OXIDATIVE PHOSPHORYLATION IN FRACTIONATED BACTERIAL SYSTEMS
XI. SEPARATION OF SOLUBLE FACTORS NECESSARY FOR OXIDATIVE PHOSPHORYLATION*

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A requirement for soluble components in addition to particles for restoration of oxidative phosphorylation with a cell-free system from Mycobacterium phlei has been reported (Brodie and Gray, 1956; Brodie, 1959). The soluble components are necessary for both oxidation and phosphorylation. Attempts to resolve the components necessary for both activities have been unsuccessful (Brodie, 1959); however, separation of the components necessary for different segments of the respiratory chain has been achieved by column chromatography (Asano and Brodie, 1963ab).

Materials and Methods

M. phlei cells were grown as previously described (Brodie and Gray, 1956) and used to obtain the particulate and supernatant fractions. The particulate fraction was washed with 0.15 M KCl and Tris buffer (pH 7.2) and used in the assay of the fractionate supernatant components. The supernatant components were partially purified by fractionation with ammonium sulfate and protamine (Brodie, 1959) and separated by column chromatography on DEAE-cellulose (Asano and Brodie, 1963a). The fractions obtained by this procedure were assayed with particles for their ability to restore oxidation, coupled phosphorylation and to support electron transport with different segments of the respiratory chain. In addition, the supernatant fractions were used to study the distribution of the partial reactions associated with oxidative phosphorylation.

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Results

The supernatant factors necessary for restoration of both oxidation and phosphorylation with different electron donors were separated by gradient elution (increasing chloride concentration) from DEAE-cellulose columns. Protein components were separated by this procedure which were necessary for restoration of coupled activity with different segments of the respiratory chain of the intact particles. One factor was obtained which was necessary for restoration of oxidation and phosphorylation with malate as substrate, Table I.

TABLE I

Resolved Soluble Components Required for Oxidative Phosphorylation with Particles

Hydrogen donor	Supernatant components	Oxidation μatoms/10 min	Phosphorylation μmoles/10 min
Malate	none*	1.3	0.0
	Malate factor	5.7	4.5
	Succinate factor	1.1	0.2
Succinate	none*	2.4	1.4
	Malate factor	1.5	0.4
	Succinate factor	5.2	3.3

* activity of particles alone

The malate and succinate factors were assayed with washed particles. The assay was similar to that described by Brodie (1959). FAD (1.7×10^{-4} M) was added for optimal activity with malate as electron donor.

This component was inactive with succinate as an electron donor. Another fraction was obtained by chromatography which stimulates both oxidation and phosphorylation with succinate. Although the succinate factor had some oxidative activity with malate further fractionation of this fraction resulted in the separation of both activities. Two additional components have been isolated which are necessary for coupled activity with DPNH and for oxidation of 6-chromanyl phosphate of vitamin K_1.

Elution pattern of these factors are shown in Figure 1. An important difference between M. phlei particles and submitochondrial particles (sonicated or digitonin treated) is that the former requires soluble components for both oxidation and phos-

