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SOME CHARACTERISTICS OF HUMAN ADRENAL MICROSOMAL 21-HYDROXYLASE ACTIVITY

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Summary—The following general characteristics of 21-hydroxylase activity were determined using pooled microsomes obtained from three glands. Enzyme activity exhibited a broad pH dependence, being optimal between pH 7.4–pH 7.8, and was maximal with NADPH in the range 2 to 4.75×10^{-4} mol/l. No microsomal 21-hydroxylase activity was detected in the absence of NADPH or substrate and when heat denatured microsomes were employed. Enzyme activity was depressed by >75% in the presence of 100% oxygen or nitrogen.

In a second set of experiments, microsomal fractions were prepared individually from 7 glands. In the presence of 17α-hydroxy progesterone (2.0×10^{-7} and 2.0×10^{-6} mol/l) product formation was linear with time for up to 90 s when the microsomal protein concentration was 5, 10 and 20 μg/ml. Between 5 and 30% of the substrate was converted during the first 60 s. In $\frac{5}{7}$ of the glands the addition of the autologous cytosol (20 μg protein/ml) was without effect, and enzyme activity (using a 60 s reaction and either 2.0×10^{-7} or 2×10^{-6} mol/l 17α-hydroxy progesterone was directly proportional to the microsomal protein concentration (range 0–20 μg/ml). With the other 2 adrenals 21-hydroxylation was not proportional to the same range of microsomal protein concentrations, although it became so upon the addition of cytosol, which significantly augmented activity.

There was considerable variation in enzyme activity between glands from different individuals (V_{max} ranging from 2.6 to 16.6×10^{-9} mol/min/mg protein) and in the apparent K_m's (from 0.22 to 1.1×10^{-6} mol/l). In the two preparations sensitive to cytosol, the V_{max} increased 2-fold, and the K_m was 3 times lower. Cytosol was without effect upon the kinetic characteristics of the other 5 microsomal preparations. Ascorbic acid (1×10^{-3} mol/l) depressed enzyme activity by 25–43% whereas oxidised and reduced glutathione (1×10^{-3} mol/l) showed a slight and variable effect upon 21-hydroxylation.

INTRODUCTION

Microsomal cytochrome P450—21-hydroxylase is a NADPH-dependent mixed function oxidase which in the adrenal catalyses the conversion of 17α-hydroxy progesterone and progesterone to 11-deoxycortisol and 11-deoxycorticosterone respectively [1]. Although there are a few reports on the characteristics of the human adrenal enzyme, [2, 3] little is known about its regulation. Recent work on bovine adrenal 21-hydroxylation (in both cell free and cell culture systems) indicates that a variety of factors may interact in the control of this enzyme [4, 5, 6]. Thus, components in cell cytosol, attributed to oxidised and reduced glutathione, can stimulate 21-hydroxylation by bovine microsomes which can also be inhibited by ascorbate [5], the role of which in adrenal and ovarian steroidogenesis has long been the subject of debate.

In the present communication, we describe the basic characteristics of human adrenal microsomal 21-hydroxylase activity. We report a kinetic analysis of 7 enzyme preparations derived from individual human glands. Finally, we have attempted to elucidate further some of the mechanisms by which 21-hydroxylase activity may be modulated, particularly with regard to the effects of cytosol (autologous to the microsomal preparation), ascorbate and glutathione. Some of this work has been presented in a preliminary from elsewhere [7, 8].

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EXPERIMENTAL

Chemicals

17α-Hydroxy[1,2,6,7-^{3}H]progesterone (55 Ci/mmol) and 17α[1,2-^{3}H(*N*)]-hydroxy-11-deoxycorticosterone, (51 Ci/mmol) were purchased from Amersham International and from New England Nuclear respectively. The steroids (confirmed to be >98% pure) were diluted in ethanol to 50 μCi/ml which served as our working standard. Non-labelled steroids and cofactors were purchased from Sigma, Fancy Road, Poole, Dorset.

