TABLE II

COUPLED PHOSPHORYLATION IN FRACTIONATED MITOCHONDRIAL RESIDUE

Pretreatment	*Protein mg*	*Cyt. c* $9 \cdot 10^{-7}$ *M*	*Oxygen µatoms*	*P/O*
None	11	—	16.9	>1.5
Dist. water	5.0	—	2.0	—
		+	10.6	0.71
0.005 *M* KCl	7.6	—	2.0	—
		+	16.7	1.19
0.001 *M* Phosphate, pH 7.0	5.1	—	1.2	—
		+	17.0	0.89
0.005 *M* Phosphate, pH 7.0	7.2	—	1.9	—
		+	17.0	1.14

The mitochondria in 0.25 *M* sucrose were centrifuged at 20,000 × *g*, and the residue was suspended in the above media for 5 minutes at 0°. One-tenth the volume of 2.5 *M* sucrose was then added and centrifuged. The residue was washed twice with 0.12 *M* KCl and resuspended in the *original* volume of sucrose. The phosphorylation coupled to succinate oxidation was measured as in Table I in the presence of $1.7 \cdot 10^{-4}$ *M* Mn^{+2}, with 0.5 ml of enzyme preparation. The protein concentration is indicated in the second column.

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1 O. LINDBERG AND L. ERNSTER, *Nature*, 173 (1954) 1038.
2 W. C. SCHNEIDER, *J. Biol. Chem.*, 176 (1948) 259.
3 W. C. SCHNEIDER, A. CLAUDE AND G. H. HOGEBOOM, *J. Biol. Chem.*, 172 (1948) 451.
4 D. E. GREEN, B. MACKLER, R. REPASKE AND H. R. MAHLER, *Biochim. Biophys. Acta*, 15 (1954) 435.

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Flavoproteins involved in the first oxidative step of the fatty acid cycle

It was recognized two years ago that the first oxidative step of the fatty acid cycle, the dehydrogenation of the saturated to the unsaturated fatty acyl CoA, is catalyzed by an enzyme system of the flavoprotein class[1-4]. Evidence was also available at that time that two distinct flavoproteins, one specific for substrates of short carbon chain and the other for substrates of intermediate or long carbon chain, are involved in this catalysis[1,2,5]. The former enzyme, a green copper-containing flavoprotein, had been obtained in high purity and was described in detail[6,7].

In attempts to isolate the flavoprotein specific for CoA derivatives of longer carbon chain it became apparent that altogether four flavoproteins are participating in the dehydrogenation of fatty acyl CoA's in pig liver. These four enzymes have now been separated from each other and obtained in a state of high purity. There is no evidence that additional enzymes are implicated in the primary dehydrogenation step. The functional relationships of the four flavoproteins are outlined in the following scheme, the arrows indicating direction of hydrogen transfer or electron flow:

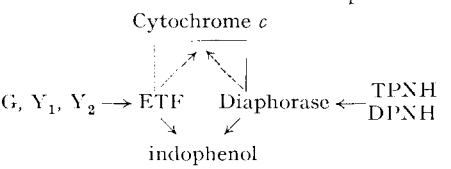
$$\text{fatty acyl CoA} \begin{cases} NC_4\text{–}C_6 \longrightarrow G \searrow \\ NC_4\text{–}C_{16} \longrightarrow Y_1 \rightarrow \text{ETF} \longrightarrow \text{acceptors} \\ NC_6\text{–}C_{16} \longrightarrow Y_2 \nearrow \end{cases}$$

G is a green copper protein similar to that described earlier[6,7]. Y_1 and Y_2 are yellow flavoproteins. Y_1 has a broad specificity range[8], whereas Y_2 does not react significantly with butyryl CoA. G, Y_1 and Y_2 accept hydrogen from saturated fatty acyl CoA's. The coincident reduction of their prosthetic

