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THE UPTAKE OF CONJUGATED BILIRUBIN BY RAT LIVER

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Abstract—Conjugated bilirubin is taken up by rat liver slices but much less is taken up by rat kidney slices. Heat denatured slices take up more than normal slices, the amount depends on the temperature of denaturation. The uptake of conjugated bilirubin is prevented by albumin and mercuric nitrate. Sodium fluoride, arsenite, iodoacetate, cyanide, dinitrophenol and ouabain do not affect the uptake. The mechanism may be important for removal of conjugated bilirubin by the liver in obstructive jaundice.

A MICROSOMAL system conjugating bilirubin with glucuronic acid has been described in animal and human liver.[1] In glucuronide synthesis in the liver, a glucuronyl group is transferred from UDP-glucuronate under the influence of UDP transglucuronylase [UDP-glucuronate glucuronyl transferase (acceptor unspecific) EC 2.4.1.17] to an acceptor, in this case bilirubin. Conjugated bilirubin is then excreted from the liver cell to the bile canaliculus. Relatively little is known about conjugated bilirubin transport by the liver and its excretion. Hargreaves and Lathe[2] drew attention to the process of excretion of conjugated bilirubin as a result of studying the effect of various substances on biliary secretion. Recently a method for the estimation of conjugated bilirubin in liver has been described,[3] this has been applied to a study of the uptake of conjugated bilirubin by rat tissues to determine whether light could be shed on the processes of uptake and excretion of conjugated bilirubin.

MATERIALS AND METHODS

Conjugated bilirubin was added as dilute human or rat bile. The concentration was determined by the method of Malloy and Evelyn.[4] Human salt-free albumin was obtained from the Lister Institute, London, England. All other chemicals were from British Drug Houses Limited.

Animals

Male Wistar rats were obtained commercially. The animals were killed by stunning and cervical dislocation, the tissues were placed immediately in phosphate-bicarbonate solution in ice. Liver slices of approximately 50 mg were cut by hand with a razor blade and weighed on a torsion balance.

Method

Approximately 200 mg rat liver or kidney slices were added to phosphate-bicarbonate solution containing 150 μg conjugated bilirubin in a total volume of 3 ml.

The flasks were stoppered and incubated for 1 hr with shaking in a water bath at 37°. The gas phase was air. All estimations were done at least in duplicate. After incubation the medium was removed, the slices washed in water and homogenised in 3 ml citric acid-phosphate buffer,[5] pH 2·2.

Phosphate-bicarbonate solution

This was 27 mM-$NaHCO_3$, 123 mM-NaCl, 5 mM-KCl, 1·2 mM-KH_2PO_4 and 1·2 mM-$MgCl_2$. The solution was gassed with O_2 + CO_2 (95:5) for 10 min.

Estimation of conjugates in liver slices

To 1 ml homogenate was added 0·5 ml freshly prepared diazotised sulphanilic acid (10 ml 1·0% sulphanilic acid in 0·25 N-HCl plus 0·3 ml 1·5% $NaNO_2$ sol.) A control tube was set up with 1 ml homogenate and 0·5 ml 0·25 N-HCl. After 30 min 0·1 ml 5% ascorbic acid solution was added to the test solution to neutralize the diazonium chloride, the tubes being inverted to ensure mixing. 0·1 ml water was added to the control. After 5 min 0·1 ml saturated $(NH_4)_2SO_4$ sol. and 3 ml ethanol were added, the tubes were mixed by inversion. The tubes were placed at −12° for at least 30 min and then centrifuged for 5 min at 2000 *g*. The extinction of the supernatant was read in a spectrophotometer (Unicam SP 600) at 525 mμ. The results were expressed as μg conjugated bilirubin/200 mg wet wt tissue. After shaking with chloroform to remove protein, the diazo pigments were extracted with butanol, concentrated at 50° *in vacuo* and chromatographed.[6]

Heat-denatured slices

Rat liver and kidney slices were denatured by heating in phosphate-bicarbonate sol. at various temperatures for 5 min. Approximately 200 mg wet wt slices were incubated in conjugated bilirubin medium and treated as described for normal tissue. The concentration of conjugated bilirubin was expressed as μg conjugated bilirubin/200 mg wet wt tissue.