Tissue preparation

Human adrenal glands (3.0–5.6 g wet wt) were obtained from kidney donors (decerebated but otherwise healthy accident victims who had been maintained on ventilators) immediately prior to transplantation (by permission of Dr R. W. Johnson, Consultant Transplant Surgeon, Manchester Royal

Infirmary). Donors had been medicated with phentolamine (α-blocker) bicarbonate and heparin. If urine volumes were low, frusemide was employed. There was no steroid treatment. The ages of the donors were 11–45 ($n = 5$, male) and 22–39 ($n = 2$, female). The glands were chilled in ice-cold NaCl (0.9%) and frozen in liquid nitrogen within 2–3 h after removal. Following thawing, individual glands ($n = 7$ five males, two females) or pooled adrenal glands ($n = 3$) were homogenised in ice-cold sucrose (2.5×10^{-1} mol/l) buffered with (5×10^{-2} mol/l) phosphate buffer (pH 7.4). Mitochondrial precipitates (10,000 *g* for 15 min), microsomal precipitates (105,000 *g* for 90 min) and cytosol (105, 000 *g* supernatant fluid) were obtained by a conventional differential centrifugation [9]. Precipitates were resuspended in glycerol buffered with (5×10^{-2} mol/l) phosphate buffer (pH 7.4), 25:75, v/v and stored in aliquots at −70°C for 1–6 weeks at a protein concentration of 5000 μg/ml. No loss of 21-hydroxylase activity was found under these conditions. Protein estimations were determined by the method of Bradford[10].

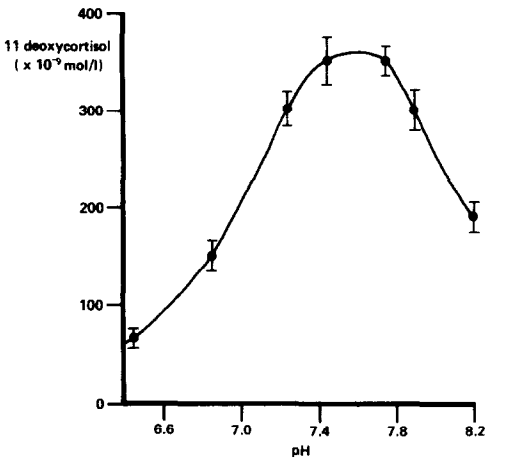

Fig. 1. Effect of pH on 21-hydroxylation of 17α-hydroxy progesterone by human adrenal microsomes. 17α-Hydroxy progesterone (5×10^{-7} mol/l) was incubated for 2 min with pooled microsomal protein (20 μg/ml) and NADPH (2.5×10^{-4} mol/l) in phosphate buffer (5×10^{-2} mol/l) over a pH range 6.4–8.2. Estimations are the mean ± SD of quadruplicate values.

The determination of 21-hydroxylase activity

Unlabelled (2.0×10^{-7} to 2.0×10^{-6} mol/l) and ^{3}H-labelled 17α-hydroxy progesterone (3.6×10^{-9} mol/l) were added to a total volume of 1 ml phosphate buffer, (5×10^{-2} mol/l, pH 7.4) containing microsomal protein (1–40 μg/ml) and saturating levels of NADPH, (2.5×10^{-4} mol/l) Cytosol (20 μg protein/ml) ascorbate (1×10^{-3} mol/l), oxidised and reduced glutathione (1×10^{-3} mol/l) were added as required. There was no loss of buffering efficiency on the addition of any of these components. Reactions were performed at 37°C in air and were initiated after 5 min preincubation by the addition of substrate. The reaction was terminated between 20 s and 10 min later by snap freezing in a cardice–acetone mixture. The steroids were later extracted with diethyl–ether (6 ml), dried overnight in air and reconstituted in ethanol (0.5 ml) containing 40 μg/ml unlabelled 17α-hydroxy progesterone and 11-deoxycortisol which acted as chromatographic carriers and aided u.v. detection of the steroids. The ethanolic extracts (50 μl) were subjected to chromatography on TLC plates pre-coated with silica gel (Whatmann LK6DF Linear K) using the system chloroform–ethanol (95:5, v/v) as the solvent.

