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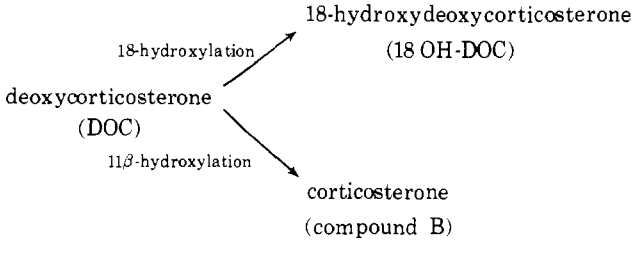
Mutant Forms of Cytochrome P-450 Controlling Both 18- and 11β-Steroid Hydroxylation in the Rat†

John P. Rapp* and Lewis K. Dahl‡

ABSTRACT: A reciprocal relationship between steroid 18- and 11β-hydroxylase activities in the salt susceptible (S) and the salt resistant (R) strains of rats was previously shown to be controlled by a single genetic locus with two alleles and inheritance by co-dominance (Rapp, J. P., and Dahl, L. K. (1972), *Endocrinology 90,* 1435). The strain specific steroidogenic patterns, characterized by the relative magnitudes of 18- and 11β-hydroxylase activities, were found to be determined by adrenal mitochondrial cytochrome P-450 particles. Carbon monoxide inhibition of 18- and 11β-hydroxylation of deoxycorticosterone in these strains showed that the CO/O_2 ratio causing 50% inhibition (i.e., Warburg's partition constant, K) was identical for 18- and 11β-hydroxylation within a strain, but different for both 18- and 11β-hydroxylation between strains. (K values were: S rats, 18-hydroxylation = 11.4 ± 1.4; S rats, 11β-hydroxylation = 11.0 ± 1.2; R rats, 18-hydroxylation = 56.4 ± 13.7; R rats, 11β-hydroxylation = 46.7 ± 11.7). This between-strain difference was unique for 18- and 11β-hydroxylation; i.e., it was not seen with cholesterol side-chain cleavage or 21-hydroxylation. Moreover, the strain-specific K values for 18- and 11β-hydroxylase and the strain-specific steroidogenic patterns due to the relative magnitudes of 18- and 11β-hydroxylase activities segregated together in an F_2 population. These data strongly suggest the same cytochrome P-450 is involved in both 18- and 11β-hydroxylation and that this cytochrome is mutated between S and R rats. K values for the reaction corticosterone → 18-hydroxycorticosterone were different between S and R strains, indicating that the mutant cytochrome was also involved in this hydroxylation, but K values for the conversion corticosterone → aldosterone were not different between strains. This was interpreted to mean that each step in the sequence corticosterone → 18-hydroxycorticosterone → aldosterone was mediated by a different cytochrome, the K value for the second step being the lower and dominating the overall reaction. It was speculated that the second step could be a second hydroxylation at position 18 to yield 18,18-dihydroxycorticosterone which would be unstable and decompose into aldosterone and water.

Rats have been selectively bred for their blood pressure response to high salt (NaCl) diet. Two strains were obtained: the susceptible or S strain and the resistant or R strain (Dahl et al., 1962). S rats respond to high salt intake with a marked increase in blood pressure, whereas R rats on the same diet show little or no blood pressure change. Recently we have described (Rapp and Dahl, 1972) a single genetic locus with two alleles inherited by co-dominance in S and R rats which controls steroidogenesis at the following branch point:



S rats show increased 18-hydroxylase activity compared with R. This increment in 18-hydroxylase activity is offset

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‡ The author notes with deep regret the death of Dr. Lewis K. Dahl, November 26, 1975.

by an equal decrement in 11β-hydroxylase activity in S compared with R. Previous work also showed that the [18OH-DOC]/[(18OH-DOC] + [B])[1] ratio was an efficient parameter for characterizing S and R adrenal phenotypes and an intermediate type obtained by crossing S and R (Rapp and Dahl, 1972).

Adrenal steroid 18- and 11β-hydroxylations are known to utilize an electron transport system involving NADPH → flavoprotein → nonheme-iron protein (adrenodoxin) → cytochrome P-450. This final electron receptor, cytochrome P-450, reacts with oxygen and substrate to yield an hydroxylated product (Suzuki and Kimura, 1965; Nakamura et al., 1966; Omura et al., 1966; Björkhem and Kalmar, 1975). Steroid hydroxylations at other positions involve similar mechanisms and cytochrome P-450 (Estabrook et al., 1963; Omura et al., 1965; Greengard et al., 1967; Simpson and Boyd, 1967; Bryson and Sweat, 1968; Wilson and Harding, 1970b). Different cytochromes P-450 are thought to exist for each specific steroid hydroxylation (Jefcoate et al., 1970; Shikita and Hall, 1973; Ramseyer and Harding, 1973). Cytochrome P-450 has the property of being inhibited by carbon monoxide and the characteristics of carbon monoxide inhibition are different for different cytochromes (Estabrook et al., 1963; Wilson and Harding, 1970a,b). In the present work the property of carbon monoxide inhibition has been exploited to obtain evidence that the steroidogenic differences in S and R rats are due to a mutation of a specific cytochrome P-450.

