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A Chromatographic Separation of Bilirubin Glucuronides from Human Bile

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A chromatographic procedure for separation of bile pigments from human bile is described. It results mainly in two fractions, bilirubin diglucuronide and a pigment with a ratio, bilirubin to glucuronic acid, equal to one. The two components are obtained free from other coloured products, but contain a considerable amount of impurities, about 50 %. A crystalline strychnine salt of diglucuronide has been prepared.

In 1956 three groups, Billing and Lathe,[1] Talafant,[2] and Schmid,[3] showed that the direct reacting bile pigment in serum and bile consists of bilirubin glucuronides. Methods for separation of these pigments are unsatisfactory and the substances have not been prepared in a pure state. Billing, Cole, and Lathe [4] introduced a reverse phase chromatography. They were able to separate bile pigments from serum into three fractions, unconjugated bilirubin, Pigment I, and Pigment II. The latter contains bilirubin diglucuronide and other conjugates, while Pigment I has a ratio, bilirubin to glucuronic acid, equal to one. The method has not led to a preparation of the compounds in a pure state. A method for preparation of a mixture of bile pigments from human bile has been worked out by Lucassen.[5] He used the different solubility of the acid and salt forms in water and acetone. Talafant [6] has used the same principle, and has also prepared a lead salt of the mixed pigments. An adsorbate of pigments from human bile to Hyflo in an ammonium sulfate solution has been prepared by Watson, Campbell and Lowry.[7]

EXPERIMENTAL

The bilirubin diglucuronide preparation according to Lucassen was the best starting material for further purification. This is prepared from fresh human fistula bile to which is added $Na_2S_2O_3$ to 1 %. pH is brought to 6.0 with 10 % oxalic acid. After centrifugation the precipitate is discarded. The pH is then lowered to about 3.5. After centrifugation once more the supernatant is discarded and the precipitate is washed with 0.01 % oxalic acid. Acetone is then added in the cold to dissolve the bilirubin glucuronide in the acid form. After filtration the pH is adjusted to 7.0. The brownish-yellow precipitate which

has a high content of bilirubin glucuronides is washed with acetone and ether and is dried in a vacuum desiccator.

Gel filtration. An alkylated, cross-linked dextran (Sephadex LH 20, Pharmacia Fine Chemicals, Sweden) was allowed to swell in a mixture of equal volumes of ethanol and water, and was then packed in a glass column (3 × 25 cm). Lucassen's preparation, dissolved in the same mixture, was applied at the top of the column and was eluted with the swelling solvent.

Estimation of bilirubin. The concentration of total bilirubin was determined with Nosslin and Michaelsson's [8] modification of the alkaline azo-coupling method of Jendrassik and Grof.[9]

Glucuronic acid was estimated by the sulfuric acid-carbazole method described by Dische.[10]

Thin layer chromatography of azo-coupling products was carried out with kieselgel as supporting medium, according to Tenhunen.[11] The solvent system used was that described by Schmid,[12] methyl ethyl ketone, propionic acid, water (15:5:6).

Spectrophotometry. Spectral curves were obtained in the range 350—650 nm with a Bausch and Lomb double beam spectrophotometer (Spectronic 505). The molar extinction coefficient for bilirubin in chloroform is 60 700 $cm^{-1}M^{-1}$ at 454 nm.[13] From this figure the molar extinction coefficient in ethanol-water (1:1) was determined. Solutions of bilirubin in chloroform and in ethanol-water (1:1) were both transformed into a mixture of chloroform-ethanol-water. To obtain the extinction coefficient for bilirubin diglucuronide in ethanol-water (1:1) the extinction was measured before and after hydrolysis in 0.1 M sodium hydroxide. For bilirubin diglucuronide the molar extinction coefficient was 59 000 $cm^{-1}M^{-1}$ at maximum, 454 nm. For the other fraction a similar figure was obtained.

RESULTS

In Fig. 1 is shown a typical elution profile from the gel filtration of Lucassen's bilirubin glucuronide preparation. The results are somewhat variable with the starting material and the age of the column.

The total bilirubin concentration and the concentration of glucuronic acid in the two main fractions are shown in Table 1. The fast fraction contains

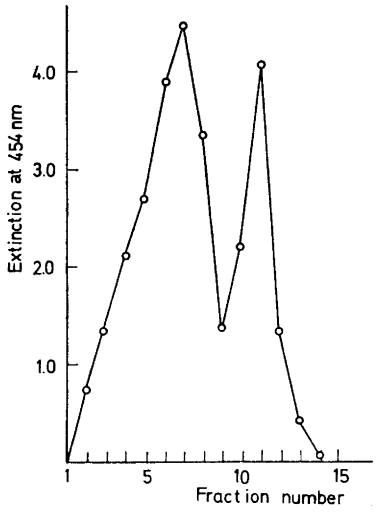

Fig. 1. Typical elution profile from the LH 20 Sephadex chromatography of bilirubin glucuronides prepared from bile according to Lucassen. Two mg dissolved in 2 ml applied. Fraction volume, 7—8 ml. Flow rate, 15 ml/h.

