarcosomes. Bücher, Klingenberg & Zebe (1958) reported that the oxidation of added Krebs-cycle intermediates by locust flight-muscle sarcosomes proceeded at a high rate when these substrates were added with pyruvate. It must be concluded, then, that housefly sarcosomes are different from locust sarcosomes in this respect, since the oxidation of external Krebs cycle intermediates is not increased by the addition of pyruvate. Three types of evidence for this conclusion can be presented:

(A) It has been shown above that added malate cannot prevent the inhibition of pyruvate oxidation caused either by the presence of malonate or by omission of P_i (or ADP) in the presence of dinitrophenol. In Table 4 it is shown that, likewise, the addition of succinate, fumarate or oxaloacetate cannot restore pyruvate oxidation inhibited by interruption of the Krebs cycle.

(B) In Table 5 two experiments are shown, in which the oxidation of a number of Krebs-cycle intermediates and of glutamate is studied in the absence and in the presence of pyruvate. By direct analysis it could be demonstrated that the oxidation of these substrates is either unaffected or decreased by the addition of pyruvate. The enhanced glutamate disappearance in the presence of pyruvate represents only an apparent contradiction to this conclusion, since a substantial amount of alanine was formed during the reaction, as could be detected by paper chromatography. Transamin-

Table 5. *Effect of the addition of pyruvate on the oxidation of other substrates*

All flasks contained the standard reaction medium (with 50 mM-glucose and 50 mM-phosphate, instead of 30 mM), 0·445 mg. (Expt. A) or 0·480 mg. (Expt. B) of sarcosome protein, and substrates as indicated. After 30 min. the reaction was stopped with $HClO_4$, which was removed again as $KClO_4$. The deproteinized reaction medium was then assayed for the substrates originally added. The substrate concentrations were: pyruvate, 10 mM; other substrates, 5 mM.

	Substrates	q_{O_2}	Δ(O) (μg.atoms)	Δ (pyruvate) (μmoles)	$\frac{\Delta(O)}{\Delta \text{ (pyruvate)}}$ ratio	Substrate disappearance (μmoles)
Expt. A	Pyruvate	685	13·64	2·84	4·81	—
	α-Oxoglutarate	16	0·32	—	—	0·26 (α-oxoglutarate)
	Pyruvate + α-oxoglutarate	677	13·49	2·75	4·90	0·20 (α-oxoglutarate)
	L-Glutamate	24	0·48	—	—	0·39 (glutamate)
	Pyruvate + L-glutamate	678	13·50	3·64	3·71	1·16 (glutamate)
	Citrate	21	0·42	—	—	0·58 (citrate)
	Pyruvate + citrate	658	13·10	2·60	5·05	0·58 (citrate)
	Pyruvate + L-malate	685	13·66	2·79	4·89	—
Expt. B	Pyruvate	753	16·16	3·39	4·77	—
	L-Isocitrate	26	0·56	—	—	0·57 (isocitrate)
	Pyruvate + L-isocitrate	772	16·57	3·24	5·11	0·57 (isocitrate)
	Succinate	31	0·67	—	—	0·48 (succinate)
	Pyruvate + succinate	768	16·41	3·32	4·95	0·53 (succinate)

ation between glutamate and pyruvate must be held responsible for the increased disappearance of both glutamate (0·77 μmole) and pyruvate (0·80 μmole). No aspartate was detectable on the paper chromatogram (see below).

(C) Under various conditions the rate of pyruvate oxidation markedly declined during the reaction period. This phenomenon was observed when either high concentrations of arsenate were present or when dinitrophenol and oligomycin were added to the reaction medium. It could be demonstrated that the decreasing respiration was caused by leaking out of the sarcosomes of Krebs-cycle intermediates under these conditions. Yet the decline in the rate of respiration could not be prevented by the addition of Krebs-cycle intermediates. In fact, only two compounds could prevent the respiratory decline, namely glutamate and aspartate (Fig. 3). In the experiment with added glutamate, it could be demonstrated that alanine was formed during the reaction (but, again, no aspartate), whereas in the experiment with added aspartate the formation of glutamate and alanine was detectable.

