Microheterogeneity of Complex Glycosides of Bilirubin from Human Bile

Previous investigations[1] have shown that bilirubin is excreted in the human bile as complex conjugates involving disaccharides. Aldobiouronic, hexuronosylhexuronic and pseudoaldobiouronic acids have been implicated as conjugating moieties (Figure 1). Evidence has been adduced by gas-liquid chromatography and mass spectrometry that the aldobiouronide type conjugate consists of three subspecies differing in the composition of the conjugating carbohydrates[1]. In an attempt to resolve these aldobiouronides into discrete entities, a chromatographic system was developed that allowed their preparative separation on polyamide sheets. In extending this method to the hexuronosylhexuronide and pseudoaldobiouronide conjugates, it was found that both also consisted of 3 subfractions.

Methods. Bilirubin conjugates were isolated from human T-tube bile as their phenylazo derivatives. Aldobiouronide (azo pigment B_4), hexuronosylhexuronide (azo pigment B_5) and pseudoaldobiouronide (azo pigment B_6) type conjugates were obtained by column chromatography using the three-step procedure described previously[1]. The preparations were freed from contaminating celite by dissolving in methanol-glacial acetic acid (3:1, v/v) and filtering through a column (2.7×9 cm) of polyamide[2]. The eluate was shaken with 1-butanol (2 vol.) and water (6 vol.). The red butanolic layer was collected, washed twice with water (4 vol.), and the pooled aqueous phases were back-extracted with 1-butanol (0.5 vol.). The combined butanolic extracts were evaporated to dryness by azeotropic distillation with water (1 vol.) using a rotary evaporator at 35 °C. The residue was dissolved in methanol, streaked on to polyamide sheets[3] (approx. 1 mg per plate) and twice developed to 15 cm with intermediate drying, using a solvent system prepared from 4-methyl-2-pentanone (methyl-*iso*-butyl-ketone)-1-butanol-acetonitrile-methanol-glacial acetic acid-water (8:2:1:1:1:1, by vol.). The separated fractions were scraped from the plates, eluted by stirring with glacial acetic acid and centrifuged at 1000 g_{max} for 5 min. The supernate was diluted with water (10 vol.) and extracted with 1-butanol (4 vol.). The butanolic layer was collected, washed twice with water (10 vol.), evaporated to dryness by azeotropic distillation with water and dried in a high vacuum.

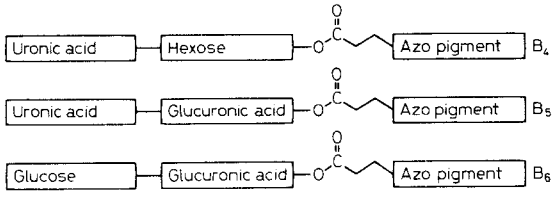

Fig. 1. Schematized structures of the complex glycosides of bilirubin isolated from human bile as their phenylazo derivatives. All compounds contain a disaccharide bound to the propionic acid side chain of azobilirubin. The non-reducing termini of the various disaccharides are at the left, and each reducing terminus is shown to be involved in an acyl glycoside linkage with the pigment moiety. In azo pigment B_4, the disaccharide is an aldobiouronic acid (uronic acid → neutral sugar); in azo pigment B_5, it is a hexuronosylhexuronic acid (uronic acid → uronic acid), and in azo pigment B_6, it is a pseudoaldobiouronic acid (neutral sugar → uronic acid).

Results. In using this system for the fractionation of the azo pigments B_4, B_5 and B_6, virtually identical chromatographic patterns were obtained. Apart from a small amount of unconjugated azo pigment originating from hydrolysis during processing, 3 azo pigment subfractions were visualized in all 3 instances (Figure 2). These were numbered in order of decreasing mobilities and were labelled azo pigments $B_{4.1}$, $B_{4.2}$ and $B_{4.3}$; $B_{5.1}$, $B_{5.2}$ and $B_{5.3}$; and $B_{6.1}$, $B_{6.2}$ and $B_{6.3}$, respectively. Gross estimation of the subfractions gave an approximate intragroup ratio of 5:60:35.

