PHOSPHORYLATION COUPLED TO ELECTRON TRANSPORT MEDIATED BY HIGH POTENTIAL ELECTRON CARRIERS

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SUMMARY

1. It is shown that with fresh preparations of rat liver mitochondria, phosphorylation coupled to electron transport to oxygen initiated by exogenous cytochrome *c*, silicomolybdate or ferricyanide, maintained reduced with ascorbate, requires the presence of factors which apparently act to stabilize the mitochondrial structure. These factors are not necessary for obtaining maximal P/O ratios in succinate oxidation. The requirements are a mildly alkaline reaction medium (pH 7.8–8.0), fluoride ($7.5 \cdot 10^{-3}$ *M*), EDTA ($3 \cdot 10^{-4}$ *M*) or phosphotungstate ($7.5 \cdot 10^{-6}$ *M*) with cytochrome *c*; Ba^{++}, Sr^{++} or Mn^{++} ($2 \cdot 10^{-4}$ *M*) or Mg^{++} ($7.5 \cdot 10^{-3}$ *M*) with silicomolybdate; and Ba^{++} or Sr^{++} ($2 \cdot 10^{-4}$ *M*) or Mg^{++} ($7.5 \cdot 10^{-3}$ *M*) with ferricyanide. The P/O ratios obtained with silicomolybdate and ferricyanide lie between 1.0 and 2.0, depending on the concentration of the complex anion.

2. It is shown that silicomolybdate can also mediate the oxidation of external DPNH by intact rat-liver mitochondria and that phosphorylation is very efficiently coupled to this oxidation in the absence of the special divalent metal ions which are required when ascorbate is used to maintain the silicomolybdate in its reduced state. Ferrocyanide is also capable of mediating DPNH oxidation but only in the presence of high ($7.5 \cdot 10^{-3}$ *M*) concentrations of Mg^{++}. Phosphorylation is efficiently coupled to this oxidation. The high potential electron donors initiate electron transport reactions more efficiently when DPNH rather than ascorbate is used to maintain the donors reduced. It is suggested that DPNH modifies the permeability of the mitochondrial surface.

3. It is suggested that the data generally support the concept that electron transport initiated by high potential electron carriers disrupts some structural feature of the mitochondria which maintains the coupling between respiration and phosphorylation and that special ionic factors can stabilize the system against such disruption.

INTRODUCTION

Phosphorylation coupled to electron transfer from exogenous ferrocytochrome *c* to oxygen catalyzed by the respiratory enzyme complex of mitochondria has been

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demonstrated by several workers[1-8]. However, the experimental conditions reported to be necessary for efficient coupling have been varied and somewhat incompatible. For example, LEHNINGER *et al.*[5] found that a preliminary exposure of the mitochondria to hypotonic media and a low ($1 \cdot 10^{-5}$ *M*) concentration of exogenous cytochrome *c* in the reaction mixture were necessary to approach P/O ratios of 1.0, whereas MALEY AND LARDY[4] used only isotonic sucrose and a 10-fold greater concentration of cytochrome *c* and found that the critical requirements were low levels of ascorbate and mitochondria. We have examined in greater detail the electron transport reactions initiated by exogenous ferrocytochrome *c* and found that a number of structurally unrelated anions (fluoride, EDTA, phosphotungstate, hydroxyl) produced significant and sometimes large increases in their P/O ratios. These same anions were not required to obtain maximal phosphorylation with succinate oxidation. It is interesting that fluoride and EDTA had been used routinely in previous work[1-8].

During the course of these studies it was found that the complex anions silico-12 molybdate[53] and ferrocyanide, both of which form high potential reversible oxidation-reduction couples, could be used in the place of exogenous cytochrome *c* to mediate electron transport to oxygen in the respiratory enzyme chain of mitochondria. In order to obtain phosphorylation coupled to these oxidations it was again necessary to supplement the reaction mixture with special factors (the divalent metal ions Mg^{++}, Ba^{++}, Sr^{++} or Mn^{++}) which were not required to obtain maximal phosphorylation with succinate oxidation. GLENN AND CRANE[6] have previously shown that the oxidation of aldehydes by the soluble aldehyde oxidase flavoprotein can be coupled to cytochrome *c* reduction with the aid of silicomolybdate. Their observations initially led us to explore the interaction of silicomolybdate with the mitochondrial electron transport chain.

In most of the above-mentioned experiments substrate quantities of ascorbate have been employed to maintain catalytic amounts of the high potential electron carriers in the reduced state during the course of the reactions. When ascorbate was replaced with exogenous DPNH it was found that the reaction pattern exhibited striking changes. In this paper we describe some characteristics of the reactions obtained when cytochrome *c*, silico-12 molybdate and ferricyanide were used to mediate the oxidations of ascorbate or DPNH by rat-liver mitochondria. The implications of the results on a relationship between mitochondrial structure and function are discussed. The preliminary experiments which have been developed in this paper were published earlier[9].

EXPERIMENTAL

The livers of 150–250 g male rats were ground in 0.3 *M* sucrose with a Teflon-glass homogenizer and the mitochondrial fraction was obtained by differential centrifugation essentially as described in the literature[10,11]. For experiments with succinate oxidation the flasks contained a basic reaction mixture defined as potassium succinate ($1.5 \cdot 10^{-2}$ *M*), potassium phosphate ($1 \cdot 10^{-2}$ *M*, pH 7.0), ATP ($1.5 \cdot 10^{-3}$ *M*), glucose ($6 \cdot 10^{-2}$ *M*), sucrose (0.3 *M*), $MgCl_2$ ($1.5 \cdot 10^{-3}$ *M*), hexokinase (0.5 mg) and freshly prepared rat-liver mitochondria (8–10 mg protein). To this was added the special components indicated in the tables or legends to the respective figures. The final volume was 3.3 ml. For experiments with ascorbate or DPNH as substrates the

flasks contained a basic reaction mixture defined as potassium ascorbate ($7.5 \cdot 10^{-3}$ *M*) or DPNH ($6 \cdot 10^{-3}$ *M*) respectively, potassium phosphate ($7.5 \cdot 10^{-3}$ *M*, pH 7.0), glucose ($6 \cdot 10^{-2}$ *M*), sucrose (0.3 *M*), hexokinase (0.5 mg) and freshly prepared rat-liver mitochondria (8–10 mg protein). To this was added the ATP, divalent metal ions, cytochrome *c*, silicomolybdate, potassium ferricyanide and other components, as indicated in the tables or figures. The final reaction volume was 3.3 ml. The center wells of all flasks contained 0.2 ml 5 *N* KOH. Oxygen consumption, for a period indicated in the text, was measured in a Warburg apparatus at 30° with air as the gas phase. The flasks were equilibrated in the bath for the first 6 min and it was assumed that the reaction during this period proceeded at the same rate as for the following 6 min. The reaction was terminated by addition of 1 ml 15% trichloroacetic acid. Inorganic phosphate in the supernatant was measured by the method of FISKE AND SUBBAROW[12]. Calibration of the assay was carried out in the presence of various quantities of ascorbate in order to include the small absorbancy correction at 750 mμ introduced by the unoxidized ascorbate remaining in the flasks at the end of the experiment. The presence of silicomolybdate in the experimental flasks, in the concentrations used in these experiments, did not significantly interfere with phosphate determinations by the FISKE AND SUBBAROW method. The presence of ferrocyanide, in concentrations up to $1.5 \cdot 10^{-3}$ *M*, also did not significantly interfere with the inorganic phosphate determinations. However, in this case, a bluish-black, rather than pure blue, color developed upon addition of reducing agent but the black component contributed only very slightly to the optical absorption at 750 mμ. Absorbancy measurements at 750 mμ were converted to phosphate concentrations by their comparison with standard curves obtained with known amounts of phosphate and ferrocyanide. With phosphate concentrations measured in this way, the accuracy of the computed P/O ratio was better than 95%. When larger concentrations (up to $1.8 \cdot 10^{-2}$ *M*) of ferrocyanide were present in the experimental flasks, inorganic phosphate was determined by the same general assay method. At these higher concentrations, however, a flocculent precipitate formed upon addition of the reducing agent. This was separated from the clear blue supernatant solution after 10 min of color development, by centrifugation. Absorbancy measurements of the clarified supernatant were again converted to phosphate concentrations by comparison with an appropriate set of standard absorption curves. Care must be taken to use freshly prepared reducing solution or a colloidal suspension, difficult to clarify by centrifugation, will form. Flocculation of the precipitate was aided by carrying out the reaction in 0.5 *M* NaCl although this procedure was not adopted routinely. With phosphate concentrations measured in this way, the accuracy of the computed P/O ratio was better than 90%.

For calculation of all P/O ratios, both inorganic phosphate disappearance and oxygen consumption were computed relative to their values observed in control flasks which contained all of the reaction components except the cytochrome *c*, silicomolybdate or ferricyanide. Oxygen uptake in the control flasks was usually less than 10% of that observed in the experimental flask with the slowest reaction rate. Inorganic phosphate remained essentially constant in the control flasks during the experiment.

