THE CHOLESTEROL SIDE-CHAIN CLEAVAGE SYSTEM OF THE ADRENAL CORTEX: A MIXED-FUNCTION OXIDASE

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In the adrenal cortex, cholesterol is metabolized to the corticosteroid hormones. The first step in this process is the cleavage of the cholesterol side-chain to form pregnenolone, proposed intermediates being 20α-hydroxycholesterol (Shimizu, Hayano, Gut and Dorfman, 1961; Constantopoulos and Tchen, 1961) and 20α, 22-dihydroxycholesterol (Shimizu, Gut and Dorfman, 1962; Constantopoulos, Satoh and Tchen, 1962). The enzyme system catalysing the cholesterol side-chain cleavage occurs in adrenal cortex mitochondria and has a requirement for molecular oxygen and reduced NADP (Halkerston, Eichhorn and Hechter, 1961); thus it has the characteristics of a mixed-function oxidase (Mason, 1957).

Studies on two other mixed-function oxidases of the adrenal cortex namely the steroid 11β- and 21-hydroxylases have shown these systems to be inhibited by carbon monoxide, this inhibition being correlated with the appearance of an

absorption maximum at 450 mμ. in the reduced difference spectrum (Estabrook, Cooper and Rosenthal, 1963; Harding, Wilson, Wong and Nelson, 1965). This carbon monoxide-binding pigment, a haemoprotein, is believed responsible for oxygen activation in these hydroxylation reactions, and has been designated 'P-450' (Omura and Sato, 1962).

In order to see if a similar situation applies in the case of the cholesterol side-chain cleavage system, the effects of carbon monoxide on this system were investigated. The evidence presented in this paper suggests that 'P-450' is indeed a component of the cholesterol side-chain cleavage system.

Methods

Bovine adrenal glands were obtained fresh from slaughter. The cortices were removed, chopped finely, and homogenised in 3 volumes of ice-cold 0.25M sucrose using an all-glass homogeniser. The mitochondria were isolated in the usual way, and washed twice with 0.154M KCl. The washed mitochondrial pellet was suspended in distilled water and sonicated as suggested by Cooper, Narasimhulu, Slade, Raich, Foroff and Rosenthal (1965) for 15 min. at 0° C with an M.S.E. Mullard Ultrasonic Disintegrator operating at 20 Kc./sec. Centrifugation of the sonicate at 105,000 x g for 30 min. gave an opalescent supernatant containing the enzymic activity. This activity was assayed by incubating cholesterol-4-C^{14}, purchased from the Radiochemical Centre, Amersham, and purified by thin-layer chromatography immediately before use, with the sonicate in the presence of an NADPH-generating system

buffered at pH 7.4 with phosphate (Table 1). Incubations were performed at 37° C for 15 min. in optical cuvettes modified to permit the bubbling of appropriate gas mixtures through the solutions. The reactions were stopped with methanol and the extracted sterols separated by thin-layer chromatography on silica gel H (Fig. 1). Analysis of the products was achieved by means of a gas-flow thin-layer radioactive scanner, and liquid

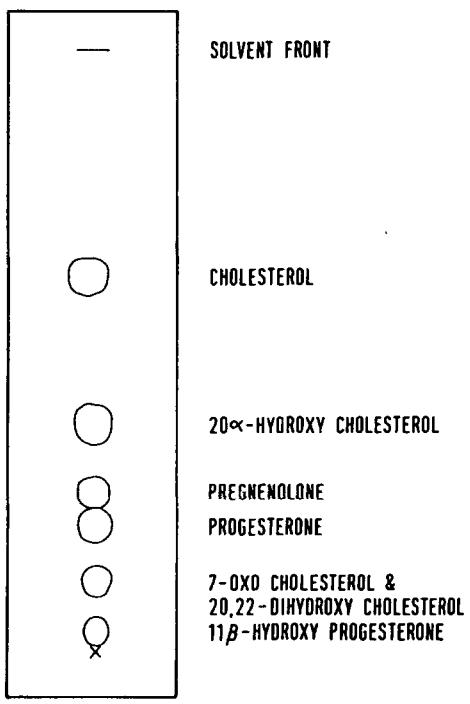

Fig. 1. Thin-layer plate showing separation of typical sterols. The plates were run in the solvent system petroleum ether: diethyl ether:acetic acid 75:25:2 until the solvent front was three-quarters of the plate length from the origin. The plates were then re-run in the solvent system petroleum ether:diethyl ether: acetic acid 65:35:2 until the solvent front reached the top of the plate.

