Activation of coupled oxidative phosphorylation in bacterial particulates by a soluble factor (s)*

In previous communications[1,2] we reported the existence in cell-free bacterial extracts of a dinitrophenol (DNP) sensitive system which coupled phosphorylation to oxidation yielding P/O ratios greater than one. These studies on *Mycobacterium phlei* extracts have now been extended to include extracts of *Corynebacterium creatinovorans*. It has also been noted[3] that the activity of these highly organized systems is dependent on the presence of particles. These particles have now been separated by differential centrifugation and studied in detail. Particular interest centers in the demonstration that oxidative phosphorylation depends not only on the retention of intact particles containing cytochromes *c*, *b*, and cytochrome oxidase[4], but also on the critical role of a factor (s) found in the supernatant which is necessary for both oxidation and esterification of inorganic phosphate.

The activities of fractionated particles and supernatant were tested for coupled oxidative phosphorylation. With succinate as electron donor neither the particles nor supernatant fractions showed activity compared to the crude extract. Recombination of these two fractions, however, restores the oxidation and phosphorylation (Table I). The reconstituted systems appear to oxidize as well as crude homogenates although the ability to esterify inorganic phosphate is somewhat reduced, resulting in lowered P/O ratios than those reported for crude extracts. Similar results are also obtained when fumarate is used as the electron donor. By dialyzing the supernatant, the dependence on the addition of a phosphate acceptor system can be demonstrated. Under anaerobic conditions inorganic phosphate is not esterified by the recombined system.

TABLE I

THE ACTIVITY OF VARIOUS FRACTIONS ON OXIDATIVE PHOSPHORYLATION

The system contained 0.4 ml particulate fraction (a) obtained from crude extracts at pH 7.3[3] by centrifugation for 2 hours at 140,000 × *g* in the Spinco centrifuge and resuspended in 0.15 *M* KCl, 0.4 ml of the resulting supernatant (b), 20 μM succinate, 16 μM inorganic phosphate, 2.5 μM AMP, 1 mg of dialyzed hexokinase, 20 μM of mannose, and H_2O to a volume of 1.3 ml. The oxygen uptake was carried out at 30° for 10 minutes after the addition of substrate and then stopped by the addition of 10% TCA and inorganic phosphate analyzed by the method of FISKE AND SUBBAROW[5].

System	*Protein mg/ml*	*Oxidation (μ atoms)*	*Δ P_i (μ moles)*	*P/O*
Crude homogenate	28	5.76	7.6	1.4
After fractionation:				
1. Particles (a)	9.1	1.6	0.9	0.56
2. Supernatant (b)	14.8	0.94	0.5	0.53
3. a + b	a 9.1 b 14.8	6.2	6.1	0.98
4. a + b + DNP ($8 \cdot 10^{-5} M$)	a 9.1 b 14.8	6.6	1.4	0.24
5. DNP treated particles + supernatant	a 4.5 b 32	2.4	1.8	0.75
6. DNP treated supernatant + untreated particles	a 9.5 b 12.3	3.94	0.4	0.12

The effect of DNP ($8 \cdot 10^{-5} M$) on oxidative phosphorylation is also demonstrated in Table I, and is similar to that reported for crude homogenates from this microorganism. Particles incubated with $1 \cdot 10^{-4} M$ DNP for 15 minutes, and then centrifuged and resuspended in 0.15 *M* KCl fail

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to show any uncoupling effects. Our inability to completely remove DNP from treated supernatant has made it impossible to demonstrate clearly the implication that the supernatant factor (s) may be the DNP sensitive component.

The ability to reconstitute activity with particulate preparations by the addition of supernatant was further investigated. The effect of various concentrations of supernatant on both oxidation and phosphorylation was demonstrated using a constant concentration of particles (Fig. 1). Usually both oxidation and phosphorylation increase in a parallel manner with increasing concentrations of supernatant. High concentrations of supernatant failed to show any inhibition. The P/O ratios do not increase significantly with increasing concentration of supernatant.

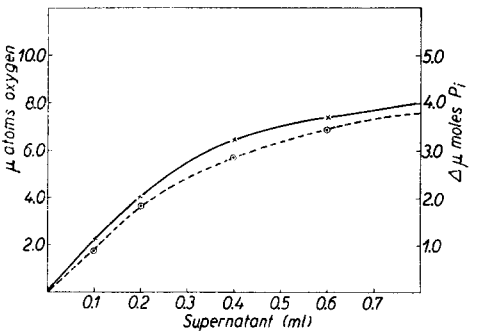

Fig. 1. The effect of supernatant concentration on oxidative phosphorylation. Same conditions as Table I. (a) Effects of concentration of supernatant on oxidation. O----O indicates μ atoms oxygen uptake per 10 minutes using constant concentration of particles (0.4 ml containing 24.2 mg protein/ml). The supernatant used contained 33.3 mg protein per ml, the amount used in each system is indicated in figure. (b) The effects of concentration of supernatant on phosphorylation $\times$——$\times$ indicates Δ μmoles of P_i per 10 minutes.

