Schachman, H. K., Pardee, A. B. & Stanier, R. Y. (1952). *Arch. Biochem. Biophys.* **38**, 245.
Schmidt, C. F. (1948). *J. Bact.* **55**, 705.
Schmidt, G. & Thannhauser, S. J. (1945). *J. biol. Chem.* **161**, 83.
Sherratt, H. S. A. & Thomas, A. J. (1953). *J. gen. Microbiol.* **8**, 217.
Smellie, R. M. S., MacIndoe, W. M., Logan, R. & Davidson, J. N. (1953). *Biochem. J.* **54**, 280.
Tyner, E. P., Heidelberger, C. & LePage, G. A. (1953). *Cancer Res.* **13**, 186.
Volkin, E. & Astrachan, L. (1956). *Virology*, **2**, 433.
Wade, H. E. (1952). *J. gen. Microbiol.* **7**, 24.
Wade, H. E. & Morgan, D. M. (1953). *Nature, Lond.*, **174**, 920.
Wade, H. E. & Morgan, D. M. (1955). *Biochem. J.* **60**, 264.
Wade, H. E. & Morgan, D. M. (1957). *Biochem. J.* **65**, 321.

The Synthesis of Bilirubin Glucuronide in Animal and Human Liver

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A system conjugating various alcohols and acids with glucuronic acid has been described by Storey & Dutton (1955) and Dutton (1956). It is located in liver microsomes and requires uridine diphosphate glucuronic acid. Isselbacher (1956) has shown that steroid glucuronides are formed in a similar manner. The finding that bilirubin is excreted as a glucuronide (Billing, Cole & Lathe, 1957; Schmid, 1957) raises the question of whether conjugation is effected by the same system. This problem was examined in order to throw light on the reduced capacity of new-born infants (Billing, Cole & Lathe, 1954) and of Gunn's strain of jaundiced rats (Malloy & Lowenstein, 1940) to excrete bilirubin. A preliminary report of this work has already been given (Lathe & Walker, 1957).

METHODS AND MATERIALS

Preliminary examination of rat bile. A 300 g. rat was anaesthetized with Nembutal and the bile duct was cannulated as described by Billing & Weinbren (1956). Bile was collected into a 0·1 ml. graduated pipette. After collection of control samples of bile, bilirubin was injected into the femoral vein. Over a period of 10 min. 3·8 ml. of a 0·2% solution of bilirubin in 0·5% Na_2CO_3 and 0·52% NaCl soln. was given. Bile was collected continuously in 0·1 ml. samples which accumulated in 3–10 min., while the secretion of pigment rose and fell again. Total and conjugated bilirubin and glucuronic acid were estimated in each sample.

Materials. *o*-Aminophenol was resublimed before use. The β-glucuronidase was an ox-liver preparation (Ketodase) from Warner-Chilcott Laboratories, New York. A solution of bilirubin (British Drug Houses Ltd. and Hoffman la Roche) was prepared by dissolving 10 mg. in a minimum amount of 0·25 N-NaOH soln. (about 0·15 ml.) with stirring. A phosphate–bicarbonate solution (see below) (0·85 ml.) was added and mixed, and the solution was centrifuged to remove any material not in solution; calcium was not included in this solution, as it precipitated bilirubin.

The uridine diphosphate glucuronic acid (UDPglucuronic acid) concentrate was prepared by a modification of the method of Storey & Dutton (1955) from a boiled extract of rabbit liver, by precipitation with acetone in acid solution. The precipitate was dried *in vacuo* over P_2O_5, and was stored at −12° in small ampoules sealed *in vacuo*.

Analytical techniques. The conjugated bilirubin in incubation media was estimated by the direct diazo reaction. Test and control (without diazo solution) values were obtained, and the difference between them gave the amount of conjugated pigment. This was determined before and after incubation.

To 1 ml. of medium in a 3 in. × ½ in. test tube was added 0·5 ml. of freshly prepared diazo reagent (10 ml. of 0·1% sulphanilic acid in 0·25 N-HCl, plus 0·3 ml. of 0·5% $NaNO_2$ soln.). A control tube was set up with 1 ml. of medium and 0·5 ml. of 0·25 N-HCl. After 30 min. 0·1 ml. of 5% ascorbic acid soln. was added to the test to neutralize the diazonium chloride, the tubes being inverted to ensure mixing; 0·1 ml. of water was added to the control. After 5 min. 0·1 ml. of saturated $(NH_4)_2SO_4$ soln. and 3 ml. of ethanol were added and the tubes were mixed by inversion. The tubes were placed at −12° for 15 min. and then centrifuged for 5 min. at 2000 *g*. The supernatant was read in a spectrophotometer (Unicam SP. 500) at 525 mμ. A solution of methyl red in sodium acetate buffer, pH 4·63, was used as a standard (Haslewood & King, 1937).