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scintillation spectrometry using a Packard Tri-Carb 314 EX.

Optical measurements were performed in an 'Optica'

CF4DR double-beam recording spectrophotometer at room temperature using the modified optical cells. Fig. 2 shows the reduced difference spectrum of the enzyme preparation in the presence of carbon monoxide. The 420 mμ. absorption may be due in part to contaminating haemoglobin which it was not possible to remove from these preparations. The 450 mμ. chromophore is characteristic of the 'P-450' pigment.

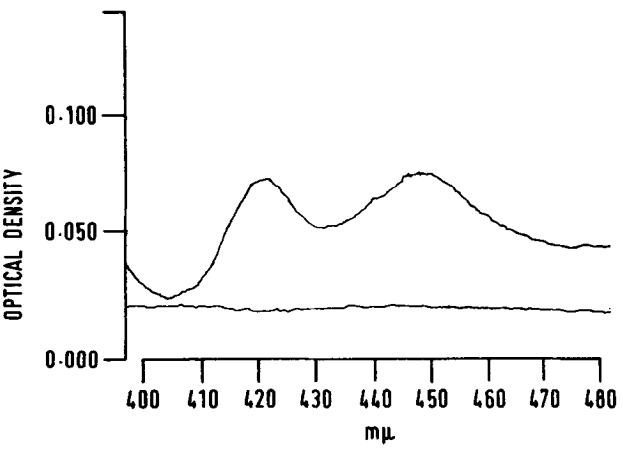

Fig. 2. Reduced difference spectrum. For the base line, each cell contained the incubation mixture shown in Table 1 but without the cholesterol-4-C^{14}. When carbon monoxide was bubbled through the experimental cell, the characteristic difference spectrum appeared.

Results and Discussion

In the presence of 10% oxygen, increasing the percentage carbon monoxide in the gas mixture produced a rapid increase in the size of the 450 mμ. absorption band. Maximum peak size was attained at 40% carbon monoxide.

When the side-chain cleavage activity was assayed in the presence of 10% oxygen and increasing proportions of carbon monoxide, the results shown on Fig. 3 were obtained. In the radioactive assays the peak on the left is the added cholesterol

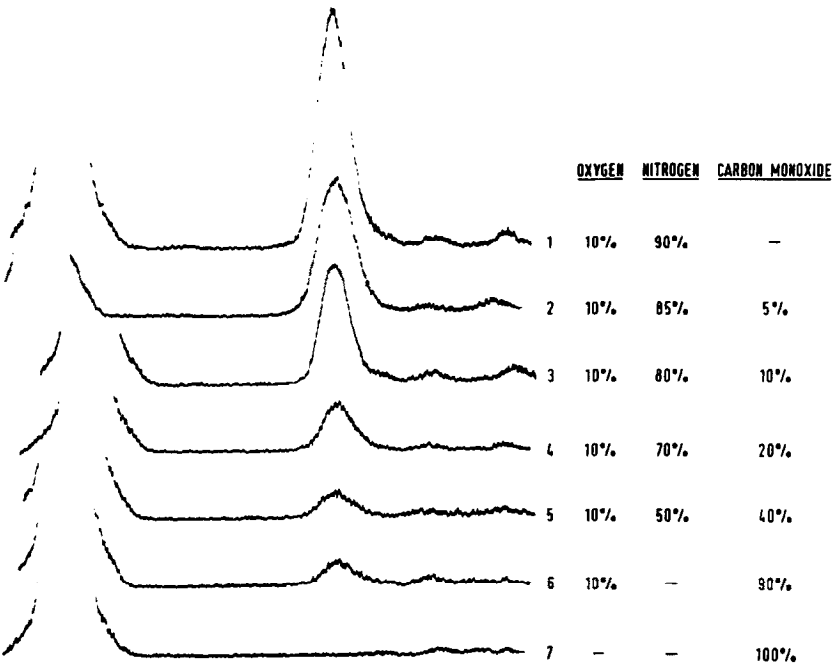

Fig. 3. Radioactive scanner traces of the thin-layer plates under the conditions shown in the accompanying Table. The peak on the left is the added cholesterol substrate and the peak in the centre the major product, pregnenolone.

substrate, and the major product is pregnenolone. There is a trace of progesterone and some 11β-hydroxyprogesterone. Some 7-oxocholesterol (formed autoxidatively) is also present. No labelled 20α-hydroxycholesterol or 20α,22-dihydroxycholesterol have been identified in these incubations. The pregnenolone peak represents a conversion of some 15% of the added radioactive cholesterol when the gas phase is 10% oxygen and 90% nitrogen, under the conditions given.

