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ELECTRON PARAMAGNETIC RESONANCE SPECTRA OF MITOCHONDRIAL AND MICROSOMAL CYTOCHROME *P*-450 FROM THE RAT ADRENAL

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SUMMARY

The electron paramagnetic resonance (EPR) spectra of rat adrenal zona fasciculata mitochondria showed peaks corresponding to low spin ferric cytochrome *P*-450 with apparent g values of 2.424, 2.248 and 1.917, and weak signals due to high spin ferric cytochrome *P*-450 with g_x values of 8.08 and 7.80. The former is attributed to cholesterol side chain cleavage cytochrome *P*-450, the latter to 11β-hydroxylase cytochrome *P*-450. On addition of deoxycorticosterone the $g = 7.80$ signal was elevated and there was an associated drop in the low spin signal. As the pH was reduced from 7.4 to 6.1, the $g = 8.08$ signal increased with again a drop in intensity of the low spin signal. Mitochondria from the zona glomerulosa showed similar spectral properties to those described above. Addition of succinate, isocitrate or pregnenolone caused a loss of the $g = 8.08$ signal. Addition of calcium increased the magnitude of the $g = 8.08$ signal, and caused a slight reduction in the magnitude of the low spin signal. Also, addition of deoxycorticosterone, pregnenolone, succinate or isocitrate caused slight shifts of the outer lines of the low spin spectrum. Interaction of mitochondrial cytochrome *P*-450 with metyrapone and aminoglutethimide modified the low spin parameters. Adrenal microsomal cytochrome *P*-450 had low spin ferric g values of 2.417, 2.244 and 1.919 and high spin ferric g_{xy} values of 7.90 and 3.85, distinct from the values obtained with mitochondria.

INTRODUCTION

EPR spectroscopy has played an important role in elucidating the operative mechanism of cytochrome *P*-450. In particular, it has been possible to show that the type 1 optical binding spectrum corresponds to a low to high spin transition of ferric haem iron and that one of the ligands of the iron atom is probably bound via a sulphur

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atom. Also it has been possible to distinguish between different forms of cytochrome *P*-450 by differences in EPR parameters, differences which are not apparent from optical spectroscopy [1].

Low spin ferric haem iron has one unpaired electron, and such haem proteins show characteristic EPR spectra in which the three principal g values are not equal, and typically lie between $g = 1.5$ and 3.5. These low spin characteristics have found more rigorous expression in the analytical procedure first applied to haem proteins by Griffiths [2] and then extended by Blumberg and Peisach [3]. The particular values obtained are determined by the nature of the fifth and sixth ligands of the haem iron and different haemoproteins can be distinguished in this way. The separation between the outer lines, $g = 2.41$, $g = 1.92$, is relatively small for cytochrome *P*-450. Similar parameters have also been obtained from model haem-thiol compounds [4]. High spin ferric haem proteins ($S = \frac{5}{2}$) show a far wider separation between g values, and in some cases, e.g. methaemoglobin, show close to axial (tetragonal) symmetry, with effective g values: g_x, $g_y = 6$; $g_z = 2$. More generally, separations of g_x, g_y around a value of $g = 6$ are observed. This is due to rhombic distortions. Cytochrome *P*-450 has the largest known rhombic distortion for an active haem protein iron centre under physiological conditions, $g = 8, 4, 2$.

During its operative cycle the haem iron of cytochrome *P*-450 undergoes both spin state and oxidation state changes. Substrate-free cytochrome *P*-450 is low spin ferric; on binding substrate it becomes high spin ferric. It then undergoes one electron reduction, binds oxygen, and following a further one electron reduction the substrate is oxidised and low spin ferric cytochrome *P*-450 is regenerated. Only in the low and high spin ferric form is the haem iron EPR active at X-band (≈ 9 GHz).

Mitochondria from the rat adrenal contain cytochrome *P*-450 systems capable of side-chain cleavage of cholesterol ($P\text{-}450_{scc}$), 11β-hydroxylation ($P\text{-}450_{11\beta}$) and 18-hydroxylation. Adrenal microsomes contain cytochrome *P*-450 involved in 21-hydroxylation. Optical spectra of these different forms of cytochrome *P*-450 fail to distinguish between them in terms of absorption peak wavelengths, but substrate specificity can be demonstrated by the additive behaviour of spectral changes on addition of pregnenolone, cholesterol and deoxycorticosterone to intact and acetone-extracted mitochondria [5].

