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The Action of 2:4-Dinitrophenol on Oxidative Phosphorylation

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In a previous paper (Judah & Williams-Ashman, 1951) the effect of a number of compounds was tested on oxidative phosphorylation in cell-free systems. 2:4-Dinitrophenol (DNP) was selected for further study because of widespread interest in this compound and because its action was particularly clear-cut and reproducible.

The starting point of the present work was the investigation of the inhibition of pyruvate oxidation by DNP; this has led to an attempt at localization of the action of DNP and to a study of the 'replacement' of inorganic orthophosphate by DNP (Loomis & Lipmann, 1948) in systems deficient in phosphate. Some experiments on the action of azide and observations on the effect of thyroxine on oxidative phosphorylation are also included.

METHODS

All methods are as described previously (Judah & Williams-Ashman, 1951) with the following exceptions.

Enzyme preparations. Preparations of mitochondria from liver and kidney of rat and rabbit were invariably carried out by the method of Schneider (1948), using 0·25 M-sucrose as the medium. This method gives a uniform preparation free of cell debris, erythrocytes and cell-nuclei, which form a large part of such preparations as 'cyclophorase' (Green, Loomis & Auerbach, 1948). For homogenization, the apparatus of Potter & Elvehjem (1936) was used. A blender yielded suspensions which were far less active and which invariably failed to show proportionality between tissue concentration and respiratory rate. Furthermore, the yields of mitochondria were low when the tissue was disintegrated in the blender. All preparations were carried out at 0°; the mitochondrial suspensions obtained were found to be stable for several hours when kept cold; preparations stored for as long as 6 hr. showed no decline in the efficiency of oxidative phosphorylation. Considerable uniformity in properties was observed from one preparation to another (O_2 uptake with pyruvate being approx. 500 μl. O_2/mg. N/hr.), and no inactive preparations have been encountered in a series of several hundred.

The mitochondria tolerated repeated resuspension and sedimentation without appreciable loss of activity, particular preparations having been subjected to a cycle of five or six such operations. In washing the particles care was taken to ensure an even suspension in the sucrose medium. This was achieved by the use of a mechanically rotated pestle which fitted the centrifuge tube loosely. A few seconds sufficed to disperse the tightly packed particles, which were dispersed in six to eight 50 ml. cellulose tubes, washings being conducted with 25–30 ml. 0·25 M-sucrose per tube.

Microsomes (submicroscopic particles) were prepared as described by Schneider (1948), a force of 25,000 *g* being applied for 1 hr. to the suspension in 0·25 M-sucrose.

Reagents. Crystalline sodium or potassium pyruvate were prepared from commercial pyruvic acid after redistillation *in vacuo*. α-Ketoglutaric acid was obtained from Nutritional Biochemicals, Inc., Cleveland, Ohio. Inorganic pyrophosphate was an analytical reagent; on chromatography in ethyl acetate-pyridine (Hanes & Isherwood, 1949), it showed only a trace of inorganic ortho-P and otherwise gave a single homogeneous spot.

Estimations. Orthophosphate was estimated by Ba and Mg precipitation (Umbreit, Burris & Stauffer, 1949) and by Ca precipitation at neutral pH by an unpublished method of the writer. Phosphate balances were determined as described by LePage in Umbreit *et al.* (1949).

Nucleic acid estimations were carried out by the method of Schmidt & Thannhauser (1945).

Pyruvate and α-ketoglutarate were determined by the method of Friedemann & Haugen (1943), using either a Beckman Model DU spectrophotometer or a Unicam diffraction-grating instrument.

Ferricyanide was determined spectrophotometrically by its absorption at 420 mμ. after deproteinization with trichloroacetic acid.

N was determined by the micro-Kjeldahl method. Steam volatile acid was estimated by the method of Long (1938).

RESULTS

The oxidation of pyruvate and its inhibition by DNP. Preliminary experiments showed that the following medium gave satisfactory results. Inorganic orthophosphate, 0·003–0·03M; adenosine-5-phosphate, 0·001M; magnesium sulphate, 0·0067M; potassium chloride, 0·025M; cytochrome *c*, 0·00001M; pyruvate, 0·01M; fumarate, malate or succinate, 0·0003–0·001M; 0·5 ml. mitochondrial suspension in 0·25M-sucrose and water to a final volume of 3·0 ml. This medium is very similar to that described by Kennedy & Lehninger (1949), and by Green *et al.* (1948).

Table 1. *Complete oxidation of pyruvate*

(The systems were as described in the text, except that pyruvate additions are as shown in the table. All values shown are corrected for blanks. Temp. 38°. Gas phase: air. The primer was fumarate in every case: 1 μg.mol. in Exp. 1, 2 μg.mol. in Exp. 2, and 3 μg.mol. in Exps. 3 and 4.)

Exp. no.	Source of mitochondria	Pyruvate added (μg.mol.)	Pyruvate at end of experiment (μg.mol.)	O_2 uptake (μg.atoms) Observed (blank subtracted)	Expected	Blanks	Time on bath (min.)
1	Rat kidney	4·0	0·0	18·0	20·0	7·0	180
2	Rat liver	4·0	0·0	19·1	20·0	14·6	165
3	Rat kidney	4·0	0·1	19·0	19·5	20·9	120
4	Rabbit kidney	10·0	0·0	48·5	50·0	17·8	110

Initially the potassium salts of all reagents were used, but it was found that sodium salts would serve equally well in the presence of 0·025M-potassium chloride. In all the experiments cited below, sodium salts were used.

Table 1 shows the complete oxidation of pyruvate by several mitochondrial preparations. The observed oxygen uptakes agree reasonably well with theory; the complete removal of pyruvate was checked by chemical estimations on the flask contents. For these experiments pyruvate was placed in the side arms of the flasks, and to avoid temperature inactivation of the enzyme in the absence of substrate, a dicarboxylic acid (malate, fumarate or succinate in the concentrations given above) was placed in the main compartment of the flask. Addition of catalytic amounts of these acids (primers) is required to initiate or prime the oxidation of pyruvate by providing oxaloacetate necessary for the condensation of pyruvate (or a degradation product of pyruvate) to form citrate. This permits its complete oxidation via the tricarboxylic acid cycle (Krebs & Johnson, 1937; Green *et al.* 1948). Blanks were run simultaneously without added pyruvate, and their oxygen consumption subtracted from that of the experimental flasks.

In Table 2 will be seen the effect of leaving out primer, adenylic acid and cytochrome *c*; loss of cytochrome *c* does not result in a great decrease of respiratory rate. The primer in this experiment was sodium bicarbonate, confirming the observation of Green *et al.* (1948) and indicating that the enzyme catalysing the reversible decarboxylation of oxaloacetic acid is present in the mitochondria. It is clear that in the presence of the enzyme, pyruvate and carbon dioxide, oxaloacetate in amounts sufficient to initiate the condensation to citrate will be formed (Utter & Wood, 1946). Determination of steam-volatile acid and of pyruvate disappearing show that a one-step oxidative decarboxylation does not occur in the absence of added primer. Several attempts were made to obtain this reaction in mitochondrial systems. None was successful. The addition of aneurin pyrophosphate, coenzyme I (Co I) and adenosinetriphosphate (ATP) failed to influence this result. Water suspensions of mitochondria were also tested, but these failed to show any activity whatever.

Table 2. CO_2 *priming of pyruvate oxidation; the effect of leaving out primer, adenosine-5-phosphate and cytochrome* c

(The complete system was as described in the text except that 30 μg.mol. of $NaHCO_3$ were added to prime the reaction; the system contained Na_2HPO_4, pH 7·40, 0·03M, as buffer. Rabbit-kidney mitochondria. Temp. 38°. Gas phase: air.)

