The Electron-transferring Flavoprotein as a Common Intermediate in the Mitochondrial Oxidation of Butyryl Coenzyme A and Sarcosine*

Dale D. Hoskins

From the Department of Biochemistry, Oregon Regional Primate Research Center, Beaverton, Oregon 97006

SUMMARY

Butyryl coenzyme A dehydrogenase and a transfer protein required for maximal activity as measured by reduction of 2,6-dichloroindophenol have been solubilized from monkey (Macaca mulatta) liver mitochondria by sonic irradiation. The dehydrogenase has been purified approximately 60-fold from the electron-transferring flavoprotein (transfer protein) on diethylamine; mucedo methyl cellulose. Purified preparations of transfer protein, measured at 450 m, were reduced by catalytic amounts of butyryl dehydrogenase and sarcosine dehydrogenase with each enzyme causing approximately one-half of the total reduction. It is concluded that the transfer protein requirement for the oxidation of butyryl coenzyme A is met by the FAD-specific electron-transferring flavoprotein.

The oxidation of radiosarcosine, in the presence of limiting amounts of transfer protein, has been shown to be inhibited 10 to 15% by reduced but not by oxidized butyryl dehydrogenase. Simultaneous measurements of 2,6-dichloroindophenol reduction have shown the combined rates of the two dehydrogenase-catalyzed reductions to be greater than the rate of the faster of the individual reactions. Purified transfer protein has been shown to be inactivated by heat treatment, yielding different curves for activity against temperature of inactivation for each activity monitoring dehydrogenase.

Presently available evidence suggests that the mitochondrial oxidations of butyryl coenzyme A and of sarcosine are both mediated by a common electron-transferring flavoprotein.

The mitochondrial systems responsible for the oxidation of the acyl coenzyme A derivatives of fatty acids and the N-methyl derivatives of glycine share several common features. The most distinctive of these is that each pathway of oxidation seems to represent an example of “coupled flavoenzymes in electron transport” (1–3). The dehydrogenases of both systems apparently are flavoproteins, although the classification of the amino acid dehydrogenases as such is clouded by inability, to date, to identify the exact nature of the flavin. Acyl-CoA dehydrogenases are flavin adenine dinucleotide-specific. Upon reduction by substrate both classes of enzymes serve as electron donors for an FAD-specific protein called the electron-transferring flavoprotein. Reducing equivalents from the transfer protein enter the terminal particulate electron transport chain at about the level of coenzyme Q (2, 3). Knowledge about this sequence of reactions has been derived from a variety of mammals, including rat liver (4, 5), monkey liver (2, 6), pig and beef liver and beef heart (7), and from microorganisms (8).

This body of information has raised the question of whether a single transfer protein is a common intermediate in the oxidative pathways of both systems. The work of Reinert and Frissell (9) has shown that the transfer proteins of the fatty acyl-CoA dehydrogenase system of pig liver, and of the sarcosine dehydrogenase system of rat liver, are able to substitute for each other in their function, while the primary dehydrogenases retain their substrate specificity. The work described in this paper is part of a series of studies designed to isolate and characterize the components of the fatty acid and amino acid oxidase systems from a single tissue in the hope that the interplay between the systems, and the biological function of the transfer protein, may be better understood. Previous reports from this laboratory (2, 6) have described the isolation and purification of sarcosine dehydrogenase and the transfer protein from rhesus monkey (Macaca mulatta) liver mitochondria and the reconstitution of the sarcosine oxidase system. The present report describes the purification and partial characterization of butyryl-CoA dehydrogenase from monkey liver mitochondria, shows a requirement for transfer protein for dehydrogenase activity as measured by reduction of 2,6-dichloroindophenol, and indicates the role of transfer protein in the acyl and sarcosine dehydrogenase pathways. The data presented suggest that a single transfer protein with substrate-specific sites mediates electron transport between both classes of substrates and the electron acceptors.

1 The abbreviations used are: transfer protein or transfer flavoprotein, the electron-transferring flavoprotein; butyryl dehydrogenase, butyryl Coenzyme A dehydrogenase; PMS, phenazine methosulfate; indophenol, 2,6-dichloroindophenol.

