Stimulation of Respiration by 2:4-Dinitrophenol

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It has been known for some time that 2:4-dinitrophenol (and other nitrophenols) stimulate the respiration of intact cells, while at the same time the energy-requiring functions of the cell are impaired.

The nature of the effect on the energy-requiring functions became clearer with the discovery by Loomis & Lipmann (1949), and Cross, Taggart, Covo & Green (1949) that dinitrophenol 'uncoupled' oxidative phosphorylation, i.e. it inhibited the synthesis of adenosine triphosphate (ATP) without inhibiting respiration. However, the mechanism of the stimulation of respiration is still obscure.

An important advance towards the elucidation of this mechanism was made by Potter & Recknagel (1951) and Lardy & Wellman (1952, 1953), who defined the conditions for demonstrating the stimulation of the respiration of isolated liver mitochondria by dinitrophenol. Carefully prepared liver mitochondria with low ATPase activity (Kielley & Kielley, 1951) respire only slowly in the absence of a phosphate-acceptor system. The respiration is greatly stimulated by adding either a phosphate-acceptor system or dinitrophenol.

Lardy's (1952) results show that these two effects are not additive, i.e. the addition of dinitrophenol to mitochondria already supplied with hexokinase and glucose has little effect on the respiration. The present paper shows that heart sarcosomes (mitochondria) behave in the same way. However, as already reported in a short communication (Slater & Lewis, 1953), the rate of oxidation of α -ketoglutarate (α -oxoglutarate) by sarcosomes isolated from the thoracic muscle of the blowfly (*Calliphora erythrocephala*) is markedly increased by 2:4-dinitrophenol, even in the presence of hexokinase and glucose. Turner (1953) has found that homogenates of the mammary gland behave similarly. The present paper elaborates and extends our previous results.

METHODS

Heart sarcosomes. These were prepared from rat heart, as described by Cleland & Slater (1953), using as isolation medium a solution of 0.32 M sucrose and 0.01 M ethylenediaminetetraacetate, adjusted to pH 7.4.

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Blowfly sarcosomes. These were prepared as described by Lewis & Slater (1954).

Activity of α -ketoglutaric oxidase system. The activities are expressed as Q_{O_2} , calculated from the α -ketoglutarate (α Kg) disappearance as described by Lewis & Slater (1954).

P:O and P: α Kg ratios. These were measured as described in previous papers (Slater, 1953*a*; Slater & Holton, 1953).

RESULTS

α -KETOGLUTARIC OXIDASE SYSTEM

Effect of dinitrophenol on rate of oxidation of α -ketoglutarate

The effect of various concentrations of dinitrophenol on the rate of oxidation of α -ketoglutarate by different sarcosomal preparations is shown in Fig. 1. Results with a larger number of preparations at a single concentration of dinitrophenol (10^{-4} M) are summarized in Table 1. In all cases, a large excess of hexokinase, together with glucose, was present in the reaction mixture.

The rate of oxidation of α -ketoglutarate by rat-heart sarcosomes was little affected by dinitrophenol. The behaviour of blowfly sarcosomes was variable, in some cases a large stimulation was observed, in other cases there was little action. The large effect was obtained only with sarcosomes made from young blowflies in media containing ethylenediaminetetraacetate (Expts. 3, 4, 6). A smaller activation was obtained with one preparation (Expt. 8), also made from young flies, isolated with sucrose. Another preparation from young flies isolated with the same medium was not activated. The effect of age of the blowflies is most clearly shown in Table 1 by comparing Expts. 6 and 5, with sucrose-ethylenediaminetetraacetate medium, since these preparations were made from the same batch of flies, at a 7-day interval. The effect of including ethylenediaminetetraacetate in the medium is best shown by comparing the two Expts. 6 in Table 1.

The influence of the age of blowfly on the effect of dinitrophenol has not yet been thoroughly studied. Whether it is an effect of age *per se* or a reflexion of some other condition (e.g. activity) cannot be stated.

Table 1. Effect of 2:4-dinitrophenol on rat-heart and blowfly sarcosomes

The figures in brackets after 'blowfly thorax' are the ages (in days) of the blowflies. Concn. of dinitrophenol, 10^{-4} M.