System	O_2 uptake (μl./30 min.)	Steam volatile acid as μg.mol. acetate
Complete	214	0·45
Adenosine-5-phosphate	57	0·90
No primer	15	—
No cytochrome *c*	180	—

Schneider & Potter (1949) have studied the oxaloacetic oxidase of rat-liver mitochondria and have claimed that the addition of the microsomes to the mitochondria gave rise to increased respiratory rates, though the microsomes were devoid of oxidase activity. These experiments were repeated to determine whether the extra oxygen uptake was associated with an increased phosphate uptake. It was found that the microsomes had a negligible action on oxygen uptake and P/O ratio, both in the presence and absence of fluoride. However, these

Table 3. *Inhibition of pyruvate oxidation by DNP*

(The systems were as described in the text. The analyses for pyruvate and acetate were carried out at the end of the experiments, when no further movement of the manometers could be observed. Exps. 1 and 2, the flask contained 10 μg.mol. pyruvate; Exp. 3, 22 μg.mol. pyruvate.)

Exp. no.	Inhibitor	Primer	O_2 uptake (μl.) 1st 20 min.	O_2 uptake (μl.) 2nd 20 min.	O_2 uptake (μl.) Total	Pyruvate disappearing (μg.mol.)	Steam volatile acid as μg.mol. acetate	Time (min.)
1	Nil	0·001 M-Fumarate	342	210	650	10·0	0·0	100
	5×10^{-5} M-DNP	0·001 M-Fumarate	300	90	480	9·7	2·2	100
2	Nil	0·01 M-$NaHCO_3$	270	190	550	10·0	0·0	100
	5×10^{-5} M-DNP	0·01 M-$NaHCO_3$	85	60	202	7·7	4·0	100
3	Nil	0·01 M-$NaHCO_3$	224	250	1000	21·0	0·0	140
	10^{-4} M-DNP	0·01 M-$NaHCO_3$	82	37	170	12·5	5·5	140

Table 4. *Reversal of DNP inhibition of pyruvate oxidation by ATP and* Co I

(The control system was as described in the text, 30 μg.mol. of pyruvate and 1 μg.mol. of succinate being substrates. The additions were made from side arms at 30 min. from the start of the experiment. ATP 160 μg. of labile P; Co I 2·5 μg.mol. Rat-liver mitochondria. Temp. 38°. Gas phase: air.)

System	Addition	O_2 uptake (μl. O_2 at 10 min. intervals after addition) 1st 10 min.	2nd 10 min.	3rd 10 min.	4th 10 min.
Control	ATP + Co I	138	139	139	135
Control	Nil	100	94	92	90
10^{-4} M-DNP	Nil	28	21	10	5
10^{-4} M-DNP	ATP + Co I	108	111	88	48
10^{-4} M-DNP	Co I	65	74	57	44

experiments differed from those of Schneider & Potter in that the bath temperatures employed were 15°, while the latter investigators worked at 38°. In the presence of sodium fluoride and at 15°, an uncorrected P/O ratio of 3·4 was obtained for pyruvate oxidation.

DNP at low concentrations (approx. 10^{-4} M) will inhibit the oxidation of pyruvate in these systems. The inhibition is variable. In some preparations immediate depression of the oxygen uptake is observed; in others the inhibition becomes apparent after some 20–30 min. incubation, during which time the rate of respiration in the DNP-poisoned systems is the same as, or slightly higher than, that in the controls. Typical examples of such experiments are shown in Table 3. No α-ketoglutarate accumulated in the flasks.

Reversal of DNP effect. Experiments were then undertaken to reverse this inhibitory effect of DNP, and it was found that reversal could be obtained by the addition of ATP or of Co I from the side arms of the flasks at the time when inhibition became apparent. The addition of aneurin pyrophosphate or of aneurin hydrochloride together or separately did not have this effect; nor did they influence the effect of ATP and Co I. Table 4 shows an experiment in which ATP (2·6 μg.mol.) and Co I (2·5 μg.mol.) were added. The figures show the oxygen uptakes from the moment when the inhibition became apparent and the additions were made. There was a considerable increase in the respiratory rate for some 20 min. after the additions.

Lipmann & Kaplan (1949) have claimed that the DNP inhibition of pyruvate oxidation can be reversed by the addition of dicarboxylic acids of the citric acid cycle. This claim has been confirmed, and in Table 5 are given interval oxygen uptakes for a system oxidizing pyruvate in the presence of 1·0 and 3·0 μg.mol. of L-malate, with and without DNP.

Table 5. *Reversal of DNP inhibition of pyruvate oxidation by extra malate and ATP*

(All systems contained 30 μg.mol. of pyruvate and 1 μg.mol. of malate. Extra malate added as shown, ATP when added was equivalent to 390 μg. of labile P.)

System	Addition	O_2 uptake (μl.) 2nd 15 min.	4th 15 min.
Control	ATP	295	250
10^{-4} M-DNP	ATP	232	183
Control	2 μg.mol. malate	296	270
10^{-4} M-DNP	2 μg.mol. malate	277	191
10^{-4} M-DNP	Nil	259	59

The extra malate had the effect of postponing the inhibitory effect of DNP, despite the fact that it was in the main compartment of the flask from the start of the experiment. In the same experiment ATP (6·3 μg.mol.) was added to an equivalent number of flasks, and it will be seen that its effect on the

inhibition was identical with that of malate, despite the fact that the adenosinetriphosphatase (ATP-ase) of the preparation would have destroyed the ATP long before its action became apparent.

It should be pointed out that the inhibition of pyruvate oxidation is fundamentally different from the inhibition of octanoate oxidation by DNP, and in keeping with this is the fact that ATP will not reverse the DNP effect on fatty acid oxidation.

To afford a single explanation for the effects of added ATP and extra malate on the inhibition of pyruvate oxidation, it must be supposed that the oxidation of malate via the tricarboxylic acid cycle gives rise to high-energy phosphate bonds even in the presence of DNP, which has been shown to inhibit, apparently completely, the phosphate uptake associated with the oxidations catalysed by the particles (Loomis & Lipmann, 1948; Cross, Covo, Taggart & Green, 1949; Judah & Williams-Ashman, 1951). This point was, therefore, further investigated, and experiments were undertaken to determine the possible site of DNP action.

Attempts to localize DNP action. A simple explanation of the problem would be to assume that DNP inhibited the net uptake of phosphate, permitting the formation of ATP which, however, was rapidly used up in side reactions before transphosphorylation to hexose could occur. One such side reaction is the breakdown of ATP by adenosinetriphosphatase (ATP-ase), but others might exist. Hunter & Hixon (1949*a*) have shown that by working at 15° very high P/O ratios can be obtained. The action of DNP on oxidative phosphorylation at this temperature was therefore tested in the hope that under optimal conditions any P uptake would become apparent. Table 6 shows an experiment with glutamate as substrate, in which no P uptake was apparent in the presence of DNP, though a P/O ratio as high as 3·8 was obtained in the controls.

Table 6. *Action of DNP on* P/O *ratio*

(The control system contained 30 μg.mol. L-glutamate as substrate. Inorganic orthophosphate, 0·02 M; adenosine-5-phosphate, 0·001 M; $MgSO_4$, 0·0067 M; NaF, 0·013 M; cytochrome *c*, 0·00001 M; KCl, 0·025 M; hexokinase, 0·2 ml.; fructose, 0·033 M; 0·5 ml. mitochondrial suspension in 0·25 M-sucrose and water to 3·0 ml. Time in bath 10 min. Temp. 15°. Gas phase: air.)

System	O_2 uptake (μg.atoms)	P uptake from inorganic orthophosphate (μg.atoms)	P/O ratio
Control	6·8	26·0	3·8
10^{-4} M-DNP	6·55	0·0	—

An attempt was therefore made to localize the steps at which phosphate uptake might occur and to determine the effect of DNP upon them.

Phosphorylation coupled to cytochrome c *oxidation; effect of DNP.* In the presence of rat-liver mitochondria, the addition of cytochrome *c* (0·0001 M) and L-ascorbic acid (0·01 M) resulted in a rapid oxygen uptake. Table 7 shows that a clear-cut phosphorylation takes place during this oxidation, the P/O ratio approaching a value of 1·0. In each experiment blanks were set up, leaving out cytochrome *c*, L-ascorbic acid or both, and the oxygen consumption and phosphate uptake in these flasks has been subtracted from the values observed in the complete systems. DNP abolishes the P uptake while causing a slight acceleration of oxygen uptake.

