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Electron Transport in Relation to Steroid Biosynthesis. Inhibition of Side-Chain Cleavage of Cholesterol by Hyperbaric Oxygen*

Peter F. Hall

ABSTRACT: Mitochondria from rat adrenals convert [^{3}H]cholesterol to [^{3}H]pregnenolone (side-chain cleavage of cholesterol). Side-chain cleavage was stimulated by succinate but not by other Krebs cycle intermediates; stimulation by succinate was prevented by malonate. Amytal inhibited side-chain cleavage in the presence or absence of succinate and neither L-malate plus amytal nor endogenous substrates plus amytal stimulated side-chain cleavage. Measurement of oxidized diphosphopyridine nucleotide (DPN^+) and reduced diphosphopyridine nucleotide (DPNH) in mitochondria from bovine adrenal cortex indicated that succinate reduced DPN^+ to a greater extent than endogenous substrates or endogenous substrates plus other Krebs cycle intermediates. This effect of succinate was prevented by dinitrophenol, amytal, and arsenate. Fluorimetric measurement of the redox state of pyridine nucleotides in adrenal mitochondria demonstrated that hyperbaric oxygen oxidized reduced pyridine nucleotides reversibly, and that succinate with or without adenosine triphosphate (ATP) protected against this effect of hyperbaric oxygen. Hyperbaric oxygen (a specific inhibitor of reversed electron transport: Chance, B., Jamieson, D., and Coles, H. (1965), *Nature 206, 257*) inhibited side-chain cleavage of cholesterol and succinate protected mitochondria against this inhibition. Finally, mitochondrial or submitochondrial systems in which side-chain cleavage requires or is stimulated by exogenous reduced triphosphopyridine nucleotide (TPNH) were not inhibited by hyperbaric oxygen. These observations suggest that electron transport associated with the side-chain cleavage of cholesterol in adrenal mitochondria follows the pathway: succinate → DPN^+ → TPN^+ → cholesterol.

Steroid biosynthesis involves the conversion of cholesterol to pregnenolone by way of two reactions which successively hydroxylate the side chain of cholesterol at C_{20} and C_{22} (Solomon *et al.*, 1956; Shimizu *et al.*, 1962; Constantopoulos *et al.*, 1962). The conversion of cholesterol to pregnenolone (side-chain cleavage of cholesterol) takes place in mitochondria (Halkerston *et al.*, 1961) and includes the rate-limiting step in steroid biosynthesis (Stone and Hechtor, 1954), a step specifically stimulated by the trophic hormones ACTH[1] (Karaboyas and Koritz, 1965) and ICSH (Hall and Koritz, 1965; Hall, 1966).

Like other steroid hydroxylations, the above reactions require TPNH (Halkerston *et al.*, 1961) and a system of electron carriers, one component of which is inhibited by carbon monoxide (Simpson and Boyd, 1966); presumably this component is the cytochrome

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[1] Abbreviations used: ACTH, adrenocorticotropic hormone; ATP, adenosine triphosphate; BSA, bovine serum albumin; ICSH, interstitial cell stimulating hormone; TPN^+ and TPNH, oxidized and reduced triphosphopyridine nucleotides; DPN^+ and DPNH, oxidized and reduced diphosphopyridine nucleotides.

TABLE I: The Influence of TPNH upon the Inhibition of Side-Chain Cleavage of Cholesterol by Mitochondria from Rat Adrenal Glands.[a]

Oxygen Pressure (psi)	Addition	[^{3}H]Pregnenolone (dpm)		
		Expt 20	Expt 21	Expt 22
Atmospheric		8,000	14,200	12,700
Atmospheric	TPN^+ and reducing system	16,000	28,000	25,100
60		2,100	3,000	3,400
60	TPN^+ and reducing system	10,300	17,000	16,400

[a] Rat adrenal mitochondria prepared in KCl (see Experimental Procedure) (1 mg of protein/ml) were incubated with [7α-^{3}H]cholesterol in oxygen at atmospheric pressure or at 60 psi for 15 min; [^{3}H]pregnenolone was isolated and measured. Where indicated TPN^+ (0.5 mM), glucose 6-phosphate (0.1 M), and glucose 6-phosphate dehydrogenase (0.5 Kornberg unit) were added in that order before addition of [7α-^{3}H]cholesterol. Other additions were those described under Experimental Procedure.

P-450 required for other steroid hydroxylations (Omura *et al.*, 1966). It also appears that in adrenal mitochondria reduction of the TPN^+ concerned in hydroxylation of the cholesterol side chain may utilize reversed electron transport from succinate to DPN^+, followed by transhydrogenation of TPN^+ (Koritz, 1966).

It has recently been shown that hyperbaric oxygen specifically inhibits reversed electron transport (Chance *et al.*, 1965). The present studies support the idea that reversed electron transport is involved in side-chain cleavage of cholesterol, by demonstrating that hyperbaric oxygen inhibits the conversion of cholesterol to pregnenolone, by establishing the occurrence of reversed electron transport in adrenocortical mitochondria, and by showing that substances which inhibit reversed electron transport also inhibit side-chain cleavage of cholesterol. Certain of these observations have been presented elsewhere in preliminary form (Hall, 1967).

