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A Possible Role for Transhydrogenation in Side-Chain Cleavage of Cholesterol†

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ABSTRACT: Three lines of evidence are presented in favor of a role for transhydrogenation in providing reducing equivalents for the side-chain cleavage of cholesterol by mitochondria from bovine adrenal cortex. Firstly, rotenone, an inhibitor of oxidation of DPNH, stimulates side-chain cleavage whether this is supported by endogenous substrates or by DPN^+-linked substrates (malate and other Krebs cycle intermediates). Secondly, a positive correlation ($r = 0.82$) was observed between levels of mitochondrial DPNH:DPN^+ and the rate of side-chain cleavage. Thirdly, DPNH supports side-chain cleavage when this nucleotide is permitted to enter mitochondria by ageing, freezing or addition of Ca^{2+} to cause swelling; this action of DPNH is enhanced by concomitant addition of TPN^+. Evidently DPNH can provide reducing equivalents for side-chain cleavage, although the purified enzyme responds more readily to TPNH by one order of magnitude. Unlike 11β-hydroxylation, side-chain cleavage is stimulated by cyanide whether this reaction is supported by endogenous substrates, by succinate, or by malate. Reasons are given for believing that 11β-hydroxylation and side-chain cleavage do not necessarily derive reducing equivalents by the same mechanism. The evidence is believed to support the view that TPNH for side-chain cleavage may be generated from DPNH by transhydrogenation.

The conversion of cholesterol to pregnenolone takes place in mitochondria of steroid-forming organs and appears to determine the rate of steroid biosynthesis (Karaboyas and Koritz, 1965; Hall and Koritz, 1965; Hall and Young, 1968). This conversion involves cleavage of a carbon–carbon bond and is referred to here as side-chain cleavage of cholesterol. Cleavage appears to involve hydroxylation of the side chain prior to or in association with cleavage of C_{20}–C_{22} bond; reducing equivalents for hydroxylation are believed to come from TPNH since the purified enzyme system shows maximal activity with TPNH at a concentration one-tenth of that required to produce maximal activity with DPNH (Satoh *et al.*, 1966). A previous report from this laboratory (Hall, 1967b) presented evidence to show that the TPNH required for side-chain cleavage can be generated by reversed electron transport from succinate to DPN^+. This conclusion suggested that either DPNH is capable of generating TPNH or that at least some of the reducing equivalents for side-chain cleavage may come directly from DPNH. The present studies were undertaken to explore these possibilities further.

Experimental Section

Preparation of Mitochondria and Incubation. Mitochondria from bovine adrenal cortex were prepared by centrifugation in sucrose (0.25 M) as described elsewhere (Hall, 1967b). The final pellet was suspended in Tris (0.1 M, pH 7.4) prepared in 0.25 M sucrose to a concentration of 8–10 mg of protein/ml (20 g of adrenal cortex provided 10 ml of such a suspension).

Incubation was performed in 25-ml erlenmeyer flasks in a buffered medium (pH 7.4) containing $MgCl_2$ (17.6 μmoles), Tris (pH 7.4, 100 μmoles), KCl (98 μmoles), and potassium phosphate (44 μmoles) in a final volume of 2 ml. The order of addition of substances to the flasks was as follows: buffered medium, cholesterol-7α-*t*, oxidizable substrates, inhibitors and mitochondria (approximately 8 mg of protein). Cholesterol-7α-*t* (2 μg; 4.4×10^6 dpm/flask) was added in *N,N*-di-

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methylformamide (20 μl). The reaction was started by addition of TPN^+ and a reducing system (Young and Hall, 1969). Rotenone was added in 5 μl of ethanol; ethanol (5 μl) was added to other flasks. Incubation was stopped by standing the flasks on ice and adding 0.1 ml of NaOH (5 N) to each flask. Zero-time controls (extracted without incubation) and incubation of heated mitochondria were performed with each experiment; zero-time values (100–300 dpm) were subtracted from values for incubated flasks. The additions of various substances to flasks for incubation are given with the accompanying tables and figures.

Mitochondria were partly denatured by incubating the final suspension of organelles at 37° for 15 min. The suspension was added directly to the flasks for determination of side-chain cleavage. Frozen mitochondria were prepared by plunging a centrifuge tube containing the final mitochondrial pellet in ethanol–Dry Ice for 30 sec. Frozen mitochondria were allowed to thaw at room temperature before the pellet was resuspended by homogenizing in an homogenizer surrounded by ice. In studies of the oxidation of DPNH, mitochondria were incubated on ice for 1 hr or at 37° for 5 min to promote the entry of DPNH into the organelles.