Received for publication, March 21, 1966

* Publication No. 126 from the Oregon Regional Primate Research Center, Beaverton, Oregon. Supported by Grant AM 08658-02 of the National Institutes of Health.
Details of the purification procedure are given in the text. Specific activity is defined as millimicromoles of indophenol reduced per min per mg of protein at 37°C. Units of activity are equivalent to specific activity X total protein and are based on extrapolated values for activity at infinite dye concentrations, i.e. $V_{\text{max}}$ (PMS).

### Table I

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Volume</th>
<th>Protein</th>
<th>Total protein</th>
<th>Protein recovery</th>
<th>Specific activity</th>
</tr>
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<tbody>
<tr>
<td>Sonic extract</td>
<td>180</td>
<td>5.43</td>
<td>980</td>
<td>100</td>
<td>86</td>
</tr>
<tr>
<td>Salt fraction, 50 to 80%</td>
<td>20</td>
<td>18.6</td>
<td>372</td>
<td>38</td>
<td>178</td>
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<tr>
<td>Ethanol fraction, 30 to 50%</td>
<td>5.9</td>
<td>2.54</td>
<td>14.4</td>
<td>1.47</td>
<td>775</td>
</tr>
<tr>
<td>DEAE-cellulose eluate (tube 28)</td>
<td>3.9</td>
<td>0.22</td>
<td>0.86</td>
<td>0.09</td>
<td>5,260</td>
</tr>
</tbody>
</table>

* Assays carried out in the presence of 0.1 mg of PMS per ml.

### EXPERIMENTAL PROCEDURE

#### Preparation of Soluble Enzymes

Monkey liver mitochondria were prepared from experimental animals of either sex as previously described (6). Mitochondrial preparations were suspended in a volume of 7.5 mM potassium phosphate, pH 7.5, equal in weight to the original liver weight, frozen overnight at -25°C, and sonically irradiated in 30-ml portions for three 10-sec periods, with a Branson model 75 Sonifier tuned to maximum intensity. The supernatant solution obtained following centrifugation at 78,000 x g for 33 hours was used as the starting material for purification of all enzymes described in this paper.

#### Substrate Synthesis

Acyl coenzyme A derivatives of butyric, caproic, caprylic and capric acids were synthesized enzymatically with the bovine liver fatty acid-activating enzyme of Mahler, Wakil, and Bock (10). The components of the system for synthesis were the same as described by these authors for assay of the activating enzyme, except for replacement of glycylglycine buffer, pH 8.0, with 0.23 M (final concentration) Tris-HCl, pH 9.0, and inclusion of 10 μg per ml of crystalline yeast inorganic pyrophosphatase (0.8 unit per μg). Incubation was carried out with stirring in a vacuum for 4 hours at room temperature. Esterified CoA derivatives were isolated from the reaction mixtures with phenol-benzyl alcohol as described by Littlefield and Sanadi (11), estimated as hydroxamates (12), titrated with iodine until free of sulfhydryl groups, and stored in the frozen state at pH 6.0.

#### Enzyme Assays

Butyryl dehydrogenase may be assayed spectrophotometrically by following the reduction of dichloroindophenol at 600 nm (7). The assay may be carried out in the presence of either phenazine methosulfate or excess transfer flavoprotein. Beinert (7) has indicated that assay in the presence of 0.1 mg of PMS per ml.

Reaction rates were calculated on the basis of the absorbance change occurring during the first 3 min of reaction. Specific activity is defined as millimicromoles of indophenol reduced per min per mg of protein at 37°C. All reported activities are corrected for endogenous indophenol reduction unless otherwise specified. Under these conditions, linearity between enzyme concentration and activity has been obtained with all butyryl dehydrogenase preparations tested, including crude extracts containing transfer protein.

Indophenol reduction in the presence of PMS is optimal in the presence of 0.12 M potassium phosphate at pH 7.4. Plots of both pH and buffer concentrations against enzyme activity, however, reveal no sharp dependence on either pH or ionic strength. At pH 7.4, for example, the relative ratios of indophenol reduction in 0.02, 0.12, and 0.24 M potassium phosphate were 88, 100, and 90. Similarly, in the presence of 0.12 M phosphate the reaction rate was 80% and 87% of maximum at pH 6.8 and 8.2, respectively. A pH of 6.8 was chosen for this study in order to minimize alkaline autoxidation of indophenol.