The u.v. absorbing 17α-hydroxy progesterone and 11-deoxycortisol areas were scraped into scintillation vials and radioactivity counted in a toluene–PPO solution (3.5 ml) using a LKB 1216 model rack–beta counter. The counting efficiency for ^{3}H was 43.0%. Total recoveries of substrate and product (assessed by the fate of [^{3}H]17α-hydroxy progesterone, and [^{3}H]11-deoxycortisol from control incubations lacking only microsomes) were 91.2 ± 11.5% and 82.7 ± 9.0% (mean ± SD, $n = 32$) respectively. Each experimental point was derived from quadruplicate estimations and the results reported were corrected for experimental losses and expressed as $\times 10^{-9}$ mol/l 11-deoxycortisol (mean ± SD). Interassay coefficient of variation was <18% for each individual adrenal gland, and intraassay coefficient of variation <12% per experiment ($n = 20$ samples, each assayed in quadruplicate).

Data analysis

Kinetic parameters were determined by weighted non-linear regression [11] using initial velocity data. Comparisons were made using unpaired *t*-tests.

RESULTS

General characteristics of 21-hydroxylase activity in microsomes derived from three pooled human adrenal glands

Enzyme activity was exhibited over a broad pH range, being optimal between pH 7.4 and 7.8 (Fig. 1) and was maximal with NADPH concentrations in excess of 2×10^{-4} mol/l (Fig. 2). No 21-hydroxylase activity was detected in the absence of NADPH or substrate and when heat denatured microsomes were employed. Enzymes activity was depressed by >75% in the presence of 100% oxygen or nitrogen. Radioactivity was distributed into two discrete areas on the chromatographic plates (R_f values 0.61 ± 0.1 and 0.36 ± 0.1, mean ± SD, $n = 25$) corresponding to the pure 17α-hydroxy progesterone and 11-deoxycortisol used as standards. There were no other detectable products. There was no 21-hydroxylation of 17α-hydroxy progesterone by the cytosolic fraction.

When the microsomal protein concentration was held at 20 μg/ml and the reaction performed at pH 7.4, and 37°C and in the presence of a saturating

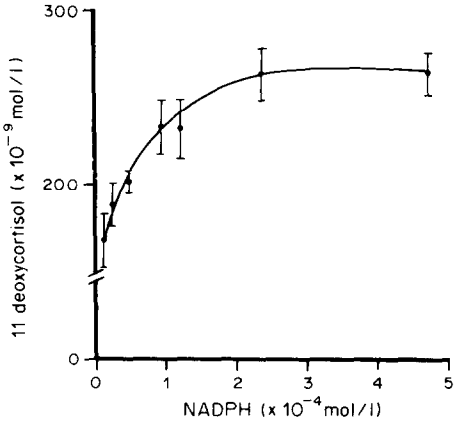

Fig. 2. Effect of NADPH (0–4.75 × 10^{-4} mol/l) concentration on 21-hydroxylase activity. 17α-Hydroxy progesterone (5 × 10^{-7} mol/l) was incubated with pooled microsomal protein (20 μg/ml) for 2 min at pH 7.4.

concentration of NADPH (2.5 × 10^{-4} mol/l) but with varying substrate concentrations, enzyme activity was linear with time for up to 8 min with the highest concentration (5 × 10^{-6} mol/l) of substrate [with 7.3, 35.4 and 52.5% being converted after 1, 5 and 8 min respectively] (Fig. 3). With the two lower concentrations (1.0 and 5.0 × 10^{-7} mol/l 17α-hydroxy progesterone) the reaction proceeded quickly to completion, being linear with time for only 1–2 min (Fig. 3). In these latter cases 14.6% (5 × 10^{-7} mol/l) and 45.1% (1.0 × 10^{-7} mol/l) of the substrate was converted into 11-deoxycortisol in the first minute, with >70% being transformed after 5 min.

21-Hydroxylase activity in microsomes from individual adrenal glands

In the presence of 17α-hydroxy progesterone (2.0 × 10^{-7} and 2 × 10^{-6} mol/l) 21-hydroxylase activity was linear with time for up to 90 s when the microsomal protein concentration was held at 5, 10 and 20 μg/ml (Fig. 4).

