CCXCV. THE BIOLOGICAL SPLITTING OF CONJUGATED BILE ACIDS.

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THE biological splitting of the conjugated bile acids into their two components, amino-acid and cholic acid, is important both from the chemical and physiological points of view. Two possible ways of effecting this reaction have to be considered: (1) by means of tissue enzymes; (2) by bacteria and moulds.

Several authors have investigated this problem but their results are of a somewhat contradictory nature.

The present work was undertaken with the intention of clarifying the position.

I. THE QUESTION OF THE SPLITTING OF CONJUGATED BILE ACIDS BY ENZYMES FROM ANIMAL ORGANS.

Smorodinzew [1923] in the course of his work on histozyme, the classical enzyme splitting hippuric acid [cf. Mutch, 1912], found that horse and canine kidney extracts which were rich in histozyme could also split glycocholic and taurocholic acids, and that preparations of dog liver, of a somewhat feeble activity against hippuric acid, hydrolysed taurocholic acid but scarcely attacked glycocholic acid. From his results he inferred that histozyme is able to split not only hippuric acid but also the conjugated bile acids. His experiments which were carried out with a rather primitive technique do not allow of more than qualitative conclusions.

Domenico [1926] working with liver pulp of various mammals did not observe splitting of the conjugated bile acids and concluded that the liver of mammals does not contain a specific enzyme capable of hydrolysing the bile constituents mentioned. Rosenthal *et al.* [1927] working with pulp of human liver and spleen state that they observed splitting of taurocholic acid after incubation for 5–6 days. Grassmann & Basu [1931] employing a modern technique confirmed the statements of Smorodinzew as regards the observed ability of kidney extracts to hydrolyse (although only to a rather limited degree) taurocholic and glycocholic acids; they did not however succeed in hydrolysing these acids by the action of liver extracts at pH 8.

On the other hand Mazza & Stolfi [1932] claimed to have found regularly in liver extracts of different animals a specific enzyme which hydrolysed the conjugated bile acids quickly and synthesized them from their two components. They attribute Grassmann's failure to observe this enzyme to the fact that it is only active in a weakly acid or neutral medium (pH optimum being 6.0); whereas Grassmann & Basu were working at pH 8, at which the enzymic action according to Mazza & Stolfi is inhibited. The latter suppose that the enzyme found by them to be regularly present in the liver is different from histozyme, though they agree with previous workers that the histozyme of the kidney is also capable of splitting the conjugated bile acids. They do not seem to have investigated however whether their specific enzyme does not split hippuric acid as well.

There seems to be general agreement among the different workers on the following points.

(1) Enzyme preparations from the pancreas and from the mucosa of the duodenum do not split the conjugated bile acids.

(2) Kidney extract which contains histozyme also hydrolyses the conjugated bile acids. This action is slow and feeble and probably has no physiological importance since the bile acids do not traverse this organ during their circulation.

As regards the activity of the liver, which from the physiological point of view is undoubtedly the most important organ in this respect, the above review reveals discrepancies in the findings of the several workers.

The experiments described below were undertaken in order to clear up this point. A comparative investigation of the activities of enzymic liver preparations upon both hippuric acid and the conjugated bile acids has been carried out for the first time.

Preparation and study of acetone-ether-dried liver extract.

Dried powder was prepared from dog liver according to Willstätter & Rohdewald [1934]. The dried material was finely ground and then sieved through a close-meshed sieve. Weighed amounts of the powder were used in the experiments. The buffer solutions employed were mixtures of citric acid ($M/10$) and Na_2HPO_4 ($M/15$). The substrates were pure hippuric acid neutralized with NaOH , and pure Na salts of glycocholic or taurocholic acid. The determinations were carried out by Sørensen's formaldehyde titration using $N/10$ NaOH , taking 4 ml. of the reaction mixture for each titration. The reaction temperature was 37° .

Enzyme experiments. (a) 0.4 g. hippuric acid dissolved and neutralized with dilute NaOH ; 1 g. of liver preparation; 10 ml. buffer solution pH 7; water to 35 ml.; 5 ml. toluene.

(b) (I): 0.7 g. Na taurocholate; 0.5 g. liver preparation; 10 ml. buffer solution pH 7; 15 ml. water; 5 ml. toluene.

