INHIBITION BY HYPERBARIC OXYGEN OF THE CONVERSION OF CHOLESTEROL TO PREGNENOLONE IN ADRENAL MITOCHONDRIA

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It is now clear that steroid biosynthesis involves the conversion of cholesterol to pregnenolone via two hydroxylated intermediates, namely: 20α-hydroxycholesterol and 20α,22ξ-dihydroxy-cholesterol (Solomon et al., 1956; Shimizu et al., 1962; Constantopoulous et al., 1962). The importance of these reactions lies in the fact that the conversion of cholesterol to pregnenolone appears to be rate-limiting for steroid biosynthesis (Stone and Hechter, 1954) and is specifically stimulated by the trophic hormones ICSH* (Hall and Eik-Nes, 1964) and ACTH (Karaboyas and Koritz, 1965)

Like other steroid hydroxylations the above reactions require reduced TPN (Halkerston et al., 1961). Moreover side-chain cleavage of cholesterol (cholesterol→pregnenolone) by adrenal mitochondria appears to require a special electron carrier which is inhibited by carbon monoxide (Simpson and Boyd, 1966). Finally, it has been suggested (Koritz, 1966) that hydroxylation of the side-chain of cholesterol, requires the transport of electrons from succinate to DPN,

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* Abbreviations used: ACTH : Adrenocorticotrophic Hormone, ICSH : Interstitial cell-stimulating hormone (also known as luteinizing hormone); Pregnenolone : 3β-hydroxypregn-5-en-20-one; p.s.i.:Pounds per square inch.

followed by transhydrogenation of TPN - the so called reverse electron transport or energy-linked reduction of pyridine nucleotides by succinate (Chance and Hollunger, 1960).

It has recently been shown that hyperbaric oxygen specifically inhibits reverse electron transport (Chance et al., 1965). The present studies support the requirement for reverse electron transport during side-chain cleavage of cholesterol by demonstrating that hyperbaric oxygen inhibits the conversion of cholesterol to pregnenolone by adrenal mitochondria.

Experimental Procedure

Rat adrenals were homogenised by means of a Dounce homogeniser and mitochondria were prepared either in 0.154 M potassium chloride as described by Peron and Koritz (1960) or in 0.25 M sucrose as described by Koritz (1966); the mitochondria were resuspended in potassium chloride or sucrose respectively, at a concentration of 1 mg protein per ml. Incubation was performed in 10 ml Erlenmyer flasks at 37° for 15 minutes. Additions were made to the flasks in the following order: potassium chloride, phosphate buffer (0.1 M pH 7.4), succinate (final concentration 10 mM) bovine serum albumin (final concentration 1%), cholesterol-7α-^{3}H (5 μc : 0.2 μg per flask unless otherwise stated) and finally mitochondria. Cholesterol-^{3}H was added in Tween-80 (Hall, 1966). Volume of incubation was 2 ml and final pH 7.4.

Following incubation the reaction was stopped by addition of methylene chloride and pregnenolone 100 μg was added to the contents of each flask. Pregnenolone-^{3}H was extracted by means of methylene chloride and purified by paper chromatography as described elsewhere (Koritz and Hall, 1964). After elution from paper chromatograms, pregnenolone-^{3}H was purified by thin layer chromatography in the system methylene chloride/ether (5:2, v/v). Pregnenolone was located by exposing the plates to iodine vapour and was eluted and measured by liquid

scintillation spectrometry (Hall, 1966). When flasks containing cholesterol-^{3}H and other additions were extracted immediately after addition of mitochondria (zero time controls) 50 - 300 dpm of tritium was recovered in the eluates from thin layer chromatograms; the value of 200 dpm (mean of six determinations) was subtracted from all values obtained by the incubation procedure described above.

Incubation under pressure was performed in a steel chamber fitted with a water-jacket. Gas was delivered through jets which entered individual flasks. The delivery of gas was regulated by a reducing-valve in such a way that the stream of gas caused the contents of each flask to be distributed as a thin layer around the walls of the flask, thereby insuring rapid equilibration with the gaseous phase.