One experiment refers to studies made with a single preparation of mitochondria. Duplicate determinations of side-chain cleavage refer to determinations on two identical flasks—the entire extract from any one flask was used to provide a single value for the reaction.

Measurement of Side-Chain Cleavage of Cholesterol. After incubation of cholesterol-7α-*t* with mitochondria, pregnenolone-*t* was isolated and measured as described elsewhere (Young and Hall, 1969). In some experiments side-chain cleavage was measured without added substrate or with exogenous cholesterol as substrate; for these purposes pregnenolone was measured by gas–liquid chromatography (Young and Hall, 1969).

Measurement of Pyridine Nucleotides. For the measurement of pyridine nucleotides, mitochondrial suspension (8.0–10 mg of protein/ml) was incubated at 0° for 5 min and the nucleotides were measured by the method of Purvis (1960). Fluorometry was performed with an Eppendorf flurometer which was set to give a full-scale deflection with 1.0–1.5 nmoles of DPNH. The minimal concentration of DPNH that could be measured under these conditions was 3×10^{-11} mole in a volume of 3 ml. Assays were performed in duplicate.

Oxygen Consumption by Mitochondria. Oxygen consumption was measured by means of a Clark electrode.

Measurement of TPNH–Cytochrome c Reductase and TPNH-Diaphorase Activities. The activities of the two proteins TPNH-diaphorase and non-heme iron (adrenodoxin), prepared from bovine adrenocortical mitochondria, were measured as described by Omura and coworkers (1966). Non-heme iron was measured by reduction of cytochrome *c* (horse heart) and values were expressed as nanomoles per minute per milligram of enzyme protein, using a millimolar extinction coefficient of 19.6 (Yonetani, 1967). TPNH-diaphorase activity was measured by reduction of dichlorophenolindophenol (0.1 mg/3 ml in each cuvet). Values were calculated for the rate of reduction of dichlorophenolindophenol on the basis of a millimolar extinction coefficient of 19 (Savage, 1957). The total volume was 3 ml in both assays and absorbance was recorded in a Gilford spectrophotometer at 550 (cytochrome *c* reductase) or 590 nm (diaphorase activity).

Liquid scintillation spectrometry was performed as described previously (Means and Hall, 1968). Values for pregnenolone-*t* were corrected to 100% efficiency of counting (expressed as disintegrations per minute) and were corrected for losses during recovery.

Determination of Protein. The protein content of mitochondrial suspensions was measured by the method of Lowry *et al.* (1951).

Chemicals. Cholesterol-7α-*t* (TRK Batch 6; 6.65 Ci/mole) was purchased from the Radiochemical Centre Amersham and purified before use by paper chromatography in ligroin–propylene glycol followed by thin-layer chromatography (Simpson and Boyd, 1966). These procedures removed several polar contaminants from the preparation with the result that zero-time controls showed little radioactivity in the final pregnenolone extracts (see Results). Rotenone, ATP, pyridine nucleotides, cytochrome *c* (horse heart), ethanol dehydrogenase, isocitric dehydrogenase, and glutamic dehydrogenase were purchased from Sigma Chemical Corp. The activities of these enzymes were examined before use by standard methods (Colowick and Kaplan, 1955; 1962; 1967). Rotenone was recrystallized three times from ethanol before use in these experiments. Other substrates were obtained from Calbiochem. Solvents of analytical grade were purchased from Byproducts and Chemicals Pty. Ltd. (Auburn, New South Wales, Australia). Bovine serum albumin was purchased from Commonwealth Serum Laboratories, Parkville, Victoria, Australia. Dichlorophenolindophenol was obtained from British Drug Houses Ltd. Oligomycin, citrate, and α-ketoglutarate were purchased from Sigma Chemical Corp.

Results

Conversion of Cholesterol to Pregnenolone. That the methods used here do in fact measure pregnenolone-*t* has been documented in previous reports (Hall, 1967a,b; Young and Hall, 1969). Conversion of cholesterol-*t* to pregnenolone-*t* is linear for at least 20 min. Zero-time and heated enzyme values for side-chain cleavage (see Methods) were in the range 100–300 dpm/flask. Values for conversion of cholesterol-*t* to pregnenolone-*t* vary considerably from one preparation of mitochondria to another, being especially influenced by levels of endogenous cholesterol.

Measurement of Pyridine Nucleotides. Recovery of DPN^+ and TPN^+ added to mitochondrial extract was 88–95% and recovery of DPNH and TPNH was 76–85% in five determinations for each nucleotide. These values and the levels of these nucleotides observed in the adrenocortical mitochondria are in keeping with those reported by other workers using bovine adrenocortical mitochondria (Purvis *et al.*, 1968). Moreover duplicate determinations agreed within 10%. Addition of the various inhibitors and substrates (including rotenone) to mitochondrial extracts did not influence the fluorometric measurement of pyridine nucleotides.