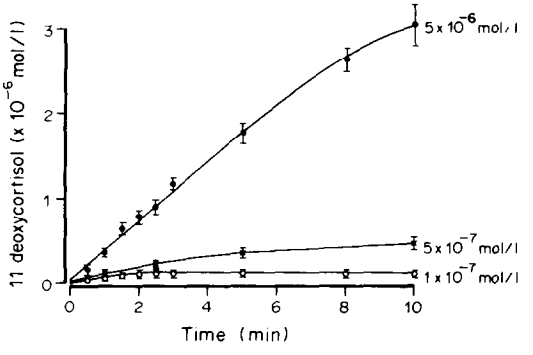

Fig. 3. The effect of time on the production of 11-deoxycortisol by 21-hydroxylase at varying substrate levels. Pooled human microsomal protein (20 μg/ml) was incubated at pH 7.4 with NADPH (2.5 × 10^{-4} mol/l) and with varying concentrations of 17α-hydroxy progesterone (1.0 × 10^{-7}, 5 × 10^{-7} and 5 × 10^{-6} mol/l) for 0–10 min.

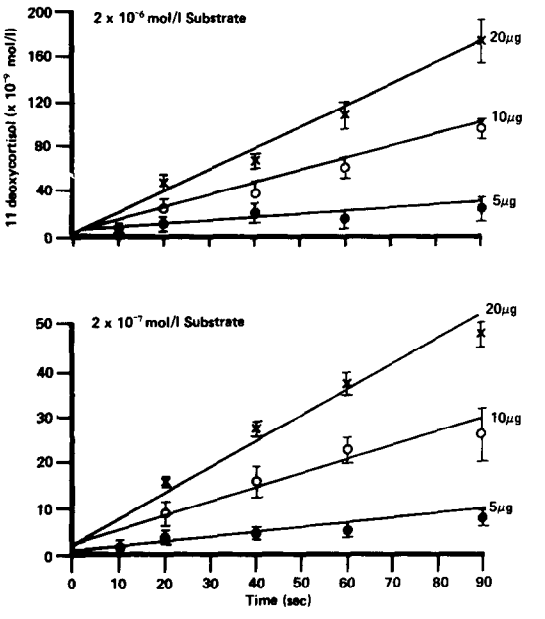

Fig. 4. The effect of time on the production of 11-deoxycortisol using microsomal fractions prepared from an individual adrenal gland. Microsomal protein (5, 10, 20 μg/ml) was incubated with 17α-hydroxy progesterone (2.0 × 10^{-7} or 2 × 10^{-6} mol/l) for up to 90 s in the presence of NADPH (2.5 × 10^{-4} mol/l) at pH 7.4.

In $\frac{5}{7}$ of the microsomal preparations, the addition of the cytosol derived from the same gland was without effect, and enzyme activity (assayed for 60 s with either 2.0 × 10^{-7} or 2 × 10^{-6} mol/l 17α-hydroxy progesterone) was directly proportional to the microsomal protein concentration over the range 0–20 μg/ml (see Fig. 5a and b). However, with the remaining two preparations, 21-hydroxylation was not proportional to the same range of microsomal protein concentrations, although it became so upon the addition of cytosol which significantly augmented activity (see Fig. 6a and b).

The effect of incresing substrate concentration (range 0–2 × 10^{-6} mol/l) under initial velocity rate conditions (pH 7.4, 37°C, 2.5 × 10^{-4} mol/l NADPH, 10 μg/ml microsomal protein, 60 second reaction) was then examined with the individual microsomal preparations. In all 7 adrenals the substrate–activity relationship was hyperbolic and demonstrated that the enzyme system was fully saturated with substrate concentrations of >1.5 × 10^{-6} mol/l. Using this type of data for the kinetic analysis it can be seen (Table 1) that there was considerable variation in enzyme activity from gland to gland with the V_{max} ranging from 2.6 to 16.6 × 10^{-9} mol 11-deoxycortisol/min/mg protein and the apparent K_m varying between 0.22 and 1.1 × 10^{-6} mol/l. The addition of cytosol was without effect on the kinetic characteristics in $\frac{5}{7}$ of the preparations (adrenals 3 to 7) but increased the V_{max} 2-fold whilst decreasing the apparent K_m 3 fold in the other 2 preparations (adrenals 1, 2, Table 1). There