Expt.	Tissue	Isolation medium	Q_{O_2}		Activation (+) or inhibition (-) (%)	P:O in absence of dinitrophenol	P:αKg in presence of dinitrophenol
			In absence of dinitrophenol	In presence of dinitrophenol			
1	Rat heart	(0.28 M Sucrose + 0.01 M ethylenediaminetetraacetate, pH 7.4	59	62	+5	2.75	0.74
2	Rat heart	(0.20 M Sucrose + 0.002 M ethylenediaminetetraacetate, pH 7.4	71	60	-16	2.72	0.64
3	Blowfly thorax (1)	(0.20 M Sucrose + 0.002 M ethylenediaminetetraacetate, pH 7.4	24	52	+118	0.95	0.91
4	Blowfly thorax (2)	(0.20 M Sucrose + 0.002 M ethylenediaminetetraacetate, pH 7.4	16	55	+251	1.03	0.83
5	Blowfly thorax (9)	(0.32 M Sucrose + 0.01 M ethylenediaminetetraacetate, pH 7.4	9.2	8.8	-3	1.68	0.71
6	Blowfly thorax (2)	(0.32 M Sucrose + 0.01 M ethylenediaminetetraacetate, pH 7.4	11.2	19.1	+71	0.88	0.74
7	Blowfly thorax (2)	(0.32 M Sucrose + 0.01 M ethylenediaminetetraacetate, pH 7.4	15.9	23.2†	+46	1.61	0.66
8	Blowfly thorax (2)	(0.36 M Sucrose + 0.005 M phosphate, pH 7.4	8.4	8.8	+5	1.79	0.69
9	Blowfly thorax (1-7)	(0.16 M Phosphate, pH 7.0 + 2.5% albumin	20.1	19.4	-3	1.03	0.70
10	Blowfly thorax (6-13)	(0.16 M Phosphate, pH 7.0 + 2.5% albumin	19.1	27.0†	+39	0.57	0.58
10	Blowfly thorax (8-15)*	(0.16 M Phosphate, pH 7.0 + 2.5% albumin	11.3	10.3	-9	0.72	0.39
10	Blowfly thorax (8-15)*	(0.16 M Phosphate, pH 7.0 + 2.5% albumin	11.6	13.0	+12	0.88	0.94
9	Blowfly thorax (8-15)*	(0.16 M Phosphate, pH 7.0 + 2.5% albumin	8.5	7.1	-16	0.58	0.32
	Blowfly thorax (8-13)	(0.16 M Phosphate, pH 7.0 + 2.5% albumin	46	46	0	0.80	0.79

* Thoraces ground in mortar for 1 min. with medium (see p. 209 of Lewis & Slater, 1954).

† 42, in presence of 10^{-5} M dinitrophenol.

Fig. 1. Effect of 2:4-dinitrophenol on the rate of oxidation of α-ketoglutarate by rat-heart sarcosomes and by various preparations of blowfly thoracic muscle sarcosomes. ●—●, Rat-heart sarcosomes isolated in sucrose + ethylenediaminetetraacetate. ○—○, blowfly sarcosomes isolated in sucrose. +—+, blowfly sarcosomes isolated in sucrose + ethylenediaminetetraacetate. The numbers against each curve refer to the Expt. nos. in Table 1.

Fig. 2. Effect of 2:4-dinitrophenol on P:αKg ratios. The symbols and numbers are the same as in Fig. 1.

Effect of dinitrophenol on phosphorylation ratios

The effect of varying concentrations of dinitrophenol on the phosphorylation ratios of the same five preparations illustrated in Fig. 1 is shown in Fig. 2. P:αKg is plotted rather than P:O because phosphorylation associated with oxidation beyond

succinate is completely uncoupled by dinitrophenol. The following points are noteworthy. (1) Phosphorylation is largely uncoupled by low concentrations of dinitrophenol, the rat-heart sarcosomes requiring higher concentrations than the blowfly sarcosomes. (2) There is a considerable dinitrophenol-resistant phosphorylation, $P:\alpha Kg$ remaining almost constant between about $5 \times 10^{-5} M$ ($10^{-4} M$ in the case of the heart sarcosomes) and $5 \times 10^{-4} M$ dinitrophenol.

One of the experiments (Expt. 4) included in Figs. 1 and 2 is shown in greater detail in Fig. 3. It should be noted that unlike Figs. 1 and 2, the concentration scale in Fig. 3 is linear, not logarithmic. The most interesting point about Fig. 3 is the effect of different concentrations of dinitrophenol on the esterification of phosphate. Low concentrations of dinitrophenol ($10^{-5} M$) sharply decrease the amount of esterified phosphate, but further additions increase it again, until with $10^{-4} M$ dinitrophenol the esterification of phosphate is actually 36% greater than in the absence of dinitrophenol. In another experiment (Expt. 3 of Table 1), this concentration of dinitrophenol increased the amount of esterified phosphate by 22%.