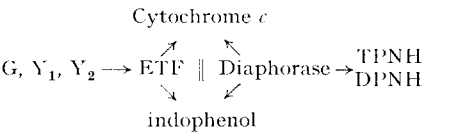
flavins can be followed spectrophotometrically. These three enzymes are therefore primary dehydrogenases operating in parallel at the substrate level. However, none of these dehydrogenases, when reduced, is reoxidized by the usual electron acceptors like 2,6-dichlorophenolindophenol, ferricyanide, cytochrome *c* or oxygen. All three enzymes require an additional enzyme in order to turn over with acceptors in continuous oxidoreductions. This enzyme is likewise a flavoprotein[9]. Because of its specific function, in transferring electrons from reduced G, Y_1 and Y_2 to acceptors, it has been called the electron transferring flavoprotein, ETF. As indicated in the scheme ETF operates in series with the three primary dehydrogenases. To our knowledge this is the first example of such coupling of flavoproteins. So far ETF has been found to be specific for reduced G, Y_1 and Y_2 as substrates*.

The natural electron acceptor which follows ETF in the electron transport chain is still unknown. Reduced ETF reacts with a variety of artificial acceptors like indophenol, ferricyanide and quinones but it does not readily reduce cytochrome *c*, when isolated under mild preparative conditions*. The ability to react with cytochrome *c* as rapidly as with indophenol can however be induced when preparations of ETF are treated with acid ammonium sulfate. The precipitate which is obtained under these conditions is eluted with solutions of decreasing salt concentration and can thus be separated into two fractions. One fraction contains almost all the ETF activity. Such an ETF preparation can now be effectively linked to cytochrome *c*. A second fraction is obtained on elution of the precipitate which strongly inhibits the interaction of ETF with cytochrome *c*. This inhibitory fraction has high diaphorase activity, equally strong with DPNH and with TPNH as substrate. When separated from ETF the dihydropyridine nucleotide dehydrogenase acquires likewise the ability to interact with cytochrome *c* as acceptor, *i.e.*, it exhibits now a DPNH and TPNH cytochrome reductase activity equal to its diaphorase activity. On recombination of the dihydropyridine nucleotide dehydrogenase fraction with ETF the cytochrome *c* reductase activity is again abolished. These observations are summarized in the following scheme, the arrows indicating direction of hydrogen transfer or electron flow:

Before acid ammonium sulfate and separation



After acid ammonium sulfate and separation



The fact that the ability to interact with cytochrome *c* is not present originally in ETF but can be artificially induced, casts considerable doubt on the significance of cytochrome *c* as the natural electron acceptor in the oxidation of fatty acyl derivatives of CoA. The conversion of a diaphorase into a cytochrome reductase by the same means is remarkable and raises the question of the significance of this class of enzymes.

The specificity of the primary dehydrogenases was tested with four substrates (C_4, C_8, C_{12}, C_{16}) at their optimal levels with the results shown in Fig. 1. When supplemented with ETF the three dehydrogenases turn over at similar rates in presence of the appropriate substrates for maximal activity.

The following criteria serve to differentiate the enzymes in crude mixtures when other specific properties of the pure enzymes are not yet discernible. G is indifferent towards palmityl CoA as substrate or as inhibitor. Y_1 acts on any one substrate regardless of chain length, but it will not oxidize butyryl CoA when traces of palmityl CoA or its product of enzymic dehydrogenation are present. Y_2 will not oxidize butyryl CoA at a significant rate under any conditions.

When octanoyl CoA was oxidized by indophenol in presence of either or Y_1 Y_2 with ETF and the product of oxidation was incubated with the remaining enzymes of the fatty acid cycle an amount of acetyl CoA was formed approximately equivalent to the amount of indophenol reduced**. Acetyl CoA was demonstrated by enzymic formation of citrate from oxalacetate.

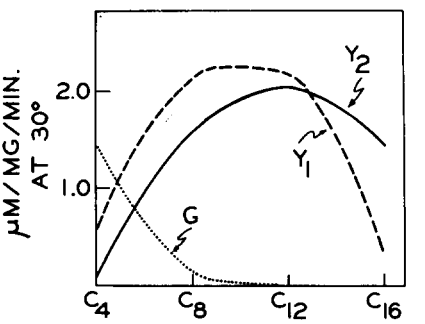


Fig. 1. Specificity of fatty acyl CoA dehydrogenases for substrates of different chain length.