Because Lehninger (1949) has suggested that dehydroascorbic acid might be further oxidized by rat-liver particles, and thus account for a phosphate uptake, the disappearance of ascorbic acid was investigated. Flasks were set up with all components; without cytochrome *c*; without particles, and without particles or cytochrome *c*. Initial values were obtained from identical flasks which were deproteinized at the end of equilibration. The results of the experiments are shown in Table 8. Very good agreement was obtained between the oxygen uptakes and chemical estimations of ascorbic acid, and this indicates a one-step oxidation of ascorbic to dehydroascorbic acid.

The enzyme preparation was of such activity as to necessitate only a short incubation in the bath. Ascorbic acid was dissolved and neutralized a few seconds before being pipetted into the ice-cold Warburg flasks. Under these conditions the spontaneous breakdown of ascorbate was negligible and the blanks small.

Table 7. *Phosphorylation coupled with oxidation of reduced cytochrome* c

(The control system contained cytochrome *c*, 0·0001 M; L-ascorbate, 0·01 M, with other components as in Table 6. Temp. 15°. Rat-liver mitochondria in every case. The figures shown have had blanks subtracted as described in the text.)

System	O_2 uptake (μg.atoms)	P uptake from inorganic orthophosphate (μg.atoms)	P/O ratio	Time in bath (min.)
Control	8·0	7·3	0·91	7
10^{-4} M-DNP	8·9	0·0	—	7
Control	11·0	6·1	0·56	15
Control	9·0	4·5	0·50	10
Control	10·6	5·3	0·50	10

Table 8. *Oxidation of* L-*ascorbic acid by rat-liver mitochondria*

(Time in bath 12 min. Initial values determined by tipping acid into duplicate flasks at end of equilibration. All components as in Table 7. Temp. 15°. Gas phase: air.)

	Complete system (blank subtracted)	No cytochrome *c*	No enzyme	No enzyme, no cytochrome *c*
O_2 uptake (μg.atoms)	15·5	0·9	0·0	0·0
Ascorbic acid disappearing (μg.mol.)	15·3	0·0	2·5	0·0

Table 9. P/O *ratio of* β*-hydroxybutyrate oxidation*

(The control system contained 30–60 μg.mol. 1-β-hydroxybutyrate and 40 μg.mol. of inorganic orthophosphate. Other components as in Table 6. Rat-liver mitochondria. Temp. 15°. Gas phase: air.)

System	O_2 uptake (μg.atoms)	P uptake from inorganic orthophosphate (μg.atoms)	P/O ratio	Time in bath (min.)
Control	11·5	29·8	2·6	20
Control	8·1	23·4	2·9	28
DNP, 10^{-4} M	8·0	0·0	—	28

The results of these experiments do not support the hypothesis that the phosphorylation recorded above is due to the oxidation of breakdown products of dehydroascorbic acid; at least within the limits of accuracy of the methods.

Effect of L-*cysteine on* P *uptake.* When L-cysteine was the reducing agent, no P uptake was observed, confirming the finding of Lehninger (1949). However, the behaviour of cysteine was anomalous. The cytochrome oxidase activity was far smaller in the presence of this compound than in the presence of ascorbate; furthermore, though it had only a slight effect in diminishing oxidative phosphorylation in the presence of β-hydroxybutyrate, it severely impaired the P uptake when it was added together with ascorbate in the cytochrome oxidase system. No obvious explanation for these anomalies can be suggested.

Phosphorylation coupled with oxidation of reduced Co I*; effect of DNP.* Lehninger & Smith (1949) have shown that rat-liver particles oxidize β-hydroxybutyrate to acetoacetate, this oxidation being coupled with a phosphorylation, the P/O ratio being about 2·0. Lehninger (1949) has given reasons for regarding this phosphorylation as associated with the oxidation of reduced Co I. At 15° the oxidation of β-hydroxybutyrate gave a P/O ratio approaching 3·0 (Table 9). DNP abolishes the P uptake without affecting oxygen uptake. This point is considered again later.

Effect of DNP on an anaerobic dismutation of α*-ketoglutarate.* Hunter & Hixon (1949*b*) have shown that in the anaerobic dismutation of α-ketoglutarate and ammonia (in which 1 mol. of α-ketoglutarate is oxidized to succinate at the expense of a second mol. which is reduced to glutamate) a phosphorylation takes place, the value of P μg.atoms taken up per μg.mol. α-ketoglutarate oxidized approaching a value of 1·0. Repetition of these experiments at 15° confirmed this figure. Table 10 illustrates experiments in which the action of DNP and azide on this phosphorylation was tested. The inhibitory effect of DNP is relatively slight, azide has no effect on the P uptake, nor do the two compounds when added together. No effect on the dismutation was observed and the disappearance of ammonia is in good agreement with the requirements of the reaction. It should be noted that in Table 10 ratios are given as P/2 α-ketoglutarate; this follows from the fact that 2 mol. of substrate disappear for each one oxidized; the P uptake is therefore related to each pair of mol., that is, to one-half of the total α-ketoglutarate disappearing.

A phosphate balance study of the reaction mixture after incubation with DNP showed that the orthophosphate taken up was incorporated into an acid-stable ester which had a soluble barium salt, which was precipitated at pH 8·2 by the addition of 4 vol. of ethanol, and may therefore be presumed to be a hexosemonophosphate. No easily hydrolysable phosphate accumulated.

To confirm that the concentrations of azide used were effective under conditions in which they had been tested previously (Loomis & Lipmann, 1949), the anaerobic oxidation of glutamate and α-ketoglutarate by ferricyanide was studied. Table 11 shows that azide and DNP powerfully inhibit P uptake while leaving ferricyanide reduction unaffected. The phosphate uptakes with a number of substrates in the presence of ferricyanide are included in Table 11. The ratio P/2 ferricyanide approaches a value of 1·0 for all. This value is not exceeded at 15°. In Table 11 the last column shows the ratio μg.atoms P taken up per pair of μg.mol. ferricyanide reduced. This follows from the fact that a single electron is transferred per mol. of ferricyanide.

Table 10. *Phosphorylation in the anaerobic dismutation of α-ketoglutarate and ammonia. Action of DNP and sodium azide*

(The control system contained 30–50 μg.mol. α-ketoglutarate; 50–80 μg.mol. of NH_4Cl; 30 μg.mol. of orthophosphate and other components as in Table 6. The experiments were carried out in Thunberg tubes. Rat-liver mitochondria in every case. Temperature varied between 15 and 25° in different experiments.)

System	α-Ketoglutarate disappearing (μg.mol.)	NH_3 disappearing (μg.mol.)	P uptake from inorganic orthophosphate (μg.atoms)	P/2 α-ketoglutarate	Inhibition (%)
Control	22·0	10·0	6·5	0·59	—
10^{-4} M-DNP	22·0	9·0	6·5	0·59	0·0
2×10^{-3} M-NaN_3	22·0	11·0	6·5	0·59	0·0
Control	14·5	7·2	6·0	0·82	—
10^{-4} M-DNP	15·7	6·2	4·9	0·62	25·0
2×10^{-3} M-NaN_3	14·5	6·2	5·7	0·78	5·0
Control	30·0	—	9·4	0·63	—
2×10^{-4} M-DNP	30·0	—	6·0	0·40	36·0
4×10^{-3} M-NaN_3	30·0	—	9·4	0·63	0·0
Control	21·7	—	9·0	0·84	—
2×10^{-4} M-DNP	19·3	—	5·4	0·56	33·0
Control	27·5	—	13·0	0·95	—
2×10^{-4} M-DNP	25·6	—	8·7	0·68	28·0
8×10^{-3} M-NaN_3	22·5	—	9·6	0·85	9·0

Table 11. *Inhibition of* P *uptake by DNP and azide in presence of ferricyanide*

(The control system contained 30 μg.mol. of substrate in the case of glutamate, α-ketoglutarate and β-hydroxybutyrate and 100 μg.mol. in the case of succinate. Ferricyanide, 50 μg.mol., was added. Other components as in Table 6; rat-liver mitochondria in every case, the experiments being carried out in Thunberg tubes at temperatures varying between 15 and 25° in different experiments.)