The final concentrations of added substances in the incubation medium were 0·01, 0·1, 1·0 and 10 mM.

RESULTS

Estimation of conjugated bilirubin

Dilute human bile was added to liver and kidney homogenates prepared by grinding normal or heat-denatured rat tissues in a teflon–glass homogeniser with citric acid-phosphate buffer, pH 2·2

The tubes contained 50, 100, 150, 200 and 250 mg wet wt tissue per tube. 0·5 ml diazotised sulphanilic acid was added to 1 ml mixture and the conjugated pigment determined. (Table 1). This shows that conjugated bilirubin can be determined in the presence of liver and that 95 per cent of the conjugated bilirubin present can be determined in the presence of kidney.

Effect of time on the uptake of conjugated bilirubin

Normal and heat-denatured rat liver and kidney slices were incubated in conjugated bilirubin medium for 15, 30, 45 and 60 min. The amount of conjugated bilirubin in

TABLE 1. ESTIMATION OF CONJUGATED BILIRUBIN IN TISSUE HOMOGENATES

Tissue (mg wet wt.)	Conjugated bilirubin mg/100 ml Liver Normal	Liver Heat-denatured	Kidney Normal	Kidney Heat-denatured
0	10	10	10	10
50	10·2	10·1	9·5	10·4
100	9·9	10·3	9·4	10·1
150	9·9	10·2	9·5	9·5
200	10·0	10·2	9·3	9·7
250	9·8	10·1	9·4	9·5

Dilute human bile was added to 50–250 mg wet wt tissue homogenate and conjugated bilirubin determined as described under methods. The tissues were denatured by heating in phosphate-bicarbonate solution at 80° for 5 min. Liver, mean of 6 experiments. Kidney, mean of 4 experiments.

the slices was determined (Fig. 1). The amount of conjugated bilirubin in rat liver increased with time but the amount in rat kidney decreased after the initial peak. Heat-denatured tissue took up more conjugated bilirubin than normal tissue, similar amounts were taken up by heat-denatured liver and kidney.

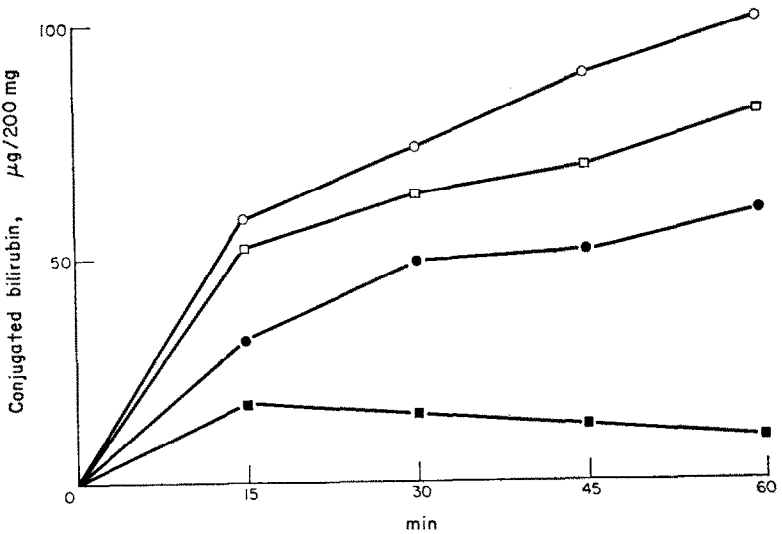

FIG. 1. Effect of time on the uptake of conjugated bilirubin uptake by ● rat liver; ■ rat kidney; ○ heat-denatured rat liver; □ heat-denatured rat kidney. The flasks contained approximately 200 mg tissue slices in 3 ml phosphate-bicarbonate solution containing 150 μg conjugated bilirubin. Incubation 1 hr at 37°. The tissues were denatured by heating at 80° for 5 min in phosphate-bicarbonate solution. Each point represents the mean of 3 experiments.

Effect of temperature on conjugated bilirubin uptake

Normal rat liver slices and slices denatured by heating at 80° for 5 min were incubated in a conjugated bilirubin medium for 1 hr at 4° and 37°. The amount of conjugated bilirubin uptake by the slice was determined at 15 min intervals (Fig. 2). Normal rat liver slices took up more than 6 times as much conjugated bilirubin at

37° than 4°. Heat denatured slices took up more than 4 times the amount of conjugated bilirubin at 37° than 4°.

