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Kinetic Studies on Substrate–Enzyme Interaction in the Adrenal Cholesterol Side-Chain Cleavage System†

Shlomo Burstein,* Jane Dinh, Nana Co, Marcel Gut, Heinz Schleyer, David Y. Cooper, and Otto Rosenthal

ABSTRACT: A study was made of the kinetics of the oxidative metabolism of (20*S*)-20-hydroxycholesterol, (22*R*)-22-hydroxycholesterol, and (20*R*,22*R*)-20,22-dihydroxycholesterol with adrenocortical heme protein P-450 preparations under various incubation conditions at relatively low substrate concentrations. When the substrate was added last to the complete system, first-order kinetics (within the experimental error) were observed until approximately 10% of the substrate was left; at longer times a decrease in the rate was seen. When the reaction was started by the addition of TPNH after the substrate had been preincubated with the heme protein P-450 system, polyphasic kinetics were observed with a much higher initial rate of substrate disappearance than that observed without equilibration of the substrate with the enzyme system. As a first-order approximation, after equilibration, the early part of the substrate disappearance curve observed could be expressed as a time function with two exponentials. From the parameters of this function could be obtained the first-order (and pseudo-first-order) rate constants of the interaction of the substrate with the enzyme system and of the reaction of the enzyme–substrate complex to irreversibly yield product. This approximation indicated that the latter rate constant was considerably higher than the rates of equilibration. The kinetics of conversion of cholesterol to pregnenolone followed a similar pattern. Under conditions without equilibration, the observed first-order rate constant of substrate disappearance thus appears to represent the overall (slower) pseudo-first-order rate of formation of the enzyme–substrate complex. The access of substrate to the enzyme system appears to be the rate-limiting step in these transformations. The theoretical approach presented may be useful for determining equilibration and reaction rates in other areas of enzymology where equilibration of an enzyme with substrate is possible prior to reaction commencement by the addition of a required cofactor.

In previous studies concerned with the elucidation of the mechanism of the conversion of cholesterol to pregnenolone with adrenocortical preparations, the kinetics of the metabolism of hydroxylated derivatives of cholesterol were studied at low substrate concentrations at which first-order kinetics appeared to hold (Burstein *et al.*, 1970a). In these studies the reaction was always commenced by adding the adrenal enzyme preparation to the substrate in the presence of a TPNH-generating system. In the course of investigations with heme protein P-450 preparations on the relationship between substrate-induced difference spectra of various oxygenated cholesterol derivatives and the enzymatic conversion of cholesterol to pregnenolone (Burstein *et al.*, 1972), it was noticed that the rate at which the spectra developed varied with the particular sterol and preparation studied. Although no definitive correlation between the magnitude (or affinity relationships) of the difference spectra and the enzymatic rates was observed, the rates at which the spectra developed indicated that some interaction between the sterols and the enzyme system was occurring at a measurable rate. This gave us the idea that perhaps the kinetics of substrate metabolism may be different if, prior to reaction commencement, the substrate is allowed to equilibrate with the enzyme preparation. Such an experimental design could be achieved, for example, by preincubating the substrate with the heme protein P-450 supplemented with nonheme iron and flavoprotein, under which conditions no reaction occurs, and by starting the reaction by adding the TPNH-generating system in the presence of oxygen.

This paper describes kinetic studies on the oxidative metabolism of (20*S*)-20-hydroxycholesterol,[1] (22*R*)-22-hydroxycholesterol, and (20*R*,22*R*)-20,22-dihydroxycholesterol with

† From the Division of Steroid Chemistry, Institute for Muscle Disease, New York, New York 10021 (S. B., J. D., and N. C.), from the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545 (M. G.), from the Harrison Department of Surgical Research, University of Pennsylvania, Philadelphia, Pennsylvania 19104 (H. S., D. Y. C., and O. R.), and from the Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania 19104 (H. S.). *Received February 24, 1972.* Supported by U. S. Public Health Service Grant CA 11291 and the Muscular Dystrophy Associations of America (S. B.), by U. S. Public Health Service Grant AM 03419 and AEC Contract AT (30-1) 918 (M. G.), U. S. Public Health Service Grant AM 04484 (D. Y. C.), and National Science Foundation Grant 8366 (D. Y. C.).

[1] The nomenclature used here conforms with the rules known as the IUPAC–IUB 1967 Revised Tentative Rules for Steroid Nomenclature (*Steroids 13*, 277 (1969)). Thus, the previous terminology for 20α-hydroxycholesterol and (22*R*)-20α,22-dihydroxycholesterol has been changed to (20*S*)-20-hydroxycholesterol and (20*R*,22*R*)-20,22-dihydroxycholesterol, respectively.

heme protein P-450, which allowed some insight into substrate–enzyme interactions under conditions under which actual transformation of these hydroxylated cholesterol derivatives to pregnenolone occurred. Some studies on the kinetics of the conversion of cholesterol to pregnenolone are also reported.

Experimental Procedure

Heme Protein P-450 Preparations and Incubation. Most of the studies reported here were done with bovine acetone-dried powder preparations made by precipitation with acetone of adrenocortical mitochondria suspended in distilled water as described previously (Burstein *et al.*, 1968). The purified heme protein P-450 preparation was a 0.2% Triton N-101 extract of acetone- and butanol-treated sonicated bovine mitochondria prepared according to the method of Schleyer *et al.* (1972). The cholesterol content of the soluble portion of the acetone-dried powder preparations used in this study was 1 nmole/mg of protein, while that of the purified heme protein P-450 preparation was 0.8 nmole/nmole of heme protein P-450. The acetone-dried powder was homogenized in 0.02 M potassium phosphate buffer and centrifuged at 50,000*g* for 1 hr; the clear, "soluble" supernatants were used. These soluble preparations were stored at −20° and all comparative experiments were done on the same day after defrosting at room temperature. The defrosted preparations were kept in ice and, as a rule, the various comparative incubations were performed within 1 to 2 hr of each other. Incubations were all done in a Dubnoff metabolic shaker at 23° in a total volume of 3.0 ml in the presence of a TPNH-generating system and air as previously described (Burstein *et al.*, 1968). In addition, the incubation mixture contained purified adrenal nonheme iron protein at a concentration of 0.8 to 1.3×10^{-6} M and adrenal flavoprotein at a concentration of 4×10^{-8} M, which were prepared from bovine adrenal glands by a procedure similar to that previously described by Omura *et al.* (1967). The concentration of the adrenal nonheme iron protein was the same in all experiments for which comparisons were made. The radioactive substrates used were those previously described (Burstein *et al.*, 1970b; Chaudhuri *et al.*, 1970) and were again purified by partition and thin-layer chromatography prior to use. The specific activities of the [1,2-^{3}H]cholesterol, (20*S*)-20-[16-^{3}H]hydroxycholesterol, (22*R*)-22-[1,2-^{3}H]hydroxycholesterol, and (20*R*,22*R*)-20,22-[1,2-^{3}H]dihydroxycholesterol were 12.6, 10.6, 22.3, and 37.7 Ci/mmole.

