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Determination of Bilirubin Glucuronide and Assay of Glucuronyltransferase with Bilirubin as Acceptor

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1. Conjugated bilirubin is conveniently determined by coupling with the diazonium salt of ethyl anthranilate. 2. This method has been used in the development of assays for UDP-glucuronyltransferase (EC 2.4.1.17), with bilirubin as substrate, in rat liver homogenates, microsomal preparations and partly purified fractions. 3. Chromatographic analysis suggests that bilirubin monoglucuronide is the product of the enzyme systems studied.

Several observations demonstrate a difference in behaviour of UDP-glucuronyltransferase (EC 2.4.1.17) when bilirubin is used instead of other acceptors (Lathe & Walker, 1958; Tomlinson & Yaffe, 1966). The study of bilirubin–UDP-glucuronyltransferase is technically difficult (Dutton, 1963; Boerth, Blatt & Spratt, 1965), and present methods are mostly unsuitable for quantitative work (van Leusden, 1963; Boerth *et al.* 1965) because small quantities of conjugated pigments have to be determined in the presence of an excess of non-conjugated bilirubin. In serum an appreciable fraction of the bilirubin reacts with diazobenzene-*p*-sulphonic acid, the usual coupling reagent (Lathe & Ruthven, 1958). Other analytical difficulties may arise; thus turbidity occurs when the colour of the azo-pigments is measured without extraction from the original mixture (Grodsky & Carbone, 1957) or an appreciable amount of azo-pigments may be lost when protein precipitation precedes optical reading (Lathe & Walker, 1958). The procedure of Weber & Schalm (1962) is inappropriate for enzymic studies (Boerth *et al.* 1965). Recent procedures employing radioactive bilirubin (Menken, Barrett & Berlin, 1966) or diazobenzene-*p*-sulphonic acid (Metge, Owen, Foulk & Hoffmann, 1964), however, offer some promise.

By using diazonium salts of aromatic amines bearing a weakly or non-ionized substituent, e.g. *p*-iodoaniline, *p*-nitroaniline or *p*-aminobenzoic acid, azo-pigments are obtained that are easily extracted by organic solvents. Extraction largely eliminates erratic blank values. Selectivity of coupling with conjugated bilirubin improves when a carboxyl group or its ethyl ester is located *ortho* to the diazo group.

The presence of a strongly binding carrier protein (Grodsky & Carbone, 1957; Lathe & Walker, 1958), though advantageous in accepting insoluble reaction products, complicates interpretation of kinetic data when used as a substrate carrier. Working with soluble bilirubinate at pH 8·5 eliminates this difficulty (Schmid, Hammaker & Axelrod, 1957), but this is at least 1·1 pH units higher than reported values for optimum glucuronic acid transfer (Lathe & Walker, 1958; Tomlinson & Yaffe, 1966).

The present paper proposes a method for measuring bilirubin glucuronide synthesis in microsomal preparations, the major innovations being a more soluble bilirubin substrate and the use of the diazonium salt of ethyl anthranilate.

MATERIALS AND METHODS

Chemicals. Bilirubin from British Drug Houses Ltd. (Poole, Dorset) was used without further purification. Bilirubin glucuronide (crude) was prepared from human post-operative fistula bile (Lucassen, 1961). Bile was collected into 10% (w/v) $Na_2S_2O_3$ solution in a dark bottle surrounded with ice. After 6hr. collection the amount of thiosulphate solution was adjusted to about 100ml./l. of bile and the mixture stored at −15° until further processing. The essential steps of the preparation of bilirubin glucuronide (Lucassen, 1961) are as follows. All manipulations were done at 0–4°. The bile–thiosulphate mixture was thawed and brought to pH 6 by adding dropwise 10% (w/v) oxalic acid solution. The precipitate formed was discarded. On adjustment of the pH of the supernatant to 3·5 a second precipitate appeared. This was isolated by centrifuging and washed twice with 0·01% oxalic acid. The washed sediment was rubbed in a mortar with 200ml. of acetone and the suspension kept for 2–4hr. at −15°. The acetone extract was filtered off and adjusted to pH 7 with 0·1N-NaOH in

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96% (v/v) ethanol. A flocculent precipitate of conjugated bilirubin was obtained. This was isolated by centrifuging and washed twice with 20ml. of dry acetone followed by two washings with 20ml. of dry ether. Bile pigment preparations, protected from light, were stored *in vacuo* at 4° over silica gel. Partly oxidized (greenish) preparations were discarded.

Pigment I and pigment II (Billing, Cole & Lathe, 1957) were isolated chromatographically (Billing, 1955) from conjugated bilirubin and from bile. Fractions were collected in 10ml. tubes at 0° in the dark. Without delay total bilirubin was determined (see below), part of the samples being kept for coupling with the diazonium salt of ethyl anthranilate. Samples were prepared for chromatography by treating 1vol. of a bile–water mixture with 2·5vol. of ice-cold ethanol for 2min., centrifuging and drying the supernatant by rotary evaporation *in vacuo* at room temperature. The residue was taken up in an adequate volume of mobile phase (Billing, 1955) and 0·5ml. of the solution subjected to chromatography. Purified preparations of conjugated bilirubin were dissolved immediately in 0·5ml. of the mobile phase.

Ethyl anthranilate (Eastman Organic Chemicals, Rochester, N.Y., U.S.A.) was stored in the refrigerator. Pentan-2-one and chloroform were redistilled before use. Ethylene glycol and ethanolamine were vacuum-distilled. Bovine serum albumin was provided by Poviet (Amsterdam, The Netherlands) and human serum albumin by Behringwerke A.-G. (Marburg/Lahn, Germany). The ammonium salt of UDP-glucuronic acid was purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.). All other chemicals were of reagent-grade quality.

Biological materials. Human serum was obtained from blood of males who had not taken food for 12–16hr. Wistar R male albino rats, fed on a standard diet, were used as source of enzyme. Rats (110–250g. body wt.) were killed by decapitation, and the livers quickly placed in ice-cold 0·25M-sucrose containing 1mM-disodium EDTA, pH7·4, cut in a few pieces, rinsed and blotted. Homogenates were prepared by the method of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) and adjusted with medium to contain the equivalent of 10g. wet wt. of liver/100ml. of suspension. After centrifuging in rotor no. 40 of a Spinco model L-50 ultracentrifuge at $41000g_{av.}$ at 4° for 7min. the supernatant was removed and centrifuged at $105000g_{av.}$ for 30min. Microsomal sediments were resuspended, adjusted to the initial volume and centrifuged a second time at $105000g_{av.}$ for 30min. The final sediments were redispersed and diluted in the above sucrose medium. Concentrations of microsomes or of other cell fractions are given in most cases as a percentage, indicating the equivalent of the g. wet wt. of liver/100ml. of suspension. In some instances a protein weight basis is used.

