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Formation of Isocaproaldehyde in the Enzymatic Cleavage of Cholesterol Side Chain by Adrenal Extract*

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ABSTRACT: The enzymatic conversion of cholesterol to pregnenolone by adrenal extracts produces isocaproaldehyde and does not normally involve 20α-hydroxy-22-ketocholesterol as an intermediate.

It is well established that the conversion of cholesterol to pregnenolone involves as intermediates 20α-hydroxycholesterol and 20α-22ξ-dihydroxycholesterol (Solomon *et al.*, 1956; Shimizu *et al.*, 1960; Constantopoulos and Tchen, 1961b; Shimizu *et al.*, 1961; Shimizu *et al.*, 1962; and Constantopoulos *et al.*, 1962). The conversion of the latter compound to pregnenolone requires TPNH[1] and we have postulated in preliminary communications that it proceeds *via* an oxygenase type reaction to yield pregnenolone and isocaproaldehyde (Constantopoulos *et al.*, 1962). On the other hand, attempts to recover isocaproaldehyde from reaction mixtures in the absence of carrier aldehyde have been unsuccessful (Lantos *et al.*, 1964) and the involvement of 20-hydroxy-22-ketocholesterol, which has been shown to be convertible to pregnenolone at a slower rate than 20α-22ξ-dihydroxycholesterol (Shimizu *et al.*, 1962), has not been rigorously excluded. We wish to report here the details of the experimental results on the formation of isocaproaldehyde and additional results obtained

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[1] Abbreviations used: TPNH, reduced triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide.

with doubly labeled 20α-hydroxycholesterol which eliminate 20α-hydroxy-22-ketocholesterol as a normal intermediate.

Materials and Methods

Isocaproic acid-1-^{14}C was reduced with tritium-labeled $LiAlH_4$ (Rabjohn, 1963), the alcohol was converted to the bromide (Black, 1950), and the latter was condensed with pregnenolone (Petrow and Stuart-Webb, 1956) to yield 20α-hydroxycholesterol-22-^{14}C-22-^{3}H. This was purified by paper chromatography freshly before use as substrate. Other materials and methods are either as previously reported (Constantopoulos and Tchen, 1961a; Constantopoulos *et al.*, 1962) or described in full under Experimental Section and Discussion.

Experimental Section and Discussion

Side-chain-labeled cholesterol was incubated with a soluble enzyme preparation in the presence of 10 μmoles/ml of isocaproate, isocaproaldehyde, or isocapryl alcohol. After 30 min or 60 min of incubation, 1 mg of emulsified unlabeled cholesterol and 5 mg of isocaproaldehyde were added and the reaction was stopped by acidification with H_2SO_4. Steam distillation was carried out until *ca.* 20 ml of distillate was obtained. Isocaproic acid and aldehyde were reextracted into a small volume of ether. The acid was then back extracted into a small volume of dilute $NaHCO_3$ solution, plated on planchets, and counted in a windowless flow counter. The ether solution was treated with a slight excess of an alcoholic solution of 2,4-dinitrophenylhydrazine and 1 drop of concentrated HCl. After standing for several hours, the solution was evaporated to dryness. The solid was redissolved in a small volume of benzene. Aliquots of this were again plated and counted in a windowless flow counter. All the counts were corrected to infinite thinness of samples. Under these conditions, a small but significant amount of cholesterol is carried over during steam distillation and contributes to the radioactivity of the samples. In routine experiments for measuring labeled-aldehyde production, a correction is made, based on the percentage of cholesterol carried over in the control. For the identification of labeled aldehyde, the benzene solution was applied to an alumina column. The dinitrophenylhydrazone was eluted by benzene, leaving behind the contaminating radioactive cholesterol. Three recrystallizations of the dinitrophenylhydrazones gave constant melting point and specific activity.

The effect of the addition of C_6 acid, aldehyde, or alcohol to the reaction mixture is shown in Table I. It is clear that the addition of isocaproaldehyde, but not of the corresponding acid or alcohol, prevented the formation of labeled isocaproic acid and led to the trapping of radioactivity in isocaproaldehyde.

In other experiments using 5 or 10 μmoles/ml of isocaproaldehyde as trapping agent, the effectiveness of trapping varied and the ratio of labeled aldehyde/acid may be as low as 0.2. Reduction of the amount of added unlabeled isocaproaldehyde to <1 μmole/ml resulted in little or no radioactivity in the aldehyde at the end of incubation, even though significant amounts of the added aldehyde can still be recovered at the end of the experiment. This is in agreement with the report of Lantos *et al.* (1964) that no isocaproaldehyde was detected by vapor phase chromatography when cholesterol was converted to pregnenolone in the absence of a trapping agent. Although the exact reason for this is not known, one may offer an explanation that the added aldehyde does not mix thoroughly and readily with the aldehyde formed in the enzymatic reaction. If the enzyme complex, dealing with lipid substrate, intermediates, and products, were strongly hydrophobic, one might envision the aldehyde produced to be attached to the enzyme complex until it is oxidized to isocaproate and released as a salt. In separate experiments, we have observed that substantial DPN-linked aldehyde dehydrogenase is present in these preparations. Assuming a fairly rapid rate of oxidation of the aldehyde bound to the cholesterol side-chain desmolase complex, it may require large amounts of unlabeled aldehyde to achieve the trapping of the labeled aldehyde.

