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ACUTE TAURINE DEPLETION AND MAXIMAL RATES OF HEPATIC CONJUGATION AND SECRETION OF CHOLIC ACID IN THE DOG

BY E. R. L. Ó'MÁILLE, T. G. RICHARDS AND A. H. SHORT
From the Physiological Laboratory, The University of Liverpool

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Previous workers (Foster, Hooper & Whipple, 1919; Virtue & Doster-Virtue, 1937) have shown that feeding cholic acid to fasting dogs with bile fistulae leads at first to an increased output of taurocholate in the bile, and that this response diminishes with repeated administration (presumably due to taurine depletion) until finally no increase at all is observed. The objects of the present research were to follow the time course of the decline in output of taurocholate during an intravenous infusion of cholic acid at a constant rate, and hence to estimate the size of the 'taurine pool'; to determine the maximal rate of secretion of free cholate in the taurine-depleted dog, and to determine the maximal rate of conjugation of cholic acid with taurine. A preliminary account of this work has been communicated to the Physiological Society (Ó'Máille, Richards & Short, 1964).

METHODS

Adult mongrel dogs premedicated with diethylthiambutene (Themalon, Burroughs Wellcome & Co.) were anaesthetized with pentobarbitone (Nembutal, Abbott Laboratories). The abdomen was opened by a median incision, the common bile duct cannulated and the cystic duct ligated. In some experiments a catheter was inserted into a radicle of the splenic vein for the purpose of infusions into the portal system. When urine was to be collected, both ureters were catheterized intra-abdominally.

Bile-acid solutions were infused into the right femoral vein by means of a motor-driven rotary burette (in earlier experiments standard hospital infusion equipment was used). Bile and urine were collected in graduated tubes; blood samples were withdrawn from the left femoral vein, transferred to tubes containing 3 drops of heparin solution (1000 I.U. per ml., Evans Medical), and gently shaken.

Cholic acid solutions which ranged in concentration from 1 to 5 g./100 ml. were made up as follows: cholic acid was first dissolved in 0·154M-NaOH (approx. 18–19 ml./g cholic acid); 1·4 ml. 0·154M-$CaCl_2$ was added per 100 ml. (final vol.) and the pH adjusted to 7·4 with 0·154M-HCl; the solutions were brought to the correct volume with either 0·3M glucose or 0·154M-NaCl. Sodium taurocholate was dissolved in water to give a 0·154M solution; Calcium chloride solution was added, 1·4 ml. of 0·154M/100 ml. (final vol.), and the pH adjusted to 7·4 with 0·154M-HCl; solutions were brought to the final volume with 0·154M-NaCl. Concentrations ranged from 3 to 8 g sodium taurocholate per 100 ml. The taurine solutions were brought to pH 7 with 0·154M-NaOH. B.D.H. chemicals were used except where other-

wise stated. The sodium taurocholate and sodium glycocholate were synthesized according to the method of Norman (1955). Part of the material used was prepared under contract for us by Mr J. F. Benford of L. Light & Co., Colnbrook, Bucks.

Analyses

Bile. Samples were diluted 201 times with water and then directly analysed for total cholate (conjugated plus free cholate) by means of the furfural-H_2SO_4 reaction as outlined by Irvin, Johnston & Kopala (1944) except that the volumes of reagent were: 0·5 ml. test solution, 3·0 ml. H_2SO_4, 0·5 ml. furfural, 1·0 ml. acetic acid; and heating in the water-bath was for 20 min instead of 13 min.

In these conditions we have found that taurocholate gives a reading 11·37 % higher than an equimolar concentration of cholate, and taurocholate concentrations have been calculated on this basis. It is therefore advisable to use standard taurocholate solutions as well as standard cholic acid solutions. Each test solution was read against its own blank at 6000 Å (Ilford filter no. 607) in an E.E.L. absorptiometer. Separation of conjugated from free cholate was carried out by descending chromatography in the Ta phase system (iso-amyl acetate 85; heptane 15, equilibrated with an equal volume of 70 % formic acid) of Sjovall (1959), after which the developed papers (Whatman 3MM) were air-dried with a fan heater. To locate the spots, reference strips were dipped in a 10 % (v/v) solution of H_3PO_4 in acetone, heated for 10–15 min in an oven at 90–100° C and examined under U.V. light, when cholic acid and its conjugates appear with a bright bluish fluorescence. The taurocholate spots were eluted with phosphate buffer (17·8 g $Na_2HPO_4.2H_2O$ plus 2 ml. conc. HCl, made up to 1 l. with water) into graduated tubes. Each free cholate spot was immersed in 7 ml. absolute ethanol overnight; 5 ml. of eluate was then removed, evaporated to dryness, and the residue redissolved in phosphate buffer. Solutions were then analysed by the furfural-H_2SO_4 reaction.