Effect of albumin

It has been shown in the previous paper (Lewis & Slater, 1954; cf. Sacktor, 1954) that the addition of low concentrations of bovine-plasma albumin to the reaction mixture markedly accelerates the rate of

oxidation of α -ketoglutarate, with little or no effect on the phosphorylation ratios. It was obviously of interest to determine the effect of dinitrophenol in the presence of albumin. Four experiments are shown in Table 2, one with a preparation giving a stimulation of the rate of oxidation of

Fig. 3. Expt. 4 of Figs. 1 and 2 and Table 1 shown in greater detail. Note that the concentration of dinitrophenol is plotted on a linear scale, not logarithmic as in Figs. 1 and 2.

Table 2. *Effect of dinitrophenol in the presence and absence of albumin*

Sarcosomes isolated in 0.20M sucrose + 0.002M ethylenediaminetetraacetate, pH 7.4, in Expt. 1; in 0.32M sucrose + 0.01M ethylenediaminetetraacetate, pH 7.4, in Expts. 2-4. The ages of the blowflies were: Expt. 1, 9 days; Expt. 2, 9 days (same batch as in Expt. 1); Expt. 3, 2 days; Expt. 4, 8 days.

Expt.	Albumin (mg./ml.)	Dinitrophenol (M)	$-\Delta O$	$-\Delta \alpha Kg$	$\Delta O:\Delta \alpha Kg$	$P:O$	$P:\alpha Kg$
1	0	0	1.73	0.73	2.37	1.68	4.00
	1.2	0	2.64	1.26	2.10	1.72	3.61
	0	10^{-4}	1.42	0.71	2.01	0.35	0.71
	1.2	10^{-4}	1.99	0.96	2.07	0.46	0.96
2	0	0	1.65	0.61	2.70	1.79	4.81
	1.2	0	2.66	1.20	2.22	1.79	3.97
	0	10^{-4}	1.35	0.64	2.10	0.33	0.69
	1.2	10^{-4}	1.99	0.94	2.11	0.44	0.94
3	0	0	1.43	0.69	2.08	1.55	3.22
	1.2	0	2.32	1.19	1.95	1.87	3.65
	0	10^{-4}	1.59	1.01	1.58	0.42	0.66
	1.2	10^{-4}	2.20	1.44	1.53	0.55	0.84
	0	2×10^{-4}	1.77	1.45	1.22	0.49	0.59
	1.2	2×10^{-4}	1.99	1.43	1.39	0.52	0.71
	0	5×10^{-4}	2.16	1.81	1.19	0.49	0.59
4	0	0	0.89	0.53	1.68	1.46	2.45
	1.2	0	1.60	0.90	1.78	1.78	3.16
	5.0	0	1.80	0.98	1.84	1.91	3.51
	10.0	0	2.06	0.89	2.31	1.99	4.60
	0	2×10^{-4}	0.74	0.33	2.26	0.39	0.88
	1.2	2×10^{-4}	0.94	0.46	2.06	0.39	0.81
	5.0	2×10^{-4}	1.51	0.75	2.01	0.35	0.71
	10.0	2×10^{-4}	1.80	0.79	2.28	0.39	0.90

α -ketoglutarate on addition of dinitrophenol (Expt. 3) and the others with preparations not showing this stimulation.

In the absence of dinitrophenol, albumin stimulated the oxidation of α -ketoglutarate to about the same degree with all four preparations (73, 97, 73 and 70 % stimulation, respectively, in Expts. 1-4 by 1.2 mg. albumin/ml.), although they differed markedly in their response to dinitrophenol. This shows that albumin and dinitrophenol do not stimulate by the same mechanism. In Expt. 3, the stimulation of α -ketoglutarate utilization by dinitrophenol (5×10^{-4} M) exceeded that given by albumin, which was used in an amount found to give maximum stimulation in the previous paper (Lewis & Slater, 1954). Although these two activators appear then to act by different mechanisms, their effects were not additive. In fact, dinitrophenol markedly depressed the activating effect of albumin, even when it had no effect on its own (Expts. 1, 2 and 4). In Expt. 3, 2×10^{-4} M dinitrophenol completely prevented the activation by 1.2 mg. albumin. Expt. 4 shows that larger amounts of albumin (5 and 10 mg.) were able to overcome this depressing effect of dinitrophenol.

Another effect of albumin is also shown in Table 2; the P: α Kg ratio with 10^{-4} M dinitrophenol is appreciably higher in the presence of albumin, approaching a value of 1 in Expts. 1 and 2.

Effect of concentration of α -ketoglutarate

It has been shown in the previous paper (Lewis & Slater, 1954) that the rate of oxidation of α -ketoglutarate by blowfly sarcosomes is markedly affected by increasing the concentration of α -ketoglutarate between 0.002 and 0.01 M, suggesting a possible permeability barrier to α -ketoglutarate.