Experimental Procedure

The Conversion of [^{3}H]Cholesterol to [^{3}H]Pregnenolone by Mitochondria from Rat Adrenals. Female rats (150–200 g) of the Buffalo strain were sacrificed by cervical dislocation and adrenal glands were removed, cleaned, and homogenized in a Potter-Elvehjem homogenizer in 0.001 M EDTA–0.25 M sucrose at pH 7.4. The homogenate was centrifuged at 600*g* for 20 min and the supernatant was centrifuged at 10,000*g* for 20 min. Mitochondria were washed twice with the EDTA–sucrose mixture and sedimented at 10,000*g* for 10 min. The final pellet was suspended in Tris (0.01 M)–sucrose (0.25 M) at pH 7.4 and magnesium chloride (5 mM).

Mitochondria were incubated in 10-ml erlenmeyer flasks at 37° for 15 min (except where stated to the contrary) in a total volume of 2 ml containing a final concentration of 1 mg of mitochondrial protein/ml. Additions were made to flasks in the following order: potassium chloride (0.154 M) to make the final volume 2 ml, potassium phosphate buffer (0.1 M, pH 7.4; 0.5 ml/flask), succinate (final concentration, 10 mM), bovine serum albumin at a final concentration of 1% (w/v) unless otherwise stated, [7α-^{3}H]cholesterol (2 μc:0.06 μg flask), and finally mitochondria. Where indicated substrates other than succinate and inhibitors were added in the concentrations stated. [7α-^{3}H]Cholesterol was added in Tween 80 (Hall, 1966).

Following incubation the reaction was stopped by mixing the contents of each flask with methylene chloride. To each flask pregnenolone (100 μg) was added and [^{3}H]pregnenolone was extracted in methylene chloride and purified by paper chromatography as described previously (Koritz and Hall, 1964). After elution from paper chromatograms, [^{3}H]pregnenolone was purified by thin layer chromatography in methylene chloride–ether (5:2, v/v). Pregnenolone was located by exposing thin layer plates to iodine vapor. [^{3}H]Pregnenolone was eluted and measured by liquid scintillation spectrometry as described in detail elsewhere (Hall, 1966). When flasks containing [^{3}H]cholesterol and other additions were extracted without incubation (zero-time controls) tritium (50–300 dpm) was recovered in the eluates from thin layer chromatography; the value 200 dpm (mean of six determinations) was subtracted from values of [^{3}H]pregnenolone obtained in the following experiments. Incubation with heated enzyme (80° for 10 min) gave values of 100–400 dpm of tritium in the final eluates. Recovery of [^{3}H]pregnenolone through this procedure was 72–80% (12 estimations). Values presented here are not corrected for these losses during isolation of [^{3}H]pregnenolone.

For the experiments reported in Table I mitochondria were prepared in KCl as described by Péron and Koritz (1960) and for expt 17–19 submitochondrial fractions were prepared from bovine adrenocortical mitochondria exactly as described by Omura *et al.* (1966). The following additions were made in the case of submitochondrial preparations to each flask: S_2 (3 mg of protein/ml), P_3 (10 mg of protein/ml), NaCl (0.058 M), KCl (0.047 M), TPN^+ (0.5 mM), glucose 6-phosphate (0.1 mM), glucose-6-phosphate dehydrogenase (0.5 Kornberg unit), $MgCl_2$ (0.0115 M), and Tris buffer

(0.01 M, pH 7.4) to 2 ml. The fractions S_2 and P_3 were prepared from sonicated adrenal mitochondria by high-speed centrifugation; the composition of these fractions is discussed at length by Omura *et al.* (1966).

Incubation of Mitochondria under High Pressure. Incubations were performed by standing erlenmeyer flasks in a steel chamber fitted with a water jacket and a Perspex window. Gas was delivered through jets which entered the top of each erlenmeyer flask. The delivery of gas (air or oxygen) was regulated by a reducing valve and an escape valve so that high pressure could be maintained while gas continued to flow into and out of the chamber. Gas jets were so arranged that the stream of gas dispersed the contents of each flask in a thin layer around the wall of the flask, thereby ensuring rapid equilibration with the gaseous phase. The in-coming gases were passed through water before entering the chamber and a vessel containing water was placed in the chamber. It was observed that during 15-min incubation at high pressure, losses due to evaporation of incubation medium amounted to less than 3% of the original volume.

Fluorescence Spectra of Mitochondria. Measurement of reduction of mitochondrial DPN^+ was performed by means of a fluorimeter as described by Chance *et al.* (1965). Mitochondria were prepared from bovine adrenal cortex as described by Omura *et al.* (1966). Bovine adrenal cortex was used in place of rat adrenals in order to provide a concentration of 10 mg of mitochondria protein/ml in the final sucrose suspension. Mitochondria were examined at room temperature in a chamber in which the capacity was approximately 90 μl. The chamber was provided with two valves in order to permit the contents to be flushed with gas and then brought rapidly to high pressure. The apparatus used is illustrated and described in detail by Chance *et al.* (1965). Oxygen uptake by a sample of each mitochondrial preparation was measured by means of the oxygen electrode. In general P:O ratios of 2 were observed with α-ketoglutarate as substrate.