The hexokinase preparation was purified through step 3a according to the procedure of BERGER *et al.*[13]. Its activity was assayed with 20 μmoles Tris buffer,

pH 7.5, 25 μmoles $MgCl_2$, 5 μmoles ATP and 200 μmoles glucose in 3 ml volume at 30°. Under these conditions 0.05 mg of the preparation catalyzed the transfer of 2 μmoles phosphate in 10 min. Protein determinations were made with the biuret reagent[14] after addition to the mitochondrial sample of an equal volume of 5% deoxycholate. Antimycin A was assayed by its absorption at 320 mμ in ethanol[15]. Cytochrome *c* (horse heart) was purchased from Sigma Chemical Co. and purified on Amberlite XE-97 cation-exchange resin according to the procedure of MARGOLIASH[16]. Crystalline ascorbic acid, supplied by Merck and Co. was neutralized with KOH immediately before use. DPNH, prepared by enzymic reduction of DPN, was obtained from Sigma Chemical Co., and used without additional purification.

A solution of $1 \cdot 10^{-3}$ *M* silicomolybdate[53] was prepared by dissolving 14.4 mg sodium metasilicate and 120 mg sodium molybdate in 50 ml water containing 0.4 ml 5 *N* HCl and used within 30–60 min. Immediately before use the pH was adjusted to 5.5 with KOH.

RESULTS

With succinate as substrate, standard aliquots (8–10 mg protein) of freshly prepared rat-liver mitochondria used in these experiments consumed 12–15 μatoms oxygen in 18 min and gave satisfactory P/O ratios (between 1.5–1.9) in the unsupplemented basic reaction mixture previously defined. Occasional preparations were obtained which did not give P/O ratios greater than 1.3 unless the basic reaction mixture was either brought to pH 7.5–7.7 or supplemented with additional magnesium (Table I). Such preparations were routinely discarded. The oxidation of ascorbate by the mitochondria in the absence of supplementary electron carriers proceeded extremely

TABLE I

EFFECT OF VARIOUS ADDITIONS ON PHOSPHORYLATION COUPLED TO SUCCINATE OXIDATION

Electron carrier	*Additions* Mg ($M \times 10^3$)	*Other*	*Oxygen* μ*atoms 18 min*	*P/O*
—	1.5	—	15.1	1.33
—	7.5	—	13.5	1.67
—	1.5	pH 7.6*	14.5	1.69
—	1.5	—	14.9	1.61
—	7.5	—	13.9	1.69
—	1.5	Ascorbate ($7.5 \cdot 10^{-3}$ *M*)	14.8	1.59
Cytochrome *c* ($2 \cdot 10^{-5}$ *M*)	7.5	—	13.5	1.65
Cytochrome *c* ($2 \cdot 10^{-5}$ *M*)	7.5	Fluoride ($7.5 \cdot 10^{-3}$ *M*)	13.1	1.66
Cytochrome *c* ($2 \cdot 10^{-5}$ *M*)	7.5	EDTA ($3 \cdot 10^{-4}$ *M*)	14.0	1.60
Silicomolybdate ($6 \cdot 10^{-5}$ *M*)	1.5	—	14.4	1.51
Silicomolybdate ($6 \cdot 10^{-5}$ *M*)	7.5	—	13.5	1.55
Ferrocyanide ($3 \cdot 10^{-3}$ *M*)	1.5	—	14.5	1.35
Ferrocyanide ($3 \cdot 10^{-3}$ *M*)	7.5	—	13.8	1.65
Silicomolybdate ($6 \cdot 10^{-5}$ *M*)	1.5	—	14.9	1.55
Silicomolybdate ($6 \cdot 10^{-5}$ *M*)	7.5	—	14.6	1.40
Ferrocyanide ($3 \cdot 10^{-3}$ *M*)	1.5	—	15.1	1.59
Ferrocyanide ($3 \cdot 10^{-3}$ *M*)	7.5	—	14.5	1.60

* pH of reaction mixture.

slowly. Oxygen consumption could be mediated by the addition of either cytochrome *c*, silicomolybdate or ferricyanide. The oxidation rates at infinite ascorbate concentration[17] were about twice those obtained with $7.5 \cdot 10^{-3}$ *M* ascorbate. The lower concentration of ascorbate usually gave slightly higher P/O ratios, in agreement with the observations of MALEY AND LARDY[4]. Both the oxidation rates and P/O ratios were additionally dependent on the presence of special factors in the basic reaction mixture. These phenomena are described for each electron donor.

Cytochrome c

When catalytic amounts of cytochrome *c* were added to the basic reaction mixture containing ascorbate, esterification of inorganic phosphate was not coupled to the subsequent electron transport to molecular oxygen unless the reaction mixture was further supplemented with either EDTA at $3 \cdot 10^{-4}$ *M*, fluoride at $7.5 \cdot 10^{-3}$ *M* or phosphotungstate at $7.5 \cdot 10^{-6}$ *M* (Table II). High concentrations of Mg^{++} ($7.5 \cdot 10^{-3}$ *M*) were necessary to demonstrate the effect of fluoride but not that of EDTA. It was also found that both the oxidation rates and P/O ratios were higher in a mildly alkaline reaction medium containing $7.5 \cdot 10^{-3}$ *M* Mg^{++} (Fig. 1 and Table II). Neither fluoride, EDTA, phosphotungstate, cytochrome *c* nor ascorbate, in the concentrations used in these experiments, had any effect on the P/O ratios accompanying succinate oxidation (Table I).

TABLE II

EFFECT OF VARIOUS ANIONS ON PHOSPHORYLATION COUPLED TO ASCORBATE-CYTOCHROME *c* OXIDATION

Anion	*Oxygen µatoms 24 min*	*P/O*
—	8.1	0.00
Fluoride ($7.5 \cdot 10^{-3}$ *M*)	6.1	0.66
EDTA ($3 \cdot 10^{-4}$ *M*)	6.0	0.86
PTA* ($7.5 \cdot 10^{-6}$ *M*)	5.9	0.35
OH (pH 8.0)	10.9	0.91
OH (pH 8.0)**	11.5	1.01
—	7.3	0.10
Fluoride ($7.5 \cdot 10^{-3}$ *M*)	5.1	0.58
EDTA ($3 \cdot 10^{-4}$ *M*)	5.8	0.91
PTA* ($7.5 \cdot 10^{-6}$ *M*)	5.0	0.53
OH (pH 7.8)**	9.8	1.10

In addition to basic reaction mixture for experiments with ascorbate as described in text, all flasks contained $7.5 \cdot 10^{-3}$ *M* $MgCl_2$, $2 \cdot 10^{-5}$ *M* cytochrome *c* and $1.5 \cdot 10^{-3}$ *M* ATP. Additions are indicated in Table.

* Added directly to the mitochondria from an unneutralized solution of phosphotungstic acid before addition of any reaction components.

** Buffered with $7.5 \cdot 10^{-3}$ *M* Tris.

We were able to confirm the observations of LEHNINGER *et al.*[5] that the P/O ratios increase as the cytochrome *c* concentration is decreased. We found this to be true with all of the factors which supported coupled phosphorylation. In their experiments the highest P/O ratios achieved were somewhat less than 1.0. However, in some of our experiments carried out in the pH range 7.8–8.0 with $1 \cdot 10^{-5}$ *M*

cytochrome *c*, P/O ratios significantly above 1.0 were obtained (Fig. 1). Combination of two or more factors occasionally improved P/O ratios but no attempt was made to find optimal combinations.

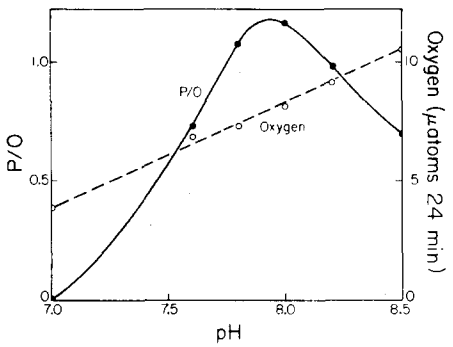

Fig. 1. Effect of pH on oxygen consumption and coupled phosphorylation mediated by exogenous cytochrome *c*. All flasks contained the basic reaction mixture for experiments with ascorbate ($7.5 \cdot 10^{-3}$ *M*) as described in the text and $7.5 \cdot 10^{-3}$ *M* Tris buffer, and $7.5 \cdot 10^{-3}$ *M* Mg^{++}.

It was found that pH effects similar to those described above could be observed under still other conditions. With mitochondrial preparations which had been aged in 0.3 *M* sucrose at 0°, phosphorylation was more efficiently coupled to succinate oxidation in mildly alkaline reaction media (Table III). For these experiments the reaction mixture was adjusted to the indicated pH by the addition of KOH. A supplementary buffer (Tris) was omitted in these experiments in order to demonstrate that the effect is primarily due to H^+ concentration. The data indicate that optimal effect is obtained close to pH 8.5.