Blood. Plasma, 1 ml., was removed from each sample and shaken up with 11 ml. acetone for 1 min; these were centrifuged and the supernatant decanted into graduated tubes which were placed in the water-bath (65°) until the volume was reduced to 0·3–0·4 ml.; water was then added to bring the volume to 1·5–1·7 ml. and the tubes were replaced in the water-bath for 15 min; 7 ml. of petroleum spirit (boiling range 40–60° C, Esso) was added and the volume of the aqueous phase (v_1) was read. After 2 min *gentle* repeated inversion the tubes were centrifuged and a layer of particulate matter which forms near the interface was removed by suction; the tubes were centrifuged once more and the upper layer (including a thin film of material at the interface) removed by suction. The volume of fluid remaining was read (v_2) and reduced by further evaporation in the water-bath to 0·5 ml., which was then analysed for total cholate by the furfural-H_2SO_4 reaction.

Samples were turbid to a variable degree, so to obtain the optical density at 6000 Å of the cholate-furfural-H_2SO_4 coloured complex *alone* the following procedure was adopted. The test solutions were read against a furfural blank at 6000 Å (Ilford filter no. 607) and 4260 Å (Ilford filter no. 601). Since the optical density reading at each wave-length is equal to the sum of the optical densities of the cholate complex and of the turbidity, and if $a = R_{6000\ Å}/R_{4260\ Å}$ of turbid solutions without the cholate colour (obtained by omitting furfural from both test and blank) and $b = R_{6000\ Å}/R_{4260\ Å}$ of the cholate complex alone (obtained from standard cholate solutions in water) then:

$$R_{6000\ Å}{}^{*} = \frac{v_1}{v_2} \times \frac{b}{(b-a)} (R_{6000\ Å} - aR_{4260\ Å})$$

* (due to cholate *alone* in test solution).

The reading of the control plasma was subtracted from the remainder of the experimental readings and the concentration of plasma total cholate resulting from experimental infusion was then read off from a calibration curve.

Alternatively, with twice the above volumes, each final-plasma extract may be divided into a test solution to which is added H_2SO_4, furfural and (after heating) acetic acid, and a blank solution which receives water instead of furfural. The test solution is read against its own blank at 6000 Å only. We have recently adopted this latter modification though it requires larger plasma samples. We regard it as otherwise preferable to the original method which, however, was the one used for the experiments described here.

RESULTS

Infusions of cholic acid at constant rate

The effects of a continuous intravenous infusion of cholic acid administered at constant rate were studied in 23 experiments; the main changes which occurred in bile are shown in Fig. 1 which is based on a typical experiment. During the first 110 min the major fraction of the infused cholic acid was conjugated with taurine and appeared in that form in the bile, together with a small amount of free cholate. Over the succeeding