Influence of Rotenone on Adrenal Mitochondria. Rotenone did not inhibit the flow of electrons from TPNH to cytochrome *c* or dichlorophenolindophenol. In one experiment values for reduction of cytochrome *c* were 198 nmoles/min per mg of protein without rotenone and 218 nmoles/min per mg of protein in the presence of rotenone; the corresponding values for reduction of dichlorophenolindophenol were 52 nmoles/min per mg of protein and 50.6 nmoles/min per mg of protein in the presence of rotenone. Six determinations with cytochrome *c* and rotenone gave values for 102 ± 4 (std dev) and eight determinations with dichlorophenolindophenol and rotenone 97 ± 5 (std dev) when control values were expressed as 100. Therefore rotenone does not inhibit the electron transport chain used for steroid hydroxylation.

TABLE I: Influence of Various Substrates and Rotenone on Redox State of Mitochondrial Pyridine Nucleotides.[a]

Expt	Substrates and Rotenone	Duration Incubn (min)	Pyridine Nucleotides (μmoles/g of Protein) DPNH	DPN+	DPNH: DPN+	TPNH	TPN+	TPNH: TPN+
5	None	5	1.9	3.2	0.6	2.5	0.3	8.4
	None + rotenone	1	1.8	2.6	0.7	2.7	0.3	9.0
		5	2.1	3.5	0.6	2.2	0.2	11.1
6	Succinate	1	2.9	1.1	2.6	1.7	0.2	8.6
		5	2.5	0.9	2.8	2.0	0.2	10.0
	Succinate + rotenone	1	1.6	2.6	0.6	1.8	0.2	9.0
		5	2.7	1.3	2.1	2.9	0.3	9.7
7	Malate + rotenone	1	5.0	1.0	5.0	1.7	0.2	8.4
		5	5.2	1.0	5.2	2.2	0.2	11.0
	Citrate + rotenone	1	5.7	1.4	4.1	3.5	0.4	8.8
		5	5.9	1.1	5.3	3.2	0.3	10.1

[a] Mitochondria (8–10 mg of protein/ml) were incubated for 1 or 5 min at 37°, with additions shown, prior to determination of pyridine nucleotides. Following incubation the reactions were stopped with acid (oxidized forms) or alkali (reduced forms) and pyridine nucleotides measured by the method of Purvis (1960). Each flask contained ATP (5 mM). Substrates were added to a final concentration of 10 mM and rotenone (3 μg/ml; final concentration 7.6 μM) was added where indicated. One preparation of mitochondria was used for each of the three experiments shown in the table. The ratio DPNH:DPN+ at 5 min in controls (no additions) was 0.7 and 0.8 for expt 6 and 7; for malate alone (expt 6), 2.2; and for citrate alone (expt 7), 2.6.

Rotenone inhibited oxidation of DPNH by adrenocortical mitochondria after the organelles had been kept for 1 hr on ice as a pellet or incubated at 37° for 5 min (Figure 1). Freshly prepared mitochondria oxidized DPNH very slowly so that rotenone inhibition was more readily demonstrated when levels of DPNH oxidation were high as in partly denatured mitochondria. The experiment was performed three times with different preparations of mitochondria aged in each case at 0 and 37° as described above. The same effect of rotenone was observed in each experiment.

Influence of Rotenone on Side-Chain Cleavage of Cholesterol. Figure 2 shows that rotenone stimulated the conversion of cholesterol to pregnenolone whether reducing equivalents were generated from endogenous sources, from malate, α-ketoglutarate, or citrate. In order to confirm these findings four additional experiments were performed along the same lines as those shown in Figure 2. Stimulation by rotenone was 2.1 ± 0.3 (std dev) with endogenous oxidizable substrates and by 2.4 ± 0.2 with malate in six experiments (p = <0.01 in each case).

Similar stimulation was observed when the conversion of endogenous and exogenous cholesterol to pregnenolone was measured (Figure 3). Adrenocortical mitochondria may sometimes contain sufficient endogenous cholesterol to saturate

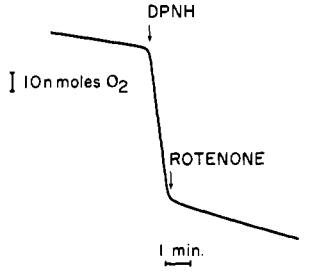

FIGURE 1: Oxygen consumption by mitochondria from bovine adrenal cortex. Mitochondria were prepared as described under Experimental Section and incubated at 37° for 15 min. The amounts of DPNH and rotenone used were 0.6 μmole and 6 μg (7.6 μM), respectively, in a final volume of 2 ml. The incubation chamber contained 0.5 mg of mitochondrial protein. The mitochondrial preparation used in this experiment was the same as that used for expt 3 of Figure 3.