TABLE III

EFFECTS OF VARIOUS ADDITIONS ON PHOSPHORYLATION COUPLED TO SUCCINATE OXIDATION IN AGED MITOCHONDRIA

Age h at 0°	*Additions*	*pH**	*Oxygen μatoms 18 min*	*P/O*
0	—	7.0	13.5	1.67
0	—	8.5	13.5	1.10
0	$MgCl_2$ ($7.5 \cdot 10^{-3}$ *M*)	7.0	13.0	1.69
24	—	7.0	15.9	0.39
24	$MgCl_2$ ($7.5 \cdot 10^{-3}$ *M*)	7.0	14.0	1.22
48	$MgCl_2$ ($7.5 \cdot 10^{-3}$ *M*)	7.0	16.9	0.10
48	$MgCl_2$ + $2 \cdot 10^{-4}$ *M* $MnCl_2$	7.0	13.9	0.83
72	$MgCl_2$ + $2 \cdot 10^{-4}$ *M* $MnCl_2$	7.0	16.1	0.00
72	$MgCl_2$ ($7.5 \cdot 10^{-3}$ *M*)	8.0	16.5	0.53
72	$MgCl_2$ ($7.5 \cdot 10^{-3}$ *M*)	8.5	15.8	0.85
72	$MgCl_2$ ($7.5 \cdot 10^{-3}$ *M*)	9.0	15.2	0.31
72	$MgCl_2$ ($7.5 \cdot 10^{-3}$ *M*)	7.0	18.2	0.00
72	$MgCl_2$ ($7.5 \cdot 10^{-3}$ *M*)	8.0	17.1	0.30
72	$MgCl_2$ ($7.5 \cdot 10^{-3}$ *M*)	8.5	17.5	0.53
72	$MgCl_2$ ($7.5 \cdot 10^{-3}$ *M*)	9.0	17.0	0.40

* pH of reaction mixture.

Silicomolybdate

The marked enhancement of oxygen consumption upon addition of catalytic amounts of silicomolybdate to the basic reaction mixture containing ascorbate is shown in Figs. 2 and 3. However, esterification of inorganic phosphate was not coupled to these oxidations unless the reaction mixture was further supplemented with Mg^{++} at $7.5 \cdot 10^{-3}$ *M* (Figs. 2 and 4) or with Ba^{++}, Sr^{++} or Mn^{++} at $2 \cdot 10^{-4}$ *M* (Fig. 5). The additional Mg^{++} also effected a concomitant increase in the oxidation rates (Fig. 2). All metal ions were added as the chloride salts. Ineffective at either low or high concentration were Co^{++}, Ni^{++}, Be^{++}, Zn^{++}, Cd^{++}, Hg^{++}, Pb^{++}, Ca^{++}, Cu^{++}, Fe^{++}, Al^{+++}, Fe^{+++}, Li^{+} and Na^{+}. Also ineffective were fluoride, EDTA, bovine serum albumin and the mildly alkaline reaction conditions (pH 7.5-8.0) which had proved successful in supporting phosphorylation coupled to the electron transport reactions mediated with cytochrome *c*. Despite these striking results with

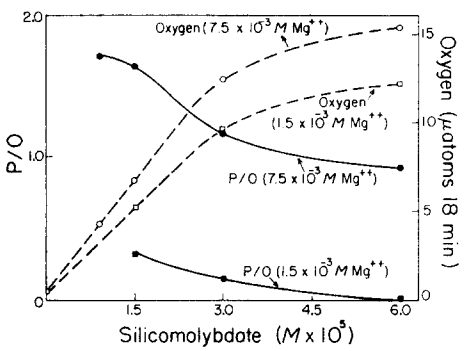

Fig. 2. Effect of $7.5 \cdot 10^{-3}$ *M* Mg^{++} on oxygen consumption and coupled phosphorylation mediated by silicomolybdate. All flasks contained the basic reaction mixture for experiments with ascorbate ($7.5 \cdot 10^{-3}$ *M*) as described in the text and the additional components indicated in the Figure.

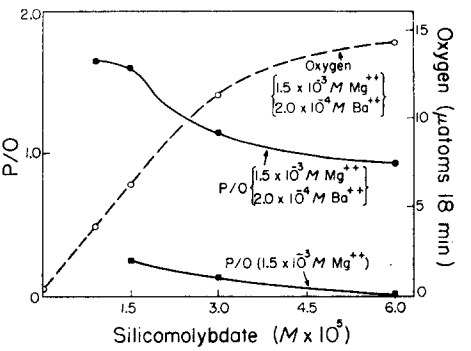

Fig. 3. Effect of $2 \cdot 10^{-4}$ *M* Ba^{++} on oxygen consumption and coupled phosphorylation mediated by silicomolybdate. Other reaction conditions are identical with those of Fig. 2.

ascorbate-silicomolybdate, neither Mg^{++} at $7.5 \cdot 10^{-3}$ *M* nor Ba^{++}, Sr^{++} or Mn^{++} at $2 \cdot 10^{-4}$ *M* had any significant effect on the P/O ratios obtained with succinate oxidation in fresh mitochondria. Fig. 4 compares the effect of Mg^{++} concentration on the P/O ratios accompanying the oxidations of succinate and silicomolybdate. In the experiments shown in Figs. 3 and 5, all flasks contained Mg^{++} at a concentration of $1.5 \cdot 10^{-3}$ *M* in addition to the specified amounts of Ba^{++}, Sr^{++}, or Mn^{++}. It can be seen from Fig. 4 that this low concentration of Mg^{++} maintained maximal P/O ratios with succinate oxidation but could not alone effectively support phosphorylation coupled to silicomolybdate oxidation. Mitochondria which had been aged for 24 h in 0.3 *M* sucrose at 0° did, however, require higher levels of Mg^{++} for maximal phosphorylation coupled to succinate oxidation (Table II). Low levels of Mn^{++} had a similar effect on mitochondria which had been so aged for 48 h (Table II). In experiments with aged mitochondria, centrifugation following the aging period was avoided since it was found that the P/O ratios obtained with preparations aged for only 24 h became extremely sensitive to centrifugal forces.

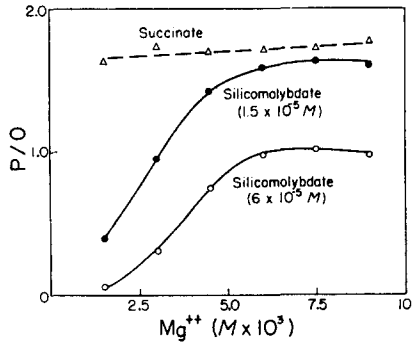

Fig. 4. Effect of Mg^{++} concentration on phosphorylation coupled to electron transport mediated by silicomolybdate. The lack of effect of Mg^{++} on phosphorylation coupled to succinate oxidation is also shown (dashed curve). Other reaction conditions are identical with those of Fig. 2.

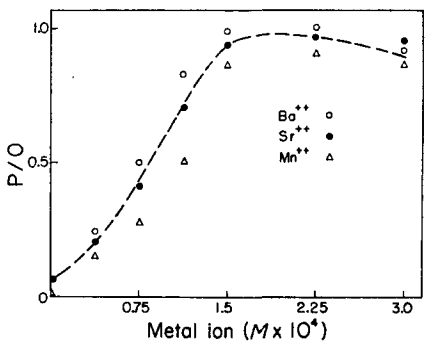

Fig. 5. Effect of Ba^{++}, Sr^{++} and Mn^{++} concentrations on phosphorylation coupled to electron transport mediated by $6 \cdot 10^{-5}$ *M* silicomolybdate. All flasks contained $1.5 \cdot 10^{-3}$ *M* Mg^{++} in addition to the divalent metal ions indicated in the figure. Other reaction conditions are identical with those of Fig. 2.

The maximum possible P/O ratio which could be attained with a single concentration of silicomolybdate was dependent on the concentration of ATP (Table IV) and to a much lesser extent on the level of hexokinase. In the experiments described in this paper, hexokinase was maintained at the level indicated in the experimental section and the optimal ATP:silicomolybdate ratios were chosen from the data of Table IV.

TABLE IV

EFFECT OF ATP CONCENTRATION ON P/O RATIOS FOR ASCORBATE–SILICOMOLYBDATE OXIDATION

Metal ion ($M \times 10^3$)	*ATP* ($M \times 10^3$)	*Silicomolybdate*		
		$1.5 \cdot 10^{-5}$ M	$3.0 \cdot 10^{-5}$ M	$6.0 \cdot 10^{-5}$ M
	0.0	0.0	0.0	0.0
	0.15	1.61	—	—
Mg^{++} (7.5)	0.30	2.12	0.77	0.30
	0.75	1.50	1.20	0.67
	1.5	1.13	1.10	0.96
	3.0	—	—	0.71
	0.0	0.0	0.0	0.0
	0.15	1.43	—	0.21
Mg^{++} (1.5) + Ba^{++} (0.2)	0.30	1.78	1.05	0.45
	0.75	1.60	1.25	0.87
	1.5	1.43	1.01	0.99
	3.0	—	—	0.80

There was some variation in the P/O ratios obtained with different mitochondrial preparations. The variation was minimal under those experimental conditions giving P/O ratios close to 1.0. For 25 mitochondrial preparations assayed with $6 \cdot 10^{-5}$ *M* silicomolybdate and $7.5 \cdot 10^{-3}$ *M* Mg^{++}, the average P/O ratio was 0.90 with a spread between 0.79 and 1.21. For 15 preparations assayed with $1.5 \cdot 10^{-5}$ *M* silicomolybdate and $7.5 \cdot 10^{-3}$ *M* Mg^{++}, the average P/O ratio was 1.49 with a spread between 1.25 and

2.50. The variation in oxidation rates was quite small and not commensurate with the variation in P/O ratios. The data plotted for each figure represent the observations on a single mitochondrial preparation which gave approximately average results. Omission of hexokinase or ATP reduced all P/O ratios to 0. Likewise, 10^{-4} *M* 2,4-dinitrophenol totally uncoupled the reactions.