In experiments with suspensions samples were pipetted directly into the diazo reagent used for the estimation of conjugated bilirubin, and allowed to react for only 5 min. before the addition of ascorbic acid. It was established by Lathe & Ruthven (1958) that the reaction of the direct pigment was almost complete within this period.

Total and conjugated bilirubin in bile were estimated by the method of Lathe & Ruthven (1958). Glucuronic acid was estimated in bile according to Fishman & Green (1955).

o-Aminophenyl glucuronide was estimated by the method of Levvy & Storey (1949).

Preparation of tissue. Wistar rats were used except where otherwise stated. Rats of the Gunn strain (Gunn, 1938, 1944) were bred from specimens supplied by Dr J. Lucey, Burlington, Vermont. Other animals were obtained commercially. All animals were fed. Rabbits, rats, mice and guinea pigs were killed by decapitation and the livers were placed immediately in the incubation medium at 4° (for slices) or in ice (for suspensions). Monkeys, immature *Macaca mulatta* weighing 2–4 kg., were anaesthetized with 65 mg. of pentobarbital given into the thorax about 30 min. before operation. They were breathing when the livers were removed. Dog livers were removed after the animal had been rapidly (within about 5 min.) exsanguinated under *cyclo*pentane anaesthesia. Cat livers were obtained from animals that had been anaesthetized with sodium pentobarbitone for 4 hr., during which other physiological experiments had been carried out. After these experiments the pulse, temperature and respiration were normal and the livers capable of conjugating bilirubin well.

Liver slices were cut by hand with a razor blade. Suspensions were prepared by grinding 1 g. of liver in a Potter–Elvehjem glass homogenizer with 9 ml. of alkaline KCl soln. as described by Dutton & Storey (1954). In some cases the suspensions were fractionated by differential centrifuging as described by Dutton (1956). The 'nuclear' fraction was obtained by spinning at 700 *g* for 10 min., 'mitochondria' were sedimented at 5000 *g* for 20 min. and 'microsomes' at 21 000 *g* for 90 min. The temperature was 4° throughout.

Incubation media. Phosphate–bicarbonate solution was 27 mM-$NaHCO_3$, 123 mM-NaCl, 5 mM-KCl, 1·2 mM-KH_2PO_4, 1·2 mM-$MgSO_4$ and 0·02 M-glucose. The solution was gassed with $O_2 + CO_2$ (95:5) for 10 min.

For standard liver-slice experiments with bilirubin, 2·5 ml. of medium was prepared from 2·1 ml. of phosphate–bicarbonate solution, 0·36 ml. of serum and 0·036 ml. of bilirubin solution. The final concentrations of serum and bilirubin were 14·6% (v/v) and 0·0146% (w/v) respectively. For liver-slice experiments with *o*-aminophenol, $MgSO_4$ in the phosphate–bicarbonate solution was replaced by $MgCl_2$ and glucose was omitted. The concentration of serum was 14·6% (v/v). The *o*-aminophenol was added in aqueous solution with ascorbic acid, as recommended by Levvy & Storey (1949). In 25 ml. of water 33 mg. of *o*-aminophenol and 50 mg. of ascorbic acid were dissolved. An approx. mM-concentration in the medium was obtained by taking 0·2 ml. of this solution in 2·5 ml. of medium. Anthranilic acid (0·36–1·80 mM) used in liver-slice experiments was added in aqueous solution.

In all experiments with suspensions the concentrations of bilirubin and serum in the mixture after addition of the suspension were 0·024% (w/v) and 24% (v/v) respectively. An aqueous solution of 0·1 M-saccharolactone was used to give a 2 mM-concentration in the incubation mixture for some liver-suspension experiments. A final concentration of 0·04 M-potassium phosphate buffer, pH 7·4, and 0·01 M-$MgCl_2$ was used in all liver-suspension experiments, following Dutton & Storey (1954) in their work on *o*-aminophenol conjugation. The lower concentrations of *o*-aminophenol (0·2 mM) were obtained by dilution of the solution containing ascorbic acid described above for liver-slice experiments. A crude preparation of UDPglucuronic acid was added (10 mg. in 5 ml. of incubation mixture). Larger amounts of this gave no increase in rates of conjugation.

Incubation. This was carried out in 50 ml. conical flasks in a water bath at 37°. The time from killing the animal to the commencement of incubation was about 1·5 hr. Controls were done in quadruplicate, specimens of serum in duplicate. Liver slices (180–220 mg.) were added to 2·5 ml. of cold medium. The gas phase was flushed with about 0·7 l. of $O_2 + CO_2$ (95:5) in 10 sec. The flasks were stoppered and incubated for 2 hr. with shaking. After incubation the medium was centrifuged free from tissue.

In experiments with liver suspensions the time from killing the animal to the commencement of incubation was about 15 min. except with the monkey, when the liver was kept on ice for 2 hr. A volume (2 ml.) of suspension was added to 3 ml. of medium. Control estimations of conjugated bilirubin were done before incubation. Flasks were incubated for 20 min. with constant shaking, the gas phase being air. The rate of reaction was linear over this period. Estimations were done without removing the tissue.