The particulate M and P fractions, as defined by Ernster, Siekevitz & Palade (1962), were prepared from microsomes by treatment with deoxycholate (Ernster *et al.* 1962). The fractions were resuspended separately, brought to the initial volume and centrifuged at $105000g_{av.}$ for 120min.

Rat bile enriched in conjugated bilirubin was obtained as follows. Bilirubin (20mg.) was dissolved in 2ml. of a mixture containing 0·05N-NaOH and 0·05M-disodium EDTA. Water (2ml.) was then added, followed by 2ml. of tris–HCl buffer, pH7·4 (0·4N-HCl), and 18ml. of human serum. Two 1ml. portions of the serum–bilirubin mixture were injected consecutively into the femoral vein of a rat weighing about 350g. After about 10min. bile was drawn from the common bile duct and introduced into a reagent tube containing 0·2ml. of 10mM-disodium EDTA. Tubes were kept in the dark at 0°. Collection of bile was continued for 30–120min.

Determination of protein. Protein was determined by the method of Daughaday, Lowry, Rosebrough & Fields (1952) with bovine serum albumin as a standard. Some advantage was found in increasing the Cu^{2+} concentration twofold.

Preparation of bilirubin and bilirubin glucuronide solutions. All manipulations (including incubations) were done in red-filtered daylight. Pigments were dissolved immediately before use. Solutions were prepared with CO_2-free water.

For most analytical work 10mg. of bilirubin was dissolved in 2ml. of 0·05N-NaOH, which was then diluted to 50–100ml. with bovine serum albumin (10mg./ml.) in water. Bilirubin glucuronide was used as an aqueous solution.

For enzymic experiments the following two types of bilirubin solutions were used for incubation systems I and II (see under 'Assay of bilirubin–UDP-glucuronyltransferase activity' below). System I: 17·4mg. of bilirubin was dissolved in 1·5ml. of 0·05N-NaOH and diluted to 10ml. with 1mM-disodium EDTA; 4ml. of human serum, previously diluted with water to a protein concentration of 50mg./ml., was mixed with 2ml. of the alkaline bilirubin solution [the human serum was replaced in some instances by human or bovine serum albumin or by serum or cell sap obtained from Gunn rats (Schmid, Axelrod, Hammaker & Swarm, 1958) or Wistar rats (Table 4)]. System II: 15mg. of bilirubin was dissolved in 15ml. of chloroform by boiling for 10sec.; the solution was filtered and 2ml. of the filtrate concentrated to dryness in a rotary evaporator; the residue was taken up in 10ml. of ethylene glycol and 0·1ml. of ethanolamine.

Standardization of bilirubin and bilirubin glucuronide solutions. For aqueous solutions total bilirubin was determined by the method of Jendrassik & Grof as modified by Lucassen (1961). A portion (2vol.) of the sample was treated with 7vol. of an accelerating mixture containing caffeine, sodium benzoate and sodium acetate (Heirwegh, Jansen & Van Roy, 1966). Colour was developed at room temperature with 1vol. of diazo reagent [0·3ml. of $NaNO_2$ (5g./l.) mixed with 10ml. of sulphanilic acid (5g./l.) in 0·15N-HCl]. A blank was prepared by omitting the $NaNO_2$ from the reaction mixture. After 10min. the E_{530} value was measured. Concentrations were calculated assuming ϵ_{530} $51{\cdot}4 \times 10^3$ $M^{-1}cm.^{-1}$. Approx. 80% of the crude preparation of conjugated bile pigments reacted without accelerator.

Bilirubin–chloroform and bilirubin–ethylene glycol–ethanolamine solutions (100μl.) were diluted with 15ml. of chloroform and the E_{450} values measured. Calculations were based on ϵ_{450} $59{\cdot}3 \times 10^3$ $M^{-1}cm.^{-1}$ for bilirubin–chloroform solutions (Fog, 1964) and ϵ_{450} $54{\cdot}3 \times 10^3$ $M^{-1}cm.^{-1}$ for diluted bilirubin–ethylene glycol–ethanolamine (F. P. Van Roy & K. P. M. Heirwegh, unpublished work).

Standardization of UDP-glucuronic acid. The concentration was calculated assuming ϵ_{261} $8{\cdot}1 \times 10^3$ $M^{-1}cm.^{-1}$; solutions were in water.

Assay of bilirubin–UDP-glucuronyltransferase activity. Incubation mixtures were prepared at 0° in 11ml. glass-stoppered centrifuge tubes with round bottoms. System I contained 50μl. of maleate–tris buffer, pH7·42 (containing 0·4M-tris and 0·4M-maleic acid adjusted with NaOH),

200μl. of bilirubin–serum mixture (final concn. 0·4mM), 10μl. of UDP-glucuronic acid solution (final concn. about 8mM), 200μl. of a suspension of a cell fraction (usually microsomes) and 40μl. of $MgCl_2$ solution (final concn. 10mM) in a final volume of 500μl. System II contained 200μl. of triethanolamine–HCl buffer, pH 7·42 (0·25N-HCl), 400μl. of a suspension of microsomes or M or P fraction (see above), 100μl. of bilirubin in ethylene glycol (see above) (final concn. 0·64mM), 20μl. of UDP-glucuronic acid solution (final concn. about 2mM) and 50μl. of $MgCl_2$ solution (final concn. 6·4mM) in a final volume of 1ml.

Incubations were at 37° with shaking for 30–60min. for system I, or 10min. (microsomal fraction) or 5min. (M or P fraction) for system II. Controls were prepared by substituting water for the UDP-glucuronic acid solution and kept at 0° for the appropriate time-intervals. Aqueous solutions of saccharo-(1→4)-lactone, when used to inhibit glucuronidase, were prepared by the method of Levvy (1952).

Determination of bilirubin glucuronide in incubation mixtures. The procedure used in conjunction with system I is described in detail, and deviations applicable to system II are given.

Reagents were as follows: (*a*) ethyl anthranilate (0·1ml. of ethyl anthranilate finely suspended in 10ml. of 0·15N-HCl); (*b*) $NaNO_2$ (a 5g./l. solution, freshly diluted from a 100g./l. solution kept in the dark at 4°); (*c*) ammonium sulphamate (171g./l.); (*d*) diazo reagent (0·3ml. of $NaNO_2$ solution mixed with the ethyl anthranilate suspension and left for 5min. at room temperature; 0·1ml. of ammonium sulphamate was then added and the reagent used 3min. afterwards); (*e*) ascorbic acid (a 40g./l. solution, freshly prepared); (*f*) glycine buffer (0·4N-HCl adjusted to pH 2·6 with glycine); (*g*) extraction solvent [pentan-2-one–*n*-butyl acetate (17:3, v/v)].

