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TRANSIENT INTERMEDIATES IN STEROIDOGENESIS

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SUMMARY

Analogs of cholesterol and pregnenolone have been synthesized and tested as hormonal precursors. Homologues of cholesterol in which C-22 is completely substituted (e.g. (20R-20-t-butyl)-5-pregnene-3β,20 diol, (20R)-20-(phenyl)-5-pregnene-3β,20 diol) were incubated with acetone powders of adrenal glands and appropriate cofactors and each was converted into pregnenolone. The side-chain fragment of the aryl derivative formed biosynthetically in this reaction was identified as phenol. In addition, another fragment, acetophenone, was isolated but the C_{19}-steroidal product of this unusual pathway has not yet been identified. In a comparable manner, the conversion of C_{21}-steroids into C_{19}-androgens was studied. An analog of pregnenolone which was devoid of oxygen at C-20, 5-pregnene-3β-ol, was synthesized and when incubated with testicular microsomes was converted into testosterone. These results suggest that the true intermediates in steroid biosynthesis may not be isolable hydroxylated compounds as traditionally conceived, but rather enzyme bound, transient, reactive species.

In several recent publications [1–4] we have recommended that consideration be given to the possibility that the true intermediates involved in the processes by which the steroid hormones are biosynthesized are transient, reactive, complexes of the precursor, oxygen and a metalloenzyme. This suggestion contrasts with the traditionally accepted view that intermediates in these pathways are stable, isolable compounds. The evidence which forms the basis for this suggestion comes from experiments in which synthetic analogs of steroids customarily considered to be intermediates in steroidogenic processes were used as precursors for some hormonal products. These analogs possess structures that would make them unfit as precursors if the traditionally accepted routes were, in fact, accurately conceived.

Figure 1 shows the generally accepted pathway leading to the formation of pregnenolone by the cleavage of cholesterol. Customarily the presumed intermediates are considered to be compounds having one or two hydroxyl groups in the cholesterol side-chain. At least two of these compounds have been isolated in crystalline form from adrenals [5]. Their presence in the media is also detected when radioactive cholesterol is incubated with adrenal mitochondria [6]. All three of these hydroxylated compounds are known to be enzymatically converted in *in vitro* experiments,

CLASSICAL PATHWAY FOR THE BIOSYNTHESIS OF PREGNENOLONE FROM CHOLESTEROL

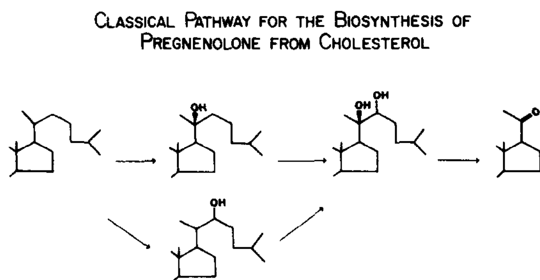

Fig. 1.

CHOLESTEROL ANALOGS BLOCKED AT C-22

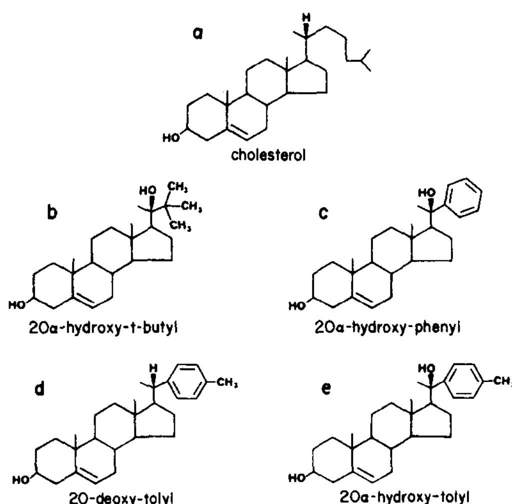

Fig. 2.

into pregnenolone [6, 7]. Elaborate kinetic experiments, however, carried out by Burstein and his collaborators [8], have shown that only a small portion of the pregnenolone formed from cholesterol could be accounted for by the involvement of these compounds. Our thesis proposes that these hydroxylated compounds are merely by-products formed from the true intermediates, and thus are only reflections of the actual intermediates that occur in the pathway.

Our first relevant experiment was that which showed that oxidation of 20α-hydroxycholesterol by lead tetraacetate results in the formation of pregnenolone [9]. There are two mechanisms by which this reaction may proceed, both involve transient intermediates, one sequence is formulated as a radical process and the other as an ionic one. The results of this experiment led to the consideration of the possibility that in the biological conversion of cholesterol

into pregnenolone, the intermediacy of the glycol, 20α 22R-dihydroxy cholesterol, was not necessary for sidechain cleavage. Generation of either a carbonium ion or an alkoxy radical at carbon 20 of 20α-hydroxycholesterol could be sufficient to rationalize the cleavage of the sidechain between carbons 20 and 22 and the formation of pregnenolone. This result led us to prepare an analog of cholesterol that could not be enzymatically hydroxylated at carbon-22 but which might, if our supposition was correct, be enzymatically converted into pregnenolone. The first compound synthesized (Fig. 2b) was one in which a tertiary butyl group replaced the isohexyl sidechain of 20α-hydroxycholesterol. The carbon atom corresponding to C-22 of cholesterol is completely substituted with methyl groups and thus may not suffer hydroxylation. When this compound (labeled with ^3H at C-7) was injected into a rabbit, tritiated pregnanediol was isolated from the urine. *In vitro* incubation of this analog of 20α-hydroxycholesterol with sonicated bovine adrenal mitochondria led to the formation of radioactive pregnenolone in about 1% yield. These results were consistent with the hypothesis, but the possibility of unblocking C-22 through enzymatic demethylation left some uncertainty. Moreover, it was apparent that the cleaved sidechain fragment would be difficult to identify since the expected C_4-moiety would undoubtedly undergo further metabolism and perhaps rearrangement and this would make characterization difficult.

Consequently a new series of analogs were synthesized. The compounds, shown in Fig. 2, have an aromatic group attached to C-20. As in the case of the t-butyl analog, what would be C-22 in cholesterol is an atom of the aromatic ring and therefore contains no replaceable hydrogen atom which is a structural feature necessary for hydroxylation. These analogs were superior for our purposes because the sidechain fragment would be easier to identify and, because of the aromatic group, they might be oxidized enzymatically more rapidly than analogs with a bulky t-butyl group.

The first of these compounds synthesized and tested as a substrate for the sidechain cleavage enzyme was the 20α-hydroxytolyl analog (Fig. 2e). This substance bearing ^3H at C-7 was incubated with an acetone powder prepared from bovine adrenal mitochondria, and both radioactive pregnenolone and progesterone were shown to be formed (Fig. 3). In some experiments, the yield of C21-steroids approached 25%. The 20-deoxy tolyl derivative (Fig. 2d) was also incubated with an acetone powder and again both radioactive pregnenolone and progesterone were isolated, the yield being a little more than 1% (Fig. 4). Surprisingly, relatively large amounts of the 20α-hydroxytolyl analog were also isolated. The yield was over 20%. This result was the first unequivocal demonstration that hydroxylation at C-20 can occur on a substrate that has no oxygen function on C-22. A styrene analog, containing a double bond between C-20 and C-21 was prepared by elimination of the hydroxyl group at C-20 of the tolyl derivative. It was tested as a substrate with adrenal mitochondria but no C_{21}-steroid was produced.

To determine the nature of the fragment cleaved from the aromatic analogs, two phenyl derivates were prepared (Fig. 2c); one was labeled with ^3H in the aromatic ring and the other labeled with ^3H in the nucleus. It was easy to demonstrate that like the tolyl analog, the nuclear-labeled phenyl derivative could be converted into radioactive pregnenolone by the cleavage enzyme. During the incubation of the sidechain labeled substrate with bovine adrenal mitochondrial acetone powder and a TPNH generating system, an effort was made to entrain volatile fragments in a stream of air. No radioactive material could be volatilized in this way. But when the incubation mixture was steam distilled, significant amounts of ^3H were found in the distillate. Most of the steam distillable ^3H was soluble both in organic solvents and in aqueous alkali. This led to the suspicion that the sidechain fragment was phenol. Non-radioactive, carrier phenol was added, the mixture was sublimed under vacuum, and a variety of specific derivatives were prepared. They were purified by chromatography, and finally recrystallized to constant specific activity. In this way, phenol was shown to be one of the sidechain fragments formed during cleavage (Fig. 5). Exper-

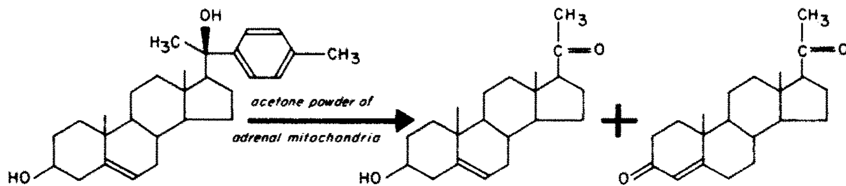

Fig. 3.

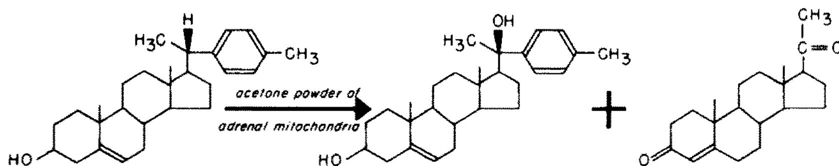

Fig. 4.

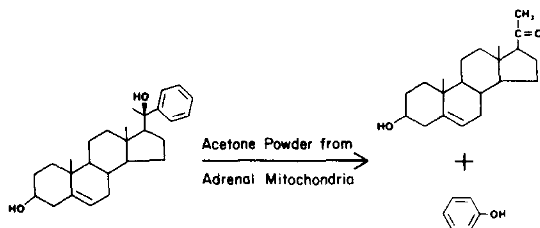

Fig. 5.

iments performed with the phenyl analog labeled in both the nucleus and sidechain demonstrated that the C21-steroid and phenol were formed in approximately stoichiometric amounts. Control experiments showed that boiled enzyme did not convert the analog into either pregnenolone or phenol. These experiments demonstrated that cleavage of the aromatic sidechains of both the tolyl and phenyl analogues had occurred without disruption of the aryl groups.

Unexpectedly some of the radioactivity found in the steam distillate following incubation of the phenyl derivative with adrenal mitochondria was not alkali soluble. It was fairly volatile and easily soluble in organic solvents. Examination of the structure of the synthetic precursor suggested that this compound might be acetophenone, the fragment that would be formed if fission had taken place between C-17 and C-20. As before, non-radioactive carrier acetophenone was added. The mixture was distilled under vacuum and the distillate derivatized with methyl hydroxylamine. The methyloxime was sublimed, chromatographed, and finally recrystallized to constant specific activity. The coincidence of radioactivity and mass proved that enzymatic fragmentation had yielded an 8-carbon moiety which was identified as acetophenone (Fig. 6). Again, control experiments demonstrated that the cleavage was indeed enzymatic and that the fragment was not an artifact of the workup. A crude estimate of the amount of acetophenone formed indicated that it constituted between 25 to 50% of the quantity of phenol formed.

The enzymatic removal of the entire C_8-sidechain of cholesterol during the formation of C_{19}-steroids had been previously postulated by others to be a possible alternate pathway for androgen biosynthesis [10, 11]. However, Shlomo Burstein and his coworkers [12] and we [13] using a variety of steroidogenic tissue have demonstrated that the scission of an C_8-fragment from cholesterol does not take place. But the formation of acetophenone clearly shows that cleavage had occurred between C-17 and C-20 and therefore, using the nuclear labeled phenyl analog an effort was made to identify the nuclear moiety

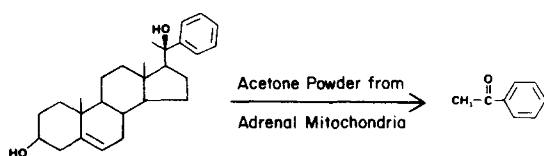

Fig. 6.

remaining after fission of acetophenone. Consideration was given first to the possibility of finding C_{19}-steroids oxygenated at both C-3 and C-17. However, no radioactivity was found in regions of the chromatogram where such products would be expected. The only significant amounts of radioactivity were found in the non-polar region of the chromatogram. Thus it seemed possible that 17-deoxy-C_{19}-compounds like 5-androsten-3β-ol and androsta-5,16-dien-3β-ol had been formed. Initial experiments with cold carriers at first seemed promising, but further purification separated counts from carrier. Experiments were then carried out using a sample of the phenyl analog that was labeled with ^3H in the nucleus at C-7 and labeled with ^{14}C in the sidechain at C-21. This doubly labeled precursor was incubated with an acetone powder from adrenal mitochondria. Carrier acetophenone was added and reisolated. After derivative formation and crystallization the methoxine contained ^{14}C, thus confirming that formation of acetophenone resulted by fission between C-17 and C-20. The steroidal fractions were purified by chromatography in an attempt to isolate a product containing only tritium. C_{21}-steroids would contain both ^{14}C and ^3H while C_{19}-steroids would have only ^3H. Pregnenolone and progesterone were isolated and as expected contained both isotopes. The fractions that contained only ^3H isotope were in the expected, non-polar area of the chromatogram. Since the common C-17-deoxy-C_{19}-compounds had already been eliminated, rearrangement products that would arise by reaction of a species having a carbonium ion or radical at C-17 are now being sought.

Two mechanisms for the transformation of the aryl derivative can be envisioned (Fig. 7). Attack at C-20 could yield a complex of the steroid, oxygen and metalloenzyme. Hydrolysis of this complex would produce the C-20 hydroxy analog. This, *via* a reversible reaction, could serve as a precursor by return to the common intermediary complex. Further oxygenation of this complex at C-22 through a mechanism involving a radical aromatic substitution or an electrophilic aromatic substitution could result in cleavage of the C-20, 22 bond. Restoration of the aromatic system and oxidation of the metalloenzyme complex would result in the formation of pregnenolone and, in this case, p-cresol.

In a similar manner, formation of the complex at carbon-20 without concomitant oxygenation of carbon-22 could lead to the formation of acetophenone. Rearrangement of the steroidal fragment would yield a C-17-deoxy-C_{19}-steroid.

In order to determine whether the hypothesis was more general, the processes involved in the cleavage of the C_2-sidechain of the C_{21}-steroid, pregnenolone, when it is converted into the C_{19}-androgen were next examined. Figure 8 depicts the pathway usually drawn for the biosynthesis of C_{19}-androgens from C_{21}-precursors. The generally accepted intermediates, 17α-hydroxypregnenolone and 17α-hydroxyprogesterone, are (as is the 20,22-glycol of cholesterol) stable, isol-

*Proposed radical (Path A) and electrophilic (Path B) aromatic substitution mechanisms for biosynthesis of pregnenolone from p-tolyl analogs **1** and **2***