It was important, therefore, to determine if dinitrophenol promoted the oxidation of α -ketoglutarate by increasing the rate of penetration of the substrate to the α -ketoglutaric dehydrogenase. This was tested by measuring the effect of dinitrophenol at different concentrations of α -ketoglutarate. The results, summarized in Table 3, show that the effect of dinitrophenol increases with increasing concentration of α -ketoglutarate. This is contrary to what would be expected if dinitrophenol were affecting the accessibility of α -ketoglutarate to its dehydrogenase, but suggests rather that dinitrophenol activates the α -ketoglutaric oxidase system at some point other than the reaction between α -ketoglutarate and the dehydrogenase.

SUCCINIC OXIDASE SYSTEM

Table 4 shows the effect of dinitrophenol on the oxidation of succinate and on the P:O ratio. In Expt. 1, the sarcosomal preparation was one which showed a marked stimulation of the α -ketoglutaric oxidase system by adding dinitrophenol; similarly, the rate of oxidation of succinate was considerably increased (by 66 %). In Expt. 2, on the other hand, in which sarcosomes from older blowflies were used, dinitrophenol had no effect on the rate of oxidation of either α -ketoglutarate or succinate. Similarly, it had no appreciable effect on the rate of oxidation of succinate by heart sarcosomes (see Table 7 of Slater & Bonner, 1952). It seems likely that the mechanisms of the stimulation of the oxidation of α -ketoglutarate and succinate are basically the same.

Expt. 2 of Table 4 shows that 10^{-4} M dinitrophenol is sufficient completely to inhibit the respiratory chain phosphorylation, even in the presence of 5 mg./ml. albumin.

Table 3. *Effect of α -ketoglutarate concentration on the activation of the oxidation of α -ketoglutarate by dinitrophenol*

Blowfly sarcosomes isolated with 0.32 M sucrose + 0.01 M ethylenediaminetetraacetate, pH 7.4, from 2-day-old blowflies.
 Ratio = $\frac{\text{value in presence of dinitrophenol}}{\text{value in absence of dinitrophenol}}$

	Dinitrophenol (M)	α -Ketoglutarate (m-moles/l.)		
		2.2	5.5	10.5
$-\Delta O$ (μ atoms)	0	1.00	1.43	1.49
	10^{-4}	0.90	1.59	1.75
<i>Ratio</i>		0.90	1.11	1.17
$-\Delta \alpha$ Kg (μ moles)	0	0.41	0.69	—
	10^{-4}	0.50	1.01	—
<i>Ratio</i>		1.22	1.46	—
Δ Esterified P (μ atoms)	0	1.66	2.22	2.41
	10^{-4}	0.38	0.67	0.83
<i>Ratio</i>		0.23	0.30	0.34
P:O	0	1.66	1.55	1.62
	10^{-4}	0.42	0.42	0.47

Table 4. *Effect of dinitrophenol on oxidation of succinate by blowfly sarcosomes*

Expt. 1, same preparation of sarcosomes as in Fig. 3; Expt. 2, sarcosomes isolated in 0.32M sucrose + 0.01M ethylenediaminetetraacetate, pH 7.4 from 8-day-old blowflies. Reaction mixture same as for α -ketoglutarate, except that 0.01M succinate replaced α -ketoglutarate, and fluoride and malonate were omitted.

Expt.	Dinitrophenol (M)	Albumin (mg./ml.)	$-\Delta O$ (μ atoms)	Δ Esterified P (μ atoms)	P:O
1	0	0	2.10	0.29	0.14
	10^{-4}	0	3.49	0.12	0.03
2	0	0	5.07	0.27	0.05
	5×10^{-6}	0	4.94	0.16	0.03
	10^{-5}	0	5.01	0.15	0.03
	2×10^{-5}	0	4.80	0.07	0.01
	5×10^{-5}	0	4.84	0.06	0.01
	10^{-4}	0	5.06	-0.05	-0.01
	0	5	5.74	1.82	0.32
	5×10^{-6}	5	6.15	1.59	0.26
	10^{-5}	5	6.17	1.45	0.23
	2×10^{-5}	5	6.10	1.22	0.20
	5×10^{-5}	5	6.16	0.63	0.10
	10^{-4}	5	5.94	0.09	0.01