Table 1. Results from three representative preparations of the two fractions from the gel chromatography.

Preparation No.	Fraction	Total bilirubin μmole/l	Bilirubin measured by μmole/l	Glucuronic acid μmole/l	Ratio glucuronic acid/ bilirubin	Dry weight μg/ml	Pigment, per cent of dry weight
I	Fast	—	27	60	2.2	55	50
	Slow	—	19	20—25	1.2	—	—
II	Fast	46	46	87	1.9	100	46
	Slow	39	39	42	1.1	90	43
III	Fast	41	48	83	1.8	94	48
	Slow	34	44	40	0.9	90	43

about 2 moles of glucuronic acid per mole bilirubin, while the slow fraction has about equal molar quantities of the two components.

Both fractions show fast coupling with diazotized sulfanilic acid, even when the ethanol is evaporated. Thin layer chromatography of the azo-coupling product from the fast fraction gives, in the best preparations, only one band, corresponding to Azo-pigment B (Billing *et al.*[4]). The slow fraction shows normally two bands, corresponding to Azo-pigments A and B. Occasionally other bands are seen, especially one with a very low R_F-value. This latter is more pronounced when Lucassen's preparation was coupled and subjected to thin-layer chromatography without preliminary gel filtration. From these results it seems reasonable to assume that the fast fraction is identical with bilirubin diglucuronide, while the slow fraction has the composition of bilirubin monoglucuronide.

A crystalline strychnine salt was prepared from the bilirubin diglucuronide fraction, by adding strychnine hydrochloride to a concentrated (evaporated) aqueous solution of bilirubin diglucuronide. The crystals had an intense yellow colour. Because of the small amount available, recrystallisation and analysis were not possible.

Both fractions contain considerable quantities of uncoloured, azo-negative impurities (Table 1). A small amount of this may be formed by degradation of bile pigments during the procedure. The yield of pigments in the chromatography was, however, in most cases above 90 %. It therefore seems more reasonable that most of the impurities originates from ethanol soluble material in the Sephadex. By evaporating and weighing eluate from a thoroughly washed column it was shown that 1 ml contained 25—50 μg solid material.

DISCUSSION

The fast fraction is bilirubin diglucuronide, which contains no other coloured substances and also no other diazo-positive components. The slow fraction corresponds to bilirubin monoglucuronide. It has been much discussed, whether such a pigment exists, or the pigment with equal molar content of bilirubin and glucuronic acid prepared from bile and serum (the so called pigment I) is a complex of bilirubin and its diglucuronide.[4,14,16] The results in the present work indicate a higher molecular weight for the diglucuronide than for the other component, but this may not be true because the adsorption to the gel plays an essential role in the chromatography of these pigments.

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REFERENCES

1. Billing, B. H. and Lathe, G. H. *Biochem. J.* **63** (1956) 6P.
2. Talafant, E. *Nature* **178** (1956) 312.
3. Schmid, R. *Science* **124** (1956) 76.
4. Billing, B. H., Cole, P. G. and Lathe, G. H. *Biochem. J.* **65** (1957) 774.
5. Lucassen, J. Thesis, Utrecht University (1961).
6. Talafant, E. and Appelt, J. *Clin. Chem.* **14** (1968) 208.
7. Watson, C. J., Campbell, M. and Lowry, P. T. *Proc. Soc. Exptl. Biol. Med.* **98** (1958) 707.
8. Michaelsson, M., Nosslin, B. and Sjölin, S. *Pediatrics* **35** (1965) 925.
9. Jendrassik, L. and Grof, P. *Biochem. Z.* **297** (1938) 81.
10. Dische, Z. *J. Biol. Chem.* **167** (1947) 189.
11. Tenhunen, R. *Acta Chem. Scand.* **17** (1963) 2127.
12. Schmid, R. *J. Biol. Chem.* **229** (1957) 881.
13. Fog, J. *Scand. J. Clin. Lab. Invest.* **16** (1963) 49.
14. Weber, A. P., Schalm, L. and Witmans, S. J. *Acta Med. Scand.* **173** (1963) 19.
15. Gregory, C. H. *J. Lab. Clin. Med.* **61** (1963) 917.
16. Schoenfield, L. J. and Bollman, J. L. *Proc. Soc. Exptl. Biol. Med.* **34** (1963) 17.

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