Short Communications and Preliminary Notes

Enzymic activation of cholic acid involving coenzyme A

It is generally accepted that cholic acid is conjugated in the liver with taurine and glycine to give taurocholic and glycocholic acids. During an investigation into the mechanism of these reactions it was observed that microsome preparations from guinea pig liver, when incubated under suitable conditions, were capable of forming a hydroxamic acid in the presence of cholic acid. There appear to be two distinct mechanisms for the synthesis of this hydroxamic acid. The first is a simple condensation of cholic acid with hydroxylamine and appears to be analagous to the lipase catalysed formation of fatty acid hydroxamates described by LIPMANN AND TUTTLE[1]. This synthesis is almost completely abolished by fluoride as shown in Table I.

TABLE I

Complete incubation mixtures contained the following: 0.2 ml of 0.25 *M* phosphate buffer, pH 7.4, 0.5 ml of 2 *M* NH_2OH, 0.1 ml of 0.04 *M* K^+ cholate, 0.1 ml of 0.3 *M* KF, 0.5 ml of a microsome suspension (equivalent to 0.5 g wet weight of liver). Final volume 2.25 ml. Incubations carried out at 37° for 1 h.

Exp. No.	*μ-mol hydroxamic acid*
1. No cholate, no KF	0.25
2. + cholate, no KF	0.6
3. + cholate, + KF	0.3

In the presence of fluoride another cholic acid-dependent formation of a hydroxamic acid can be demonstrated. As shown in Table II this requires the addition of coenzyme A (CoA), ATP and cysteine for maximum activity.

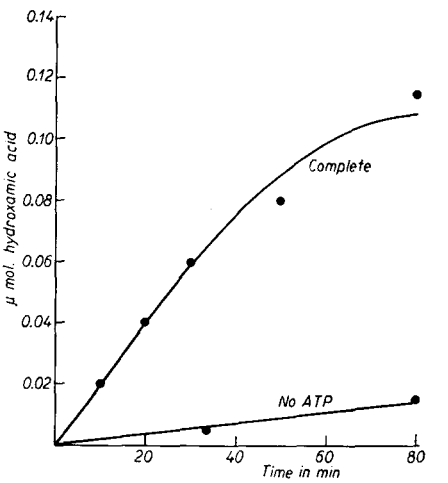

Fig. 1.

TABLE II

Complete incubation mixtures contained the following: 0.2 ml of 0.25 *M* phosphate buffer, pH 7.4, 0.5 ml of 2 *M* NH_2OH, 0.5 ml of 0.04 *M* K^+ ATP, 0.1 ml of 0.04 *M* K^+ cholate, 0.05 ml of 0.2 *M* cysteine, 0.1 ml of 0.1 *M* $MgSO_4$, 0.1 ml of 0.3 *M* KF, 100 Lipmann units of CoA, 0.5 ml of microsome suspension. Final volume, 2.25 ml. Incubations were carried out at 37° for 80 min.

Exp. No.	*μ-mol cholylhydroxamic acid*
1. Complete	1.45
2. Complete, reaction stopped at time 0	0.3
3. No cholate added	0.5
4. No ATP added	0.45
5. No CoA added	0.6
6. No cysteine added	0.9

Fig. 1 shows the time course of the reaction in the presence and absence of ATP.

The optimum concentration of cholic acid is about 0.002 *M*. High concentrations of hydroxylamine are required; this suggests that a non-enzymic interaction between cholyl-CoA and hydroxylamine is occurring. Relatively high concentrations of ATP are also required for full activity.

An incubation mixture in which a hydroxamic acid had been synthesised enzymically was dried *in vacuo* and an ethanol extract of this was chromatographed in a solvent containing *iso*propyl ether

(100) *n*-propanol (45) acetic acid (15) H_2O (10). A single hydroxamic acid spot was found after spraying the paper with $FeCl_3$ with the same R_F value (approx. 0.6) as a sample of the hydroxamic acid prepared from methyl cholate.

These results suggest that cholyl-CoA is an intermediate in the synthesis of conjugated bile acids. Since this work was started BERGSTRÖM AND GLOOR[2] have reported the synthesis of taurocholic acid in rat liver slices and homogenates.