Low spin cytochrome *P*-450 from bovine adrenal mitochondrial preparations has been examined by Mitani and Horie [6], Harding et al. [5, 7] and by Schleyer et al. [8]. These results differ in one important aspect, namely that the first two sets of authors find that addition of deoxycorticosterone results in a reduction of intensity, whereas the latter authors did not detect such an effect.

High spin cytochrome *P*-450 from bovine adrenal mitochondria has been examined by Jefcoate et al. [1]. These workers found a large signal at $g = 8.1$ which was titrated out by adding 20 α-hydroxycholesterol, and which they attributed to cytochrome $P\text{-}450_{scc}$ bound to endogenous cholesterol. Subsequent addition of deoxycorticosterone gave rise to a new signal at $g = 7.9$ which was attributed to cytochrome $P\text{-}450_{11\beta}$ bound to this substrate.

The purpose of this communication is to determine the EPR properties of low and high spin ferric cytochrome *P*-450 from subcellular fractions of rat adrenal zones, in order to provide a basis for examining the effect of trophic stimuli on the behaviour of cytochrome *P*-450 in the rat adrenal (see accompanying paper).

MATERIAL AND METHODS

Female rats of the Sprague-Dawley strain weighing around 150–250g were used throughout. The rats were killed by cervical dislocation and the adrenals removed and placed in ice-cold 0.25 M sucrose. The adrenals were trimmed free of adhering fat and decapsulated according to the method of Tait et al. [9]. The decapsulated adrenals (zona fasciculata-reticularis) and adrenal capsules (mainly zona glomerulosa) were homogenised with three passes of a Teflon-glass homogeniser in icecold 0.25 M sucrose containing 10 mM triethanolamine hydrochloride, 100 μM ethylenediamine tetracetic acid and 1 % bovine serum albumin, pH 7.1. The ratio of homogenising medium to tissue was 1 ml per rat equivalent in the case of decapsulated adrenals and 0.2 ml per rat equivalent in the case of capsular mitochondria. The contamination of the capsules with fasciculata cells was assessed to be routinely between 2–5 % in terms of cell number [9].

The homogenates were centrifuged for 10 min at $600 \times g$ at 4 °C, the resulting supernatant was then pipetted off and recentrifuged at $10\,000 \times g$ for 10 min. After discarding the supernatant, the centrifuge tubes were wiped free of adhering fat and the pellet resuspended in 0.25 M sucrose and recentrifuged at $10\,000 \times g$ for 10 min. The resulting washed mitochondrial pellet was resuspended in 0.25 M sucrose to give a protein concentration of 5–8 mg/ml. For the preparation of microsomes, the mitochondrial supernatant was centrifuged at $15\,000 \times g$ for 10 min and the resulting supernatant centrifuged at $100\,000 \times g$ for 60 min at 4 °C. The microsomal pellet was resuspended in 0.25 M sucrose.

When making additions, the mitochondria were diluted in 0.25 M sucrose containing 10 mM triethanolamine hydrochloride, 20 mM KCl and 5 mM $MgCl_2$, pH 7.1 at room temperature to give a protein concentration of about 1 mg/ml. All steroids were added in acetone (8 μl acetone/ml suspension) to give a final concentration of 50 μM. Acetone was also added to the control mitochondria. It was used in preference to ethanol because of the reported effects of the latter on EPR spectra of cytochrome *P*-450 [10]. Cyanoketone (2 α-cyano-4,4,17α-trimethyl-androstane-17β-hydroxy-3-one) was also added at a concentration of 3 μM to prevent metabolism of pregnenolone. At this concentration, this compound had no effect on the EPR spectra. Metyrapone and aminoglutethimide were added as aqueous solutions. After incubating for 5 min the mitochondria were centrifuged at $15\,000 \times g$ for 10 min. The mitochondrial pellets, containing 8–10 rat equivalents and about 4 mg protein per tube were resuspended in 120 μl of the appropriate incubation medium and transferred by means of syringes into EPR tubes and frozen in liquid nitrogen.

EPR spectra were obtained with a Varian Associates E9 X-band EPR spectrometer equipped with either a liquid nitrogen cryostat or an Oxford Instruments variable temperature liquid helium cryostat. Calibration of the magnetic field of the spectrometer was achieved with a modified Newport Instruments proton magnetometer and an Advance Instruments timer counter. The apparent low spin g values were obtained from the field corresponding to the maximum (g_1), minimum (g_3) of the 1st derivative spectrum, and g_2 from the zero crossing point. Apparent effective high spin g values g_x, g_y were obtained from the maximum and zero crossing points. Microwave frequencies varied between 9.2–9.1 GHz, and were calibrated using a free radical sample of known g value.