System	Substrate	Ferricyanide reduced (μg.mol.)	P uptake from inorganic orthophosphate (μg.atoms)	P/2 ferricyanide
Control	Glutamate	16·8	4·9	0·58
NaN_3, 2×10^{-3} M	Glutamate	16·8	0·8	0·10
Control	Glutamate	20·5	8·1	0·79
Control	α-Ketoglutarate	25·0	11·3	0·90
DNP, 10^{-4} M	α-Ketoglutarate	25·0	0·0	—
NaN_3, 4×10^{-3} M	α-Ketoglutarate	25·0	1·3	0·14
Control	Succinate	34·8	7·4	0·43
Control	β-Hydroxybutyrate	22·4	9·9	0·89

As the anaerobic dismutation studied involves two oxidations:

$$\alpha\text{-ketoglutarate} \rightarrow \text{succinate}, \quad (1)$$
$$\text{Co I } H_2 \rightarrow \text{Co I}, \quad (2)$$

it is not possible to state precisely which reaction is coupled with the phosphorylation, though it is probably reaction (1). To obtain evidence on this point, α-ketoglutarate and β-hydroxybutyrate were incubated aerobically with rat-liver mitochondria in the presence of DNP. The purpose of this experiment was twofold: (1) to determine the effect of DNP on the oxidation of reduced Co I, as exemplified by the oxidation of β-hydroxybutyrate, and (2) to determine whether the aerobic oxidation of α-ketoglutarate would show a phosphate uptake in the presence of DNP, as suggested by the dismutation and contrary to previous experience (Cross *et al.* 1949). The inorganic orthophosphate addition was limited to 20 μg.mol. and the incubation times prolonged to make it possible to detect relatively small changes in orthophosphate content. Table 12 shows that no phosphate uptake was observed in the presence of β-hydroxybutyrate, while with α-ketoglutarate phosphorylation was easily demonstrable. It will be seen that while the P/O ratio for the latter substrate was 0·2, the ratio μg.atoms P taken up/μg.mol. α-ketoglutarate disappearing was 0·4, which is in fair agreement with the figures obtained in the dismutation in the presence of DNP. To confirm that the systems oxidizing the substrates were functioning properly, P/O ratios were determined in the absence of DNP. Figures of 3·4 and 2·9 were obtained for α-ketoglutarate and β-hydroxybutyrate respectively.

It thus appears likely that the phosphorylation in the anaerobic dismutation of α-ketoglutarate and ammonia is coupled with reaction (1) and that a mechanism exists whereby ATP can be formed in the presence of DNP. The site of action of the latter can also be inferred from these data; it is suggested as a working hypothesis that DNP inhibits phosphate

Table 12. *Phosphate uptake in presence of DNP in aerobic systems*

(The systems contained 30 μg.mol. α-ketoglutarate or 60 μg.mol. β-hydroxybutyrate, DNP, 2×10^{-4} M; inorganic orthophosphate, 20 μg.mol.; other components as in Table 6. Temp. 15°. Gas phase: air.)

Substrate	O_2 uptake (μg.atoms)	P uptake from inorganic orthophosphate (μg.atoms)	α-Ketoglutarate disappearing (μg.mol.)	P/α-ketoglutarate (μg.atoms/μg.mol.)
α-Ketoglutarate	21·0	4·7	12·0	0·40
	26·6	6·8	16·0	0·43
β-Hydroxybutyrate	16·0	Nil	—	—
	18·6	Nil	—	—
	16·7	Nil	—	—

Table 13. Co I *requirement for α-ketoglutarate oxidation in aged preparations of rat-liver mitochondria*

(The systems contained adenosine-5-phosphate, 0·001 M; inorganic orthophosphate, 0·01 M; $MgSO_4$, 0·0067 M; α-ketoglutarate, 0·01 M; KCl, 0·025 M; 0·5 ml. rat-liver mitochondria suspended in 0·25 M-sucrose and additions as indicated. Final volume 3·0 ml. Temp. 38°. Gas phase: air. For details of preparation, see text.)

Exp. no.	System	O_2 uptake (μl.) Methylene blue $0·8 \times 10^{-4}$ M	Methylene blue Nil	Cytochrome *c*, 10^{-5} M	No cytochrome *c*	No aneurin pyrophosphate	α-Ketoglutarate disappearing (μg.mol.)
1	Co I (5 μg.mol.)	233	235	—	—	—	—
	No Co I	33	26	—	—	—	—
2	Co I (5 μg.mol.) + malonate (0·03 M)	—	—	142	141	128	15·0
	Malonate (0·03 M), no Co I	—	—	65	70	—	7·5
3	Co I (5 μg.mol.)	—	—	280	—	—	18·0
	Alkali-inactivated Co I (5 μg.mol.)	—	—	80	—	—	12·0
	No Co I	—	—	98	—	—	12·0
	ATP (6·5 μg.mol.)	—	—	80	—	—	9·0

uptake in the respiratory sequence from pyridine nucleotide level upwards. Since no evidence exists that pyridine nucleotides play any part in the oxidation of α-ketoglutarate, it was decided to investigate this possibility.

Co I *requirement for α-ketoglutarate oxidation.* Rat-liver mitochondria were aged for 24–30 hr. after preparation. During this time they were maintained at 0°. On incubation after this period, with α-ketoglutarate as substrate, it was found that the oxygen uptake fell rapidly after 10 min. and soon became negligible. Table 13 shows that if Co I were added a great enhancement of respiration is observed. When the experiment is carried out in the presence of malonate, it will be seen that the changes are not so great, but an increased removal of α-ketoglutarate is confirmed by the chemical estimations. It can be seen that this increased removal amounts to 30–50%. The results in Table 13 also show that alkali-inactivated Co I is ineffective in stimulating the oxygen uptake, and that ATP will not replace pyridine nucleotide. There is no obvious requirement for aneurin pyrophosphate or cytochrome *c*; nor is methylene blue required as a carrier. When these aged preparations were tested for their ability to carry out oxidative phosphorylation, no activity could be detected in the experiments carried out at 38°. However, in a single experiment carried out at 25°, a P/O ratio of 1·5 was obtained with α-ketoglutarate as substrate; this experiment was conducted with a different preparation to those illustrated above.

In order to shorten the times of ageing, several experiments were done in which mitochondria were aged in the presence of DNP (2×10^{-4} M) for 1 hr. at 0°. In no case did this procedure have any effect on the ability of the particles to oxidize L-glutamate or α-ketoglutarate. The addition of large amounts of Co I did not increase oxygen consumption significantly, whether DNP were present in the flasks or not, and whether the systems were deficient in inorganic orthophosphate or not.

These experiments do not prove that the enzyme oxidizing α-ketoglutarate is a Co I linked dehydrogenase or a pyridinoprotein, in the sense that triosephosphate dehydrogenase may be considered one; Dr M. Dixon has pointed out that in mitochondrial systems the Co I may quite well be concerned in the regeneration of another co-factor essential for α-ketoglutarate oxidation.

The mechanism of phosphate 'replacement' by DNP

Loomis & Lipmann (1948) showed that in the absence of added inorganic orthophosphate, DNP caused a stimulation of respiration which raised the oxygen uptake of such deficient systems to the same value as that for the controls. This finding was confirmed by Judah & Williams-Ashman (1951). Teply (1949) claimed that pre-incubation of the particles with DNP destroyed this stimulating effect, and suggested that 'replacement' was due to liberation of inorganic orthophosphate from labile esters firmly bound to the particles (gel-P). This 'gel-P' could not be washed away with large volumes of 0·9 % (w/v) potassium chloride.

It has been found in the present work that washing of purified mitochondrial preparations with 0·25 M-sucrose reduces the phosphate concentration to a level where replacement is no longer observed. Figures for a typical experiment are presented in Table 14 and show that in the presence of α-ketoglutarate as substrate, and in a well washed preparation, DNP causes slight stimulation of respiration in the absence of added inorganic orthophosphate. The stimulating power of DNP may be increased by the addition of small amounts of inorganic orthophosphate. Figures are also given for initial and final inorganic orthophosphate levels in the flasks, and show that no output of inorganic orthophosphate has taken place. On the contrary, a marked uptake is seen, even in the presence of DNP. This could be expected from evidence presented earlier in this paper, which shows that DNP permits P uptake in α-ketoglutarate oxidation. Loss of 'replacement' by DNP could also be shown when L-glutamate was substrate. With β-hydroxybutyrate as substrate, the restoration of 'replacing' activity is achieved with much lower phosphate concentrations than is the case with α-ketoglutarate as substrate (Table 15) as might have been expected, as DNP completely blocks P uptake during this oxidation.