Incubation mixtures were quickly cooled on ice, treated immediately with 0·3ml. of glycine buffer and placed in a thermostat at 25°. After 10min. 0·5ml. of diazo reagent was added and coupling allowed to proceed for 20min. The reaction was stopped by adding 4ml. of ascorbic acid solution. After 10min. the content of the tubes was shaken vigorously (30 times) with 3–5ml. of extraction mixture. The extraction was continued by mechanical inversion of the tubes for 30min. The upper layer was separated by centrifuging and its E_{530} value measured, with extraction solvent as reference. Control mixtures were treated in exactly the same way as the incubation mixtures.

For incubation system II the following changes were applied: 0·5ml. of a 250g./l. ascorbic acid solution was used, and the volumes of the glycine buffer and the diazo reagent were 1ml. Azo-pigments were extracted with 5ml. of pentan-2-one–*n*-butyl acetate.

Separation of azo-pigments. (*a*) Thin-layer chromatography. About 2ml. of the azo-pigment extract was concentrated to dryness in a rotary evaporator and dissolved in 0·2ml. of *n*-butyl acetate. Plates were prepared with a 0·2mm. layer of aluminium oxide G and silica gel G (1:1, w/w) (E. Merck A.-G., Darmstadt, Germany) and dried in air. Chromatograms were developed for 10cm. with butan-1-ol–3% (v/v) acetic acid–propan-1-ol (4:3:3, by vol.).

(*b*) Column chromatography. About 3ml. of the azo-pigment extract was washed twice with 5ml. of ascorbic acid (1g./l.) in 1mN-HCl. Then 2ml. was placed on a column (15cm. × 1cm.) of CM-cellulose (Na^+ form) in pentan-2-one. The column was washed with pentan-2-one to elute the less polar azo-pigments (azo-pigment A fraction) followed by pentan-2-one–formamide–ethanol (44:5:1, by vol.) to elute the more polar azo-pigments (azo-pigment G fraction). Ethyl anthranilate was eluted together with azo-pigment A. The azo-pigment fractions were collected in 10ml. volumetric flasks each containing 0·5ml. of ethanol, brought to the mark with pentan-2-one and their E_{530} values read in 4cm. cuvettes. The purity of the fractions was monitored by thin-layer chromatography.

Measurement of pH. A glass electrode (G202C) was used with a model TTT1 Titrator (Radiometer, Copenhagen, Denmark).

Spectrophotometry. Extinctions were measured at 25° with a Beckman model DB spectrophotometer (Fullerton, Calif., U.S.A.). Spectra were determined in conjunction with a recorder.

RESULTS

Reaction of bilirubin compounds with diazonium salts

In a comparative study a very slow reaction of the diazonium salt of anthranilic acid with β-naphthol (Lucassen, 1961) and with *N*-(1-naphthyl)ethylenediamine (Brodersen, 1960) was observed. Even with conjugated bilirubin coupling is slow in the absence of an accelerator. As shown in Table 1 this is characteristic of the *o*-carboxyl group. With the *o*-carboxylic acid ethyl ester, however, coupling is rapid. Both *ortho* derivatives react very slowly with non-conjugated bilirubin.

Analytical study with the diazonium salt of ethyl anthranilate

Effect of pH *on coupling in aqueous media.* As shown in Fig. 1, colour development with bilirubin is minimal below pH 3·0, but is optimum for bilirubin glucuronide at pH 2·4–3·1. Similar results were found for reaction systems containing equal volumes of human serum, glycine–hydrochloric acid buffer (0·25N-hydrochloric acid) and diazo reagent. To allow neutralization of buffer components present in enzymic incubation mixtures, coupling with diazonium salt was done as a routine in the presence of more concentrated glycine buffer, pH 2·6 (0·4N-hydrochloric acid). For work with other sources of conjugated bilirubin (bile diluted 20-fold or more, or serum) the concentration and the pH of the glycine buffer were adapted to obtain final pH 2·7–2·8 in the reaction mixtures.

Effect of reaction time. The reaction with conjugated bilirubin was investigated at 0° and 25° both in aqueous medium and in the complete incubation system I, containing microsomes, buffer and serum. At various times after addition of diazo reagent ascorbic acid was added and the colour

Table 1. *Effect of nature and position of activating groups on coupling rate of diazonium salts*

Reaction mixtures contained 0·5ml. of 10mM-diazonium salt in 0·15N-HCl, 0·5ml. of glycine buffer, pH2·6, and either 0·5ml. of a human serum albumin (10mg./ml.)–bilirubin (110mμmoles) mixture or 0·5ml. of bilirubin glucuronide (60mμmoles). The reaction was stopped with 4ml. of ascorbic acid (50mg./ml.) and the azo-pigments were transferred to 4ml. of extraction mixture (see the text).

	Reaction time (min.)	$E^{4\,cm.}_{530}$ obtained with diazonium salt of: Aminobenzoic acids (*ortho*)	(*meta*)	(*para*)	Ethyl anthranilate (*ortho*)
Bilirubin glucuronide	5	0·304	0·89	1·05	0·75
	30	0·428	1·13	1·11	0·73
Bilirubin	30	0·010	0·287	0·497	0·036

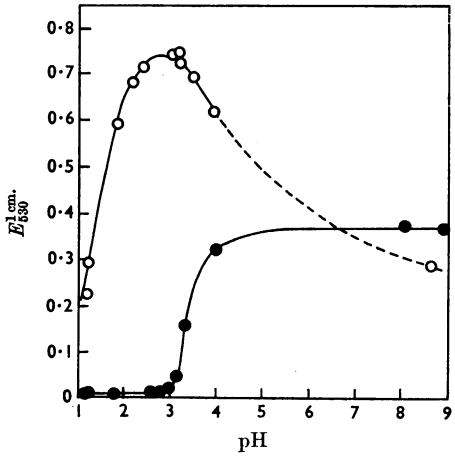

Fig. 1. Effects of pH on coupling with bile pigments. Samples (0·4ml.) containing 270mμmoles of bilirubin (●) in bovine serum albumin (10mg./ml.) or 220mμmoles of conjugated bilirubin (○) were mixed with 0·6ml. of one of the following solutions: 0·15N-NaOH, water, glycine solutions of various concentrations, glycine–NaOH mixtures of various compositions. Then 1ml. of ethyl anthranilate diazo reagent was added and coupling allowed to proceed for 30min. at 25°; the reaction was stopped with 8ml. of ascorbic acid soln. (50mg./ml.). The mixtures were adjusted to pH2 and water was added to 15ml. Azo-pigments were extracted with 8ml. of solvent and their E_{530} values read (1cm. light-path). Determinations of pH were done on mixtures, prepared in parallel, that contained bile pigment, diluent and diazo reagent.