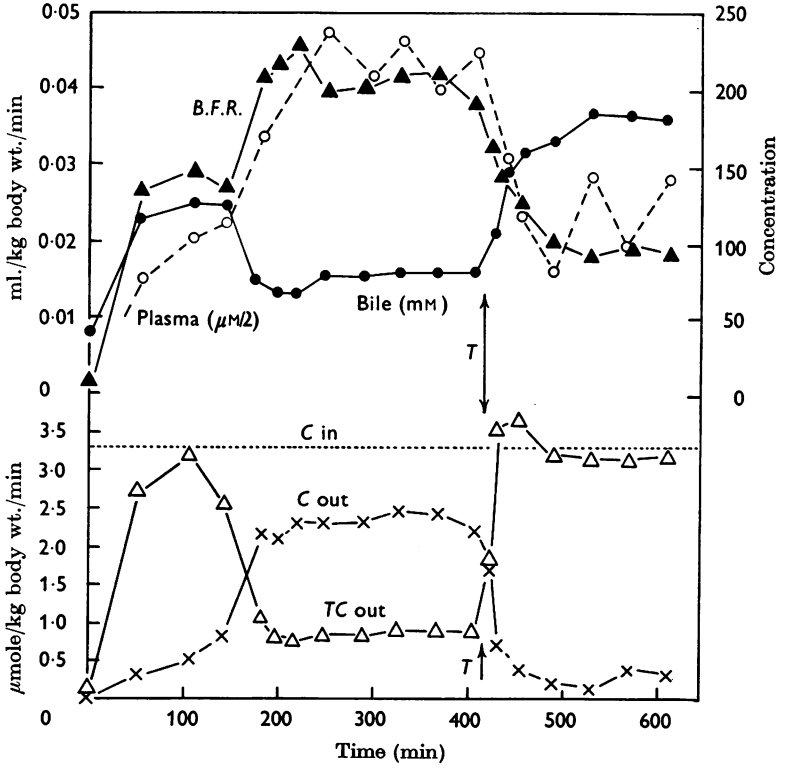

Fig. 1. Male dog, 11·6 kg. Effects of constant infusion of cholic acid at 3·3 μmole/kg body wt./min. Cholate output in bile (*C* out ×——×), taurocholate output (*TC* out △——△), total cholate concentration of plasma (plasma ○- - - - -○) and bile (bile ●——●), and bile flow-rate (*B.F.R.* ▲——▲) plotted against time after beginning infusion. Taurine (dose given in text) administered portally from *T*.

100 min the output of taurocholate showed a continuous decline to reach a steady level of 0·85 μmole/kg body wt./min which was maintained for the next 3 hr (in 15 expts. this was 0·85 ± 0·3 (S.D.) μmole/kg body wt./min). If this fall in taurocholate excretion were due to taurine depletion, as seems likely, then the size of the 'taurine pool' can be calculated approximately from the area under this (taurocholate) curve up to the end of the decline; the value of this pool in 13 experiments was 673 ± 146 (S.D.) μmole/kg body wt. Coincident with the decline in taurocholate output the output of free cholate steadily increased and also reached a stable level. The choleresis which resulted from the cholic acid infusion remained fairly constant from 45 to 140 min but then increased sharply. This increase in bile flow-rate signalled the appearance of increasing amounts of free cholate in bile.

The administration of taurine, taurine precursors, and glycine to the taurine-depleted dog

At *T* in Fig. 1 taurine (priming dose of 880 μmole/kg body wt., followed by infusion of 25·6 μmole/kg/min) was given portally; it caused a prompt reversal of the earlier changes in outputs of taurocholate and cholate, total bile salt concentration and bile flow-rate. The effects of some taurine precursors (cysteine, methionine and serine), and of glycine, given at the corresponding point in other experiments have already been reported (Ó'Máille *et al.* 1964). It is interesting that glycine could not be used for conjugation with cholic acid by the dog even in the taurine-depleted state. When, however, sodium glycocholate was administered, it was readily excreted in the bile.

Plasma total cholate concentration

Even when cholic acid was administered at a constant rate well within the excretory capacity of the liver, the plasma cholate concentration did not remain unchanged throughout the experiment, but increased at the point where the output of conjugated acid fell (and that of free acid rose) to attain a new equilibrium level in the taurine-depleted phase. In Fig. 2 it can be seen that during the first part of the experiment (when the bile acid being excreted was almost exclusively taurocholate) the plasma total cholate concentration was approximately 74 μM (hepatic clearance = 25·9 ml./kg/min); in the latter part (when free cholic acid predominated in the bile) the plasma concentration was about 313 μM (clearance = 5·5 ml./kg/min). The same phenomenon is seen in Fig. 1 which shows in addition the fall in plasma total cholate concentration following the administration of taurine. The cholate clearance was about 19·0 ml./kg/min before, and 7·5 ml./kg/min after depletion and again after

saurine administration it was 13·2 ml./kg/min. When synthetic taurotcholate was administered the clearance in the same range of output was of the same order as that of cholate when conjugation was proceeding. Following taurine administration (Fig. 1) the output of bile acid temporarily exceeded the input. This 'off-loading' was a constant feature of these experiments. The possible reasons for the changes in clearance are discussed below.