The reduction of stimulating ability could also be achieved when the oxidation of α-ketoglutarate and glutamate were carried out anaerobically in the presence of ferricyanide, but restoration was not satisfactory on addition of phosphate in small amounts. These results are presented in Table 16. They make it reasonable to doubt the hypothesis of Loomis & Lipmann (1949) that azide inhibits a transphosphorylation between a 'primary ester' and adenosine-5-phosphate, while DNP prevents the formation of 'primary ester'. This hypothesis was based on the inability of azide to 'replace' orthophosphate, a finding which has been confirmed.

Table 14. *Effect of DNP on oxidation of α-ketoglutarate in phosphate-deficient systems*

(The mitochondria were washed by five successive centrifugations at 15,000 *g* in 0·25 M-sucrose after an initial sedimentation at 9000 *g*. Substrate α-ketoglutarate, 0·01 M; other components as in Table 12, except that phosphate additions were as shown below. Temp. 25°. Rat liver in every case. Where phosphate was omitted NaCl was added to maintain salt concentration.)

Phosphate (μg.)			O_2 uptake (μl.)			
Initial	Final	Difference	1st 10 min.	2nd 10 min.	3rd 10 min.	DNP × 10^{-4} M
944	—	—	67	79	81	0·0
14	7·0	7·0	20	17	17	0·0
14	7·0	7·0	26	21	20	2·0
76	7·0	69·0	30	23	19	0·0
76	16·0	60·0	58	38	35	2·0
107	7·0	100	28	22	21	0·0
107	28·0	79	64	49	44	2·0
138	7·0	131	33	23	21	0·0
138	50·0	88	69	56	48	2·0

Table 15. *Effect of DNP on oxidation of* L-*glutamate and* 1-β-*hydroxybutyrate in phosphate-deficient system*

(Details as for Table 14, except for substrates. 30 μ.mol. L-glutamate or 30 μ.mol. 1-β-hydroxybutyrate added. Rat-liver mitochondria at 25°. Where phosphate was omitted NaCl was added to maintain salt concentration.)

Substrate	Phosphate (μg.)			O_2 uptake (μl.)	DNP × 10^{-4} M
	Initial	Final	Difference		
L-Glutamate	944	—	—	104	0·0
	14·0	14·0	0·0	29	0·0
	14·0	13·0	−1·0	42	2·0
1-β-Hydroxybutyrate	940	—	—	78	0·0
	10·0	—	—	13·0	0·0
	10·0	—	—	41·0	1·0
	950	—	—	51·0	0·0
	21·5	7·0	−14·5	24·0	0·0
	21·5	34·0	+12·5	40·0	1·0
	37·0	7·0	−30·0	24·0	0·0
	37·0	48·0	+11·0	46·0	1·0

Table 16. *Failure of DNP and azide to 'replace' P in presence of ferricyanide*

(In the first two experiments CO_2 output was determined manometrically; the flasks containing 0·02 M-$NaHCO_3$ with 95% N_2–5% CO_2 in the gas phase. The third experiment was carried out in Thunberg tubes. All components as in Table 11. 30 μg.mol. α-Ketoglutarate as substrate in Exps. 1 and 3; L-glutamate in Exp. 2; no hexokinase or fructose present. Rat-liver mitochondria. Temp. 25°. Where phosphate was omitted NaCl was added to maintain salt concentration.)

Exp. no.	System	Control	No orthophosphate added	No orthophosphate + DNP, 10^{-4} M	No orthophosphate + NaN_3, 8×10^{-3} M	No orthophosphate + DNP and NaN_3
1	CO_2 output (μl./4 min.)	253	147	168	160	181
2	CO_2 output (μl./10 min.)	187	128	157	—	—
3	Ferricyanide reduced (μg.mol./45 min.)	21·8	15·0	13·4	—	—

In order to explain the discrepancy between the present work and the findings of Teply (1949) several experiments were carried out. A lead to the correct interpretation was obtained by an experiment in which exhaustive washing of the mitochondria was not attempted. In this case it was found that sufficient phosphate remained in the preparation to show a typical replacement effect by DNP. However, when the particles were pre-incubated with DNP, this ability was lost. It is concluded that in the absence of proper washing, pre-incubation with DNP will reduce the potential inorganic orthophosphate. The source of this inorganic orthophosphate is not certain, but quite clearly there is no evidence to suggest 'gel-P' as being responsible. An experiment which suggests the contrary is shown in Table 17. This indicates that

Table 17. *Failure of DNP to 'replace' inorganic orthophosphate in the anaerobic dismutation of α-ketoglutarate and ammonia in phosphate-deficient systems*

(The reaction was followed manometrically. The complete system was as described in Table 10, but no fructose or hexokinase were present: in addition $NaHCO_3$, 0·02 M, was added. Gas phase, 95% N_2–5% CO_2. Temp. 25°. Rat-liver mitochondria. Where phosphate was omitted NaCl was added to maintain salt concentration.)

System	Complete	No added inorganic ortho-phosphate	No added inorganic ortho-phosphate + 10^{-4} M-DNP
CO_2 output (μl./5 min.)	102	15·0	28·0

the anaerobic dismutation of α-ketoglutarate and ammonia is very sensitive to phosphate deficiency. DNP did not 'replace', though 2·5 μg.mol. inorganic orthophosphate were present and the preparation had been subjected to only the normal washing procedure (two washes). As this dismutation is very sensitive to phosphate lack and as DNP affects the phosphorylation only slightly, it is clear that this experiment is a reasonable test for the hypothesis of Teply (1949) that DNP stimulation of phosphate-deficient systems is due only to liberation of inorganic orthophosphate from the particles.

It should be pointed out that exhaustive washing of mitochondria does not reduce their ability to esterify inorganic orthophosphate. Experiments on preparations subjected to six successive washings on the centrifuge at 15,000 *g* revealed that the efficiency of oxidative phosphorylation was unimpaired, P/O ratios of approx. 2·0 being obtained at 25°, this being the temperature at which all the experiments on phosphate replacement were carried out. From these experiments it is concluded that 'replacement' of inorganic orthophosphate by DNP represents a sparing or economizing of inorganic orthophosphate and that it is only manifest when a certain critical concentration of inorganic orthophosphate is present in the medium.

The synthesis of ATP from inorganic pyrophosphate and Co I

Judah & Williams-Ashman (1951) showed that inorganic pyrophosphate when added to respiring systems did not change in amount. However, it seemed certain that mitochondrial systems are capable of maintaining their coenzyme levels, and it was decided to investigate the possibility that the reaction described by Kornberg (1950) takes place in such systems. Kornberg showed that reversible synthesis of Co I can take place as follows:

$$\text{ATP} + \text{NMN} \rightleftharpoons \text{Co I} + \text{P.P},$$

where NMN is nicotinamide mononucleotide and P.P is inorganic pyrophosphate. The formation of ATP from Co I and inorganic pyrophosphate was readily demonstrated in rabbit-kidney mitochondrial systems. Table 18 illustrates an experiment in which hexokinase and fructose were added to the system, together with Co I and inorganic pyrophosphate. Under these conditions the terminal phosphate atom of ATP is removed as soon as formed and the

Table 18. *Synthesis of ATP from* Co I *and inorganic pyrophosphate*

(The complete system contained Co I, 0·001 M; inorganic pyrophosphate, 0·001 M; hexokinase, 0·02 ml.; fructose, 0·02 M; $MgSO_4$, 0·0067 M; NaF, 0·013 M; 0·5 ml. 0·1 M-veronal-HCl buffer; pH 7·40, and water to 3·0 ml. Temp. 38°. Time in bath 20 min. Rabbit-kidney mitochondria. All figures are corrected for small changes in orthophosphate content which occurred.)