Glutamate oxidation and transamination in housefly sarcosomes. From Fig. 3 and Table 5 it is clear that added glutamate is oxidized very slowly by housefly sarcosomes. This may be explained either by a low activity of the enzymes responsible for the oxidation of glutamate or by an insufficient permeability of the sarcosomes towards glutamate. But in the same experiments it has become clear that the glutamate–pyruvate transaminase in the sarcosomes is very active towards external glutamate. Moreover, Fig. 3 shows that one of the products of the transamination, namely α-oxoglutarate, is formed inside the sarcosomes, or at least in such a place as to enable it to restore the

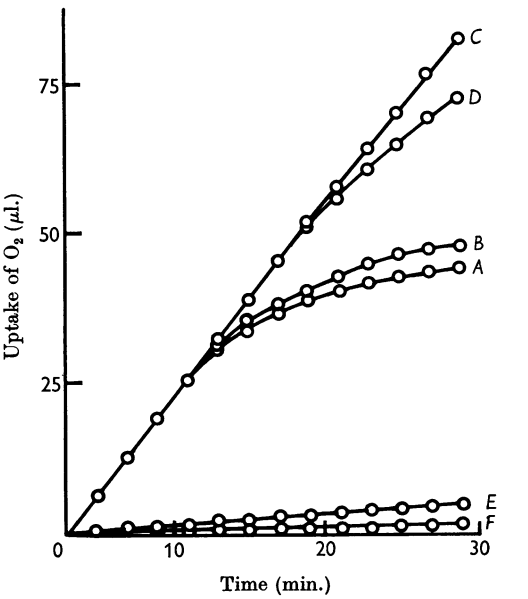

Fig. 3. Prevention of the decline of respiration with pyruvate by the addition of glutamate or aspartate. All flasks contained the standard reaction medium, sarcosomes (0·56 mg. of protein), dinitrophenol (0·1 mM), arsenate (30 mM), oligomycin (3 μg.) and the following substrates (each 20 mM): *A*, pyruvate; *B*, pyruvate + α-oxoglutarate; *C*, pyruvate + glutamate; *D*, pyruvate + aspartate; *E*, glutamate; *F*, aspartate.

declining pyruvate oxidation, whereas external α-oxoglutarate is inactive in this respect.

The similar effect of aspartate might be attributed to an aspartate–pyruvate transamination, which has often been observed in insect prepara-

tions (Kilby & Neville, 1957; Fukuda, 1957; Price & Moriya, 1962), but it has never been demonstrated that the reaction was catalysed by a single enzyme. In the present work aspartate–pyruvate-transaminase activity could not be detected in either intact housefly sarcosomes or in sarcosomes that had been treated with the ultrasonic disintegrator. If, however, catalytic amounts (as low as 0·01 mM) of glutamate or α-oxoglutarate were added, aspartate and pyruvate were formed from alanine and oxaloacetate, and vice versa. It must be assumed, then, as suggested by Kilby & Neville (1957), that transamination between aspartate and pyruvate resulted from linking glutamate–oxaloacetate transaminase and glutamate–pyruvate transaminase.

These two enzymes were demonstrated to be present in housefly sarcosomes (in agreement with Price, 1961) as well as in the supernatant fluid. Whereas the highest glutamate–oxaloacetate-transaminase activity was present in the supernatant, the glutamate–pyruvate-transaminase activity resided mainly in the sarcosomes.

The activity of the glutamate–pyruvate transaminase of housefly sarcosomes towards externally added substrates can also be seen from the experiment shown in Table 6. The combination of α-oxoglutarate and alanine as substrates for housefly sarcosomes led to a respiratory rate far in excess of the sum of the rates with the separate substrates.

Availability of adenosine triphosphate, generated by substrate-level phosphorylation, for the dinitrophenol-stimulated adenosine triphosphatase. Azzone & Ernster (1961) have suggested that in rat-liver mitochondria the ATP generated by the α-oxoglutarate-linked substrate-level phosphorylation is not readily available to the dinitrophenol-stimulated adenosine triphosphatase (see, however, Charles, Tager & Slater, 1963). The experiment shown in Fig. 4 shows that this conclusion of Azzone & Ernster (1961) does not hold for housefly sarcosomes. In the absence of hexokinase, the addition of dinitrophenol could restore pyruvate oxidation (cf. Tables 1 and 3). Evidently, the dinitrophenol-stimulated adenosine triphosphatase could provide the substrate-linked phosphorylation with sufficient ADP for optimum operation. In separate experiments it was found that oligomycin completely inhibited the dinitrophenol-stimulated as well as the Mg^{2+} ion-stimulated adenosine triphosphatase of the sarcosomes (Van den Bergh, 1962). Therefore, when oligomycin was added, the dinitrophenol-stimulated pyruvate oxidation declined sharply as soon as all the available ADP was phosphorylated. In the standard reaction medium 1 μmole of ADP was always included. Since the break in the curve occurred after about 71 μl. of oxygen had been consumed, the observed P:O ratio is 0·16, in good agreement with the value obtained in experiments with added hexokinase (Table 3). From the fact that in the absence of oligomycin there is no break in the curve, it can be concluded that the ATP generated by the substrate-linked phosphorylation is readily available for the dinitrophenol-stimulated adenosine triphosphatase of housefly sarcosomes. (Glutamate was added in this experiment to prevent the decline in respiratory rate that was normally observed when pyruvate was oxidized in the presence of dinitrophenol and oligomycin.)