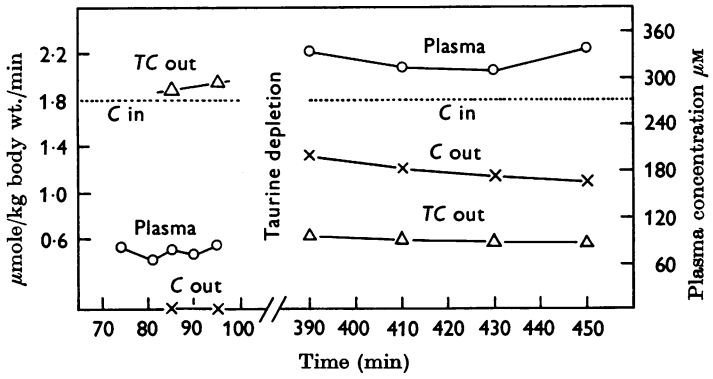

Fig. 2. Male dog, 25·2 kg. Plasma total cholate concentration and cholate and taurocholate outputs before and after acute taurine depletion. Constant infusion of cholic acid at 1·8 μmole/kg body wt./min (0–100 min and from 235 min onwards). Taurine depletion produced by cholic-acid administration at 7·1 μmole/kg/min(100–135 min) and 5·3 μmole/kg/min(135–235 min). Symbols and notation as in Fig. 1. When conjugation is greatly diminished by taurine depletion the plasma cholate level is markedly higher than in the presence of virtually complete conjugation.

Maximal rate of secretion of free cholate

This has been determined by experiments of two kinds: (*a*) Cholic acid was administered throughout at a constant rate of 10·9 μmole/kg/min (Fig. 3); the taurine pool was depleted and the output of free cholate reached a maximum of about 3·4 μmole/kg/min. (*b*) The taurine pool was first washed out by means of a continuous infusion of cholic acid at a rate of 4·16 μmole/kg/min after which the infusion was stopped for 50 min; cholic acid was then administered at five ascending rates (Fig. 4*a*) each lasting for 30–45 min, towards the end of which period bile and blood samples were taken. The secretion of free cholate was again limited to a rate of 3·4 μmole/kg/min. In these circumstances there was still a fairly steady output of conjugated bile acid at a low rate. In six experiments the maximum secretory rate of free cholate was found to be $3{\cdot}62 \pm 0{\cdot}26$ (S.D.) μmole/kg body wt./min.

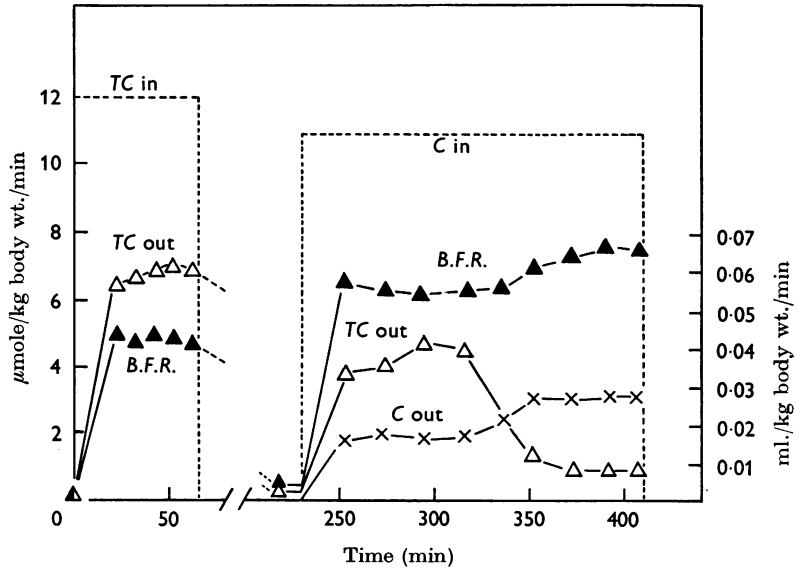

Fig. 3. Female dog, 21·4 kg. Constant infusion of synthetic taurocholate at 12·0 μmole/kg/min for 65 min. Pause for 150 min to permit return to resting state. Cholic-acid infusion at 10·9 μmole/kg/min from 231 min. Symbols and notation as in Fig. 1. Note that bile flow-rate during maximal taurocholate secretion is significantly less than during maximal free cholate excretion.