Determination of Substrate Disappearance Curves. This was done by removing at appropriate time intervals 0.1-ml aliquots (with the aid of an Eppendorf 100 microliter pipet) from the incubation mixture into glass-stoppered tubes containing ethyl acetate and 20 μg each of the unlabeled substrate and of [4-^{14}C]pregnenolone (approximately 2000 cpm). After shaking, the two layers were filtered through a column of glass wool (approximately 3 cm long) loosely packed in a Pasteur pipet. The glass-stoppered tubes were quantitatively washed with ethyl acetate and filtered through the glass wool column. This procedure removed the aqueous layer and afforded a relatively clean extract which was then chromatographed on thin-layer silica gel G plates (250 μ) which contained Rhodamine 6G prepared according to the procedure of Avigan *et al.* (1963) as described previously (Burstein *et al.*, 1970b). The steroid spots were visible on these thin-layer plates by the naked eye or more clearly when viewed under ultraviolet (366 nm) light. For the separation of the substrates from their products the system hexane–ether–glacial acetic acid (85:15:10)(modified after Simpson and Boyd, 1967) was used. This afforded a complete separation of (20*S*)-20-hydroxycholesterol and of (22*R*)-22-hydroxycholesterol from the (20*R*,22*R*)-20,22-dihydroxycholesterol and pregnenolone as well as of the dihydroxycholesterol from pregnenolone. To separate the latter two compounds, however, 2 to 3 runs in the same system were required. For this reason, in most studies on the metabolism of the (20*R*,22*R*)-20,22-dihydroxycholesterol the system benzene–ethyl acetate (67:34) was used, in which a complete separation of the dihydroxycholesterol from pregnenolone was obtained in 1 run This system, however, was not good for the resolution of the other sterols from their products. The efficiency of the separation was checked by partition chromatography as described previously (Burstein *et al.*, 1970b) and also by the fact that the recovered substrates were devoid of pregnenolone (the major product) as evidenced by the lack of ^{14}C in the eluates of the chromatographic spots. The chromatographic zones were removed from the plates by loosening the demarcated silica gel with a spatula followed by vacuum suction into a Pasteur pipet filled to approximately 4 cm with glass wool. The sterols were eluted by pouring 2 ml of methanol into the Pasteur pipets placed directly over scintillation vials containing 10 ml of 0.4% of 2,5-diphenyloxazole in toluene. After the addition of 2.0 ml of dimethyl sulfoxide and 4.0 ml of distilled water, the vials were thoroughly shaken. They were counted in a Packard Model 3320 Tricarb scintillation spectrometer after keeping the vials in the counter at 5° for 1–2 hr during which time the two layers were sufficiently separated to afford stable counts. The technique used gave efficiencies for both single- and dual-label counting that were not significantly different from those observed with the scintillation mixture alone. The great advantage of this procedure, therefore, was that it did not require evaporation of the methanol or separation from the dye, which stayed in the aqueous layer. The efficiency of tritium counting was 54.5% with a background of 23 cpm when single-label counting was done. Dual-label counting was done at an efficiency of 26.3% for ^{3}H and 62.7% for ^{14}C with corresponding backgrounds of 20 cpm and 13 cpm, and a carry-over of ^{14}C into the ^{3}H channel of 0.089 and no significant carry-over of the ^{3}H into the ^{14}C channel.

The radioactivity present in the incubation medium was determined by removing 0.1-ml aliquots directly into scintillation vials filled with the toluene scintillation mixture and counting in the two-phase procedure outlined above.

Procedural loss was determined from the ^{14}C recovered in the pregnenolone area and all the results were accordingly adjusted. This implied the assumption that, up to the elution procedure from the plates, the various compounds studied behaved in a manner precisely equivalent to that of the added [^{14}C]pregnenolone. This was ascertained in several comparative experiments in which the recoveries of the pregnenolone and that of the various substrates were not found to be significantly different. Furthermore, the technique used afforded virtually a quantitative recovery, and the adjustment for technical loss rarely exceeded 10%.

The counting error was below 5% and in most cases 1-min counts were sufficient to determine the substrate disappearance curves. All the counts per minute given in this paper were expressed for the higher (tritium) efficiency of 54.5%.

The results were expressed as fraction of substrate left at the specified times. Curve fitting was done using the experimental data obtained *after equilibration* with substrate.

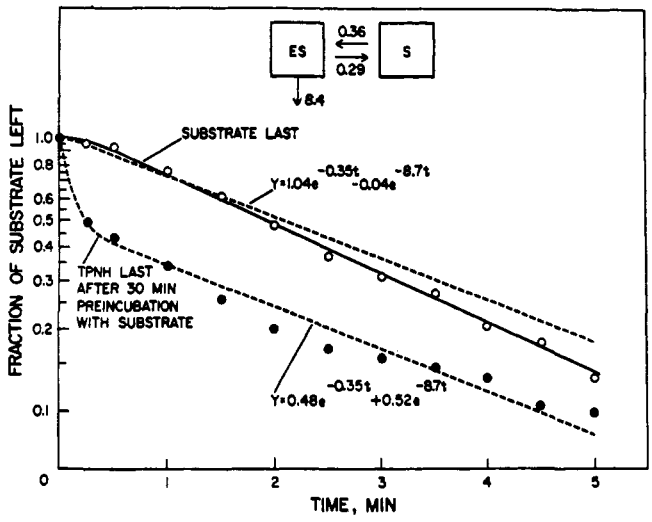

FIGURE 1: Kinetics of (22*R*)-22-hydroxycholesterol metabolism with an adrenocortical acetone powder preparation. ○, Substrate and TPNH (in 0.2 ml) were added after the heme protein P-450 system was incubated in air for 30 min (A). ●, TPNH (in 0.2 ml) was added after the substrate was incubated with the heme protein P-450 system in air for 30 min (B). The initial substrate concentration in A and B was 99,000 cpm/ml (3.8×10^{-9} M) and 110,000 cpm/ml (4.2×10^{-9} M), respectively. The protein concentration was 0.095 mg/ml. (- - -) Theoretical y functions. (—) Line through the experimental (○) points; a corresponding line through the (●) points was not drawn.

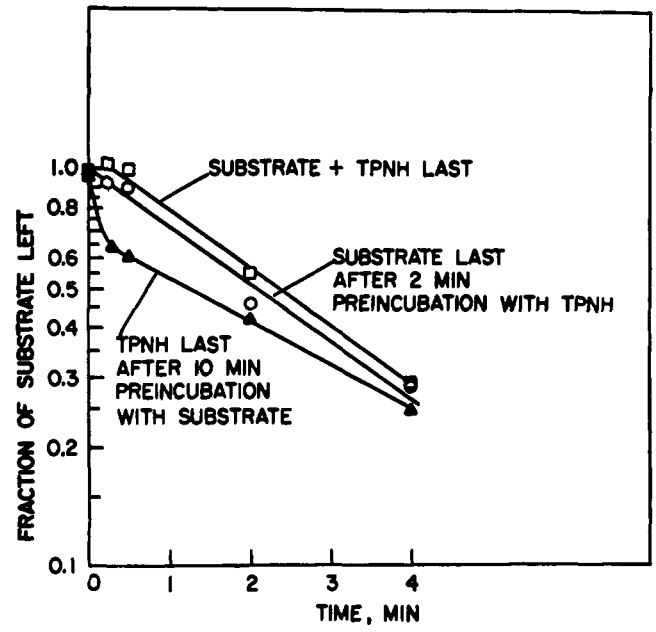

FIGURE 2: Kinetics of (22*R*)-22-hydroxycholesterol metabolism with a purified heme protein P-450 preparation. □, Substrate and TPNH (in 0.2 ml) were added after the heme protein P-450 system was incubated in air for 10 min (A). ○, Substrate (in 0.1 ml) was added last after the heme protein P-450 system was incubated for 8 min, followed by incubation with TPNH (added in 0.1 ml) for 2 min (B). ▲, TPNH (in 0.2 ml) was added after the substrate was incubated with the heme protein P-450 system in air for 10 min (C). The initial substrate concentrations in A, B, and C were 80,000 cpm/ml (3.1×10^{-9} M), 110,000 cpm/ml (4.2×10^{-9} M), and 100,000 cpm/ml (3.8×10^{-9} M). The heme protein P-450 concentration was 10^{-8} M (0.008 mg of protein/ml).

The double exponential function

$$y = Ce^{-\alpha t} + De^{-\beta t}$$

approximating the experimental results was obtained using the slope of the slower linear (logarithmic) portion of the curve and its intercept on the y axis (for example: $\alpha = 0.35$ min^{-1} and $C = 0.48$, Figure 1). With the aid of an Olivetti 101 computer the best second exponential was then obtained by iteration using $(1 - C)$ for the parameter D. Because sufficient points of the early phase could not be obtained with the methodology employed, the curve-fitting technique entailed a $\pm 30\%$ uncertainty regarding β and, for this reason, also of the calculated constant k_3 (see Discussion). The constants k_1' and k_2 were less sensitive to this uncertainty and the best fit afforded a possible spread of $\pm 5\%$ for these constants. The theoretical function expected for the experimental design in which the enzyme was not equilibrated with substrate prior to reaction commencement was obtained from α and β using the expression $y = [\beta/(\beta - \alpha)]e^{-\alpha t} + [\alpha/(\alpha - \beta)]e^{-\beta t}$ (see eq 8, Discussion).

The counts and the molar concentration of the substrate present at the beginning of the incubation are given in the legends to the figures.

Results

Kinetic Observations. No significant substrate metabolism occurred in the presence of Tween 80, nonheme iron protein, and flavoprotein alone, without added heme protein P-450, and no blank correction was, therefore, necessary. The products with the monohydroxycholesterol derivatives studied were pregenenolone and (20*R*,22*R*)-20,22-dihydroxycholesterol; with the latter sterol, pregnenolone was the only product identified. As previously shown, these products accounted (within 10%) for the consumed substrate (Burstein *et al.*, 1970a,b). In this paper emphasis was placed on the kinetics of metabolism of the hydroxylated cholesterol derivatives, and the formation of the products was not reported. Because of the difficulties in determining accurately the relatively slow disappearance rates of cholesterol, studies with this substrate were done by determining the formation of the main product—pregnenolone.

Representative results of the kinetics of (22*R*)-22-hydroxycholesterol metabolism under two experimental conditions differing in respect to whether or not the heme protein P-450 system was preincubated with the substrate are shown in Figure 1. It may be seen from Figure 1 that, whereas, without substrate equilibration, the rate of disappearance followed a first-order logarithmic decay (within the experimental error), in the experiment in which the sterol was allowed to equilibrate with the heme protein P-450 system, a sharp initial drop in the substrate concentration occurred followed by a much slower rate of disappearance. In the experiment in which no equilibration was allowed, there appeared to be a lag of about 15 sec before the first-order decay curve was realized. The kinetics of the metabolism of (22*R*)-22-hydroxycholesterol with the purified heme protein P-450 preparation under three different conditions is given in Figure 2. It appeared that when the substrate was added last after the heme protein P-450 system was preincubated with TPNH for 2 min there was no lag of 15–30 sec before the logarithmic substrate decay phase ensued. Although the relatively small lag observed when the substrate was not preincubated with the heme protein P-450 was seen on numerous occasions, in some experiments this

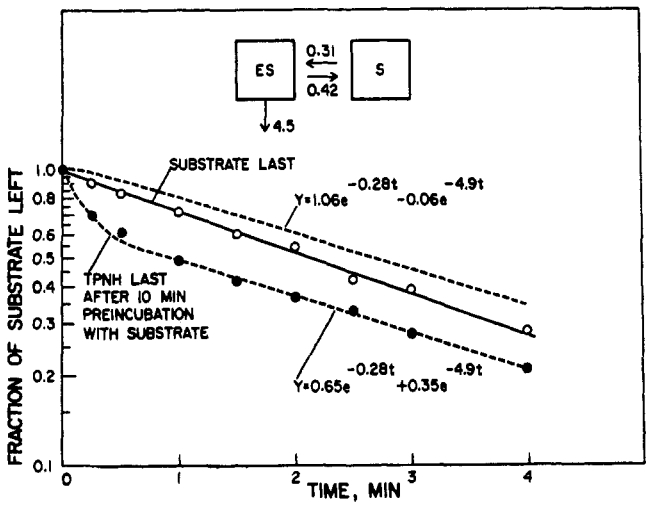

FIGURE 3: Kinetics of (20*R*,22*R*)-20,22-dihydroxycholesterol metabolism with an adrenocortical acetone-dried powder preparation. O, Substrate and TPNH (in 0.2 ml) were added after the heme protein P-450 system was incubated in air for 10 min (A). ●, TPNH (in 0.2 ml) was added after the substrate was incubated with the heme protein P-450 system in air for 10 min (B). The initial substrate concentration in A and B was 32,000 cpm/ml (7.2×10^{-10} M) and 37,000 cpm/ml (8.3×10^{-10} M), respectively. The protein concentration was 0.05 mg/ml. (- - -) Theoretical *y* functions. The meaning of the solid line is given in the legend of Figure 1.