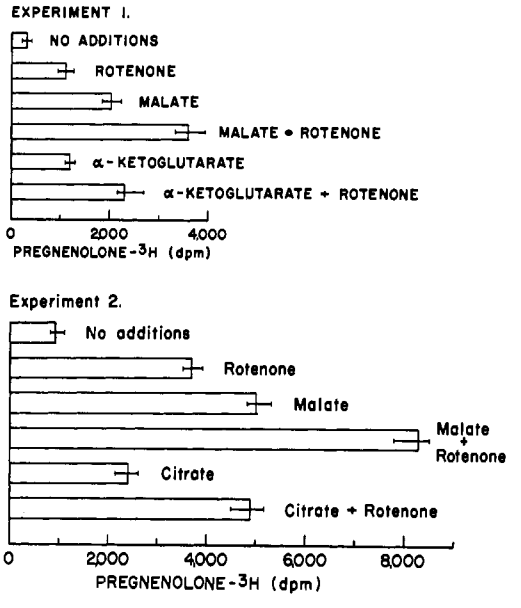

FIGURE 2: Two experiments in which bovine adrenocortical mitochondria were incubated with cholesterol-7α-t and the production of pregnenolone-t was measured. Rotenone (6 μg; final concentration 7.6 μM) was added in 20 μl of ethanol; ethanol was added to flasks not containing rotenone. Malate, α-ketoglutarate, and citrate were added where shown to a final concentration of 10 mM; mitochondrial protein concentration was 3.8 and 4.2 mg per ml for expt 1 and 2, respectively. Final volume was 2 ml. Bars show means and ranges for triplicate determinations.

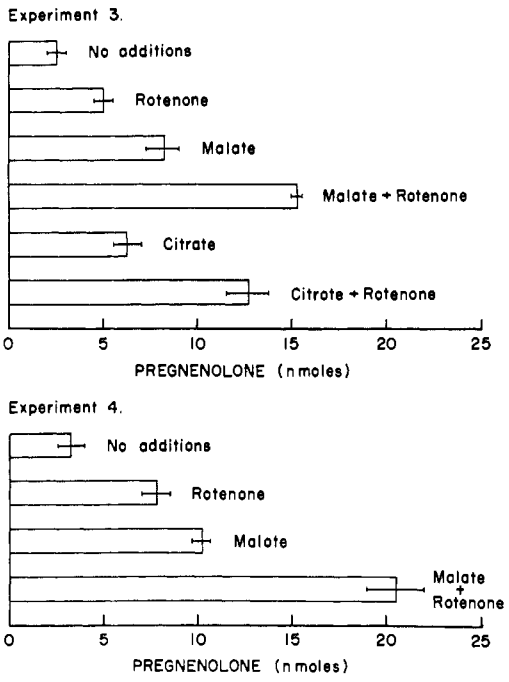

FIGURE 3: Two experiments performed exactly as shown under Figure 2 except that in expt 3 no exogenous substrate was added and in expt 4 cholesterol (final concentration 0.1 m*M*) was added in *N,N*-dimethylformamide; in both experiments the production of pregnenolone was measured by gas–liquid chromatography (Young and Hall, 1969). Mitochondrial protein was 4.3 and 4.9 mg per ml for expt 3 and 4, respectively. Bars show means and ranges for duplicate determinations.

the side-chain cleavage system for short periods of incubation in which case exogenous cholesterol appears to be without influence on this system.

Influence of Rotenone on Redox State of Mitochondrial Pyridine Nucleotides. Table I shows levels of pyridine nucleotides in bovine adrenocortical mitochondria in the presence of ATP and various substrates with and without rotenone. ATP was added in these studies before it was discovered that the influence of rotenone does not require an exogenous source of energy. Succinate, malate, and citrate caused rapid reduction of DPN^+ and this reduction was slower than with succinate alone (Table II). The ratio $TPNH:TPN^+$ was in general higher than the corresponding ratio for DPN^+ and

TABLE II: Influence of Succinate on Mitochondrial DPN^+ [a]

Additions	Duration of Incubn (min)	DPNH:DPN+ Expt 1	DPNH:DPN+ Expt 2
None	1	0.5 ± 0.2	0.6 ± 0.2
	5	0.6 ± 0.2	0.7 ± 0.2
Succinate	1	2.4 ± 0.2	2.6
	5	2.6 ± 0.3	2.3 ± 0.3
Succinate + rotenone	1	0.9	1.0 ± 0.2
	5	2.3 ± 0.2	2.7 ± 0.3

[a] Experiments were performed with two mitochondrial preparations as described under Table I except that ATP was not added to the flasks. Values for DPNH: DPN^+ are means and ranges for duplicate determinations.