Mitochondrial suspension (60 μl) was introduced into the chamber and succinate (final concentration 15 mM) and ATP (final concentration 1.5 mM) were added where shown under Results. Fluorescence emission spectra with incident light (366 mμ) and emission measured at 450 mμ were recorded continuously.

Measurement of DPN^+ and DPNH. DPN^+ and DPNH were measured fluorimetrically as described by Purvis (1960). Mitochondria (4–8 mg of protein/ml) were incubated at 0° for 5 min with the substrates and inhibitors shown. Incubation was stopped by means of trichloroacetic acid (DPN^+) or boiling sodium carbonate (DPNH). Thereafter the procedure described by Purvis (1960) was followed. The reliability of this procedure has been demonstrated by Snoswell (1962).

Identity and Radiochemical Purity of [^{3}H]Pregnenolone. Recrystallization of [^{3}H]pregnenolone and determination of specific activities of crystals and mother liquors were performed by methods already published (Kortiz and Hall, 1964). Other procedures used to identify this steroid including preparation of derivatives and countercurrent distribution have also been reported previously (Rosner *et al.*, 1965).

Chemicals. [7α-^{3}H]Cholesterol was obtained from Nuclear-Chicago Corp. (lot no. TRV 122; 11 c/mmole) and was purified before use in these experiments as described previously (Hall and Koritz, 1964). Pregnenolone was purchased from Calbiochem; amytal, L-malate, malonate, and α-ketoglutarate from Sigma Chemical Corp.; and citrate, isocitrate, and pyruvate from British Drug Houses Ltd.

Results

Identity and Radiochemical Purity of [^{3}H]Pregnenolone. Table II shows the results of recrystallizing a sample of [^{3}H]pregnenolone from four different solvent systems after addition of 10 mg of authentic pregnenolone. The [^{3}H]pregnenolone was isolated as described above after incubation of rat adrenal mitochondria with [7α-^{3}H]-cholesterol. It will be seen that the specific activities of crystals and mother liquors remained constant within the limits of experimental error. Moreover, the specific activities of another sample of [^{3}H]pregnenolone isolated as before was submitted to the following procedures in turn: paper chromatography in ligroin–propylene glycol, acetylation followed by paper chromatography in hexane–formamide, deacetylation followed by paper chromatography in ligroin–propylene glycol, 99 transfer countercurrent distribution, oxidation by chromic acid followed by paper chromatography in hexane–formamide, and finally gas chromatography on SE 30 with collection of the effluent for measurement of radioactivity. The specific activities of pregnenolone and its

TABLE II: Recrystallization of [^{3}H]Pregnenolone Isolated Following Incubation of Adrenal Mitochondria with [7α-^{3}H]Cholesterol.[a]

Recrystallization	[^{3}H]Pregnenolone Sp Act. (dpm/mg) Crystals	Mother Liquor
After addition of Pregnenolone	430	
Ligroin–acetone (1st)	440	450
Hexane–chloroform (2nd)	430	420
Pentane–ethyl acetate (3rd)	440	420
Aqueous methanol (4th)	460	430

[a] A sample of [^{3}H]pregnenolone was isolated following incubation of rat adrenal mitochondria with [7α-^{3}H]cholesterol and 10 mg of authentic pregnenolone was added. The mixture was recrystallized from the solvents shown and the specific activities of crystals and mother liquors were determined as described elsewhere (Koritz and Hall, 1964).

TABLE III: Reduction of DPN^+ by Substrates and Inhibitors in Mitochondria from Bovine Adrenal Cortex.[a]

Expt	Substrate	Inhibitor	μmoles/g of Protein			
			DPN^+	DPNH	DPN^+ + DPNH	DPNH: DPN^+ + DPNH
1			7.1	1.3	8.4	0.15
	Malate		6.5	1.3	7.8	0.17
	Pyruvate		6.1	1.0	7.1	0.14
	α-Ketoglutarate		7.3	1.8	9.1	0.20
	Succinate		2.1	4.8	6.9	0.70
2			4.8	0.6	5.4	0.11
	Malate		5.5	0.8	6.3	0.13
	Succinate		2.5	4.4	6.9	0.64
	α-Ketoglutarate		5.7	1.4	7.1	0.19
	Succinate	Malonate	5.3	0.6	5.9	0.10
3			6.0	0.1	6.1	0.20
	Succinate		1.5	6.4	7.9	0.81
	Succinate	Arsenate	5.7	1.5	7.2	0.21
	Succinate + ATP		1.3	4.3	5.6	0.76
	Succinate + ATP	Arsenate	4.8	1.1	5.9	0.19
4			3.9	1.2	5.1	0.24
	Succinate		0.8	6.3	7.1	0.88
	Succinate	Amytal	5.8	1.6	7.4	0.21
	Succinate	Amytal	4.7	1.7	6.4	0.26
	Glutamate		6.5	1.4	7.9	0.19
5			4.9	1.3	6.2	0.21
	Succinate		1.4	5.7	7.1	0.80
	Succinate	DNP	5.8	1.6	7.4	0.22
	Succinate	DNP	5.2	1.6	6.8	0.23
	Glutamate		5.3	1.3	6.6	0.20