Oxygen consumption. Liver slices were examined by the Warburg technique, a phosphate medium with the usual addition of serum, and a gas phase of pure O_2, being used; CO_2 was absorbed by 20% (w/v) KOH soln. in the central well.

Identification of the product formed in rat-liver-slice experiments. The final ethanolic solution (about 500 ml.) from the determination of conjugated bilirubin in 100 experiments with liver slices was brought to pH 5·5 with N-NaOH. After evaporation under reduced pressure to about 120 ml. the mixture was saturated with $(NH_4)_2SO_4$, when an ethanol layer containing the pigment rose to the surface. This layer was separated and evaporated until the pigment began to precipitate, when it was extracted with butan-1-ol and the solvent was evaporated. The pigment, dissolved in a minimum of the mobile phase, was chromatographed on a column of silicone-treated kieselguhr with the solvent system described by Billing (1954). The more polar pigment was collected as it came off the column with the leading edge. The eluate (2 ml.) was incubated for 1 hr. at 37° with 0·5 ml. (2500 units) of β-glucuronidase and rechromatographed.

RESULTS

Nature of the bilirubin excretion product in the rat. After the intravenous injection of bilirubin into a rat the concentration of both bilirubin and glucuronic acid in the bile rose. The molar ratio of the increments of glucuronic acid and bilirubin varied, and in the most concentrated specimen was 1·7. Almost all of the bile pigment in rat bile gave the direct van den Bergh reaction. The maximum rate of excretion was 65 μg. of bilirubin/100 g. rat/min. The polar azo pigment obtained from experiments with rat-liver slices was changed to a non-polar azo pigment by treatment with β-glucuronidase, and the *R* values of the azo pigments agreed with those of the corresponding azo pigments of human bile (Billing, 1954).

Optimum conditions for bilirubin conjugation by rat-liver slices. Rat-liver slices incubated in phosphate–bicarbonate solution containing bilirubin, but without serum, conjugated the pigment very

slowly (5 μg./g. of wet liver/hr. at 0·0146% of bilirubin). Bilirubin was largely precipitated and adhered to lipid droplets. The addition of serum, for the proteins of which bilirubin has a strong affinity, increased the rate of conjugation. The highest rate of conjugation was obtained with serum concentrations of 8–32%, and bilirubin concentrations of 0·012–0·028%. The effect of varying salt concentrations was not examined in detail. Sulphate (0–12 mM) did not affect the rate of conjugation, which was, however, reduced by higher concentrations of magnesium.

Male rat-liver slices conjugated bilirubin equally well when incubated in fresh pooled male rat sera, frozen pooled male rat sera, fresh human male sera or pooled frozen or dehydrated (and reconstituted) human male sera. Pooled human male sera stored at −12° were used in further experiments. Sera from pregnant women, placental blood, new-born infants and domestic animals gave lower rates of conjugation (Lathe & Walker, 1958). Livers from male and female rats gave the same rates of conjugation.

Optimum conditions for bilirubin conjugation by rat-liver suspensions. The addition of UDPglucuronic acid (10 mg. of the crude preparation/5 ml. of medium) to rat-liver suspensions increased the rate of conjugation from about 140–400 μg./g. of wet liver/hr. The optimum final concentrations of bilirubin and serum, in the presence of added UDPglucuronic acid, were 0·016–0·028% and 8–24% (v/v) respectively. In a number of experiments the pH of the mixture was varied. Conjugation was greatest at a final pH of 6·6. The medium adopted had a pH of 6·4 after the addition of the tissue suspension. After incubation with the liver suspension it was 6·7. The optimum concentration of bilirubin for conjugation with slices or suspensions was in the same range for rats and mice.

Optimum conditions for o-aminophenol conjugation. UDPGlucuronic acid was essential to obtain maximal rates of conjugation by mouse-liver suspensions. The optimum concentration of *o*-aminophenol for conjugation was determined for slices and suspensions (extra UDPglucuronic acid being added) of rat, mouse, rabbit and human livers (Table 1). The presence of serum did not affect the rate of *o*-aminophenol conjugation. The optimum concentrations for conjugation by monkey, cat and dog liver were not determined.

Inhibition studies. Attempts were made to inhibit the conjugation of bilirubin by the addition of other substrates for glucuronide conjugation. Anthranilic acid (0·36–1·80 mM) caused no inhibition of bilirubin glucuronide formation in rat-liver slices incubated with 0·25 mM-bilirubin. Bilirubin conjugation was inhibited by relatively high concentrations of *o*-aminophenol in liver slices of rat (2·5 mM), rabbit (1·5 mM) and by low concentrations in suspensions of mouse liver (Table 2). Bilirubin failed to inhibit *o*-aminophenol conjugation in mouse-liver suspensions when 0·20–0·48 mM-bilirubin was added in the presence of *o*-aminophenol at a concentration (0·2 mM) which is optimum for this substrate alone.