Table 6. *Oxidation of α-oxoglutarate and alanine by housefly sarcosomes*

Experimental details are given in the text. The concentration of each substrate was 20 mM.

Substrates	q_{O_2}
α-Oxoglutarate	17
L-Alanine	4
α-Oxoglutarate + L-alanine	177

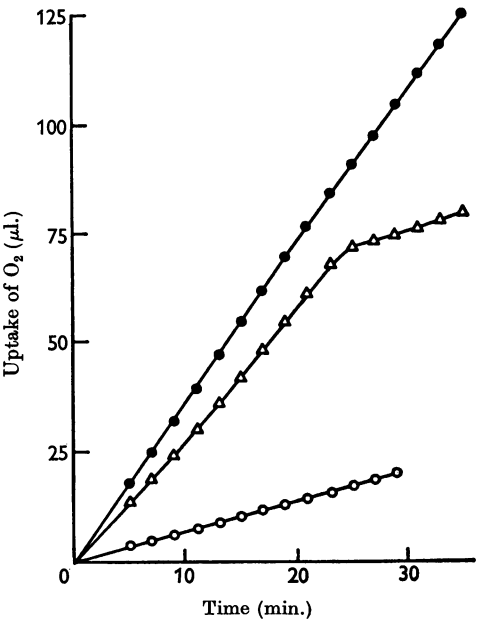

Fig. 4. Effect of oligomycin on the dinitrophenol-stimulated oxidation of pyruvate + glutamate in the absence of hexokinase. All flasks contained the standard reaction medium (without hexokinase), pyruvate (20 mM), L-glutamate (20 mM) and sarcosomes (0·387 mg. of protein), with the following additions: ○, none; ●, dinitrophenol (0·1 mM); △, dinitrophenol (0·1 mM) and oligomycin (1 μg.).

DISCUSSION

Pyruvate oxidation without added primers. Sarcosomes, isolated from the thoracic muscles of the housefly, have been shown to catalyse the complete oxidation of pyruvate to carbon dioxide and water. The inhibition of this oxidation by malonate proves that it occurs by way of the Krebs cycle.

Although it has been amply demonstrated that the mitochondrial fraction of cells contains all the enzymes necessary for the oxidation of pyruvate, most isolated mitochondria oxidize pyruvate poorly unless succinate, fumarate or malate is added. Some isolated mitochondria oxidize pyruvate without added primers by carboxylating pyruvate to malate or oxaloacetate. Two enzymes have been described that can bring about such a carboxylation [malic enzyme by Ochoa, Mehler & Kornberg (1948) and pyruvate carboxylase by Utter & Keech (1963)], and others are known to work with phosphoenolpyruvate (Bandurski & Greiner, 1953; Utter & Kurahashi, 1954; Siu & Wood, 1962), but it appears that these enzymes are not operating at an appreciable rate in most isolated mitochondria. As pointed out above, the almost complete malonate-sensitivity of pyruvate oxidation in our housefly sarcosomes demonstrated that carboxylation reactions were very slow, if present at all, under the conditions of the present experiments.

Apart from housefly sarcosomes, no mitochondrial preparations from animal sources have been reported to oxidize pyruvate in the absence of added primers or of a carboxylating system. Plant mitochondria also need catalytic amounts of Krebs-cycle intermediates to initiate pyruvate oxidation (Millerd, 1956).

It is clear that four conditions have to be fulfilled for the oxidation of pyruvate in the absence of added primers or of an active carboxylating system:

(1) The operation of all the steps of the Krebs cycle must be very rapid.

(2) No side reactions may occur, that drain away intermediates of the cycle.

(3) Oxaloacetate or some precursor of it must be present in the sarcosomes to initiate pyruvate oxidation. This condition is fulfilled in housefly sarcosomes by the presence of a high content of endogenous citrate. The occurrence of high citrate concentrations in insects has often been observed (see, e.g., Levenbook & Hollis, 1961), but no physiological function has been found.