Crude preparations of butyryl dehydrogenase containing transfer protein may be assayed in the absence of PMS. Optimal conditions for such systems have not been defined. Proportionality between enzyme concentration and activity, however, is obtained with all preparations, including crude extracts. This observation is in contrast to the results obtained with preparations of the sarcosine dehydrogenase system (4, 6).

Sarcosine dehydrogenase and the transfer flavoprotein of monkey liver mitochondria were purified and assayed as previously reported for "purified" monkey liver sarcosine dehydrogenase (6). Similar high specific activity preparations ($V_{\text{max}}$ (PMS) > 20,000), obtained by rechromatography on DEAE-cellulose, have been obtained from rat liver mitochondria.

4 Several of the sarcosine dehydrogenase preparations used in this paper possess specific activities considerably greater than that previously reported for "purified" monkey liver sarcosine dehydrogenase (6).
viously described (2, 6). Sarcosine dehydrogenase activities, unless otherwise specified, were determined in the presence of 0.2 mg of PMS per ml. Specific activities of dehydrogenase and transfer protein are expressed as micromoles of indophenol reduced per hour per mg of protein at 37°.

Chemical Assays—Soluble protein was determined either by the biuret method (14) or with the Folin phenol reagent as described by Lowry et al. (15). Mitochondrial protein was determined by the biuret method after solubilization with deoxycholate.

Materials—Coenzyme A, acetyl-CoA, palmitoyl-CoA, antimony A, FAD, FMN, and phenazine methosulfate were purchased from Sigma. Fatty acids were obtained from Mann. Yeast inorganic pyrophosphatase was a product of Worthington. Sarcosine-\(^{14}\)CH\(_3\) was obtained from New England Nuclear. All other reagents were of the highest purity obtainable.

Results

Purification of Butyryl Dehydrogenase—Butyryl dehydrogenase was purified from sonic extracts of monkey liver mitochondria by a combination of ammonium sulfate fractionation, precipitation with zinc ethanol, and chromatography on DEAE-cellulose (Table I). A total of 180 ml of extract were fractionated with solid ammonium sulfate between the limits 0 to 30, 35 to 45, 45 to 50, and 50 to 80% saturation. This and all subsequent operations were carried out at 0-4°. Solutions were stirred for 15 min after the addition of each increment of salt. The fraction precipitating at the highest salt concentration was centrifuged at 75,000 x g for 30 min, dissolved in 5 ml of 50 mM potassium phosphate, pH 6.6, and dialyzed overnight against 2 liters of this same buffer. Residual ammonium sulfate was subsequently removed on a Sephadex G-25 column (2.5 x 22 cm), equilibrated with 20 mM Tris acetate, pH 7.2, and the protein concentration of the eluate (containing 95 to 100% of the protein applied to the column) was adjusted to 15 mg per ml with Tris-acetate buffer. Fractionation with zinc-ethanol was carried out essentially as described by Beinert (7) for the butyryl dehydrogenase of pig liver. A 50 mM zinc-lactate solution was added slowly with rapid stirring until a final Zn\(^{++}\) concentration of 1 mM was obtained. The pellet obtained on centrifugation was discarded, and the supernatant solution used for ethanol fractionation. Cold ethanol (4°) was subsequently added so that fractions between 0 to 10, 10 to 20, 20 to 30, and 30 to 50% alcohol were obtained. The temperature during precipitation was maintained at 15 to 20° with an ethanol-Dry Ice bath. Enzyme solutions were stirred vigorously during, and for exactly 1 min after, addition of each increment of ethanol. Precipitated protein was centrifuged at 0° for 15 min at 66,000 x g. The fraction precipitating at the highest ethanol concentration was dissolved in 5 ml of 0.4 M sodium citrate, pH 7.2, and dialyzed overnight against 2 liters of 25 mM potassium phosphate, pH 6.6, containing 1 mM EDTA. The distinctly green enzyme solution was then placed on a DEAE-cellulose column (1.1 x 20 cm, packed under 7 pounds pressure) equilibrated with 250 mM phosphate. Maximum enzyme activity was found in tube 28. The over-all purification from crude extracts was found to be 60-fold. This value may represent a minimum purification factor due to an anticipated oxidation of butyryl-CoA by the general acyl dehydrogenase (Cd to Cr6) assumed to be present in crude extracts.