To elucidate further the intermediate involved, specifically, to test whether 20α-hydroxycholesterol is a normal intermediate, 20α-hydroxycholesterol labeled in the C_{22} position with both ^{14}C and ^{3}H was synthesized and used as substrate. The reaction mixture contained 6 mg of soluble enzyme, 0.4 mg of labeled 20α-hydroxycholesterol, 100 μmoles of carrier isocaproaldehyde, and the usual buffer and cofactors in a total volume of 10 ml. Reaction was stopped by acidification

TABLE I: Effect of Isocaproic Acid, Isocaproaldehyde, and Isocapryl Alcohol on the Side-Chain Desmolase Reaction.[a]

Addn	Isocaproic Acid (cpm)	Isocapro-aldehyde (cpm)
None	8000	...
Sodium isocaproate	9000	...
Isocaproaldehyde	200	6300
Isocapryl alcohol	8100	...

[a] Each reaction mixture contained 2 × 10^5 cpm (0.1 μmole) of cholesterol-26-^{14}C, 20 μmoles of potassium phosphate buffer, pH 7.4, 8 μmoles of DPNH, and dialyzed extract from adrenal mitochondria acetone powder equivalent to 0.4 g of fresh tissue in a total volume of 2.0 ml. Incubation was carried out for 2 hr at 37° in an automatic shaker. The isocaproaldehyde counts per minute was obtained by counting an aliquot of ether solution after extraction with $NaHCO_3$ and applying a correction factor for the amount of labeled cholesterol carried over during steam distillation.

TABLE II: $^{3}H/^{14}C$ Ratio in the Isocaproaldehyde Derived from 20α-Hydroxycholesterol-22-^{14}C,^{3}H.

	Substrate	Product
^{3}H (cpm)[a]	3.448	270
^{14}C (cpm)	4.246	724
$^{3}H/^{14}C$	0.8	0.4

[a] The counts per minute reported here represent the total amounts. Actual counting was carried out with 15 mg of the labeled dimedone and with approximately one-tenth of the total substrate in the presence of 15 mg of unlabeled dimedone which exerted a considerable degree of quenching. The dimedone derivative, instead of the dinitrophenylhydrazone of the aldehyde, was prepared and used as the dinitrophenylhydrazone caused complete quenching of tritium counts. The extent of side-chain cleavage, determined by the amount of residual substrate at the end of reaction, was *ca.* 23%. The yield of ^{14}C in the dimedone was 37% of theoretical value based on the above figure of 23% conversion.

with H_2SO_4 after 30 min of incubation at 37°. After steam distillation, the aldehyde was converted to its semicarbazone and recrystallized three times to reach constant melting point and constant specific activity. The $^{3}H/^{14}C$ ratio was determined by counting in a liquid scintillation counter and compared to the $^{3}H/^{14}C$ ratio in 20α-hydroxycholesterol counted under identical conditions (with same amount of unlabeled semicarbazone). The results are shown in Table II. It is clear that, since the aldehyde has retained approximately half of the ^{3}H, 20α-hydroxy-22-ketocholesterol cannot be an obligatory intermediate. The same conclusion had been reached previously (Constantopoulos *et al.*, 1962) by entirely different experiments using analogs of 20α-hydroxycholesterol in which the isovaleryl group attached to C_{20} was replaced by isopropyl or *sec*-butyl groups. These analogs, with a tertiary carbon atom at position 22 barring the oxidation to a 22-keto group, were, nevertheless, converted to pregnenolone at rates comparable to that shown by derivatives having an ethyl group on C_{20}, leading to the conclusion that 22-keto derivatives are not obligatory intermediates. It remains, therefore, only to consider whether 20α-hydroxy-22-ketocholesterol, although not an obligatory intermediate, might still be a normal intermediate in the cleavage of the cholesterol side chain.

The elegant work of the Worcester group has shown that one can isolate from incubation mixtures only one of the two isomers of 20α,22ξ-dihydroxycholesterol (Shimizu *et al.*, 1961, 1962). Since this natural isomer is a better substrate for the desmolase than the other unnatural isomer not found in reaction mixtures (Shimizu *et al.*, 1962), it may be concluded that the absence of the latter cannot be due to a more rapid rate of further conversion to pregnenolone, but must be due to the lack, or to a very slow rate, of formation from 20α-hydroxycholesterol. In other words, although both isomers can serve as substrate for the desmolase, only one of the two is the normal intermediate resulting from a stereospecific oxygenation at C_{22} of 20α-hydroxycholesterol. It is also known that, during the oxidation of cholesterol to pregnenolone the enzyme-bound 20α-hydroxycholesterol has little tendency to dissociate from the enzyme and to undergo exchange with added 20α-hydroxycholesterol, as indicated by the poor efficiency of trapping experiments (Solomon *et al.*, 1956). One may thus expect that, when 20α-hydroxycholesterol is used as substrate, those molecules which become attached to the desmolase will have little tendency to dissociate from the enzyme and will be largely further oxidized. From these two considerations, namely, the stereospecificity of the 22-oxygenation reaction and the lack of tendency for enzyme-bound 20α-hydroxycholesterol to dissociate from the enzyme, one can make the following conclusions: If 20α-hydroxy-22-ketocholesterol is not an intermediate, and if the cleavage of the C–H bond on C_{22} is not the rate-limiting step so that no kinetic isotope effect would be present, then the isocaproaldehyde obtained from the doubly labeled 20α-hydroxycholesterol should retain half of the tritium. If the 22-keto compound is an intermediate, the aldehyde should contain no tritium. Experimentally, it was found that the semicarbazone of the aldehyde, after recrystallization to constant melting point and specific activity, had a $^{3}H/^{14}C$ ratio exactly one-half the $^{3}H/^{14}C$ ratio of the starting material. It is, therefore, clear that the cleavage of the cholesterol side chain does not normally involve the intermediary formation of 20α-hydroxy-22-ketocholesterol. The results also suggest that in this complex oxygenase reaction, the cleavage of the C–H bond on C_{22} is not the rate-limiting step.

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