The data clearly show that P/O ratios higher than 1.0 can be obtained at lower concentrations of silicomolybdate. The high P/O ratios are related to the fact that the net amount of phosphate esterification decreases less rapidly than the oxidation rate when the concentration of silicomolybdate is lowered. In Fig. 6 it is shown that antimycin A, at levels which completely inhibit succinate oxidation (0.3–10 μg), reduced P/O ratios greater than 1.0 to a value close to 1.0. With higher concentrations of silicomolybdate, where the P/O ratios were no higher than 1.0, antimycin A had no effect at the above levels (Fig. 7). The complete inhibition of phosphorylation at much higher levels of antimycin, seen in Figs. 6 and 7, has also been observed by

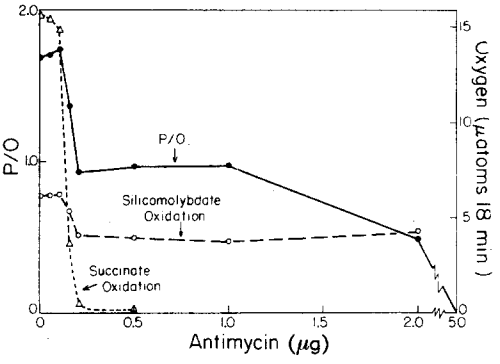

Fig. 6. Effect of antimycin A on oxygen consumption and coupled phosphorylation mediated by $1.5 \cdot 10^{-5}$ *M* silicomolybdate. The effect of antimycin A on succinate oxidation is also shown (△—△). All flasks contained $7.5 \cdot 10^{-3}$ *M* Mg^{++}. Other reaction conditions are identical with those of Fig. 2.

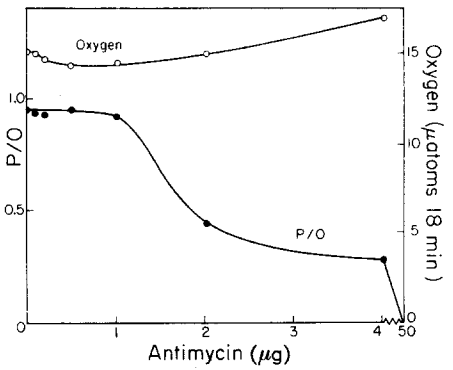

Fig. 7. Effect of antimycin A on oxygen consumption and coupled phosphorylation mediated by $6 \cdot 10^{-5}$ *M* silicomolybdate. Other reaction conditions are identical with those of Fig. 6.

NIELSEN AND LEHNINGER[6] with phosphorylation coupled to the oxidation of ferrocytochrome *c*.

The data of Table I demonstrate that phosphorylation coupled to succinate oxidation was not significantly inhibited by silicomolybdate in both the absence and presence of excess Mg^{++}. In these experiments also the silicomolybdate was effectively in the reduced form, since it was rapidly reduced by the mitochondrial electron transport system in the presence of succinate.

When the mitochondria were allowed to carry out electron transport mediated by silicomolybdate in the absence of metal ions other than Mg^{++} at $1.5 \cdot 10^{-3}$ *M*, their coupling mechanism suffered irreversible damage. The addition of excess Mg^{++} to mitochondria which had been allowed to so respire for 6 min did not subsequently reactivate coupled phosphorylation (Table V). The coupling mechanism sustained a similar fate when conditions of respiratory control (absence of ATP and hexokinase) were imposed on the oxidations (Table V).

TABLE V

INSTABILITY OF PHOSPHORYLATION COUPLED TO SILICOMOLYBDATE OXIDATION IN THE TEMPORARY ABSENCE OF METAL IONS OR ATP

Omitted from complete reaction mixture during first 6 min*	*Oxygen** μatoms 18 min*	*P/O***
—	14.2	0.95
Mg^{++} ($6 \cdot 10^{-3}$ *M*)	12.2	0.06
Hexokinase + ATP	12.6	0.00

* In addition to basic reaction mixture for experiments with ascorbate oxidation as described in text, all flasks contained $6 \cdot 10^{-5}$ *M* silicomolybdate and $7.5 \cdot 10^{-3}$ *M* $MgCl_2$.

** Oxygen consumption and P/O ratio for 18 min reaction period immediately following the addition of the temporarily omitted components.

It was observed that mitochondria have a marked tendency to bind silicomolybdate and that the binding was greatly enhanced in the presence of divalent metal ions. Such information was obtained by the following type of experiment: Silicomolybdate (final concentration of $1.8 \cdot 10^{-4}$ *M*) was added directly to a suspension of mitochondria in 0.3 *M* sucrose containing 8–10 mg protein/ml and metal ions as indicated in Figs. 8 and 9. The mitochondria were then washed by centrifugation (9000 × *g* for 10 min) and resuspension in the original volume of fresh cold sucrose containing metal ions as before but no additional silicomolybdate. Subsequent washings were repeated in the same manner. Figs. 8 and 9 show oxygen uptake by 1 ml (8–10 mg protein) of the washed mitochondria, in a reaction medium containing ascorbate but no additional silicomolybdate, as a function of the number of washings. Repeated washing with the medium which lacked divalent metal ions removed the silicomolybdate and thereby reduced the oxidation rates. In contrast, repeated washing in the presence of divalent metal ions left the oxidation rates essentially unchanged, presumably because the silicomolybdate was strongly bound to the mitochondria under these conditions. Monovalent ions were found to be ineffective in this respect. It should be noted that Co^{++} simulates the action of Mn^{++} in promoting the binding although it does not support phosphorylation coupled to electron transport mediated by silicomolybdate.

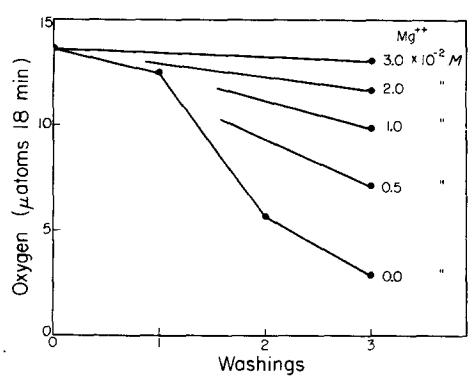

Fig. 8. Effect of Mg^{++} on the binding of silicomolybdate by whole mitochondria. The complete experimental procedure is described in the text.

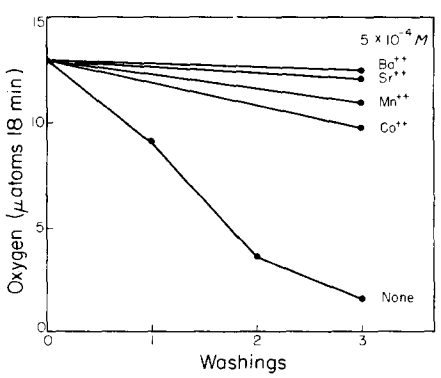

Fig. 9. Effect of various divalent cations on the binding of silicomolybdate by whole mitochondria. Other experimental conditions are identical with those of Fig. 8.

Ferrocyanide

The stimulation of oxygen consumption by addition of ferricyanide to the basic reaction mixture containing ascorbate is shown in Figs. 10 and 11. Phosphorylation was not coupled to these oxidations unless the reaction mixture was further supplemented with Mg^{++} at $7.5 \cdot 10^{-3}$ *M* (Fig. 10) or Ba^{++} (Fig. 11) or Sr^{++} at $2 \cdot 10^{-4}$ *M*. Inactive in this respect were all of the other metal ions tested including Mn^{++}. High levels of Mg^{++} produced greater stimulation of oxidation (Fig. 10) than did the other metal ions.

P/O ratios were somewhat (about 20 %) dependent on the concentration of ATP. Maximal ratios were usually obtained when the ATP : ferricyanide ratio was unity in the presence of Mg^{++} and 0.2 in the presence of Ba^{++} or Sr^{++}. These relative concentrations were maintained throughout.

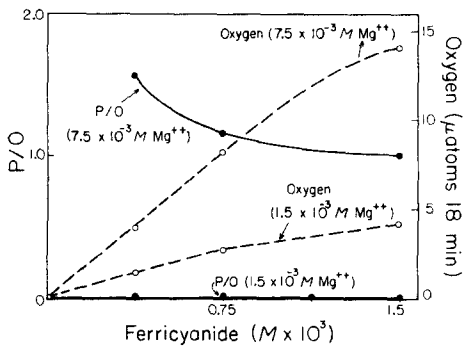

Fig. 10. Effect of $7.5 \cdot 10^{-3}$ *M* Mg^{++} on oxygen consumption and coupled phosphorylation mediated by ferricyanide. Other reaction conditions are identical with those of Fig. 2.

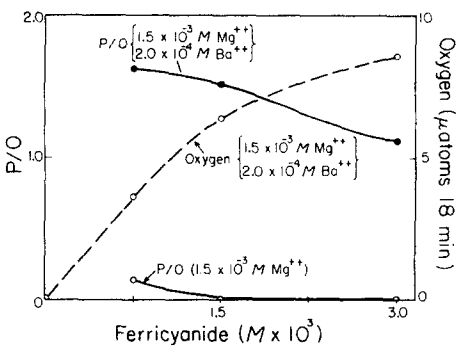

Fig. 11. Effect of $2 \cdot 10^{-4}$ *M* Ba^{++} on oxygen consumption and coupled phosphorylation mediated by ferricyanide. Other reaction conditions are identical with those of Fig. 2.