Nature of Prosthetic Group—The flavin nature of the prosthetic group is indicated by the visible absorption spectrum of the purified enzyme shown in Fig. 1. Absorption maxima are found at 340 to 350 and 490 to 440 nm. The absorbance in the flavin regions is considerably reduced on addition of butyryl-CoA. The addition of dithionite to the enzymatically reduced protein results in only a slight additional reduction. At 450 nm 85% of the total reduction may be attributed to substrate and 15% to added dithionite. As shown in Table I, the activity of butyryl dehydrogenase, as measured in the presence of 0.1 mg of PMS per ml, is enhanced at all stages of purity by the addition of FAD. This enhancement is specific; neither flavin mononucleotide nor riboflavin is capable of replacing FAD. The specific requirement for FAD has been routinely noted following chromatography on DEAE-cellulose, but has not always been apparent with less pure enzyme preparations. The butyryl dehydrogenase of monkey liver mitochondria, along with the dehydrogenase from a variety of other species and tissues (3), may thus be considered as an FAD-specific flavoprotein. If one assumes that the stimulation by FAD shown for the crude DEAE-cellulose eluate in Table I represents a proportionate loss of FAD from the holoenzyme, then calculation from the reduction by substrate, at 450 nm, for this purified enzyme preparation, shown in Table I and Fig. 1, indicates a minimum molecular weight for the dehydrogenase of approximately 45,000.

Substrate Specificity and Kinetic Constants—Purified butyryl dehydrogenase preparations exhibit marked specificity for
butyryl-CoA. At substrate concentrations of 0.4 μM, CoA de-

rivatives of acetic, caprylic, capric, and palmitic acids are not

oxidized to a measurable degree. Caproyl-CoA at a concentra-
tion of 0.4 μM is oxidized at a rate approximately 15% that of

butyryl-CoA. The Kₘ for butyryl-CoA is 4.7 μM.

Fractionation and Reconstitution of Dehydrogenase Activity—
Following ethanol fractionation, enzyme activity, assayed in the
absence of PMS, drops markedly in comparison to that measured
in the presence of dye (Table I). This observation suggested
the separation of dehydrogenase activity into two or more com-
ponents, one being replaceable by PMS. Such fractionation
during purification of the N-methylamino acid and acyl-CoA
derhydrogenases is well documented for a variety of tissues
(4, 6, 17). Separation of monkey liver butyryl dehydrogenase
and a protein fraction required for maximum enzyme activity in
the absence of PMS, by column chromatography of a crude
dehydrogenase preparation, is shown in Fig. 2. A portion, 141
mg, of a 50 to 80% ammonium sulfate fraction of mitochondrial
extract in 4.5 ml of 2.5 mM phosphate buffer, pH 7.5, was applied
to a DEAE-cellulose column (1.1 x 19 cm, packed under 7
pounds pressure) equilibrated with this same buffer. Protein
was subsequently eluted under 2 pounds pressure with 30-ml
portions of 2.5, 10, 25, 100, and 200 mM potassium phosphate
buffer, pH 6.6, 4-ml fractions being collected. Under these
conditions 80% of the protein applied to the column was eluted.
Butyryl dehydrogenase was assayed under standard conditions
in the presence of PMS (0.1 mg per ml). Dehydrogenase was
eluted from the column, beginning with 100 mM phosphate
buffer. The distribution of the synergistic factor was monitored with 25 μg of the butyryl dehydrogenase of
maximum activity (DEAE-cellulose eluate, tube 34). Indophenol reduction in this case was measured in the absence of
PMS, serum albumin, and phosphate. Phosphate buffer was
replaced with 10 μmoles of Tris-HCl, pH 7.8. These changes
from the standard assay method resulted in a 6- to 7-fold in-
crease in activity despite the fact that such conditions are far
from optimal for assay in the presence of PMS (see enzyme assays
in “Experimental Procedure”). Under these conditions maxi-

mum activity (labeled electron-mediating protein in Fig. 2) was
obtained in eluate tube 19 following elution with 25 mM phos-
phate.