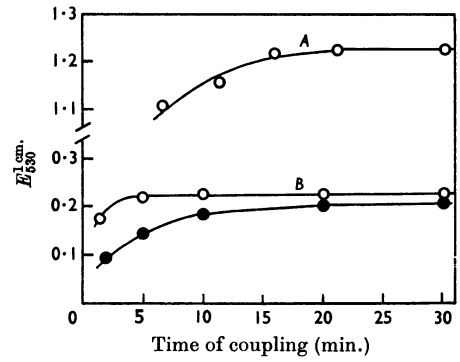

Fig. 2. Rate of azo-pigment formation. Each sample contained 110mμmoles of conjugated bilirubin in incubation mixture I (curve *A*) or 21mμmoles of conjugated bilirubin in aqueous solution (curve *B*). Colour was developed at 25° (○) or at 0° (●).

extracted (Fig. 2). In the presence of microsomes (equivalent to 0·3g. of liver) diazo-coupling was slower than in aqueous solution; for practical work a 20min. reaction time at 25° was considered adequate. At 0° coupling was still incomplete after 30 min. With diluted rat bile (36mμmoles of conjugated bilirubin), glycine–hydrochloric acid buffer and diazo reagent (1:1:1, by vol.) reaction at 25° was complete after 4min., colour yields being 88·5% after 1min. and 97·5% after 2min.

Efficiency of extraction of azo-pigments. The partition coefficient between glycine buffer–ascorbic acid and pentan-2-one–*n*-butyl acetate was found to be more than 1000 in favour of the organic phase for pigments derived from either free or conjugated bilirubin.

Recovery experiments with incubation system I (see above) showed that 200μl. of microsomal suspension (equivalent to up to 400mg. of liver) or homogenate (equivalent to 40mg. of liver) did not affect the recovered yield of 20–30mμmoles of conjugated bilirubin, but with more concentrated tissue preparations the yield was decreased.

Influence of incubation at neutral pH *(at* 37°*) and at* pH2·6 *(at* 25°*) on colour development.* Incubation system I was used with 200μl. of a cytoplasmic extract (10%) and 20mμmoles of conjugated bilirbuin. Mixtures were incubated at 37°, with

Table 2. *Reproducibility of azo-pigment colour yield*

Colour was determined on conjugated bilirubin in aqueous solution: (*A*) in enzymic incubation mixture (system I, see the text); (*B*) after storage at −15°; (*C*) after enzymic production.

	Conjugated bilirubin in sample (mμmoles)	±S.D.	No. of observations
(*A*)	53·3	0·03	9
(*B*) First day	9·8		2
Second day	9·5		2
Third day	9·6		3
		0·008	7
(*C*) Microsomal concn. in final incubation mixture (g. wet wt. of liver equiv./ml.)			
0·08	17·3		3
0·16	34·3		3
0·24	51·2		3
		0·006	9

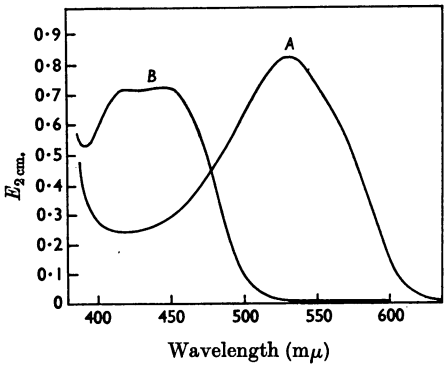

Fig. 3. Absorption spectrum of azo-pigment (curve *A*) and conjugated bilirubin (curve *B*). A 0·5ml. sample containing 39mμmoles of conjugated bilirubin in aqueous solution was treated as described in the text (curve *A*); for curve *B* extract was obtained by application of the same procedure except for the omission of $NaNO_2$ from the diazo reagent.

and without saccharo-(1→4)-lactone (final concn. approx. 0·2mM), for 0, 15, 30, 45 and 60min. and further incubated with glycine buffer (but no diazo reagent) at 25° for 0, 10 and 30min. before the diazo-coupling was started. Total colour yield was unaffected by these treatments.

Reproducibility of the determination and stability of azo-pigment extracts. The reproducibility of the determination was tested with conjugated bilirubin both in incubation system I without added bilirubin and in aqueous solution. Determinations were also done on conjugated bilirubin produced by enzyme in system I. As shown in Table 2 reproducibility of the assay is satisfactory. When the extracts were kept at 4° colour readings obtained from conjugated bilirubin in aqueous medium and in the incubation mixture remained unchanged for 24hr. and 3hr. respectively.

Absorption curve, linearity of extinction with concentration, and the molar extinction coefficient. The shapes of the absorption curves, with a maximum at 529mμ (Fig. 3), were identical for conjugated and non-conjugated azo-pigments. At 530mμ interference by the yellow colour of bilirubin was negligible. Beer's Law was followed up to $E^{1cm.}_{530}$ 1·5.

The molar extinction coefficient was obtained as follows. To 0·5ml. of standardized solutions of bilirubin in purified chloroform (Fog, 1964) were added 4ml. of propan-1-ol and 0·5ml. of diazo reagent. After 60min. at room temperature the azo-pigments were dried in a rotary evaporator. Water (0·5ml.), 0·5ml. of glycine buffer, pH 2·6, and 4ml. of ascorbic acid were added and the colour was extracted in 4–10ml. of extraction mixture. The ϵ_{530} value found was 44·36 $(\pm 0{\cdot}53) \times 10^3$ $M^{-1}cm.^{-1}$ (S.E.M., 16 determinations). The same value was obtained when aqueous solutions of bilirubin were used.