The errors in g values quoted in the text represent the experimental variations in the complex preparations used. They do not necessarily represent the absolute errors, since there may be non-random instrumental errors, such as that introduced by measuring the magnetic field external to the cavity.

Aminoglutethimide was a gift of Ciba, Horsham. Metyrapone was obtained from Aldrich Chemical Co. Ltd; Cyanoketone was a gift from Dr. J. I. Mason. Other reagents were B.D.H. Analar grade. Protein estimations on samples after EPR were by the method of Lowry et al. [11].

RESULTS

Control mitochondria

In Table I are listed the g values obtained for low spin ferric cytochrome *P*-450 in capsular and decapsulated adrenal mitochondria. The 2.424 signal was generally broad and in some cases there was evidence of a slight upfield shoulder, but this was never further upfield than $g = 2.415$. At helium temperatures the EPR spectra of decapsulated adrenal mitochondria also show lines with g_x values of 7.80 and 8.08 (Table II) due to high spin ferric cytochrome *P*-450s. These components were generally about equal in magnitude, with sometimes one, and sometimes the other, predominating in size. In addition, species with broad signals at around $g = 6$ and $g = 4.3$ were also present in these mitochondria and a large sharp signal at

TABLE I

APPARENT g VALUES OF LOW SPIN FERRIC CYTOCHROME *P*-450 IN RAT ADRENAL FRACTIONS

Results expressed as mean ±S.E.M. (No. of observations): see Materials and Methods. N.S. = not significant; n.m. = not measurable.

	g_1	g_2	g_3
Decapsular mitochondria			
Control, pH 7.1	2.424±0.001 (11)	2.248±0.001 (11)	1.917±0.001 (10)
Pregnenolone, 50 μM	2.413±0.002 (4) $P < 0.001$	2.248±0.0003 (4) N.S.	1.921±0.001 (4) $P < 0.01$
Deoxycorticosterone, 50 μM	2.422±0.002 (5) N.S.	2.248±0.0002 (5) N.S.	1.920±0.002 (5) $P < 0.02$
Isocitrate, 5 mM	2.413±0.004 (3) $P < 0.001$	2.247±0.0004 (3) N.S.	n.m.
Succinate, 2.5 mM	2.418±0.003 (4) $P < 0.02$	2.250±0.001 (4) $P < 0.05$	n.m.
Ca^{2+}, 5 mM	2.426	2.249	1.917
pH 6.1	2.424	2.247	1.918
Aminoglutethimide, 2 mM	2.437	2.248	1.919
Metyrapone, 2 mM	2.460	2.264	1.894
Capsular mitochondria			
Control, pH 7.1	2.426	2.248	1.915
pH 6.2	2.426	2.247	1.915
Decapsular microsomes			
Control, pH 7.1	2.417±0.003 (3)	2.245±0.0003 (3)	1.918±0.0003 (3)