[a] Mitochondria from bovine cortex (4–8 mg of protein/ml) were incubated at 0° for 5 min. DPN^+ and DPNH were measured by the method of Purvis (1960). Additions were made to the following final concentrations: succinate (10 mM), malate (40 mM), α-ketoglutarate (40 mM), pyruvate (40 mM), ATP (4 mM), glutamate (40 mM), arsenate (24 mM), DNP (0.5 mM), amytal (5 mM), and malonate (20 mM).

derivatives were determined after each procedure and were found to remain between 690 and 730 dpm/mμmole throughout these procedures. It is concluded that the method of isolating [^{3}H]pregnenolone described above, yields this steriod in radiochemically pure form.

Reversed Electron Transport in Bovine Adrenocortical Mitochondria. Table III shows the results of measurement of DPN^+ and DPNH in mitochondria from bovine adrenal cortex. It will be seen that succinate increased the proportion of DPNH. Malate appeared to cause slight reduction of DPN^+, although insufficient observations have been made to allow statistical analysis. Pyruvate and glutamate were without demonstrable effect upon the proportion of DPNH observed. It would appear that α-ketoglutarate caused a small increase in reduction of DPN^+ (perhaps as the result of some conversion to succinate) and that ATP did not increase the effect observed with succinate. Finally, reduction of DPN^+ by succinate was prevented by malonate, arsenate, amytal, and dinitrophenol (DNP).

Figure 1 shows records of fluorescence emission spectra from a suspension of mitochondria prepared from bovine adrenal cortex. Graph a shows that rapid increase in oxygen pressure from atmospheric pressure to 90 psi was accompanied by a sharp decrease in fluorescence at 450 mμ, indicating increased oxidation of reduced pyridine nucleotides. The effect of hyperbaric oxygen was reversible since return to atmospheric pressure was followed by reduction of pyridine nucleotides and subsequent increase in oxygen pressure to 90 psi again caused oxidation of reduced pyridine nucleotides. Graph b shows that when exposure to hyperbaric oxygen was followed by addition of succinate and ATP with oxygen at atmospheric pressure, reduction of pyridine nucleotides was observed, and the subsequent increase in oxygen pressure to 90 psi caused

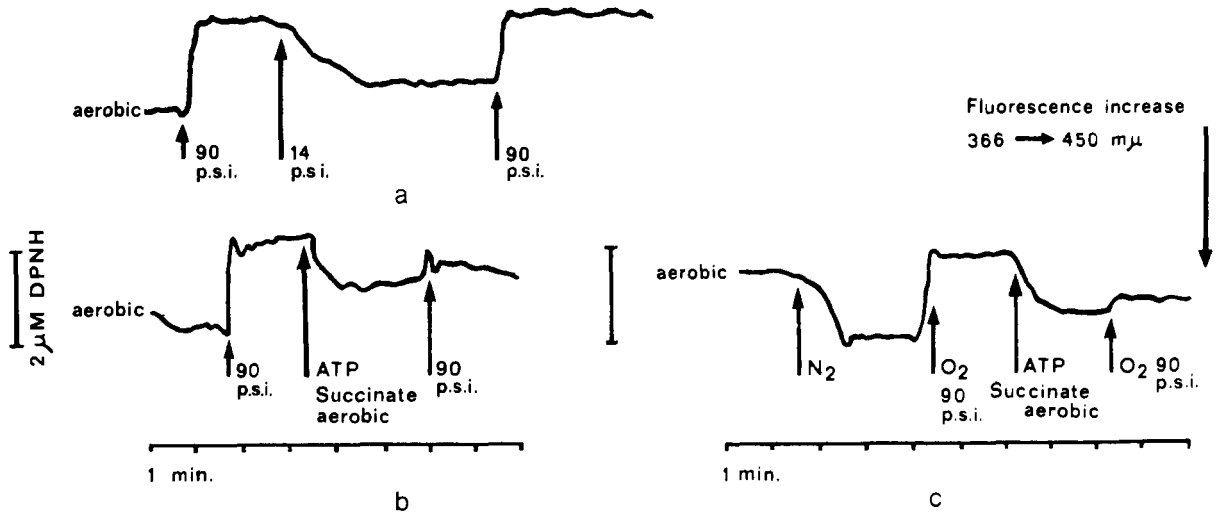


FIGURE 1: Recordings of fluorescence emission spectra of suspensions of bovine adrenocortical mitochondria. Graph a shows oxidation of reduced pyridine nucleotides (decreased fluorescence) by hyperbaric oxygen. In b, ATP (1.5 mM) and succinate (15 mM) were added before oxygen pressure was increased to 90 psi for the second time. Graph c shows the same procedure as that followed in b except that mitochondria were first flushed with nitrogen. The relevant methods are described under Experimental Procedure.