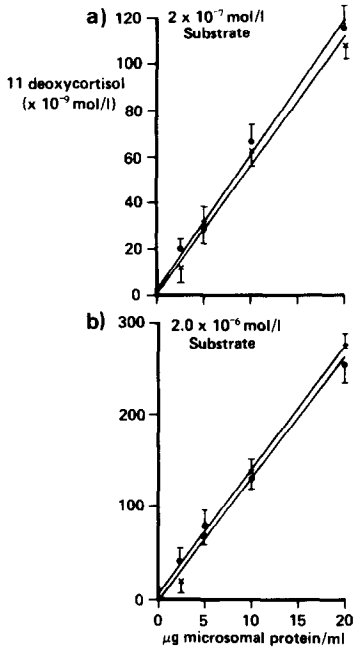

Fig. 5a and b. The effect of cytosol on 21-hydroxylase activity in microsomes from adrenal 7. 17α-Hydroxy progesterone (2.0×10^{-7} or 2×10^{-6} mol/l) was incubated with microsomal protein (0–20 μg/ml) for 60 s in the presence [●] and absence [×] of cytosolic protein (20 μg/ml).

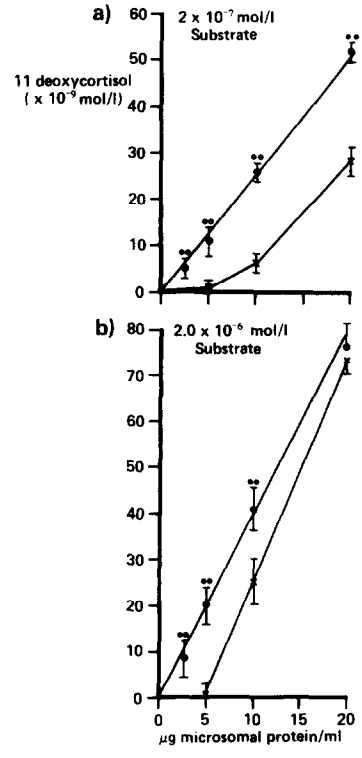

Fig. 6a and b. The effect of cytosol on 21-hydroxylase activity in microsomes from adrenal 1. 17α-Hydroxy progesterone (2.0×10^{-7} or 2×10^{-6} mol/l) was incubated with microsomal protein (0–20 μg/ml for 60 s in the presence [●] and absence [×] of cytosolic protein (20 μg/ml).

was no correlation between enzyme activity and the HLA status of the adrenal donor.

Effect of ascorbate and glutathione

Ascorbate (1×10^{-3} mol/l) caused a significant ($p < 0.01$) inhibition (between 25–43%) of 21-hydroxylase activity in five of the six microsomal preparations tested (Table 2). These included a preparation sensitive to cytosolic stimulation (adrenal 2). Both oxidised and reduced glutathione (1×10^{-3} mol/l) showed variable effects on 21-hydroxylation. In two cases, (adrenals 3 and 4), the presence of oxidised glutathione, significantly stimulated enzyme activity (11.7 and 38.8% activation respectively), with other preparations (adrenal 2 and 7), slight inhibition was found (30.8 and 16% loss of activity respectively). Similarly reduced glutathione both stimulated (adrenal 4, 30.3% increase) and inhibited enzyme activity (adrenal 3, 11.5% loss).

DISCUSSION

Inevitably in a study of this kind, the availability and control of the source material (i.e. fresh human adrenals) was less stringent than in normal laboratory circumstances. Thus, the adrenals (taken at the time of kidney removal for transplantation purposes) were obtained from subjects in a variety of hospitals over a fairly large geographical area (i.e. the North-West of England). Nevertheless, the adrenals which were stored on ice during transportation were frozen to −70°C within 3 h of removal from the body. However, although the biological interpretation of data obtained from such a *in vitro* study should be made with caution, useful information has been obtained.