Chromatographic Identification of Transfer Flavoprotein—The
above result gave rise to the speculation that the activity-en-
hancing factor was either similar to or identical with the FAD-
specific electron-transferring flavoprotein (transfer protein)
required for reduction of indophenol in the presence of monkey
liver mitochondria sarcosine dehydrogenase (2). This enzyme
is known to be adsorbed on a DEAE-cellulose column prepared
as described above, and is also eluted with 25 mM phosphate at
pH 6.6. The elution pattern from the column used in obtaining
the data for Fig. 2 for transfer protein, as monitored by sarcosine
dehydrogenase, is shown in Fig. 3. The elution pattern of the
factor monitored by butyryl dehydrogenase is reproduced from
Fig. 2 on an enlarged scale. Both dehydrogenases were present
at a concentration twice that required to give maximum dye

![Butyryl-CoA Dehydrogenase](Image)

![Electron-mediating Protein](Image)

**Fig. 2.** Separation of butyryl dehydrogenase and an electron-mediating protein on DEAE-cellulose. Butyryl dehydrogenase was as-

sayed in the presence of 0.1 mg of PMS per ml. The electron-mediating protein was monitored with 25 μg of butyryl dehydrogenase (Fra-
ction 34) in the absence of PMS. For experimental details see text. Specific activity is defined as millimicromoles of indophenol re-
duced per min per mg of protein at 37°C.
reduction in the presence of a limiting amount of eluate Fraction 19, thus insuring a rate-limiting function for the activity-stimulating factors. The patterns of elution, as monitored by the dehydrogenases, are seen to bear a strong resemblance to each other.

**Reduction of Purified Transfer Protein by Sarcosine and Butyryl Dehydrogenases**—More substantial evidence for the possible involvement of a single, or of structurally similar, transfer flavoproteins in the oxidative pathways of both sarcosine and butyryl systems was obtained with purified preparations of monkey liver transfer protein. This enzyme may be brought to such a state of homogeneity that it moves as an essentially single component in the analytical ultracentrifuge and possesses an activity equal to or greater than that of the protein prepared from other tissues (1, 17). The visible spectrum of transfer protein (solid line), and spectra of the reduction of transfer protein by catalytic amounts of reduced butyryl dehydrogenase (dashed line) and of sarcosine dehydrogenase (bottom line), are shown in Fig. 4. Reduction upon addition of butyryl-CoA was complete within 15 min. Excess substrate caused no further spectral changes. The addition of sarcosine and sarcosine dehydrogenase, however, resulted in considerable further reduction. Of the total change in absorbance in the flavin region at 450 mp approximately one-half was accounted for by the action of butyryl dehydrogenase, and the remaining portion by sarcosine dehydrogenase. It should be noted that this latter enzyme has previously been reported to effect only a partial reduction of the FAD of transfer protein (2). The lower spectrum shown in Fig. 4, the result of reduction by both dehydrogenases, strongly resembles that obtained upon addition of dithionite (2). This chemical reducing agent was not, unfortunately, added to the preparation shown in the figure. Independent experiments, however, carried out in the Beckman model DU spectrophotometer, failed to detect additional reduction of transfer protein by dithionite following reduction by both dehydrogenases.

**Competition between Butyryl and Sarcosine Dehydrogenases for Transfer Protein**—Butyryl and sarcosine dehydrogenases compete with each other for purified transfer protein to a slight but significant degree as measured by the formation of radioformaldehyde from HCH3-sarcosine (Table II). The amount of each dehydrogenase used in the experiment shown in Table II was determined by first ascertaining the concentration required to give half-maximal velocity in the presence of 9.2 mg of transfer protein (specific activity, 2700). In this experiment, each cuvette contained, in addition to transfer protein, one dehydrogenase, 0.1 pmole of FAD, 40 pmoles of Tris-HCl buffer, pH 7.8, 1.6 pmoles of semicarbazide, indophenol, and water to a volume of 0.98 ml. Butyryl-CoA and sarcosine (20 ml) were added to the final concentrations of 80 mum and 2 mm, respectively.

The above experiment was next duplicated, except that each cuvette contained 4 times the half-maximal velocity concentration of butyryl dehydrogenase (170 mg, specific activity, 450), and a similar concentration of sarcosine dehydrogenase (412 mg, specific activity, 1220). Unlabeled sarcosine was replaced with 1.98 pmoles of sarcosine-1C3, containing 4.47 cC per pmole. The reaction in cuvettes containing radiosarcosine was stopped after 11.0 min by addition of 0.2 ml of 50% trichloroacetic acid, and the supernatant solution obtained on centrifugation was used for formation of the radioformaldehyde derivative of formaldehyde. Conditions for counting solid samples of radioformaldehyde have been described (6). Activities shown in Table II represent the averages of 5.0-mg samples counted in triplicate. The data shown indicate that the oxidation of sarcosine, which has an absolute dependence on transfer protein under the conditions of assay, was inhibited approximately 18% by the reduced butyryl dehydrogenase. Butyryl-CoA and oxidized dehydrogenase, added to the sarcosine dehydrogenase system individually, resulted in 6% and 1% inhibition in radioformaldehyde production, respectively. The butyryl dehydrogenase system thus exerts a slight but real inhibitory action.