There was some variation in the observed P/O ratios. For 20 different preparations assayed with $1.5 \cdot 10^{-3}$ *M* ferricyanide and $7.5 \cdot 10^{-3}$ *M* Mg^{++}, the average P/O ratio was 0.94 with a spread between 0.81 and 1.22. With $3.75 \cdot 10^{-4}$ *M* ferricyanide and $7.5 \cdot 10^{-3}$ *M* Mg^{++}, the average P/O ratio for 12 preparations was 1.51 with a spread between 1.82 and 1.34. The data chosen for the figures represent observations on a single mitochondrial preparation which gave approximately average results.

The effects of antimycin A on the P/O ratios obtained with high and low concentrations of ferricyanide were similar to those obtained with silicomolybdate; sensitivity was clearly limited to those ratios above 1.0. Omission of hexokinase or ATP from the reaction mixtures or inclusion of 10^{-4} *M* 2,4-dinitrophenol reduced all P/O ratios to 0.

Table I shows that ferrocyanide itself does not strongly affect the phosphorylations coupled to succinate oxidation. In light of our finding that high levels of Mg^{++} stimulated the rate of ferrocyanide-mediated oxidation it became of interest to see whether the rapid enzymic reduction of ferricyanide by succinate also required high levels of Mg^{++}. Ferricyanide has been widely used as an electron acceptor for oxidative–enzyme systems. Phosphorylation coupled to the reduction of ferricyanide by rat-liver mitochondria in the presence of a variety of substrates of the Krebs cycle has been demonstrated[3,18,62] but no unusual requirements for the reaction

have yet been reported. PRESSMAN[62] obtained somewhat higher P/O ratios than COPENHAVER AND LARDY[3] with a spectrophotometric assay system but was unable to find a reason for the discrepancy. Table VI shows the unexpected finding that high levels of Mg^{++} had no effect at all on the anaerobic reduction of $6 \cdot 10^{-3}$ *M* ferricyanide by succinate. Identical results were obtained with lower ($1.5 \cdot 10^{-3}$ *M*) concentrations of ferricyanide.

TABLE VI

EFFECT OF Mg^{++} ON ANAEROBIC REDUCTION OF FERRICYANIDE BY SUCCINATE AND OXIDATION OF ASCORBATE-FERROCYANIDE

*Reduction**			*Oxidation***		
Mg^{++} (M × 10^3)	*Ferricyanide (μmoles reduced 5 min)*	*P:2e*	*Mg^{++} (M × 10^3)*	*Oxygen μatoms 18 min*	*P/O*
1.5	14.1	0.43	1.5	3.2	0.0
7.5	12.0	0.45	7.5	12.8	0.92
1.5	10.4	0.31	1.5	4.1	0.0
7.5	11.9	0.30	7.5	13.0	0.89
7.5	0.9***	0.00	7.5	12.5***	0.95

* All flasks contained basic reaction mixture for experiments with succinate oxidation together with 20 μmoles potassium ferricyanide, under anaerobic conditions. Mg^{++} concentration is indicated in Table.

** All flasks contained basic reaction mixture for experiments with ascorbate and $1.5 \cdot 10^{-3}$ *M* ferricyanide.

*** In the presence of 1γ antimycin A.

The binding of ferri- and ferro-cyanide anions by mitochondria is apparently rather weak since their retention could not be demonstrated with the technique of repeated washing, even in the presence of divalent metal ions. Oxygen uptake mediated by residually bound ferricyanide was negligibly small after the very first washing. However, because of this and the fact that ferrocyanide is stable in neutral solution, it was possible to study the characteristics of reactions in which ascorbate was absent and ferrocyanide present in substrate quantities (Table VII). The data clearly confirm that phosphorylation was not efficiently coupled to the oxidation of substrate amounts of ferrocyanide unless special divalent metal ions were added to the system. In these experiments, the rates of oxygen consumption were much less than when ascorbate was used to maintain the ferricyanide totally reduced. The rates decreased slowly with time and consequently about 60-min reaction periods were employed in order to obtain reliable measurements of oxygen consumption. Ferrocyanide (60 μmoles) was placed in the side arms of the reaction vessels and tipped into the reaction mixtures after a 3-min thermal equilibration in the bath. A conservative estimate of maximum error in the P/O ratios of Table VII would be about 10%.

The dependence of the electron transport reactions mediated by complex anions on endogenous cytochrome c

It was found that electron transport mediated by either silicomolybdate or ferricyanide proceeded to molecular oxygen *via* endogenous cytochrome *c*. The mitochondria were depleted of their endogenous cytochrome *c* by suspension in 0.015 *M* KCl and subsequent washing with 0.15 *M* KCl. As judged by the concomitant

TABLE VII

PHOSPHORYLATION COUPLED TO THE OXIDATION OF FERROCYANIDE IN THE ABSENCE OF ASCORBATE

Metal ions ($M \times 10^4$)	*Oxygen uptake* (*µatoms/60 min*)	*Pi uptake* (*µmoles/60 min*)	*P/O*
Mg^{++} (15)	2.6	0.8	0.31
Mg^{++} (15) ferrocyanide omitted	0.0	0.0	—
Mg^{++} (75)	6.4	7.6	1.10
Mg^{++} (75) ferrocyanide omitted	0.9	0.0	—
Ba^{++} (2)	5.6	6.8	1.22
Sr^{++} (2)	3.0	3.2	1.07
Mn^{++} (2)	3.1	0.8	0.26
Mg^{++} (15)	4.4	0.9	0.20
Mg^{++} (15) ferrocyanide omitted	0.8	0.0	—
Mg^{++} (75)	6.0	6.3	1.06
Mg^{++} (75) ferrocyanide omitted	0.9	0.1	—
Ba^{++} (2)	6.6	8.2	1.24
Sr^{++} (2)	5.0	5.5	1.10
Mn^{++} (2)	4.8	1.4	0.30

All flasks contained basic reaction mixture for experiments with ascorbate except that ascorbate was omitted and $1.8 \cdot 10^{-2}$ *M* $K_4Fe(CN)_6$ was present. Other additions are indicated in Table.

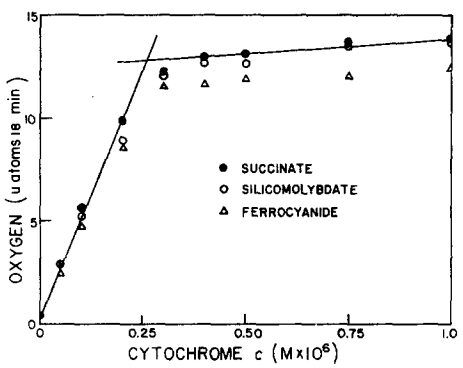

Fig. 12. Restoration by externally-added cytochrome *c* of succinate, ascorbate–silicomolybdate and ascorbate–ferricyanide oxidations in mitochondria depleted of their endogenous cytochrome *c*. All flasks contained the basic reaction mixtures for the respective substrates, $7.5 \cdot 10^{-3}$ *M* Mg^{++}, and where indicated, $6 \cdot 10^{-5}$ *M* silicomolybdate or $1.5 \cdot 10^{-3}$ *M* ferricyanide.

loss of succinate oxidation, this treatment removed more than 95 % of the endogenous cytochrome *c*. Fig. 12 shows that the oxidations of succinate, ascorbate-silicomolybdate and ascorbate-ferricyanide in the extracted preparations were restored with equal efficiency by low concentrations of added cytochrome *c*.

Experiments with DPNH

The literature contains conflicting reports regarding the capacity of intact rat-liver mitochondria to catalyze the direct oxidation of exogenous DPNH. CHANCE AND WILLIAMS[19] and LEHNINGER[20,21] were unable to observe this reaction. ERNSTER *et al.*[22] obtained slow oxidation with P/O ratios approaching 1.0. MALEY[23] observed good oxidation rates with P/O ratios approaching 3.0. LINNANE AND ZIEGLER[24]

have concluded that undamaged beef-heart mitochondria also do not carry out the direct oxidation of DPNH at any significant rate. We did observe the inception of this oxidation, however, following a long and somewhat variable incubation time ranging from 18–60 min. The length of the latent period was considerably prolonged by high levels of Mg^{++} ($7.5 \cdot 10^{-3}$ *M*) or by low levels of Mn^{++}, Sr^{++} or Ba^{++} ($2 \cdot 10^{-4}$ *M*). The oxidation rates progressively increased during the course of the reaction which followed the latent period. Phosphorylation was usually coupled to this delayed oxidation but the maximal P/O ratios (about 1.0) were always associated with the initial (1–2 μatoms) oxygen consumption and extended reaction times invariably led to low ratios. This phenomenon may account for the observations of ERNSTER *et al.*[22] but the high P/O ratios reported by MALEY[23] were never approached in our experiments. Previous workers[20–23] also reported a marked stimulation of DPNH oxdiation upon addition of supplementary cytochrome *c* with final P/O ratios ranging between 0 and 2. ERNSTER *et al.*[22] found that Amytal reduced these P/O ratios close to zero while MALEY[23] reported that antimycin A reduced them to 0.5. Their experiments made in the presence of cytochrome *c* and antimycin A or Amytal show oxidation and phosphorylation rates very similar to ours (Fig. 1, Table VIII) which were generally insensitive to these inhibitors.

