The role of fumarate and TPN in steroid enzymic 11β-hydroxylation

We have shown[1] that suspensions of ox-adrenocortical mitochondria prepared in 0.25 *M* sucrose catalyse 11β-hydroxylation of 11-deoxycorticosterone (DOC) if a member of the citric acid cycle is oxidized at the same time. The "intact" mitochondria do not show the specific requirements for fumarate referred to by HAYANO AND DORFMAN[2]. The requirement for fumarate and for triphosphopyridine nucleotide (TPN) becomes evident when the mitochondria are treated with hypotonic solutions of electrolytes or if they are acetone-dried[3]. The role of fumarate has now been investigated further using enzymes extracted from acetone-dried mitochondria, and it has been found that this substance is required for the reduction of added TPN^+ probably via malate and with the intervention of OCHOA's malic enzyme[4]. TPNH is required for 11β-hydroxylation of DOC.

Ox-adrenal mitochondria were prepared in sucrose, dried with acetone and the acetone removed with ether. Enzymes were extracted from the dry powder with ten times its weight of 0.154 *M* KCl, and were obtained as a clear red solution by centrifuging at 0° for 0.5 hour at $2 \cdot 10^4$ g and occasionally for 1 hour at 10^5 g. Reaction mixtures contained 2 ml enzyme solution, 40 m*M* TRIS buffer (pH 7.4), 10 m*M* fumarate and about 0.1 m*M* TPN^+ in a total volume of 3 ml. These mixtures were incubated with 500 μg DOC for 1 hour at 37° in air.

The general arrangement of controls and methods of steroid analysis have been described elsewhere[1]. Under these conditions 80–90 % of the added DOC was converted to a product which was almost exclusively corticosterone as judged by paper chromatography and direct determination of this steroid. Hydroxylation did not occur in the absence of oxygen. It was markedly inhibited if the TRIS buffer was replaced by phosphate, in the presence of 10 m*M* versene or by dialysing the enzyme solution. The activity of the dialysed enzyme was partly restored by 1 m*M* $MnCl_2$. KCN 10 m*M* was slightly inhibitory. Fumarate could be replaced by L-malate or L-*iso*citrate but not by α-oxoglutarate which is the most potent "activator" of 11β-hydroxylation with intact mitochondria[1].

On addition of fumarate to reaction mixtures containing TPN^+ but no DOC a marked increase in optical density at 340 mμ was observed (Fig. 1, II and III). When DOC was present from the start the increase in optical density was less pronounced (Fig. 1, IV). These results suggest that TPN^+ is reduced by reactions involving fumarate. Evidence for the formation of pyruvate in the reaction mixture containing fumarate and TPN^+ was obtained by the paper chromatographic method of EL HAWARY AND THOMPSON[5]. This supports the suggestion that the malic enzyme[4] is involved in the reduction of TPN^+. In the absence of fumarate added TPNH is reoxidized (Fig. 2).

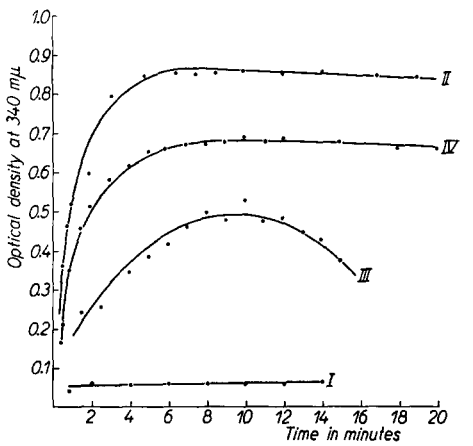

Fig. 1. The reaction mixtures contained 2 ml enzyme, 40 m*M* TRIS buffer pH 7.4, 0.1 m*M* TPN^+ in a final vol. of 3 ml. I contained no fumarate, IV contained 500 g DOC in 0.04 ml propyleneglycol. At zero time fumarate was added to II and IV to give 10 m*M* final concentration and to III to give 1 m*M* final concentration. The cell contents were maintained at 37° and were stirred at 2 min intervals to assist oxygenation.

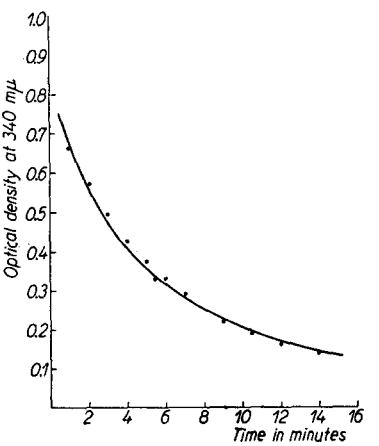

Fig. 2. The reaction mixture contained 2 ml enzyme and 40 m*M* TRIS buffer pH 7.4, in a final vol. of 3 ml. At zero time TPN^+ reduced by $Na_2S_2O_4$ was added to give a final concentration of about 0.1 m*M*. The cell contents were maintained at 37° and were stirred at 2 min intervals to assist oxygenation.