Amount of tissue

50, 100, 150, 200 and 250 mg wet wt normal and heat-denatured rat liver slices were incubated for 1 hr in 3 ml phosphate-bicarbonate solution containing 150 μg conjugated bilirubin. Heat-denatured slices were prepared by heating the slices at

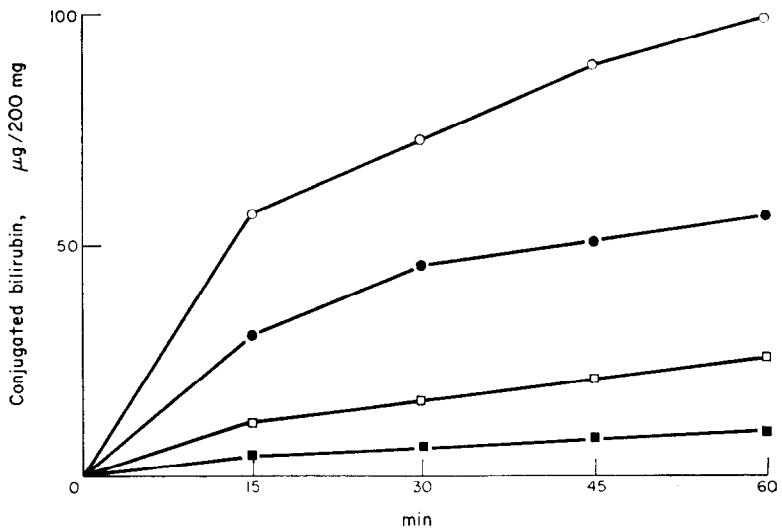

FIG. 2. Effect of temperature on conjugated bilirubin uptake. Uptake by ■ rat liver at 4°; ● rat liver at 37°; □ heat-denatured rat liver at 4°; ○ heat-denatured rat liver at 37°. The flasks contained approximately 200 mg wet wt liver slices in 3 ml phosphate-bicarbonate solution containing 150 μg conjugated bilirubin. The slices were denatured by heating at 80° for 5 min in phosphate-bicarbonate solution. Incubation 1 hr at 4° or 37°. Each point represents the mean of 3 experiments.

80° for 5 min in phosphate-bicarbonate solution. The concentration of conjugated bilirubin in the slice reached a maximum at 200 mg normal slices and 150 mg heat-denatured slices (Fig. 3).

Concentration of conjugated bilirubin

Approximately 200 mg wet wt normal and heat-denatured rat liver slices were incubated in a conjugated bilirubin phosphate-bicarbonate medium containing 100, 200, 400, 600 and 800 μg conjugated bilirubin in 3 ml. The results (Fig. 4) confirmed that heat-denatured rat liver slices took up more conjugated bilirubin than normal slices.

Effect of pH

The phosphate-bicarbonate solution was replaced by 1/15 M phosphate buffer, pH 6·5, 7·0, 7·4 and 8·0. Approximately 200 mg normal and heat-denatured rat liver slices were incubated in 3 ml buffered medium containing 150 μg conjugated bilirubin

