Adrenal Gland Steroid C-21 Cytochrome P-450 Reductase*

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ABSTRACT: The adrenal gland mitochondrial cytochrome P-450 reductase system (adrenal flavoprotein and adrenodoxin) is shown in this investigation to enhance steroid 21 hydroxylation in carefully prepared microsomal suspensions and acetone powder extracts. Examination of the reductase components separately reveals that the enhancement can be accounted for on the basis of the flavoprotein moiety alone. This implicates flavoprotein as an electron carrier in steroid 21 hydroxylation. While it seems likely that the mitochondrial and microsomal flavoproteins are identical, there remains a possibility that different, yet compatible, species may exist for the two organelles. The current studies indicate that adrenodoxin is inhibitory to microsomal 21 hydroxylation. This suggests that, either steroid 11β hydroxylation occurs at a different electron potential level than 21 hydroxylation, or that adrenodoxin relates to enzyme specificity factors of 11β hydroxylation as well as electron supply.

Consistent with these evaluations is the observation that methodology, which resolves relatively large quantities of adrenodoxin from adrenal gland mitochondria, gives rise to only insignificant quantities of this entity when employed with adrenal gland microsomes. It is concluded that appropriately prepared adrenal microsomes are essentially lacking in adrenodoxin.

Steroid 11β hydroxylation was early shown to occur in adrenal gland cortex mitochondria (Sweat, 1951) and to require TPNH (Sweat and Lipscomb, 1955; Grant and Brownie, 1955) and molecular oxygen (Sweat *et al.*, 1955, 1956; Hayano *et al.*, 1955a,b) as participants in the reaction. Shortly thereafter, 21 hydroxylation was shown to be associated with elements of the adrenal gland cortex endoplasmic reticulum (Plager and Samuels, 1954) and to also require TPNH (Ryan and Engel, 1956) and molecular oxygen (Cooper *et al.*, 1963; Nakano *et al.*, 1968). Both 11β- and 21-hydroxylation reactions have been classified in the category of mixed-function oxidases (Mason, 1957). While it has been suggested that steroid hydroxylations may occur as a generalized process, Mason (1957) has pointed out that nonspecific reactions characteristic of free radicals are not apparent in the steroid hydroxylations, and that these reactions must thus proceed under enzymic control in respect to both position and stereospecificity.

Several dissimilarities exist between the chemical groupings attacked by hydroxylating mechanisms: steroid 11β hydroxylation occurs on an alkyl ring; 17 hydroxylation at the juncture of an alkyl ring and an aliphatic chain; 21 hydroxylation at the terminal end of an aliphatic chain. Cholesterol hydroxylation at C-20 and C-22 occurs within the body of an aliphatic chain (prior to side-chain cleavage). In liver tissues, hydroxylations occur on aromatic ring compounds and unsaturated aliphatic chains such as squalene (initiation of cyclization to form lanosterol).

Distinctly different characteristics are observed between the several hydroxylation reactions in the response to various inhibitors. Adrenochrome strongly inhibits 11β hydroxylation (Sweat and Bryson, 1965) and cholesterol side-chain cleavage (Bryson and Kaiser, 1965), but has little influence on 17 or 21 hydroxylation. Metyrapone behaves similarly to adrenochrome in being specific for 11β hydroxylation, and having little effect on either 17 or 21 hydroxylation (Levy *et al.*, 1965). *p*-Mercuribenzoate is inhibitory to 11 and 21 hydroxylation, but less effective on 17 hydroxylation (Young *et al.*, 1965). The rate of 17 hydroxylation increases as the pH is increased between the ranges of 7 and 7.9, whereas 21 hydroxylation decreases through this range (Young *et al.*, 1965).

Marked differences between the nature of the 11- and 21-hydroxylating systems are revealed during processing with acetone and ammonium sulfate. Phosphate buffer extracts of adrenal cortex mitochondrial acetone powder may be readily fractionated into components of the 11β-hydroxylating system by ammonium sulfate. Sweat and Bryson (1962, 1964) reported resolution of two active fractions at 40 and 80% ammonium sulfate saturation and designated them, respectively, "factor 40" and "factor 80." Factor 80 was further resolved on a diethylaminoethylcellulose column with a severalfold increase in specific activity (Sweat, 1962). It since has been shown (Bryson and Sweat, 1968; Sweat and Young, 1966) to be almost pure nonheme iron–protein (NHIP–adrenodoxin) as characterized and identified by Suzuki and Kimura (1965), Kimura and Suzuki (1967), and Omura *et al.* (1965a). Factor 40 is now known to have consisted of cytochrome P-450 and a lightly bound adrenal flavoprotein (Sweat and Young, 1966) which have been characterized and shown to be participants (along with the NHIP) in a specialized electron transfer chain supporting 11β hydroxylation by Omura *et al.* (1965a) and cholesterol side-chain cleavage (Bryson and Sweat, 1968). Harding *et al.* (1964), earlier to these latter reports, suggested that cytochrome P-450 of rat adrenal mitochondria was likely associated with steroid 11β hydroxylation.

Extracts of microsomal acetone powders do not lend themselves to fractionation as do the mitochondria. Not only is

* From the Department of Obstetrics and Gynecology, University of Utah School of Medicine, Salt Lake City, Utah 84112. *Received April 21, 1969.* These studies were supported by Grant AM-01803 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U. S. Public Health Service, and by Grant P-248 from the American Cancer Society.

there a tenfold decrease in 21 hydroxylation in these preparations (in respect to fresh microsomes), but only traces of extractable nonheme iron–protein or flavoprotein moieties have been detected.[1] Previous to the present report, steroid 21 hydroxylation has been associated only with a cytochrome P-450 and TPNH. In the presence of TPNH, microsomal cytochrome P-450 complexes with carbon monoxide to form a characteristic difference spectrum (Omura *et al.*, 1965a). Carbon monoxide complexes with mitochondrial cytochrome P-450 only in the presence of added flavoprotein, nonheme iron–protein, and TPNH (Omura *et al.*, 1965a). As the reconstructed 11β-steroid-hydroxylating system requires relatively large quantities of nonheme iron–protein, and this is readily extractable from mitochondrial preparations, it is concluded by the present investigators that this moiety is essentially lacking in the microsomal system. It has been generally suggested that a flavoprotein is involved in microsomal 21 hydroxylation (Cooper *et al.*, 1965). From these foregoing observations, it is apparent that a number of basic aspects of the various hydroxylations remain unclear.

The literature is generally suggestive that requirement of nonheme iron–protein in hydroxylation reactions is not universal. Liver microsomes possess large quantities of cytochrome P-450 but evidence for nonheme iron–protein has not been found. In the present investigation, experiments indicate that the flavoprotein electron carrier isolated from adrenal cortex mitochondria markedly enhances steroid 21 hydroxylation in microsomal preparations. The compatibility of this entity with the microsomal steroid hydroxylation system suggests that both mitochondria and the endoplasmic reticulum may utilize the same species of flavoprotein. Concurrently with these observations it has been found that nonheme iron–protein distinctly inhibits steroid 21 hydroxylation in contrast to its obligatory requirement for steroid 11β hydroxylation. Figure 1 integrates these findings with background energy supply systems and contemporary concepts of adrenal steroid hydroxylation processes.

Experimental Procedure

Preparative Methods. Critical in the evaluation of the present experiments is the degree to which adrenal gland microsomal elements may be resolved from mitochondria. Criteria for the differential centrifugation of adrenal gland tissues have not been established. Examination of the literature relative to *in vitro* adrenal steroid synthesis reveals the employment of a wide latitude of centrifugation conditions extrapolated from the classical work of hepatic cell fractionation. Obstacles encountered in adrenal gland homogenate differential centrifugation stem not only from the nature of adrenal gland histology, which, in contrast to hepatic tissue, is coarse and stringy in consistency and consists of several specialized zones, but also the question of the degree to which mitochondria are fragmented and sedimented with the microsomal fraction. Electron microscopic examination of differentially centrifuged fractions prepared in this laboratory according to several differential centrifugation methods reported in the literature have consistently shown intercontamination of mitochondria and microsomes. Irrespective of back-washing, intact mitochondria persist to a significant degree up through centrifugation speeds greater than 17,500*g* for 20 min. As noted by Spiro and Ball (1961), who quote a number of investigators who have examined this problem, the lipid content of organelles likely plays a prominent role in the sedimentation rates of organelles. A number of other aspects of the differential centrifugation of adrenal gland tissue homogenates have also been examined in this laboratory. It has been found that neither morphological differences as monitored by electron microscopy nor quantitative differences in enzymic steroid conversion, as studied by *in vitro* incubation methods, are demonstrable between pestle and blade homogenation providing the latter process does not exceed 0.08 sec/ml for 20% homogenates. Further, as will be extended below and has been shown in other studies of this laboratory (R. B. Young, M. J. Bryson, and M. L. Sweat, 1969, unpublished data), the customary procedure of back-washing microsomal suspensions leads to preparations with less 11β hydroxylation not because of more thorough separation of mitochondria and mitochondrial fragments but because of extraction of components of the cytochrome P-450 reductase system. From these experiments it is apparent that either microsomes possess an innate mitochondrial-like cytochrome P-450 or are significantly contaminated with small mitochondria or mitochondrial fragments.

In some of the preparative procedures described below, it will be noted that the microsomal fractions are collected through much higher ranges of differential centrifugation than conventional procedures. With such procedures, up to 20% of the bulk of microsomes prepared by conventional procedures is sacrificed in the interest of greater homogeneity of the microsome fraction. However, even here, mitochondrial-like activity remains demonstrable when the mitochondrial cytochrome P-450 reductase system is reinstated.

Preparation of Fresh Adrenal Microsomes. Bovine adrenals (400 g) were transported on ice to the laboratory trimmed of fat and demedulated. The cortices were minced in a meat grinder, homogenized in two 625-ml aliquots of 0.25 M sucrose for 50 sec with a Waring Blendor (not over 0.08 sec/ml), and centrifuged at 700*g* for 15 min to remove nuclei and cellular debris. After centrifugation at 7000*g* for 30 min and 14,500*g* for 30 min to remove heavy and light mitochondria, respectively, the supernatant fraction was centrifuged at 105,000*g* for 1 hr. The resulting pellet was suspended in the 0.25 M sucrose, homogenized with a Teflon pestle homogenizer, and sedimented at 105,000*g* for 1 hr. After the washing process was repeated three additional times, the sedimented material was suspended in 0.25 M sucrose at a concentration of 8 mg of protein/ml.

Preparation of Mitochondrial and Microsomal Acetone Powders. Bovine adrenal glands collected within 30 min after slaughter were transported to the laboratory on Dry Ice and stored at −80°. All procedures were performed at 0–4°. The thawed adrenal (400 g) was demedullated, minced in a meat grinder, and added to 1 l. of 0.25 M sucrose. After

[1] Miyake *et al.* (1967) reported finding an electron spin resonance signal at G 1.94 characteristic of nonheme iron protein in microsomes sedimented between 9000*g* for 25 min and 104,000*g* for 60 min. However, the present authors have been unable to resolve this entity from microsomal preparations sedimented above 34,000*g* for 1 hr. It is felt that the NHIP[2] reported by the referred to authors can be accounted for in the presence of significant quantities of mitochondria in the fractions sedimented at the lower centrifugation speeds.

[2] Abbreviations used are: NHIP, nonheme iron protein; AFP, adrenoflavoprotein.

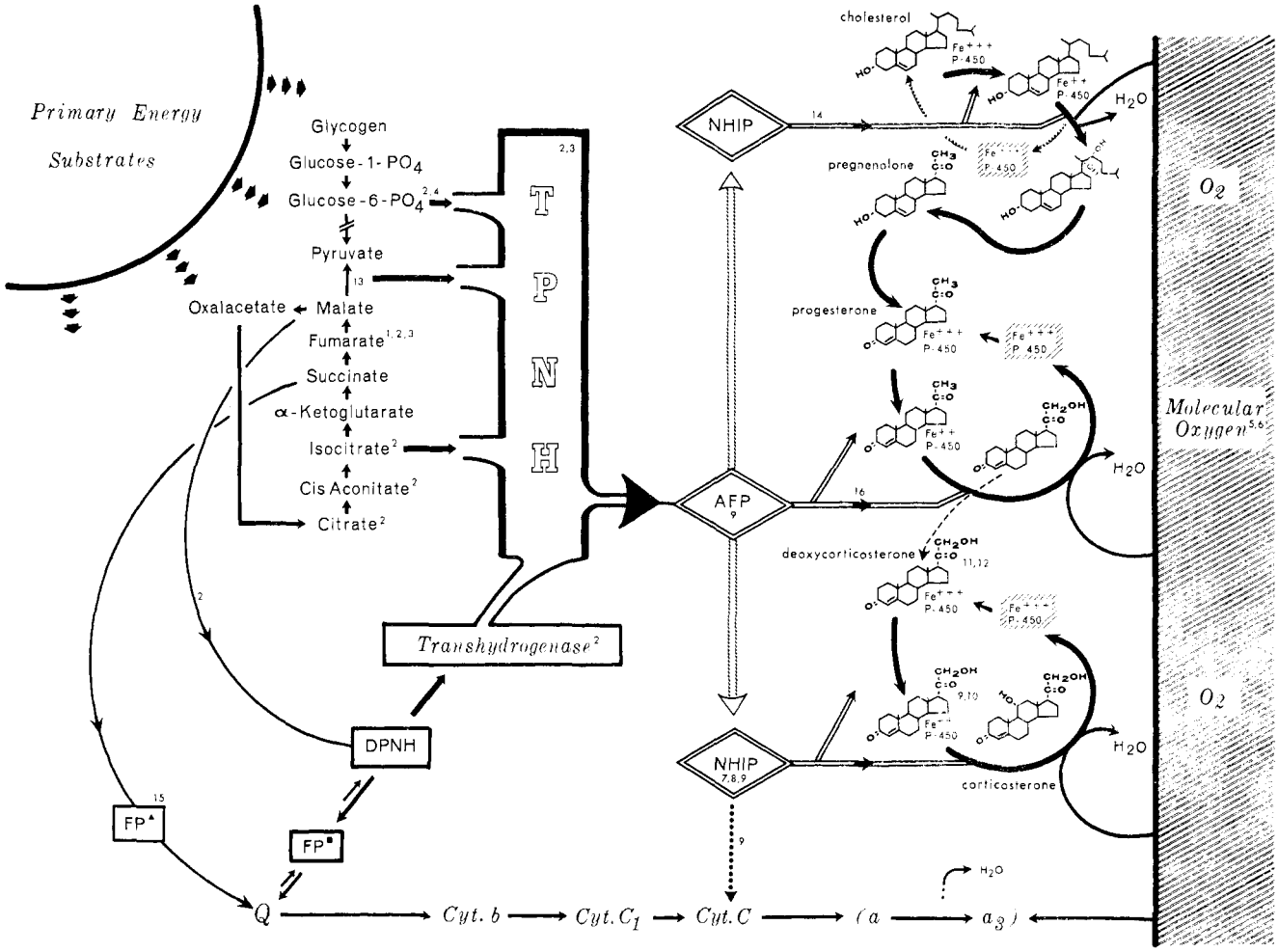

FIGURE 1: Integration of steroid 21 hydroxylation with classical and adrenal gland respiratory systems. Experiments of the present study associate the adrenoflavoprotein resolved by Omura *et al.* (1965a,b) with steroid 21 hydroxylation. This is graphically depicted in relationship to the source of the steroid precursor and reduction-oxidation equivalents relating to cytochrome P-450 in the above graph. The observations of Cammer and Estabrook (1967b) and Sweat *et al.* (1969) that the 11-hydroxylatable substrate associates with cytochrome P-450 in the oxidized state is considered as a prototype of steroid 21 hydroxylation. Footnotes refer to original observations associating particular steps with the over-all scheme: 1, Hayano *et al.* (1949); 2, Sweat (1951) and Sweat and Lipscomb (1955); 3, Grant and Brownie (1955); 4, Kelly *et al.* (1955); 5, Sweat *et al.* (1955, 1956); 6, Hayano *et al.* (1955a); 7, Kimura and Suzuki (1967); 8, Omura *et al.* (1965a); 9, Omura *et al.* (1965b); 10, Harding *et al.* (1964); 11, Cammer and Estabrook (1967b); 12, Sweat *et al.* (1969); 13, Grant (1956) Cammer and Estabrook (1967b) and Simpson and Estabrook (1969); 14, Bryson and Sweat (1968); 15, Cammer and Estabrook (1967a); and 16, current paper.

blade homogenization for 50 sec, the mixture was centrifuged for 10 min at 600*g* to remove the cellular debris (pellet discarded) and at 10,800*g* for 20 min to sediment the mitochondria.

The mitochondrial pellet was resuspended in a minimum quantity of water and slowly poured into 20 volumes of −5° acetone. After settling of the solid material, the acetone was decanted and additional volumes of acetone were added. The procedure was carried out three times and the desiccated mitochondria were collected by filtration. The acetone was removed in a vacuum desiccator, and the dry powder was stored at −80°.

To prepare microsomal acetone powder, the supernatant from the sedimented mitochondria was centrifuged at 34,000*g* for 1 hr to remove the light mitochondria and an intermediate fraction of microsomes. Microsomes of the supernatant fraction were then sedimented at 105,000*g* for 90 min. The pellet was resuspended in 0.25 M sucrose and recentrifuged at 105,000*g* for 90 min. The final pellet was then subjected to procedures identical with those described for the preparation of mitochondrial acetone powder.

Preparation of Adrenal Mitochondrial Flavoprotein (AFP). Mitochondrial acetone powder (10 g) was suspended in 100 ml of 0.001 M phosphate buffer (pH 7.4) and sonicated for 5 min in a MSE ultrasonic disintegrator at 10 kc. The suspension was then centrifuged at 32,000*g* for 1 hr (pellet discarded), 105,000*g* for 1 hr (pellet discarded), and finally at 368,000*g* for 90 min. The supernatant from the final centrifugation was introduced on a 2.0 × 30 cm chromatographic column containing DEAE-cellulose (0.42 mequiv/g) and equilibrated with 0.001 M phosphate buffer (pH 7.4). After a red band was eluted from the column with 0.001 M phosphate buffer, a yellow zone, consisting of flavoprotein, was eluted with 0.2 M NaCl in 0.01 M phosphate buffer. The slower moving brown zone (consisting primarily of NHIP) was eluted with 0.4 M NaCl in 0.01 M phosphate buffer.

Preparation of Adrenal Mitochondrial Nonheme Iron-Protein (F-80). Acetone powder (10 g) was suspended in 100

ml of 0.01 M phosphate buffer, sonicated in a MSE ultrasonic disintegrator at 10 kc for 5 min, and centrifuged at 33,000*g* for 1 hr. Saturated ammonium sulfate solution (pH 7.4) was slowly added to the supernatant solution to effect protein precipitation at 55%. The suspension was centrifuged at 10,000*g* for 30 min and the sediment was discarded. The clear supernatant fraction was further treated with ammonium sulfate to attain 80% saturation and the resulting precipitate was collected by centrifugation at 10,000*g* for 30 min, redissolved in 0.05 M phosphate buffer, and dialyzed against 0.025 M phosphate buffer for 24 hr to remove the ammonium sulfate. An alternative method (largely based on the method of Omura *et al.*, 1965a)) for preparing this component is to remove the cytochrome P-450 initially by centrifugation.

The above dialyzed solution was placed on a column as described above for the purification of the flavoprotein. Red pigments and trace quantities of flavoproteins were eluted with 0.2 M NaCl in 0.01 M phosphate buffer (pH 7.4) after which 0.4 M NaCl in 0.01 M phosphate buffer (pH 7.4) eluted the remaining nonheme iron–protein. Further removal of nonactive proteins and concentration of the nonheme iron–protein was accomplished by dialysis of the above fraction to remove sodium chloride, repeated column chromatography, and elution with gradient sodium chloride (0.1–0.4 M NaCl in 0.01 M phosphate buffer). The resolved dark brown, sulfur odorous product, on the basis of its distinctive precipitation at 80% ammonium sulfate saturation, was originally referred to by us as factor 80.[3] It has since been shown to be almost pure nonheme iron–protein (Sweat and Young, 1966; Bryson and Sweat, 1968) conforming to the preparations of "adrenodoxin" by Suzuki and Kimura (1965) and Omura *et al.* (1965a). Both this preparation and the flavoprotein have been purified free of intercontamination in this laboratory (Bryson and Sweat, 1968).

Preparation of Microsomal Cytochrome P-450. Microsomal acetone powder (1.5 g) was homogenized in 25 ml of 0.01 M (pH 4.7) phosphate buffer and sonicated at 20 kc for 5 min. After centrifugation at 198,000*g* for 2 hr, the supernatant was processed for resolution of the reductase components described above. The pellet was resuspended in 25 ml of 0.01 M phosphate buffer and recentrifuged at 198,000*g* for 2 hr. The final pellet (cytochrome P-450) was suspended in 0.01 M phosphate buffer at a concentration of 8.2 mg of protein/ml.

Incubation Method. Radiochemically pure progesterone-4-^{14}C (30 nmoles; specific activity 0.073 μCi/μmole) was incubated for 15 min at 37° with the appropriate concentration of enzyme preparation. The incubation medium consisted of 0.001 M TPN, 0.001 M glucose 6-phosphate, 0.5 kornberg unit of glucose 6-phosphate dehydrogenase, 0.0005 M $MgCl_2$, 0.04 M sodium–potassium phosphate buffer (pH 7.4), and the indicated amounts of metyrapone, flavoprotein, and NHIP in a total volume of 1 ml.

[3] As the activity of F-80 was originally observed through enzymic assay and the preparations employed were in a relatively high order of dilution, only the protein bands at 280 mμ were detected. However, when the preparations were concentrated, the dark brown color and sulfur odor were readily discernible and characteristic absorption peaks were observed at 325, 415, and 450 mμ. This spectrum was originally reported at the 46th Annual Meeting of the Federation of American Societies for Experimental Biology, April 14–18, 1962, Atlantic City, N. J. (Sweat, 1962).

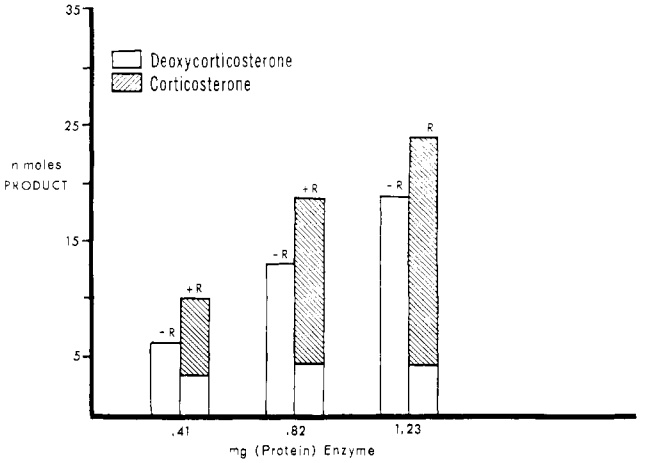

FIGURE 2: Enhancement of 21 hydroxylation in microsomal acetone powder extracts by the mitochondrial cytochrome P-450 reductase system. Chromatographically pure progesterone-4-^{14}C (30 nmoles) and 0.4, 0.82, or 1.23 mg of protein content of microsomal cytochrome P-450 preparation were incubated in the presence and absence of the mitochondrial cytochrome P-450 reductase system consisting of a mixture equivalent to 15 mg of flavoprotein and 15 mg of nonheme iron–protein. The incubation medium contained 0.5 nmole of $MgCl_2$, 0.04 M sodium–potassium phosphate buffer at pH 7.4, and a TPNH-generating system consisting of 1 nmole of TPN, 1 nmole of glucose 6-phosphate, and 0.5 kornberg unit of glucose 6-phosphate dehydrogenase. Samples were incubated 15 min at 37° in a total volume of 1 ml. The three sets of bar graphs represent the effect of 0.1 ml of the reductase preparation on incubation mixtures containing the designated quantities of the hydroxylating enzyme. "−R," without reductase. "+R," in the presence of reductase.

Incubations were terminated by the addition of 5 ml of chloroform and then extracted with seven additional 5-ml portions. After evaporation of the chloroform with the aid of a stream of nitrogen, the residue was chromatographed in the benzene–formamide system of Zaffaroni (1953). The products 11-deoxycorticosterone and corticosterone and unconverted substrate were located on the paper chromatogram by means of a Micromil window gas-flow scanner equipped with a ratemeter and an automatic recorder. Quantitation was accomplished by area calculation of the radioactive peaks on the recordings in comparison with standard chromatographs. Extent of conversion was determined by calculation of the quantity of substrate originally added and the final ratio between substrate and products recovered.

Results

Attempts to resolve a microsomal cytochrome P-450 reductase from adrenal cortex microsome acetone powder preparations have been unsuccessful. Significant enhancement of C-21 hydroxylation could not be shown upon recombination of chromatographed fractions of microsomal acetone powder extracts with the sedimented microsomal cytochrome P-450. Further, in contrast to mitochondrial acetone powder extracts in which the 11β-hydroxylating system is retained with only a small loss in specific activity, the microsomal extracts showed a marked drop in steroid 21-hydroxylation activity.

Incubation of progesterone-4-^{14}C with microsomal acetone powder extracts results in C-21 hydroxylation as shown in the "R−" bars of Figure 2. With the addition of mitochondrial

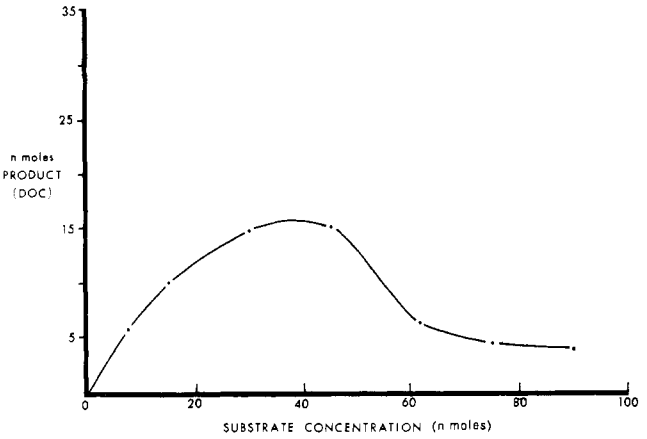

FIGURE 3: Evaluation of optimum substrate concentration. Adrenal microsomes (0.13 mg of protein) and the quantities of progesterone-4-^{14}C designated on the graph were added to separate flasks containing the medium described in Figure 2. The combined components were incubated for 15 min at 37° and processed as described in the text.

cytochrome P-450 reductase (AFP and NHIP), C-21 hydroxylation is significantly increased as indicated by the "R+" bars which record both deoxycorticosterone and corticosterone as total 21-hydroxylated products. A similar increase of C-21 hydroxylation associated with 11β hydroxylation has been observed previously (Young and Sweat, 1967). As the increased 21 hydroxylation occurs as the 11,21-dihydroxy product, a question arose as to whether the enhancement in C-21 hydroxylation is due to a direct influence of the reductase on the 21-hydroxylating system or is due to a synergistic effect of 11β hydroxylation, possibly by increasing the rate of release of deoxycorticosterone from the enzymic complex thus resulting in an increased turnover rate.

With the observation that the mitochondrial reductase system enhances 21 hydroxylation in extracts of microsomal acetone powder and that the microsomes themselves were devoid of significant quantities of an extractable reductase system, attention was redirected toward suspensions of intact microsomes, which, as referred to above, are tenfold more active than corresponding protein equivalents of acetone powder extracts.

To establish appropriate substrate concentrations and avoid characteristic steroid product inhibition, the capacity for 21 hydroxylation of the microsomal suspensions was examined as depicted in Figure 3. The typical product inhibition curve originally noted by Hayano and Dorfman (1953) is evidenced. The point of optimum activity without product inhibition chosen was 30 nmoles for the designated protein equivalent. Examination of various incubation time intervals with this quantity of enzyme and substrate verified that an incubation time of 15 min as employed in the experiment of Figure 3 aligned appropriately on the ascending arm of the time curve.[4]

[4] As preparations vary and deteriorate rapidly, appropriate kinetic data cannot be determined for each preparation. It has therefore been the practice in this laboratory to employ the above values as points of embarkation and confirm whether, through appropriate controls in the various experiments, approximately 50% transformation of the substrates occurs within a 15-min period.

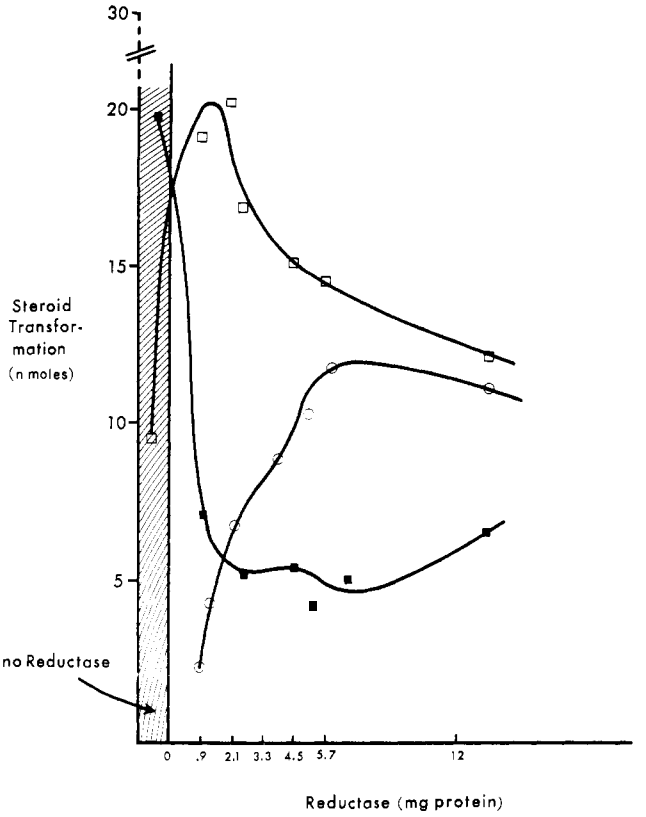

FIGURE 4: Progesterone metabolism in adrenal microsomes fortified with adrenal mitochondrial cytochrome P-450 reductase system. Adrenal microsomes (0.8 mg of protein), 30 nmoles of progesterone-4-^{14}C, phosphate buffer, the TPNH-generating system, and the designated quantity of mitochondrial cytochrome P-450 reductase (equal quantity of adrenal flavoprotein and nonheme iron–protein) were combined in a total volume of 1 ml and incubated for 15 min at 37°. The crosshatched area indicates quantities of steroids isolated after incubation without the reductase; the clear area, the quantities isolated after incubation in the presence of the reductase system. (■) Progesterone, (□) deoxycorticosterone, and (○) corticosterone.

Microsomes employed in experiments of Figure 3, even though sedimented above 14,500g for 30 min, showed significant 11β-hydroxylation activity. This activity was essentially eliminated by subjection to the four washings indicated in the preparation procedure. However, during processing, the specific activity for 21 hydroxylation was markedly decreased. To attain the recordings of Figure 4 an equivalent of 0.8 mg of protein was required in contrast to 0.13 of Figure 3.

Activity of the microsomal suspension in the presence of mitochondrial reductase is depicted in Figure 4. After 15-min incubation, approximately 9 nmoles of 11-deoxycorticosterone was formed (without the addition of mitochondrial reductase). Corticosterone could not be detected as a product. With the addition of 0.9 mg of protein reductase, formation of 17 nmoles occurred. Small quantities of corticosterone were also indicated. As the reductase was increased, total 21 hydroxylation progressively increased. 11β Hydroxylation occurred latent to 21 hydroxylation resulting in the formation of corticosterone. With 12 mg of the reductase preparation the quantity of the 11β-hydroxy product formed almost equaled that of deoxycorticosterone at the prescribed time interval. These data show a distinct response of 21 hydroxyla-

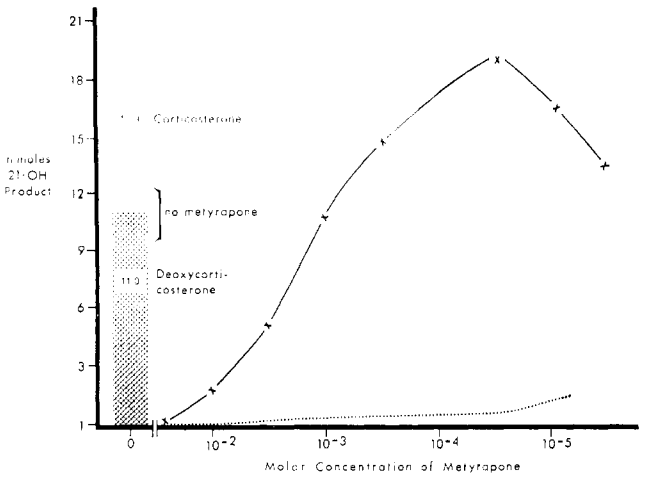

FIGURE 5: Influence of metyrapone on adrenal steroid 11β and 21 hydroxylation. To ensure the presence of adequate quantities of mitochondria and microsomes for both steroid 11 and 21 hydroxylation, a differentially centrifuged fraction sedimented between 8,000g and 17,000g (30 min) was employed. A 3.2-mg protein equivalent of the preparation was added to each flask containing the TPNH-generating system and 30 nmoles of progesterone-4-^{14}C in phosphate buffer. The designated concentration of metyrapone was added and the incubations were carried out at 37° for 15 min. The crosshatched areas indicate the proportional quantities of corticosterone and deoxycorticosterone formed in the control flasks with no metyrapone, the solid line indicates the quantity of deoxycorticosterone, and the broken line indicates the quantities of corticoste one formed in the presence of the designated concentrations of metyrapone.

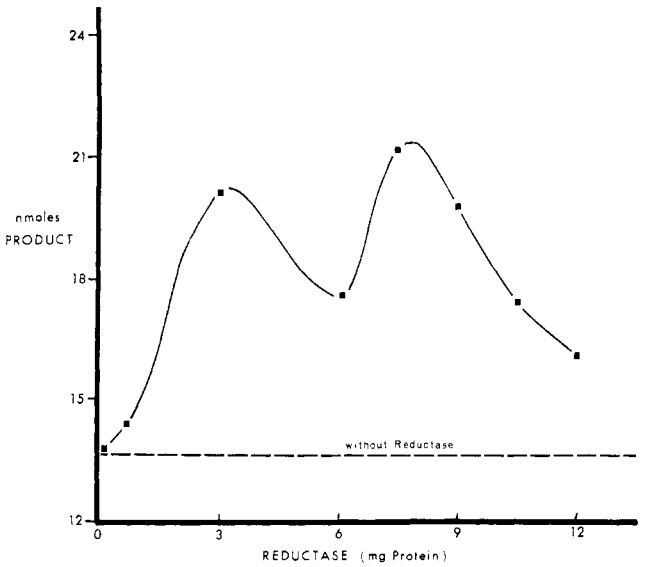

FIGURE 6: The effect of the mitochondrial reductase system on microsomal 21 hydroxylation. The incubations were carried out with 30 nmoles of progesterone-4-^{14}C, a 0.8-mg protein equivalent of the microsomal preparation and the indicated quantities of cytochrome P-450 reductase (AFP–NHIP, 1:1) in the previously described incubation medium containing 10^{-3} M metyrapone. The broken line indicates the level of deoxycorticosterone formation in the unfortified control incubations. The solid line indicates the influence of the reductase at various concentrations.

tion to mitochondrial cytochrome P-450 reductase. From these observations and the steroid-induced spectral studies (R. B. Young, M. J. Bryson, and M. L. Sweat, 1969, unpublished data), it is concluded that conventional methods of preparing adrenal microsomes effectively extract mitochondrial reductase from unwashed microsomal preparations and that, if the cytochrome steroid 11 P-450 moiety arises from mitochondrial fragments, these also are extracted free of reductase components during preparative procedures.

These observations prompted an examination of the influence of metyrapone, the extensively used 11β-hydroxylation inhibitor. To examine the range of effectiveness, a preparation consisting of both microsomes and mitochondria was examined with the results recorded in Figure 5. Whereas in the absence of added inhibitor, biosynthesis of corticosterone and 11-deoxycorticosterone represented, respectively, 38.0 and 38.2%, conversion of progesterone, addition of the inhibitor (5×10^{-4} M) depressed the formation of corticosterone to less than 5% and allowed over-all 21 hydroxylation to proceed with only a moderate inhibition. Under these conditions greater accumulation of deoxycorticosterone *per se* occurred due to blocking of 11β hydroxylation. As additional experiments indicated a moderate variation between preparations in response to metyrapone, the concentration employed in the subsequent experiments was increased to 1×10^{-3} M even though at this concentration a greater inhibition of total 21 hydroxylation could be expected. Effects of increasing the concentration of the reductase system in microsomes with this concentration of metyrapone is shown in Figure 6. A marked increase in 21 hydroxylation is demonstrated initially. Surprisingly, however, a biphasic curve was evidenced as the reductase was increased to a 6-mg equivalent of protein in the presence of metyrapone. Partial explanation of the biphasic response resolves in experiments represented by the data plotted in Figure 7. Nonheme iron–protein was added in varying quantities to incubation media containing the microsomal preparation, a constant quantity of flavoprotein, and 1×10^{-3} M metyrapone; and, conversely, flavoprotein was varied with a constant quantity of nonheme iron–protein and metyrapone. A marked inhibition is noted as the nonheme iron–protein concentration is increased. The biphasic response remains in evidence in the figure as a plateau near the intersection point of the two graphs and in this respect implies yet other implications. This phenomenon does not seem due to a relationship with the classical respiratory chain as the presence of cyanide does not alter the nature of the curve. Recently, Kraulis *et al.* (1968) have reported the reduction of metyrapone in adrenal tissue. The biphasic nature of the curves may thus be due to a competition between these two systems. The nature of the underlying reactions leading to this manifestation is under further study. It is noted that the quantity of nonheme iron–protein chosen for the constant component in the second experiment of the graph was sufficient to depress 21 hydroxylation significantly below the control values of the unfortified microsomes. This demonstrates a marked inhibitory effect of nonheme iron–protein against steroid 21 hydroxylation. The inhibition in these experiments is not overcome until the adrenal flavoprotein is increased to concentrations greater than 4.5 mg of protein. At a concentration of 7.75 mg of protein, 21 hydroxylation still did not attain the level of that in the first experiment. When the flavoprotein moiety is employed separately,

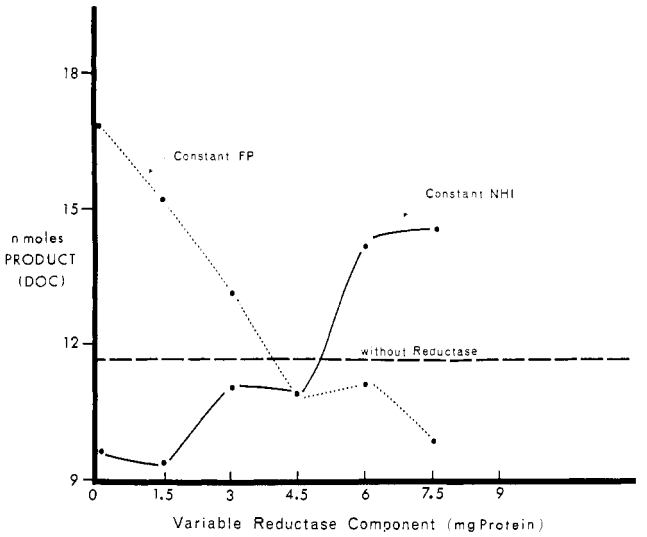

FIGURE 7: Influence of individual mitochondrial cytochrome P-450 reductase components on microsomal 21-steroid hydroxylation. Incubation was carried out in the presence of 10^{-3} M metyrapone and a 0.8-mg protein equivalent of microsomes in the medium described for Figure 2. Progesterone-4-^{14}C (30 nmoles) was employed as the substrate. One series of flasks contained a 4.5-mg protein equivalent of NHIP with varying amounts of flavoprotein (solid line). The other series contained a 4.5-mg protein equivalent of flavoprotein with varying amounts of NHIP (dotted line). Incubation was for 15 min at 37°. The broken line indicates the level of steroid transformation without addition of the reductase preparation.

a progressive curve plateauing with no indication of a secondary feed-off mechanism occurs.

Discussion

It is well documented that adrenal microsomes require only the addition of exogenous TPNH for reduction of their cytochrome P-450, whereas TPNH, adrenal flavoprotein, and adrenodoxin are required for reduction of mitochondrial cytochrome P-450. Liver microsomes exhibit characteristics similar to those of adrenal microsomes. These distinguishing characteristics have been the subject of inquiry by several laboratories. Omura *et al.* (1965a), as well as this laboratory (unpublished data), have attempted without success to substitute hepatic TPNH–cytochrome C reductase for the adrenoflavoprotein in mitochondrial cytochrome P-450 reduction. Miyake *et al.* (1967) carefully examined liver microsomes for the low-temperature electron spin resonance signal at G 1.94, characteristic of reduced nonheme iron–protein, and found no indication of its presence. They suggest that a simpler electron transport system is likely involved for liver microsomal cytochrome P-450 reduction than that for adrenal gland mitochondrial cytochrome P-450 reduction. However, conclusions by these authors that adrenal microsomes contain NHIP would seem subject to further evaluation as they employed a differential centrifuged fraction which would retain significant quantities of mitochondria. Attempts in this laboratory to resolve either a hepatic nonheme iron–protein or an adrenal microsomal NHIP by prototype methodology developed for the preparation of adrenal mitochondrial NHIP has met with failure. Recently, Miyake *et al.* (1968) have reported a preparation of a liver submicrosomal particle containing cytochrome P-450 free from cytochrome b_5. Although these particles contained quantities of a flavoprotein in essentially the same concentration as intact microsomes, the hepatic cytochrome P-450 was not reduced by the singular addition of TPNH. The retained flavoprotein was suggested to be the cytochrome C reductase associated with cytochrome b_5 reduction. When a crude preparation of adrenal gland mitochondrial cytochrome P-450 reductase was added to the submicrosomal particles, the cytochrome P-450 was reduced. However, the extent to which the adrenal mitochondrial cytochrome P-450 is retained in preparations treated with 20% ammonium sulfate saturation influenced the reported results does not seem to have been considered. On the basis of the present work, it would seem likely that if the mitochondrial reductase system is effective for hepatic cytochrome P-450 reduction, it would be restricted to the adrenal flavoprotein moiety.

In the present study, by employing metyrapone to inhibit mitochondrial activity of microsome preparations, it has been shown that the adrenoflavoprotein is compatible with microsomal steroid C-21-hydroxylation mechanism. Marked enhancement of C-21 hydroxylation occurs. Striking in the present study is the observation of the inhibition of this reaction by adrenodoxin. As indicated in the initial experiments, the complete mitochondrial cytochrome P-450 reductase system was found to increase 21 hydroxylation. Upon extended study, the enhancement could be accounted for on the basis of the flavoprotein moiety only. The above observations afforded new parameters for the evaluation of hydroxylating systems but do not answer the basic question of whether the adrenoflavoprotein is the penultimate carrier carrying electrons to cytochrome P-450 in microsomes or whether there exists another as yet undetected intermediate carrier corresponding to mitochondrial adrenodoxin. Although it would seem most likely that a common flavoprotein functions for both mitochondrial and microsomal cytochrome P-450, a possibility remains that separate species exist. The scheme in the beginning of the paper assumes a single species.

As adrenodoxin seems to be lacking in carefully fractionated microsomes and is not required for steroid 21 hydroxylation, it appears that electrons of the specialized adrenal respiratory chain may leave the chain at more than one E_0 potential. Further, as the ionic state of the basic oxygenating species for hydroxylation reactions remains in a state of conjecture, there remains the possibility that there are different oxygenator species for each hydroxylatable position. As pointed out earlier, a wide diversity in chemical groupings exist at sites where molecular oxygen is incorporated. It is apparent that with these diverse groupings, markedly different energy barriers relating to both stereoarrangement and chemical properties confront the activated oxygen atom during its presentation to the substrate. It would thus seem possible that to effect the final incorporation of oxygen, not only are specific enzymic entities required for each individual site of attack, but also appropriate and different electron potential levels.

Acknowledgments

The authors express appreciation to Helga Baumgart for technical assistance and to Brigitte B. Foster for assistance in preparing the manuscript and art work.

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