Selectivity of the determination. The selectivity of the determination was tested by changing the concentration of conjugated bilirubin in the presence of an excess of bilirubin, and by varying the concentration of bilirubin in the presence of a constant amount of conjugated bilirubin. As illustrated in Fig. 4, colour yields obtained for conjugated bilirubin (curve *C*) only slightly increased in the presence of an excess of unconjugated pigment (curve *B*). The amount of colour developed

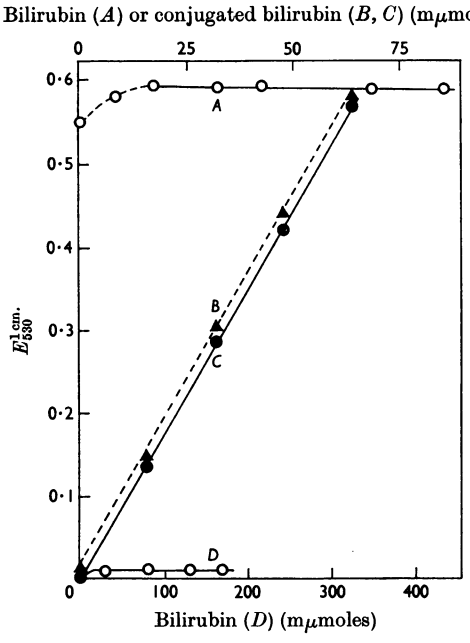

Fig. 4. Selectivity of coupling. Reaction systems contained 50μl. of triethanolamine buffer, pH 7·4, 200μl. of bovine serum albumin (dialysed, 5mg./ml.), 100μl. of microsomes (equivalent to 50mg. of liver), 40μl. of $MgCl_2$ (125mM) and 100μl. of disodium EDTA (1mM) in a final volume of 490μl. Bilirubin was added dissolved in EDTA, and conjugated bilirubin added with the serum albumin. The mixtures were incubated for 30min. at 37° before colour development. Curve *A*, increasing amounts of bilirubin in the presence of 68mμmoles of conjugated bilirubin; curve *B*, increasing amounts of conjugated bilirubin in the presence of 170mμmoles of bilirubin; curve *C*, increasing amounts of conjugated bilirubin; curve *D*, increasing amounts of bilirubin. The upper abscissa refers to curves *A*, *B* and *C*, the lower abscissa to curve *D*.

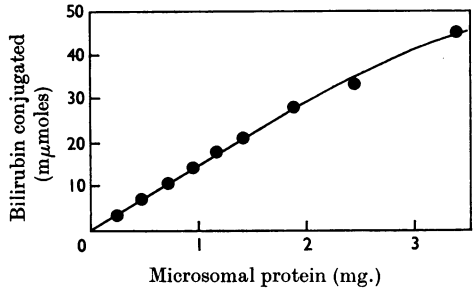

Fig. 5. Effect of enzyme concentration. Incubation system I was used with 200mμmoles of bilirubin and 3·9μmoles of UDP-glucuronic acid. The incubation time was 45min.

with bilirubin (curve *D*) was nearly independent of pigment concentration.

Exactly similar results were obtained when 0·5ml.

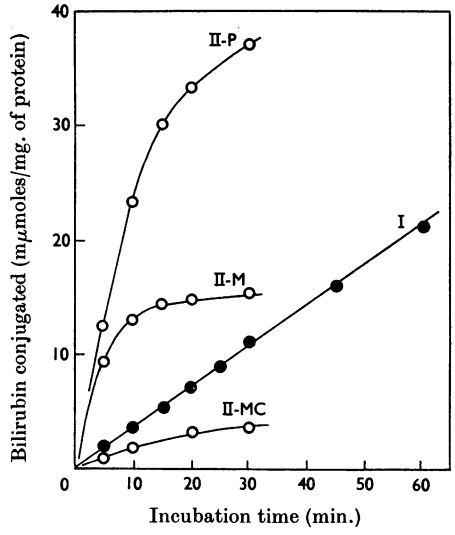

Fig. 6. Effect of incubation time. Curve I, system I (●) with 200mμmoles of bilirubin, 3·9μmoles of UDP-glucuronic acid and 200μl. of microsomal fraction (2·3mg. of protein). Curves II, system II (○) with 65mμmoles of bilirubin and 2μmoles of UDP-glucuronic acid at a final ethylene glycol concn. of 10% (v/v), together with 400μl. of microsomal preparation (9·5mg. of protein) (curve II-MC), M fraction (2·3mg. of protein) (curve II-M) or P fraction (0·41mg. of protein) (curve II-P). The M and P fractions were obtained from the assayed microsomal preparation.

samples of diluted human bile (69mμmoles of conjugated bilirubin in each sample) containing 0–170mμmoles of unconjugated bilirubin were treated for 20min. at 37° with 1vol. of glycine buffer, pH 2·6, and 1vol. of diazo reagent (final pH of the reaction mixtures 2·7). Preincubation of the bile–bilirubin mixtures for 30min. at 37° before colour development had no detectable influence on the final colour yields.

Coupling of pigment I and pigment II. The pigment fractions were obtained from conjugated bilirubin. By comparison of the colour yields found with diazobenzene-*p*-sulphonic acid (i.e. in the presence of an accelerating mixture) and with the diazonium salt of ethyl anthranilate (i.e. without an accelerator) mean ϵ_{530} values found for pigment I and pigment II were 42×10^3 and 43×10^3 M^{-1} $cm.^{-1}$ respectively. These values agree reasonably well with the more accurate value found for bilirubin, namely $44{\cdot}4 \times 10^3$ $M^{-1}cm.^{-1}$.

Characteristics of the bilirubin–UDP-glucuronyltransferase system

Effect of enzyme concentration. The velocity of the reaction was directly proportional to the amount of microsomal protein present (Fig. 5).

Table 3. *Values of maximal velocity and Michaelis constant*

Microsomal fractions were incubated for 45min. in system I or for 10min. in system II. Human serum albumin (not dialysed) at a constant final concn. of 8mg./ml. replaced the serum in system I. The bilirubin or UDP-glucuronic acid concentrations were varied to obtain the kinetic constants, at fixed (standard) concentration of the other substrate.

	Substrate	Bilirubin carrier	pH	$10^3 \times K_m$ (M)	V (μmole/g. wet wt. of liver/10min.)
System I	Bilirubin	Human serum albumin	8·2	0·17	0·048
	UDP-glucuronic acid	Human serum albumin	8·2	0·68	0·051
System II	Bilirubin	Ethylene glycol (30%, v/v)	7·4	0·09	0·098

Table 4. *Efficiency of carrier proteins*

Incubation system I was used with or without added Mg^{2+} (final concn. 10·9mM). Activities are compared with the value found in the presence of undialysed human serum albumin (taken as 100%). Samples of the carrier proteins were dialysed for 24hr. against 100vol. of 1mM-disodium EDTA, pH7·4. Values are means±S.D., with numbers of determinations in parentheses.

