On the oxidation of cholesterol by mouse liver mitochondria. Bile acids and Steroids 88

It has been established that cholesterol is catabolized to bile acids in the liver of the intact animal[1,2], but the sequence of reactions has not been fully elucidated. By investigating the ability of the bile fistula rat to convert hypothetical intermediates to cholic acid, BERGSTRÖM AND LINDSTEDT[3-5] have obtained convincing evidence that the steroid nucleus is hydroxylated, first at C-7 and then at C-12 before the side-chain oxidation is completed. MEIER *et al.*[6] have reported on the formation of $^{14}CO_2$ from [26-^{14}C]cholesterol in rat liver slices. ANFINSEN AND HORNING[7] as well as GURIN and coworkers[8] have studied the same reaction in cell-free preparations of liver, and both groups of workers[9,10] have characterized the co-factor requirements of the mitochondrial system for $^{14}CO_2$ formation from [26-^{14}C]cholesterol.

In connection with these studies GURIN *et al.*[8] reported the formation of labeled 25-dehydrocholesterol, a labeled C_{27} steroid aldehyde and acid, after incubation with [26-^{14}C]cholesterol. FREDRICKSON[11] investigated the oxidation of [4-^{14}C]-cholesterol in the mitochondrial system originally described by ANFINSEN AND HORNING[7], and found that of the 25 % of cholesterol metabolized, 15 % was present as acidic products not identical with cholic or deoxycholic acid, 7 % as 25- and 26-hydroxycholesterol and the remainder as cholesterol esters.

Using the mitochondrial system recently described by HORNING *et al.*[10] we have investigated the oxidation of [4-^{14}C]- and [26-^{14}C]cholesterol and isolated the products formed by reversed-phase partition chromatography[12].

Mouse (Danish State Serum Institute strain) liver mitochondria and the soluble co-factor were prepared as previously described[10]. [4-^{14}C]cholesterol (Radiochemical Centre, Amersham, England) and [26-^{14}C]cholesterol (Beta Lab., Philadelphia, USA) were checked prior to use for absence of autooxidation products by chromatography on reversed-phase partition columns[12]. Incubations were carried out under the conditions already described[10]. Incubations were stopped by the addition of ethanol and the resulting precipitate filtered off. The ethanol was evaporated, the aqueous phase was acidified with HCl and extracted twice with water-saturated butanol. The residue from the neutral washed butanol extracts was then subjected to chromatography[12] to separate cholesterol from more polar products.

After preliminary experiments had shown the consistent formation of the same polar products, the following experiment was performed. 60 μC [4-^{14}C]cholesterol (1.2 mg) and 8.8 μC [26-^{14}C]cholesterol (8.8 mg) were each incubated with liver mitochondria from 12 mice, weighing approx. 25 g. The reaction mixtures were worked up as described above and the butanol extracts were chromatographed with phase system I[12]. It was found that 8 % of the [4-^{14}C]- and 3 % of the [26-^{14}C]cholesterol had been converted to more polar products. There was no appreciable formation of cholesterol esters. The polar products appearing as one major peak in phase system I (Fig. 1) were separated into several peaks with phase system III. Fig. 2 shows a chromatogram with phase system III of the more polar products formed from [4-^{14}C]cholesterol. Most of the isotope was present in a distinct peak with a maximum at 28 ml of effluent. This material was chromatographed together with various known compounds and was found to be somewhat more polar than 25-hydroxycholesterol and considerably more polar than 7α-hydroxycholesterol, but not polar enough to

be an acid. As the compound was formed also in the incubation with [26-^{14}C]cholesterol, it is probably a C_{27}-steroid, having a chromatographic mobility similar to a neutral trihydroxylated C_{27}-steroid.

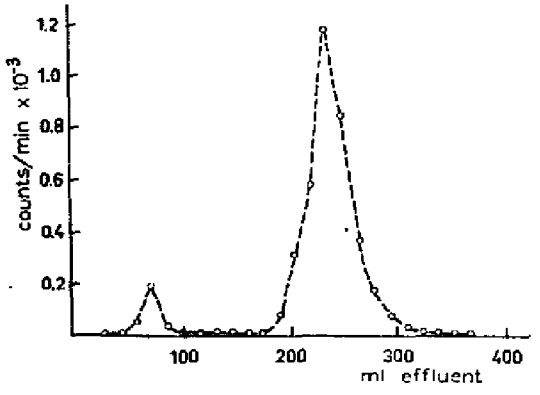

Fig. 1. Chromatogram of the residue from the butanol extract of the incubation of 60 μC [4-^{14}C]cholesterol with liver mitochondria from 12 mice. 18 g column. Mobile phase, 55% aq. isopropanol; stationary phase, 20% chloroform in heptane.