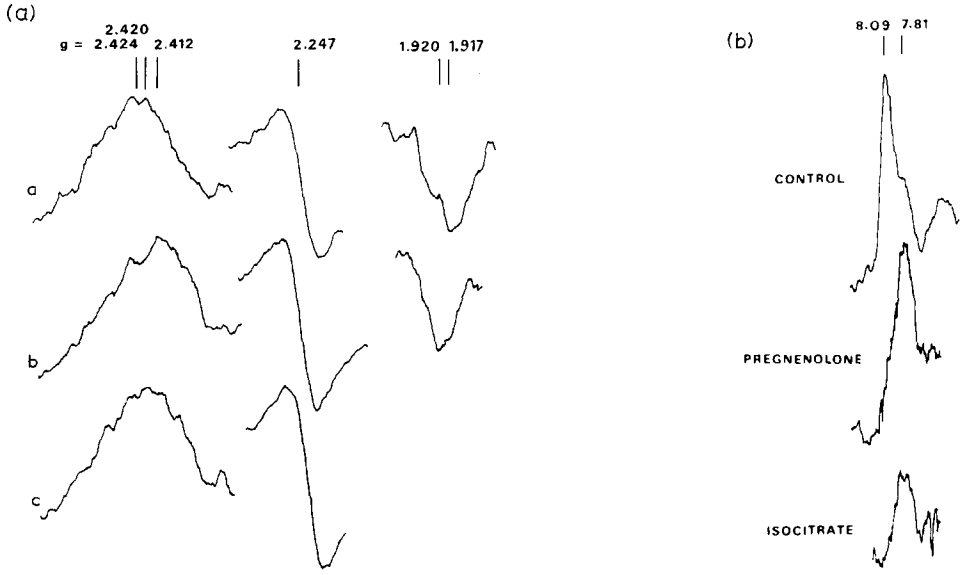

Fig. 1. (a) EPR spectra at high field of rat decapsulated adrenal mitochondria. (a) control; (b) after addition of pregnenolone (50 μM); (c) after addition of DL-isocitrate (5 mM). Microwave frequency = 9.127 GHz; temperature 77 K, pH 7.1. (b) EPR spectra at low field of rat decapsulated adrenal mitochondria. Concentration of additions: pregnenolone 50 μM, DL-isocitrate 5 mM. Microwave frequency = 9.150 GHz; temperature 7.1 K, pH 7.1.

TABLE II

APPARENT EFFECTIVE g VALUES OF HIGH SPIN FERRIC CYTOCHROME *P*-450 IN RAT ADRENAL FRACTIONS

Results expressed as mean ±S.E.M. (No. of observations). For concentrations, Table I. Microwave frequencies varied between 9.13 and 9.17 GHz. n.m. = not measurable.

	g_x	g_y
Decapsular mitochondria		
Control, pH 7.1	8.08 ±0.02 (7)	n.m.
	7.80 ±0.01 (7)	n.m.
Pregnenolone	7.81 ±0.03 (4)	n.m.
Deoxycorticosterone	7.782±0.004 (3)	3.96
Calcium	8.085 (2)	n.m.
pH 6.1	8.10 ±0.01 (4)	3.53
Decapsular microsomes		
Control, pH 7.1	7.91	3.85
Capsular mitochondria		
Control, pH 7.1	n.m.	n.m.
Deoxycorticosterone	7.8	n.m.
pH 6.2	8.1	n.m.

$g = 2.01$ which was only present at liquid helium temperatures, and was not present in intact adrenal glands. The capsular mitochondria showed no detectable signals around the $g = 8$ region attributable to high spin ferric cytochrome *P*-450; however, a broad, weak signal at $g = 6$ was visible.

Effect of pregnenolone and isocitrate

Table I shows the effects of 50 μM pregnenolone and 5 mM DL-isocitrate on the g values of low spin ferric cytochrome *P*-450 in decapsulated adrenal mitochondria for a number of experiments. Typical spectra are shown in Fig. 1a. It can be seen that small shifts in the g_1 and g_3 lines of the EPR spectrum were produced without affecting the central g_2 line. Succinate (2.5 mM) produced similar effects to isocitrate. At the same time, neither pregnenolone nor isocitrate produced any noticeable effects on the magnitude of the EPR spectrum of low spin cytochrome *P*-450. Fig. 1b shows the corresponding effects on the $g = 8$ region of the cytochrome *P*-450 spectrum of decapsulated adrenal mitochondria. Addition of pregnenolone resulted in loss of the $g = 8.1$ signal without any decrease of the $g = 7.8$ signal (Table II). Addition of isocitrate also resulted in a loss of the $g = 8.1$ signal, while the $g = 7.8$ signal remained unaffected.

Effect of pH and deoxycorticosterone

Figs. 2a, 3 show the effect of deoxycorticosterone on the spectrum of high spin ferric cytochrome *P*-450 in decapsulated adrenal mitochondria. Titration with deoxycorticosterone resulted in a concentration dependent increase of the $g_{xy} = 7.78$, 3.96 signal (Table II) with no change in the $g = 8.1$ signal. There was a corresponding decrease in the amount of low spin ferric cytochrome *P*-450 to a lower limit of around 80 per cent of the control low spin value (based on the size of the $g = 2.248$ signal). From optical measurements it is expected that a 30% conversion to high spin should occur in the presence of 50 μM deoxycorticosterone in rat adrenal mitochondria and 27% in bovine adrenal cortex mitochondria [12]. These and our numbers are probably not significantly different given the assumptions used in both sets of calculations, which include the absence of temperature-dependent spin-state transitions; the absence of ferrous cytochrome *P*-450 in the mitochondria and the assumptions made in the calculation of the extinction coefficient of the type 1 difference spectrum [12].

The effect of lowering the pH on the spectrum of high spin ferric cytochrome *P*-450 is shown in Figs. 2b, 3. Lowering the pH resulted in an incremental increase specifically in the magnitude of the $g_{xy} = 8.08$, 3.53 signal, with no change in the $g = 7.78$ signal, and again there was a corresponding decrease in the magnitude of the low spin signal. This pH dependent spin state change provides direct evidence supporting the interpretation of previous optical experiments [12] where it was found that lowering the pH resulted in an increase in the magnitude of the pregnenolone induced inverted type 1 difference spectrum with no effect on the deoxycorticosterone-induced type 1 difference spectrum, and we conclude that the $g = 8.08$ signal corresponds to *P*-450$_{scc}$.