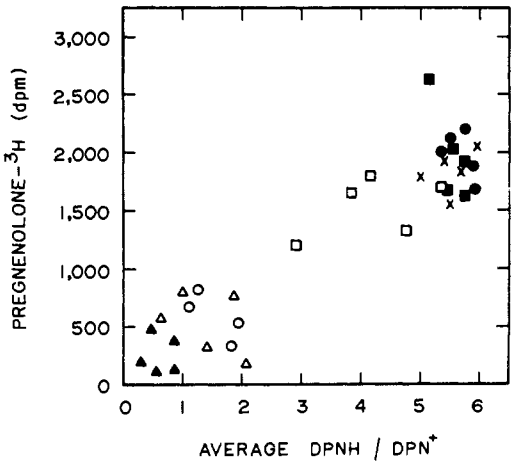

FIGURE 4: Relationship of DPNH:DPN^+ (average of values for zero time and 5 min) to side-chain cleavage of cholesterol with various substrates. Incubation procedure was that shown in Table I. Substrates were present at a final concentration of 10 mM and rotenone, 3 μg/ml (final concentration 7.6 μM). Values on the abscissa represent the average for the ratio DPNH:DPN^+ at zero time and 5 min. Samples of mitochondria from the same preparations as those used to calculate the ratios were incubated with cholesterol-7α-*t* (2 μg; 4.4×10^6 dpm/flask) and pregnenolone-*t* was isolated and measured as described under Experimental Section. (×) α-Ketoglutarate, rotenone; (□) citrate, rotenone; (■) malate, rotenone; (●) succinate; (△) α-ketoglutarate; (○) citrate; (▲) malate.

appeared to be little influenced by addition of substrates (Table I).

In order to explore this relationship more closely, both side-chain cleavage and levels of DPN^+ were measured with a variety of substrates at zero time and after 5-min incubation. Both the measurements of cleavage and of DPN^+ were made on samples of the same preparation of mitochondria incubated simultaneously under the same conditions. Average values for the ratios DPNH:DPN^+ at zero time and after 5-min incubation were plotted against the extent of side-chain cleavage during 5-min incubation (Figure 4). The relationship is approximately linear, the value of the correlation coefficient *r* being 0.82.

Influence of Cyanide upon Side-Chain Cleavage. Cyanide stimulated side-chain cleavage of cholesterol whether the reaction was supported by endogenous sources of reducing equivalents or by addition of succinate (Figure 5). Figure 6 shows similar studies with malate as the source of reducing equivalents; it will be seen that cyanide enhances the stimulation of side-chain cleavage produced by malate. The concentration of cyanide used in these experiments (4 mM) is sufficient

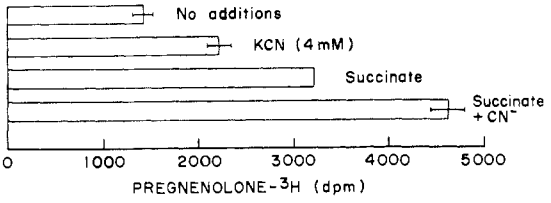

FIGURE 5: The influence of cyanide on side-chain cleavage. Cholesterol-7α-*t* was added to mitochondria and the production of pregnenolone-*t* was measured. Conditions used were those shown under Figure 2. Concentration of mitochondrial protein was 4.2 mg/ml; no oxidizable substrate was used except where shown. Bars show means and ranges for duplicate determinations.

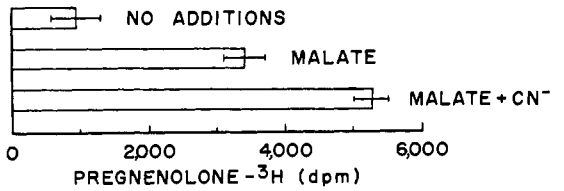

FIGURE 6: This experiment was performed as described under Figure 2. Malate (final concentration 10 mM) was added to each flask except in the case marked "no additions." Bars represent means and ranges for duplicate determinations. The concentration of mitochondrial proteins added was 4.8 mg/ml.

to inhibit respiration by adrenocortical mitochondria completely; similar results were observed with 1 mM cyanide (data not shown).