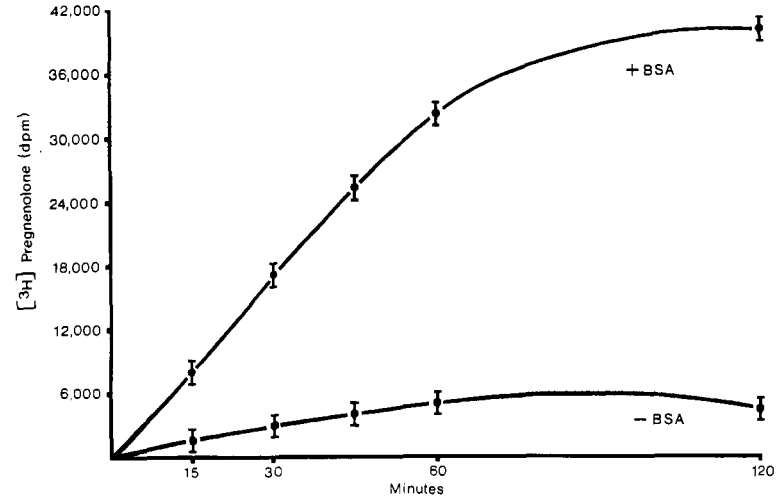


FIGURE 2: Conversion of [7α-^{3}H]cholesterol to [^{3}H]pregnenolone by mitochondria from rat adrenals. Mitochondria (1 mg of protein/ml) were incubated with [7α-^{3}H]cholesterol (2 μc:0.06 μg/flask) for the times shown with and without bovine serum albumin (final concentration 1%, w/v). [^{3}H]Pregnenolone was measured as described under Experimental Procedure.

no more than a small upward deflection of the pen, indicating slight oxidation of pyridine nucleotides.

Graph c of Figure 1 shows that the transition from aerobic to anaerobic conditions (N_2) was accompanied by reduction of pyridine nucleotides. Oxygen at 90 psi again caused oxidation of pyridine nucleotides. As before, the addition of succinate and ATP greatly decreased the oxidation of pyridine nucleotides when the pressure of oxygen was raised to 90 psi. Evidently succinate and ATP cause reduction of pyridine nucleotides in adrenocortical mitochondria as reported for other tissue (Chance and Hollunger, 1960). Oxygen at 90 psi produced oxidation of reduced pyridine nucleotides; succinate and ATP decreased this response to hyperbaric oxygen. It was also observed that succinate alone exerted the same effect but that ATP alone did not prevent the effect of hyperbaric oxygen (data not shown).

Conversion of [7α-^{3}H]Cholesterol to [^{3}H]Pregnenolone by Rat Adrenal Mitochondria. Figure 2 shows that rat adrenal mitochondria convert [7α-^{3}H]cholesterol to