By preparing similar fractions from *C. creatinovorans* it is possible to activate both *M. phlei* and *C. creatinovorans* particles with the supernatant obtained from either organism.

Attempts to replace the supernatant factor (s) with either DPN, TPN, cytochrome *c* or Kochsaft have been unsuccessful. The factor (s) was also found to be nondialyzable and heat labile, indicating that at least one component is protein in nature. The supernatant material could be lyophilized and stored at $-2°$ without loss of activity. Furthermore, treatment of the supernatant with activated norit does not lower its activity. The particulate preparations, however, were found to be more labile, requiring isotonicity, and were destroyed by freezing and resonication.

Recent work by NASON[6] indicates that menadione reductase is unique in its sensitivity to DNP and other uncoupling agents, while MARTIUS[7] has presented evidence of the role of this enzyme in oxidative phosphorylation. Since the esterification of inorganic phosphate is inhibited 50% by $1 \cdot 10^{-4} M$ dicumarol, the menadione reductase activity of the supernatant was further fractionated by the method of NASON[8]. This partially purified fraction of greatly increased menadione reductase activity is capable of reactivating the oxidation and phosphorylation of the resuspended particles (Table II). Furthermore, in the presence of DNP the reoxidation of DPNH in the presence of menadione is inhibited. Although both activities can be fractionated together, direct involvment of menadione reductase as a factor for oxidative phosphorylation must await further fractionation and analysis.

TABLE II

EFFECTS OF FRACTIONATED SUPERNATANT (CONTAINING MENADIONE REDUCTASE) ON PARTICLES

System similar to Table I; the supernatant was fractionated according to NASON[8], and 0.4 ml used containing 6.6 mg protein/ml. The fractionated supernatant reoxidized 40 μg of DPNH/min/mg protein in the presence of menadione.

System	*Oxygen (μ atoms)*	*Δ P_i (μ moles)*	*P/O*
Particles	1.52	0.6	0.39
Supernatant	0.8	0	0
Particles + supernatant	4.92	3.8	0.77
Particles + fractionated supernatant	5.6	3.9	0.70

Since preparation of this manuscript a report by TISSIERES AND SLATER[9] provided evidence of a similar activation between particles and supernatant of *Azotobacter vinlandii*. Attempts in

the past to reconstitute the *M. phlei* particles with *Azotobacter* supernatant were unsuccessful by our methods of preparation. This may also reflect on the differences in DNP sensitivity of these various supernatants.

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Demonstration of direct effects of insulin on the isolated perfused diabetic rat liver*

Although administration of insulin to a diabetic animal is followed by correction of the characteristic defects in hepatic lipogenesis and glycogenesis, liver slices from diabetic rats are not influenced by insulin which is added *in vitro*[1,2]. Furthermore, no direct effect of insulin on net glucose uptake by isolated normal or diabetic liver tissue has yet been reported. LUNDSGAARD, in whose laboratory liver perfusions of short duration (three hours or less) were performed[3,4], states in a recent symposium, "I am not convinced that it has ever been proved that insulin has a direct effect on liver tissue"[5].

Development of a method in this laboratory for maintaining the isolated perfused rat liver for at least 6 hours[6] has permitted us to reinvestigate hepatic metabolism in diabetes with special reference to the action of insulin.

Rats of the Wistar strain, fasted 48 hours and weighing between 175 and 225 grams, were made diabetic by a single subcutaneous injection of 5% alloxan, 140 mg per kg, one hour after an injection of 1 unit/kg of insulin[7]. They were then maintained on daily injections of protamine zinc insulin for at least three weeks before use as liver donors, with no insulin being given them for 40 hours preceding an experiment. Normal and diabetic rats were allowed to eat a stock Purina pellet ration until sacrifice.

Liver perfusion apparatus and operative technique were essentially as described in previous reports from this laboratory[6,8]. Prior to incision of the abdomen, a specimen of tail blood was removed for determination of blood sugar content. Ketonuria was estimated semi-quantitatively through the use of Acetest tablets (Ames Co., Elkhart, Indiana). The initial liver glycogen was determined from the right lateral lobe which was ligated and removed during the operation. At the end of the perfusion two pieces of tissue weighing about 300 mg each were removed from different lobes of the liver for estimation of the final glycogen content[9]. The NELSON method[10] was used for all glucose determinations.

Total liver fatty acids were isolated by the usual methods and their radioactivity measured with a dynamic vane electrometer after oxidation to carbon dioxide[11].

In all experiments cited in the accompanying table, livers from rats weighing between 200 and 330 grams were perfused with approximately 140 ml of rat blood diluted one-third with Ringer's solution and containing a total of 60 mg of heparin. To the perfusing blood were added 500 mg of glucose and 35 mg of sodium acetate-1-^{14}C which had a total radioactivity of 2200 arbitrary units**. In experiments No. 173, 197 and 274 the entire labelled substrate was added

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** Each unit is equivalent to $2.2 \cdot 10^4$ disintegrations/minute.