System	Complete	No Co I	No inorganic pyrophosphate	No hexokinase
Decrease in easily hydrolysable P (μg.)	60	15	0·0	5·0

Table 19. *Phosphate balance of ATP synthesis from* Co I *and inorganic pyrophosphate*

(The system was as described in Table 18, but on five times the scale. The figures for easily hydrolysable P are corrected for orthophosphate.)

Barium-insoluble fraction

	Orthophosphate (μg.)	Easily hydrolysable P (μg.)	Total P (μg.)	Inorganic pyrophosphate (as orthophosphate) (μg.)
Initial values	440	920	1880	800
After incubation	360	360	1240	Nil
Difference	−80	−560	−640	−800

Barium-soluble: ethanol-insoluble fractions

	Orthophosphate (μg.)	Easily hydrolysable P (μg.)	Total P (μg.)	Reducing power in terms of fructose-6-P barium salt (mg.)
Initial values	Nil	Nil	1460	Nil
After incubation	Nil	Nil	820	9·5
Difference	—	—	−640	+9·5

Table 20. *Oxidative phosphorylation; pyruvate and octanoate oxidation after thyroxine treatment of rats*

(The mitochondria were prepared from equal amounts of tissue from control and thyroxine-treated rats (see text), except in the last experiment where no precautions were taken to obtain equal amounts. System as described in Tables 2 and 6. Temperature 38° except in the last experiment when it was 20°. 30 μg.mol. of substrate added except in the case of octanoate where 4 μg.mol. of fatty acid and 3 μg.mol. of succinate were used and in the case of pyruvate where 1 μg.mol. of succinate was added as primer to 30 μg.mol. of pyruvate.)

		Oxidative phosphorylation				
Mitochondria from	Substrate	O_2 uptake (μg.atoms)	P uptake from inorganic orthophosphate (μg.atoms)	P/O ratio	Pyruvate oxidation O_2 uptake (μl./20 min.)	Octanoate oxidation O_2 uptake (μl./20 min.)
Control	L-Malate	17·7	33·0	1·87	276	279
Thyroxine treated	L-Malate	21·4	26·6	1·24	395	393
Control	L-Malate	18·6	36·2	1·95	—	—
Control + 0·3 unit insulin	L-Malate	18·6	35·4	1·91	—	—
Thyroxine treated	L-Malate	22·1	36·2	1·65	—	—
Thyroxine treated + 0·3 unit insulin	L-Malate	22·2	36·2	1·64	—	—
Control	L-Glutamate	7·0	16·8	2·4	—	—
Thyroxine treated	L-Glutamate	6·5	13·0	2·0	—	—

reaction may be followed by the decline in readily hydrolysable phosphate (i.e. hydrolysable by incubation at 100° in N-hydrochloric acid for 10 min.). It will be seen that such a decline takes place in the presence of all components, but not in the absence of Co I, inorganic pyrophosphate or of hexokinase. Table 19 gives the result of a balance experiment carried out on a larger scale with an identical preparation; a satisfactory balance is obtained. The formation of ATP is indicated by the accumulation of hexose monophosphate as a result of the hexokinase reaction.

Further experiments showed that adenosine-5-phosphate will not substitute for Co I in this reaction.

It has been stated that no change in added inorganic pyrophosphate level is observed in freely respiring systems; however, if DNP (10^{-4} M) is added to a rabbit-kidney mitochondrial preparation while freely respiring, a decline in readily hydrolysable phosphate takes place when inorganic pyrophos-

phate is present. It may be inferred that during oxidative phosphorylation the maintenance of a high ATP level forces the reaction in the direction of Co I synthesis.

The action of thyroxine on oxidative phosphorylation. It has been shown that the addition of L-thyroxine or of thyroglobulin to systems *in vitro* affected neither their oxygen uptake nor the esterification of inorganic orthophosphate (Judah & Williams-Ashman, 1951). Mr E. V. Rowsell, of the School of Biochemistry, Cambridge, has kindly informed me of unpublished experiments in which he observed a diminution of the P/O ratio during β-hydroxybutyrate oxidation by particles prepared from rat livers after the rats had been rendered hyperthyroid by thyroxine injections.

These experiments were therefore repeated. All animals used in this work were litter mates and were maintained on high-vitamin diets; food intake was controlled by pair-feeding. The animals were treated with DL-thyroxine, injected subcutaneously in 1·0 mg. doses every other day. The controls were injected with solvent. After seven consecutive injections, the animals were killed and mitochondria prepared from their livers in the usual manner. The results in Table 20 show that the effect of thyroxine on oxidative phosphorylation was not considerable under these conditions, and that the effect on fatty acid oxidation was the reverse of that of DNP. The oxidation of pyruvate is also enhanced by thyroxine treatment. It is of some interest that turbidimetric determination of the number of mitochondria and estimation of ribonucleic acid phosphate showed that equal numbers of particles were present in the flasks containing control and thyroxine-treated tissue. These experiments were not extended, but they indicate that the action of thyroxine both *in vitro* and *in vivo* is essentially different from that of DNP.

DISCUSSION

From the experiments quoted in this paper, the site of action of DNP can be inferred. It has been shown that DNP only slightly reduces the phosphate uptake coupled with the anaerobic dismutation of α-ketoglutarate and ammonia; it has also been shown that in the aerobic oxidation of the same substrate in the presence of DNP, a phosphate:α-ketoglutarate ratio approximating to that obtained in the dismutation is found. Since DNP causes complete abolition of phosphate uptake associated with β-hydroxybutyrate oxidation, it seems reasonable in the light of present knowledge to assume that DNP inhibits phosphate uptake in the respiratory sequence from pyridine nucleotide upwards. In making this suggestion, it is assumed that Co I takes part in the oxidation of α-ketoglutarate. Though this has never been shown conclusively, evidence is presented in this paper that it may in fact participate. It has also been assumed that the oxidation of reduced Co I (as exemplified by the oxidation of β-hydroxybutyrate) involves an identical mechanism whatever the substrate. There is no evidence for this, but it has been accepted for want of evidence to the contrary. It is obvious that these assumptions must make the conclusion tentative.

It seems clear that DNP does not uncouple oxidation from phosphorylation in the sense that arsenate uncouples these processes in the oxidation of triosephosphate. (Mr E. V. Rowsell has kindly informed me of experiments conducted by Dr G. D. Greville and himself (1949, unpublished observations) in which it was found that DNP has no effect on phosphorylation coupled with the anaerobic oxidation of triosephosphate, using pyruvate as hydrogen acceptor and muscle acetone powder as source of enzyme.) While no proof is forthcoming, it is suggested that DNP inhibits a transphosphorylation between an unstable ester or esters and adenosine-5-phosphate at the three successive steps from pyridine nucleotide, through flavoprotein to the cytochrome system. Some evidence is available that DNP may act in the manner indicated. It is clear that the methods normally used to estimate inorganic orthophosphate (deproteinization with trichloroacetic acid followed by an estimation of the molybdenum blue colour in strongly acid solution) might well fail to demonstrate the presence of a very labile phosphate compound. Some experiments were undertaken to test this point. Deproteinization was carried out by half-saturation with ammonium sulphate, followed by estimation of inorganic orthophosphate by the method of Lowry & Lopez (1946). When this method was applied to systems poisoned with DNP, it was found that colour development was delayed some 15 min. beyond the time required in the control and initial estimations. This suggested the presence and breakdown of an unstable ester. Extrapolation to zero time gave values for P/O ratio of approx. 2·0. The requisite control estimations indicated that no simple artifact was involved. Unfortunately, such results were not reproducible. Numerous attempts were made to precipitate inorganic orthophosphate under the mildest conditions in the hope that they might show the presence of the suspected labile compounds, but were not successful. Unfortunately, this work has to be suspended for the time being, and the preliminary observations given in this paper are recorded in the hope that further work may be stimulated.