Table 1. *Optimum concentration of o-aminophenol for conjugation by liver slices and suspensions*

Liver slices (180–220 mg.) were incubated for 2 hr. in 2·5 ml. of sulphate-free phosphate–bicarbonate solution containing 14·6% (v/v) of serum. Suspensions [1 ml. of 10% (w/v) suspension] were incubated for 20 min. in a medium containing 24% (v/v) of serum, 0·2 ml. of 0·5 M-potassium phosphate buffer, pH 7·4, 0·1 ml. of 0·3 M-$MgCl_2$ and 5 mg. of UDPglucuronic acid in a final volume of 2·5 ml. pH at start of incubation was 6·4. Conditions are described in the section on methods.

Animal	Concn. of *o*-aminophenol (mM)	
	Slices	Suspensions
Rat	1·25–2·50	—
Mouse	1·5	0·2
Rabbit	2·5	0·5
Human (premature)	1·0	0·3

Table 2. *Effect of o-aminophenol on bilirubin conjugation by mouse-liver suspensions*

Conditions are described in the section on methods. Bilirubin was 0·41 mM (0·024%, w/v), serum 24% (v/v). Additions of buffer, $MgCl_2$ and UDPglucuronic acid were as described in Table 1.

Concn. of *o*-aminophenol (mM)	Bilirubin conjugation (μg./g. of wet liver/hr.)	Inhibition (%)
0	273	—
0·05	192	30
0·10	120	56
0·20	72	74
0·50	30	89
1·00	24	91

Saccharolactone (2 mM), a specific and powerful inhibitor of β-glucuronidase, was added to liver suspensions from young animals. The addition produced no significant change in *o*-aminophenyl glucuronide formation in the new-born and adult mouse, or in bilirubin glucuronide formation in the new-born human or young rabbit (Table 3). One experiment of this type with adult rabbit-liver suspensions showed an increase in the yield of conjugated bilirubin, but this was not confirmed on repetition.

Intracellular location of the bilirubin-conjugating system. Cell fractions were separated from three liver suspensions in alkaline KCl soln. and two suspensions in sucrose. The activity was fairly

evenly distributed between the particulate fractions but none remained in the high-speed supernatant. As the nuclear and mitochondrial fractions had not been washed and respun it is possible that the activity was due to contamination with microsomes. These were probably not contaminated with other fractions.

Comparative studies of conjugation. Some observations on the rates of bilirubin and *o*-aminophenol conjugation by liver slices and suspensions from different animals are given in Table 4.

Premature human infants. The livers of three premature human infants were examined for their capacity to conjugate bilirubin. The conjugation of *o*-aminophenol was examined in one. Precautions were taken to preserve the enzyme activity during the period between death and incubation. In case no. 1 the abdomen was packed in ice and the liver removed within 20 min. of death. In the other two cases the liver was removed to iced medium 5 min. after death. As far as could be determined death in infants no. 1 and no. 2 occurred quickly but was

Table 3. *Effect of uridine diphosphate glucuronic acid and saccharolactone on conjugation of bilirubin and o-aminophenol by liver suspensions under standard conditions*

Concentrations of bilirubin and serum were as in Table 2; other constituents of medium were as in Table 1. Conditions are given in the section on methods. Each horizontal line of figures is the result of an experiment done in duplicate on a single adult animal, or on a single litter of baby mice comprising 6–10 animals. UDPGlucuronic acid was added in a concentration of 2 mg. of crude preparation/ml. Saccharolactone was 2 mM.

Substrate	Animal	Age	Rate of conjugation (μg./g. of wet liver/hr.) Without supplement	With UDPglucuronic acid	With UDPglucuronic acid and saccharolactone
Bilirubin	Rat, Wistar	Adult	140	400	—
	Rat, Gunn (homozygous)	Adult	81	71	—
	Guinea pig	Adult	60	120	—
	Human	Premature	7	8	18
	Rabbit	6 days	55	75	80
	Rabbit	7 days	24	33	63
	Rabbit	5 weeks	—	75	81
	Rabbit	Adult	18	66	123
	Rabbit	Adult	183	187	177
	Rabbit	Adult	159	138	153
	Rabbit	Adult	168	198	201
	Mouse	2 days	30	288	—
	Mouse	Adult	108	288	—
o-Aminophenol	Mouse	3 days	12	37	35
	Mouse	5 days	12	44	43
	Mouse	Adult	18	54	50

Table 4. *Conjugation of bilirubin and o-aminophenol by liver preparations*

Bilirubin conjugation was determined under conditions which were optimum for the rat and mouse. Concentrations of *o*-aminophenol for rat, mouse, rabbit and new-born human liver preparations are given in Table 1; for dog-, cat- and monkey-liver slices and suspensions mM-*o*-aminophenol was used. UDPglucuronic acid was added in the experiments with suspensions. Figures in parentheses represent numbers of animals. Other figures are μg. of aglycone conjugated/g. of wet liver/hr.

Animal	Bilirubin Slices	Bilirubin Suspension	*o*-Aminophenol Slices	*o*-Aminophenol Suspension
Rat, Wistar	57 (34)	439 (13)	176 (4)	0 (3)
Rat, Gunn, homozygous	0 (2)	71 (1)	85 (1)	0 (1)
Rat, Gunn, heterozygous	—	—	100 (1)	0 (1)
Monkey (*Macaca mulatta*)	59 (15)	48 (1)	96 (1)	39 (1)
Human, premature	0 (2)	9 (3)	8 (1)	15 (1)
Mouse	48 (1)	336 (4)	104 (1)	54 (3)
Rabbit, adult	53 (10)	115 (5)	412 (1)	190 (1)
Rabbit, age 1 week	23 (1)	33 (1)	—	—
Rabbit, age 5 weeks	31 (1)	75 (1)	316 (1)	192 (1)
Guinea pig	17 (6)	120 (1)	—	—
Dog	54 (3)	48 (3)	195 (3)	—
Cat	30 (2)	54 (2)	0 (1)	—

Table 5. *Observations on livers of premature human infants*

Slices and suspensions of liver were examined in the standard way described for animals. The *o*-aminophenol concentration was mM for slices, 0·3 mM for suspensions.

Case no.	Birth wt. (g.)	Length of gestation (weeks)	Cause of death and age at death	Q_{O_2} of liver slices (μl./g. of wet liver/hr.)	Bilirubin conjugation (μg./g. of wet liver/hr.)		*o*-Aminophenol conjugation (μg./g. of wet liver/hr.)	
					Slices	Suspensions	Slices	Suspensions
1	878	26	Intraventricular haemorrhage; 98 hr.	1070	0	0	—	—
2	1342	32	Anencephaly; 5 min.	725	0	0	—	—
3	766	25	Intraventricular haemorrhage and bilateral atelectasis; 65 min.	650	—	27	8	15

more prolonged in infant no. 3. Incubation of slices and suspensions commenced within 1·5–2·5 hr. of autopsy. Particulars of the individual cases and the results are given in Table 5.

In order to ascertain whether delay in removal of the liver, and an extended period in ice, would reduce the bilirubin-conjugating capacity of the liver, a control experiment with a rat was carried out. The liver was left *in situ* for 10 min. after death and was then removed and packed in ice for 2 hr. Samples were examined at 10 min., 1 hr. 10 min. and 2 hr. 10 min. after death. The loss of activity was about 25 %/hr.

Excretion of glucuronic acid by infants. The glucuronic acid concentration of 151 specimens of urine from 45 male infants (of whom about a third were premature and about a third became jaundiced) was determined during the first 10 days of life. Cases of jaundice attributable to haemolytic disease were excluded from this study. The urine-glucuronic acid concentration rose from a mean value of 9 mg./100 ml. on day 1 (range 0–18 mg./100 ml.) to 29 mg./100 ml. on day 2 (range 2–74 mg./100 ml.), and thereafter fell to a mean value of 12 mg./100 ml. (range 1–41 mg./100 ml.) on day 5. The values for infants of less than 5·5 lb. birth weight were not strikingly lower than those with a birth weight greater than 6·5 lb., nor did the jaundiced infants have much lower concentrations than the unpigmented ones.

DISCUSSION

The appearance of increased amounts of glucuronic acid in rat bile after the injection of bilirubin and the hydrolysis of the azo pigment by β-glucuronidase suggest that the rat excretes bilirubin as a glucuronide. In this respect it resembles the human (Billing *et al.* 1957; Schmid, 1956) and the dog (Talafant, 1956). Grodsky & Carbone (1957) have reached the same conclusion and state that the theoretical amount of glucuronic acid is present in the polar azo-pigment formed by rat-liver suspensions.

In seeking conditions in which slices and suspensions would conjugate bilirubin and glucuronic acid the work of Dutton & Storey (1954) on the conjugation of *o*-aminophenol was followed closely. The most striking difference in experimental conditions which we observed was the necessity for including plasma protein in the medium for conjugating bilirubin. Earlier work (Lathe, 1954; Day, 1954) suggested that bilirubin had a deleterious effect on the oxygen consumption of tissues, and recently Zetterström & Ernster (1956) showed that it 'uncoupled' oxidative phosphorylation. In our experiments serum to which bilirubin was attached was added in order to provide substantial amounts of bilirubin in the medium without reducing the oxygen uptake of the cells. The bilirubin concentrations used in this work caused no fall in oxygen consumption. We have found that serum has no effect on the rate of conjugation of *o*-aminophenol.