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[1] F. LIPMANN AND L. C. TUTTLE, *Biochim. Biophys. Acta*, 4 (1950) 301.
[2] S. BERGSTRÖM AND U. GLOOR, *Acta Chem. Scand.*, 8 (1954) 1373.

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Conversion of 19-hydroxy-Δ^4-androstene-3,17-dione to estrone by endocrine tissue

Ever since the similarity in structure of the steroidal estrogens and androgens was recognized in the middle thirties, speculations as to the biological conversion of C_{19} into C_{18} steroids have been advanced[1]. The most direct experimental foundation for such a hypothesis was arrived at in the observation of an increase in the urinary estrogens after the administration of androgens. The possibility that these androgens functioned indirectly by stimulating the production or release of the estrogens, however, could not be ruled out[2]. The recent failure to detect a substantial incorporation of injected radioactive cholesterol (a precursor of androgens) into the urinary estrone of the pregnant mare[3], left the problem wide open[4].

With the discovery of an enzyme in mammalian tissue capable of hydroxylating androstenedione at the angular methyl group which is absent in the estrogens, the possibility of a biosynthesis of the natural estrogens from non-aromatic steroids became more plausible. The elimination of an oxygenated methyl group is compatible with biological concepts and such a course was consequently postulated[5]. Subsequently the conversion by human ovary of radioactive testosterone to estradiol, deduced by the use of a carrier dilution technique, was announced[6]. The utilization of acetate in the production of estradiol and estrone by dog ovaries[7] and by human term placenta[8] has also been reported recently.

In the present experiments, 400 μg of steroid were incubated, while shaking at 37.5° for 4 h in air, with 0.80 g of tissue slices suspended in 3.0 ml of aqueous medium (pH 7.3) containing the following additions: phosphate buffer 0.09 *M*, sodium fumarate 0.012 *M*, nicotinamide 0.02 *M*, and diphosphopyridine nucleotide 0.0006 *M*. The incubated slices were homogenized and the total incubate deproteinized with acetone. The filtered extract was brought to dryness by lyophilization and the residue re-extracted with fresh acetone. This second extract was dried and separated into three fractions on a silica gel adsorption column. The solvent mixture used in the first fraction (5% hexane in benzene) eluted lipid material only and was not processed further. The second fraction (40% ether in benzene) contained substances of the polarity of estrone and estradiol, and the third fraction (25% isopropanol in ethyl acetate) the more polar compounds such as Δ^4-androsten-19-ol-3,17-dione.

The second fraction was placed on a 3–4 cm wide paper strip (Whatman No. 1) which was impregnated with a solution of propylene glycol in 90% aqueous methanol (40:60 by volume) and blotted after 3 minutes. When chromatographed at 31° with propylene glycol saturated toluene[9], the R_F for estrone was 0.20 and for estradiol 0.03. These zones were eluted and suitable aliquots rechromatographed on paper at 31° (after a 4 h equilibration) with a two phase mixture of benzene-hexane-methanol-2 *N* ammonium hydroxide (65:35:15:15)[10]. In this system the R_F for estrone was 0.85 and for estradiol 0.65. Evidence for the presence of estrogens was considered acceptable when the very sensitive Turnbulls blue reagent for phenolic substances[11] produced a notable color at the correct location (sensitivity 0.5 μg/sq. cm). The quantity of phenolic estrogens could be easily estimated visually by comparing the color intensities with those of known amounts (accuracy $\pm$ 10%).

Of the tissues tested, human placenta appeared to convert 19-hydroxy-Δ^4-androstene-3,17-dione (I) to estrone (II) most efficiently. A production of II was also observed with cow ovarian follicular fluid (1.5 ml of fluid in a buffered and balanced salt solution with 400 μg of steroid) and to a lesser extent with cow adrenals. Rabbit placenta, removed after 15 days gestation period, and ovaries of rabbits which had been stimulated with gonadotropic hormone 7 h before sacrifice, did not show a pronounced conversion.

Human placentae were obtained by Caesarean section from healthy women after gestation periods of 7 months to term. Slices were incubated 1–3 hours after collection. A conversion of I $\rightarrow$ II amounting to 2–6% was observed. The formation of estradiol (III) was of a smaller order of magni-