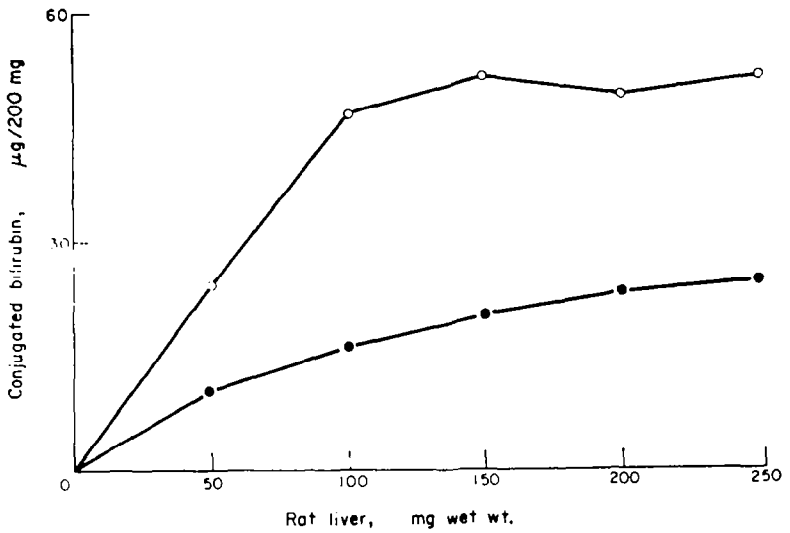

FIG. 3. Uptake of conjugated bilirubin by rat liver. Uptake by ● rat liver; ○ heat-denatured rat liver. The slices were denatured by heating at 80° for 5 min in phosphate-bicarbonate solution. The flasks contained 50–250 mg wet wt liver slices in 3 ml phosphate-bicarbonate solution containing 150 μg conjugated bilirubin. Incubation 1 hr at 37°. Each point represents the mean of 3 experiments.

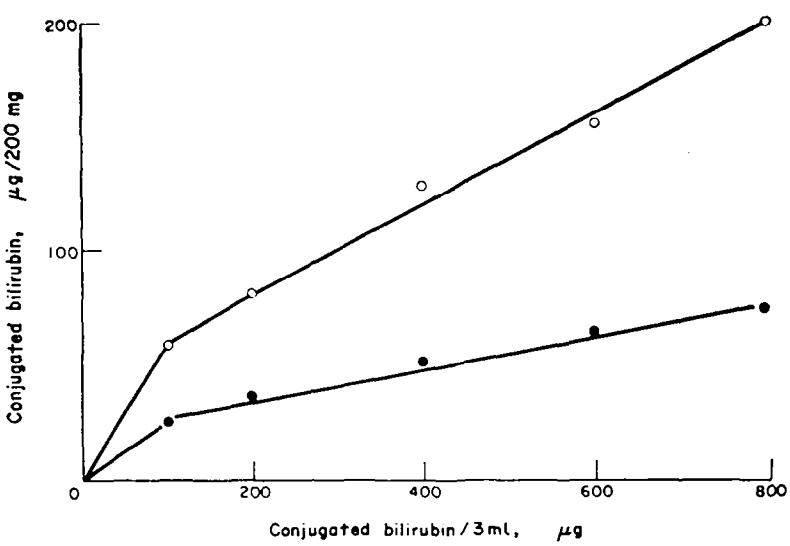

FIG. 4. Effect of conjugated bilirubin concentration on conjugated bilirubin uptake by rat liver. Uptake by ● normal rat liver ○ heat denatured rat liver. The liver was denatured by heating at 80° for 5 min in phosphate-bicarbonate solution. The flasks contained approximately 200 mg wet wt liver slices in 3 ml phosphate-bicarbonate solution containing 100, 200, 400, 600 and 800 μg conjugated bilirubin. Incubation 1 hr at 37°. Each point is the mean of 3 experiments.

for 1 hr at 37°. The conjugated bilirubin in the slices was then determined. Over this limited range, pH did not affect conjugated bilirubin uptake (Table 2).

Effect of albumin

Approximately 200 mg wet wt normal and heat-denatured rat liver slices were incubated in 3 ml phosphate-bicarbonate solution containing 150 μg conjugated bilirubin and 1, 2, 3, 4 and 5 g human salt-free albumin/100 ml and the conjugated bilirubin uptake into the slices determined. Albumin retarded the uptake of conjugated bilirubin by normal and heat denatured liver slices (Fig. 5). This effect was investigated

TABLE 2. EFFECT OF pH ON UPTAKE OF CONJUGATED BILIRUBIN BY RAT LIVER SLICES

pH	Conjugated bilirubin (μg/200 mg wet wt liver slices) Normal	Heat-denatured
6·5	24·6	67
7·0	24·4	66·8
7·4	26·1	67·6
7·6	26·1	68
8·0	32·8	69

The flasks contained approximately 200 mg wet wt normal and heat denatured liver slices in 3 ml 1/15 M phosphate buffer containing 150 μg conjugated bilirubin. Liver slices were denatured by heating at 80° for 5 min in phosphate-bicarbonate solution. Mean of 3 experiments.