	Relative activity (%)		
		Dialysed	
Bilirubin carrier	Not dialysed Mg^{2+} added	Mg^{2+} added	No Mg^{2+} added
Human serum albumin	(100) (3)	133, 168 (2)	0 (1)
Bovine serum albumin	74 (1)	150 (1)	0 (1)
Normal rat serum*	109±43 (3)	140 (1)	
Gunn rat serum*	130±43 (3)	155 (1)	
Normal rat cell sap*	110±31 (3)	44±2 (5)	0 (1)
Gunn rat cell sap*	125±49 (3)	47±15 (3)	0 (1)

* Sera and cell-sap preparations from male and female rats in each group.

Effect of incubation time. Zero-order kinetics were followed for 60min. in system I and for shorter times in system II (Fig. 6). Additional experiments with system II showed a constant velocity for 10min. with the microsomal fraction and for 5min. with the M and P fractions.

Effect of substrate concentration. The UDP-glucuronyltransferase activity of microsomal preparations and of the M and P fractions measured with bilirubin as the glucuronic acid acceptor shows typical Michaelis–Menten kinetics. Values of the Michaelis constants, K_m, for substrates and of the maximal velocity, V, obtained by the graphical method of Lineweaver & Burk (1934), are given in Table 3.

Properties of different substrate forms. As already observed by Lathe & Walker (1958), very low activities were found when an aqueous solution of bilirubin was used as a substrate (system I without added serum protein). The efficiencies of various carrier proteins are compared in Table 4. For preparations obtained from Gunn rats some endogenous bilirubin will be present, but at the high substrate concentrations used this was probably of minor importance. Some inhibitory

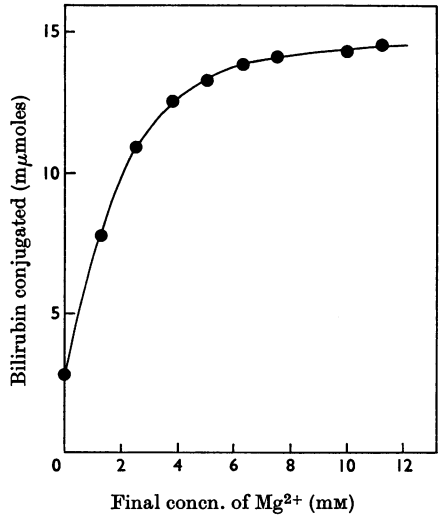

Fig. 7. Influence of Mg^{2+} concentration on the activity of the P fraction. System II was used with 84mμmoles of bilirubin, 2μmoles of UDP-glucuronic acid and 400μl. of P fraction (0·48mg. of protein). The final concn. of ethylene glycol was 10% (v/v). The incubation time was 30min.

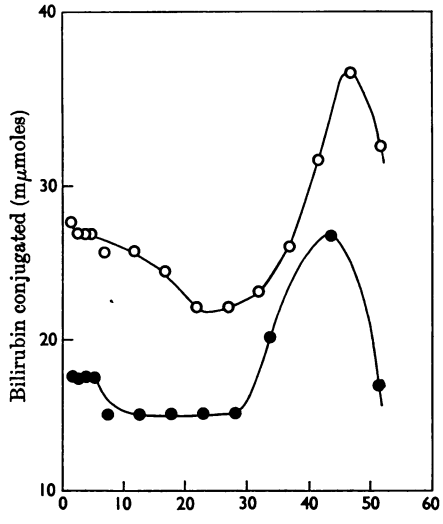

Fig. 8. Influence of ethylene glycol concentration. System II was used with 2 μmoles of UDP-glucuronic acid and either (○) 37 mμmoles of bilirubin and 11·5 mg. of microsomal protein (incubation time 15 min.) or (●) 38 mμmoles of bilirubin and 8·6 mg. of microsomal protein (incubation time 10 min.).

material was removed from rat serum (normal rats and Gunn rats) and from commercial serum albumin preparations by dialysis; as the increases in enzyme activity were less pronounced when the buffer used for dialysis was 0·05 M-phosphate instead of 1 mM-disodium EDTA (both at pH 7·4) the inhibition may have been due to metal ions. A noticeable decrease in activity was observed when dialysed cell-sap preparations were used, and the rates could only partly be restored by the addition of Mg^{2+}.

Though system I worked equally well with cell homogenates, the ethylene glycol substrate (system II) was found to be useful only with isolated cell fractions.

Activation by Mg^{2+}. Data from Table 4 (system I) suggest an important activating role for Mg^{2+} in bilirubin–UDP-glucuronyltransferase activity. The same effect was observed with the P fraction described by Ernster *et al.* (1962) (Fig. 7).

Influence of ethylene glycol on enzyme activity. After a decrease the UDP-glucuronyltransferase activity of the microsomal fraction reached a maximum at an ethylene glycol concentration of 45% (v/v) (Fig. 8).

Subfractionation of microsomes with deoxycholate. In five experiments microsomes (final protein concn. 3·3–4·3 mg./ml.) were treated with sodium deoxycholate (final concn. 2·36 mg./ml.) and the M and P fractions (Ernster *et al.* 1962) prepared. Bilirubin–UDP-glucuronyltransferase activity was determined in the microsomes and in the M and P fractions. Total activity recovered in the M plus the P fractions amounted to 2·2–3·5 times that of the corresponding microsomal preparations. A variable and sometimes considerable amount of the enzyme activity (5–20%) sedimented with the P fractions; in two experiments with starved rats the amounts were 41 and 43% respectively.

On a protein basis the activity of the M and the P fractions were eight- to 15-fold higher than in the corresponding microsomes (Table 5); it was roughly equal in both subfractions.

Chromatography of azo-pigments

Thin-layer chromatography. Thin-layer chromatography of the azo-pigments derived from rat bile revealed two major spots called azo-pigment A (R_F approx. 0·7) and azo-pigment G (R_F 0·34). The faster-moving spot was identified as azobilirubin by parallel and mixed runs with authentic azobilirubin. With conjugated bilirubin prepared from human bile azo-pigment G dominated, whereas azo-pigment A and azo-pigment G were about equally important when azo-pigment extracts obtained after enzymic incubations were studied. Only a faint line, with the R_F of azobilirubin, was found when 2 μmoles of bilirubin (system I without UDP-glucuronic acid) were analysed.

Azo-pigment G was eluted and proved to resist a 30 min. treatment with glycine buffer, pH 2·6. A slight increase of azo-pigment A, observed after incubation of conjugated bilirubin from human bile with cell homogenate (for 60 min. at 37° at pH 7·4), could be prevented by adding saccharo-(1→4)-lactone (final concn. approx. 0·2 mM).