Materials and Methods

Rats used were females from the salt susceptible (S) and salt resistant (R) strains (Dahl et al., 1962). Animals were killed by decapitation and adrenals collected in incubation buffer at 4 °C. Different incubation systems were used for the various steroid hydroxylations studied. For 18- and 11β-hydroxylations, adrenals were homogenized 12 mg/ml in Krebs-Ringer bicarbonate media (Dawson et al., 1959) with increased Ca^{2+} concentration (3.81 mM). Homogenate was preincubated in bicarbonate media in air without additives for 15 min at 37 °C to swell mitochondria (Peron et al., 1965). One-half milliliter of preincubated adrenal homogenate (6 mg of tissue) was mixed with 1 ml of bicarbonate media containing per milliliter: 18.75 μg of deoxycorticosterone (DOC), 0.2 μCi of [4-^{14}C]DOC (60 mCi/mM), and 1.5 mg NADPH. Incubation time was 5 min, 37 °C. The reaction was stopped by adding the incubate to 5 ml of ethyl acetate, followed by a 5-ml ethyl acetate wash of the reaction flask. The sample was extracted once in the 10 ml of ethyl acetate, dried under nitrogen, and chromatographed on paper in the butyl acetate–formamide–water system (Mattox and Lewbart, 1958). Paper strips were scanned for radioactivity and peak sizes measured by digital integration. The identity of the labeled products, 18-hydroxydeoxycorticosterone (18OH-DOC) and corticosterone, was established previously by crystallization to constant specific activity (Rapp and Dahl, 1972).

The conversion of ^{14}C-labeled corticosterone (B) to 18-hydroxycorticosterone (18OH-B) and aldosterone was also studied using the Krebs-Ringer homogenate system above. Adrenal capsules (zona glomerulosa) were obtained from S and R rats fed sodium deficient diet (Nutritional Biochemical Corp.) for 2 to 4 weeks to increase zona glomerulosa enzyme activity. Tissue was homogenized so that 0.5 ml contained approximately the equivalent of 1 adrenal capsule. Homogenate (0.5 ml) was mixed with 1 ml of media containing 1.5 mg of NADPH and 0.2 μCi of [4-^{14}C]corticosterone (57 mCi/mM) without added unlabeled corticosterone. Incubation time was 20 min, 37 °C. The reaction was stopped and samples were extracted by addition of ethyl acetate, followed by chromatography on paper in chloroform–formamide with quantitation by radiochromatographic scanning. The identity of the labeled product peaks 18OH-B and aldosterone was established by oxidizing two representative samples of each to their respective γ-lactones and demonstrating that the labeled peaks ran with the standard γ-lactones on paper chromatography (hexane–formamide, butyl acetate–formamide–water) and silica gel thin-layer chromatography in benzene–acetone 6:1.

Cholesterol side-chain cleavage (i.e., the conversion of cholesterol to pregnenolone) was studied in the system described by Simpson and Boyd (1967). Adrenal mitochondria were prepared from pooled rat adrenals and sonicated in distilled water for 10 min. One-half milliliter of sonicate containing mitochondria from 60 mg of adrenal was mixed with 1.5 ml of 0.027 M sodium phosphate buffer containing 20 μmol of magnesium sulfate, 0.75 mg of NADPH, 5.98 mg of glucose 6-phosphate, 0.5 unit of glucose-6-phosphate dehydrogenase, and 0.35 μCi of [4-^{14}C]cholesterol (56 mCi/mM). Incubation time was 40 min at 28 °C. The reaction was stopped and samples extracted by addition of ethyl acetate, followed by chromatography on paper in hexane–formamide, with quantitation by radiochromatographic scanning. Only one product peak was seen corresponding to the position of pregnenolone and progesterone which run together in hexane–formamide. Representative pregnenolone + progesterone areas were eluted, acetylated, and rerun in hexane–formamide. Only one radioactive peak was seen corresponding to the position of pregnenolone acetate; no progesterone peak was seen. Also, additional pregnenolone + progesterone areas from the original hexane–formamide chromatograms were crystallized to constant specific activity with pregnenolone.

21-Hydroxylation (i.e., conversion of progesterone to DOC) was studied using the system of Cooper and Rosenthal (1962). Pooled adrenals were homogenized in 0.25 M sucrose, 20 mg/ml, and centrifuged at 12 000g for 15 min at 4 °C. For incubation 0.5 ml of supernatant was added to 1.5 ml of 0.025 M glycylglycine buffer, pH 7.4, containing 0.12 M NaCl, 0.88 mM $MgCl_2$, 4.8 mM KCl, 50 μg of progesterone, 0.2 μCi of [4-^{14}C]progesterone (59 mCi/mM), 0.88 mg of NADPH, 1.86 mg of glucose 6-phosphate, and 0.5 unit of glucose-6-phosphate dehydrogenase. Incubation time was 15 min at 37 °C. The reaction was stopped and samples were extracted by addition of ethyl acetate followed by chromatography on paper in hexane:benzene (1:1)–formamide with quantitation by radiochromatographic scanning. Only one product radioactive peak was seen in this system which ran with DOC standard. Representative DOC peaks were eluted and either acetylated or oxidized with periodic acid and esterified with diazomethane. In each case the labeled peak ran with similarly treated authentic DOC standards on paper chromatography.

[1] Trivial names used were: progesterone (pregn-4-ene-3,20-dione); pregnenolone (3β-hydroxypregn-5-en-20-one); DOC or deoxycorticosterone (21-hydroxypregn-4-ene-3,20-dione); 18OH-DOC or 18-hydroxydeoxycorticosterone (18,21-dihydroxypregn-4-ene-3,20-dione); B or corticosterone (11β,21-dihydroxypregn-4-ene-3,20-dione); 18OH-B or 18-hydroxycorticosterone (11β,18,21-trihydroxypregn-4-ene-3,20-dione); aldosterone (11β, 21-dihydroxy-18-al-pregn-4-ene-3,20-dione).