* Even at the highest purity level (*cf.* ref. 9) ETF exhibits diaphorase activity, specifically when isolated under relatively mild conditions. Evidence indicates that this activity is due to an associated flavoprotein.

With the D-amino acid oxidase test it was shown that flavin-adenine dinucleotide is the prosthetic flavin of Y_1, Y_2 and ETF**. Small amounts of iron were consistently found in Y_1 and ETF at a ratio of one iron to six or more flavins. The significance of this small amount of iron is not clear. No other metals were found in the flavoproteins at a significant level, except for copper in G. In view of the findings reported above the actual role of copper in G will have to be reinvestigated.

G, Y_1 and Y_2 can be separated and prepared in high purity from an extract of mitochondrial acetone powder in one procedure involving only seven steps. ETF of high purity is obtained in a separate four step procedure from the same source. Details of these procedures, spectral characteristics and criteria of purity of the new flavoproteins will be reported elsewhere.

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1 H. BEINERT, R. M. BOCK, D. S. GOLDMAN, D. E. GREEN, H. R. MAHLER, S. MII, P. G. STANSLY AND S. J. WAKIL, *J. Am. Chem. Soc.*, 75 (1953) 4111.
2 H. R. MAHLER, *Federation Proc.*, 12 (1953) 694.
3 W. SEUBERT AND F. LYNEN, *J. Am. Chem. Soc.*, 75 (1953) 2787.
4 F. LYNEN, *Federation Proc.*, 12 (1953) 683.
5 S. MII AND D. E. GREEN, *Biochim. Biophys. Acta*, 13 (1954) 425.
6 D. E. GREEN, S. MII, H. R. MAHLER AND R. M. BOCK, *J. Biol. Chem.*, 206 (1954) 1.
7 H. R. MAHLER, *J. Biol. Chem.*, 206 (1954) 13.
8 H. BEINERT AND F. L. CRANE, *Federation Proc.*, 13 (1954) 181.
9 F. L. CRANE AND H. BEINERT, *J. Am. Chem. Soc.*, 76 (1954) 4491.

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The occurrence of a direct oxidative pathway of carbohydrate metabolism in the fly *Musca domestica* L.

The carbohydrate metabolism of Diptera has been shown to be similar to that found in other animals. In this group of insects, the oxidation of glucose to CO_2 and water is mediated by the universally accepted glycolytic scheme[1] and citric acid cycle[2]. An investigation of the metabolism of the flight muscles of the housefly has revealed the presence of another route by which glucose may be metabolized. This alternative direct oxidative pathway appears to be essentially similar to that operating in higher animals[3-6] and plants[7].

The enzymic activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were assayed spectrophotometrically according to GLOCK AND MCLEAN[6]. The reactions proceed as follows:

$$\text{(1) Glucose-6-phosphate} + \text{TPN} \xrightarrow[\text{dehydrogenase}]{\text{Glucose-6-phosphate}} \text{6-phosphogluconate} + \text{TPNH}_2$$

$$\text{(2) 6-phosphogluconate} + \text{TPN} \xrightarrow[\text{dehydrogenase}]{\text{6-phosphogluconate}} \text{ribulose-5-phosphate} + \text{CO}_2 + \text{TPNH}_2$$

Fig. 1 indicates that both dehydrogenases were active in the presence of their respective substrates. When to the system was added lactic dehydrogenase which is capable of reacting with $TPNH_2$[8] pyruvate was rapidly reduced due to the oxidation of $TPNH_2$. It can be seen that glucose-6-phosphate dehydrogenase is approximately twice as active as 6-phosphogluconate dehydrogenase. Both enzymes were specific for TPN.

The reaction mixture with 6-phosphogluconate as substrate accumulated a product which reacted positively on the orcinol test and was probably a pentose.

The breakdown of ribose-5-phosphate to sedoheptulose-7-phosphate is catalyzed by pentose phosphate isomerase and transketolase[3,4]. Sedoheptulose-7-phosphate is then converted to hexose monophosphate by transaldolase[5]. The activity of transketolase was measured according to HORECKER *et al.*[4]. The reaction proceeds as follows: