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BASIC STUDIES OF STEROID HORMONE METABOLISM

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1) INTRODUCTION AND RESEARCH ACCOMPLISHMENTS

The enzymatic transformation of cholesterol to pregnenolone by steroid hormone-producing tissues has been presented as a consecutive, irreversible reaction scheme, involving the sequences (A) cholesterol $\rightarrow$ (20S)-20-hydroxycholesterol $\rightarrow$ (20R,22R)-20,22-dihydroxycholesterol $\rightarrow$ pregnenolone, and (B) cholesterol $\rightarrow$ 22S-hydroxycholesterol $\rightarrow$ (20R,22R)-20,22-dihydroxycholesterol $\rightarrow$ pregnenolone.¹⁻⁴ Since it has been demonstrated that (22R)-22-hydroxycholesterol is formed from cholesterol and efficiently transformed to pregnenolone, unlike the 22S isomer, which is neither formed from cholesterol nor efficiently converted to pregnenolone,⁵⁻⁸ sequence B should be more precisely written as entailing the 22R isomer. Sequence A has been advanced most frequently and also has served as the textbook version of the mechanism by which cholesterol is transformed into pregnenolone. The studies of Koritz and Hall^{9,10} and Simpson and Boyd¹¹ cast some doubt on whether sequence A is a valid mechanism for the conversion of cholesterol to pregnenolone, because these workers were unable to demonstrate that (20S)-20-hydroxycholesterol is formed from cholesterol *in vitro*. Hall and Koritz¹⁰ proposed that multienzyme complexes may exist in their systems, in which there occurs transfer of a product from one enzyme to the next without the release of the intermediate into the medium. Direct experimental evidence for such multienzyme complexes has hitherto not been provided, however.

In previous studies with adrenocortical mitochondrial acetone-dried preparations, at relatively small substrate concentrations, under which first-order kinetics appeared to obtain, a quantitative attempt was made to evaluate the question of whether sequences A and B account for the formation of pregnenolone from cholesterol.^{5,7,8} These studies have revealed that sequence B is occurring at a much higher rate than A, but that these sequences, when treated as homogeneous consecutive reactions, accounted for but a small fraction of the pregnenolone formed. The total formation of pregnenolone from cholesterol could be represented by a combination of several direct (or one-step) pathways, such as cholesterol $\rightarrow$ (20R,22R)-20,22-dihydroxycholesterol $\rightarrow$ pregnenolone or (22R)-22-hydroxycholesterol $\rightarrow$ pregnenolone, of which the former appeared to be the most significant. Although the hypothesis of the existence in these preparations of multienzyme complexes would explain these results, again no direct proof of their existence was offered. Furthermore, these results could also be explained, for example, by the postulation of the existence of other intermediates such as cholesterol 20 α -hydroperoxide, as suggested by Van Lier and Smith,¹² or of free radical intermediates, as suggested by Lieberman and colleagues.¹³ Recent experimental data on the conversion of (20R)-20-*t*-butyl-5-pregnene-3 β ,20-diol¹⁴ to pregnenolone strongly suggest that enzymatic reactions may involve transient free radical (or ionic) intermediates, although again the existence of such intermediates has not been demonstrated in the case of the conversion of cholesterol to pregnenolone.

A correlative study was made between the enzymatic rates of conversion of cholesterol to pregnenolone and the substrate-induced difference spectra (in the 390- to 420-nm range) observed with cholesterol-oxygenated derivatives using various heme protein P-450 preparations from bovine

adrenocortical mitochondria. With acetone-dried powder extracts at 24 μ M (22 β)-20 α ,22-dihydroxycholesterol, 20 α -hydroxy-22-oxocholesterol, 20 β -hydroxy-20-isocholesterol, and 25-hydroxycholesterol exhibited type I difference spectra. Type II spectra were observed with 20 α -hydroxycholesterol, (22 β)-22-hydroxycholesterol, (22 S)-22-hydroxycholesterol, 20 α ,21-dihydroxycholesterol, (22 S)-20 α ,22-dihydroxycholesterol, and 22-oxocholesterol. With some acetone-dried powder preparations, no difference spectra were observed with (22 β)-20 α ,22-dihydroxycholesterol. The type of the difference spectra observed with some of the sterols depended on the technique used in the preparation of the heme protein P-450 fractions. There appeared to be no consistent correlation between the ability of the preparations to catalyze the conversions of cholesterol

to pregnenolone and the magnitude of the observed difference spectra. Although with the acetone-dried powder preparations the apparent binding affinity of the (22 β)-22-hydroxycholesterol and that of the (22 β)-20 α ,22-dihydroxycholesterol (as determined from the difference spectra) was higher than that exhibited by 20 α -hydroxycholesterol, the converse was seen with some of the other partially purified heme protein P-450 preparations studied. These results suggest that great caution must be exercised in deriving mechanistic enzymatic conclusions from substrate-induced difference spectra as these may drastically vary both with respect to magnitude and affinity without significantly affecting, in certain cases, the enzymatic activities.

A study was made of the kinetics of the oxidative metabolism of (20 β)-20-hydroxycholesterol, (22 β)-22-hydroxycholesterol, and (20 β ,22 β)-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 has 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.

The question whether cholesterol is an obligatory intermediate in the biosynthesis of cholesterol required the availability of desmosterol which is expensive and often unavailable. We have elaborated several syntheses of this important cholesterol precursor: 6 β -Hydroxy-22-iodo-3 α ,5-cyclo-5 α -23,24-bisnorcholeane 6-methyl ether, readily produced from stigmaterol, coupled with π -dimethylallyl nickel complex gives, after hydrolysis of the 6-methoxy-i moiety, desmosterol in high yield.

Desmosterol has also been produced by dehydrating 25-hydroxy-cholesterol. This diol has been produced by the reaction of 6 β -hydroxy-22-iodo-3 α ,5-cyclo-5 α -23,24-bisnorcholane 6-methyl ether with lithiated 3-methyl-1-butyn-3-ol tetrahydropyranyl ether. The reaction product can now readily be converted to 25-hydroxycholesterol and hence to desmosterol by the following reaction sequence: Hydrogenation of the 23,24-triple bond, followed by the hydrolysis of the ethers at C-6 and C-25.

A third synthesis starts from 3 β -tetrapyranyloxy-24-norchol-5-en-23-al which was reacted with 2-methylpropenemagnesium bromide to give the complete cholestane structure. The reduction of the 23-methyl ether, followed by the hydrolysis of the tetrahydropyranyl ether completes the synthesis of desmosterol.

Desmosterol-1,2-³H was prepared from cholesta-1,4,24-trien-3-one by selective catalytic tritiation of the 1,2-double bond, followed by conversion of the cholesta-4,24-dien-3-one-1,2-³H to its enol acetate and hydride reduction to the desired cholesta-5,24-dien-ol-1,2-³H.

Desmosterol-26-¹⁴C was prepared from 3 β -tetrapyranyloxy-26-norcholest-5-en-25-one by a Grignard reaction with methylmagnesium iodide-¹⁴C, followed by elimination of the 25-hydroxyl. Unlabelled and labelled desmosterol are being used in a study on the catabolism of cholesterol and cholesterol precursors, and may be considered an extension of our preliminary study.¹⁵

In order to get a more tangible proof for the existence of short lived reactive species,¹⁴ and especially in view of the claim of Van Lier and Smith¹⁶ regarding the intermediacy of (20S)-20-hydroperoxycholesterol, we attempted to elaborate a synthesis of this peroxide which might be adapted to a radiosynthesis in order to study its metabolism. The syntheses of (Z)-3 α ,5-cyclo-5 α -cholest-17(20)-en-6 β -ol 6-methyl ether and of its 6-benzoate is indicated on the flow sheet (below).

It has already been established that the (Z)-17(20) olefin gives on hydroboration a mixture containing the 20-borane of the desired configuration in ca. 50%. However, neither the 3,5-cyclo-6-benzoate nor the 3,5-cyclo-6-methoxy compound resisted attack by dichloroborane diethyl etherate, reagent¹⁷ of choice, as shown below.

Efforts are now underway to react (Z)-6 β -hydroxy-3 α ,5-cyclo-5 α -cholest-17(20)-ene 6 β -methyl ether with borane and to oxidize this with oxygen. We have not tried this sequence in the past because of the known instability of such boranes and their tendency to explode (on contact with oxygen).

Sterols could conceivably be hydroxylated at carbons 17, 20 and 22 and then undergo desmolytic cleavage between C-17 and C-20 to yield dehydroepiandrosterone, as suggested for a direct biosynthetic pathway from cholesterol to the C₁₉ hormones.¹⁸ Alternatively, oxidative cleavage between C-17 and C-20 would yield 17 α -hydroxypregnenolone. The synthesis of (20S, 22R)-17 α , 20, 22-trihydroxycholesterol and also of (20S, 22S)-17 α , 20, 22-trihydroxycholesterol was carried out by the addition of a vinyl Grignard to the known 16 α , 17 α -oxidopregnenolone acetate, followed by reduction of the epoxide, conversion of the product to a

3 α ,5-cyclo steroid, and epoxidation of the remaining double bond. This gave a mixture of C-22 epimeric epoxides which, when condensed with isobutyllithium and recovered to the 3 β -hydroxy- Δ^5 -sterols gave the desired 3,17,20,22-tetrols. It appears that neither 17 α -hydroxycholesterol nor (20S,22R)-17 α ,20,22-trihydroxycholesterol are metabolites of cholesterol.

Since we have developed a large variety of synthetic methods, specifically applicable to the side chain of sterols, it seemed tempting to construct unnatural structures as an approach to therapeutic agents. The stereospecific syntheses of the two epimeric 22-aminosteroids

from the two known epimeric 22-hydroxycholesterols via tosylate $\rightarrow$ azide $\rightarrow$ amine have been elaborated. These amines are also obtained by the reduction of 22-ketocholesterol oxime. Preliminary results indicate a complete inhibition of the oxidation of cholesterol to pregnenolone in the presence of (22*R*)-22-aminocholesterol in epimolar amounts (10^{-10} M).

2) PLANS FOR CONTINUATION

As already mentioned, only a relative small (30-50) fraction of the pregnenolone formation from cholesterol could be ascribed to the known enzymatic reaction sequences.

We plan further studies whether cholesterol can be degraded to pregnenolone by a pathway which includes radicals.

Verification for a suggested "direct" mechanism of oxidation of cholesterol, simultaneously at C-20 and C-22, to the known (20 β ,22 β)-20,22-dihydroxycholesterol will be carried out with acetone dried powders of mitochondria and ^{18}O . This is patterned in close analogy to mechanisms formulated and proved by Samuelsson¹⁹ and Nugteren *et al.*²⁰ that in the biosynthesis of prostaglandin E_1 the oxygen atoms at C-9 and C-11 originate from the same molecule of oxygen. Work is now in progress to develop a preparation which will give a sufficient yield of (20 β ,22 β)-20,22-dihydroxycholesterol (for formation of derivative and mass anal.) to make the use of $^{18}\text{O}_2$ technically and financially feasible.

The fragments represented by the above values (217 and 185) refer to $^{16}\text{O}_2$. For $^{18}\text{O}_2$ the numbers would be 221 and 189, respectively. If, however, the attacking species would be an oxygen atom (instead of molecular oxygen), the values 219 and 187 would be obtained. Preliminary experiments revealed that the 20,22-acetonide²¹ are suitable for the mass analysis of a fragment with intact side chain (C-20 to C-27, without loss of the oxygen functions). The results of $^{18}\text{O}_2$ / $^{16}\text{O}_2$

experiments, specifically the comparison of the isotope ratio in the gas and in the (20R,22R)-20,22-dihydroxycholesterol will then answer the question whether the oxygen atoms at C-20 and at C-22 are derived from the same molecule of oxygen. If both atoms do come from the same molecule then the involvement of a reactive cyclic peroxide intermediate would seem plausible. Most likely this intermediate would be bound to an enzyme. However, whether this oxygenation reaction is due to a single enzyme complex or dependent upon multiple enzymes, whether the mechanism proceeds via a free radical or whether ionic species are involved, are all questions which will have to be answered eventually.

We intend to elaborate a synthesis of (20S)-20-hydroperoxycholesterol. This will involve more labor than its isolation from aged cholesterol²² but, if the synthesis is successful, it will make the desired compound available in larger amounts and hopefully, with some modification, can be adapted to a synthesis with a radioactive isotope. It is important to us to have pure, labeled and unlabeled, 20-hydroperoxycholesterol since this compound could be the primary enzymatic oxidation product, just as it is certainly the primary autooxidation product. The decomposition of this hydroperoxide could then lead to pregnenolone as the final product.

A method by Abraham and Davies²³ and recently elaborated on many compounds by Brown and Midland²⁴ described the preparation of alkylperoxides of different elements. The indication that boron trialkyls could be converted by autooxidation to alkyl boric acid diester was of particular interest.

However, compounds of type RB(OR)OOR can be hydrolyzed without loss of peroxide, if the last B-C bond is oxidized with perbenzoic acid to a B-O-C bond. This method will be used for the preparation of the desired hydroperoxide as follows.

Since (E)- $\Delta^{17(20)}$ -5 α -cholestenol gives upon hydroboration mostly a 20-hydroxy product of the wrong configuration²⁵, our starting material must be its double bond isomer. (Z)-6 β -Hydroxy-3 α ,5-cyclo-5 α -cholest-17(20)-ene 6-methyl ether will be hydroborated with borane and the organoborane oxidized with oxygen. The separation of the hydroperoxide from the alcohol will be done by extraction of the former as its potassium salt.²⁶ Mild acid treatment could give the desired (20S)-20-hydroperoxycholesterol.

Although considerable efforts were made to trap a radical and to identify it by addition of a radical scavenger, we were unable to isolate any such material from incubations of side chain labeled cholesterol.

We now plan to incubate (20S)-20-hydroperoxycholesterol with our adrenal powder preparations and observe the ESR spectrum. The absence of any resonance would indicate that the degradation of cholesterol proceeds without the intermediacy of any stable free radicals. If we are able to observe a resonance we shall determine the character of the radical and investigate its metabolism.

Our investigation will thus investigate a possible analogy between the photocatalyzed production of the 3β-hydroxy-5α-hydroperoxycholest-6-ene and the formation of cholesterol-20-hydroperoxide in the absence of photocatalysis. The photochemical investigation of simple models has become an important tool in the interpretation of photobiological phenomena at the molecular level. Recently the formation of 3β-hydroxy-5α-hydroperoxycholest-6-ene in red cell membranes and photohemolysis in Erythropoietic Protoporphyrin have been observed after irradiation in the presence of oxygen.²⁷ It is possible that a similar reaction sequence to that advanced for photobiologically triggered reaction may also occur under stimulation which excludes light.

3) GRADUATE STUDENTS

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5) SIGNIFICANCE

The precursor-product relationship of cholesterol and adrenocortical steroid hormones was established some thirty years ago. The quantitative aspects however are still controversial and need further work.

The progress obtained after the availability of labeled cortisol and labeled aldosterone²⁸ is a good illustration of the importance of the availability of starting materials and metabolites in this and similar studies. The failure of Shimizu *et al.*²⁹ to properly produce and identify the hypothetical 17 α ,20 α -dihydroxycholesterol illustrates the pitfall of neglecting this aspect. Only with the availability of (20S)-20-hydroperoxycholesterol will we be able to assess the pertinence of models advanced by e.g. Lattrell *et al.*¹⁴ and Tan and Falardeau.³⁰ Also, with the availability of this hydroperoxide we will use electron spin resonance for the observation of the so far hypothetical free radicals, since attempts at trapping radicals (with dibenzyl disulfide-³⁵S) were not successful (unpublished observations). Our results to date could not have been obtained unless all intermediates were available to us, in labeled as well

as in unlabeled form. Further difficulties arise because the differential equations of the Michaelis type cannot be directly solved. The availability of tracer methodology allows the determination of the disappearance rates of each intermediate and thereby gives the possibility for the determination of the individual rates of each step under first order kinetics (low substrate concentration and short incubation time). On the basis of the overall substrate and product disappearance rates the differential equations can be solved completely and the individual rates calculated from the net formation of each product. With the help of these mathematical models the importance of a suspected intermediate in a given sequence can be determined.

It is obvious that an answer to the question of the relative importance of an intermediate in a sequence must involve the knowledge of its stability in that system. Thus, failing to observe an intermediate must not be an indication of its insignificance; it may just be the result of its extremely rapid removal. So far, no quantitative studies have been published either to substantiate or to refute the importance of the role of various possible intermediates in the transformation of cholesterol to pregnenolone.

Thus, quantitative studies of the overall reaction sequence cholesterol $\rightarrow$ pregnenolone will establish base lines for the assessment of corticoid production. In general terms, only such or similar theoretical approaches allow the determination of equilibria reaction rates and will also apply in other areas of enzymology, where equilibration of enzyme and substrate is possible prior to the beginning of the reaction by the addition of a prerequisite cofactor. Only with this knowledge in hand can an attempt be made to delineate the possible loci of trophic hormone action. The question of cause-effect relationship between essential familial hypercholesterolemia and hormone production or hormone therapy may find a solution from such studies. Furthermore, such studies may add to our knowledge of adrenal carcinoma.

Although the amount of endogenous cholesterol present is generally higher than the dietary cholesterol, its role and the role of other nutritional sterols on hypocholesterolemia has been considered and sitosterol has been investigated by Schettler *et al.*³¹ as a therapeutic agent for treatment of hypercholesterolemic cardiovascular disease and of essential hypercholesterolemia. A clarification of the qualitative and quantitative aspects of the catabolism of sitosterol and stigmasterol may shed some light on the relationship between these dietary sterols and the above mentioned pathologies.

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