Addition of deoxycorticosterone resulted in slight shifts in the outer lines (g_1, g_3) of the low spin ferric spectrum (Table I), similar in direction to those produced by pregnenolone and isocitrate, but smaller in magnitude. On the other hand,

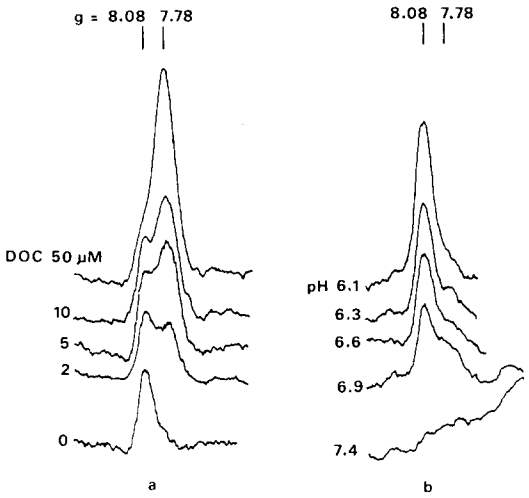

Fig. 2. EPR spectra of high spin ferric cytochrome *P*-450 in rat adrenal mitochondria generated by (a) increasing concentrations of deoxycorticosterone (DOC) (pH 7.1); (b) successive lowering of pH. Microwave frequency = 9.147 GHz; temperature 9.0 K.

varying the pH did not produce any detectable shifts in the g values of low spin ferric cytochrome *P*-450.

Whilst no signals could be detected from high spin ferric cytochrome *P*-450 in control capsular mitochondria, lowering the pH again resulted in the appearance of a $g_x = 8.1$ signal and on addition of deoxycorticosterone a $g_x = 7.8$ signal was obtained. Thus, although restricted by the small amount of zona glomerulosa tissue in the

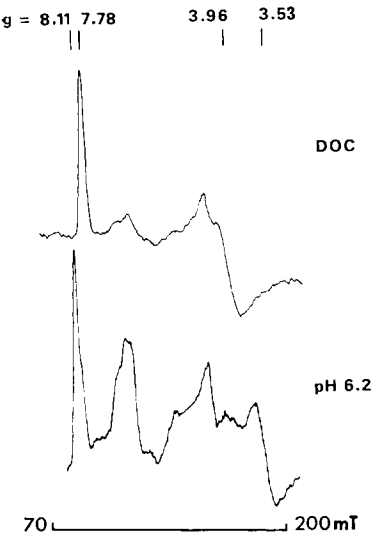

Fig. 3. Low field EPR spectra of rat decapsulated adrenal mitochondria in the presence of 50 μM deoxycorticosterone pH 7.1, and at pH 6.2. Temperature 7.7 K; microwave frequency = 9.147 GHz.

rat (the spectra shown here represent material from 40 rat equivalents of adrenal capsules), it appears that the properties of cytochrome *P*-450 in zona glomerulosa mitochondria are similar to those of fasciculata-reticularis mitochondria *P*-450.

Effect of calcium

Fig. 4 shows the effect of addition of 5 mM calcium phosphate on the high and low spin ferric cytochrome *P*-450 EPR spectra in decapsulated adrenal mitochondria. As can be seen calcium produced a substantial increase in the $g_x = 8.08$ line (Table II) with no change in the $g_x = 7.78$ line. There was no change in the g values of the low spin ferric cytochrome *P*-450 spectrum (Table I) but again there was a small decrease in the magnitude of this spectrum consistent with the increase in the high spin spectrum. These results confirm previous conclusions about the mechanism of action of calcium in stimulating cholesterol side chain cleavage [13, 14] which were based on the calcium induced increase in the magnitude of the pregnenolone inverted type 1 difference spectrum, namely that calcium acts by causing an increase in the amount of cytochrome $P\text{-}450_{scc}$ bound to cholesterol within the mitochondria.