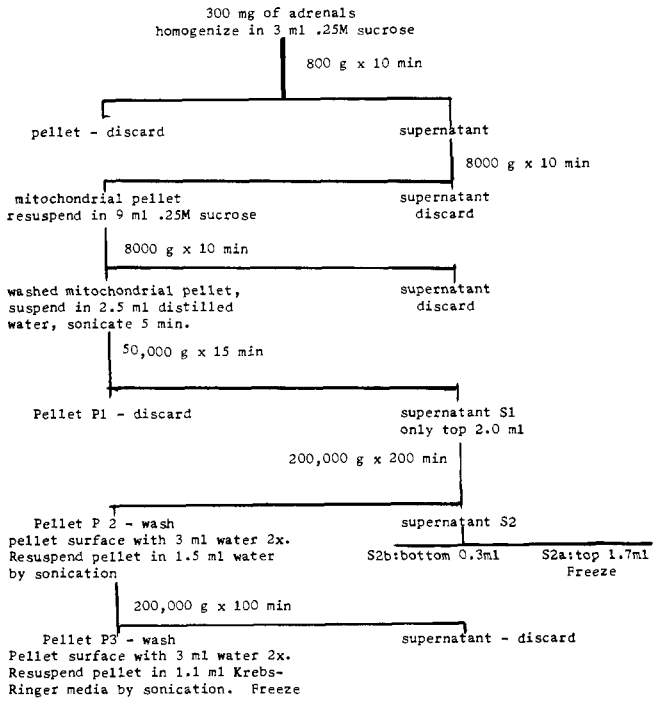

FIGURE 1: Flow diagram for isolation of rat adrenal mitochondrial cytochrome P-450 particles (pellet P3) and supernatant (S2a), the latter containing flavoprotein and nonheme-iron protein. Adapted from Omura et al. (1966).

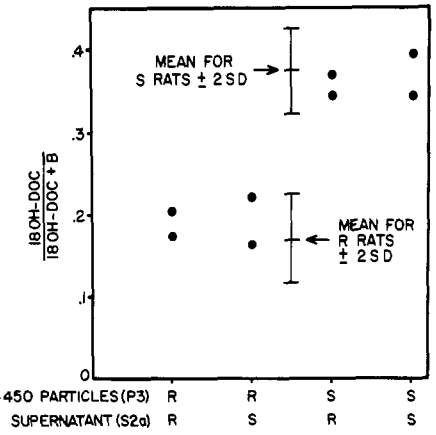

FIGURE 2: Adrenal mitochrondrial cytochrome P-450 particles (P3) and supernatant (S2a) isolated from S or R rats (Figure 1) were mixed in various combinations. For incubation 0.25 ml of double-strength Krebs-Ringer media, containing 0.65 mg of NADPH, 0.2 μCi of [4-^{14}C]DOC (60 mCi/mM), but no added unlabeled DOC, was mixed with 0.25 ml of S2a fraction and 0.15 ml of P3. Incubation was in air for 30 min at 37 °C. Extracted samples were chromotographed on paper in butyl acetate–formamide–water and scanned for radioactivity. The peak areas for DOC, 18OH-DOC, and B were determined and the ratio [18OH-DOC]/([18OH-DOC] + [B]) is plotted on the vertical axis. This ratio was previously shown to characterize S and R adrenal phenotypes (Rapp and Dahl, 1972). The mean ±2SD for the [18OH-DOC]/([18OH-DOC] + [B]) ratio for whole adrenal homogenates from S or R rats are shown in the figure.

All incubations were done in Warburg flasks with the center well removed. Adrenal enzyme preparation was placed in the side arm, and media were placed in the body of the flask. Flasks were gassed with 50 ml of various mixtures of CO, O_2, and N_2 prepared in gas burets. Incubations were done in a room lit only by red light to reduce the possibility for reversal of CO inhibition by light. Flasks were brought to temperature equilibrium in the water bath for 3.5 min before mixing the contents.

Adrenal mitochondria were sonicated and fractionated by ultracentrifugation into cytochrome P-450 particles and a supernatant fraction containing flavoprotein and nonheme-iron protein by a modification of the methods of Omura et al. (1966; Figure 1). All steps are carried out at 4 °C.

In order to study carbon monoxide (CO) inhibition of cytochrome-catalyzed reactions, theoretical considerations developed by Warburg 1949 were employed. Warburg's equations have been applied to steroid hydroxylations previously (Estabrook et al., 1963; Simpson and Boyd, 1967; Wilson and Harding, 1970a,b). If the competition between oxygen and carbon monoxide for an oxygen utilizing enzyme (E) is given by

$$E \cdot O_2 + CO \rightleftarrows E \cdot CO + O_2$$

then Warburg's partition constant is defined as:

$$K = \frac{[E \cdot O_2][CO]}{[E \cdot CO][O_2]} \quad (1)$$

If the E·CO complex is enzymatically inactive and if

$$n = \frac{\text{rate of reaction in presence of CO}}{\text{rate of reaction in absence of CO}} \quad (2)$$

then in the presence of CO, n is a measure of [E·O_2] and (1 − n) is a measure of [E·CO]. Substituting these values in eq 1 yields

$$K = \frac{n[CO]}{(1-n)[O_2]} \quad (3)$$

from which K can be determined experimentally.

If $r = CO/O_2$, then substituting r in eq 3 and rearranging gives

$$(1-n)/n = (1/K)r \quad (4)$$

which indicates that a plot of r vs. $(1-n)/n$ should be a straight line through the origin with slope $1/K$. Solving eq 4 for n gives:

$$n = K/(K+r) \quad (5)$$

From eq 5, n is obviously inversely related to r. If, however, r is held constant, then the right side of eq 5 is a constant and n is unchanged. Thus it should be experimentally verified that n is constant as long as the $[CO]/[O_2]$ ratio is constant regardless of the absolute concentrations of CO and O_2. Equation 5 also shows that, if $r = K$, then $n = 0.5$; i.e., Warburg's partition constant, K, is that ratio of $[CO]/[O_2]$ which causes a 50% reduction in the reaction rate obtained in the absence of CO.

Results

Adrenal mitochondria from S and R rats were fractionated as shown in Figure 1. Incubation of pellet P_3 (cytochrome P-450 particles) alone or supernatant S2a (flavoprotein + nonheme-iron protein) alone as the only source of enzyme in the Krebs-Ringer system with labeled DOC precursor yielded no detectable conversion. Mixing P_3 and S2a yielded a fully active enzyme preparation producing both 18OH-DOC and corticosterone (B). The adrenal phenotype, i.e., S type or R type, as defined previously (Rapp and Dahl, 1972) in terms of the [18OH-DOC]/([18OH-DOC] + [B]) ratio was determined entirely by the source (S or R rats) of cytochrome P-450 particles and was independent of the source (S or R rats) of the flavoprotein + nonheme-iron protein fraction (Figure 2).

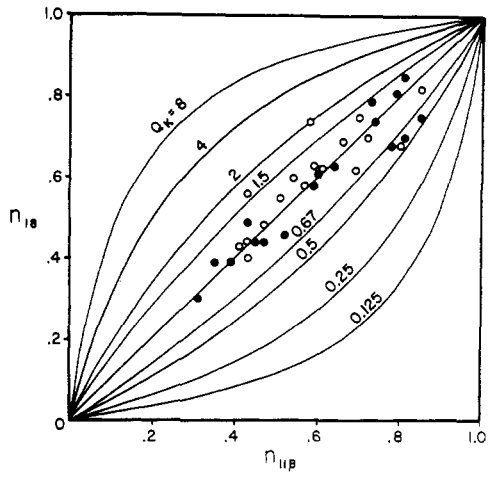

FIGURE 3: Relationship between CO inhibition of 18-hydroxylase and 11β-hydroxylase activity within the same flasks. Each point (n value) was determined by dividing the mean conversion for four flasks inhibited by CO, by the mean conversion for four flasks without CO. [CO]/[O_2] ratios varied from 2 to 99 and absolute O_2 concentration from 1 to 12%. For calculation of curves at various Q_k values, see text; $Q_k = K_{18}/K_{11\beta}$. (●) S rats; (○) R rats.

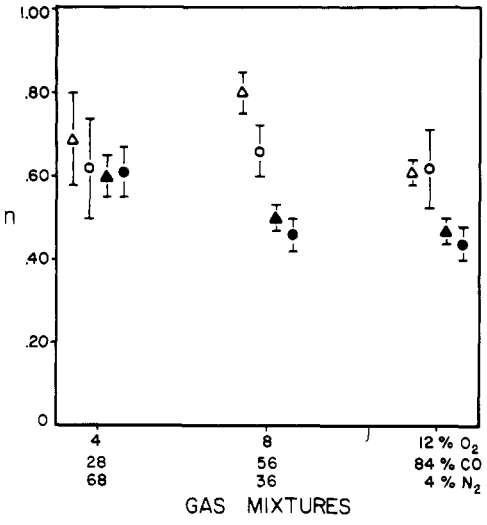

FIGURE 4: n vs. various gas mixtures with variable O_2 and CO concentrations but constant [CO]/[O_2] = 7. Each point is the mean of three or four determinations ± standard error. (●) S rats, 18-hydroxylation; (▲) S rats, 11β-hydroxylation; (○) R rats, 18-hydroxylation; (△) R rats, 11β-hydroxylation.

Preliminary studies with CO inhibition using [4-^{14}C]DOC precursor at various [CO]/[O_2] ratios strongly indicated that 18- and 11β-hydroxylase reactions (i.e., production of 18OH-DOC and B, respectively) were always identically inhibited. This fact is shown in Figure 3 where n values for 18-hydroxylase (n_{18}) and 11β-hydroxylase ($n_{11\beta}$) activities from the same flasks are plotted. If both activities are identically inhibited (i.e., if $n_{18} = n_{11\beta}$), the points will fall on a straight line through the origin. This of course can only be the case if K values from 18-hydroxylase (K_{18}) and 11β-hydroxylase ($K_{11\beta}$) are identical; i.e., $K_{18} = K_{11\beta}$. The relationship between n_{18} and $n_{11\beta}$ in the same flasks for the condition where $K_{18} \neq K_{11\beta}$ is found as follows. Solving equation 4 for r

$$r_{18} = K_{18}[(1 - n_{18})/n_{18}]$$

$$r_{11\beta} = K_{11\beta}[(1 - n_{11\beta})/n_{11\beta}]$$

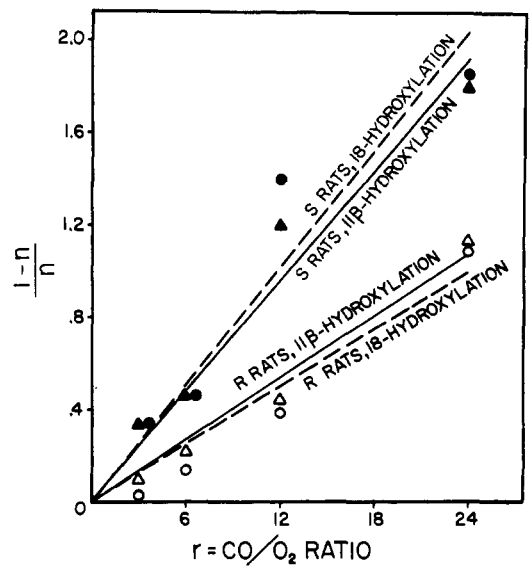

FIGURE 5: Plot of $(1 - n)/n$ vs. [CO]/[O_2] (see eq 4). Regression lines were calculated on the assumption that the lines pass through the origin. K values calculated from the relationship K = 1/slope of line were: S rats, 18-hydroxylase 11.6; S rats, 11β-hydroxylase 12.4; R rats, 18-hydroxylase 24.0; R rats, 11β-hydroxylase 22.2. (●) S rats, 18-hydroxylation; (▲) S rats, 11β-hydroxylation; (○) R rats, 18-hydroxylation; (△) R rats, 11β-hydroxylation.

but $r_{18} = r_{11\beta}$ in the same flask so that

$$K_{18}\frac{(1 - n_{18})}{n_{18}} = K_{11\beta}\frac{(1 - n_{11\beta})}{n_{11\beta}}$$

Solving for $n_{11\beta}$ in terms of n_{18} yields

$$n_{11\beta} = \frac{1}{1 + Q_k[(1 - n_{18})/n_{18}]} \quad (6)$$

where $Q_k = K_{18}/K_{11\beta}$. The expected relationships between $n_{11\beta}$ and n_{18} for various values of Q_k are also drawn in Figure 3. Inspection of Figure 3 suggests $Q_k = 1$ in both S and R rats.

In order to calculate K values for 18- and 11β-hydroxylase activities, it should be shown that the incubation system has the properties expected from the theoretical development. Figure 4 shows no consistent changes in n when the absolute gas concentrations are changed but the [CO]/[O_2] ratio is constant. This is the result expected from eq 5. But Figure 4 also suggests that 18- and 11β-hydroxylations are more easily inhibited by CO in S than in R rats. Data for n with variable [CO]/[O_2] ratio are given in Figure 5 which shows reasonably linear relationships based on eq 4. The data in Figure 5 also strongly suggest greater inhibition of both 18- and 11β-hydroxylation by CO in S compared with R rats.

In order to obtain statistically meaningful comparisons of K values for S and R rats, a different experimental design from that shown in Figure 5 was necessary. K was calculated using eq 3, each K value being derived from one CO inhibited and one uninhibited incubate. Using 24 samples per experiment, 12 K values were obtained: 6 for S rats and 6 for R rats. Such an experiment was repeated two to four times for a given hydroxylation reaction, and K values for S and R rats were compared by two-sided t tests utilizing the appropriate test (Sokal and Rohlf, 1969) where the variances of K values to be compared were unequal.

Before these experiments could be done, however, the theoretical question arose: what [CO]/[O_2] ratio should be

Table I: Values for Warburg's Partition Constant (K) for Various Adrenal Steroid Hydroxylase Reactions in S and R Rats.[a]

Reaction	Substrate	Product	Enzyme Source	K Values S Rats	K Values R Rats	t-test S vs. R Significance Level
18-Hydroxylation	DOC	18OH-DOC	Whole adrenal	11.4 ± 1.4	56.4 ± 13.7	0.005–0.01
11β-Hydroxylation	DOC	Corticosterone	Whole adrenal	11.0 ± 1.2	46.7 ± 11.7	0.01–0.025
Cholesterol side-chain cleavage	Cholesterol	Pregnenolone	Whole adrenal	4.89 ± 0.71	4.41 ± 0.79	>0.5
21-Hydroxylation	Progesterone	DOC	Whole adrenal	0.84 ± 0.094	0.97 ± 0.123	>0.5
18-Hydroxylation	Corticosterone	18OH-Cortico-sterone	Adrenal capsules	22.4 ± 4.5	49.2 ± 9.6	0.01–0.025
B → aldosterone	Corticosterone	Aldosterone	Adrenal capsules	8.49 ± 0.85	8.34 ± 0.94	>0.5

[a] K values given are mean ± standard error of 12 replicate determinations, or 24 replicates in the case of adrenal capsules. Experiments were done in the dark (red light) to reduce the possibility of light reversal of CO inhibition and concomitant increase in K value. Gaseous phase used for 18- and 11β-hydroxylation contained $[CO]/[O_2]$ = 14 and 4% O_2; cholesterol side-chain cleavage $[CO]/[O_2]$ = 5.25 and 16% O_2; 21 hydroxylation $[CO]/[O_2]$ = 0.8519 and 54% O_2; adrenal capsular enzymes $[CO]/[O_2]$ = 6.12, 14% O_2 (12 replicates) or $[CO]/[O_2]$ = 14, 6% O_2 (12 replicates). Rats used for studies on adrenal capsular zone (zona glomerulosa) were fed sodium deficient diet for 2–4 weeks before sacrifice to increase enzyme activities.

used in order to increase the chance of finding statistically different K values between strains if in fact these values are different? It is desirable to operate under the experimental constraint that the same $[CO]/[O_2]$ ratio be applied to both S and R samples so that both kinds of sample, handled in randomized order, are gassed from the same gas burets. K values for S and R rats can only be different, if n values for S and R rats at the same $[CO]/[O_2]$ ratio are different (see eq 3). Thus a logical $[CO]/[O_2]$ ratio (r) to compare S and R strains is that ratio which would maximize the difference in n values between strains. We wish to find the r value at which the difference $d = n_R - n_S$ is a maximum. From eq 5

$$n_R = K_R/(K_R + r),\ n_S = K_S/(K_S + r)$$

Substituting for n_R and n_S yields

$$d = [K_R/(K_R + r)] - [K_S/(K_S + r)] \qquad (7)$$

where $r \geq 0$; $K_R \geq K_S$; $d \geq 0$. If the first derivative of d with respect to r is set equal to zero, a solution occurs at $r = (K_R K_S)^{1/2}$. This critical point is a maximum since the second derivative at this point is <0. Preliminary data from Figure 5 indicate an r value on the order of 14–16 to be appropriate for comparing both 18- and 11β-hydroxylase between S and R rats. As more data accumulated (see below), this value was increased in later experiments up to 19.