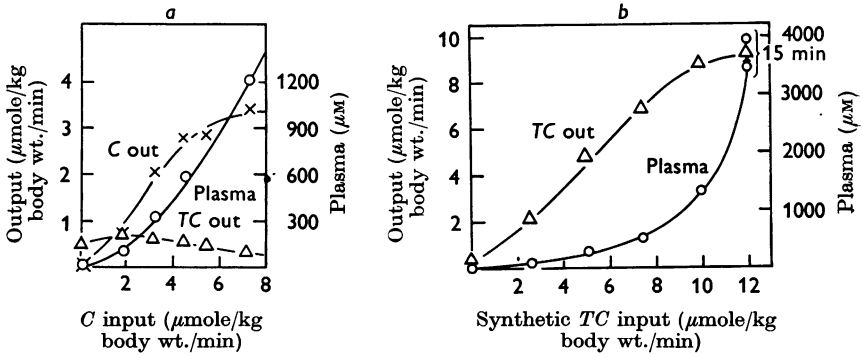

Fig. 4. *a*. Free cholate excretory maximum. Male dog, 17·8 kg. Free cholate and taurocholate outputs plotted against cholic acid input for five levels of infusion, each stage lasting about 38 min, *in the taurine depleted state*. *b*. Taurocholate excretory maximum. Male dog, 11·5 kg. Taurocholate output plotted against synthetic taurocholate input for five increasing levels of infusion, each stage lasting about 55 min. Symbols and notation as in Fig. 1.

Maximal rate of secretion of taurocholic acid

This has been determined both by continuous infusion of synthetic taurocholate at a rate above the expected maximum (Fig. 3) and by stepwise increase in the administration rate of taurocholate (Fig. 4*b*). The maximal rate of secretion of taurocholate in six experiments was 8·5 ± 1·6 (S.D.) μmole/kg body wt./min. This is somewhat higher than that reported in conscious dogs with chronic bile fistulae by Wheeler, Mancusi-Ungaro & Whitlock (1960) who used a bile salt preparation containing significant proportions of dihydroxycholanic acids (Wheeler & Ramos, 1960).

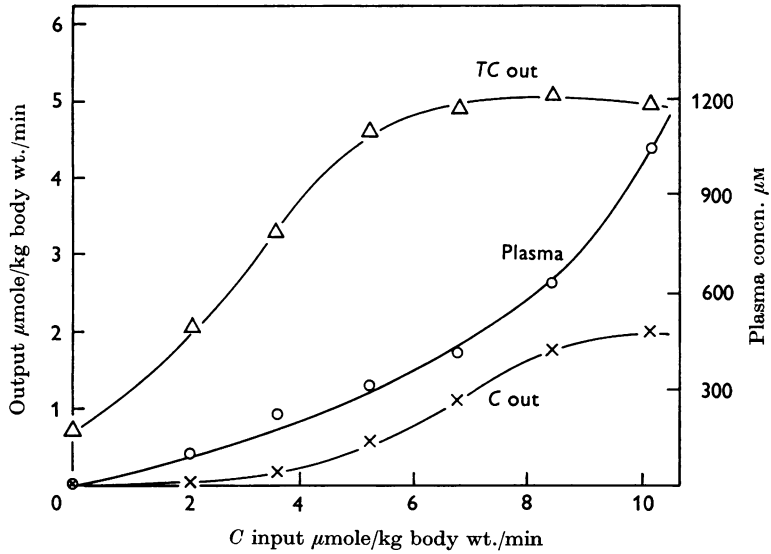

Fig. 5. Male dog, 13·1 kg. Maximal conjugation rate demonstrated by cholic-acid administration at six rates of constant infusion (each stage lasting about 70 min) together with taurine at six times (molar) the corresponding cholic acid rate. Symbols and notation as in Fig. 1.

Conjugation of cholic acid with taurine therefore enables a higher maximal rate of bile-acid secretion to be attained. It can also be seen in the early phase of the experiment depicted in Fig. 3 (i.e. before taurine depletion occurred) that active conjugation of the infused cholic acid with endogenous taurine enabled a higher rate of output of bile acid (mainly as taurocholate) to be reached as compared with the later taurine-depleted phase.