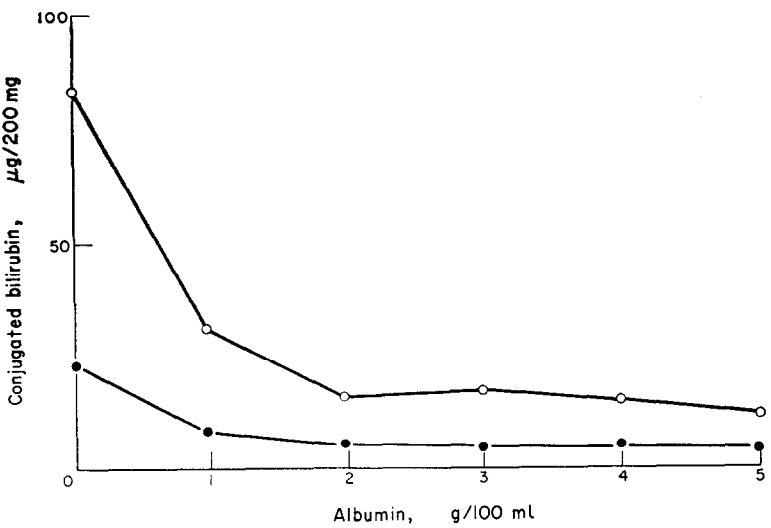

FIG. 5. Effect of albumin on conjugated bilirubin uptake by rat liver slices. Uptake by ● normal rat liver. ○ heat-denatured rat liver. The liver was denatured by heating at 80° for 5 min in phosphate-bicarbonate solution. The flasks contained approximately 200 mg wet wt liver in 3 ml phosphate-bicarbonate solution containing 150 μg conjugated bilirubin and 1, 2, 3, 4 and 5 g human salt-free albumin/100 ml. Incubation 1 hr at 37°. Each point represents the mean of 3 experiments.

further by incubating rat liver slices in a medium containing conjugated bilirubin and heated human salt-free albumin. The albumin was heated to 45°, 50°, 55°, 60° and 65° for 5 min before incubation. The results (Table 3) showed that when rat liver slices were incubated in the medium containing 0·5 g and 5 g/100 ml heat-denatured salt-free albumin more conjugated bilirubin was taken up when albumin was

TABLE 3. EFFECT OF HEAT-DENATURED ALBUMIN ON CONJUGATED BILIRUBIN UPTAKE BY RAT LIVER

Albumin g/100 ml medium Temperature of albumin denaturation	0·5 Conjugated bilirubin (μg/200 mg wet wt liver slices)	5·0
37	14·0	1·6
45	13·2	2·0
50	13·7	2·1
55	15·9	2·8
60	15·7	2·7
65	13·1	1·4
Control (no albumin)	29·2	28·9

The flasks contained approximately 200 mg wet wt normal liver in 3 ml phosphate-bicarbonate solution containing 150 μg conjugated bilirubin and 0·5 and 5·0 g albumin/100 ml heated to 37, 45, 50, 55, 60 and 65° for 5 min. Incubation 1 hr at 37°. Conjugated bilirubin determined in slices as described under methods.

heated to 55–60° than at other temperatures. This suggested that at this point heat has destroyed some of the receptors for conjugated bilirubin in the albumin molecule, the conjugated bilirubin is then more readily available for uptake by the liver.

Temperature of denaturation of liver

The effect of heat-denaturation of the liver slice was investigated further by denaturing the rat liver slices at different temperatures. Rat liver slices were heat-treated by immersing in phosphate-bicarbonate solution for 5 min at 45°, 50°, 55°, 60°, 65°, 70°, 80° and 100°. The slices were then immersed in 3 ml of conjugated bilirubin medium containing 150 μg conjugated bilirubin for 1 hr at 37° and the uptake into the slice determined (Fig. 6). Denaturation at 55–60° prevented the uptake of conjugated bilirubin to some extent, the amount taken up was then increased as the temperature of denaturation was increased.

Rat liver slices were heat denatured by heating at 60° for 5, 10, 15, 20, 25 and 30 min. The slices were then immersed in 3 ml of conjugated bilirubin medium containing 150 μg conjugated bilirubin for 1 hr at 37° and the uptake into the slice determined (Fig. 7). Continued heating at 60° enabled the rat liver slice to take up more bilirubin, probably by increasing the number of receptor sites.