Cholesterol-7α-^{3}H was obtained from Nuclear Chicago (Lot No. TRK122, 11 C/m mole) and was purified before use in these experiments by paper chromatography as described previously (Hall and Koritz, 1964).

Results and Discussion

Table I shows the results of recrystallizing pregnenolone-^{3}H after addition of authentic pregnenolone. This sample of pregnenolone-^{3}H was isolated after incubating adrenal mitochondria with cholesterol-7α-^{3}H as described above. The specific activity remained constant through four recrystallizations from different solvent systems thereby establishing the identity and radiochemical purity of the compound measured in the following experiments.

Figure 1 shows the result of five experiments in which the influence of hyperbaric oxygen (60 p.s.i.) upon the conversion of cholesterol to pregnenolone was examined. It will be seen that in each experiment hyperbaric oxygen inhibited the side-chain cleavage of cholesterol. Succinate enhanced side-chain cleavage in confirmation of the findings of Koritz (1966). Moreover succinate appeared to

Table I

Recrystallization of Pregnenolone-^{3}H

Recrystallization	Solvent	Specific Activity (dpm/mg) Crystals	Mother Liquors
After addition of carrier	-	780	-
1st	Aqueous methanol	770	790
2nd	Ethyl acetate/ ligroin	780	770
3rd	Hexane/benzene	790	780
4th	Ligroin/acetone	780	770

Pregnenolone-^{3}H was isolated as described under Experimental Procedure and authentic pregnenolone (20 mg) was added. Crystals were allowed to form from the solvents shown and specific activities of crystals and mother liquors were determined after each crystallization by methods described elsewhere (Koritz and Hall, 1963).

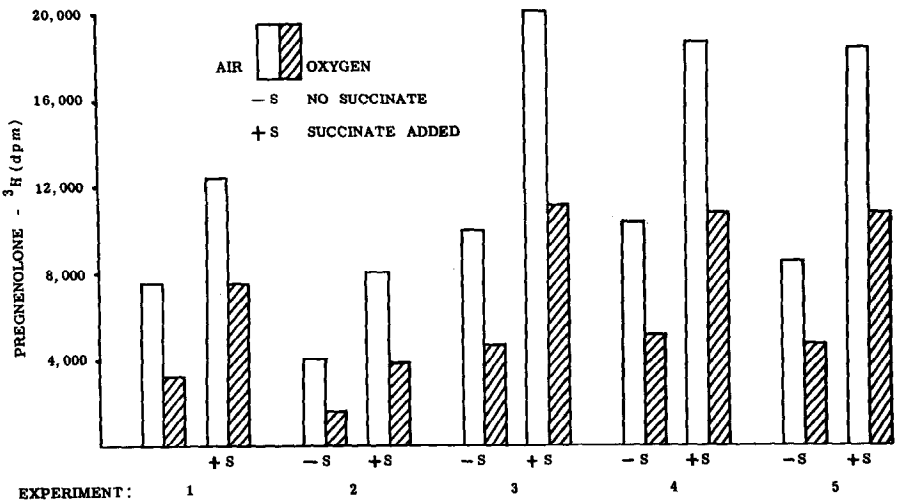

Figure 1. The influence of hyperbaric oxygen (60 p.s.i.) upon the conversion of cholesterol-^{3}H by mitochondria from rat adrenal incubated with and without succinate (10 mM). Experiments 1 and 2 cholesterol-7α-^{3}H 2 μc : 0.08 μg per flask was used, while in Experiments 3-5, 5 μc : 0.2 μg per flask was used.

decrease the extent of inhibition by hyperbaric oxygen since the ratio of pregnenolone-^{3}H formed in air to that formed in hyperbaric Oxygen is significantly greater in the absence of succinate than in the presence of succinate ($p<0.05$).