(II): like (I), except that the buffer solution was of pH 8.

(c) (I): like (b) (I), except that the substrate was Na glycocholate.

(II): like (b) (II), except that the substrate was Na glycocholate.

Controls: liver preparation + corresponding buffer solutions; substrates + corresponding buffer solutions. All controls have the same concentration as the corresponding enzyme experiments.

The figures given in Table I are the observed changes after the corresponding control values are deducted.

Table I. *Action of dog liver preparation (dried with acetone-ether) on hippuric, glycocholic and taurocholic acids.*

		Increase in ml. $N/10$ NaOH per 4 ml. reaction mixture after the indicated number of days											
	pH	1	2	3	4	5	6	7	8	9	10	11	12
Na hippurate	7	0.16	0.36	—	—	1.06	—	—	—	—	—	—	1.96
		(6.3%)	(14.1%)			(41.5%)							(77%)
Na taurocholate	7	0	0	0.08	—	0.08	—	0.16	—	0.14	0.14	—	0.08
	8	0	0	—	0.0	—	-0.18	—	-0.18	—	—	—	—
Na glycocholate	7	0.04	—	0.06	0.08	—	0.06	—	—	—	0.06	—	—
	8	0.0	—	-0.10	—	-0.14	—	-0.14	—	—	—	—	—

Table I shows that the liver preparation contains an enzyme capable of splitting hippuric acid, i.e. the classical histozyme. Neither at pH 7 nor 8 were the conjugated bile acids split. The slight irregularities observed are due to the presence of small solid liver particles in the reaction mixtures and are within the experimental error. This result suggests (1) that the enzyme contained in this liver and splitting hippuric acid is not able to split taurocholic or glycocholic acid; (2) that it must therefore be different from that found by other authors in the kidneys which splits both hippuric and conjugated bile acids and (3) that this liver preparation does not contain the specific enzyme of Mazza & Stolfi capable of splitting taurocholic and glycocholic acids.

At the time these experiments were carried out the paper of the last-mentioned authors was unknown to us. Since the technique employed by these authors differed from that used by us in that they did not use the dried liver preparation directly but extracted it with glycerol, we repeated their experiments with fresh ox liver and made an acetone-ether-dried preparation as well as a direct glycerol liver extract according to the details given by Mazza & Stolfi. According to these authors both preparations should be active.

Preparation and study of glycerol liver extracts.

Liver of a freshly slaughtered ox was used for both preparations according to Mazza & Stolfi.

(a) *Glycerol liver extract (direct extraction).* The liver was roughly ground, minced and triturated with quartz in 90 % glycerol in a porcelain mortar. The liquid was then filtered off or centrifuged.

(b) *Acetone-ether preparation and subsequent glycerol extraction.* The material was dried by acetone-ether treatment according to Willstätter & Rohdewald and was then extracted with glycerol-water mixture (containing 80 % glycerol) at 37° during 24 hours.

The following substrates were used: (1) glycylglycine; (2) *dl*-leucylglycine; (3) hippuric acid; (4) Na glycocholate; (5) Na taurocholate. Buffer mixtures: the same as used by Mazza & Stolfi, i.e. phosphate buffers (*M*/15). Titrations according to Sørensen, employing 2 ml. of the reaction solutions and *N*/10 NaOH.

(A) *Enzymic experiments with glycerol liver extracts.* (1) 0.3 g. *dl*-leucylglycine; 8 ml. buffer pH 8; 5 ml. glycerol liver extract; water to 25 ml.

(2) 0.3 g. glycylglycine, all other components as in (1).

(3) 1.4 g. hippuric acid neutralized with *N* NaOH; 8 ml. buffer pH 7; 4 ml. liver extract; water to 25 ml.

(4) 1.5 g. Na glycocholate; 8 ml. buffer pH 6.2; 4 ml. liver extract; water to 25 ml.

(5) 1.5 g. Na taurocholate, all other components as in (4).

(B) *Experiments using glycerol extract of acetone-ether-dried liver.* (1) 0.3 g. glycylglycine; 8 ml. buffer pH 8.0; 5 ml. liver extract; water to 25 ml.