In ten experiments of a design similar to the above in which taurine was administered throughout with the cholic acid, the maximal output of conjugated acid was 5·2 ± 0·8 (S.D.) μmole/kg/min, which was associated

with 1·4 ± 0·8 (S.D.) μmole/kg/min of free cholic acid. Figure 5 is an illustration of one of these experiments. Here cholic acid was administered at six different rates, each lasting between 55 and 75 min; taurine was administered portally throughout at a rate six times (molar) the corresponding cholic acid rate. Blood, bile and urine samples were taken at the end of each stage. The maximal output of taurocholate was about 5·1 μmole/kg/min: this would appear in this instance to be also a maximal rate of

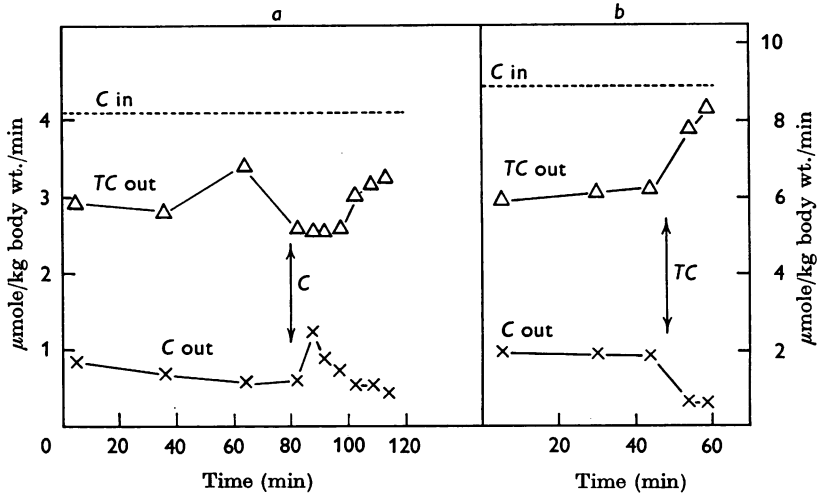

Fig. 6. Transport competition between cholate and taurocholate. *a*. Female dog, 17·7 kg. 41·2 μmole/kg body wt. cholic acid injected at *C* during constant infusion of cholic acid. Time 0 arbitrary. *b*. Female dog, 15·7 kg. 103·2 μmole/kg body wt. synthetic sodium taurocholate injected at *TC* during constant infusion of cholic acid and taurine. Time 0 arbitrary. Symbols and notation as in Fig. 1.

conjugation and not an excretory limit, as it can be seen that, when the output of taurocholate had become virtually constant, the secretion of free acid continued to increase. Excretory competition between cholate and taurocholate has been demonstrated (Fig. 6) so that conjugation itself must have been at a maximum in these circumstances. Experiments of this type are further discussed below.

Bile flow rates associated with the excretion of free and conjugated cholic acid

The presence of free cholic acid in bile was associated with a higher bile flow-rate than its molar equivalent of taurocholic acid. This has been briefly mentioned in the description of the effects of continuous infusion of cholic acid at constant rate. Figure 7 is a plot of total bile-acid output against bile flow-rate in which the points are derived from eighteen different ex-

periments. Biles containing taurocholic acid alone are contrasted with biles in which free acid largely predominated.

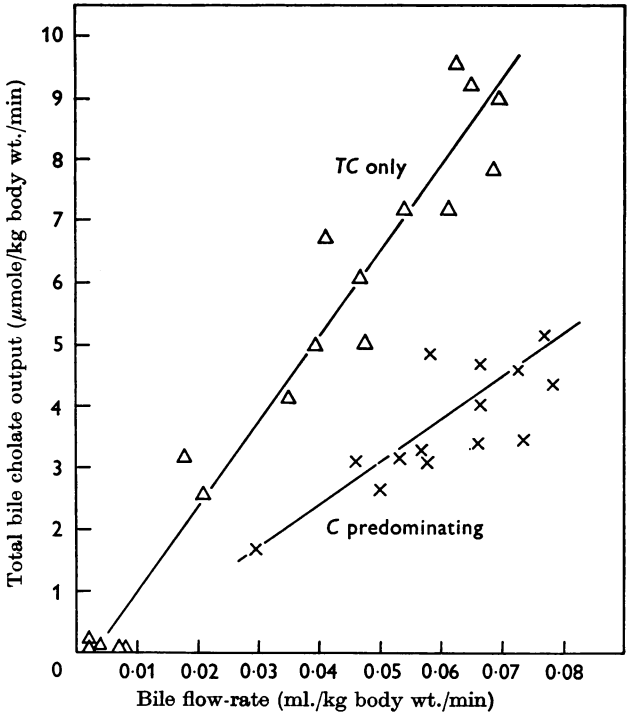

Fig. 7. Total cholate output rate(μmole/kg/min) plotted against bile flow-rate (ml./kg/min). Bile containing exclusively taurocholate, △. Bile containing at least 50 % free cholate ×. Data obtained from 18 experiments.