TABLE VIII

PHOSPHORYLATION COUPLED TO DPNH OXIDATION MEDIATED BY VARIOUS ELECTRON CARRIERS

Catalyst	*Mg^{++} ($M \times 10^3$)*	*Additions*	*Oxygen (μatoms 18 min)*		*P/O*	
			Expt. 1	*Expt. 2*	*Expt. 1*	*Expt. 2*
—	1.5	—	0.95	1.1	—	—
—	7.5	—	0.0	0.9	—	—
Silicomolybdate ($1.5 \cdot 10^{-5}$ *M*)	1.5	—	12.7	10.5	1.03	1.00
Silicomolybdate ($1.5 \cdot 10^{-5}$ *M*)	7.5	—	12.5	10.4	1.01	1.05
Silicomolybdate ($1.5 \cdot 10^{-5}$ *M*)	1.5	ascorbate ($7.5 \cdot 10^{-3}$ *M*)	12.1	12.5	1.10	1.09
Silicomolybdate ($1.5 \cdot 10^{-3}$ *M*)	1.5	antimycin (1 μg)	12.0	10.0	1.05	0.95
Silicomolybdate ($6 \cdot 10^{-5}$ *M*)	1.5	—	12.0	9.8	1.01	1.00
Silicomolybdate ($6 \cdot 10^{-5}$ *M*)	7.5	—	9.7	8.5	0.78	0.84
Ferricyanide ($3 \cdot 10^{-3}$ *M*)	1.5	—	0.90	1.2	—	—
Ferricyanide ($3 \cdot 10^{-3}$ *M*)	7.5	—	12.1	11.9	1.14	1.08
Ferricyanide ($3 \cdot 10^{-3}$ *M*)	1.5	Ba^{++} ($2 \cdot 10^{-4}$ *M*)	1.0	1.9	—	—
Cytochrome *c* ($1.5 \cdot 10^{-5}$ *M*)	7.5	—	10.6	11.5	0.35	0.41
Cu^{++} ($1.5 \cdot 10^{-5}$ *M*)	7.5	—	13.5	12.6	0.10	0.05
Menadione ($1.5 \cdot 10^{-5}$ *M*)	7.5	—	11.4	9.1	0.20	0.43

All flasks contained the basic reaction mixture for experiments with DPNH as indicated in the text. Other additions are indicated in Table.

We have found that a variety of substances could mediate the oxidation of exogenous DPNH by rat-liver mitochondria. Figs. 13 and 14 compare the capacity of silicomolybdate, ferrocyanide, menadione, Cu^{++} and cytochrome *c* to act in this fashion. Fig. 14 shows that the electron-transport reactions mediated with ferrocyanide could only proceed in the presence of high levels of Mg^{++} ($7.5 \cdot 10^{-3}$ *M*). No other metal ions could replace Mg^{++} in this respect. The electron-transport

reactions mediated by each of these five carriers were totally blocked by 10^{-3} *M* KCN but insensitive to antimycin A and $1.8 \cdot 10^{3}$ *M* Amytal. Phosphorylation was efficiently coupled only to those oxidations mediated by silicomolybdate or ferro-

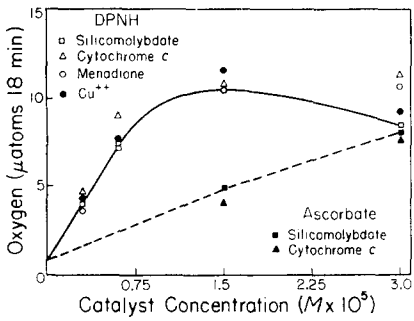

Fig. 13. Electron transport mediated by various electron carriers with $6 \cdot 10^{-3}$ *M* DPNH and $1.5 \cdot 10^{-2}$ ascorbate. All flasks contained the basic reaction mixtures for the respective substrates as described in the text, $7.5 \cdot 10^{-3}$ Mg^{++} and the concentrations of the electron carriers indicated in the figure. The concentrations of ATP used with DPNH were identical with those for ascorbate–silicomolybdate oxidations.

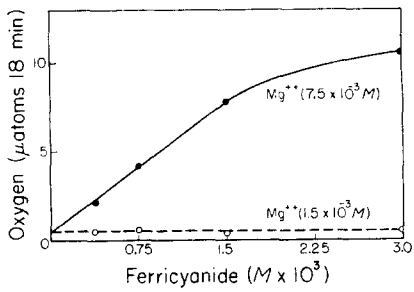

Fig. 14. Electron transport mediated by ferricyanide in the presence of DPNH. Data are shown for two concentrations of Mg^{++}. Other experimental conditions are identical with those of Fig. 13 that the concentrations of ATP were identical with those for ascorbate–ferricyanide oxidations.

cyanide (Table VIII). The data of Table VIII clearly demonstrate that the P/O ratios obtained with DPNH-silicomolybdate are maximal even at levels of Mg^{++} ($1.5 \cdot 10^{-3}$ *M*) for which the corresponding ratios obtained with ascorbate-silicomolybdate were close to zero. Fig. 13 also shows that silicomolybdate and cytochrome *c* were much less effective in mediating electron-transport reactions when maintained reduced with ascorbate rather than with DPNH. The concentration of ascorbate ($1.5 \cdot 10^{-2}$ *M*) used in these experiments produced oxidation rates close t(those obtained at infinite ascorbate concentration. Addition of ascorbate to the DPNH system did not inhibit oxidation or coupled phosphorylation.

The non-enzymic reduction of cytochrome *c*, silicomolybdate and ferricyanide by DPNH proceeded at a negligibly slow rate. Upon addition of mitochondria, under either aerobic or anaerobic conditions, each of these electron carriers was rapidly reduced, as shown by instantaneous development of the characteristic color of the reduced compound. The rate of reduction of ferricyanide by exogenous DPNH in the presence of the mitochondria was found to be totally independent of Mg^{++} concentration.

The reductase activity responsible for the observations reported in this section could presumably originate with the microsomes which invariably contaminate mitochondrial preparations. However, extensive washing of the mitochondria with 0.3 *M* sucrose failed to reduce this activity. The possibility must also be considered that an "external" DPNH-coupled reductase, capable of rapidly reducing a number of compounds, resides on the outer surface of the mitochondria. The existence of such an enzyme was first suggested by LEHNINGER[20].

RAW *et al.*[25] have recently published an account of the purification and properties of a DPNH-cytochrome *c* reductase extracted from the mitochondria of pig, guinea-

pig, and rabbit livers. These workers were able to resolve the enzyme into a "556" cytochrome and a flavoprotein. Their purified flavoprotein was able to reduce directly ferricyanide and cytochrome 556 but not cytochrome *c*. Presumably menadione and cytochrome *c* were reduced via the cytochrome 556. Functionally related reductases[26,27] have also been obtained from beef- and pig-heart mitochondria.

The possible role of an "external" DPNH-cytochrome *c* reductase in the interaction of both DPNH and the complex anions with the terminal electron-transport system of the mitochondria was further investigated by controlled removal of the antimycin-insensitive reductase activity from the mitochondria. LEHNINGER[21] and PRESSMAN AND DE DUVE[57] had previously observed that mitochondria exposed to hypotonic media oxidized DPNH much more rapidly than mitochondria maintained in an isotonic environment. The oxidations induced by the hypotonic exposure were considerably insensitive to antimycin A. In our experiments rat-liver mitochondria were exposed to distilled water containing 0.002 *M* histidine, 0.001 *M* phosphate and 0.002 *M* Tris, adjusted to pH 6.0. A sufficient volume of this solution was used such that solubilized proteins did not alter the initial pH (6.0). The pH of the mitochondrial suspension was then gradually increased by slow addition of dilute KOH. Aliquots were taken out at various points and centrifuged at 100,000 × *g* for 10 min. The supernatant was stored and the residue resuspended in unbuffered 0.3 *M* sucrose. Table IX shows the oxidation rates and P/O ratios effected by these residues with succinate, β-hydroxybutyrate and DPNH. Table IX also shows the relative DPNH-cytochrome *c* reductase activity in the supernatants. The data clearly show an inverse relationship between this activity and the capacity of the residue to oxidize rapidly the exogenous DPNH via an antimycin-insensitive pathway. The mitochondria exposed to the hypotonic medium at pH 6.0 showed the greatest increase in DPNH

TABLE IX

OXIDATIONS AND COUPLED PHOSPHORYLATION IN MITOCHONDRIA EXPOSED TO HYPOTONIC MEDIA OF INCREASING pH

pH of hypotonic medium	*Succinate*		*β-Hydroxybutyrate*		*DPNH*		*DPNH-cytochrome c reductase activity*** of supernatant*
	Oxygen (μatoms/ 18 min)	*P/O*	*Oxygen* (μatoms/ 18 min)*	*P/O*	*Oxygen (μatoms/ 18 min)*	*P/O*	
Control (0.3 *M* sucrose)	13.1	1.69	5.8	2.65	0.9	—	
6.0	12.1	0.95	5.1	1.69	14.2	0.2	0.04
6.0	0.0**	—	0.0**	—	10.9**	0.0	
6.5	13.5	0.91	4.9	1.65	9.3	0.51	0.13
7.0	13.2	0.90	4.8	1.61	5.9	1.08	0.18
7.5	15.1	0.81	5.2	1.49	4.3	1.39	0.26
7.5	0.0**	—	0.0**	—	0.5**	—	
8.0	20.1	0.61	6.1	1.11	4.4	1.08	0.25
8.5	16.3	0.20	5.9	0.39	3.9	0.30	0.29
9.0	14.8	0.11	6.9	0.10	4.5	0.0	0.35

All flasks contained basic reaction mixture for experiments with succinate oxidation and the residue derived from 8 mg protein of whole mitochondria. Where indicated $7.5 \cdot 10^{-3}$ *M* β-hydroxybutyrate or $6 \cdot 10^{-3}$ *M* DPNH replaced succinate.