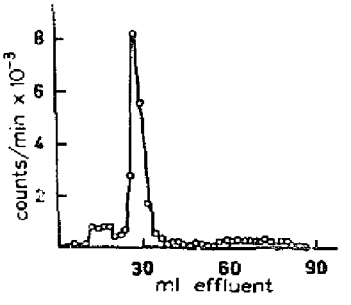

Fig. 2. Chromatogram of the polar products (first peak, Fig. 1) formed from [4-^{14}C]cholesterol incubation. 4.5 g column. Mobile phase, 50% aq. isopropanol; stationary phase, 20% chloroform in heptane.

To test the conversion of this unknown compound to bile acids, 0.23 μC was injected intraperitoneally as a serum-albumin suspension into a bile-fistula rat. 62.5% of the injected activity was excreted in the fistula in 19 h. The saponified bile was chromatographed on phase system[13] C. The unknown compound was not to any appreciable extent converted to cholic acid but to three other acids, two of which exhibited mobilities similar to the 6-hydroxylated acids formed from chenodeoxycholic acid in the rat. The eluate from this column was then chromatographed on phase system[14] F and the main portion of the isotope coincided with the titration peak of chenodeoxycholic acid. The identity of the third isotopically labeled acid with chenodeoxycholic acid was established by crystallization to constant specific activity with unlabeled chenodeoxycholic acid.

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Evidence for a new oxidative pathway for tryptophan

The conversion of tryptophan to kynurenine is a well established biochemical reaction and with the recent work showing the conversion of kynurenine to 3-hydroxykynurenine[1,2] the overall sequence of metabolic steps from tryptophan to niacin appears to be substantially complete: tryptophan → kynurenine → 3-hydroxykynurenine → 3-hydroxyanthranilic acid → niacin.

The existence of these intermediates has been demonstrated repeatedly. Indeed, so much work has been reported in this area that it has almost obscured the possibility that tryptophan could also be metabolized by a second and hitherto unknown pathway. Although the sequence of reactions outlined above does not account for the ready oxidation of the benzene ring of tryptophan[3], it has been suggested that the enzymic opening of the 3-hydroxyanthranilic acid ring could lead to an oxidizable product instead of to niacin[4].

We wish to report evidence for a new pathway of tryptophan oxidation in rat liver which appears to be independent of kynurenine. The materials used for these experiments were L-[7a-^{14}C]tryptophan and L-[^{14}C]kynurenine singly labeled in the equivalent position of the benzene ring. The tryptophan was synthesized chemically, and resolved enzymically. The L-[^{14}C]kynurenine was prepared enzymically from the L-[7a-^{14}C]tryptophan. Both materials were pure as determined by paper chromatography and radioautography in two sets of solvents. The specific activity of each compound was 1 μC/μmole. Homogenates prepared from the livers of female Sprague-Dawley rats weighing approximately 200 g were used in these experiments. The livers were homogenized in four times their weight of 0.9 % KCl and then centrifuged at 2000 × *g* for 10 min to remove whole cells and debris. Incubations were carried out for 2–2.5 h at 37° in a Dubnoff shaking incubator. CO_2 was collected by means of alkali in a center well and precipitated and counted as $Ba^{14}CO_3$.

It can readily be seen that although tryptophan and kynurenine are quantitatively balanced in the medium, only the tryptophan is significantly oxidized under these conditions. Therefore, kynurenine cannot be an intermediate in the total oxidation observed in these experiments. The possibility exists, of course, that an activated form of kynurenine is produced from tryptophan; one which cannot readily be derived from free kynurenine itself.

Expt. 3 indicates that a reversal of oxidative rates for tryptophan and kynurenine occurred when 0.25 *M* sucrose was used for the medium instead of 0.9 % KCl. This result was also obtained in a second set of experiments. There is no question but that L-[^{14}C]kynurenine *can* be oxidized by broken cell preparations under appropriate conditions. However, in 0.9 % KCl, the evolved CO_2 never amounted to more than 0.05 % and was usually 0.02 % of the starting radioactivity.