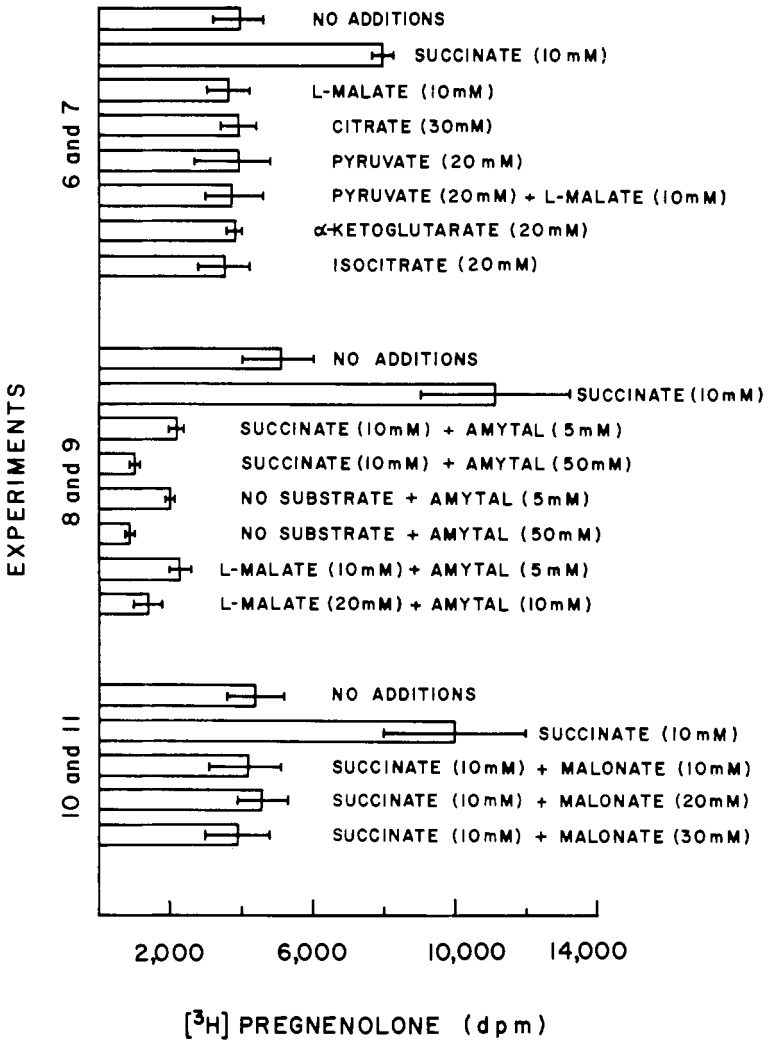


FIGURE 3: The influence of substrates and inhibitors upon the conversion of [7α-^{3}H]cholesterol to [^{3}H]pregnenolone by mitochondria from rat adrenals. Mitochondria (1 mg of protein/ml) were incubated with [7α-^{3}H]cholesterol for 15 min with the substrates and inhibitors shown and [^{3}H]pregnenolone was isolated and measured as described under Experimental Procedure. Each group of bars represents the mean and range of two separate experiments, each experiment involving single determinations for each condition. Each experiment was performed with a different mitochondrial preparation.

[^{3}H]pregnenolone in confirmation of findings reported by Halkerston *et al.* (1961). The rate of this conversion declined after 60 min; subsequent incubations were performed for 15 min. It is clear that bovine serum albumin increased the conversion of [^{3}H]cholesterol to [^{3}H]-pregnenolone in this system (Figure 2); this protein was therefore added at a final concentration of 1% (w/v) in subsequent experiments.

It can be seen from Figure 3 that succinate increased the conversion of [^{3}H]cholesterol to [^{3}H]pregnenolone and that other Krebs cycle intermediates did not increase conversion above levels observed with endogenous substrate. Figure 3 also shows that side-chain cleavage of cholesterol was inhibited by amytal as reported by Koritz (1966) and that succinate did not produce stimulation of side-chain cleavage in the presence of amytal. Moreover, neither endogenous substrates plus amytal nor malate together with amytal stimulated the conversion of [^{3}H]cholesterol to [^{3}H]pregnenolone. Finally, malonate prevented the increase in side-chain cleavage observed in the presence of succinate (Figure 3). It was repeatedly observed that TPN^+ with a reducing system was without demonstrable effect upon side-chain cleavage by mitochondria (data not shown).

Influence of Hyperbaric Oxygen upon Side-Chain Cleavage of [^{3}H]Cholesterol by Mitochondria from Rat Adrenals. The influence of hyperbaric oxygen upon side-chain cleavage of cholesterol is shown in Figure 4 where it will be seen that increasing pressure of oxygen caused increasing inhibition of side-chain cleavage. In six additional experiments with air at 60 psi and oxygen at 60 psi, it was found that oxygen produced significant

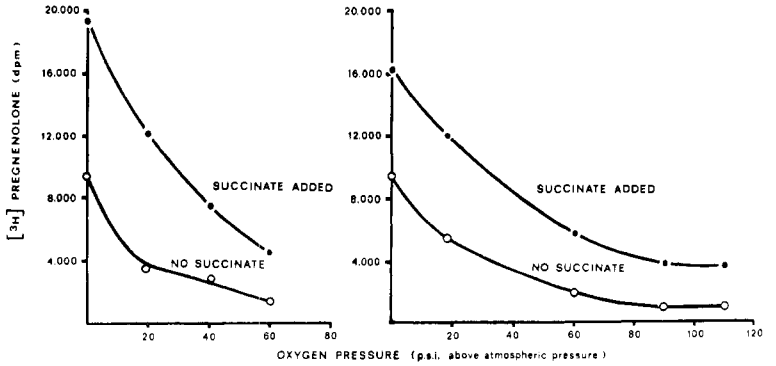


FIGURE 4: The influence of hyperbaric oxygen upon the conversion of [^{3}H]cholesterol to [^{3}H]pregnenolone by rat adrenal mitochondria. Mitochondria (1 mg of protein/ml) were incubated with [7α-^{3}H]cholesterol (2 μc:0.05 μg/flask) for 15 min and [^{3}H]pregnenolone was isolated as described under Experimental Procedure.

inhibition ($p < 0.001$) and that succinate protected against this inhibition, since the ratio of cleavage in air to cleavage in oxygen was significantly greater in the absence than the presence of succinate ($p < 0.01$).

The inhibitory influence of oxygen upon side-chain cleavage of cholesterol is further indicated by the data of Figure 5 where it will be seen that cleavage was greater in air than in oxygen at atmospheric pressure (significant at $p < 0.001$). Moreover, side-chain cleavage in air at 60 psi was less than that in air at atmospheric pressure (significant at $p < 0.01$).