* Plus $3 \cdot 10^{-4}$ *M* DPN.

** Plus 1γ antimycin A.

*** ΔA 550 mμ/min/mg protein in 0.01 potassium phosphate, pH 7.0.

oxidation activity, over 2/3 of which was insensitive to antimycin A. The data of Table IX are consistent with the idea that when the pH of the hypotonic medium is raised, the antimycin-insensitive reductase is obtained in the centrifuged supernatant while the sensitive pathway remains in the residue. The residual oxidation appears coupled to phosphorylation.

Attempts were made to purify the reductase activity released into the soluble phase between pH 6.0 and 8.0 by protamine and ammonium sulfate precipitation. A 10- to 20-fold purification was usually achieved in this way. Further purification of the reductase as a soluble enzyme proved to be extraordinarily difficult. Additional ammonium sulfate fractionation produced a precipitate which could not be redissolved below pH 10. The same phenomenon was observed with isoelectric precipitation at pH 5.1. Attempts to purify the enzyme by adsorption on cellulose anion-exchange resins were generally unsuccessful; the activity collected in a narrow, intensely yellow band at the top of the column which subsequently could not be eluted with phosphate or Tris at various pH values and concentrations. The enzyme displayed strong reductase activity in either the adsorbed, precipitated or soluble states. The absorption spectrum of preparations purified more than 10 times always showed a characteristic cytochrome "*b*" absorption peak at 556 mμ upon reduction with DPNH. A broad flavin absorption at 450–460 mμ and a Soret band at 415 mμ were also evident Further work on the purification and properties of this reductase is in progress since the experiments described above are not sufficient reason to exclude the possibility that the reductase activity is of microsomal origin.

DISCUSSION

The data presented in this paper demonstrate that phosphorylation is efficiently coupled to electron transport mediated by high potential electron carriers only if special factors, not required to obtain maximal P/O ratios with succinate oxidation, are present in the reaction medium. When mitochondria are depleted of their endogenous cytochrome *c*, oxidation of both succinate and the reduced complex anions is dependent on addition of external cytochrome *c* (Fig. 12), suggesting that the pathway of terminal electron transport is likely to be the same in both systems. Previous workers[1–8] who studied electron transport reactions mediated by cytochrome *c* overlooked the general scope of this phenomenon, perhaps because they routinely included fluoride or EDTA in their experimental media. NIELSEN AND LEHNINGER[6] attributed the EDTA requirement for phosphorylation coupled to the oxidation of ferrocytochrome *c* to contamination of their mitochondria by heavy metal ions. Although this explanation can not be excluded, the similar activity of a variety of other ions in the several related reaction systems described in this paper now seems to indicate the existence of a more basic requirement for factors which can stabilize some critical feature of the mitochondrial structure characteristically unstable to electron transport mediated by high potential electron carriers.

There is considerable evidence in the literature[28–42] supporting the concept that structural stabilization is an essential factor for optimal phosphorylation in intact rat-liver mitochondria. BALTSCHEFFSKY[41] has shown that the stability of respiratory control of succinate oxidation by fresh mitochondria in a magnesium-free medium is markedly enhanced under mildly alkaline (pH 8.0) reaction conditions.

This phenomenon is undoubtedly related to our observation (Table I) that a mildly alkaline reaction medium (pH 7.6) containing low ($1.5 \cdot 10^{-3}$ *M*) levels of Mg^{++} is as optimal an environment for fresh mitochondria oxidizing succinate as a neutral medium containing high ($7.5 \cdot 10^{-3}$ *M*) levels of Mg^{++}. As the mitochondrial structure becomes progressively weakened by aging, however, excess Mg^{++} ions apparently can no longer provide the required degree of stabilization during electron transport. Under these conditions BSA[42], Mn^{++} and OH^- (Table III) are partially effective in maintaining phosphorylation coupled to succinate oxidation. Apparently the structure of even freshly prepared mitochondria is excessively strained during electron transport mediated by high potential electron carriers since, as shown in this paper, phosphorylation coupled to such electron transport depends on the presence of factors which are not required for phosphorylation coupled to the oxidation of succinate which is a natural source of electrons for reducing the endogenous cytochromes. This phenomenon does not appear to be due to an inhibitory action of ascorbate, cytochrome *c*, silicomolybdate or ferrocyanide since these compounds do not seriously affect phosphorylation coupled to succinate oxidation (Table I), even though mitochondria bind exogenous cytochrome *c*[43,63] and silicomolybdate (Figs. 8 and 9).

BRENNER-HOLZACH AND RAAFLAUB[33], RAAFLAUB[31,32], and ERNSTER AND LÖW[28] have advanced the concept that some structural feature of mitochondria, critically related to phosphorylative capacity, is maintained by endogenous ATP and magnesium. BALTSCHEFFSKY[41] postulated the existence of some mitochondrial component with a p*K* around 7.5 whose ionization greatly influences the stability of mitochondrial structure and function. SIEKEVITZ AND POTTER[39] offered evidence that fluoride forms a complex with bound Mg^{++} and thereby prevents its discharge from the mitochondria. It is conceivable that hydroxyl, EDTA, phosphotungstate and divalent metal ions can also form complexes with endogenous components and thereby stabilize the mitochondrial structure under the present conditions of electron transport.

The data of Fig. 13 and Table VIII show that electron transport from substrate to oxygen is more efficiently mediated through silicomolybdate and cytochrome *c* when the substrate is exogenous DPNH rather than ascorbate, and that maximal rates of oxidation and phosphorylation no longer require addition of special factors. Our data suggest that with DPNH these electron carriers are first reduced by a non-phosphorylative DPNH-coupled reductase which is presumably localized on the outer surface of a mitochondrion and subsequently donate their electrons to the internal cytochromes. LEHNINGER[20] has suggested that both of these steps proceed *via* an external pathway, presumably because he found oxygen consumption initiated under these conditions to be essentially non-phosphorylative. Our observations, however, with silicomolybdate and ferrocyanide (Table VIII) tend to support the suggestion that some segment of the internal phosphorylative electron transport pathway is included in the oxidation reactions.

The higher oxidation rates obtained with DPNH compared to those with ascorbate imply that the former substrate is able to maintain a higher local concentration of reduced carrier in the region of the internal cytochromes. This could be due to an increase in the permeability of the mitochondrial surface induced by the DPNH or a capacity of DPNH to effect reduction of the carrier molecules behind the permeability barrier. SLATER[17] previously discussed this situation with respect to the capacity of ascorbate to reduce effectively the exogenous cytochrome *c*. Since the rapid

interaction of ferrocyanide with the internal cytochromes requires high levels of Mg^{++} whether maintained reduced with ascorbate or DPNH (Figs. 10 and 14), it can be further suggested that Mg^{++} also has the capacity to effect changes in the mitochondrial surface. The anomalous behavior of ferrocyanide in this respect may be related to the fact that mitochondria generally bind it much less strongly than cytochrome *c* or silicomolybdate.

The mode of action of divalent metal ions in the electron transport reactions mediated by complex anions may be related to their capacity to enhance the binding of silicomolybdate by the mitochondria. The data are inadequate to decide whether the metal ions form a direct bridge between silicomolybdate and mitochondrial protein or induce structural or electrostatic modifications of the protein which subsequently favor enhanced binding and increased stability. The former explanation is analogous to the postulated[28,31] binding of ATP to mitochondria through the intermediary of Mg^{++} or Mn^{++}. Stabilization of phosphorylation coupled to succinate oxidation in Ca^{++}-inactivated mitochondria by Mg^{++}, Mn^{++}, Co^{++} and Ni^{++} has been reported by ERNSTER AND LÖW[28]. Likewise, stabilization of phosphorylation coupled to DPNH oxidation in sonicates of mitochondria by Mn^{++}, Co^{++} and Fe^{++} has been observed by BRONK AND KIELLEY[44]. It would appear as if stabilization of phosphorylation by divalent metal ions may be quite a general effect. In contrast, however, COOPER AND LEHNINGER[45] observed an uncoupling action of exogenous Mg^{++} with digitonin extracts of mitochondria. These preparations were reported to contain considerable bound Mg^{++}. The failure of Mn^{++} to support phosphorylation coupled to the oxidation of ascorbate mediated by ferricyanide but not that mediated by silicomolybdate may be related to the tendency of Mn^{++} to form insoluble salts with ferrocyanide. The inability of Co^{++} and Ni^{++} to elicit phosphorylation with either complex anion may be due to a similar phenomenon. It has previously been shown[46,47] that Mg^{++} and Mn^{++} antagonize the uncoupling of oxidative phosphorylation by iodo-thyronines—a close parallel to their effect on the phosphorylations coupled to silicomolybdate oxidation. Since it now appears[48–50] that thyroxine tends to labilize mitochondrial structure, a somewhat similar role for silicomolybdate may be inferred from this body of information. The action of silicomolybdate, however, seems additionally related to its capacity to mediate electron transport since phosphorylation coupled to succinate oxidation is not significantly affected by the presence of silicomolybdate (Table I), suggesting that the act of binding *per se* does not elicit loss of phosphorylative capacity.