Fig. 4 shows a graphical presentation of the results of Fig. 3 together with the variation in size of the 450 mμ.

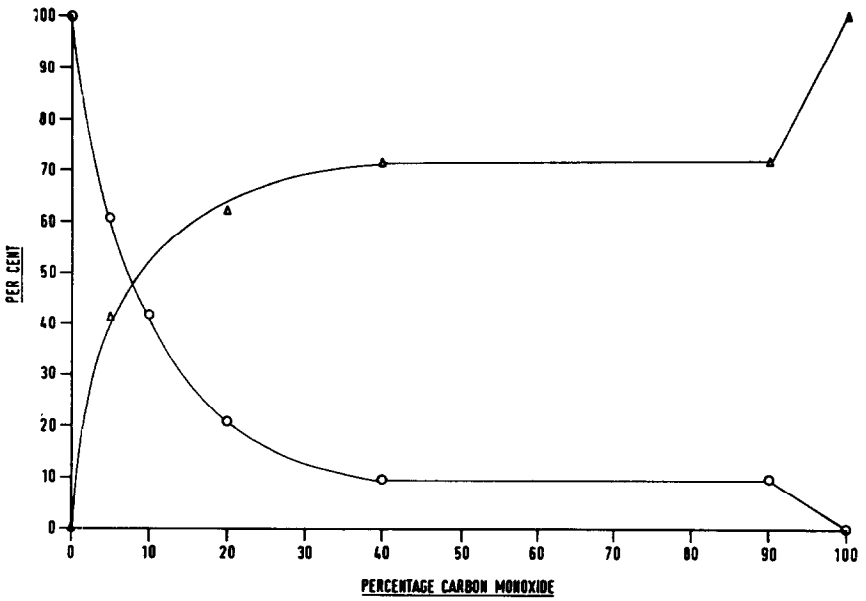

Fig. 4. O - Enzymic activity (percentage of that occurring in the absence of CO).

Δ - Height of 450 mμ. peak (percentage of that obtained in 100% CO).

Graph of enzymic activity and height of 450 mμ. chromophore versus percentage carbon monoxide. The oxygen content was constant at 10% in all studies except when the gas phase was 100% carbon monoxide.

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absorption band with increasing carbon monoxide content. As the 'P-450' peak increases in size, there is a corresponding decrease in the side-chain cleavage activity. With 10% oxygen in the gas phase, maximum inhibition and maximum size of the 'P-450' peak were attained at 40% carbon monoxide content. With 100% carbon monoxide there is complete inhibition of side-chain cleavage activity and a further increase in size of the 'P-450' peak. This implies that the carbon monoxide binding, which gives rise to the 450 mμ. chromophore, is responsible

for the inhibition of the side-chain cleavage activity. Thus 'P-450' is implicated as a component of the cholesterol side-chain cleavage system. These results are quite similar to those obtained by Harding et al. (1965) for the steroid 11β-hydroxylase.

Table 1

Incubation Mixture

SONICATE SUPERNATANT	15 mg. protein/ml.	1 ml.
PHOSPHATE BUFFER	0.1M pH 7.4	1.2 ml.
MAGNESIUM SULPHATE	200 μ moles/ml.	0.125 ml.
NADP (in distilled water)	37.5 mg./ml.	0.05 ml.
G-6-P (in distilled water)	125 mg./ml.	0.05 ml.
G-6-P DEHYDROGENASE	1 unit/0.1 ml.	0.05 ml.
CHOLESTEROL-4-C^{14}	0.25 μ C in 0.05 ml. ACETONE	0.05 ml.

This 'P-450' pigment although accepted as being responsible for oxygen activation in several steroid mixed-function oxidases has not yet been isolated in a pure form. It is believed to be a cytochrome, and in keeping with other cytochrome systems responsible for oxygen activation, it would be expected to lie at the end of an electron transport pathway. That this is so in the case of the steroid 11β-hydroxylase has been shown by Omura and co-workers (Omura et al. 1965). In this, electrons pass from the reduced pyridine nucleotide

to the 'P-450' reaction centre via an electron transport pathway consisting of flavoprotein and a non-haem iron protein. We tentatively suggest a similar scheme for the cholesterol side-chain cleavage system.

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