Attempts to correlate radioformaldehyde formation with dye reduction in the above experiment were unsuccessful because of contamination of sarcosine dehydrogenase preparations with proteins, presumably nonspecific thioesterase, which bring about the hydrolysis of butyryl-CoA. The results, however, suggest that transfer protein (or proteins) possesses more than a single site, since, in the course of this and several other experiments with sarcosine dehydrogenase preparations of varying states of purity, the activity of the combined dehydrogenase systems, corrected for contaminating enzyme activities, has always been found to be considerably greater than that of the faster of either of the dehydrogenase systems assayed individually.

**Dehydrogenase-Monitored Temperature Lability Curves of Transfer Protein**—Further evidence for independence of action of the two dehydrogenases towards transfer protein is given by the temperature lability curves shown in Fig. 5. In this experiment, a sample of purified transfer protein, specific activity, 2700, was incubated for 10 min at the temperature indicated, immediately immersed in an ice bath, and a sample containing 4.0 mg of pro-
Fig. 4. Spectra of oxidized and enzymatically reduced forms of transfer protein. ——, oxidized enzyme, 418 μg per ml, specific activity, 8330; ---, transfer protein reduced with 20 μg of butyryl dehydrogenase (specific activity, 740) and 40 μmole of butyryl-CoA; ----, transfer protein reduced with 32 μg of sarcosine dehydrogenase, specific activity, 5300, and 1 μmole of sarcosine after reduction with butyryl dehydrogenase and butyryl-CoA. Spectra were taken at 15-min intervals and corrected for absorption of substrates and catalytic amounts of dehydrogenases. Volume, 0.4 ml. Solvent system, 75 mM phosphate, pH 6.8. Other conditions are described in Fig. 1.

Table II

<table>
<thead>
<tr>
<th>Assay components</th>
<th>Radioformaldehyde formation from radiosarcosine by reduced butyryl dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcosine dehydrogenase</td>
<td>1.38 ± 0.01</td>
</tr>
<tr>
<td>Sarcosine dehydrogenase + butyryl-CoA</td>
<td>1.30 ± 0.01</td>
</tr>
<tr>
<td>Sarcosine + butyryl-CoA dehydrogenase</td>
<td>1.37 ± 0.01</td>
</tr>
<tr>
<td>Sarcosine + butyryl-CoA dehydrogenase + butyryl-CoA</td>
<td>1.11 ± 0.01</td>
</tr>
</tbody>
</table>

The dependence of butyryl dehydrogenase on its transfer protein for maximum rates of dye reduction was used in this study for determining the extent of solubilization. As described above, it is possible to add increasing amounts of butyryl dehydrogenase to transfer protein until a saturation condition obtains. Assay under these conditions gives a measure of the amount of transfer protein present. A system not stimulated by dehydrogenase is assumed to contain a limiting amount of transfer protein. This latter condition was found to be the case for intact monkey liver mitochondria, sonically irradiated mitochondria, and the soluble system obtained after centrifugation for 3½ hours at 78,000 × g. Consequently, 20-μl samples of the above preparations containing 218, 219, and 109 μg of protein, respectively, were assayed under standard assay conditions, except for deletion of PMS and addition of 10 μl of antimycin A (in 95% ethanol) to a final concentration of 50 μM.
Precipitated at high salt concentration (Table I). Transfer protein activity. Such separation has not been possible with protein for butyryl dehydrogenase is also contained solely in the detectable upon recombination with any other fraction obtained on precipitation with salt from 0 to 100% saturation. The experiments indicate that a transfer protein, or proteins required for the butyryl dehydrogenase system since this latter enzyme is also identical.

This level of antibiotic has been shown to inhibit almost completely the oxidation by molecular oxygen of reduced transfer protein in a reconstituted sarcosine oxidase preparation (2). Sonically irradiated mitochondria, and the supernatant fraction obtained after centrifugation, were found to possess 62 and 83%, respectively, of the butyryl dehydrogenase activity of untreated mitochondria. The degree of solubilization of transfer protein for both dehydrogenase systems would thus appear to be almost identical.