Effect of enzyme inhibitors on conjugated bilirubin uptake

Approximately 200 mg normal and heat-denatured rat liver slices were incubated in 3 ml phosphate-bicarbonate solution containing 150 μg conjugated bilirubin. Sodium cyanide, fluoride, arsenite, iodoacetate, mercuric nitrate, dinitrophenol and ouabain were added in final concentrations 0·01, 0·1, 1·0 and 10 mM. The uptake was retarded

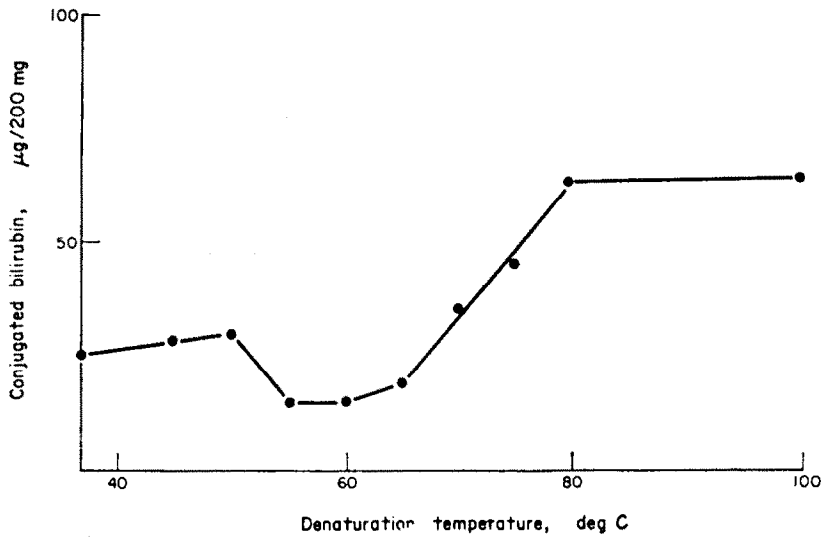

FIG. 6. Effect of temperature of denaturation of the liver slice on conjugated bilirubin uptake by liver slices. The liver was denatured by heating at 37°, 45°, 50°, 55°, 60°, 65°, 70°, 80° and 100° for 5 min. 200 mg wet wt rat liver slices were incubated in 3 ml phosphate-bicarbonate solution containing 150 μg conjugated bilirubin. Incubation 1 hr at 37°. Each point represents mean of 3 experiments.

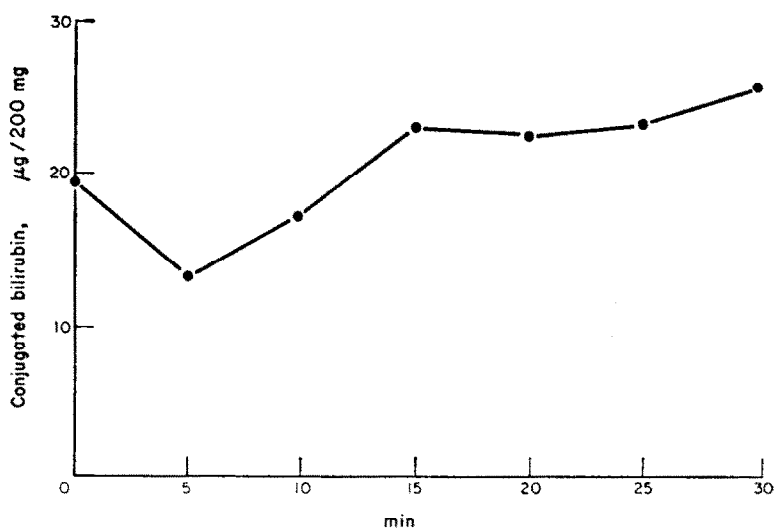

FIG. 7. Effect of time of denaturation at 60° of uptake of conjugated bilirubin by rat liver slices. The liver was heated for 5, 10, 15, 20, 25 and 30 min at 60°. 200 mg wet wt liver slices were incubated in 3 ml phosphate-bicarbonate solution containing 150 μg conjugated bilirubin and the amount of conjugated bilirubin in the slice determined as described in methods section. Incubation 1 hr at 37°.