Dutton & Storey (1954) showed that the addition of a boiled-liver extract was necessary for optimum conjugation of *o*-aminophenol by mouse-liver homogenates, and identified the agent as UDP-glucuronic acid (Storey & Dutton, 1955). Grodsky & Carbone (1957) found that boiled-liver extract increased the conjugation of bilirubin by rat-liver suspensions. Our crude UDPglucuronic acid concentrate increased the rate of bilirubin conjugation by liver suspensions of all normal adult animals except in one rabbit (Table 3). The stimulating effect was very marked in the new-born mouse and in the adult rat.

After the intravenous administration of bilirubin to a rat the maximum rate of excretion was about 1·2 mg./g. of wet liver/hr. This agreed with the rate given by Billing & Weinbren (1956). The rate of conjugation by rat-liver suspensions was 0·4 mg./g. of wet liver/hr., i.e. one-third of the rate obtained in the living animal. When allowance is

made for tissue damage in preparing the suspensions the agreement between results *in vivo* and *in vitro* is satisfactory.

The procedure for estimating the rate of conjugation of bilirubin by suspensions was developed for rat liver, and accurate estimations were not possible when liver suspensions conjugated less than 90 μg./g. of wet liver/hr. and liver slices less than 15 μg./g./hr. The accuracy for conjugation of *o*-aminophenol was low with less than 60 μg./g./hr. (suspensions) and 15 μg./g./hr. (slices).

Fishman & Green (1957) have maintained that β-glucuronidase may synthesize glucuronides *in vivo*. Dutton & Storey (1954) were unable to find any inhibition of *o*-aminophenol conjugation by the addition of the specific inhibitor, saccharolactone. The effect of added saccharolactone on bilirubin conjugation by liver suspensions in young animals (Table 3) which have high levels of β-glucuronidase was not significant. The one positive result with adult-rabbit liver could not be repeated. Glucuronidase appears neither to conjugate bilirubin nor to reduce the yield of conjugated pigment by hydrolysis, under the conditions of our experiments.

There are striking similarities between the systems conjugating *o*-aminophenol and bilirubin. If the two processes were mediated by the same enzyme, however, competitive inhibition should be demonstrable, there should be a parallel between the relative amounts of the two substrates conjugated by different animals and different types of preparation, and the two systems should show a similar stability. Although inhibition can be demonstrated, it is not clear that this is due to competition for the enzyme transferring the glucuronyl radical. In mouse-liver suspensions bilirubin conjugation was much more sensitive to *o*-aminophenol concentration than in rat and rabbit experiments with slices.

It was noted that twice the optimum concentration of bilirubin (0·41 mM) did not affect *o*-aminophenol conjugation at its optimum concentration (0·2 mM). It was not possible to examine the data quantitatively according to Lineweaver & Burk (1934), since the effective concentration of bilirubin in a serum–bilirubin mixture was unknown. Grodsky & Carbone (1957) obtained inhibition of bilirubin conjugation by borneol, but this may be due to competition for a limited amount of UDPglucuronic acid. The highest rates of conjugation they obtained were about a third of the values in our Wistar rats. Among different animals there was no parallel between the capacities to conjugate the two substrates (Table 4). Thus the rat-liver suspension, which had the highest bilirubin-conjugating capacity, had negligible activity with *o*-aminophenol, though G. J. Dutton (personal communication) reports a small degree of activity. The rabbit-liver suspension conjugated *o*-aminophenol two to three times as fast as bilirubin. The homozygous Gunn rat-liver slices conjugated *o*-aminophenol but not bilirubin, whereas the cat conjugated bilirubin but not *o*-aminophenol. The latter observation confirms the finding of Dutton & Grieg (1957). K. J. Isselbacher (personal communication) has identified bilirubin glucuronide in cat bile. Rupturing the cells had a marked effect on the two systems in some animals. It was noted that in the rat and mouse, bilirubin conjugation was increased by preparing suspensions, but in these and other animals, breaking up the cells had a depressing effect on *o*-aminophenol conjugation. The enhancement of bilirubin conjugation may have been due to easier penetration of bilirubin to the intracellular site of enzyme activity.

Some of these conflicting results might be explained by bilirubin conjugation with substances other than glucuronic acid, since glucuronide formation has been established for only five species (human, dog, rat, guinea pig, cat), but there is as yet no evidence on this point. The same consideration applies to *o*-aminophenol, though to a less extent since sulphate would not be estimated by the method used. The differences in the distribution and behaviour of the two conjugating systems raises the question whether glucuronides of bilirubin and *o*-aminophenol are formed by different enzyme systems. The position may be clearer when investigations have been carried out with pure UDPglucuronic acid; although firm conclusions about different enzymes are premature, this possibility is not to be excluded.