It should be pointed out that the control flasks in this experiment (i.e. those incubated in air) were subjected to air at the same pressure (60 p.s.i.) as that in the samples exposed to hyperbaric oxygen. Figure 2 shows the inhibition of the conversion of cholesterol to pregnenolone as a function of oxygen pressure. It is seen that increasing pressure produces increasing inhibition up to approximately 60 p.s.i. and that (as observed in Figure 1) succinate protects mitochondria to some extent from this inhibition. Finally, it is clear from Table II that oxygen at atmospheric pressure causes pronounced inhibition of the conversion of cholesterol to pregnenolone ($p< 0.001$). Similar experiments in which mitochondria were prepared in sucrose (0.25 M) gave similar results.

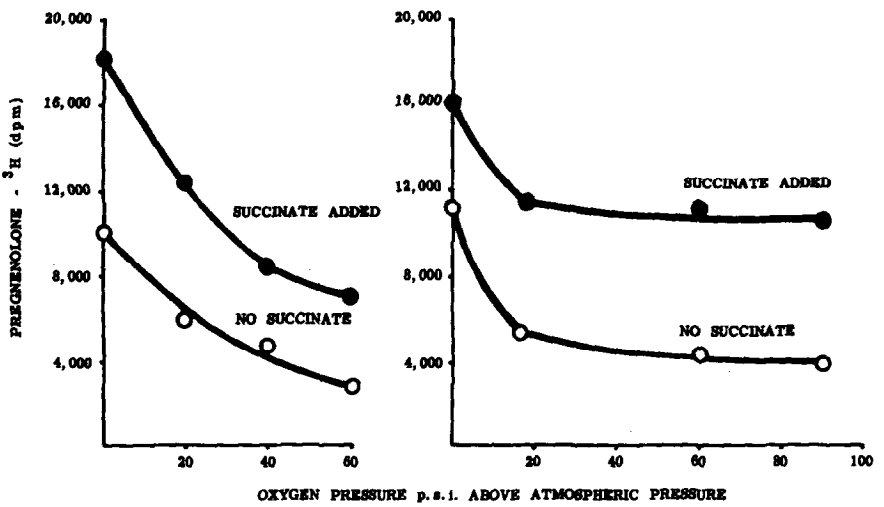

Figure 2. The influence of oxygen upon side-chain cleavage of cholesterol as a function of oxygen pressure. In two separate experiments, mitochondria were incubated with and without succinate at the pressures shown.

Table II

Incubation of Adrenal Mitochondria in Air or Oxygen

Experiment	Gas	Pregnenolone-^{3}H (dpm)	% Inhibition
8	Air	40,000	
	Air	38,900	
	100% Oxygen	26,000	34
	100% Oxygen	26,600	
9	Air	36,400	
	Air	35,800	
	100% Oxygen	19,000	50
	100% Oxygen	19,200	
10	Air	29,000	
	Air	27,000	
	100% Oxygen	17,000	47
	100% Oxygen	18,000	

Adrenal mitochondria were prepared in potassium chloride and incubated with cholesterol-^{3}H (5 μc : 0.2 μg per flask) as described under Experimental Procedure. Incubation was performed in an atmosphere of air or 100% oxygen at atmospheric pressure and following incubation at 37° for 15 minutes pregnenolone-^{3}H was isolated and measured.

Chance and coworkers (1965) have presented evidence to show that hyperbaric oxygen causes selective inhibition of reverse electron transport and does not inhibit either the usual "forward" flow of electrons or the energy-transfer reactions associated with it. The present observation that hyperbaric oxygen inhibits the conversion of cholesterol to pregnenolone is in keeping with a recent suggestion that the rate-limiting step in steroid biosynthesis requires reverse electron transport to generate the reduced TPN required for hydroxylation of the cholesterol side-chain (Koritz, 1966). Further

support for the flow of electrons: Succinate → DPN → TPN in relation to the side-chain cleavage, is to be found in the protective effect of succinate which provides electrons and high-energy compounds to maintain pyridine nucleotides in a state of reduction, in competition with hyperbaric oxygen which promotes oxidation of pyridine nucleotides (Chance et al., 1965).

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