TABLE 4. EFFECT OF SODIUM CYANIDE, SODIUM FLUORIDE AND MERCURIC NITRATE ON UPTAKE OF CONJUGATED BILIRUBIN BY RAT LIVER

Concn. (mM)	Conjugated bilirubin μg/200 mg wet wt liver Normal NaCN	NaF	$Hg(NO_3)_2$	Heat-denatured NaCN	NaF	$Hg(NO_3)_2$
0	24·9	27·8	27·0	54·4	63·5	54·2
0·01	26·5	26·2	22·2	60·4	62·0	46·0
0·1	29·8	29·8	24·6	57·0	62·3	44·2
1·0	31·8	29·7	10·9	58·7	57·8	27·2
10·0	27·3	35·0	7·0	44·3	57·6	8·3

The flasks contained approximately 200 mg wet wt rat liver slices in 3 ml phosphate-bicarbonate solution containing 150 μg conjugated bilirubin. Sodium cyanide, sodium fluoride and mercuric nitrate were added to give final concentrations 0·01, 0·1, 1·0 and 10·0 mM. Incubation 1 hr at 37°. Mean of 3 experiments with each substance.

TABLE 5. EFFECT OF DINITROPHENOL AND SODIUM ARSENITE ON UPTAKE OF CONJUGATED BILIRUBIN BY RAT LIVER SLICES

Conc. (mM)	Conjugated bilirubin μg/200 mg wet wt liver Normal Dinitrophenol	Sodium arsenite	Heat-denatured Dinitrophenol	Sodium arsenite
0	38·5	38·1	84·7	70
0·01	37·8	32·8	78·2	80
0·7	36·6	26·2	80	72·4
1·0	36·7	34·2	86·6	70
10·0	48·8	51·2	73·6	68·4

The flasks contained approximately 200 mg wet wt rat liver slices in 3 ml phosphate-bicarbonate solution containing 150 μg conjugated bilirubin. Dinitrophenol and sodium arsenite were added to give final concentrations 0·01, 0·1, 1·0 and 10 mM. Incubation 1 hr at 37°. Mean of 3 experiments with each substance.

TABLE 6. EFFECT OF OUABAIN AND SODIUM IODOACETATE ON UPTAKE OF CONJUGATED BILIRUBIN BY RAT LIVER SLICES

Concn. (mM)	Conjugated bilirubin μg/200 mg wet wt liver Normal Ouabain	Sodium iodoacetate	Heat-denatured Ouabain	Sodium iodoacetate
0	25·7	24·7	60	56·1
0·01	27	23·4	54·1	56·3
0·1	24	24·5	54	56·2
1·0	22·7	22·5	55·8	57·2
10·0	24	16·5	54·2	58·6

The flasks contained approximately 200 mg wet wt rat liver slices in 3 ml phosphate-bicarbonate solution containing 150 μg conjugated bilirubin, ouabain and sodium iodoacetate were added to give final concentrations 0·01, 0·1, 1·0 and 10 mM. Incubation 1 hr at 37°. Mean of 3 experiments.

by mercuric nitrate (Table 4) and by iodoacetate (Table 6). The uptake was increased by sodium fluoride (Table 4) and by sodium arsenite (Table 5). The other substances did not alter the uptake of conjugated bilirubin.

DISCUSSION

Conjugated bilirubin was taken up by normal and by heat denatured rat liver and kidney slices when the tissues were immersed in a solution containing conjugated bilirubin. The uptake by rat liver was much greater than rat kidney. The progressive decrease in the amount of conjugated pigment in rat kidney with time is not due to the presence of β-glucuronidase because experiments have shown that the free bilirubin does not correspondingly increase as the conjugated bilirubin content falls. The uptake of conjugated bilirubin by heat-denatured liver and kidney was approximately the same. The results suggested that rat liver takes up conjugated bilirubin preferentially to rat kidney in *in vitro* experiments. The uptake by rat liver was retarded by human salt-free albumin and by mercuric nitrate. Mercuric nitrate retarded the uptake in normal and denatured slices suggesting that this was a physical phenomenon. The results obtained with the other inhibitors suggested that the uptake mechanism does not depend on respiratory processes, thiol groups or ATPase.