Discussion. In preparative work the system was not entirely satisfactory for two reasons. First, the azo pig-

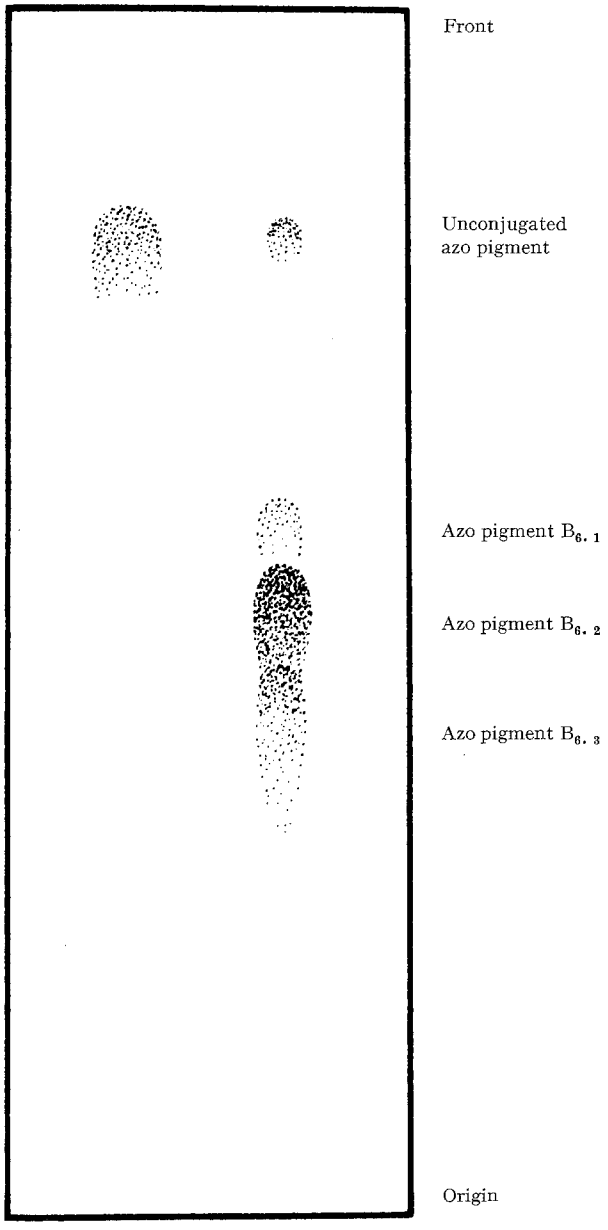

Fig. 2. Analytical thin-layer chromatogram on polyamide showing subfractionation of azo pigment B_6 (right lane). Unconjugated azo pigment (left lane) was run as a marker. Virtually identical chromatographic patterns were obtained with azo pigments B_4 and B_5.

[1] C. C. KUENZLE, Biochem. J. *119*, 387 (1970); Biochem. J. *119*, 395 (1970); Biochem. J. *119*, 411 (1970).

[2] M. WOELM, Eschwege, Germany.

[3] Polygram®, polyamide 11-UV_{254}, pre-coated sheets for thin-layer chromatography, 20×20 cm, thickness, 0.1 mm; Macherey-Nagel & Co, Düren, Germany.

ments of lowest mobility (azo pigments $B_{4\cdot3}$, $B_{5\cdot3}$ and $B_{6\cdot3}$) were incompletely separated from those of intermediate mobility (azo pigments $B_{4\cdot2}$, $B_{5\cdot2}$ and $B_{6\cdot2}$) and were therefore not obtained pure. Second, elution of the subfractions from the plates resulted in partially solubilizing the chromatographic support, thus contaminating the preparations with depolymerized material.

Summary. A thin-layer chromatographic system on polyamide was developed that allowed a further fractionation of previously isolated azo pigments from bile. Aldobiouronic, hexuronosylhexuronic and pseudoaldobiouronic acids involved in bilirubin conjugation thus appear to be heterogeneous. Structural elucidation is in progress[4].

Zusammenfassung. Die Aldobiouronid-, Hexuronosylhexuronid- und Pseudoaldobiouronidkonjugate des Bilirubins lassen sich durch Polyamid-Dünnschichtchromatographie in je 3 Subfraktionen unterteilen.

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Evidence of Major Role of the Intestine in Cholesterol Synthesis in the Adult Male Rat

It has recently been shown by means of indirect proof that the digestive tract is the source of 65% of the cholesterol synthesized per day in the rat[1]. Usually the liver has been considered responsible for producing most of the body cholesterol[1]. We felt that it was essential, in order to challenge this traditional theory, to provide a direct proof of the major role played by the intestine.