(2) 1.4 g. hippuric acid neutralized with *N* NaOH; 8 ml. buffer pH 7; 3 ml. liver extract; water to 25 ml.

(3) 1.5 g. Na glycocholate; 8 ml. buffer pH 6.2; 3 ml. liver extract; water to 25 ml.

In all cases described under A and B the appropriate controls were conducted, and the control values observed were deducted from the figures given in Table II. The reaction temperature throughout was 37°.

The results summarized in Table II are in accordance with those of Mazza & Stolfi as regards the presence of dipeptidase in both preparations but do not

Table II. *Action of glycerol or liver extract preparations on dipeptides, hippuric acid and conjugated bile acids.*

		Increase in ml. <i>N</i> /10 NaOH per 2 ml. reaction mixture after the indicated number of hours								
Substrate	pH	1	2	3	19	20	48	68	96	168
Experiments with extract obtained by direct extraction of liver with glycerol.										
Glycylglycine	8	0.50	0.60	0.65	—	1.40	—	—	—	—
<i>dl</i> -Leucylglycine	8	0.22	0.30	—	0.46	0.48	—	0.54	—	—
Na hippurate	7	—	—	—	0.0	—	0.0	—	0.02	0.03
Na glycocholate	6.2	—	—	—	0.0	—	0.0	—	0.0	0.0
Na taurocholate	6.2	—	—	0.03	—	—	0.0	—	0.0	—
Experiments with extract obtained by previous treatment of liver with acetone-ether and subsequent extraction with glycerol.										
Glycylglycine	8	0.0	0.10	—	—	1.15	—	—	—	—
Na hippurate	7	0.0	—	—	0.0	—	0.0	—	0.0	0.04
Na glycocholate	6.2	0.0	0.0	—	—	—	0.0	—	0.0	0.0

support the findings of these authors as regards the presence also of a specific enzyme splitting the conjugated bile acids. The preparations hydrolysed *dl*-leucylglycine and glycylglycine but, as in the previous case with dog liver, failed to split glycocholic or taurocholic acid, although the experiments were carried out at the optimum pH or within the pH range given by Mazza & Stolfi for the activity of their specific enzyme. Thus no cholic acid could be detected by qualitative test in the reaction mixtures. Our results are in agreement with those of Domenico and Grassmann & Basu and moreover cover a broader pH range.

II. THE EXISTENCE OF BACTERIA CAPABLE OF HYDROLYSING CONJUGATED BILE ACIDS.

As early as 1876 Hoppe-Seyler supposed that taurocholic acid could be hydrolysed by bacteria [1876]. Since cholic acid is regularly found in the faeces, it has been assumed that bacterial hydrolysis of the conjugated bile acids occurs during their passage through the intestines. This has never been proved experimentally. Several authors [Licht, 1924; Rosenthal *et al.*, 1927], working mainly with pure strains of bacteria known to be present in the intestines, failed to find hydrolysis of the conjugated bile acids into their components; they supposed that the molecule of the bile acids was attacked by bacteria at other places, the .CONH. linkage between the two components remaining intact.

Isolation of bacteria splitting conjugated bile acids.

The bacteriological part of the following experiments was carried out by Dr M. Aschner.

A synthetic nutrient medium was used containing 1–2 % of either glycocholic or taurocholic acid as sole source of C and N, and the following inorganic salts: K₂HPO₄ (0.1 %), MgSO₄ (0.1 %), CaCO₃ (0.2 %) and traces of iron. The medium was inoculated with small amounts of different biological materials, such as soil, contents of the lower part of canine intestines, human faeces and in a few cases also human intestinal contents taken during autopsy. After keeping at about 20–25° under aerobic conditions in positive cases bacterial growth was observed. By re-inoculating into the same medium pure cultures of various strains were finally obtained, which, as will be shown, are able to hydrolyse glycocholic and taurocholic acids, liberating cholic acid.

The bacteria are Gram-negative, mobile and produce thick membranes and pigments in media containing bile acids. They do not grow *in vitro* at 37°. A detailed description of the bacteriological properties of the strains will be given elsewhere by Dr M. Aschner.