Presence of unidentified substance in bile after cholic acid administration

Whenever free cholic acid was administered an unidentified substance, thought to be a cholate derivative, appeared in bile. It has an R_F slightly greater than taurocholic acid and reacts with H_3PO_4 similarly to cholic and taurocholic acids (see Methods). It was at first thought to be an additional conjugate of cholic acid (Ó'Máille *et al.* 1964) but we have been unable to substantiate this. It is quantitatively unimportant.

Urinary losses

The urinary total cholate output where measured only accounted for a very small fraction of the infused bile acid. In general the urinary excretion varied with the plasma concentration, but in a non-linear fashion.

DISCUSSION

The rapid phase of taurine depletion demonstrated in the present experiments is not exposed in the procedure of earlier workers who fed cholic acid to dogs with chronic bile fistulae. The mass of material readily available for conjugation has been found to be the equivalent of 673 μmole/kg ± 146 (S.D.) of taurine. Since the administration of cysteine promptly leads to a considerable increase in taurocholate output in the depleted dog, part of this available mass may be in the form of cysteine. The steady rate of taurocholate output, after exhaustion of the rapidly available conjugant, of 0·85 μmole/kg/min may be due to either the transfer of taurine or cysteine from other tissues to the liver or to synthesis of taurine from cysteine precursors in the liver itself. Although it appears reasonable to suppose the immediate conjugant reservoir to be the liver itself, analyses of the exchangeable amino acids in the liver and other tissues of the dog do not seem to have been reported. Awapara (1956) has estimated the free taurine concentrations of the important tissues of the rat, rabbit, guinea-pig, ox, sheep and pig. In these species the liver contains only a small proportion of the total body taurine and, if the dog is similar in this respect, a loss of the order of 0·8 μmole/kg/min from the extrahepatic taurine pool might well fail to give detectable evidence of decay in rate over the period of observations reported here.

The secretion of bile containing a high proportion of free cholic acid is associated with higher plasma cholate levels than equivalent outputs of taurocholic acid. This is the case whether the taurocholate is synthesized within the liver or administered ready-made. The hepatic clearance of cholate is thus chiefly determined not by its form in plasma but by whether it is conjugated or free in the bile. The fall in clearance of cholate following taurine depletion is rapidly reversed by the administration of taurine or cysteine. The reduced clearance when free cholate is present may be explained by one or more of three mechanisms, a fall in hepatic blood flow without proportionate increase in extraction, elevated canalicular hydrostatic pressure owing to the higher bile flow-rate associated with free-acid secretion, or a less efficient membrane transport of cholate compared with taurocholate such that a relatively higher cellular concentration is required to achieve a given secretory rate. Since no measurement of hepatic blood flow was made in these experiments, it is not possible to assess the first possibility but it is hard to suggest a mechanism by which blood flow changes of the required order could be causally related to the state of cholic acid in the bile. The effect of hydrostatic pressure on bile secretion was studied by Richards & Thompson (1961) and it was shown that when the liver was caused to secrete bile against elevated pressure, recovery of

the original secretion rate was achieved by the accumulation within the body of a quantity of bile acid related to the magnitude of the pressure increment. The 'load' accumulated was deduced from the additional efflux of bile acid when the back pressure was removed. It was subsequently confirmed (Richards, unpublished experiments) that the plasma cholate concentration showed the expected rise in response to imposed back pressure. Since at present we cannot even approximate to the canalicular pressures required to induce the range of bile flows experimentally seen, this factor is of uncertain importance. Finally, any difference in the efficiency of excretion of the two species of bile-acid molecule must give rise to a change in cell content, and hence plasma concentration, as first taurocholate and then cholate predominates in bile. Such a difference in efficiency could arise either from a disparity in the number of carrier sites available to each species, or from difference in the turnover time of each species on a carrier available to either.

Although in general a rise in plasma level was associated with a rise in secretion rate of bile acid, very high plasma cholate levels appeared to be deleterious. When, for instance, the rate of bile-acid input rose above the rate of output during experiments to demonstrate maxima, the plasma cholic acid concentration, and presumably also the liver content, continued to rise with time. It was frequently observed in these circumstances that secretion reached peak values and subsequently gradually declined. This apparently inhibitory effect of increasing cholic-acid concentration resembles the results reported by Elliott (1956) which show that the reaction rate of cholic acid with hydroxylamine carried out by liver-microsome preparations *in vitro* was reduced by concentrations of cholic acid above a certain level. It was suggested by Elliott that this phenomenon was related to the detergent properties of cholate. Sometimes high plasma cholate levels were also associated with significant haemolysis.