The confirmation of Hunter & Hixon's (1949*a*) observations on the P/O ratio of α-ketoglutarate oxidation needs further comment. No attempt has been made to correct the ratios obtained in this work, though Hunter has introduced corrections which tend to lower his high initial figures. It would appear

that such corrections may well be necessary and may be exemplified by the case of the figures obtained for glutamate oxidation. In the latter, P/O ratios of 3·0 and over have regularly been found. Now the E'_0 of the glutamate-α-ketoglutarate system has been given as −0·03 V. at pH 7·0 by Kalckar (1941), and assuming a value of 12,000 cal. for the high-energy phosphate bond of ATP at pH 7·0 (Lipmann, 1941), this only gives room for a P/O ratio of 3·0, if one accepts the observed value of 1·0 for the P/O ratio for electron transport over the cytochrome system (cf. Dixon, 1949). Furthermore, the oxidation of β-hydroxybutyrate gives a P/O ratio of 3·0, this system having E'_0 of −0·293 V. at pH 7·0, while that of Co I is given as −0·281 V. It is therefore difficult to account for a P/O ratio of 4·0 for the oxidation of glutamate to α-ketoglutarate.

For the latter substrate, however, a case can be made for a P/O ratio as high as 4·0. The phosphorylations can be apportioned as follows: (1) The oxidation of α-ketoglutarate to succinate yields one phosphorylation; the evidence for this is presented in the present work. (2) In the presence of ferricyanide, α-ketoglutarate yields P/2 ferricyanide approaching 1·0. This phosphorylation cannot be that associated with (1) above, because it is shown that both azide and DNP inhibit in the presence of ferricyanide, but do not affect (1). It would seem that ferricyanide itself must inhibit at least one phosphorylation, which is most likely that associated with (1) above. (3) and (4) In the aerobic oxidation of β-hydroxybutyrate, a P/O ratio approaching 3·0 is observed, and if it is accepted that the oxidation of reduced cytochrome *c* gives a P/O ratio of 1·0, and it has been shown that β-hydroxybutyrate gives a P/2 ferricyanide of 1·0, the third phosphorylation probably occurs at flavoprotein level, assuming a value of 12,000 cal. for the high-energy phosphate bond. Thus, the third and fourth phosphorylations in α-ketoglutarate oxidation would be due to oxidations of reduced flavoprotein and cytochrome respectively. It is obvious that the nature of the assumptions made in this argument is such that the conclusions are open to the gravest doubts. However, it has been considered worth while making these points to formulate a working hypothesis which has the merit of linking together a series of isolated observations. The P/O ratio for β-hydroxybutyrate oxidation has been accepted in this argument because it is known that rat-liver particles oxidize this substance quantitatively to acetoacetic acid, and side reactions cannot contribute to a falsely high ratio.

Hummel & Lindberg (1949) have demonstrated that when ^{32}P is incubated with rabbit-kidney particles carrying out oxidative phosphorylation the indicator is incorporated into flavine-adenine dinucleotide (FAD) and ATP but not into Co I. Though these results are suggestive they are by no means conclusive; thus the marker will not enter Co I if its synthesis follows the reaction described by Kornberg (1950), unless the ^{32}P has been incorporated into the phosphate group of adenosine-5-phosphate. Similarly, the incorporation of the label into FAD may simply indicate that the mechanism of synthesis of the latter does not follow an identical course to that described by Schrecker & Kornberg (1950).

Evidence is presented in this paper that the reaction between Co I and inorganic pyrophosphate does occur in rabbit-kidney mitochondria, and since it is reasonable to suppose that this reaction is reversible (as is that described by Kornberg), a mechanism exists for the synthesis of Co I in these systems. It is suggested that mitochondrial systems can maintain their own coenzyme levels so long as they are capable of oxidative phosphorylation. The observations recorded in this paper on the reversal of DNP inhibition of pyruvate oxidation and on the coenzyme requirements of aged preparations support this view. Such a hypothesis would also explain the fact that the only known coenzyme requirement of freshly prepared particles is that for adenosine-5-phosphate. There is therefore no need to postulate the existence of a new type of adenyloprotein (Cross *et al.* 1949). Furthermore, the formation of inorganic pyrophosphate may be readily explained without suggesting the breakdown of hypothetical 'coenzyme pyrophosphates' (Cross *et al.* 1949).

Much of the evidence given above is unsatisfactory. It has been assumed that α-ketoglutarate oxidation involves pyridine nucleotide, but the demonstration of Co I requirement in this oxidation leaves open the possibility that Co I is playing an indirect part. The coupling of a phosphorylation with the oxidation of reduced cytochrome *c* is by no means certain. The experiments presented in this paper show that it is difficult to explain a phosphate uptake in the presence of cytochrome *c* and ascorbate as being due to the further breakdown of dehydroascorbic acid; on the other hand, the absence of effect in the presence of cysteine is an anomaly, further complicated by the fact that while cysteine has no effect on the overall P/O ratio of β-hydroxybutyrate oxidation, it certainly reduces the phosphate uptake when it is present together with ascorbate. The problem of this phosphorylation must be solved by a direct experiment. Indirect methods which attempt to bypass the cytochrome system are open to several criticisms. Friedkin & Lehninger (1949), who attempted to analyse the oxidative phosphorylation system, used carriers such as methylene blue and brilliant cresyl blue, but were unable to find any phosphate uptake in so doing. Judah & Williams-Ashman (1951) showed that both these dyes inhibit

oxidative phosphorylation. Friedkin & Lehninger (1949) also attempted to show a phosphate uptake under anaerobic conditions when excess cytochrome *c* was used as hydrogen acceptor. They failed to show any phosphorylation. Slater (1950) has recently repeated these experiments using α-ketoglutarate as substrate and has obtained P/O ratios which appear to be exactly the same whether cytochrome *c* was used as acceptor anaerobically or the system was allowed free access to oxygen. The source of enzyme in his experiments was a mitochondrial preparation isolated from cat heart. An experiment of this type is open to an obvious fallacy when it is used to determine the presence or absence of phosphorylation through the cytochrome system. As the two efficiencies have to be compared, it has to be assumed that each one represents a near approach to a maximum value. That is, a value between 2·0 and 2·9 can be taken as approaching a value of 3·0, and so on. Therefore, fractional differences between the ratios may be missed. However, this simplification is not justified; to take an extreme example it can be supposed that a series of four steps yield phosphorylations, the maximum possible P/O ratio being 4·0. Leaks and side reactions may result in 50% efficiency at each step, so that the observed P/O ratio is near 2·0. If it is a little below, it will be taken as 2·0, if a little above, it will be taken as 3·0. A greater possibility of error arises when one step is more liable to inhibition or fractional efficiency than the others. In this case, the increment due to the step in question may easily be overlooked, whereas if a direct determination were made, such an increment would be readily recognized. It seems reasonable to assume, therefore, that overall values for P/O ratios are not of great value in deciding the possible number of phosphorylations. An example which supports this belief can be quoted from determinations made in the present work and those reported in a previous paper (Judah & Williams-Ashman, 1951) for the oxidation of β-hydroxybutyrate by mitochondria. In the earlier determinations values for the P/O ratio of just over 1·0 were observed in experiments at 38°, and we believed that the maximum ratio was 2·0; at a bath temperature of 15°, values of well over 2·0 have been found, and it appears that a maximum value of 3·0 is not unreasonable. The preparations of enzyme and other components were identical in the two investigations.

As the present work has covered much the same ground as that of Friedkin & Lehninger (1949), though by different means, their results are of considerable interest in this discussion. Using ^{32}P as an indicator, these workers investigated the phosphate uptake associated with the oxidation of reduced Co I. In contrast to the work of Ochoa (1943), they found a marked incorporation of the label into ATP during the oxidation; though the results cannot supply an index of the P/O ratio, it was obvious that dihydrocozymase oxidation gave rise to as great an esterification as did the oxidation of malate. They explained the lack of phosphate uptake in Ochoa's experiments as being due to the high concentrations of nucleotide used by the latter. These have been found to be inhibitory, and it is suggested that the inhibition is not due to heavy metal contaminants but to the nucleotide itself. In order to facilitate analysis, Lehninger (1949) then used β-hydroxybutyrate as substrate, arguing from the standard oxidation-reduction potentials of this system and that of Co I that a phosphorylation at substrate level was unlikely, and that β-hydroxybutyrate oxidation represented to all intents an oxidation of reduced Co I. Indirect experiments with a dismutation between oxaloacetate and β-hydroxybutyrate and direct experiments where excess Co I was used as hydrogen acceptor supported this view. It has been confirmed in this laboratory that the anaerobic oxidation-reduction of β-hydroxybutyrate and Co I does not give rise to a phosphate uptake, and the new experiments dispose of a possible criticism of Lehninger's experiment that in the absence of sodium fluoride, hexokinase and a hexose acceptor, the small phosphate exchange would not be observable. Nevertheless, it cannot be regarded as certain that there is no phosphate uptake coupled to the oxidation of β-hydroxybutyrate to acetoacetate. It should be mentioned that the latter substance is not further oxidized by rat-liver particles. Lehninger followed these observations by an attempted analysis of the steps at which phosphorylation might occur. Despite the pronounced phosphate uptake in the whole system, he was unable to show any uptake when the cytochrome system was by-passed. The use of carriers such as methylene blue has already been mentioned, but it was also impossible to show any phosphate uptake when ferricyanide was used as hydrogen acceptor.