The complete separation of sarcosine dehydrogenase and transfer protein may be accomplished by ammonium sulfate fractionation of crude mitochondrial extracts, with the former enzyme precipitating between 35 and 45% saturation, and the latter between 50 and 80%. Recombination of these fractions results in restoration of the capacity for dye reduction. The fraction with the higher concentration of salt contains all of the transfer protein activity. Such separation has not been possible with the butyryl dehydrogenase system since this latter enzyme is also precipitated at high salt concentration (Table I). Transfer protein for butyryl dehydrogenase is also contained solely in the 50 to 80% fraction as indicated by the fact that, in the presence of limiting amounts of dehydrogenase, no synergistic effect is detectable upon recombination with any other fraction obtained on precipitation with salt from 0 to 100% saturation. The experiments indicate that a transfer protein, or proteins required for reduction of dye for both classes of dehydrogenases, is solubilized to the same degree by sonic irradiation, and is precipitated with ammonium sulfate under the same conditions.

**Discussion**

The results of this study establish that a coupled flavoprotein system exists in monkey (Macaca mulatta) liver mitochondria for the oxidation of butyryl coenzyme A. The primary dehydrogenase has been shown to be an FAD-specific flavoprotein, which exhibits dependency on a previously described (2) FAD-specific transfer protein for reduction of indophenol. The ubiquitous nature of this system in animal and bacterial tissue is well documented (3, 7). The data reported in this paper, however, along with the previous reports that the sarcosine dehydrogenase system of monkey liver mitochondria involves a coupled flavoprotein reaction (6), represent the first description of two such coupled systems isolated from mitochondria of the same tissue.

Preliminary evidence that a single transfer protein is involved in the oxidation of both butyryl-CoA and sarcosine has come from the work of Beinert and Frisell (9), who have shown that the transfer proteins derived from the rat and the pig are “functionally equivalent.” The present paper represents an extension of the idea put forth by these workers, and serves to strengthen their hypothesis. Specifically, it has been shown that the transfer protein for both systems is solubilized to the same degree by sonic irradiation of the mitochondrion, is separated from crude extracts by ammonium sulfate precipitation in the same manner, and is adsorbed on and eluted from DEAE-cellulose with identical elution patterns, as monitored by both dehydrogenases. No other transfer protein activity has been found in fractions obtained either by salt fractionation or chromatography. Furthermore, purified transfer protein is reduced by both reduced sarcosine and butyryl dehydrogenases with each of the latter enzymes causing approximately one-half of the total reduction at 150 μμ.

If one accepts the “purified” transfer protein used in this report as a single functional species of protein, then this reduction by two substrates gives considerable support to the concept of a role for the enzyme as a common intermediate in both oxidative pathways. Unfortunately, the purity of the transfer protein is open to question. The enzyme has been shown, however, to move essentially as a single component in the analytical ultracentrifuge, and to possess an activity equal to or greater than the activities of preparations from other tissues (1, 17).

Several observations reported in this paper support the view that transfer protein (or proteins) possesses separate binding
sites for each dehydrogenase. First, purified transfer protein is partially reduced by each reduced dehydrogenase. Secondly, separate transfer protein temperature lability curves exist for each dehydrogenase. Finally, the rate of reduction of indophenol in the presence of both dehydrogenases and limiting amounts of transfer protein is greater than the more rapid of the individual reactions. The observation that reduced butyryl dehydrogenase brings about a slight but real depression in the formation of radioformaldehyde from radiosarcosine, while the oxidized enzyme fails to do so, suggests steric hindrance. Such a result would not appear likely if more than one transfer protein were involved. In order to pursue the problem further, preparations of transfer protein more completely tested for homogeneity and of highly purified dehydrogenases devoid of all contaminants which interfere with indophenol reduction are needed. The data presented in the present paper can best be interpreted as giving support to the view that the mitochondrial oxidation of both butyryl coenzyme A and sarcosine involves a single transfer protein possessing binding sites at least partially specific for each reduced dehydrogenase.

Acknowledgments—The author is indebted to Mr. W. Zimmer- man, Mrs. Esther Lee, and Miss D. L. Patterson for technical assistance during the course of this investigation.

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