Influence of Reduced Pyridine Nucleotides upon Side-Chain Cleavage by Treated Mitochondria. Figure 7 shows the influence of reduced pyridine nucleotides upon side-chain cleavage by mitochondria treated in three different ways, namely, partial denaturation at 37° (A), freezing (B), and treatment with Ca^{2+} at concentrations which produce swelling (11 mM) (C) (Guerra *et al.*, 1966). Each treatment was performed with a different mitochondrial preparation. It will be seen that with all three treatments DPNH causes pronounced stimulation of side-chain cleavage. It is interesting to notice that the maximal side-chain cleavage was observed with TPNH at a final concentration of 0.1–0.2 mM and at 1.0–1.5 mM with DPNH. These values are in the general range of those observed by Satoh *et al.* (1966) for a partly purified enzyme system. Maximal conversion with DPNH was less than maximal conversion with TPNH (Figure 7).

The top panel of Figure 7 shows that DPNH was not effective in untreated mitochondria, *i.e.*, mitochondria freshly prepared by the standard procedure (Experimental Section). Similar studies were performed with cholesterol-7α-*t* as substrate; the results were qualitatively similar to those shown in Figure 7 (data not shown). Table III shows the results of experiments in which the influence of DPNH upon side-chain cleavage by partly denatured mitochondria was shown to be enhanced by addition of TPN^+.

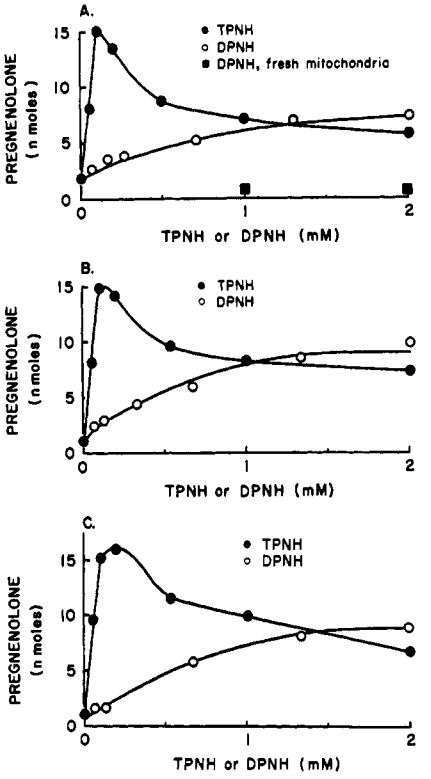

FIGURE 7: Mitochondria were incubated without added cholesterol for 15 min at 37° and side-chain cleavage was measured by gas–liquid chromatography (Young and Hall, 1969). Mitochondria were aged (A) or frozen (B) as described under Experimental Section. Calcium chloride was added to a final concentration of 11 mM (C). Fresh mitochondria (A) refers to a separate batch of mitochondria feshly prepared (according to standard procedure) using the same adrenocortical tissue.

Discussion

The experiments reported here provide evidence for a role for DPNH in generating reducing equivalents for the side-chain cleavage of cholesterol. The evidence is threefold. Firstly, a specific inhibitor of DPNH oxidation, namely rotenone, stimulates side-chain cleavage with endogenous substrates and increases the stimulation produced by DPN^+-linked substrates (Figures 2 and 3). Rotenone, in the concentrations used here, does not inhibit the transport of electrons which is associated with side-chain cleavage. This substance appears to inhibit DPNH dehydrogenase (Ernster *et al.*, 1963; Chance and Hollunger, 1963). Under certain conditions rotenone may also inhibit electron transport at a second site between non-heme iron and cytochrome *c* (Palmer *et al.*, 1968). Presumably the stimulation of side-chain cleavage reported here results from increased levels of DPNH: DPN^+ resulting from inhibition of DPNH oxidation.

The second line of evidence is based upon the demonstration of significant correlation between the ratio DPNH: DPN^+ and the rate of side-chain cleavage in various preparations of mitochondria with a number of different substrates (Figure 4). Rotenone permits rapid reduction of DPN^+ with DPN^+-linked substrates; with succinate a lag period is observed before DPN^+ is reduced, suggesting prior conversion of succinate to some DPN^+-linked substrate, *e.g.*, malate (Table II). A similar suggestion was proposed for the same observation in heart mitochondria (Hull and Whereat, 1967). Thirdly, although the purified enzyme system for side-chain cleavage uses TPNH in preference to DPNH by an order of magnitude (Satoh *et al.*, 1966), DPNH serves as a source of reducing

TABLE III: Influence of Pyridine Nucleotides upon Side-Chain Cleavage in Partly Denatured Mitochondria.[a]