Effect of aminoglutethimide and metyrapone

Fig. 5 shows the effect of aminoglutethimide and metyrapone on low spin cytochrome *P*-450 in decapsulated adrenal mitochondria. The g values obtained

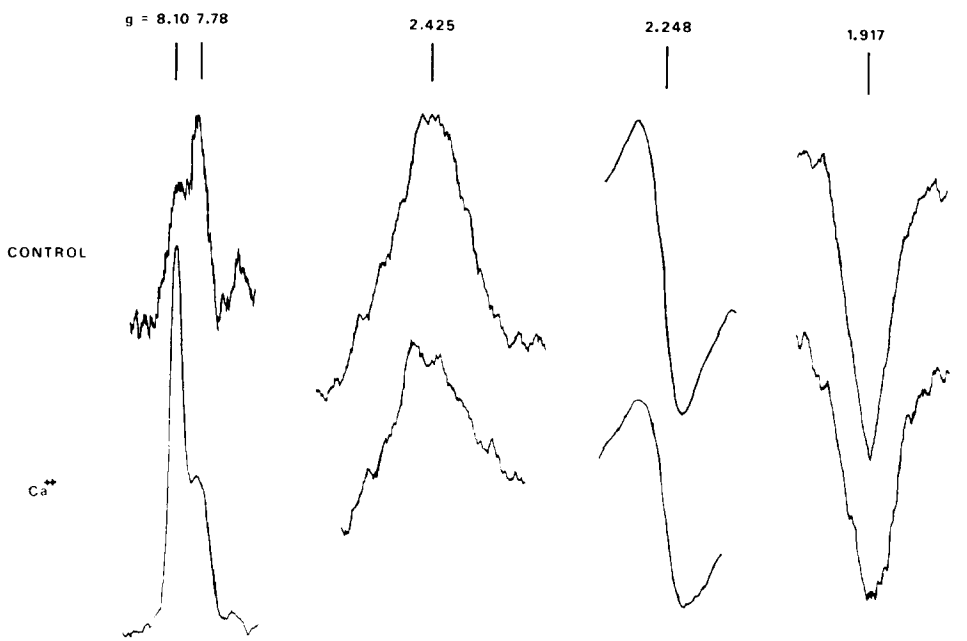

Fig. 4. Effect of calcium on the EPR spectra of the high spin and low spin lines of ferric cytochrome *P*-450 from rat decapsulated adrenal mitochondria. Calcium phosphate concentration 5 mM. High spin measurements were made at 7.6 K, microwave frequency = 9.139 GHz, low spin 110 K, 9.132 GHz.

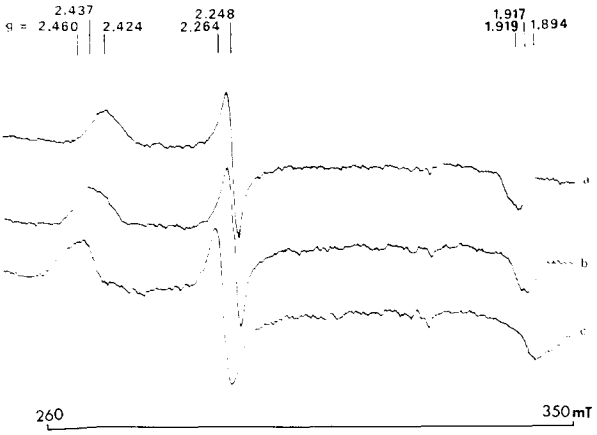

Fig. 5. EPR spectra at 77 K of rat decapsulated adrenal mitochondria. (a) control, microwave frequency = 9.157 GHz; (b) after addition of aminoglutethimide (2 mM), microwave frequency = 9.157 GHz; (c) after addition of metyrapone (2 mM), microwave frequency = 9.159 GHz.

(Table I) are similar to those produced by aniline and metyrapone in liver microsomes [10, 15] and in a bovine adrenal preparation [8]. The introduction of a nitrogenous base perturbs the heme iron, presumably by substituting for a native ligand derived from the protein.

Microsomes from decapsulated adrenals

Low spin ferric cytochrome *P*-450 from decapsulated adrenal microsomes showed *g* values which were different from those of mitochondria of both decapsulated and capsular adrenals (Table I). Fig. 6 shows a spectrum of low spin ferric cytochrome *P*-450 from both microsomes and mitochondria of decapsulated adrenals for comparison.