Brauer and Pessotti[7] showed that anoxia and various enzyme poisons failed to reduce the uptake of bromsulphthalein by rat liver slices and suggested that uptake was due to intracellular protein binding. Barber-Riley[8] showed that denaturation of liver slices increased the uptake of bromsulphthalein and suggested this was due to unfolding of the protein molecule. He also suggested that the liver passively accepted a large fraction of the dye presented.

Heat denatured slices take up more conjugated bilirubin than normal slices. Nosslin[9] found that denatured protein had a high affinity for bilirubin. The experiments in which different temperatures of denaturation were used suggest that the uptake of conjugated bilirubin may be due to two components. The first component is partially destroyed by heating to 55°. Mackay and Martin[10] showed that human albumin was partially denatured at 56°, the amount of denaturation increased at 60°. Higher temperatures cause unfolding of protein chains making available more binding sites for conjugated bilirubin on the liver slices.

Experiments utilising rat liver slices conjugating bilirubin[1] depend on the measurement of conjugated bilirubin in the medium in which the slice is immersed. The living slice secretes conjugated bilirubin, the amount of conjugated bilirubin in the slice depends on the amount absorbed and the amount excreted. Heat-denatured slices take up more conjugated bilirubin than normal slices, the difference between the amounts of conjugated pigment in the normal and the heat-denatured slice depends on the increased uptake due possibly to the increased availability of receptor sites and a failure of excretion. High concentrations of fluoride and arsenite increased the conjugated pigment in the normal slice but not the denatured slice suggesting that these substances may block the excretion of conjugated pigment.

The mechanism of excretion of conjugated bilirubin is not known. It is possible that the excretory process is governed by the orientation of the excreted molecules across a lipid water interphase and the rate of biliary secretion depends on the relative affinity of the metabolite for the two phases.[11] It is thought that effective biliary secretion depends upon mechanisms preventing or limiting biliary or intestinal reabsorp-

tion of bilirubin glucuronide. The lipid soluble unconjugated bilirubin is readily reabsorbed from the bowel,[12] bilirubin glucuronide is not reabsorbed from the bowel because conjugation converts the pigment into a larger water soluble molecule.[13]

In obstructive jaundice excess of conjugated bilirubin is found in the serum. The amount of conjugated bilirubin used in these experiments (5 mg/100 ml) is about the same or slightly less than the serum level in obstructive jaundice. The experiments described here together with the observation that injected conjugated bilirubin is excreted in the bile[14] show that liver can excrete conjugated bilirubin as well as that normally excreted by the kidney.[15]

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REFERENCES

1. G. H. LATHE and M. WALKER, *Biochem. J.* **70**, 705 (1958).
2. T. HARGREAVES and G. H. LATHE, *Nature, Lond.* **200**, 1172 (1963).
3. T. HARGREAVES, *Clin. chim. Acta.* **11**, 278 (1965).
4. H. T. MALLOY and K. A. EVELYN, *J. biol. Chem.* **119**, 481 (1937).
5. T. C. MCILVAINE, *J. biol. Chem.* **49**, 183 (1921).
6. R. SCHMID, *Science* **124**, 76 (1956).
7. R. W. BRAUER and R. L. PESSOTTI, *Am. J. Physiol* **162**, 535 (1950).
8. G. BARBER-RILEY, *S. Afr. J. med. Sci.* **26**, 91 (1962).
9. B. NOSSLIN, *Scand. J. clin. Lab. Invest.* **12**, Suppl. 49 (1960).
10. M. E. MACKAY and N. H. MARTIN, *Biochem. J.* **65**, 284 (1957).
11. R. LESTER, *New Engl. J. Med.* **270**, 779 (1964).
12. R. LESTER and R. SCHMID, *J. clin. Invest.* **42**, 736 (1963).
13. R. LESTER and R. SCHMID, *New Engl. J. Med.* **269**, 178 (1963).
14. I. M. ARIAS, *Annls. N.Y. Acad. Sci.* **104**, 1014 (1963).
15. D. K. WALLACE and E. E. OWEN, *J. Lab. clin. Med.* **64**, 741 (1964).