Column chromatography. Cation exchange has been used by Vegas (1963) for the chromatography of azo-pigments. Column chromatography with organic solvents permits ready separation and spectrophotometric evaluation of the extracted pigments. Azo-pigment A was readily eluted with pentan-2-one (see the Materials and Methods section); recovery was 99·6%. Azo-pigment G, which was quantitatively adsorbed on the column in pentan-2-one, was eluted with pentan-2-one–formamide–ethanol. The azo-pigments from conjugated bilirubin were completely recoverable from the columns. Tenfold dilution of samples of azo-pigment A (in the extraction mixture) with either solvent caused no change in E_{530}.

The ratio of azo-pigment A to azo-pigments A + G was nearly 0·5 for conjugated bilirubin produced *in vitro* by enzymic incubation (Table 6), whereas for rat bile the value was 0·37. In the extracts prepared from conjugated bilirubin obtained from human bile azobilirubin was much less important.

Table 5. *Partial solubilization of the microsomal fraction with deoxycholate*

In five experiments microsomes (final protein concn. 3·3–4·3mg./ml.) were treated with sodium deoxycholate (final concn. 2·36mg./ml.) and the M and P fractions prepared; UDP-glucuronyltransferase activity was determined in the washed M and P fractions and in the untreated microsomes. Incubation system II was used; the incubation temperature was 37°, except in Expt. 2, where it was 25°.

	Non-starved animals						Starved animals			
	Protein concn. in assay (mg./ml.)			Conjugation (μmoles/g. of protein/10min.)			Protein concn. in assay (mg./ml.)		Conjugation (μmoles/g. of protein/10min.)	
Expt. no.	1	2	3	1	2	3	4	5	4	5
Preparation										
Microsomal fraction	9·53	9·00	7·20	1·9	0·64	2·10	7·48	7·24	1·49	2·90
M pellet	2·31	2·30	1·57	18·8	5·5	20·7	1·48	1·66	14·9	22·9
P pellet	0·41	0·44	0·056	24·0	7·7	24·2	1·43	1·23	11·9	21·3

Table 6. *Separation of azo-pigments on CM-cellulose column*

Azo-pigment extracts were obtained from bilirubin and from conjugated bilirubin (CB) prepared from human post-operative fistula bile (CB–H) or from rat bile (CB–R) collected over a 15–30min. period after intravenous injection of bilirubin–serum. Extracts prepared after incubation of microsomal protein in system I (various protein carriers) are indicated by CB–I. A 1ml. sample of the extracts was diluted to 10ml. with pentan-2-one to assess colour recovery from columns; corresponding values of the extinction are given under the heading 'Reference'. Values are means ± S.D., with numbers of determinations in parentheses.

	$E_{530}^{4\,cm.}$			
		Azo-pigment fraction		
Source	Reference	A	G	$\frac{A}{A+G}\times 100$
Bilirubin	0·241	0·240 ± 0·002 (5)		100
CB–H	0·281 ± 0·017 (6)	0·025 ± 0·007 (6)	0·258 ± 0·008 (6)	9
CB–R				36·6 ± 3·5 (6)
CB–I				49·1 ± 2·9 (8)

Isolation of conjugated bilirubin after its production in vitro

The column-chromatographic technique of Billing (1955) was used. Glucuronyl transfer to bilirubin was allowed to proceed for 120–180min. in incubation system I, scaled up to a final volume of 1·5ml. [final concns.: microsomal protein, 12·5mg./ml.; human serum albumin, 9·6mg./ml.; saccharo-(1→4)-lactone, 0·7mM]. After incubation and chilling 6·25ml. of ice-cold ethanol and 0·45ml. of saturated ammonium sulphate solution were added. After gentle mixing for 2min. the suspensions were centrifuged. Each supernatant was concentrated to dryness in a rotary evaporator. The residue was taken up in 0·5ml. of the mobile phase described by Billing (1955) and 0·5ml. of a suspension of silicone-treated kieselguhr and placed on a chromatographic column (see below). Reference material was obtained from incubation systems lacking either bilirubin or UDP-glucuronic acid. For comparison parallel runs were done with UDP-glucuronic acid alone and with conjugated bilirubin.

Columns (diam. 0·9cm.) containing 1·5g. or 3g. of silicone-treated kieselguhr were used. Seven to ten 2ml. fractions were collected at 0° and analysed as soon as possible.

After enzymic incubation only one mobile yellow peak was observed, and this had an absorption spectrum ($\lambda_{max.}$ 450mμ) superimposable on that of pigment II prepared chromatographically from purified conjugated bilirubin. The typical absorption band ($\lambda_{max.}$ approx. 530mμ) was found on reaction with diazobenzene-*p*-sulphonic acid (in the presence of an accelerating mixture) or with the diazonium salt of ethyl anthranilate, but the enzymic incubation product was eluted later than pigment II.

When UDP-glucuronic acid was omitted from the incubation system the material eluted from the column had a very low E_{450} value (3–5% of that found with the complete system). As control runs

with UDP-glucuronic acid alone showed the presence of large amounts of material absorbing at 261 mμ in the fractions corresponding to the pigment peak determination of the ratio of total glucuronic acid to total bilirubin in the isolated enzymic reaction product was not attempted.

DISCUSSION

Conjugated bilirubin can be coupled selectively with the diazonium salt of ethyl anthranilate at pH 2·6 (Fig. 1), even in the presence of a large excess of unconjugated bilirubin (Fig. 4). The increased selectivity as compared with the classical diazo reagent diazobenzene-*p*-sulphonic acid seems to be related to the presence of a sterically hindering group in the *ortho* position relative to the diazo group (Table 1). In contrast with the *m*- and *p*-carboxylic acid derivatives of diazobenzene the *o*-carboxylic acid derivative couples extremely slowly with unconjugated bilirubin in aqueous medium, and the reaction is incomplete even with conjugated bilirubin after 30 min. at room temperature. A similar degree of selectivity is found with the diazonium salt of ethyl anthranilate, but with this reagent coupling with conjugated bilirubin is completed rapidly (Figs. 2 and 3). According to Fog & Jellum (1963) conjugation of bilirubin with glucuronic acid confers mobility to both dipyrryl moieties, allowing them to rotate about the central methene bridge. Bilirubin itself, on the other hand, is probably a rather rigid molecule as a result of intramolecular hydrogen bonding. If this hypothesis is correct a diazo reagent carrying a sterically hindering group in the *ortho* position would be expected to react more selectively with conjugated bilirubin than the corresponding *meta* and *para* derivatives.