(4) No Krebs-cycle intermediates may leak out of the sarcosomes during the operation of the cycle. Since the intermediates play the role of catalysts in pyruvate oxidation, a decrease in their concentration leads to a decreased rate of pyruvate oxidation.

Physiological function for the low permeability of the sarcosomes. Van den Bergh & Slater (1962) showed that Krebs-cycle intermediates cannot freely enter the sarcosomes, and in the present paper strong additional evidence for a very limited permeability of the sarcosomes is presented. To date no physiological function for this low permeability of the sarcosomes, experimentally demonstrated by the limited inward migration of Krebs-cycle intermediates, could be found. The fourth condition listed above suggests, however, that the true physiological function of the permeability barrier in the sarcosomes may be the prevention of the outward leakage of Krebs-cycle intermediates from the sarcosomes.

As pointed out above, under various conditions Krebs-cycle intermediates tended to leak out of the sarcosomes.

It is not clear what caused this loss of Krebs-cycle intermediates from the sarcosomes. Conditions that promoted the leaking of the Krebs-cycle intermediates from the sarcosomes failed to promote the penetration of added substrates into the sarcosomes, as could be demonstrated by the inability of added Krebs-cycle intermediates to prevent the declining rate of pyruvate oxidation (Fig. 3).

Substrate-level phosphorylation linked with pyruvate oxidation. Ochoa (1943) indicated that 15 phosphorylations occur for each molecule of pyruvate oxidized. Only one of these is substrate-linked (Hunter, 1949), but, since the oxidation of α-oxoglutarate to succinate is an obligatory step in the oxidation of pyruvate (and not just further oxidation of a product of pyruvate oxidation), it follows that the properties of this step of the Krebs cycle are properties of pyruvate oxidation. Consequently, the mechanism of the phosphorylation concomitant with the oxidation of pyruvate has all the properties of substrate-linked phosphorylation, such as the inability of dinitrophenol to stimulate respiration in the absence of ADP or P_i. It is clear, therefore, that the factor required for the stimulation by dinitrophenol of pyruvate oxidation in the absence of ADP is not ATP, as suggested by Remmert *et al.* (1961), but ADP, derived from the added ATP by the dinitrophenol-stimulated adenosine triphosphatase of the sarcosomes.

The experiments described in the present paper refute the conclusion of Sacktor & Cochran (1958) that no substrate-linked phosphorylation was coupled to the oxidation of α-oxoglutarate in housefly sarcosomes.

Since the respiratory control found in the oxidation of pyruvate + malate was the only experimental foundation for the conclusion (Van den Bergh & Slater, 1962) that respiration in housefly sarcosomes is tightly coupled to phosphorylation, it

might now be argued that all respiratory control, demonstrated experimentally, is caused by the requirement of the substrate-linked phosphorylation step for ADP and P_i, and that respiratory-chain phosphorylation is only loosely coupled. However, Table 3 shows the extensive inhibition of pyruvate oxidation by oligomycin (average inhibition 97 %). Since oligomycin does not affect substrate-linked phosphorylation, this inhibition is a reliable criterion for the tightness of the coupling of respiratory-chain phosphorylation in housefly sarcosomes (cf. Huijing & Slater, 1961).

Amino acid oxidation by housefly sarcosomes. Work in a number of Laboratories (Krebs & Bellamy, 1960; Borst & Slater, 1960; Chappell & Greville, 1961; Jones & Gutfreund, 1961; Borst, 1962) has shown that, with mitochondria from a variety of animal tissues, glutamate is oxidized mainly or exclusively via a pathway initiated by transamination with oxaloacetate and resulting in the formation of aspartate. In this Laboratory aspartate has never been found as a product of glutamate oxidation with housefly sarcosomes. Yet the presence of glutamate–oxaloacetate transaminase in the sarcosomes was established and its activity towards external glutamate and oxaloacetate was demonstrated. These experiments show clearly that the oxaloacetate that is formed endogenously in the sarcosomes is not readily available for transamination with external glutamate. This conclusion is supported by the observation (Fig. 3) that added glutamate supports pyruvate oxidation when the amount of endogenous Krebs-cycle intermediates becomes rate-limiting. It must, therefore, be concluded that glutamate oxidation in isolated housefly sarcosomes occurs via the classical pathway involving glutamate dehydrogenase. The presence of this enzyme in the sarcosomes could easily be demonstrated, since glutamate was formed when the sarcosomes were incubated in the standard reaction medium with 20 mM-α-oxoglutarate and 20 mM-ammonium chloride.