DISCUSSION

Dinitrophenol-resistant oxidative phosphorylation

Judah (1951) and Hunter (1951) independently and at about the same time made the important observation that dinitrophenol did not completely uncouple phosphorylation when α -ketoglutarate was substrate. Since the phosphorylation (Hunter & Hixon, 1949) accompanying the Krebs & Cohen (1939) dismutation of α -ketoglutarate was little affected by concentrations of dinitrophenol which completely uncoupled phosphorylation associated with the oxidation of other substrates, Judah concluded that the substrate-linked phosphorylation was not affected by dinitrophenol. More recently this has been confirmed by Kaufman, Gilvarg, Cori & Ochoa (1953) and by Hift, Ouellet, Littlefield & Sanadi (1953), using partially purified soluble enzymes. These enzymes bring about the following reactions (CoA = coenzyme A; DPN = diphosphopyridine nucleotide; DPN_{red} = reduced diphosphopyridine nucleotide):

Both the rat-heart and the blowfly sarcosomes also have a dinitrophenol-resistant phosphorylation with α -ketoglutarate as substrate. In this respect, the blowfly sarcosomes differ from mung-bean mitochondria (Bonner & Millerd, 1953), which also give low P:O ratios. However, the P: α Kg ratio in the presence of dinitrophenol is considerably below unity, the value expected if the substrate-linked

phosphorylation were completely unaffected by dinitrophenol. Moreover, the P: α Kg ratio in the presence of 10^{-4} M dinitrophenol varied considerably with different preparations. Hunter (1951) also obtained a low P: α Kg ratio (about 0.5) with liver mitochondria in the presence of dinitrophenol.

Three possible explanations of the low ratios for the dinitrophenol-resistant phosphorylation will be considered. (1) The substrate-linked phosphorylation reaction is inhibited by dinitrophenol. (2) Dinitrophenol increases the ATPase activity, so that the phosphorylative activity is not completely measured. (3) In the presence of dinitrophenol, α -ketoglutarate is oxidized by a non-phosphorylating pathway as well as by that described in equations (a) and (b).

The first possibility is eliminated by the report of Hift *et al.* (1953) that the enzyme catalysing reaction (b) is not inhibited by up to 10^{-3} M dinitrophenol and also by the shape of the curves in Fig. 2. (Hunter's (1951) graph is very similar.) If dinitrophenol inhibited the substrate-linked phosphorylation reaction, the P: α Kg ratio would decline progressively as the concentration of dinitrophenol increased. In Fig. 2 (it should be noted that the concentration is plotted on a logarithmic scale), it can be seen that further addition of dinitrophenol beyond 5×10^{-5} M in the case of blowfly sarcosomes or 10^{-4} M in the case of heart sarcosomes caused no further decline in the P: α Kg ratio, until a concentration of 5×10^{-4} M was exceeded. Between 5×10^{-5} M and 5×10^{-4} M dinitrophenol, there was in fact a slight rise in the P: α Kg ratio with the blowfly preparations, isolated in sucrose.

The second possibility can be excluded, so far as the heart sarcosomes are concerned, by the finding of Slater & Cleland (1953a) that 10^{-4} M dinitrophenol increased the ATPase activity by only 58%. The

amount of hexokinase used was considerably in excess of that required to react with virtually all the ATP, according to the analysis of Slater & Holton (1953), and a 58% increase in ATPase activity would cause a negligible loss. In any case, it is possible by adding albumin to obtain P:αKg ratios near unity in the presence of 10^{-4} M dinitrophenol.

This leaves the third possibility considered above. It is probable that the alternative pathway is provided by the substitution of reaction (b) by the hydrolysis of succinyl-CoA, catalysed by an enzyme isolated from heart by Gergely, Hele & Ramakrishnan (1952) and by Kaufman *et al.* (1953). This raises an important question. Does the succinyl-CoA deacylase operate in the untreated sarcosomes and so decrease the yield of phosphorylation available from respiration or does it become active only in the presence of dinitrophenol? The succinyl-CoA deacylase is probably present in the intact sarcosome, but it may not be accessible to the succinyl-CoA formed by the oxidative decarboxylation of α-ketoglutarate. While it is not possible to make a final choice between these two possibilities, the ability of dinitrophenol to increase the rate of other reactions catalysed by the sarcosomes (respiration, hydrolysis of ATP) suggests that it might increase the deacylase activity in the same way. This is given some support by the finding that although the P:αKg ratio in the presence of 10^{-4} M dinitrophenol is increased by albumin, the phosphorylation ratios in the absence of dinitrophenol are little affected by the addition of albumin (Lewis & Slater, 1954; see also Table 2 of the present paper).

On the basis of this explanation, it would be concluded from Fig. 2 that the deacylase would be completely activated (or become completely accessible to the succinyl-CoA) at about 5×10^{-5} M dinitrophenol. The slight increase of the P:αKg ratio often found with higher concentrations of dinitrophenol could be because the deacylase is inhibited by these concentrations.