Additions	Expt 1	Expt 2
None	2200	900
DPN^+	1900	700
DPNH	4100	2100
TPN^+	2100	800
$DPN^+ + TPN^+$	2400	1000
DPNH + TPN^+	6800	5300

[a] Mitochondria were partly denatured by incubation at 37° (Experimental Section) and then incubated for 20 min with cholesterol-*t*. Pregnenolone-*t* was isolated and measured. In each case the final concentration of the pyridine nucleotide was 1 mM.

equivalents for this reaction when mitochondria are prepared under conditions which permit DPNH to enter these organelles, *i.e.*, ageing, freezing, and Ca^{2+} (Figure 7). Failure of DPNH to act in freshly prepared mitochondria is presumably due to failure of DPNH to enter the organelles. Moreover, the action of DPNH in accelerating side-chain cleavage was enhanced by addition of TPN^+ (Table III).

Several features of these studies are worthy of mention. Firstly rotenone stimulates side-chain cleavage with endogenous (oxidizable) substrates so that whatever sources of reductive energy are present when the mitochondria are prepared (and hence presumably present *in vivo*), these substrates behave in a manner consistent with a role for transhydrogenation in relation to side-chain cleavage. The findings with exogenous oxidizable substrates, which are consistent with the occurrence of transhydrogenation in connection with side-chain cleavage, cannot therefore be dismissed as nonspecific or indirect responses to unphysiological concentrations of these substrates. Secondly the experiments were performed with endogenous cholesterol alone as substrate, with large concentrations of exogenous cholesterol (Figure 3) and in some cases with trace amounts of cholesterol-7α-*t* (Figure 2). In every case the results are consistent with the hypothesis advanced and cannot be attributed to nonspecific inhibition of electron transport by the massive concentrations of exogenous substrates usually used in such experiments (Sauer, 1970).

If DPNH plays some part in generating reducing equivalents for side-chain cleavage, there would seem to be two ways in which this could occur. DPNH itself may serve as the electron donor for the enzyme reaction or DPNH may act by generating TPNH *via* transhydrogenation. The evidence suggesting that the enzyme system uses TPNH in preference to DPNH is based upon the fact that maximal activity is seen with concentrations of TPNH lower by an order of magnitude than the concentrations of DPNH which produce maximal activity and upon certain calculations of the concentrations of pyridine nucleotides in the adrenal cortex which indicate that insufficient DPNH would be available to the enzyme if the distribution of the pyridine nucleotide is homogeneous throughout the adrenocortical mitochondria (Satoh *et al.*, 1966). If TPNH is the true electron donor, the present studies suggest that some form of transhydrogenation must occur to convert DPNH to TPNH. In mitochondria from heart muscle, two distinct transhydrogenase systems are known; these are distinguished by the fact that only one requires energy (Teixeria da Cruz *et al.*, 1971; Rydström *et al.*, 1971). If transhydrogenase does support side-chain cleavage it must be a system not requiring a source of energy, since activity is seen when rotenone is present.

Since levels of mitochondrial TPNH:TPN^+ remained high and seemed largely uninfluenced by the experimental conditions studied (Table I), it would appear likely that if TPNH is the electron donor for side-chain cleavage, the equilibrium of transhydrogenation is such as to maintain these high ratios, in spite of the fact that TPNH is being used for side-chain cleavage. This would be consistent with the observations of Flint and Denton (1970) that TPNH:TPN^+ is not altered by hormonal stimulation of the corpus luteum which greatly enhances the production of steroids by that tissue.

It is not possible, from data available at this time, to determine whether DPNH exists at high concentration in the vicinity of the side-chain cleavage enzyme and acts as a source of reducing equivalents without the intervention of TPN^+. However it is interesting to notice that not only do maximal rates of side-chain cleavage require higher concentrations of DPNH than TPNH but the maximal rates achieved with DPNH are less than those seen with TPNH (Figure 7). This would make TPNH a more attractive candidate for the role of electron donor *in vivo* and would in turn suggest that DPNH and DPN^+-linked substrates act by a mechanism which involves transhydrogenation. These suggestions would be consistent with previous findings that side-chain cleavage and 11β-hydroxylation compete, under certain conditions, for limiting supplies of TPNH (Young and Hall, 1971) and with the observation that TPN^+ enhances the effect of exogenous DPNH on side-chain cleavage (Table III).