The average q_{O_2} (μl. of oxygen consumed/mg. of sarcosome protein/hr.) observed with glutamate as respiratory substrate was 21. Of the other amino acids tested, only proline could be oxidized with q_{O_2} values higher than 10, and this never exceeded 32. For this reason we cannot agree with Winteringham's (1958) suggestion that free amino acids, present in high concentrations in the haemolymph (blood) and in the thoracic tissues of insects, may act as energy-furnishing reserves for flight. [The same role has been suggested for proline in tsetse flies by Bursell (1963).] If amino acids have a function in energy production for flight activity, their only possible contribution could be the supply of Krebs-cycle intermediates by the transamination of glutamate or aspartate with pyruvate. In doing so they do not supply oxidizable substrates, but cofactors for the oxidation of pyruvate. Moreover, they do so at the expense of readily oxidizable substrate, since one molecule of pyruvate is converted into alanine for every molecule of Krebs-cycle intermediate formed in this way.

Another remarkable conclusion that may be drawn from the experiments described in the present paper (e.g. Fig. 3 and Table 6) is that added substrates that cannot freely penetrate towards their oxidase systems in the sarcosomes are readily available for the transaminases of the sarcosomes. In fact, the transaminases seem to operate as a selective transport mechanism through the permeability barrier in the sarcosomes.

SUMMARY

1. Housefly sarcomes have been shown to oxidize added pyruvate by way of the Krebs cycle. This oxidation occurred in the absence of added Krebs-cycle intermediates and in the absence of carboxylating reactions that might have formed malate or oxaloacetate from the added pyruvate. Various conditions that have to be fulfilled before this unique phenomenon can occur are discussed.

2. The inability of 2,4-dinitrophenol to stimulate pyruvate oxidation in the absence of adenosine diphosphate or inorganic phosphate is explained by the requirement for these compounds of the substrate-level phosphorylation coupled to the oxidation of α-oxoglutarate.

3. Additional evidence is presented in support of the suggested permeability barrier in the sarcosomes towards externally added substrates. It is suggested that the physiological function of this barrier might be to prevent the Krebs-cycle intermediates from leaking out of the sarcosomes.

4. Isolated housefly sarcosomes oxidize glutamate via the glutamate-dehydrogenase pathway, although glutamate–oxaloacetate transaminase is present in the sarcosomes and is active towards external glutamate and oxaloacetate.

The author thanks Professor E. C. Slater for stimulating discussions and helpful advice and Dr W. C. Hülsmann for generous gifts of L-isocitrate, isocitrate dehydrogenase and aconitase. The expert technical assistance of Miss Betty Kelder is gratefully acknowledged. This work was supported in part by a grant from the Life Insurance Medical Research Fund.

REFERENCES

Azzone, G. F. & Ernster, L. (1961). *J. biol. Chem.* **236**, 1501.

Bandurski, R. S. & Greiner, C. M. (1953). *J. biol. Chem.* **204**, 781.

Bellamy, D. (1962). *Biochem. J.* **82**, 218.

Borst, P. (1962). *Biochim. biophys. Acta*, **57**, 256.