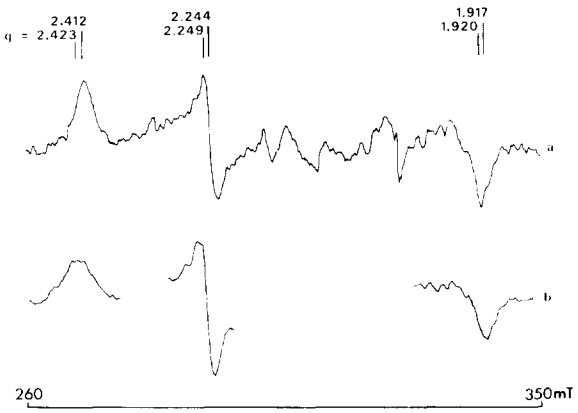

Fig. 6. EPR spectra of low spin ferric cytochrome *P*-450 at 77 K in rat adrenal microsomes (upper trace) and in rat decapsulated adrenal mitochondria (lower trace). Microwave frequencies = 9.116 GHz, 9.118 GHz, respectively. Temperature 77 K.

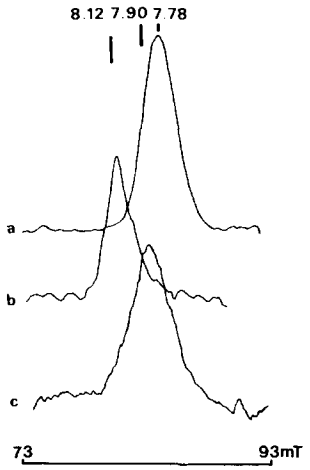

Fig. 7. Comparison of the low field regions of the EPR spectra of high spin ferric cytochrome *P*-450 from (a) rat decapsulated adrenal mitochondria in the presence of deoxycorticosterone (50 μM) pH 7.1; (b) rat decapsulated adrenal mitochondria pH 6.2; (c) rat adrenal microsomes, pH 7.0. Microwave frequency = 9.167 GHz, temperature 9.0 K.

Microsomes showed a single signal in the low field region attributable to high spin ferric cytochrome *P*-450, with g_{xy} values of 7.91, 3.85 distinct from either signal present in mitochondria. This is shown in Fig. 7.

Relative quantitation of cytochrome P-450

The relative amounts of the high and low spin forms of cytochrome *P*-450 were approximately quantitated using the very simple procedure of comparing increments in high spin signals caused by addition of deoxycorticosterone, or lowering the pH, with the corresponding decrease in size of the low spin signal at $g = 2.248$. Using the deoxycorticosterone titration as a quantitative basis the high spin signal intensity on lowering pH could be predicted from the low spin spectrum to within 20 %, and vice versa. It was then estimated that the high spin ferric cytochrome P-450 in control decapsulated adrenal mitochondria accounts for only 1.5 ± 0.5 per cent of the total EPR detectable cytochrome *P*-450. Since there is no detectable reduced adrenodoxin signal present in the mitochrondria, there is presumably no ferrous cytochrome *P*-450 present either, and the EPR detectable cytochrome *P*-450 therefore represents all of the *P*-450 present, and all but a few per cent of this is low spin ferric.

By comparing the relative magnitudes of the *P*-450 spectra of the different cell fractions, it was estimated that the zona glomerulosa mitochondria accounted for about 3 per cent of the recovered low spin ferric cytochrome *P*-450, and the zona fasciculata-reticularis microsomes, about 15 per cent. This compares with an estimate of 4.5 per cent for the zona glomerulosa mitochondria derived from optical studies [14]. The remainder is accountable essentially in terms of zona fasciculata-reticularis mitochondria. Since high-spin ferric cytochrome *P*-450 accounts for only a few per cent of the total, these numbers represent to a first approximation the distribution of total cytochrome *P*-450 within the rat adrenal gland.

DISCUSSION

Low spin ferric cytochrome P-450 of rat adrenal fractions

The g values for rat adrenal low spin ferric cytochrome P-450 presented here agree favourably with those reported for other cytochrome P-450 preparations. Thus Schleyer [8] quoted g values of 2.414. 2.240 and 1.904 for bovine adrenal mitochrondria and Jefcoate et al. [1] quoted values of 2.426, 2.24 and 1.91, also for bovine adrenal cortex mitochondria. Values of 2.425, 2.254 and 1.915 were presented by Stern et al. [16] for liver microsomes from normal rats.