Side-Chain Cleavage of Cholesterol by a Submitochondrial System in Oxygen. A submitochondrial system was prepared by sonication of bovine adrenocortical mitochondria. This system consisted of TPN$^+$ with a reducing system, a fraction with TPNH–cytochrome P-450 reductase activity (S_2) and P-450 (P_3) prepared as described by Omura *et al.* (1966). It can be seen from Figure 5 that hyperbaric oxygen was without demonstrable effect upon side-chain cleavage by this system. It will be shown elsewhere that both components of this system (S_2 and P_3) are necessary for side-chain cleavage of [^{3}H]cholesterol.

Influence of TPNH upon Inhibition of Side-Chain Cleavage of Cholesterol by Mitochondria of Rat Adrenals. Table I shows that when mitochondria were prepared from rat adrenals with 0.154 M KCl in place of sucrose, TPNH produced increased side-chain cleavage of cholesterol, in contrast to the behavior of mitochondria prepared in sucrose (see above). Hyperbaric oxygen inhibited side-chain cleavage in the absence and in the presence of TPN$^+$ with a reducing system. However, percentage inhibition was less in the presence of the nucleotide. In fact cleavage in the presence of hyperbaric oxygen and TPNH exceeded that in hyperbaric oxygen without TPN$^+$ by an amount approximately equal to the additional cleavage produced by TPNH at atmospheric pressure (Table I, line 4 minus line 3 approximately equals line 2 minus line 1), indicating that the additional cleavage produced by TPNH was not inhibited by hyperbaric oxygen.

Discussion

The present data include measurements of the conversion of [7α-^{3}H]cholesterol to [^{3}H]pregnenolone by mitochondria from rat adrenals. Table II demonstrates that the methods used measure radiochemically pure [^{3}H]pregnenolone and Figure 2 shows that conversion of [^{3}H]cholesterol to [^{3}H]pregnenolone in this system is linear with respect to time.

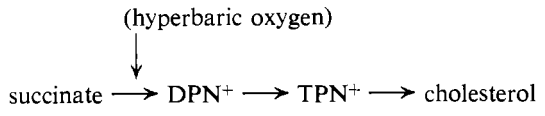
The experiments reported here demonstrate that succinate reduces DPN$^+$ of adrenal mitochondria and that this reduction requires energy since it is inhibited by DNP and by arsenate (Table III). Moreover, reduction of DPN$^+$ by succinate is also inhibited by amytal (Table III). Reduction of DPN$^+$ by succinate is inhibited by malonate, indicating that the effect on DPN$^+$ is not indirect (*e.g.*, by conversion to some other Krebs cycle intermediate) but involves the action of succinic dehydrogenase. These observations are in accord with the view that succinate causes an energy-dependent reversal of electron transport (Chance and Hollunger, 1961). In this respect mitochondria from the adrenal cortex behave like those from rat liver, pigeon heart (Chance and Hollunger, 1961), and rabbit heart (Snoswell, 1962).

Measurements of the state of oxidation of pyridine nucleotides in bovine adrenocortical mitochondria show that hyperbaric oxygen produces rapid but reversible oxidation of reduced pyridine nucleotides and that succinate protects mitochondria from this effect of hyperbaric oxygen (Figure 1). These findings are compatible with conclusions reached by Chance *et al.* (1965), in the cases of mitochondria from liver and heart, namely that hyperbaric oxygen inhibits reversed electron transport.

The influences of various substrates and inhibitors on side-chain cleavage of cholesterol are those expected of a system requiring reversed electron transport (Figure 3). In the first place, succinate stimulates the conversion of cholesterol to pregnenolone by adrenal mitochondria, whereas other Krebs cycle intermediates (citrate, L-malate, isocitrate, α-ketoglutarate, and pyruvate) fail to

stimulate this conversion above levels observed with endogenous substrate. In the second place, stimulation by succinate involves succinic dehydrogenase, since it is prevented by malonate. In the third place, both the conversion of [^{3}H]cholesterol to [^{3}H]pregnenolone and the stimulation by succinate were inhibited by amytal, a substance known to inhibit reversed electron transport (Chance and Hollunger, 1960). In the fourth place, two procedures which would be expected to promote reduction of DPN$^+$ *via* DPN$^+$-linked substrates (namely endogenous substrates plus amytal and L-malate plus amytal) did not accelerate side-chain cleavage of cholesterol.

In keeping with this evidence suggesting that the conversion of cholesterol to pregnenolone utilizes reversed electron transport, are observations of side-chain cleavage in oxygen at high pressure. Hyperbaric oxygen inhibits side-chain cleavage and inhibition can be attributed to increased partial pressure of oxygen (Figures 4 and 5); succinate protects against this inhibition (see Results). Not only does succinate stimulate side-chain cleavage but it protects against inhibition by hyperbaric oxygen. In view of the observations of Chance and coworkers (1965) that hyperbaric oxygen specifically inhibits reversed electron transport and that succinate specifically protects against this inhibition, the present findings strengthen the suggestion by Koritz (1966) that side-chain cleavage of cholesterol utilizes reversed electron transport. If side-chain cleavage of cholesterol utilizes reversed electron transport, the flow of reducing equivalents is presumably as follows.