Results of comparisons of partition constants for various adrenal steroid hydroxylations are given in Table I. Using whole adrenals and DOC precursor, K values for 18- and 11β-hydroxylation were identical within strains ($p > 0.5$ by t tests), confirming inferences made from Figure 3. K values between S and R strains were markedly different for both 18- and 11β-hydroxylation, confirming inferences made from Figures 4 and 5. The between-strain differences were, however, not a generalized phenomenon since K values between strains were identical for cholesterol side-chain cleavage, 21-hydroxylation, and the conversion of corticosterone to aldosterone. K values for 18-hydroxylase in the zona glomerulosa (adrenal capsules) also did show a strain difference (Table I).

It has previously been shown that S and R rats can be phenotypically classified in terms of the [18OH-DOC]/([18OH-DOC] + [B]) ratio formed in vitro from [4-^{14}C]DOC precursor, and that phenotypes are controlled by

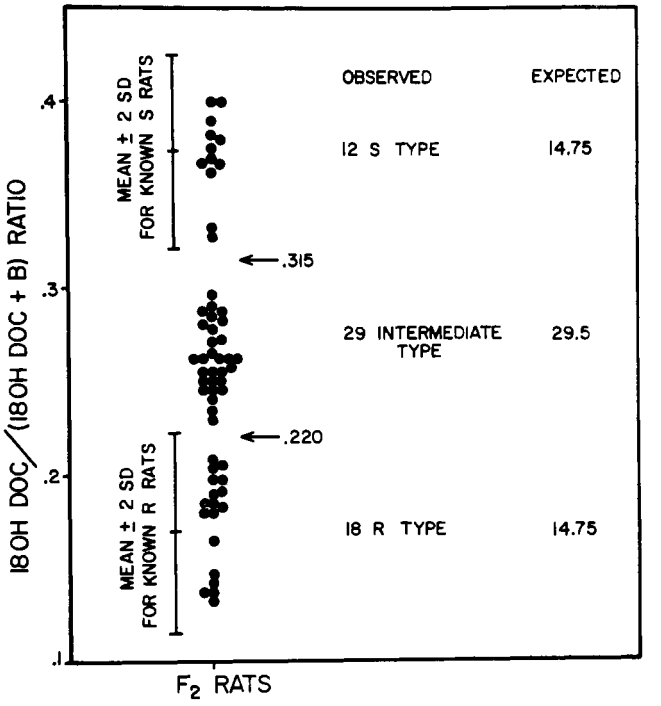

FIGURE 6: Scatter diagram of the [18OH-DOC]/([18OH-DOC] + [B]) ratio formed in vitro from ^{14}C-labeled DOC precursor for F_2 rats. The arrows indicate division points between phenotypes. Expected values are calculated on the premise that the phenotypes segregate in a 1:2:1 ratio which is compatible with the data in this figure and previous results (Rapp and Dahl, 1972). The observed and expected frequencies were not significantly different by a chi-square test ($0.5 < p < 0.75$).

a single genetic locus with two alleles inherited by co-dominance (Rapp and Dahl, 1972). If the different phenotypes and the different K values for 18- and 11β-hydroxylase activities are pleiotropic effects of the same genetic locus, then they should segregate together. Figure 6 shows the phenotypic classification of a large F_2 population derived by crossing S and R strains. The 18 R-type F_2 rats identified in Figure 6 were killed and their adrenals pooled, and the 12 S-type F_2 rats identified in Figure 6 were killed and their adrenals pooled. K values for these pools were determined concomitantly and the results are given in Table II. If K values segregated independently of the [18OH-DOC]/

([18OH-DOC] + [B]) adrenal phenotype, then the K values for pooled S and pooled R types from the F_2 population would be identical for 18- and 11β-hydroxylations. These K values (Table II) do show, however, the same difference seen when comparing parental S and R strains (first two lines Table I). This suggests control of both adrenal phenotype and K values by the same genetic locus.

Discussion

It is important to realize that the adrenal phenotypes (S, intermediate, and R types), characterized in terms of the [18OH-DOC]/([18OH-DOC] + [B]) ratio for the sake of removing variability (due to sex and experimental errors), arise from equal but opposite changes in 18- and 11β-hydroxylase activities.[2] But these reciprocal enzyme activity changes are controlled by a single genetic locus (Rapp and Dahl, 1972). It was originally speculated that this situation arose from the mutation of some structural mitochondrial protein controlling the stoichiometric assembly of 18- and 11β-hydroxylase enzymes into functional aggregates. This interpretation is no longer appropriate since the present data suggest a mutation in the enzyme molecules (cytochrome P-450) themselves.

Steroid hydroxylase reactions at different positions are thought to utilize different cytochromes P-450 since: (1) separations of mitochondrial cytochromes P-450 supporting cholesterol side cleavage and 11β-hydroxylation have been reported (Jefcoate et al., 1970; Isaka and Hall, 1971; Shikita and Hall, 1973; Ramseyer and Harding, 1973); (2) microsomal cytochrome P-450 supporting 21-hydroxylase activity does not require nonheme-iron protein (adrenodoxin) in electron transport whereas mitochondrial cytochromes P-450 do (Masters et al., 1971; Baron et al., 1972); (3) different steroid hydroxylations show different partition constants (K) for competitive binding of CO and O_2 (Wilson and Harding, 1970a,b). The data in Table I show different K values for different hydroxylation reactions. The marked and specific difference of K values for 18- or 11β-hydroxylase activities between S and R rat strains (Table I) then logically suggests different (i.e., mutated) cytochromes P-450 between strains. But Figure 3 and K values (Table I) also suggest that, within a strain, both 18- and 11β-hydroxylase activities are supported by the same cytochrome P-450, which violates the principle that hydroxylase activities at different positions have different cytochromes P-450. Still the evidence for the same cytochrome P-450 serving both 18- and 11β-hydroxylases is compelling since: (1) the change in K value from S to R for 11β-hydroxylase is identical with the change in K value from S to R for 18-hydroxylase; (2) these changes are apparently mediated by the same genetic locus. A model is needed which explains the fact that the same genetic locus which causes parallel changes in K values for 18- and 11β-hydroxylase enzymes, also changes 18- and 11β-hydroxylase activities in opposite directions.