Results obtained with pigment I and pigment II demonstrate that coupling with both pigment fractions is virtually complete under the reaction conditions allowed. The implications of this observation depend on the nature of pigment I. If it consists of bilirubin monoglucuronide(s) (Billing *et al.* 1957) then the observed behaviour would mean that the unconjugated dipyrryl moiety reacts completely after rupture of the central methene bridge. If pigment I is a 1:1 molecular complex formed between bilirubin and bilirubin diglucuronide (Weber, Schalm & Witmans, 1963; Gregory, 1963) then the bilirubin in the complex must be in an unusually reactive state. It is difficult to imagine such an activation process, as coupling with diazobenzene-*p*-sulphonic acid (Billing *et al.* 1957; Brodersen, 1960, 1962) and with the present diazo reagent obviously starts with the conjugated part of the molecule. As a consequence only a relatively small fraction of unconjugated bilirubin should be present in the form of a complex in the early stages of the coupling reaction. However, unconjugated bilirubin added in large excess to purified preparations of conjugated bilirubin in serum (Fig. 4) or to diluted human bile did not react with the present reagent, even after preincubation of the mixtures for 30 min. at 37° before colour development. These observations render activation of unconjugated bilirubin unlikely.

Though formation of molecular complexes between tetrapyrroles very probably can occur (Brodersen, 1966), perhaps by intermolecular hydrogen bonding (Fog & Jellum, 1963), the results reported in the present work are more easily interpreted in terms of a monoglucuronide structure for pigment I. Some of the discrepancies with respect to this matter (Schoenfield & Bollman, 1963) may perhaps be related to species differences (Heirwegh, Van Roy & De Groote, 1967).

As found by Schoenfield & Bollman (1963), bile pigments present in rat bile behave mainly as pigment II. If pigment II had arisen as an artifact by disruption of pigment I (Weber *et al.* 1963; Gregory, 1963) then equivalent amounts of unconjugated bilirubin should have been found at the top of the chromatographic columns. This was not the case. In contrast, azo-pigment analysis (Table 6) suggests a predominance of monoconjugated material. In the azo-pigment G fraction small amounts of azo-pigments (about 5% of the total) differing from the main component can be detected (Heirwegh *et al.* 1967). If these contaminants are neglected, estimates of about 73% monoglucuronide and 27% diglucuronide are obtained. Acid-lability of some of the glucuronic acid residues present in the conjugated bilirubin of rat bile may explain the contradictory azo-pigment patterns. When samples of rat bile were preincubated for 5 min. at pH 2·6 (the pH used for coupling with the diazo reagent), readjusted to pH 7·7 and chromatographed, only 57% of the total diazo-positive material now moved as pigment II; 26% of the bile pigments moved appreciably more slowly and 17% behaved as unconjugated bilirubin.

Transfer of glucuronic acid from UDP-glucuronic acid to bilirubin in the presence of various UDP-glucuronyltransferase preparations has been demonstrated (Schmid *et al.* 1957; Grodsky & Carbone, 1957; Lathe & Walker, 1958). According to the present results bilirubin monoglucuronide is formed by rat liver microsomes in incubation systems with bilirubin either bound to carrier protein (Table 6) or solubilized in ethylene glycol. β-Glururonidase activity was shown to be negligible in the systems used and could thus not be responsible for the production of monoconjugated bilirubin.

The low activity found when an aqueous solution of bilirubin was used as a substrate for microsomal

UDP-glucuronyltransferase can probably be explained by non-specific binding of bilirubin to microsomal material. Non-specific binding of an insoluble substrate can be prevented by using a carrier protein (Heymann & Fieser, 1948), and bilirubin can be largely removed by an amount of human serum albumin slightly greater than 1 mole/2 moles of bilirubin (Heirwegh, Van Roy & De Groote, 1964). For the study of UDP-glucuronyltransferase *in vitro* with bilirubin as the glucuronic acid acceptor systems have been developed with bovine albumin (Grodsky & Carbone, 1957) or human serum preparations (Lathe & Walker, 1958) as bilirubin carriers. Other serum albumin and serum preparation proved to be very efficient in this respect, especially after dialysis against 1mM-EDTA (Table 4).

Organic solvents are commonly used in work with enzyme systems that metabolize hydrophobic molecules such as steroids (Dutton, 1966). With microsomes and with derived fractions (M and P fractions described by Ernster *et al.* 1962) ethylene glycol solutions of bilirubin were at least as active in accepting glucuronic acid as protein-bound pigment (Table 3). The activity of UDP-glucuronyltransferase varied in a rather complex way with the concentration of the alcohol (Fig. 8). Contradictory observations on the effects of ethanol and propylene glycol on enzyme systems (Dutton, 1966) can possibly be explained by a similar behaviour. The pattern observed with ethylene glycol resembles that found with myosin adenosine triphosphatase (Kay & Brahms, 1963). These effects of solvent on UDP-glucuronyltransferase may well occur with other membrane-bound enzymes.

Effects of Mg^{2+} on UDP-glucuronyltransferase activity vary, depending on the source of enzyme and the acceptor substance used (Dutton, 1966). Transfer of glucuronic acid to *p*-nitrophenol seems to be inhibited by this ion (Storey, 1965; Tomlinson & Yaffe, 1966), whereas stimulation is apparent with *o*-aminophenol. In the present work a requirement for Mg^{2+} was noted with bilirubin (Table 4), as already observed by Tomlinson & Yaffe (1966). Activation by Mg^{2+} is still apparent with a purified enzyme preparation and ethylene glycol as the substrate solvent (Fig. 7).

Attempts to obtain soluble preparations of microsomal UDP-glucuronyltransferase by the use of deoxycholate have resulted in loss of activity (Leventer, Buchanan, Ross & Tapley, 1965; Tomlinson & Yaffe, 1966). In the present study roughly 300% of the enzyme activity and 30% of microsomal protein were recovered in the M plus the P fractions. At the relatively low deoxycholate/microsomal protein ratios used more than 100% recovery of several other microsomal enzymes has been observed by Ernster *et al.* (1962) and by Dallner (1963). Treatment of rat liver microsomes with ethylene glycol (Fig. 8) or with diethylnitrosamine (Greenwood & Stevenson, 1965) also leads to similar activation of UDP-glucuronyltransferase.

The reaction velocity (110mμmoles/10min./g. of liver) at saturating concentrations of bilirubin obtained with rat liver homogenates by Lathe & Walker (1958) is similar to the rates found in the present work with microsomes (Table 3), if the fact that a considerable fraction of the enzyme activity sediments with the mitochondrial fraction is taken into account. The maximum rate of excretion of conjugated bilirubin into rat bile was found to be about threefold higher (Lathe & Walker, 1958).

As already discussed, the evidence suggests that during incubation *in vitro* bilirubin monoglucuronide is produced. Even after prolonged incubation no bilirubin diglucuronide was formed. This suggests that in the formation of diglucuronide an additional mechanism may be involved.

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