Borst, P. & Slater, E. C. (1960). *Biochim. biophys. Acta*, **41**, 170.
Buchanan, J. M. & Anfinsen, C. B. (1949). *J. biol. Chem.* **180**, 47.
Bücher, T., Klingenberg, M. & Zebe, E. C. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, vol. 12, p. 153. London: Pergamon Press Ltd.
Bursell, E. (1963). *J. Insect Physiol.* **9**, 439.
Chance, B. & Connelly, C. M. (1957). *Nature, Lond.*, **179**, 1235.
Chance, B. & Hagihara, B. (1961). *Symp. Intracellular Respiration: Phosphorylating and Non-phosphorylating Oxidation Reactions; Proc. 5th int. Congr. Biochem., Moscow*, vol. 5, p. 19. London: Pergamon Press Ltd.
Chance, B. & Williams, G. R. (1954). *Fed. Proc.* **13**, 190.
Chance, B. & Williams, G. R. (1955). *J. biol. Chem.* **217**, 383.
Chappell, J. B. & Greville, G. D. (1961). *Nature, Lond.*, **190**, 502.
Charles, R., Tager, J. M. & Slater, E. C. (1963). *Biochim. biophys. Acta*, **74**, 33.
Cleland, K. W. & Slater, E. C. (1953). *Biochem. J.* **53**, 547.
Darrow, R. A. & Colowick, S. P. (1961). In *Methods in Enzymology*, vol. 5, p. 226. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Fukuda, T. (1957). *Nature, Lond.*, **180**, 245.
Gale, E. F. (1945). *Biochem. J.* **39**, 46.
Grafflin, A. L. & Ochoa, S. (1950). *Biochim. biophys. Acta*, **4**, 205.
Gregg, C. T., Heisler, C. R. & Remmert, L. F. (1960). *Biochim. biophys. Acta*, **45**, 561.
Gregg, C. T., Johnson, J. R., Heisler, C. R. & Remmert, L. F. (1964). *Biochim. biophys. Acta*, **82**, 343.
Huijing, F. & Slater, E. C. (1961). *J. Biochem., Tokyo*, **49**, 493.
Hunter, F. E. (1949). *J. biol. Chem.* **177**, 361.
Hunter, F. E. (1951). In *Symposium on Phosphorus Metabolism*, vol. 1, p. 297. Ed. by McElroy, E. D. & Glass, B. Baltimore: The Johns Hopkins Press.
Jones, E. A. & Gutfreund, H. (1961). *Biochem. J.* **79**, 608.
Judah, J. D. (1951). *Biochem. J.* **49**, 271.
Kilby, B. A. & Neville, E. (1957). *J. exp. Biol.* **34**, 276.
Krebs, H. A. & Bellamy, D. (1960). *Biochem. J.* **75**, 523.
Lardy, H. A., Johnson, D. & McMurray, W. C. (1958). *Arch. Biochem. Biophys.* **78**, 587.
Levenbook, L. & Hollis, V. W. (1961). *J. Insect Physiol.* **6**, 52.
Millerd, A. (1956). In *Encyclopedia of Plant Physiology*, vol. 2, p. 573. Ed. by Ruhland, W. Berlin: Springer-Verlag.
Ochoa, S. (1943). *J. biol. Chem.* **151**, 493.
Ochoa, S. (1948). *J. biol. Chem.* **174**, 133.
Ochoa, S., Mehler, A. H. & Kornberg, A. (1948). *J. biol. Chem.* **174**, 979.
Price, G. M. (1961). *Biochem. J.* **80**, 420.
Price, G. M. & Moriya, S. (1962). *Biochem. J.* **84**, 98P.
Pucher, G. W., Abrahams, M. D. & Vickery, H. B. (1948). *J. biol. Chem.* **172**, 579.
Remmert, L. R., Heisler, C. R. & Gregg, C. T. (1961). *Fed. Proc.* **20**, 50.
Sacktor, B. & Cochran, D. G. (1958). *Arch. Biochem. Biophys.* **74**, 266.
Siu, P. M. L. & Wood, H. G. (1962). *J. biol. Chem.* **237**, 3044.
Slater, E. C. (1953). *Biochem. J.* **53**, 157.
Slater, E. C. & Holton, F. A. (1953). *Biochem. J.* **55**, 530.
Utter, M. F. & Keech, D. B. (1963). *J. biol. Chem.* **238**, 2603.
Utter, M. F. & Kurahashi, K. (1954). *J. biol. Chem.* **207**, 787.
Van den Bergh, S. G. (1962). *Respiration and Energy Production in the Flight Muscle of the Housefly* Musca domestica *L.* Amsterdam: Rototype.
Van den Bergh, S. G. & Slater, E. C. (1962). *Biochem. J.* **82**, 362.
Winteringham, F. P. W. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, vol. 12, p. 201. London: Pergamon Press Ltd.

Biochem. J. (1964), **93**, 136

The Use of a Gel-Diffusion Technique for the Determination of Metal Ion–Protein Association Constants, and its Application to the Zinc Ion–Collagen System

BY B. G. NEWSOM* AND I. G. F. GILBERT
Physics Department, Institute of Cancer Research, Royal Cancer Hospital, London, S.W. 3

(*Received* 16 *January* 1964)

The precise measurements needed for the determination of metal ion–protein association constants have usually been carried out in dilute aqueous solutions, and it is often not clear if the results can be applied to solid or gel-like protein systems. Bone, cartilage and arterial walls are obvious examples where metal ion deposition is both important and possibly more complex than would be shown by a study of the proteins in free solution. We have therefore attempted to deter-

* Present address: Division of Protein Chemistry, C.S.I.R.O., Victoria, Australia.