This observation has not been confirmed in the present work, and it is suggested that the earlier failure was due to the fact that no fluoride and no hexokinase and hexose were added; with the low ratios found in the presence of ferricyanide, there is no doubt that these omissions would have a decisive effect.

Experiments in which excess cytochrome *c* was added as hydrogen acceptor under anaerobic conditions have already been referred to. These were negative probably for the same reasons as the ferricyanide experiments. These experiments of Friedkin & Lehninger (1949) and of Lehninger (1949) clearly showed that while the over-all oxidation of reduced Co I gave rise to a well marked phosphate uptake, any attempt under their conditions to demonstrate the participation of the partial reactions was negative; later work has equally well shown that some of these

defects could be remedied, and the results underline the comments made earlier with regard to the differing efficiencies of the whole system and its parts.

The results obtained in the present work on the stimulation of phosphate-deficient systems provide reasonable evidence that DNP does not substitute for inorganic orthophosphate in these systems. This, in effect, confirms the view of Teply (1949); on the other hand, the suggestion of the latter that DNP exerts this effect by discharging 'gel-P' is not supported. It has been found that reduction of the residual inorganic orthophosphate to about 0·5 μg.mol./vol. of particles added to the flasks is sufficient to abolish the 'replacement' effect with all the substrates tested. When α-ketoglutarate is the substrate, the amount of phosphate required for full replacement is higher than when β-hydroxybutyrate or L-glutamate are used. Examination of the protocols of Teply (1949) reveals that the concentrations of 'gel-P' that he found are probably insufficient to account for DNP action. Thus he quotes the following values for 'gel-P': initial values of 0·55 μg.mol. for an untreated suspension and 0·09 μg.mol. for a DNP pre-incubated suspension. After incubation with substrate and other components, the 'gel-P' dropped to a level of 0·04 μg.mol. in the presence of DNP in phosphate-deficient systems, and rose to a value of 0·15 μg.mol. in phosphate-deficient systems in the absence of DNP. In the complete system the final value was 0·49 μg.mol. No values are recorded for complete systems in the presence of DNP, nor are inorganic phosphate levels in the suspension media. The substrate was α-ketoglutarate in this experiment. While it is possible that differences in preparations might account for differences in phosphate requirement, it is suggested that gross variations are unlikely. In the absence of figures for inorganic orthophosphate actually present, rather than values for 'gel-P', it is suggested that Teply's hypothesis is doubtful.

The significance of the 'gel-P' described by Green, Atchley, Nordmann & Teply (1949) is also doubtful. Though these workers provide evidence that 'cyclophorase' preparations become radioactive upon incubation with ^{32}P and that this phenomenon is associated with oxidative processes, there is no evidence whatever that the tracer has really been incorporated into the particles, whether in a labile coenzyme-linkage or not. Thus, 'gel-P' might well represent an ester or esters formed (free) in the suspension medium, and difficult to remove from the particles. There is nothing in the inability to wash the particles free of this material to suggest that it is actually incorporated into them. It is difficult to wash out all traces of substrate from such preparations also, as has been found both in the present work and by Green *et al.* (1948). Another point which has not been satisfactorily established is whether the 'gel-P' is actually concerned with the active particles and not with the debris associated with 'cyclophorase' preparations. Though an experiment is quoted in which it is claimed that 'gel-P' is found in mitochondrial preparations, an examination of the protocols reveals that the tissue was homogenized in a Waring blender, a process known both to damage the mitochondria and to result in nuclear disintegration (Potter, 1950). Furthermore, it is not at all clear from this experiment to what washing procedure the mitochondria had been subjected; nor is there any cytological evidence to show that the suspension was indeed one of mitochondria alone. This is a precaution which has been taken regularly in this laboratory. These experiments must, therefore, be accepted with reserve, a situation common to many experiments with particle systems, whether purified cytologically or not.

A solution to many of the problems discussed in this paper must await the purification of the multienzyme systems concerned. A start in this direction has already been made, but the temporary suspension of this work has made a detailed investigation impossible. It has been found that soluble proteins may be obtained from isolated mitochondria by a simple method. Treatment of the particles with 5 vol. ethanol at $-20°$ for 30 min. resulted in their complete precipitation. The particles were recovered by centrifugation in the cold and then extracted with 0·1M-phosphate buffer, pH 7·4. The cloudy extract contained much soluble protein. Ammonium sulphate fractionation of the crude extract yielded a soluble glutamic dehydrogenase, which rapidly decolorized methylene blue in the presence of L-glutamic acid and CoI. It has, so far, proved impossible to obtain a cytochrome oxidase in solution, nor have tests so far carried out revealed the presence of the phosphokinases concerned in oxidative phosphorylation. These observations are recorded in the hope that improvements of the method may result in the purification of these important systems. It should, at all events, be clear that the enzymes of insoluble particles are by no means insusceptible to ordinary methods of isolation, and this justifies the hope that the fundamental problems of oxidative phosphorylation may be solved in the near future by a direct approach, rather than by the doubtful methods hitherto employed.

SUMMARY

1. Mitochondrial systems catalysing the complete oxidation of pyruvate have been described.

2. The mechanism of the inhibition of pyruvate oxidation by 2:4-dinitrophenol has been investigated; the reversal of such inhibition has been demonstrated. Adenosinetriphosphate, coenzyme I and added dicarboxylic acids of the citric acid cycle will bring about this reversal.

3. Phosphorus/oxygen ratios for pyruvate, α-ketoglutarate, L-glutamate and β-hydroxybutyrate have been determined at 15°. Values approaching 4·0 have been obtained for the first three substrates and approaching 3·0 for the fourth. The significance of these findings has been criticized and discussed. A requirement for coenzyme I in α-ketoglutarate oxidation has been indicated, but not proved conclusively.

4. The phosphorylation coupled with the anaerobic dismutation of α-ketoglutarate and ammonia has been investigated. It is shown that DNP and azide scarcely affect this phosphorylation.

5. A phosphorylation has been shown to accompany the oxidation of reduced cytochrome *c* by rat-liver mitochondria in the presence of ascorbic acid. Anomalous results have been obtained in the presence of cysteine; the existence of a phosphorylation in the oxidation of reduced cytochrome *c* cannot be regarded as finally established.

6. Phosphorylation during anaerobic oxidation of a variety of substrates in the presence of ferricyanide has been investigated.

7. A site of action is proposed for 2:4-dinitrophenol. It is suggested that this involves all transphosphorylations from pyridine nucleotide level upwards.

8. The 'replacement' of inorganic orthophosphate by 2:4-dinitrophenol has been reinvestigated. In the case of purified mitochondria, there is no need to postulate that the breakdown of labile esters plays any part in the 'replacement'. It is suggested that 'replacement' is the consequence of a sparing of inorganic orthophosphate and evidence is presented to support this hypothesis.

9. The synthesis of adenosinetriphosphate from coenzyme I and inorganic pyrophosphate has been demonstrated in mitochondrial systems.

10. The action of thyroxine on oxidative phosphorylation, pyruvate oxidation and fatty acid oxidation has been investigated. This action is entirely different from that of 2:4-dinitrophenol, both *in vivo* and *in vitro*.

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