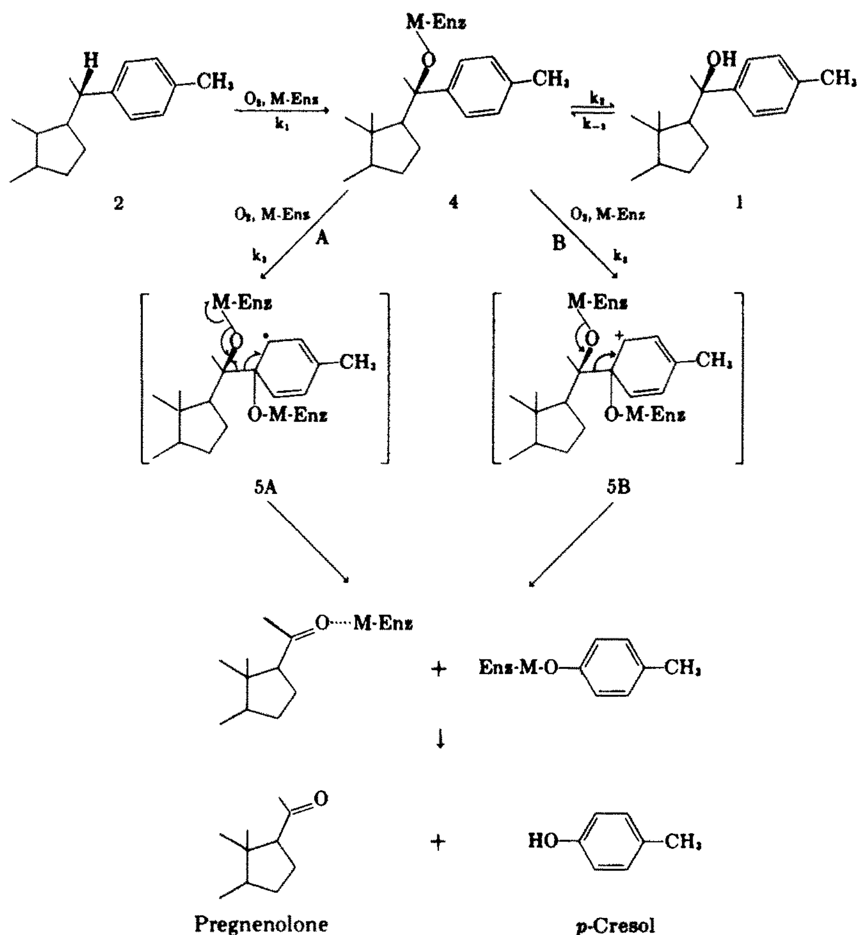

Fig. 7.

able compounds which bear oxygen atoms on the two adjacent carbon atoms between which cleavage occurs. Both of these compounds serve as precursors of C_{19}-steroids in *in vitro* experiments.

To determine whether either compound is an obligatory intermediate, an analog of pregnenolone, which is devoid of oxygen in the sidechain was synthesized. This compound, 5-pregnen-3β-ol, presumably cannot be converted into a steroid having a 17α-hydroxy-20-keto sidechain since a 20-hydroxylase has never been found in microsomes of testes. When the tritiated, C-20 deoxysteroid was incubated with microsomes from rat testes and a TPNH generating system, tritiated testosterone was formed in nearly 5% yield (Fig. 9). The presence of an oxygen function at C-20 was not an essential structural feature of the substrate since in spite of its absence 5-pregnen-3β-ol was converted into testosterone.

These results lead to the following conclusions about the pathway by which 20-deoxypregnenolone is converted into testosterone. From control experiments, it appears likely that pregnenolone is not an intermediate in this conversion, and, apparently, initial oxygenation at C-20 is not required to prepare

CLASSICAL PATHWAY FOR THE BIOSYNTHESIS OF C-17 OXYGENATED C_{19}-STEROIDS (ANDROGENS) FROM C_{21}-STEROIDS

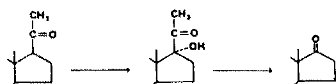

Fig. 8.

BIOSYNTHETIC CONVERSION OF A 20-DEOXY-C_{21}-STEROID TO A 17-OXYGENATED-C_{19}-STEROID

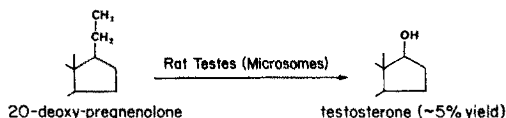

Fig. 9.

the molecule for cleavage. Thus, it may be considered that the side-chain cleavage of 20-deoxypregnenolone occurs by a mechanism analogous to that for the corresponding transformation of pregnenolone. Certainly, oxygenation at C-17 occurs, since the final product in each case contains a 17-oxygen function. Thus, the formation of a carbon-17-oxygenated enzyme bound complex could be followed directly by oxidative attack, perhaps at carbon-20, concerted with cleavage of the C-17 to C-20 bond. Additional evidence about the actual mechanism may come from the identification of the respective 2-carbon fragments formed in each of these processes, and a search for these moieties is being made now.

In conclusion, we interpret these results as additional support for the hypothesis presented a few years ago that holds that the true intermediates in steroid hormone biogenetic processes involving carbon-carbon fission are not stable hydroxylated compounds like 20α-hydroxycholesterol or 17α-hydroxyprogesterone, but rather are reactive complexes of the steroid, oxygen, and a metallo-enzyme. In this view, the precursor is normally converted into the product via such complexes by a concerted mechanism without the obligatory formation of isolable intermediates.

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