It would thus appear that a curve of the type III, Fig. 1 might be explained by a higher rate of TPN^+ reduction during the first 10 minutes whereafter fumarate concentration becomes the limiting factor and TPNH oxidation is the dominant reaction.

The requirement for TPNH for 11β-hydroxylation of DOC was confirmed by observations (Table I) that this reaction occurred on the slow addition of TPNH (final concentration 0.6 mM) to a reaction mixture containing no fumarate. No reaction occurred when TPN or DPNH were added under the same conditions.

TABLE I

THE 11β-HYDROXYLATION OF DOC BY OX-ADRENAL ENZYMES IN THE PRESENCE OF REDUCED TPN^+

Additions to reaction mixtures containing 2 ml enzyme + 40 mM TRIS pH 7.4	*DOC added (μmoles)*	*DOC hydroxylated (μmoles)*	*% hydroxylation*
0.1 mM TPN^+ + 10 mM fumarate	1.33	1.33	100
0.6 mM TPN^+	1.33	0	0
0.6 mM TPNH	1.33	0.57	43
0.6 mM DPNH	1.33	0	0

Incubations were for 1 hour at 37g in air. Coenzymes were added at intervals to give the final concentrations shown.

The results described above show a remarkable similarity to those of BRODIE *et al.*[6] who found that TPNH and oxygen are required for the metabolism of certain drugs by liver microsomes. They suggested that TPNH oxidation in their system could involve the production of hydrogen peroxide which might be used in the drug transformations observed. MITOMA AND UDENFRIEND[7] have shown a requirement for DPNH and oxygen for the oxidation of phenylalanine to tyrosine.

BRODIE[7] has confirmed the production of H_2O_2 on TPNH oxidation in his enzyme system and has found that TPNH cannot be replaced by H_2O_2 generating enzyme systems[8], although this may be due to failure of H_2O_2 to enter the microsomes. A mechanism of 11β-hydroxylation of steroids involving H_2O_2 might offer an explanation of the observations of HANAYO AND DORFMAN[9] with D_2O and $H_2{}^{18}O$ that water does not take part in the reaction. Experiments to investigate the possible role of H_2O_2 in 11β-hydroxylation are in hand. It is important, however, to point out that the production of H_2O_2 on TPNH oxidation may be a normal occurrence, when the reduced flavoprotein formed reacts directly with oxygen in microsomal particles which are deficient in cytochrome oxidase[10]. "Intact" mitochondria however which catalyse steroid 11β-hydroxylation will normally oxidize TPNH via the flavoprotein and cytochrome systems without the production of H_2O_2. The saline washed residues of HAYANO AND DORFMAN[2] show a special requirement for fumarate and cannot be considered to contain "intact" mitochondria. Acetone drying as used in our preparations is known to destroy cytochrome oxidase[11]. The possibility must therefore be considered that under these very artificial conditions steroid 11β-hydroxylation may proceed by mechanisms not involved in "intact" mitochondria.

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1 A. C. BROWNIE AND J. K. GRANT, *Biochem. J.*, 50 (1954) 255.
2 M. HAYANO AND R. I. DORFMAN, *J. Biol. Chem.*, 201 (1953) 175.
3 A. C. BROWNIE AND J. K. GRANT, *Biochem. J.*, (1955), in press.
4 S. OCHOA, A. H. MEHLER AND A. KORNBERG, *J. Biol. Chem.*, 174 (1948) 978.
5 M. F. S. EL HAWARY AND R. H. S. THOMPSON, *Biochem. J.*, 53 (1953) 340.
6 B. B. BRODIE, J. AXELROD, J. R. COOPER, L. GAUDETTE, B. N. LA DU, C. MITOMA AND S. UDENFRIEND, *Science*, 121 (1955) 603.
7 B. B. BRODIE, personal communication.
8 D. KEILIN AND E. F. HARTREE, *Biochem. J.*, 39 (1945) 293.
9 M. HAYANO AND R. I. DORFMAN, personal communication.
10 W. C. SCHNEIDER, *J. Biol. Chem.*, 165 (1946) 585.
11 D. KEILIN AND E. F. HARTREE, *Proc. Roy. Soc.*, B.125 (1938) 171.

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