Billing *et al.* (1957) suggested that transformation to a glucuronide was an essential step in the excretion of bilirubin. Our experiments and those of Schmid, Axelrod, Hammaker & Rosenthal (1957) point to the microsomes as the site of this transformation. This observation raises some interesting questions about the movement of bilirubin in the cell. It is present at the cell surface as a bilirubin–albumin complex, is conjugated in the microsomes and transferred to the intercellular biliary canaliculi. Other aspects of intracellular localization are involved, too, for Munch-Peterson, Kalckar & Smith (1955) have found that uridine diphosphate glucose is synthesized in the nucleus, Strominger, Maxwell, Axelrod & Kalckar (1957) observed its oxidation to UDPglucuronic acid in the particle-free cytoplasm, and UDPglucuronic acid appears to be utilized in or on the surface of the microsomes. Clearly an attempt to reconstruct the movements of bilirubin is dependent on further studies in the biochemical architecture of the liver cell.

These studies were undertaken with a view to devising means of increasing the capacity of the

new-born infant to excrete bilirubin. Our observations on premature-infant livers strongly suggest, by analogy with the system of Storey & Dutton (1955), that the primary defect in the new-born liver involves an enzyme transferring the glucuronyl radical to bilirubin, since the addition of UDPglucuronic acid did not correct the defect. In two of the three infants no conjugated bilirubin was detected with slices and suspensions, and the rate of conjugation by suspensions in the third liver was so low that it was of doubtful accuracy. Whether there is also a reduced synthesis of UDPglucuronic acid is less certain. The studies of the concentration of glucuronic acid in the urine of new-born infants are open to criticism since we were not able to obtain the total urine excretion. The rapid fall in concentration during the first 10 days may be due to the rapid increase in the output of urine. In addition part of the excreted glucuronic acid may have been of maternal origin. Nevertheless, that there was no marked difference in the concentration of glucuronic acid, when jaundiced infants were compared with normal infants or small with large infants, suggests that the availability of glucuronyl radical was not the decisive factor determining the ability to excrete bilirubin. One new-born-human liver was examined for *o*-aminophenol conjugation. The rate was lower than could be measured accurately under our conditions, but was quite distinct, and approximately half or a third of that found in the adult monkey. Unfortunately we do not yet know the rate of conjugation of these substances by adult-human liver for comparison. Indirect calculations, based on the rate of removal of intravenously injected bilirubin, suggest that the rate of conjugation of bilirubin by the human liver is similar to that in the rat relative to unit weight of liver. If this is so then the defect in capacity of the new-born-human liver to conjugate bilirubin is much more marked than with other subtrates.

The homozygous Gunn rats also showed a striking departure from normal, as far as bilirubin conjugation was concerned (Table 4). This confirms the prediction made by Malloy & Lowenstein (1940) to explain the accumulation in plasma of a bile pigment giving the indirect van den Bergh reaction, and the absence of the direct-reacting pigment. Schmid *et al.* (1957) have suggested that the defect also includes a greatly reduced production of other glucuronides. In the one homozygous animal in which the *o*-aminophenol conjugation of slices was determined the conjugation was about half the normal for Wistar rats and about the same as the rate in a heterozygous rat which was not jaundiced. The amount of UDPglucuronic acid used would have been several times that required for conjugation of bilirubin by slices of normal liver. Yet the homozygous Gunn rat-liver slices do not conjugate bilirubin. It follows that the production of UDPglucuronic acid is not the limiting factor. These animals, which are permanently jaundiced, present a contrast with the premature human infant, in which jaundice is temporary. Both lack, or are deficient in, one enzyme. In the Gunn rat this is genetically determined, whereas in the premature infant it is controlled by developmental or environmental factors which have not yet been defined. This fact suggests a possibility of treating jaundice in the new-born infant by accelerating the production of the enzyme in the liver cells.

SUMMARY

1. Evidence has been obtained that normal rat liver conjugates bilirubin with glucuronic acid.

2. Conditions have been determined for examining the synthesis of bilirubin glucuronide by liver slices and suspensions.

3. In view of the lack of parallelism between rates of conjugation of bilirubin and of *o*-aminophenol in tissue slices and suspensions from a number of animals, the possibility is discussed that the enzyme concerned in bilirubin conjugation is not identical with the one involved in *o*-aminophenyl glucuronide formation.

4. The bilirubin-conjugating system is present in cell particles, and absent from the soluble phase of the cytoplasm.

5. Preliminary results suggest that livers of human new-born infants and of homozygous Gunn rats are deficient in the enzyme required for bilirubin glucuronide formation.

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REFERENCES

Billing, B. H. (1954). *Biochem. J.* **56**, xxx.
Billing, B. H., Cole, P. G. & Lathe, G. H. (1954). *Brit. med. J.* **2**, 1263.
Billing, B. H., Cole, P. G. & Lathe, G. H. (1957). *Biochem. J.* **65**, 774.
Billing, B. H. & Weinbren, K. (1956). *Brit. J. exp. Path.* **37**, 199.
Day, R. L. (1954). *Proc. Soc. exp. Biol., N.Y.*, **85**, 261.
Dutton, G. J. (1956). *Biochem. J.* **64**, 693.
Dutton, G. J. & Grieg, C. G. (1957). *Biochem. J.* **66**, 52P.
Dutton, G. J. & Storey, I. D. E. (1954). *Biochem. J.* **57**, 275.
Fishman, W. H. & Green, S. (1955). *J. biol. Chem.* **215**, 527.
Fishman, W. H. & Green, S. (1957). *J. biol. Chem.* **225**. 435.