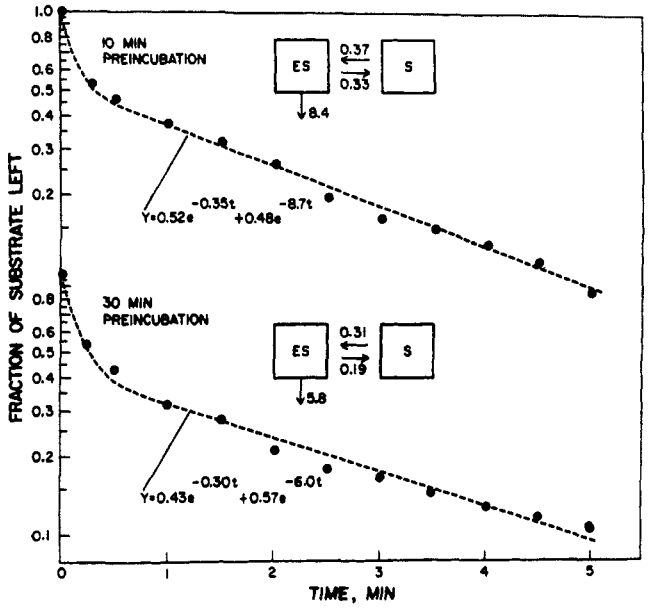

FIGURE 5: Effect of duration of substrate equilibration on the kinetics of (22*R*)-22-hydroxycholesterol metabolism. After the specified equilibration times, TPNH (in 0.2 ml) was added. The initial substrate concentrations in the experiments depicted in the upper and lower curves were 116,000 cpm/ml (4.4×10^{-9} M) and 107,000 cpm/ml (4.1×10^{-9} M), respectively. The protein concentration was 0.095 mg/ml. (- - -) Theoretical *y* functions.

lag was not clearly apparent and it was uncertain whether this was sometimes obscured by the experimental error of the determinations or because of technical mixing differences from one experiment to the other after the addition of the TPNH (see for example Figure 3). The initial drop, however, observed after substrate equilibration was always seen. The behavior of the (20*S*)-20-hydroxycholesterol and that of the (20*R*,22*R*)-20,22-dihydroxycholesterol followed a similar pattern to that observed with the (22*R*)-22-hydroxycholesterol and representative results are given in Figures 3 and 4. Again after equilibration there was a significant sharp initial drop in the substrate concentration. Although with the (20*S*)-20-hydroxycholesterol (Figure 4) in the experiment in which the substrate was added last the heme protein P-450 system was preincubated with TPNH for 2 min prior to substrate addition there still appeared to be a slight lag.

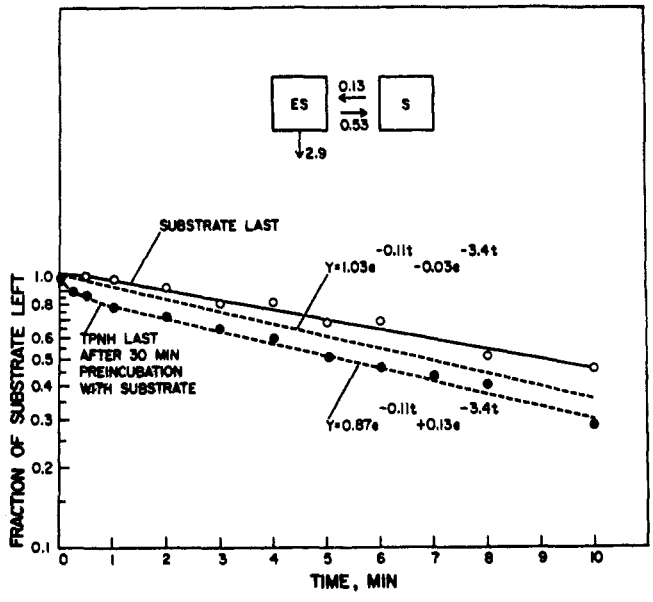

FIGURE 4: Kinetics of (20*S*)-20-hydroxycholesterol metabolism with an adrenocortical acetone powder preparation. O, Substrate (in 0.1 ml) was added after the heme protein P-450 system was incubated for 28 min by itself and for 2 min with TPNH (added in 0.1 ml). (A) ●, TPNH (in 0.2 ml) was added after the substrate was incubated with the heme protein P-450 system in air for 30 min. (B) The initial substrate concentration in A and B was 43,000 cpm/ml (3.3×10^{-9} M) and 42,000 cpm/ml (3.2×10^{-9} M), respectively. The protein concentration was 0.098 mg/ml. (- - -) Theoretical *y* functions. The meaning of the solid line is given in the legend of Figure 1.

The effect of equilibration time on the kinetics is illustrated in Figure 5 for the (22*R*)-22-hydroxycholesterol. The two experiments illustrated in this figure were done with the same heme protein P-450 preparation on the same day. (In these two experiments a different preparation was employed from that used in the experiments described in Figure 1 which were done on another day, and the similarity of the two sets of results is fortuitous.) It appeared that longer equilibration periods resulted in a somewhat larger initial drop in the substrate disappearance. In addition, the kinetics following this initial stage of reaction appeared to deviate more from first-order kinetics the longer the duration of the equilibration. However, more data would be required to definitively establish the effect of equilibration time on the kinetics.

As could be seen from the figures, with the exception of (20*S*)-20-hydroxycholesterol, the first-order rate was somewhat larger when the substrate was added last than the slower first-order rate observed (after the initial rapid phase of substrate disappearance subsided) when substrate equilibration was allowed. This effect was even more pronounced at higher heme protein P-450 concentrations or activities. This is illustrated in Figure 6 for the (20*R*,22*R*)-20,22-dihydroxycholesterol with a more active preparation. As may be seen, the two curves crossed each other because of the diminishing