Since 10^{-4} M dinitrophenol was sufficient completely to inhibit the respiratory chain phosphorylation associated with the oxidation of succinate, even in the presence of 5 mg. albumin/ml., it is clear that the higher P:αKg ratios obtained in the presence of 10^{-4} M dinitrophenol by adding 1.2 mg. albumin/ml. are due to a protection of the substrate-linked phosphorylation. This might be due to an effect on the structure of the sarcosome (cf. Watanabe & Williams, 1953) which results in the succinyl-CoA and the deacylase remaining inaccessible to one another. It has been shown (Lewis & Slater, 1954) that grinding the thoracic muscle in a mortar for only 1 min. caused a marked decline in the Q_{O_2} and in the P:O ratio of the isolated sarcosomes. Expt. 10, Table 1, shows that the P:αKg ratio of such a preparation in the presence of 10^{-4} M dinitrophenol

was very low (0.32). This is probably associated with structural damage to the sarcosome.

Stimulation of phosphorylation by dinitrophenol

Fig. 3 illustrates an apparently anomalous situation in which the rate of esterification of inorganic phosphate by oxidative phosphorylation is increased by dinitrophenol, the typical inhibitor of oxidative phosphorylation. This result is a consequence of three factors: (1) the substrate-linked phosphorylation is little affected by dinitrophenol; (2) the dinitrophenol-sensitive respiratory chain phosphorylation contributes a relatively small proportion of the total phosphorylation in this preparation of sarcosomes from young blowflies; (3) dinitrophenol increases the rate of oxidation of α-ketoglutarate.

This experiment provides evidence in favour of the feasibility of the mechanism suggested by Lardy (1952) to explain the stimulation of metabolism by certain hormones. Lardy suggested that these hormones acted by partially uncoupling phosphorylation from respiration in such a way that the rate of the respiration was increased to a greater degree than the decrease of the P:O ratio. Thus, although the efficiency of the utilization of respiration for the synthesis of ATP would be decreased, the actual rate of synthesis of ATP would be increased.

Chin & Bueding (1954), working with a particulate fraction of *Ascaris lumbricoides*, have recently also found an increase of the rate of phosphorylation on the addition of dinitrophenol. The explanation in this case must be more complicated than that given above, since in Chin & Bueding's experiments the P:O ratio was considerably increased by dinitrophenol.

Mechanism of stimulation of respiration

The mechanism of respiratory chain phosphorylation previously proposed (Slater, 1953*b*) provides an explanation of the stimulation of respiration by dinitrophenol. In the following equations:

AH_2 and B are adjacent members of the respiratory chain and C is an additional component or grouping required for their interaction. A and C must be regenerated from the compound $A \sim C$ before hydrogen-transfer can continue. In the intact mitochondria or sarcosome, this is brought about primarily by the transphosphorylating reaction (2), which leads to the esterification of inorganic phosphate. It is suggested that reaction (3) is relatively slow in the intact mitochondria, but can be greatly accelerated by dinitrophenol. In this way, $A \sim C$

would be hydrolysed and be unavailable for the synthesis of ATP by reaction (2).

When reaction (2) is fast compared with reaction (1), the addition of dinitrophenol by promoting reaction (3) will stop the esterification of phosphate but since it increases the rate of a process which is not rate-limiting, it will not increase the rate of hydrogen-transfer to any extent. This is presumably the situation in liver mitochondria or heart sarcosomes. It would be consistent with our experience with heart sarcosomes that the P:O ratio is largely unaffected by treatments which considerably affect the oxidative activity (Slater & Cleland, 1953*b*).

When, on the other hand, reaction (2) is slow compared with reaction (1), the rate of hydrogen-transfer will be increased by increasing the rate of breakdown of $A \sim C$, for example by the addition of dinitrophenol. This is probably the case with the sarcosomes of young blowflies isolated with media containing ethylenediaminetetraacetate.

Only preparations of blowfly sarcosomes which were isolated from young blowflies with media containing ethylenediaminetetraacetate showed the stimulation of respiration by dinitrophenol (Table 1), and different preparations within this group varied considerably. Although there was a considerable variation in the oxidative activity in the absence of dinitrophenol, only in one case (sarcosomes isolated in 0.16*M* phosphate, pH 7.0, 2.5% albumin) could the lack of stimulation be ascribed to the fact that the activity without adding dinitrophenol was already high. Table 1 shows no obvious correlation between the activation of the oxidation of α -ketoglutarate by dinitrophenol and the P:O ratio in the absence of dinitrophenol.