Although the characteristics of adrenal microsomal hydroxylase activity are reasonably well described for some species [12], relatively little is known about the regulation of the human enzyme, changes in the activity of which are the commonest cause of the human adrenal deficiency states [13]. The aim of the

Table 1. K_m and V_{max} determinations using individual preparation of adrenal microsomes in the presence and absence of autologous cytosol

	K_m ($\times 10^{-6}$ mol/l)		V_{max} ($\times 10^{-9}$ mol 11-deoxycortisol/min/mgprotein)	
Adrenal	No cytosol	With cytosol	No cytosol	With cytosol
1 (11, M)	1.1	0.35	2.6	5.7
2 (30, M)	0.99	0.34	3.4	8.6
3 (45, M)	0.28	0.27	14.5	15.2
4 (31, M)	0.22	0.24	15.9	20.4
5 (39, F)	0.34	0.36	3.8	3.8
6 (—, M)	0.32	0.15	8.5	7.2
7 (22, F)	0.44	0.37	16.6	16.6

Microsomal preparations (10 μg/ml protein) were incubated with 17α-hydroxy progesterone (0 to 2.0×10^{-6} mol/l) and NADPH (2.5×10^{-4} mol/l) for 1 min at 37°C in the presence and absence of autologous cytosol (20 μg protein/ml). The age and sex of each donor are indicated in the brackets.

Table 2. The effect of glutathione and ascorbate on the 21-hydroxylation of 17α-hydroxy progesterone using microsomes prepared from individual adrenal glands

Adrenal	Control	Ascorbate	Oxidised glutathione	Reduced glutathione
2†	95.4 ± 17.0	64.8 ± 2.8**	66.0 ± 8.3*	78.2 ± 7.2
3	162.0 ± 8.2	92.0 ± 10.0**	181.0 ± 8.0*	143.4 ± 12.0**
4	67.6 ± 8.0	34.2 ± 1.2**	93.8 ± 8.6**	88.1 ± 3.8*
5	59.3 ± 4.2	38.2 ± 2.0**	54.0 ± 4.0	50.0 ± 7.1
6	161.2 ± 17.0	140.0 ± 14.8	164.9 ± 11.0	186.3 ± 12.0
7	189.8 ± 5.4	140.8 ± 6.8**	173.8 ± 5.4**	189.9 ± 6.8

The results are expressed as $\times 10^{-9}$ mol/l 11-deoxycortisol formed/min ($n = 4$). $P^* < 0.05$. $P^{**} < 0.01$.
†Sensitive to cytosol. Microsomal protein (10 μg/ml) was incubated with 17α-hydroxy progesterone (2.0×10^{-6} mol/l) for 1 min in the presence of either ascorbate, oxidised or reduced glutathione (1×10^{-3} mol/l.)

present study was to describe some of the general characteristics of human adrenal 21-hydroxylase activity, to analyse the kinetics of substrate binding and finally to examine the effects of possible intra-adrenal modulators of this hydroxylation system.

Optimum activity of the human enzyme was seen over the pH range 7.4–7.8 and the NADPH concentration required to saturate the system was 2×10^{-4} mol/l. In both cases, these values are slightly higher than that described for the bovine enzyme [12, 14]. The dependence of 21-hydroxylation on molecular oxygen is as described earlier [12] although the inhibitory effect of 100% oxygen is at variance with previous work [12].

The V_{max} of 17α-hydroxy progesterone to 11-deoxycortisol transformation seen in the present study ($2.6–16.6 \times 10^{-9}$ mol/min/mg) was similar to that reported for the foetal human adrenal enzyme (8.9×10^{-9} mol/min/mg), [15] but higher than that seen with the adult human ($0.55 \rightarrow 3.3 \times 10^{-9}$ mol/min/mg) [2] or bovine system (1.9×10^{-9} mol/min/mg) [4]. The apparent K_m for 17α-hydroxy progesterone binding in our work ranged between 0.22 to 1.1×10^{-6} mol/l compared with 4 and 13×10^{-6} mol/l (human foetal), [15, 16] and 0.3×10^{-6} mol/l (bovine) [4] and with a K_s value of 1.5×10^{-6} mol/l for the human adult [3].