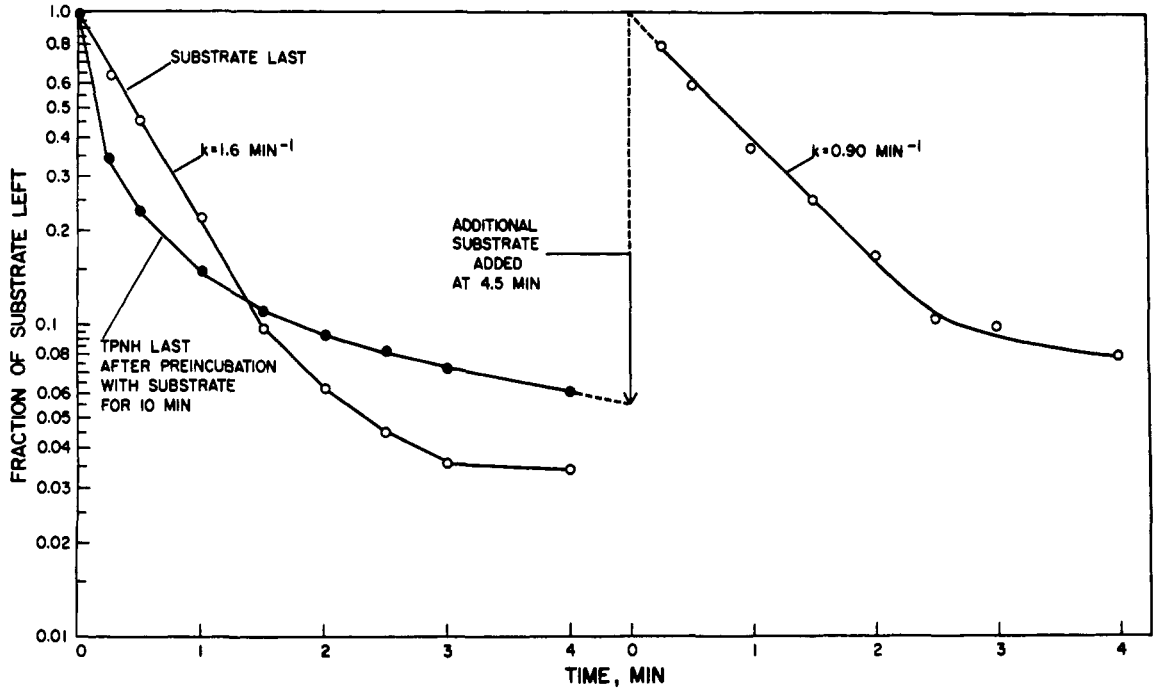

FIGURE 6: Kinetics of (20*R*,22*R*)-20,22-dihydroxycholesterol metabolism with an adrenocortical acetone-dried powder preparation. ○, Substrate and TPNH (in 0.2 ml) were added after the heme protein P-450 system was incubated in air for 10 min (A). ●, TPNH (in 0.2 ml) was added after the substrate was incubated with the heme protein P-450 system in air for 10 min (B). The initial substrate concentration in A and B was 32,000 cpm/ml (7.2×10^{-10} M) and 24,000 cpm/ml (5.4×10^{-10} M). To experiment B additional substrate was added (to give approximately 30,000 cpm/ml) after 4.5 min had elapsed from the start of the reaction, and the disappearance of substrate was measured again. In this case the exact initial substrate concentration could not be determined and the first-order rate constant given was calculated from the curve starting at 15 sec. The protein concentration was 0.095 mg/ml.

rate in the experiment in which the substrate had been equilibrated. It is noteworthy that in the experiment in which the reaction was commenced by adding the substrate last the first-order rate also decreased when the substrate concentration decayed to about 10%. The reason for the slowdown was investigated by adding more substrate after the reaction had proceeded for 4.5 min (Figure 6). As may be seen, the first-order rate constant was decreased to 56%, but it is evident that the system was still able to metabolize the substrate at a considerably higher rate than that seen from the kinetics at a time when the substrate concentration was exhausted below 10%.

The effect of equilibration on the kinetics of conversion of cholesterol to pregnenolone with the acetone powder preparation and with the purified heme protein P-450 is shown in Figures 7 and 8. As may be seen with both preparations, when the substrate was equilibrated with the enzyme system the initial rate of pregnenolone formation was considerably higher than that realized when the substrate was added last. In the latter case there was a 30-sec lag before the formation of the pregnenolone became linear. This lag would not be usually observed unless samples were taken at these initial short time intervals. The (slower) linear portion of the kinetics observed when the substrate was not equilibrated with the enzyme prior to reaction commencement with TPNH appeared to be almost parallel to the linear rate observed when the substrate was added last. These results are similar to those described for the disappearance curves of the hydroxylated cholesterol derivatives.

Discussion

The interpretation of the observations on the substrate disappearance curves under the experimental conditions used of necessity depends on what kind of a model system of the enzyme reaction is postulated. As yet, the heme protein P-450 system of adrenal mitochondria has not been extensively purified to allow the assumption of a definitive model. Likewise, the mode of the interactions with the other enzyme components such as the nonheme iron protein and the flavoprotein of the steroid hydroxylase multienzyme system is not sufficiently understood. The exact physical state in the medium of the sterol which was dispersed with Tween 80 is likewise not precisely known.[2] These questions are further complicated by the fact that these reactions must be carried out in an oxygen atmosphere.

The most significant observation presented here, namely, that equilibration of the substrate with the heme protein P-450 system resulted in much higher initial rates of substrate disappearance (or in the case of cholesterol to pregnenolone formation) than when the substrate was added last, may be explained by assuming that the rate of equilibration of the enzyme with the substrate is slower than the rate of reaction of the enzyme–substrate complex to give product irreversibly. After the enzyme–substrate complex formed by equilibration has reacted, one observes the slower rate of the enzyme–substrate complex formation. If the substrate is added last, it is the latter rate that is actually seen and except for a short (15–30 sec, experimentally sometimes difficult to determine) lag, the reaction appears to be of first-order kinetics. The very

[2] This question has been previously discussed by K. Savard and L. L. Engel (*cf.* Burstein and Gut, 1971). Dr. Engel pointed out the possibility that the observed first-order rates of transformation (measured at that time without substrate equilibration) may represent the rates of release of the precursors from their micellar form rather than the enzymatic rates.

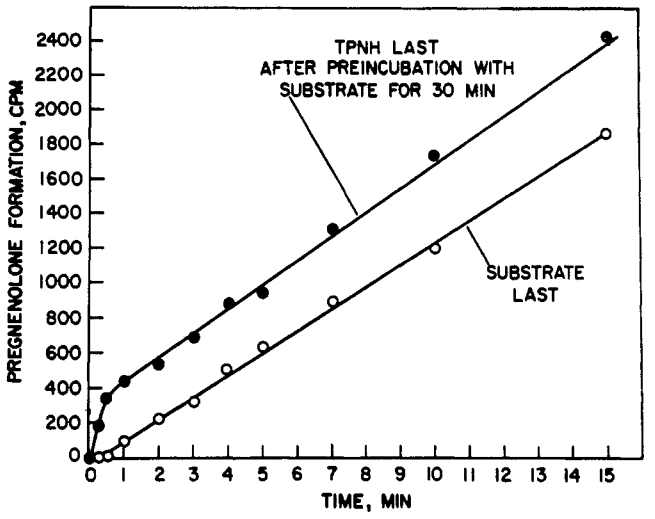

FIGURE 7: Kinetics of conversion of cholesterol to pregnenolone with an adrenocortical acetone-dried powder preparation. ○, Substrate (in 0.1 ml) was added after the heme protein P-450 system was incubated in air for 28 min, followed by incubation for 2 min with TPNH (added in 0.1 ml) (A). ●, TPNH (in 0.2 ml) was added after the substrate was incubated with the heme protein P-450 system in air for 30 min (B). The initial substrate concentration in A and B was 359,000 cpm/ml (1.15×10^{-7} M) and 347,000 cpm/ml (1.14×10^{-7} M). The molar concentration brought into account the endogenous cholesterol concentration. The protein concentration was 0.091 mg/ml.

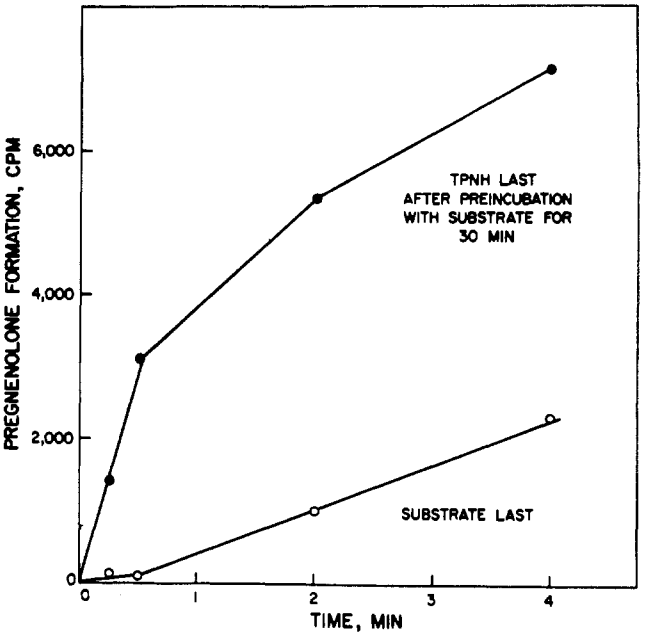

FIGURE 8: Kinetics of conversion of cholesterol to pregnenolone with a purified heme protein P-450 preparation. ○, Substrate (in 0.1 ml) was added last after the heme protein P-450 system was incubated in air for 28 min, followed by incubation for 2 min with TPNH (added in 0.1 ml) (A). ●, TPNH (in 0.2 ml) was added last after the substrate was incubated with the heme protein P-450 system in air for 30 min (B). The initial substrate concentration in A and B was 366,000 cpm/ml (3.2×10^{-8} M) and 389,000 cpm/ml (3.4×10^{-8} M), respectively. The heme protein P-450 concentration was 10^{-8} M (0.008 mg of protein/ml).

rapid initial reaction when equilibration is allowed is most probably the result of a complicated series of reactions which requires, on the one hand, the formation of an "active oxygen" moiety and, on the other, its reaction with the enzyme-bound steroid moiety within a topological arrangement in which reaction between these components may occur. It is generally assumed that the formation of the "active oxygen" entails the reduction of the nonheme iron protein by TPNH, mediated by a flavoprotein reductase; the reduced nonheme iron protein in turn reduces the heme protein P-450 to the ferrous state. That nonheme iron protein is required in 11β-hydroxylation and the cholesterol side-chain cleavage reaction has been shown by Cooper *et al.* (1970a) and Huang and Kimura (1970). There is reasonably good evidence that the reduced nonheme iron protein is the electron source in the final irreversible step leading to the hydroxylated product (Schleyer *et al.*, 1971). During equilibration the steroid most likely binds to the heme protein P-450 and as soon as the system is reduced, reaction of the bound steroid with oxygen ensues. A general hypothetical scheme of some of these possible reactions is represented in Figure 9. It is presumed that during preincubation the substrate which is added to the solution equilibrates with the heme protein P-450 compartment at rates expressed by $\bar{k}_1$ and $\bar{k}_2$. Inside the compartment the substrate combines with the enzyme $[E]^{3+}$ [3] to form an enzyme–substrate complex $[ES]^{3+}$ ($\bar{k}_1$, $\bar{k}_2$).

The rate of equilibration in the experimental design used in this study (when preincubation with substrate was done with $[E]^{3+}$) is a complicated function of the specific rates $\bar{k}_1$, $\bar{k}_2$, $\bar{k}_1$, and $\bar{k}_2$. When TPNH is added further equilibrations occur involving the specific rates $\bar{\bar{k}}_1$, $\bar{\bar{k}}_2$, $\bar{\bar{k}}_1$, and $\bar{\bar{k}}_2$, causing a perturbation of the originally established equilibrium. The *initial* rapid rate observed when reducing equivalents are added is likewise a complicated function of all the rate constants involved in the formation of the hypothetical moiety $[ESO_2]^{2+}$ from $[ES]^{3+}$ and its specific rate of decomposition $\bar{k}_3$ to the product [SO]. This function involves the rates $\bar{\bar{k}}_1$, $\bar{\bar{k}}_2$, $\bar{\bar{k}}_1$, and $\bar{\bar{k}}_2$ as well as the specific rates of the oxido-reduction of $[E]^{3+}$ and $[ES]^{3+}$, and the reaction of $[E]^{2+}$ and $[ES]^{2+}$ with oxygen. Because relatively little knowledge of these various rates is currently available, a quantitative representation of our data can only be made on a first approximation basis. To this end a simple model is assumed, indicated in Figure 9 by the dashed lines and again given in eq 1. In this

$$[E] + [S] \underset{k_2}{\overset{k_1}{\rightleftarrows}} [ES] \downarrow k_3 \qquad (1)$$

simplified scheme the equilibration rates k_1 and k_2 represent overall second-order and first-order rate constants to express the rates of substrate–enzyme interaction. These, of course, are complicated functions of $\bar{k}_1$, $\bar{k}_2$, $\bar{k}_1$, and $\bar{k}_2$. Although strict evidence is unavailable at this time, there are indications (*cf.* Schleyer *et al.*, 1971) that $\bar{k}_1$ and $\bar{k}_2$ may be relatively much larger than $\bar{k}_1$ and $\bar{k}_2$, thus probably making the latter constants—the rates of communication between the two compartments—the "rate-limiting" steps. In this treatment, the specific rate constant k_3 represents the irreversible formation of product [SO] and is again a complicated function of some of the other rate constants. From the model studies of Cooper *et al.* (1970b) and Ando and Horie (1971) with adrenocortical

[3] For convenience the term enzyme [E] is here used to represent the heme protein P-450. It represents the "free" moiety as well as that which may be combined with the nonheme iron or flavoprotein.

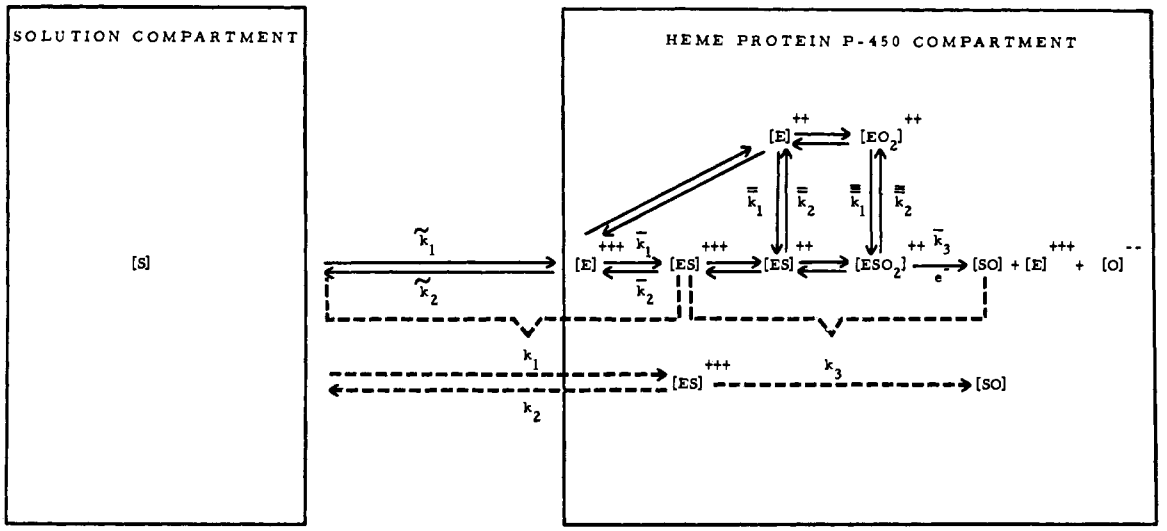

FIGURE 9: General hypothetical scheme of some of the possible heme protein P-450–substrate–oxygen interactions. The system is represented by two compartments: the outside solution interacting with the heme protein P-450 compartment. The electrons (or their source) involved in the oxidoreductive equilibria given in this diagram are not explicitly indicated. The atmospheric oxygen and the substrate in the enzyme compartment are also not shown. $[E]^{3+}$ and $[E]^{2+}$ represent the heme protein P-450 in its two oxidation states; the [ES] moieties represent the heme protein P-450–substrate complexes; the $[ESO_2]$ moieties represent the heme protein–substrate–oxygen complexes, and the $[EO_2]^{2+}$ represents the oxygenated reduced heme protein P-450. e^- is the second electron required in the hydroxylation reaction and is most probably delivered to the system through the reduced nonheme iron protein.

mitochondrial preparations and of Gigon *et al.* (1969), Sasame and Gillette (1969), and Diehl *et al.* (1970) with hepatic microsomes it may be speculated that k_3 actually represents the rate of reduction of the heme protein P-450 moiety which is probably the rate-limiting step in this series of reactions. (In the first-order approximation presented, no allowance was made for the perturbation that occurs in the equilibrium once $[E]^{3+}$ and $[ES]^{3+}$ are reduced.) The differential equations describing eq 1 are given in eq 2 and 3. Under conditions

$$\frac{d[S]}{dt} = -k_1[E][S] + k_2[ES] \tag{2}$$

$$\frac{d[ES]}{dt} = k_1[E][S] - k_2[ES] - k_3[ES] \tag{3}$$

under which the substrate concentration is small compared to that of enzyme, the unbound enzyme concentration will remain approximately constant and $k_1[E]$ may be expressed as a pseudo-first-order rate k_1' and eq 2 and 3 assume expressions 2a and 3a which have the general solutions shown in eq 4 and

$$\frac{d[S]}{dt} = -k_1'[S] + k_2[ES] \tag{2a}$$

$$\frac{d[ES]}{dt} = k_1'[S] - (k_2 + k_3)[ES] \tag{3a}$$

5 where $m_1 = \{k_1' + k_2 + k_3 - [(k_1' + k_2 + k_3)^2 - 4k_1'k_3]^{1/2}\}/2$ and $m_2 = \{k_1' + k_2 + k_3 + [(k_1' + k_2 + k_3)^2 + 4k_1'k_3]^{1/2}\}/2$

$$[S] = A_1e^{-m_1t} + A_2e^{-m_2t} \tag{4}$$

$$[ES] = B_1e^{-m_1t} + B_2e^{-m_2t} \tag{5}$$

The following relationships between m_1 and m_2 hold: $m_1m_2 = k_1'k_3$ and $m_1 + m_2 = k_1' + k_2 + k_3$.

The coefficients A and B depend on the initial boundary conditions. If no equilibration is allowed, *i.e.*, at $t = 0$, $[S] = 1$, and $[ES] = 0$, eq 6 and 7 hold. The time function

$$[S] = \frac{m_2 - k_1'}{m_2 - m_1}e^{-m_1t} + \frac{m_1 - k_1'}{m_1 - m_2}e^{-m_2t} \tag{6}$$

$$[ES] = \frac{k_1'}{m_2 - m_1}e^{-m_1t} + \frac{k_1'}{m_1 - m_2}e^{-m_2t} \tag{7}$$

of the *total* (extractable) substrate which is actually the variable experimentally determined will be given by $[S]_T$ (eq 8).

$$[S]_T = \frac{m_2}{m_2 - m_1}e^{-m_1t} + \frac{m_1}{m_1 - m_2}e^{-m_2t} \tag{8}$$

For initial boundary conditions, such that reaction is allowed only after equilibration of enzyme with substrate, the initial fractions of the free substrate ($[S]_0^{eq}$) and of the substrate–enzyme complex ($[ES]_0^{eq}$) (under the pseudo-first-order conditions assumed above) are given below

$$[S]_0^{eq} = \frac{k_2}{k_1' + k_2}\ ;\ [ES]_0^{eq} = \frac{k_1'}{k_1' + k_2}$$

and the time functions of [S], [ES], and $[S]_T$ are given in eq 9, 10, and 11.

$$[S]^{eq} = \frac{k_2m_2}{(k_1' + k_2)(m_2 - m_1)}e^{-m_1t} + \frac{k_2m_1}{(k_1' + k_2)(m_1 - m_2)}e^{-m_2t} \tag{9}$$

$$[ES]^{eq} = \frac{(k_1' - m_1)m_2}{(k_1' + k_2)(m_2 - m_1)}e^{-m_1t} + \frac{(k_1' - m_2)m_1}{(k_1' + k_2)(m_1 - m_2)}e^{-m_2t} \tag{10}$$

$$[S]_T^{eq} = \frac{(k_1' + k_2 - m_1)m_2}{(k_1' + k_2)(m_2 - m_1)} e^{-m_1 t} + \frac{(k_1' + k_2 - m_2)m_1}{(k_1' + k_2)(m_1 - m_2)} e^{-m_2 t} \quad (11)$$

The experimentally observed substrate disappearance curves obtained *after substrate equilibration* could be approximated by functions containing two exponentials: $y = Ce^{-\alpha t} + De^{-\beta t}$ (see Experimental Procedure). From the experimental parameters α, β, C, and D, the constants k_1', k_2, and k_3 could be calculated using eq 11 and the relationships between m_1 ($=\alpha$) and m_2 ($=\beta$) given after eq 5: $k_1' = [(C/D)\cdot \beta\alpha^2 + \beta^2\alpha]/[\beta^2 + (C/D)\alpha^2]$; $k_2 = \alpha + \beta - k_1' - k_3$; $k_3 = \alpha\beta/k_1'$.

The calculated rate constants for the (22*R*)-22-hydroxycholesterol, (20*S*)-20-hydroxycholesterol, and (20*R*,22*R*)-20,22-dihydroxycholesterol are given in the block diagrams in Figures 1, 3, 4, and 5. The first-order rate constants of the enzyme–substrate complex ([ES]) reaction to give product (k_3) were approximately 23, 22, and 15 times higher than the corresponding pseudo-first-order rate constants (k_1'), describing the formation of the enzyme–substrate complex. A similar relationship was observed in several other experiments (*cf.* Figure 5: the [ES] reaction rate constants of the (22*R*)-22-hydroxycholesterol were, for the upper and lower experiments, 23 and 19 times larger than the pseudo rate constant of [ES] formation). The methodology employed did not allow the determination of the substrate disappearance during the first 15 sec or so, which constituted the relatively very rapid reaction phase after equilibration. As pointed out in Experimental Procedure, this imposed a ±30% uncertainty regarding the initial reaction rates described. Further studies with automatic rapid sampling devices such as that described by Eisenhardt (1964) may further extend our knowledge regarding this phase. Such experiments may also clarify the uncertainty concerning the lag in the early kinetics in the experiments in which the substrate was not allowed to equilibrate with the enzyme.

It must be emphasized that the double-exponential representation of our data given in Figures 1, 3, 4, and 5 is an approximation. The deviation from a biphasic curve appeared to be greater with either more active preparations (*cf.* Figure 6) or when longer equilibration times were used (*cf.* Figure 5). These results may be explained by the assumption that in addition to the active site where the enzymatic transformations occur there are other nonspecific sites to which the substrate binds. When most of the substrate in the form of the enzyme–substrate complex and that present in solution is exhausted, substrate from these nonspecific sites slowly diffuses to the active site at which reaction occurs. However, further elaboration at this time without additional data would be speculative.

The results reported here suggest that, under the experimental conditions employed, the rate of access of the substrate to the active site of the enzyme may be the rate-limiting step in the transformations involved in the conversion of cholesterol or its hydroxylated derivatives to pregnenolone. These results may provide a basis for the explanation of some of our previous observations on consecutive reaction sequences such as A → B → C → D (Burstein *et al.*, 1970a) in which B, for example, after being formed from A in the enzyme compartment may be expected to further transform to C at the much higher rate k_3 and not k_1'.

The question of the existence of a sterol-binding protein (Ritter and Dempsey, 1971; Scallen *et al.*, 1971) in the adrenal cholesterol side-chain cleavage system has been recently studied by Kan and Ungar (1972). Such proteins would be expected to affect k_1' and k_2 of the kinetics described here if the action of such carriers is to facilitate the transport of the sterol to the active site of the enzyme.

A biphasic formation of corticosterone from endogenous precursors has been described by Koritz and Kumar (1970) in succinate-supplemented rat adrenal mitochondria. A similar time course for pregnenolone formation was also seen by Simpson *et al.* (1971) with bovine adrenocortical mitochondria following the addition of malate. This biphasic behavior was attributed by Koritz and Kumar (1970) to a possible feedback inhibition by pregnenolone, and by Simpson *et al.* (1971) also to a rate-limited transport of cholesterol to the heme protein P-450.

Biphasic kinetics have been observed by Sasame and Gillette (1969), by Gigon *et al.* (1969), and by Diehl *et al.* (1970) for the formation of the CO complex of hepatic microsomal heme protein P-450 after the addition of appropriate reducing agents. Similar biphasic kinetics for the CO complex formation were observed by Ando and Horie (1971) with adrenocortical mitochondrial heme protein P-450. The interpretation of these results, however, has been most difficult and has been recently discussed by Ando and Horie (1971). No unequivocal explanation of this phenomenon was provided (*cf.* also Diehl *et al.*, 1970).

It is noteworthy that the initial rapid phase of formation of the CO complex was completed within 10–30 sec after the addition of the reducing agent, which is of the same order of magnitude observed in our studies for the initial phase of substrate disappearance. Since the CO complex formation is only indirectly related to the actual hydroxylation process, it is uncertain whether our experiments reported here bear more than a formal relationship to the kinetics of the CO complex formation.

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A Possible Role for Transhydrogenation in Side-Chain Cleavage of Cholesterol†

Peter F. Hall*

ABSTRACT: Three lines of evidence are presented in favor of a role for transhydrogenation in providing reducing equivalents for the side-chain cleavage of cholesterol by mitochondria from bovine adrenal cortex. Firstly, rotenone, an inhibitor of oxidation of DPNH, stimulates side-chain cleavage whether this is supported by endogenous substrates or by DPN^+-linked substrates (malate and other Krebs cycle intermediates). Secondly, a positive correlation ($r = 0.82$) was observed between levels of mitochondrial DPNH:DPN^+ and the rate of side-chain cleavage. Thirdly, DPNH supports side-chain cleavage when this nucleotide is permitted to enter mitochondria by ageing, freezing or addition of Ca^{2+} to cause swelling; this action of DPNH is enhanced by concomitant addition of TPN^+. Evidently DPNH can provide reducing equivalents for side-chain cleavage, although the purified enzyme responds more readily to TPNH by one order of magnitude. Unlike 11β-hydroxylation, side-chain cleavage is stimulated by cyanide whether this reaction is supported by endogenous substrates, by succinate, or by malate. Reasons are given for believing that 11β-hydroxylation and side-chain cleavage do not necessarily derive reducing equivalents by the same mechanism. The evidence is believed to support the view that TPNH for side-chain cleavage may be generated from DPNH by transhydrogenation.

The conversion of cholesterol to pregnenolone takes place in mitochondria of steroid-forming organs and appears to determine the rate of steroid biosynthesis (Karaboyas and Koritz, 1965; Hall and Koritz, 1965; Hall and Young, 1968). This conversion involves cleavage of a carbon–carbon bond and is referred to here as side-chain cleavage of cholesterol. Cleavage appears to involve hydroxylation of the side chain prior to or in association with cleavage of C_{20}–C_{22} bond; reducing equivalents for hydroxylation are believed to come from TPNH since the purified enzyme system shows maximal activity with TPNH at a concentration one-tenth of that required to produce maximal activity with DPNH (Satoh *et al.*, 1966). A previous report from this laboratory (Hall, 1967b) presented evidence to show that the TPNH required for side-chain cleavage can be generated by reversed electron transport from succinate to DPN^+. This conclusion suggested that either DPNH is capable of generating TPNH or that at least some of the reducing equivalents for side-chain cleavage may come directly from DPNH. The present studies were undertaken to explore these possibilities further.

Experimental Section

Preparation of Mitochondria and Incubation. Mitochondria from bovine adrenal cortex were prepared by centrifugation in sucrose (0.25 M) as described elsewhere (Hall, 1967b). The final pellet was suspended in Tris (0.1 M, pH 7.4) prepared in 0.25 M sucrose to a concentration of 8–10 mg of protein/ml (20 g of adrenal cortex provided 10 ml of such a suspension).

Incubation was performed in 25-ml erlenmeyer flasks in a buffered medium (pH 7.4) containing $MgCl_2$ (17.6 μmoles), Tris (pH 7.4, 100 μmoles), KCl (98 μmoles), and potassium phosphate (44 μmoles) in a final volume of 2 ml. The order of addition of substances to the flasks was as follows: buffered medium, cholesterol-7α-*t*, oxidizable substrates, inhibitors and mitochondria (approximately 8 mg of protein). Cholesterol-7α-*t* (2 μg; 4.4×10^6 dpm/flask) was added in *N,N*-di-

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* Present address: Department of Physiology, California College of Medicine, University of California, Irvine, Calif. 92664.