In contrast to our finding with whole mitochondria that P/O ratios associated with the oxidations mediated by cytochrome *c* are maximal near pH 8, where oxidation rates are also large (Fig. 1), COOPER AND LEHNINGER[51] observed that with digitonin extracts derived from whole mitochondria, corresponding P/O ratios were maximal at pH 5.5, where oxidation rates were minimal. HÜLSMANN AND SLATER[8] reported a maximum near pH 6 in guinea pig heart mitochondria, but their experiments were made in the presence of $6 \cdot 10^{-4}$ *M* EDTA which we found (Table II) would independently enhance the P/O ratio. A comparison of these data, however, may not be justified since the pH curves probably represent different phenomena. The stimulation of cytochrome *c*-mediated oxidation by hydroxyl ions (Fig. 1) may be related to the induction of mitochondrial swelling, known[52] to occur at pH 8, with a concomitant increase in permeability. It is not clear why this would also lead to higher

P/O ratios. A similar change in permeability was shown by LEHNINGER *et al.*[5] to accompany exposure of mitochondria to hypotonic media.

The redox potential of the silicomolybdate couple, extrapolated to pH 7.0 from measurements made between pH 2.5–4.5 with a Beckman platinum electrode, was found to be $E'_0 = +0.45$ V. STRICKLAND[53] estimated the value to be near $+0.56$ V. The potential level of the anion bound to mitochondrial protein may vary somewhat from this value. The redox potential of the ferri- ferro-cyanide couple[54] (E'_0) corrected for potassium-ion effects, is $+0.44$ V. Although the potentials are comparable, the concentration of ferrocyanide required to catalyze maximal rates of oxygen consumption with the mitochondria in the presence of ascorbate was found to be 25–50 times greater than that of silico-12 molybdate. Assuming that the electron-transport processes can be initiated only by those complex anions which enter or become bound to some special region of the mitochondria, this phenomenon can be ascribed to a difference in binding or rate of penetration of the two complex anions. This is supported by the data in Figs. 8 and 9. Such differences could be related to the nature of the charged groups on the surfaces of these large anions.

The data of Table VII clearly demonstrate that the oxidation and coupled phosphorylation observed with ferrocyanide is independent of ascorbate. It seems reasonable to assign a similar passive role to ascorbate in the reactions mediated by other high-potential donors like silicomolybdate and cytochrome *c*. The P/O ratios reported in Table VII indicate a remarkably efficient coupling between phosphorylation and electron-transport reactions initiated by ferrocyanide. We have demonstrated (Fig. 12) that electron transport mediated by ferrocyanide can only proceed to molecular oxygen *via* endogenous cytochrome *c*. In order to get an estimate of the potential level of the mitochondrial component to which ferrocyanide in the absence of ascorbate donates electrons, a kinetic analysis was made of the time course of oxygen consumption with ferrocyanide as substrate, using an appropriate simple reaction scheme*. An E'_0 value close to that of cytochrome *c* was obtained, suggesting that phosphorylation coupled to electron transport initiated by ferrocyanide in the absence of ascorbate is primarily that associated with the oxidation of an endogenous component near the potential level of cytochrome *c*, which has been non-enzymically reduced *in situ* by ferrocyanide. In the presence of ascorbate, mitochondrial components with redox potentials considerably below that of cytochrome *c* could be reduced by ferrocyanide. The P/O ratios greater than 1.0 and approaching 2.0, obtained with ascorbate and the complex anions clearly indicate that the complex anions can feed electrons into the respiratory chain at two different levels. The characteristic increase in P/O ratios with decreasing concentrations of electron carrier were observed in all of the electron transport reactions studied.

LEHNINGER[20] has previously suggested that the surface of intact mitochondria is impermeable to external DPNH but contains an enzyme which can couple its oxidation to the reduction of external cytochrome *c*. He also suggested that exposure of the mitochondria to hypotonic media removed this permeability constraint and thereby created a pathway for the direct oxidation of external DPNH by the internal cytochrome chain. The following more complicated picture of DPNH oxidation seems to be consistent with our data:

(1) The outer surface of intact mitochondria contains an enzyme or enzyme

* Unpublished work of M. Gellert and E. Jacobs.

complex which can couple the oxidation of external DPNH to the rapid reduction of external cytochrome *c*, silicomolybdate, menadione and ferrocyanide, but not to the reduction of the internal electron transport chain which itself appears to reside on a well-defined structure[60]. The first three of the above carriers can freely donate electrons to the internal electron transport chain in the presence of external DPNH alone while ferrocyanide additionally requires high levels of Mg^{++} in order to do this. In our experiments the pathway between external DPNH and oxygen which was completed by each of the above electron carriers was totally insensitive to antimycin A and Amytal. These results are consistent with previous studies using cytochrome *c* as the mediator[20,22,23]. Menadione oxidation in heart preparations, on the other hand, have been reported to be sensitive to antimycin A[55]. The significance of this difference is not discernable at present. In our experiments with external DPNH oxidation, the antimycin-insensitive electron transport to oxygen mediated by silicomolybdate and ferricyanide always gave P/O ratios of 1.0 whereas that catalyzed by external cytochrome *c* and menadione always resulted in P/O ratios less than 0.5.

(2) Exposure of the mitochondria to hypotonic media at pH 6 induces swelling and structural damage, thereby permitting direct interaction between the external reductase and the internal electron-transport chain. Since extraction of endogenous cytochrome *c* inactivates this newly created pathway and since the reaction is not inhibited by antimycin A, the point of interaction would appear to be around cytochrome *c*. Under these conditions we have observed that the rate of oxidation of external DPNH in the absence of supplementary electron carriers is maximal and that very little esterification of inorganic phosphate accompanies the oxidation. We have also always observed that the P/O ratios accompanying oxidation of substrates of the Krebs cycle drop by about 1.0 when this situation obtains (Table IX). Assuming that the cytochrome oxidase chain is identical for all of the above-mentioned oxidations we are led to postulate that hypotonic exposure rapidly inactivates the coupled phosphorylation in this segment of the respiratory chain. PRESSMAN[62] had previously suggested that some loss of phosphorylative capacity accompanies exposure of mitochondria to hypotonic media. Whether or not the observed inactivation can be attributed to a collapse of two juxtaposed surfaces is at present being investigated.

(3) The external reductase is stripped off from the mitochondrion as the pH of the hypotonic medium is raised from 6.0 to 8.0, thereby destroying the artificially-created shunt pathway between DPNH and oxygen. After the external reductase has been removed in this manner, oxidation of added DPNH becomes slower and more commensurate with the rates of oxidation of endogenous DPN-linked substrates, antimycin-sensitive and efficiently coupled to phosphorylation (Table IX). Presumably the oxidation of external DPNH is now proceeding by the same pathway as that utilized by the DPN-linked substrates. Previous investigations[56–58] of DPNH oxidation in relation to DPNH-cytochrome *c* reductase activity and its localization following exposure of mitochondria to distilled water have not described the above mentioned effect of pH, possibly because the pH of unbuffered distilled water found in many laboratories lies between 5.5–6.0. We have consistently observed that whenever mitochondria were subjected to any treatment which enables them to oxidize rapidly external DPNH in the absence of supplementary electron carriers, a concomitant decrease of about 1.0 appears in the P/O ratios for all substrate oxidations. LINNANE AND ZIEGLER[24] have suggested that DPNH oxidation can give P/O

ratios of only 2 and that the third phosphorylation step may lie between substrate and DPN. It would appear as if these authors have not considered the possibility that the loss of a phosphorylation in the cytochrome oxidase segment of the respiratory chain may be induced by treatments which degrade the mitochondrial structure. COOPER AND LEHNINGER[45] have reported that particles which can directly oxidize DPNH obtained from digitonin extracts of mitochondria show P/O ratios approaching 3.0 for β-hydroxybutyrate oxidation. However, more recent work by DEVLIN AND LEHNINGER[59] indicates that these P/O ratios approach 2.0 rather than 3.0 as an upper limit. Table VIII shows that the corresponding P/O ratios obtained with mitochondria disrupted by exposure to hypotonic media below pH 7.8 also approaches 2.0. SIEKEVITZ AND WATSON[60] have shown that low concentrations of deoxycholate induce mitochondrial swelling in a fashion similar to that effected by distilled water. It would appear, therefore, that structural changes produced in mitochondria by the usual type of mild physical or chemical treatment, including exposure to digitonin, generally lead to a lowering of P/O ratios by 1.0.

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FURTHER OBSERVATIONS ON THE ADENOSINE PHOSPHATASES OF COBRA VENOM

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SUMMARY

Dried cobra venom has been dialysed under various conditions. The effect on the $\alpha\beta$-ATP-ase, 5'-nucleotidase and DPN-*pyro*phosphatase, also that of chelating agents and of certain metal ions, is reported. The effect of specific antiserum has been investigated. The venom hydrolyses inosine triphosphate and coenzyme A in a manner analogous to the breakdown of ATP and DPN respectively. Flavine mononucleotide, ribose-5-phosphate and nicotinamide mononucleotide are hydrolysed at only 0.5, 1 and 2 % of the rate of adenosine monophosphate hydrolysis. Thiamin *pyro*phosphate is not hydrolysed.