Previous studies with liver microsomes [15] and bovine adrenal mitochondria [1] have indicated that low spin ferric cytochrome P-450 is heterogeneous and contains more than one species with differing g values. Jefcoate et al. [1] presented evidence that two forms of low spin ferric cytochrome P-450 in bovine adrenal cortex mitochondria were the low spin forms of $P\text{-}450_{11\beta}$ and $P\text{-}450_{scc}$. In rat adrenal mitochondria however, the situation is not so clear cut. From the results presented here there is some evidence of heterogeneity in low spin ferric cytochrome P-450. This stems from the occasional appearance of shoulders on the broad 2.424 peak (e.g. Fig. 1a). However, both pregnenolone and deoxycorticosterone, which are believed to bind specifically to high spin ferric cytochrome $P\text{-}450_{scc}$ and low spin ferric cytochrome $P\text{-}450_{11\beta}$, respectively, caused shifts in rat adrenal mitochondrial low spin ferric cytochrome P-450 in the same direction, and the shifts caused by deoxycorticosterone were less than those caused by pregnenolone (Table 1 and Fig. 1a), in spite of the fact that deoxycorticosterone caused a considerable decrease in the total low spin ferric cytochrome P-450. These results suggest then that in rat adrenal mitochondria the low spin forms of cytochrome $P\text{-}450_{scc}$ and $P\text{-}450_{11\beta}$ have more or less identical g values, and that the steroid-induced shifts in the g values may be due rather to interaction of the steroids with all of the low spin ferric cytochrome P-450 in a manner unrelated to the specific high-low spin state transitions induced by pregnenolone and deoxycorticosterone in $P\text{-}450_{scc}$ and $P\text{-}450_{11\beta}$ respectively.

That such non-specific interactions can occur is supported by the fact that addition of reducing equivalents in the form of isocitrate and succinate, while not causing any significant reduction of cytochrome P-450 under the conditions described here, also brought about shifts in the g values of low spin ferric cytochrome P-450 similar to those brought about by pregnenolone. Conceivably, however, this could have been caused by the initiation of cholesterol side-chain cleavage induced by addition of reducing equivalents, with resulting pregnenolone formation.

In contrast, microsomal P-450 is clearly distinguishable from mitochondrial P-450 on the basis of its low spin spectrum.

High spin ferric cytochrome P-450 of rat adrenal fractions

In previous studies [1] it has been reported that bovine adrenal cortex mitochondria contain a large EPR signal at $g = 8.1$ identified as high spin ferric cytochrome P-450. The presence of this component in purified preparations containing cholesterol side chain cleavage activity led to its identification as the cholesterol complex of $P\text{-}450_{scc}$. On the other hand, addition of deoxycorticosterone to intact mitochondria gave rise to a new signal with a g value of 7.9 thus establishing that this component represents the substrate complex of $P\text{-}450_{11\beta}$.

As reported here and previously [17] in rat adrenal mitochondria, the proportion of cytochrome *P*-450 present as the $g = 8.1$ component, i.e. considered to be cytochrome $P\text{-}450_{scc}$ bound to cholesterol, is much less than in bovine adrenal cortex, and is present at about the same level as the $g = 7.8$ component, considered to be cytochrome $P\text{-}450_{11\beta}$ and 18-hydroxylase cytochrome *P*-450 bound to endogenous substrate such as deoxycorticosterone or progesterone. Addition of steroids does not cause shifts in the g values of high spin ferric cytochrome *P*-450 (Table II), but causes spin state transitions. Thus addition of deoxycorticosterone converts a proportion of the low spin ferric cytochrome *P*-450 to the high spin component with $g = 7.78$. On the other hand, addition of pregnenolone (Fig. 1b) specifically removes the component with the $g = 8.08$ signal, presumably by converting it to low spin ferric cytochrome *P*-450, although the small change in the magnitude of the $g = 2.248$ signal could not be detected. Addition of 20α-hydroxycholesterol to bovine adrenal cortex mitochondria [1] produced a loss of the $g = 8.1$ signal with corresponding increase in the low spin form, due to the much greater concentration of the high spin form originally present. These observations then provide direct evidence for the previous interpretation of the inverted type 1 difference spectrum produced by adding pregnenolone to adrenal mitochondria, namely that the optical and EPR spectra produced by addition of pregnenolone are the result of displacement of endogenous substrate from cytochrome $P\text{-}450_{scc}$ [1, 5]. Similarly, the loss of the EPR spectra of high spin cytochrome *P*-450 produced on addition of isocitrate (Fig. 1b) provides direct evidence for the previous interpretation of the inverted type 1 difference spectrum produced by addition of isocitrate, namely that this was caused by conversion of endogenous cholesterol to pregnenolone.

Whilst in the liver it has not yet proved possible to distinguish different cytochromes *P*-450, *P*-448 by the EPR spectra of their high spin forms a further heterogeneity is here observed with $P\text{-}450_{scc}$, $P\text{-}450_{11\beta}$ and microsomal *P*-450 each having a distinct high spin spectrum. Whether this is due to differences in *P*-450 protein or to substrate induced perturbations of *P*-450 heme cannot yet be decided.

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