As regards moulds it should be noted that Grassmann & Basu [1931] found that an aqueous extract of *Aspergillus oryzae* splits glycocholic acid very slightly but taurocholic acid not at all. We have more than once observed growth of mould organisms on synthetic nutrient containing bile acids.

Detection and isolation of cholic acid liberated from conjugated bile acids by bacteria.

Pure Na taurocholate and glycocholate were used in these as well as in the enzymic experiments. Many commercial samples contain significant amounts of other substances which caused us much trouble until this source of complications was traced. In these experiments the nutrient medium contained besides the salts mentioned above 2% of the conjugated bile acid. In order to avoid prolonged heating which favours hydrolysis of the taurocholic acid the solutions were sterilized by filtering through a Seitz filter. Solid CaCO_3 was afterwards added. To this solution was added a small amount of one of the previously mentioned pure cultures of bacteria grown on conjugated bile acid and washed when necessary. The solution was kept at 20–25° until bacterial growth was sufficiently developed. For the qualitative detection of cholic acid the Mylius reaction which is not given by the conjugated bile acids and is specific for cholic acid was used: some of the solution to be tested was acidified with *N* HCl, and after decanting the supernatant fluid the precipitate was dissolved in alcohol; iodine solution was added and then water drop by drop with shaking. In the presence of cholic acid, green-blue needles suddenly appear which often fill the whole test-tube as a voluminous fluorescent mass. In order to isolate the cholic acid, ether was added to the solution in a separating funnel and then, with continuous shaking, *N* HCl until acid to Congo red. Under such conditions cholic acid generally passes into the ether layer. Only when greater volumes are handled does some remain undissolved. The ether layer was dried with CaCl_2 and evaporated. The crystalline residue was dissolved in a little hot absolute alcohol and kept for some days in the ice-box with repeated scratching with a glass rod. The crystals were then collected, washed several times with ice-cooled alcohol and dried *in vacuo* at 100°. They gave all the reactions of cholic acid. In most cases crystallization and drying had to be repeated, before the melting point of pure cholic acid (197.5°, not corrected) was reached. Identity of this product with cholic acid was finally confirmed by mixed melting point.

Such experiments were carried out with both glycocholic and taurocholic acids on a number of strains isolated from the different sources indicated above: human faeces, human intestines, dog intestines and soil. The yields of cholic acid were relatively considerable. Since the operations of isolation and recrystallization involve considerable losses and also since the cholic acid seems to be used as a nutrient by the bacteria, no quantitative data are given. Hitherto it has not been possible to separate the enzyme from the bacterial cell.

As mentioned above the participation of the bacterial flora of the intestines in the process of splitting conjugated bile acids has been discussed several times in the past, although previous workers have not found such bacteria.

As regards the physiological interpretation of the present findings the following should be noted.

(1) It was not possible to isolate the bacteria described from every sample of human faeces.

(2) The bacteria described here do not grow at body temperature *in vitro*.

It was attempted to ascertain whether these bacteria are able to hydrolyse glycocholic and taurocholic acids at 37°. Cultures of strains from human and dog intestines and grown at about 25° were washed free of their medium until they did not give the test for cholic acid. They were then introduced into media containing either Na taurocholate or glycocholate and kept at 37° under sterile conditions, together with appropriate controls consisting of the same media without inoculation of the different strains. The controls remained negative. In inoculated mixtures it was possible within 24 hours to detect and, where it was attempted, also to isolate cholic acid.

It is therefore clear that the bacteria, although they do not grow at 37° *in vitro* are capable of hydrolysing the conjugated bile acids at this temperature.

SUMMARY.

1. A dog liver preparation capable of splitting hippuric acid did not hydrolyse the conjugated bile acids. Liver histozyme therefore does not necessarily split the conjugated bile acids.

2. No enzyme splitting the conjugated bile acids (or hippuric acid) was found in an ox liver preparation.

3. Bacteria were isolated from soil, human and dog intestines and from human faeces which grow on synthetic nutrient media containing conjugated bile acids as sole source of carbon and nitrogen and which split these acids into their two components.

4. Although not growing at 37° *in vitro* tested strains hydrolysed the conjugated bile acids at this temperature *in vitro*.

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