Cytochromes P-450 bind specific steroid substrates with consequent well-known light spectral changes (for example see Cooper et al., 1968; Cammer et al., 1968; Isaka and Hall, 1971) and must therefore be involved in any final enzyme–substrate complex which has substrate as well as position specificity. One can speculate that a single cytochrome P-450 could participate in both 18- and 11β-hydroxylase activities but that position specificity was determined by interaction with other unidentified molecules (enzyme subunits?). The relative affinity of P-450 for either 18- or 11β-position specific factors would then determine the relative rates of 18- and 11β-hydroxylase activities and of course these relative affinities could change for different mutant forms of P-450. Different mutant forms of P-450 would account for different K values. An alternate hypothesis is that a single cytochrome P-450 can bind DOC substrate in two stereospecific ways, and that binding one way results in 18-hydroxylation and binding the other way results in 11β-hydroxylation. The relative probabilities of binding substrate in each way would correspond to the relative rates of 18- and 11β-hydroxylase activities. A mutant form of P-450, as well as showing altered CO and O_2 binding (altered K values), might also show altered affinities for binding substrate in either of two ways. Interestingly Michaelis constants (K_m), obtained previously for 18- and 11β-hydroxylase activities in S and R rats, showed the same symmetry seen with the partition constants; that is, K_m values for 18- and 11β-hydroxylase were essentially identical within a strain but were different between strains (Rapp and Dahl, 1971). This is not necessarily surprising since K_m values presumably reflect P-450 binding to DOC and partition constants reflect P-450 binding to CO and O_2.

In the above models, rats with intermediate-type adrenals would of course arise from 1:1 mixtures of S and R forms of the P-450 involved. Theoretical plots of eq 4 for such a mixed population of molecules do not yield a straight line and the K value for such a mixture of molecules would be expected to vary for different $[CO]/[O_2]$ ratios.

Nakamura et al. (1966) have shown that the species-specific ratios of 18- to 11β-hydroxylase activities in the rat and pig are controlled by mitochondrial P-450 particles and not by the soluble factors. Thus the result that P-450 particles control the strain specific ratio of 18- to 11β-hydroxylase activity was expected. Species differences in the structure of macromolecules presumably result from a series of mutations. Generalizing the present results in rats suggests that K values for 18- and 11β-hydroxylase activities should be identical within a species, but K values for 18- or 11β-hydroxylase compared between species which show different proportions of 18- and 11β-hydroxylase activities are

Table II: Values for Warburg's Partition Constant (K) for 18- and 11β-Hydroxylation in S and R Adrenal Phenotypes from an F_2 Population.[a]

	S Type	R Type	t-Test S-Type vs. R-Type Significance Level
18-Hydroxylation	13.6 ± 1.5	36.9 ± 9.8	0.01–0.025
11β-Hydroxylation	13.3 ± 1.5	30.4 ± 5.1	<0.005

[a] K values given are mean ± standard error of 18 replicate determinations. Whole adrenal homogenates were used and DOC was the labeled precursor. Gaseous phase contained $[CO]/[O_2] = 19$ and 5% O_2.

[2] The 18OH-DOC/(18OH-DOC + B) ratio was preferred to the 18OH-DOC/B ratio in genetic phenotyping. Although both parameters contain essentially the same information the former gives equal incremental changes between phenotypes whereas the latter does not. This scale effect arises from the equal but opposite changes in 18- and 11β-hydroxylase activity between phenotypes, which means that the sum 18OH-DOC + B is constant between phenotypes as shown previously (Rapp and Dahl, 1972).

likely to be different.

If the relative magnitudes of 18- and 11β-hydroxylase activities are determined by a single cytochrome P-450, possibly other branch points in steroidogenesis are similarly controlled. A logical place to look for such a phenomenon is the branch point where progesterone is either 21- or 17α-hydroxylated.

The production of 18OH-B in the rat has been variously reported to take place only in the zona glomerulosa (capsular zone) (Lucis et al., 1965) or in both capsular and inner zones (Sheppard et al., 1964; Baniukiewicz et al., 1968). Changes in dietary salt, however, cause parallel changes in both 18OH-B and aldosterone production by rat adrenal capsules in vitro (Rapp, unpublished observations), which strongly implies that 18OH-B is mainly a product of the zona glomerulosa. Secretion rates of 18OH-B and aldosterone were also parallel under a wide variety of conditions in humans (Ulick et al., 1964). We feel confident, therefore, that the studies reported herein on adrenal capsule tissue and 18-hydroxylation of B in rats on low sodium diet represent zona glomerulosa function, and that we are not merely seeing marginal 18-hydroxylation of B by contaminating inner zone 18-hydroxylase. The fact that the K values in Table I for the conversion of B to 18OH-B by capsular tissue showed the same mutation between S and R as seen for the conversion of DOC to 18OH-DOC by inner zones allows one to conclude that the same cytochrome P-450 is involved in 18-hydroxylations by both capsular and inner zones.