One of the more striking results in these experiments has been the difference in the maximal rates of secretion of taurocholate (8·5 μmole/kg/min) and free cholate (3·6 μmole/kg/min). The value of the latter could not be obtained in the complete absence of taurocholate, and so is likely to be a slight underestimate of the true value. The bile volume associated with each maximum sheds some light on the problem of the influence of canicular pressure on secretory performance. In a single animal (Fig. 3), in which it is possible to assume that a constant pressure–flow relation obtains throughout, the bile-water flow was much lower during maximal secretion of taurocholate than during maximal secretion of mixtures of bile salt in which free cholate predominated. Secretion pressure cannot therefore be decisive in determining the maximal secretory rate of taurocholate, though it may play a part in limiting free cholate output. It is far more likely that

the maxima reflect the influence of molecular structure on secretory kinetics discussed above in relation to plasma cholate levels.

The demonstration of a maximal rate of conjugation depends on the fulfilment of certain conditions, viz. that free acid excretion continues to rise after taurocholate output has become constant. One of two such experiments is shown in Fig. 5, in which a maximum conjugation rate of 5·1 μmole/kg/min was found. The other experiment gave a value of 4·7 μmole/kg/min. When, as commonly occurred, taurocholate and free cholate outputs levelled out *together* in the face of augmented administration of free acid and taurine, it was not possible to conclude that the taurocholate output represented the maximum conjugation rate, but it did at least give a minimal value for it. Ten such experiments suggest that this value is 5·24 ± ·81 (S.D.) μmole/kg/min. This is always in excess of the free-acid secretory maximum, and less than the taurocholate secretory maximum.

The relation between bile-water output and bile-acid species has been noted. Sperber (1959) has suggested that bile-water flow is determined by the osmotic effect of the actively secreted bile acid. The greater choleresis which accompanies the appearance of free cholate in bile may be due to the exertion by the bile acid of a greater osmotic activity owing to a decrease in micelle size.

These experiments may suggest that conjugation of bile acids confers a biological advantage on the animal, but they offer substantial evidence against the view (Coombes, 1964) that conjugation is obligatory for bile-acid excretion. The biological advantage postulated stems from the markedly higher hepatic clearance of taurocholate (or cholate which can be conjugated) than the clearance of cholate when conjugation is much reduced. With a higher clearance a smaller proportion of the total bile-acid pool will be present at all times in the blood and liver, and more in the biliary tree and gut. It would follow that for the same pool size, conjugation would enable an animal to enjoy a greater nutritional advantage with a lower circulating bile-acid concentration.

SUMMARY

1. Cholic acid, synthetic taurocholate, or cholic acid together with taurine, were intravenously infused into anaesthetized dogs in which the common bile duct had been cannulated.

2. Acute taurine depletion was produced by constant infusion of cholic acid. The quantity of taurine readily available (possibly also as cysteine) for conjugation was 673 ± 146 μmole/kg body wt./min (13 expts.). After this depletion free cholate appeared unchanged in bile together with a low, steady output of taurocholate.

3. The maximal rate of secretion of free cholate in the taurine depleted state was about 3·6 μmole/kg body wt./min.

4. The maximal rate of secretion of synthetic taurocholate was 8·5 ± 1·6 (S.D.) μmole/kg body wt./min (6 expts.).

5. When taurine was administered throughout with cholic acid, the maximal rate of taurocholate output was 5·2 ± 0·8 (S.D.) μmole/kg body wt./min (10 expts.) which was associated with 1·4 ± 0·8 (S.D.) μmole/kg body wt./min of free cholate. In two of these experiments a maximal conjugation rate was demonstrated. The remainder provide a minimal value for it.

6. The hepatic clearance of taurocholate was significantly higher than that of cholate excreted unchanged after taurine depletion. When active conjugation was possible the cholate clearance was of the same order as that of taurocholate.

7. The bile flow-rate during the excretion of free cholate was higher than that associated with its molar equivalent of taurocholate.

8. Possible explanations for the above findings are discussed and a biological advantage of conjugation of bile-acid is postulated.

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