Two possible explanations of the failure of preparations made without ethylenediaminetetraacetate or from older blowflies to be activated suggest themselves:

(i) The potential activity of the α -ketoglutaric oxidase system, i.e. the activity of reaction (1) when $A \sim C$ is decomposed as fast as it is formed, is much lower in these preparations. This would imply that the potential activity of the α -ketoglutaric oxidase system in the blowfly sarcosomes declines rapidly during the first week of life. It would also mean that the potential activity was decreased when preparations were isolated in sucrose without ethylenediaminetetraacetate, in agreement with the experience with heart sarcosomes (Slater & Cleland, 1953*a*).

(ii) The activity of the enzyme catalysing reaction (3) is inadequate even when fully activated by dinitrophenol. This explanation is not necessarily inconsistent with the uncoupling of the respiratory chain phosphorylation by dinitrophenol, since it is possible that the $A \sim C$ block envisaged above occurs at a point in the respiratory chain where

little phosphorylation occurs, because of the absence of the enzyme catalysing reaction (2). This is not unreasonable in view of the low P:O ratios of blowfly sarcosomes.

Fig. 2 shows that the concentration of dinitrophenol required to uncouple respiratory chain phosphorylation is less in the case of blowfly sarcosomes than in the case of the better phosphorylating heart sarcosomes. Moreover, the preparation of blowfly sarcosomes with the highest P:O ratio in the absence of dinitrophenol required the highest concentration of dinitrophenol for uncoupling. These relationships can be explained by the relative activities of the enzymes catalysing reaction (2); the greater the activity, the greater has reaction (3) to be stimulated so that all the $A \sim C$ is hydrolysed.

The concentrations of dinitrophenol required in the present study for stimulating respiration are much higher than those used by other workers using intact cells. This is understandable, since in the intact cell where $[ATP]/[ADP]$ is high, the back reaction of equation (2) will be an additional factor limiting the breakdown of $A \sim C$. Under these circumstances, it can be shown by solving the kinetic equations appropriate to reactions (1), (2) and (3) that a moderate increase in the rate of reaction (3) will markedly increase the rate of oxidation. These equations also make it clear why higher concentrations of dinitrophenol are required to stimulate respiration than to uncouple phosphorylation.

In studies with the intact cell, concentrations above about 10^{-5} to 10^{-4} *M* dinitrophenol (e.g. Simon, 1953) inhibit respiration. No inhibitory effects were detectable with isolated sarcosomes below 10^{-3} *M*. This different behaviour is probably a reflexion of the different degrees of complexity of the two systems. In the intact cell, respiration may be governed by processes which require the supply of a small amount of ATP. In the simpler systems used in the present study, the medium contains all the components which are required for respiration and which are not in the sarcosome.

It has been found by a number of authors that respiration can proceed in the absence of phosphate acceptor, although usually at a lower rate (e.g. Lardy & Wellman, 1952; Slater & Holton, 1953). For respiration to occur, reaction (1) must proceed continuously in the one direction. This can be brought about only by introducing an irreversible reaction causing the decomposition of $A \sim C$ to A and C . There are only two possible irreversible reactions: (a) the irreversible hydrolysis of ATP by ATPase, or (b) the irreversible hydrolysis of $A \sim C$ (reaction (3)). There is no evidence which allows a choice between the two possibilities.

Lee & Eiler (1953*b*) have proposed a mechanism of respiratory chain phosphorylation which can be

rewritten in terms of the nomenclature used in this paper as follows:

These authors believe that inorganic phosphate is returned to the system by reversal of the reactions leading to its incorporation into ATP. Since this mechanism does not include the essential irreversible reaction mentioned above it is a closed circuit, describing the overall equilibrium

The forward reaction is oxidative phosphorylation, the reverse is named reductive dephosphorylation. Even if the overall reaction describing the oxidation of substrate (SH_2) through A and B by oxygen is made irreversible by introducing reactions (7) and (8),

the overall reaction (9) (which is the sum of reactions (6), (7) and (8))

will stop as soon as all the ADP is phosphorylated. Thus Lee & Eiler's experiments, in which a steady respiration was obtained in the presence of added ATP, are not explained by their mechanism.

If the irreversible reaction permitting respiration is reaction (3), we can rewrite equations (1), (2) and (3) as follows

This shows that reaction (2) will remain at the position of equilibrium throughout the reaction and consequently $[ATP]/[ADP]$ will remain constant. This will be the case even after the addition of dinitrophenol, which will increase the rate of reaction (3) and consequently decrease the steady-state concentration of $A \sim C$. As a result, the rate of incorporation of ^{32}P into ATP will be decreased without altering the concentration of ATP. This is what Lee & Eiler (1953*a*) found experimentally and explained (Lee & Eiler, 1953*b*) in essentially the same way.