If this is the case, TPNH should protect side-chain cleavage from inhibition by hyperbaric oxygen, since TPNH is beyond the site of inhibition. Two lines of evidence are presented here to demonstrate such a protective effect of TPNH. In the first place, a submitochondrial system prepared as described by Omura *et al.* (1966) produces side-chain cleavage of cholesterol; this aspect of side-chain cleavage will be reported subsequently. The system of Omura *et al.* (1966) consists of TPN$^+$ and a reducing system, TPNH–cytochrome P-450 reductase, nonheme iron, and cytochrome P-450; all these components (including TPN$^+$ and a reducing system) are necessary for side-chain cleavage. Figure 5 shows that hyperbaric oxygen (60 psi) did not produce detectable inhibition of side-chain cleavage by this system. By contrast it can be seen from Figure 4 that oxygen at 60 psi causes inhibition of cleavage by intact mitochondria.

In the second place, when mitochondria were prepared in 0.154 M KCl, side-chain cleavage was stimulated by addition of TPN$^+$ with a reducing system (Table I). Hyperbaric oxygen inhibited side-chain cleavage by mitochondria prepared in KCl but incubated without TPN$^+$ and a reducing system. In the presence of

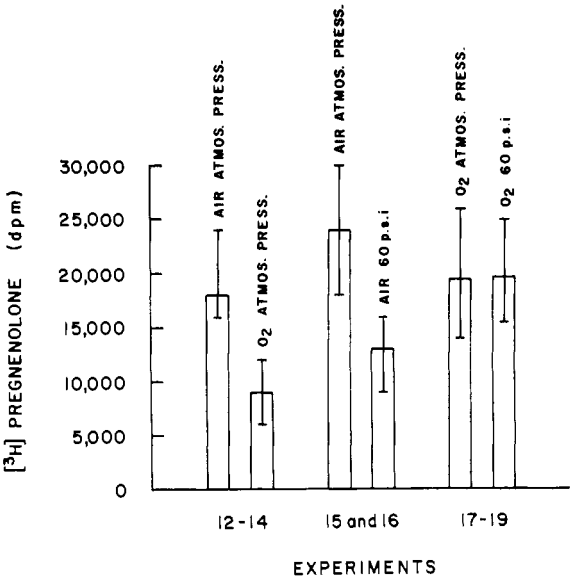


FIGURE 5: The influence of air and oxygen at various pressures upon side-chain cleavage of [7α-^{3}H]cholesterol by mitochondria (expt 6–16) and by a submitochondrial system (expt 17–19). Mitochondria prepared from rat adrenals (1 mg of protein/ml) were incubated with [7α-^{3}H]cholesterol and [^{3}H]pregnenolone was isolated and measured. A submitochondrial system was prepared by sonication of bovine adrenocortical mitochondria exactly as described by Omura and co-workers (1966). This system consisted of the fractions referred to by these authors as S_2 (0.3 ml/flask) and P_3 (0.1 ml/flask) together with additions given under Experimental Procedure. Following incubation for 15 min in oxygen under the pressure shown, [^{3}H]-pregnenolone was isolated and measured. The nature of the fractions S_2 and P_3 is discussed in detail by Omura *et al.* (1966). Before incubation each flask was briskly gassed with the appropriate gas for 1 min. The bars show means and ranges of the experiments shown and in each experiment every condition was measured in duplicate. Each experiment was performed with a separate preparation of mitochondria (expt 6–16) or a separate submitochondrial preparation (expt 17–19).

TPNH, hyperbaric oxygen reduced side-chain cleavage by an amount approximately equal to the activity in the absence of TPNH. It seems clear that the side-chain cleavage stimulated by TPN$^+$ is resistant to hyperbaric oxygen. It should be pointed out here that mitochondria prepared in sucrose do not show increased side-chain cleavage in the presence of TPNH (see Results) presumably because these mitochondria do not allow entry of TPNH and are to this extent more nearly "intact" than mitochondria prepared in KCl.

Although energy-dependent reversal of electron transport is now a well-recognized phenomenon, doubt has been expressed concerning the physiological significance of this effect of succinate upon the reduction of DPN$^+$ (*e.g.*, Krebs, 1965). The studies reported here suggest

the possibility that steroid biosynthesis represents a physiological role for reversed electron transport, although no evidence has been presented to show that succinate can stimulate steroidogenesis in the whole cell. However, it seems likely that the reducing equivalents required for the hydroxylation of cholesterol must be provided in some compartment of the mitochondrion which is protected from the otherwise highly oxidative environment within that organelle.

Acknowledgments

The author is grateful to Mr. H. A. S. van den Brenk of the Cancer Institute Board, Melbourne, for helpful advice and for the use of the pressure chamber and the fluorimeter with which these experiments were performed. The technical assistance of Miss Virginia Gates and valuable discussions with Dr. Alan Snoswell of the School of Vetinary Science, University of Melbourne, are gratefully acknowledged.

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