It has been suggested that transhydrogenation may be involved in generating reducing equivalents for another mitochondrial hydroxylation, namely 11β-hydroxylation (Harding and Nelson, 1966; Guerra *et al.*, 1966), but this suggestion has met with disagreement (Simpson and Estabrook, 1969). The present studies point to certain important differences between these two mitochondrial reactions—side-chain cleavage and 11β-hydroxylation. Firstly cyanide, which inhibits 11β-hydroxylation when reducing equivalents are generated by succinate, does not inhibit side-chain cleavage supported by malate or succinate (Figures 5 and 6). Secondly, rotenone does not simulate 11β-hydroxylation. The studies of Simpson and Estabrook (1969) point to a third difference. These authors claim that the production of pyruvate from malate by mitochondrial malic enzyme is sufficient to account for the observed 11β-hydroxylation. Side-chain cleavage from endogenous cholesterol normally proceeds at approximately half the rate of 11β-hydroxylation in such mitochondria (Young and Hall, 1971) and uses at least twice as much TPNH per mole of substrate cleaved (Shimizu *et al.*, 1962). Therefore if the activity of malic enzyme were responsible for the reducing equivalents used for both 11β-hydroxylation and side-chain cleavage, at least twice as much pyruvate would have been produced in the experiments of Simpson and Estabrook (1969). Whether 11β-hydroxylation requires transhydrogenation or not, the same arguments cannot be applied to side-chain cleavage because of these differences between the two reactions.

The studies reported here do not demonstrate the existence of a specific transhydrogenase enzyme, since they are compatible with any form of transhydrogenation, *e.g.*, that resulting from the successive actions of the DPN^+-linked enzyme and a TPN^+-linked enzyme. Moreover it should be realized that more than one mechanism may be responsible for generating TPNH for steroid hydroxylation and that studies *in vitro* may accentuate the relative importance of one or more alternatives at the expense of others. Some reducing equivalents may be provided by reversed electron transport with transhydrogenation, some by malic enzyme and some by other mechanisms. It remains to determine which of these pathways is used *in vivo*, whether all steroid hydroxylations use the same mechanism and whether the source of TPNH is influenced by the physiological state of the adrenal.

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Proteolytic Digestion of Erythrocytes, Resealed Ghosts, and Isolated Membranes†

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ABSTRACT: The organization of the major proteins of the erythrocyte membrane was studied by proteolysis of intact red cells, resealed ghosts, and isolated membranes followed by electrophoretic analysis of membranes from the treated samples using acrylamide gel electrophoresis in sodium dodecyl sulfate. Trypsin digestion of intact human erythrocytes or resealed ghosts cleaves only the glycoprotein of the major membrane proteins. Pronase or chymotryptic digestion results in cleavage of a second component of mol wt 108,000 to yield apparently identical fragments of mol wt 70,000. Treatment of isolated erythrocyte membranes with proteolytic enzymes results in extensive degradation of the membrane proteins. The differences in the digestibility between the membrane of the intact cell and that of the isolated ghost appears to be primarily due to the ability of the protease to penetrate into the ghost, as shown by the permeability of the ghost to inactivated, labeled chymotrypsin. Neither tryptic nor chymotryptic digestion gives any discernible cleavage of the major membrane proteins (exclusive of the glycoproteins) for intact erythrocytes of several species (bovine, dog, sheep, and horse) under the same conditions used for digestion of human cells. Bovine erythrocytes are also quite resistant to Pronase digestion, but cleavage of the 108,000 molecular weight component of dog cells is noted with Pronase.

In a previous study of bovine and human red blood cells by chemical modification and proteolytic digestion we have shown that the membrane glycoprotein(s) is the most readily accessible of the major proteins of the erythrocyte membranes (Carraway *et al.*, 1971). A second protein with a molecular weight near 100,000 was also shown to be present at the cell surface, but less readily accessible. Similar results have been obtained with intact cells using different modification (Bretscher, 1971a; Phillips and Morrison, 1971b) or proteolytic digestion (Bender *et al.*, 1971; Phillips and Morrison, 1971c) methods. However, a different orientation of the membrane proteins has been proposed by Steck *et al.* (1971), based on the proteolytic digestion of right-side-out and inside-out vesicles. A major problem in the interpretation of protease studies arises from the difference in the digestibility of the membrane proteins in the intact cell when compared to the isolated membrane. In the current study we have shown that this difference results from the increased permeability of the ghost to protease. In addition variations in the digestibility of membrane proteins of intact erythrocytes have been shown to be dependent on both the type of protease used and species of animal from which the erythrocytes were obtained.

Experimental Section

Materials. Chemicals for electrophoresis were obtained from Eastman (highest purity grade) or Canalco. PMSF,[1]

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[1] Abbreviations used are: PMSF, phenylmethanesulfonyl fluoride; mosm, milliosmolar (ideal).