But this is not the only possible explanation of Lee & Eiler's findings. If, as is more generally believed, the irreversible reaction permitting respiration is the hydrolysis of ATP, it would certainly be expected that dinitrophenol in an amount sufficient to depress the phosphorylation would decrease the concentration of ATP. But if, as

is the case in Lee & Eiler's experiments, the ratio $[ATP]/[ADP]$ is, in the absence of dinitrophenol, so high that ADP cannot be detected, an inhibition of the phosphorylation by 40 % (the value obtained by Lee & Eiler (1953*a*)) might result in a considerable decrease in the ratio $[ATP]/[ADP]$, without decreasing $[ATP]$ sufficiently to be detected experimentally.

SUMMARY

1. The rate of oxidation of α -ketoglutarate and succinate by some preparations of blowfly sarcosomes was markedly increased by 2:4-dinitrophenol, even in the presence of excess hexokinase and glucose.

2. Only preparations isolated from young blowflies in media containing ethylenediaminetetraacetate showed this effect. The respiration of sarcosomes isolated from older blowflies, or from younger flies in sucrose medium, was little affected by dinitrophenol. The respiration of heart sarcosomes was also unaffected.

3. In agreement with earlier work of others, it was found that there was a dinitrophenol-resistant phosphorylation step in the oxidation of α -ketoglutarate. This is undoubtedly the substrate-linked phosphorylation, involving succinyl-coenzyme A.

4. The phosphorus: α -ketoglutarate ratio in the presence of sufficient dinitrophenol completely to inhibit respiratory chain phosphorylation was substantially below the theoretical value of 1, but could be raised to near 1 by the addition of albumin, which did not prevent the inhibition of respiratory chain phosphorylation by dinitrophenol.

5. With some preparations, the increase of respiration brought about by dinitrophenol more than counterbalanced the decrease in the phosphorylation ratios, with the result that the rate of phosphorylation was actually increased by dinitrophenol.

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Synthesis of Cellulose by *Acetobacter xylinum*

2. PREPARATION OF FREEZE-DRIED CELLS CAPABLE OF POLYMERIZING GLUCOSE TO CELLULOSE*

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Non-proliferating cells of *Acetobacter xylinum* acting on glucose in the presence of oxygen are known to synthesize cellulose (Hestrin, Aschner & Mager, 1947; Hestrin, 1953). Hitherto, however, the factors determining rate and yield of the production of cellulose in this system have not been described quantitatively. In an earlier communication, a micromethod for the specific determination of cellulose in a biological mixture was presented (Schramm & Hestrin, 1954a). The present paper describes the procedure for the separation of *A. xylinum* bacteria from their cellulose pellicle and the preparation of an essentially cellulose-free suspension of non-proliferating freeze-dried cells which polymerize glucose to high-polymer cellulose. Optimum conditions for the synthesis are described and the specific synthetic activity of the cells is evaluated. Some observations which relate to the mechanics of the growth of cellulose gel bodies are also presented.

The chemical mechanism of the synthesis of cellulose by *A. xylinum* will be the subject of a later communication.

* Experiments described in this communication were carried out by M.S. in the course of work leading to the Ph.D. degree.

METHODS AND MATERIALS

Culture technique

The following medium was used (% w/v): glucose, 2.0; peptone (Difco Bactopeptone), 0.5; yeast extract (Marmite), 0.5; disodium phosphate (anhydrous), 0.27; citric acid (monohydrate), 0.115; pH adjusted to 6.0 with dil. HCl or NaOH. The solution was cleared by filtration and sterilized by autoclaving. A shallow-layer quiescent-culture technique which favours rapid appearance of a surface pellicle and growth of the vigorously cellulose-synthesizing *A. xylinum* wild type (Schramm & Hestrin, 1954b) was used. The medium was inoculated 1:10 with fluid from the liquid phase of a 3-day starter culture, distributed aseptically into large Petri dishes as a shallow fluid layer and incubated at 30° at rest. The pellicles were harvested after 48 hr.

Preparation of freeze-dried bacteria

The separation of *A. xylinum* bacteria from their matrix of cellulose was based on the observation that cheesecloth retains the cellulose fibrils but allows the bacteria to pass (Hestrin *et al.* 1947). Sodium phosphate-citrate mixture (pH 6.0; final phosphate concn., 0.01 M) was used as buffer. At all stages of washing, the temperature of the milieu was kept below 6° with ice. The use of this low temperature both conserved activity and protected the bacteria from agglutination in bodies of cellulose gel which might otherwise have formed during the early stages of the washing process from the traces of glucose in the cell milieu.