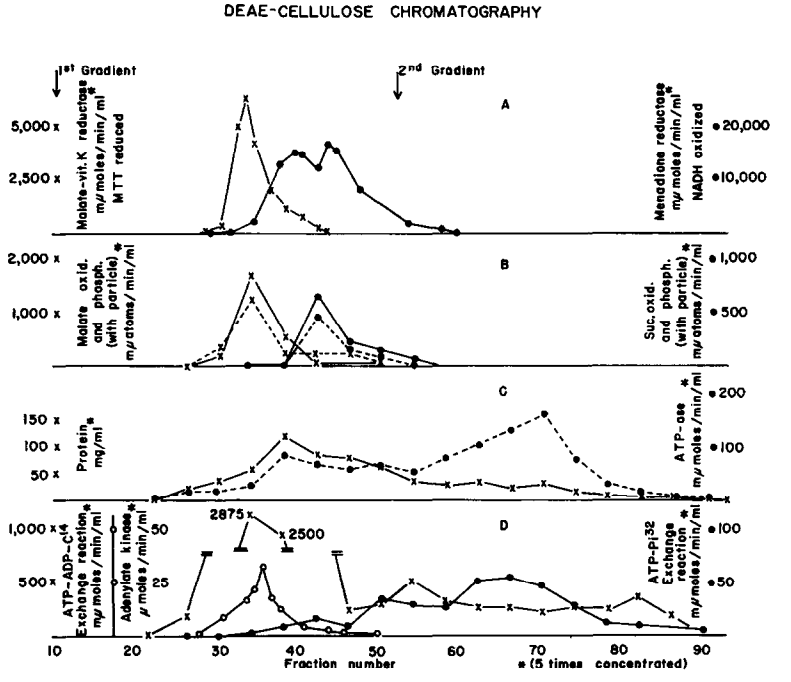

Figure 1

The crude supernatant fluid obtained following removal of particles was fractionated with ammonium sulfate (Brodie, 1959), dialyzed, and absorbed on a DEAE-cellulose column which was previously treated with 0.005 M Tris-HCl buffer (pH 8.2). The column was washed with 10 ml. of 0.005 M Tris buffer (pH 8.2) and developed with an increasing chloride gradient. Malate-K reductase and menadione reductase activities were determined spectrophotometrically at 565 and 340 mμ respectively. MTT-tetrozolium was used as a final electron acceptor in the malate K-reductase assay. ATPase was determined at pH 7.2 by following the liberation of orthophosphate from ATP whereas the adenylate kinase activity was assayed spectrophotometrically (340 mμ) following the addition of hexokinase, TPN, and glucose-6-phosphate dehydrogenase. The Pi-ATP exchange activity and ADP-ATP exchange activity were measured by the method of Cooper and Kulka (1961). Dotted line in (B) shows phosphorylation.

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phorylation (Brodie, 1959) but the latter requires soluble components for only phosphorylation (Penefsky et. al, 1960; Linnane and Titchner, 1960; Lehninger, 1960; Webster, 1962; Smith and Hansen, 1962). The distribution of a number of oxidative enzymes which are associated with supernatant fluid was studied following fractionation on DEAE-cellulose. The soluble menadione reductases which are associated with an electron transport bypass reaction are shown on the first column. There appear to be two different menadione reductases which differ in flavin requirement and sensitivity

to pCMB (Asano and Brodie, in preparation); however, neither reductase was associated with the components necessary for malate oxidation. Both reductases overlap the fractions which contain the succinate factor.

An enzyme corresponding to or identical with the malate coupling factor of the supernate has been isolated. This enzyme utilizes malate as an electron donor and requires the presence of vitamin K_1*, Figure 1A. The properties of this enzyme (malate-vitamin K reductase) will be described in a following paper (Asano and Brodie, 1963c). The supernatant fraction also contains a DPN-dependent malic dehydrogenase; however, the distribution and properties of this enzyme were found to differ from those of the malate-vitamin K reductase or the factors necessary for coupled activity with malate.

The distribution of protein, ATPase and the exchange enzymes is shown in Figure 1, C and D. Although both ATPase and the Pi^{32}-ATP exchange reaction were detected in all fractions obtained by chromatography, the bulk of this activity was separated from both coupling factors. Furthermore, the rate (activity/mg/min) of both enzymes was less than one-tenth of the overall rate of phosphorylation. Neither activity was affected by DNP. The particles also exhibited both ATPase and Pi^{32}-ATP exchange activity, but these activities were low when compared to the overall rate of phosphorylation and only partially sensitive to uncoupling agents. Adenylate kinase activity can be separated from the succinate factor but partly overlaps with the malate factor fraction. ADP-C^{14}-ATP exchange reaction was detected in all fractions, Figure 1D; the bulk of this activity overlaps the fractions containing the malate and the succinate factors. Although adenylate kinase is known to have ADP-ATP exchange activity the distribution of both activities was found to be different in M. phlei extracts. Furthermore, two different ADP-ATP exchange enzymes were obtained by chromatography of the malate factor on Sephadex G-200. By this procedure the malate-vitamin K reductase fraction containing the ADP-ATP exchange activity was separated from adenylate kinase.

* Vitamin K_1 was purified before use and suspended in the supernatant fluid or in phospholipid. Tween 80 was not used for suspension of this vitamin since it was found to inhibit the major oxidative pathways.

Discussion

The malate-K reductase is of particular interest since this activity and the malate oxidation factor appear in the same fraction following purification by ammonium sulfate precipitation, protamine fractionation, DEAE-cellulose chromatography and Sephadex G-200 chromatography. Furthermore, the rate of malate-K reductase activity is always two to three times higher than that of the overall rate of oxidation with the particles. Both activities are inhibited to the same degree by amytal, atebrin, Dicumaro pentachlorophenol, Tween 80 and BRIJ 35. The identification of the factors necessary for succinate and DPN H oxidation are now in progress; however, it should be mentioned that the primary succinic dehydrogenase as assayed by the PMS method (Singer and Kearney, 1957) and cytochrome oxidase as assayed by p-phenylenediamine method are completely particle-bound, and are not stimulated by the addition of supernatant fluid (Brodie, 1959).

ATPase and ATP-Pi^{32} exchange reaction were separated from both malate and succinate oxidation factors and have low rates compared with the overall rate of phosphorylation of the reconstituted system. In contrast the ADP-ATP exchange activity and phosphorylation factor(s) have not been separated from the components necessary for restoration of oxidation. The rate of the ADP-ATP exchange reaction in both malate and succinate fractions are almost the same magnitude as the overall rate of phosphorylation with the reconstituted phosphorylating systems. Although it appears that the ADP-ATP exchange enzymes are associated with the phosphorylation factors further study is necessary to establish the nature of the phosphorylation factors of this bacterial system.

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