Material and methods. Our argument runs as follows: acetyl CoA is used by several pathways, some of which are irreversible, such as oxydation and cholesterol synthesis, whereas other pathways, such as the synthesis of fatty acids coupled with their breakdown, are reversible. If we label acetyl CoA at a starting point 0, no labelled acetyl CoA can be produced by breakdown of fatty acids, provided the experimental time is sufficiently short. From the radioactivity standpoint, therefore, reversible systems can be linked to irreversible systems. Hence the distribution of labelled acetyl CoA in each metabolic route where it is used depends on the rate of utilization alone. If m_S is the rate of cholesterol synthesis, m_U the sum of the rates of all types of acetyl CoA utilisation, R_S the radioactivity of the synthesized sterols and R_U the radioactivity of all compounds in all the metabolic routes involved, we obtain

$$\frac{m_S}{m_U} = \frac{R_S}{R_U}$$

The calculation of m_S is valid no matter what variations there may be in the specific radioactivity of the acetyl CoA or in the distribution of radioactivity amongst the various compounds within one and the same metabolic chain.

Employing this method, we used adult male rats weighing 350 g on a control feed[2]. Acetate-1-^{14}C was infused by a catheter placed into the jugular vein some days before[3]. Only rats having a normal food intake during the days preceeding the experiment were used. Specific radioactivity of the expiratory $^{14}CO_2$ was recorded continuously[4]. An equilibrium value was attained from the 3rd h of infusion onwards (Figure 1). 3 groups of 5 rats each were killed at the end of the following periods of infusion respectively: 3.5 h, 6 h, and 10 h. We then determined the radioactivity of free sterols (R_F) and esterified sterols (R_E) from 22 organs or fragments of tissue[5]. These crude radioactivities were corrected to a same specific equilibrium radioactivity of the animal's $^{14}CO_2$ (1 μCi/1% CO_2). From this it was possible to calculate the activities of the free and esterified sterols of the whole rat[2] and their sum (R_S) (Table I). Furthermore, R_U is the difference between the radioactivity of acetate administered and that of the unused labelled acetyl CoA. The latter value can be determined, since we know the pool of acetyl CoA (M) (see below), and its specific radioactivity calculated from that of the $^{14}CO_2$[6].

Finally, to determine the m_U value with precision, we injected 50–100 μCi of 1-^{14}C-acetate into the jugular vein of 8 additional rats and continuously recorded the logarithm of the specific radioactivity of the $^{14}CO_2$ (Figure 2). In a first approximation, the curves can be decomposed into 2 straight lines[6]. The first (steep slope) corresponds to the overall utilization of acetyl-CoA. From this line the fractional turnover rate of acetyl CoA (K = 1.37 h^{-1}) and the size of its pool (M = 3.08 mM) can be determined, and from this the rate of turnover

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[4] F. CHEVALLIER, M. BRIÈRE, F. SERELL and M. CORNU, J. Physiol., Paris *54*, 701 (1962).
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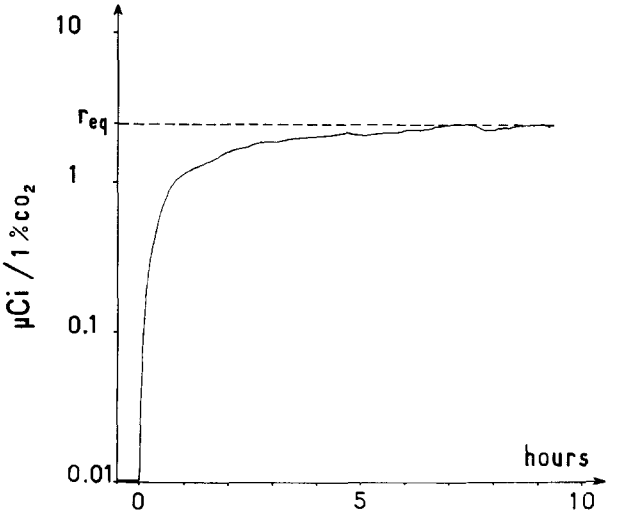

Fig. 1. Logarithm of specific activity (μCi/1% CO_2) of $^{14}CO_2$ expired by a rat as a function of time (h) during an i.v. infusion of 1-^{14}C-acetate. r_{EQ}: specific activity at equilibrium.