The biosynthetic pathway from corticosterone to aldosterone is thought to be B → 18OH-B → aldosterone and the conversion of 18OH-B → aldosterone is usually assumed to be a dehydrogenation. Although the conversion of labeled B → aldosterone is readily demonstrated, the conversion of labeled 18OH-B → aldosterone is inordinantly low (see Müller (1971) for review). To explain this, Greengard et al. (1967) have suggested that 18OH-B once formed either remains associated with an enzyme complex to be converted directly to aldosterone or dissociates from the enzyme to yield 18OH-B. If this is so, the 18OH-B accumulating in an incubation is not the precursor pool for aldosterone but is essentially an end product. In calculating K values for the reaction, we have therefore used the conversion of B to 18OH-B and not the sum of the conversions 18OH-B + aldosterone in applying eq 2 and calculating partition constants for 18-hydroxylation of B in Table I.

The partition constants in Table I indicate mutant cytochromes between S and R for the step B → 18OH-B but in the B → aldosterone conversion S and R are identical. This implies that two cytochromes are involved in converting B to aldosterone, presumably one for B → 18OH-B and one for 18OH-B → aldosterone. K values in Table I for the capsular zone are internally compatible with such a scheme. Since the overall conversion of B to aldosterone is more sensitive to CO inhibition (i.e., lower K value) than the step B → 18OH-B, this implies that the step 18OH-B → aldosterone is the more sensitive of the two steps and dominates the K value for the overall B to aldosterone conversion. Since it is the second step which is the more sensitive to inhibition, the first step still goes well enough under conditions inhibiting the second. Recently Marusic et al. (1973) showed that NADPH and air were required for the conversion of 18OH-B to aldosterone which also suggests that this reaction is a mixed function oxidase utilizing a cytochrome rather than a dehydrogenation. We speculate that the 18OH-B → aldosterone step is an hydroxylation of 18OH-B to form 18,18-dihydroxy-B, which would be unstable and decompose into aldosterone and water. Similar dihydroxy intermediates have been postulated for the formation of kaurenal from kaurenol in plants (Dennis and West, 1967) and in the aromatization of the A ring in estrogen biosynthesis by placental microsomes (Akhtar and Skinner, 1968).

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The Arrangement of Subunits in Cholera Toxin†

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ABSTRACT: Cholera toxin consists of five similar B subunits of apparent molecular weight about 10 600 and one A subunit (29 000) consisting of two peptides (A_1 23 000–24 000 and A_2 about 5500) linked by a single disulfide bond. Each B subunit also contains one internal disulfide bond which is readily reduced but is protected from carboxymethylation unless the reduced subunits are heated in urea. Tyrosine residues in A_1 and in B subunits are readily iodinated, but the intact B assembly does not react with iodine. Upon reaction with the cross-linking reagent dimethyl suberimidate, B subunits may be covalently connected to each other, to A_1 and to A_2. A_1 and A_2 may also be cross-linked. The B subunits are probably arranged in a ring with A on the axis. A_2 is required for the re-assembly of toxin from its subunits and may serve to hold A_1 on the B ring. The maximum activity of cholera toxin in vitro is obtained only when the active peptide, A_1, is separated from the rest of the molecule. Such separation, and the insertion of A_1 into the cytosol, must follow the binding of the complete toxin, through component B, to the exterior of intact cells. This binding increases the effective concentration of the toxin in the vicinity of the plasma membrane. Possible ways in which A_1 then crosses the membrane are considered in the Discussion.

Recent evidence has changed our concept of the mode of action of cholera toxin. This protein can interact with intact vertebrate cells of many types and elevate the activity of the membrane enzyme adenylate cyclase (reviewed by Pierce et al., 1971; Finkelstein, 1973). It was formerly considered possible that the toxin might act stoichiometrically while bound to, or present in, the plasma membrane. However, for the past 2 years it has been possible to activate adenylate cyclase in disrupted cells and thereby analyze in some detail the events following addition of toxin. It is becoming increasingly clear that the toxin must act enzymically, that it catalyzes some intracellular reaction between one or more cytoplasmic compounds and that portion of the adenylate cyclase which protrudes into the cytosol.

The most rapid activation of adenylate cyclase in vitro requires the presence, apart from cell membranes, of NAD, ATP, and an unidentified soluble cellular protein (Gill, 1975a, 1976). Cholera toxin then changes the adenylate cyclase in such a way that the enzyme's activity rises considerably (at least 30-fold in the case of pigeon erythrocyte ghosts), its stability increases (Gill and King, 1975), its response to epinephrine is enhanced (Field, 1974), and the extent of its stimulation by fluoride ions is reduced (Sharp et al., 1973; Field, 1974). None of these changes are reversed by avid antibodies directed against the toxin.

Of the several peptides that compose cholera toxin, only peptide A_1 is required for the activation of adenylate cyclase in vitro (Gill and King, 1975). The adenylate cyclase activity of a pigeon erythrocyte lysate starts to increase immediately upon the addition of peptide A_1 and continues to increase at a constant rate until a certain maximum activity is reached. The rate of activation is proportional to the A_1 concentration and significant activation can be achieved with less than one copy of A_1 per erythrocyte ghost (Gill, 1976).

Since peptide A_1 has little effect on intact cells, it is supposed that the remainder of the toxin molecule is involved in the insertion of A_1 through the plasma membrane and into the cytosol. This paper is concerned with experiments that relate to the arrangement of, and the interaction between, the various peptides of the toxin. The results suggest an arrangement of subunits in the toxin that, in turn, suggests possible ways in which A_1 may be assisted across a plasma membrane.

Methods

Cholera Toxin. Cholera toxin and choleragenoid were purified by R. A. Finkelstein, Ph.D., The University of

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