The activation of 21-hydroxylation by cytosol has been previously described for a bovine system [4] although it was not observed for a similar rat microsomal preparation [17]. In the present study, cytosol was found to increase enzyme activity in two of the seven human microsomal preparations, particularly at low substrate and microsomal protein concentrations. This cytosolic effect was characterised by a doubling of the V_{max} and a 3-fold decrease in the K_m and appeared not to be related to the HLA status of the donor. To our knowledge this is the first time such a finding has been described for the human enzyme.

Greenfield *et al.*[5] have suggested that there is a differential cytosolic control of 21-hydroxylase activity involving glutathione (activating) and ascorbate (inhibiting). We were unable to show any consistent effect on 21-hydroxylation by either reduced or oxidised glutathione, even with a preparation which showed clear activation by cytosol. Failure to demonstrate such a stimulatory effect on the human enzyme may be a reflection of the short incubation time used in our experimental work. Matthijssen[18] has reported that sheep adrenal homogenates depleted of glutathione are capable of 21-hydroxylation suggesting that this antioxidant is not essential for maintaining the hydroxylating system over short time periods. However, the fact that the glutathiones failed to activate the microsomal preparation sensitive to cytosol indicates that these antioxidants are unlikely to be the main modulators in the cytosol enhancing the activity of the human enzyme.

We were able to confirm the well-known inhibitory effect of ascorbate on 21-hydroxylase activity [14, 19]. The *in vivo* relevance of this observation is still unclear. Indeed, the role of this vitamin in steroidogenesis and in the maintenance of adrenal cortical and medullary function is still a fascinating area of study. Ascorbate is sequestered in the human adrenal gland at 135 mg/100 g cortex [20] and it is the ACTH-stimulated release of this vitamin which formed the basis of the first bioassay of this hormone [21]. Various mechanisms have been suggested for the involvement of ascorbate in steroidogenesis. It could be that the efflux of the vitamin releases a "breaking effect" on 21-hydroxylase activity [5]. Another suggestion is that the removal of ascorbate from the cortex allows molecular oxygen to interact with cytochrome P450 and NADPH during steroid hydroxylation rather than with ascorbate, which is itself an efficient oxygen scavenger [22]. More recently another role for ascorbate has been described in a bovine adrenal cell culture system [23]. It has been proposed [24] that the ascorbate eliminates the free radicals (lipid peroxides) formed when steroids bind in the presence of molecular oxygen to cytochrome P450 during hydroxylation reactions and in doing so protects the hydroxylation system against free radical damage [24]. Such a role for ascorbate would imply that the vitamin is required within the cell during steroidogenesis, rather than released from the cell prior to steroid production. Thus there appears to be two apparently contradictory roles for ascorbate in the functioning of the adrenal cell. However, it could be that the two mechanisms act together in regulating steroidogenesis in the following way.

It has been suggested that ascorbate exists as two pools, [25, 26] protein bound and "free" [26] and that

only the protein-bound pool is depleted on ACTH stimulation [26]. Upon ACTH activation of the gland, the protein-bound ascorbate is lost from the cells and in doing so releases the block on 21-hydroxylation by either of the two mechanisms described earlier [5, 22]. The "free" ascorbate, being unaffected by ACTH, remains within the cell and in doing so acts on the anti-oxidant which protects the cytochrome P450 system against lipid peroxide damage. Thus, ascorbate can both control and protect the steroidogenic function of the gland.

In conclusion, there is considerable gland to gland variation in human adrenal 21-hydroxylase activity. In some cases, cytosol changed enzyme activity, leading to an increased V_{max} and decreased K_m. This effect did not appear to be related to HLA. This activation was not due to glutathione. In addition ascorbate and some steroids, in concentrations found within the gland, are capable of acutely affecting 21-hydroxylation and these substances may play a role in the control of the enzyme's activity, changes in which form the continuum of 21-hydroxylase deficiency states (i.e. classical, late onset and cryptic congenital adrenal hyperplasia and the more recently described minor variant of 21-hydroxylase deficiency) [27, 28]. In addition to the possibility of mutations leading to a modified enzyme it may be that regulatory defects involving the factors described in this paper form the basis of some of these deficiency states.

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