Grodsky, G. M. & Carbone, J. V. (1957). *J. biol. Chem.* **226**, 449.
Gunn, C. K. (1938). *J. Hered.* **29**, 137.
Gunn, C. K. (1944). *Canad. med. Ass. J.* **50**, 230.
Haslewood, G. A. D. & King, E. J. (1937). *Biochem. J.* **31**, 920.
Isselbacher, K. J. (1956). *Recent Progr. Hormone Res.* **12**, 134.
Lathe, G. H. (1954). *Symp. biochem. Soc.* **12**, 34.
Lathe, G. H. & Ruthven, C. R. J. (1958). *J. clin. Path.* **11**, 155.
Lathe, G. H. & Walker, M. (1957). *Biochem. J.* **67**, 9P.
Lathe, G. H. & Walker, M. (1958). *Quart. J. exp. Physiol.* **43**, 257.
Levvy, G. A. & Storey, I. D. E. (1949). *Biochem. J.* **44**, 295.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Malloy, H. T. & Lowenstein, L. (1940). *Canad. med. Ass. J.* **42**, 122.
Munch-Petersen, A., Kalckar, H. M. & Smith, E. E. B. (1955). *Dan. Biol. Medd.* **22**, 1.
Schmid, R. (1956). *Schweiz. med. Wschr.* **86**, 775.
Schmid, R. (1957). *J. biol. Chem.* **229**, 881.
Schmid, R., Axelrod, J., Hammaker, L. & Rosenthal, I. M. (1957). *J. clin. Invest.* **36**, 927.
Storey, I. D. E. & Dutton, G. J. (1955). *Biochem. J.* **59**, 279.
Strominger, J. L., Maxwell, E. S., Axelrod, J. & Kalckar, H. M. (1957). *J. biol. Chem.* **224**, 79.
Talafant, E. (1956). *Nature, Lond.*, **178**, 312.
Zetterström, R. & Ernster, L. (1956). *Nature, Lond.*, **173**, 1335.

Trace Elements in Human Tissue

3. STRONTIUM AND BARIUM IN NON-SKELETAL TISSUES*

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It has been found previously (Sowden & Stitch, 1957) that barium and strontium were regularly present in human bone in concentrations of 7 and 100 μg./g. of ashed tissue respectively. This work has been extended to the estimation of these trace elements in other human tissues, to determine which organ in the body concentrated the elements to the greatest extent, and to estimate the total body content of barium and strontium. These points are significant in the estimation of the radiation hazard to man from contamination with radioactive forms of these elements. It has been generally assumed, from the pattern of distribution of activity within the body after injection of radioactive isotopes, that strontium accumulates to a greater extent in bone than in soft tissues (Pecher, 1941; Lisco, Finkel & Brues, 1947; Kidman, Tutt & Vaughan, 1950; Comar, Wasserman, Ullberg & Andrews, 1957). Three reports issued by Tipton *et al.* (1957) give extensive details of the barium and strontium content of human organs taken from autopsies of American subjects. Other references to the concentrations of these elements in tissues are isolated. Strontium was detected in human tissues by Sheldon & Ramage (1931), Gehrlach & Muller (1934) and Forbes, Mitchell & Cooper (1956). In a single case of barium chloride poisoning reported by Mancini (1943) 5 mg. of barium was found in the liver and kidneys, with traces in the spleen and adrenals. Spectrographic analysis of eye tissue from various animals has established that barium is concentrated in the pigmented parts of the eye (Bowness, Morton, Shakir & Stubbs, 1952; Scott & Canaga, 1940; Ramage & Sheldon, 1931; Oksala, 1954). The degree of concentration in cow-, rabbit- and human-eye tissue has been established in this Laboratory by neutron-activation analysis (Sowden & Pirie, 1958).

* Part 2: Sowden & Stitch (1957).

METHODS

Estimations of strontium and barium concentrations were made by neutron-activation of the tissue in ashed form. Radioactive strontium and barium were chemically separated from the ash for comparison of their activity with that of irradiated standard samples of pure barium and strontium salts. Details of the techniques of tissue collection and preparation, neutron-activation and radioassay are given in a previous paper on bone analysis (Sowden & Stitch, 1957), but the more varied mineral composition of non-skeletal tissue made certain modifications of this method necessary. A more elaborate separation was required to obtain radiochemically pure fractions of strontium and barium. To obtain a sufficient activity in the barium and strontium for radioassay, larger amounts of tissue ash were required and these were subjected to a preliminary concentration of strontium and barium, in order to avoid the high radioactivity induced in gram quantities of ash after neutron-activation.

Reagents

Modifications of the technique used for bone analysis involved the use of certain additional reagents.