The Synthesis of Bile Acids in Perfused Rat Liver Subjected to Chronic Biliary Drainage

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(Received 9 March 1970)

1. Isolated rat liver was perfused with heparinized whole blood under physiological pressure resulting in the secretion of bile at about the rate observed *in vivo*. 2. The preparation remained metabolically active for 4 h and was apparently normal in function and microscopic appearance. 3. When the perfusate plasma and liver cholesterol pool was labelled by the introduction of [2-¹⁴C]mevalonic acid the specific radioactivity of the perfusate cholesterol increased. The biliary acids (cholic acid and chenodeoxycholic acid) were labelled and had the same specific radioactivity. 4. Livers removed from rats immediately after, and 40 h after, the start of total biliary drainage, were perfused; increased excretion rates of both cholic acid and chenodeoxycholic acid were found when the liver donors had been subjected to biliary drainage. 5. The incorporation of [2-¹⁴C]mevalonic acid or rat lipoprotein labelled with [¹⁴C]cholesterol into bile acids was studied. 6. A dissociation between the mass of bile acid excreted and the rate of incorporation of ¹⁴C was found. This was attributed to the changing specific radioactivity of the cholesterol pool acting as the immediate bile acid precursor.

Although the evidence for the conversion of cholesterol into bile acids in the rat is now well established (Siperstein & Chaikoff, 1952; Bergstrom & Norman, 1953; Danielsson & Tchen, 1967) the study of factors affecting bile acid synthesis *in vivo* is complicated by a number of interrelated phenomena. The bile acids present in rat bile consist predominantly of cholic acid (3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid) and chenodeoxycholic acid (3 α ,7 α -dihydroxy-5 β -cholan-24-oic acid), which are conjugated with taurine. About 95% of the bile acids secreted into the intestine is later reabsorbed; these acids are removed from the portal blood and re-excreted by the liver establishing an entero-hepatic circulation (Bergstrom, 1962). The remaining 5% is excreted in the faeces after degradation by the intestinal bacterial flora (Norman & Sjoval, 1958).

The bile acids present in the bile consist of both newly synthesized and reabsorbed compounds; assessment of the rates of biliary secretion, therefore, does not provide a precise method for measuring rates of bile acid synthesis. The rate of bile acid turnover and conversion of cholesterol into bile acids may be determined by techniques involving

study of the excretion of radioactive bile acids and their metabolites in faeces, (Lindstedt & Norman, 1956), or the rate of decay in specific radioactivities of bile acids in the intestine (Hellstrom & Lindstedt, 1964). These techniques provide only an indirect means of assessing the synthesis rates, and depend on rapid and complete mixing of the radioactive bile salts with the intestinal pool.

The formation of a complete biliary fistula in rats results in a decrease in the rate of excretion of total bile acids; about 24 h after the start of drainage the rate rises to values similar to those found during the first 3-4 h (Eriksson, 1957; Strand, 1962; Percy-Robb & Boyd, 1967). The increased rates of excretion that occur after 24 h of biliary drainage have been interpreted as indicating rising rates of bile acid synthesis from cholesterol (Eriksson, 1957).

Perfusion studies with isolated livers from nine rats have been reported in which both chenodeoxycholic acid and cholic acid have been excreted in bile and incorporation of radioactive cholesterol into both of these compounds has been demonstrated (Danielsson, Insul, Jordan & Strand, 1962). This technique therefore provides a unique method for the study of hepatic bile acid synthesis in isolation from the entero-hepatic circulation. However, the livers were anoxic for 4-6 min before the start of

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the perfusions and perfusion pressures used were at least twice the physiological levels. This study was essentially an investigation, to demonstrate the qualitative conversion of cholesterol into bile acids in perfused rat liver preparations. Kay & Entenman (1961) studied the production of biliary cholic acid in the perfused rat liver preparation, but the experimental design did not permit assessment of the rate of conversion of cholesterol into bile acids but merely the rate of biliary excretion and the assumption was made that this reflected the synthesis rate.

It was decided to perfuse rat livers under physiological conditions, and to monitor the viability of the preparation throughout the perfusion. By using this isolated perfused rat liver preparation the rates of excretion of both chenodeoxycholic acid and cholic acid have been studied. The rate at which livers from rats subjected to 40 h bile drainage utilize cholesterol synthesized from added DL-[2-¹⁴C]-mevalonic acid or from added [¹⁴C]cholesterol-containing lipoprotein has been compared with that of livers perfused immediately after bile duct cannulation.

METHODS

Analytical techniques. Biliary bile acid analysis was carried out by using a modification of the method of Reid & Boyd (1959) (also Boyd, Eastwood & MacLean, 1966). The modifications consisted of adjustment of the size of the apparatus to allow extraction and hydrolysis of small bile samples. The partition coefficient for cholic acid between the aqueous phase (pH 2–3) and the chloroform phase was shown to be 0.84 and the three stage extraction procedure gave a 99.9% extraction. Recovery values for added ¹⁴C-labelled bile acids were shown to vary between 80 and 85%. When portions of a single sample of bile were analysed on different days the method was found to have a coefficient of variation of 4.3% for cholic acid and 5.5% for chenodeoxycholic acid. Plasma and hepatic cholesterol contents were measured by the method of Abell, Levy, Brodie & Kendall (1952). In some experiments the liver was finely chopped and extracted with 95% (v/v) ethanol under reflux for 1 h. The extracted material was then applied to silicic acid columns (Hirsch & Ahrens, 1958) and radioactivity and cholesterol were measured in the eluates. Radioactivity of bile acid and cholesterol samples was measured in a Packard Tri-Carb model 314ex liquid-scintillation spectrometer. The scintillation system consisted of 2,5-diphenyloxazole (5 g/l) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene (0.3 g/l) dissolved in toluene. The toluene had been washed with sulphuric acid and with water to a final pH of 7 and dried over anhydrous CaCl₂ for 24 h. T.l.c. of bile acids was carried out on layers of silica gel G (E. Merck A.-G., Darmstadt, Germany), 0.25 mm thick, activated in a hot air oven at 110°C for 15 min. The chromatograms were developed with the systems of Eneroth (1963) and the bile acids were located by spraying the developed chromatogram with a 10% ethanolic

phosphomolybdic acid solution and heating at 80°C for 10 min (Kritchevsky & Kirk, 1952). Chromatograms of radioactive material were scanned with a scanner constructed after the design of Ravenhill & James (1967).

Bromsulphthalein concentration in bile was determined after the addition of 0.1 M-NaOH (Varley, 1958), by reading the final extinction at 580 nm. Plasma urea, Na⁺, K⁺ and total CO₂ concentrations were measured by using a multichannel Technicon AutoAnalyzer (Campbell & Annan, 1966). Blood glucose concentration was assayed by a glucose oxidase method (Marks, 1959). Perfusate pH values were measured by means of a micro electrode pH meter by using a capillary glass electrode (capacity approx. 0.1 ml). Perfusate samples were taken anaerobically from the reservoir at the lower end of the oxygenator and the pH-measurement was carried out within 30 min (Andersen & Engel, 1960). Haemoglobin oxygen saturation was measured by direct reflectometry by using samples equilibrated with pure oxygen as 100% saturation reference values. Blood haematocrit values were determined in capillary tubes in a microhaematocrit apparatus (Hawksley, Lancing, Sussex, U.K.). A standard 10 min centrifugation time was used. Perfusate haemolysis rates were measured by determining the plasma haemoglobin concentration and referring the value to the concentration found after 100% haemolysis by addition of water (Burton, St George & Ishida, 1960).

Samples of rat plasma containing [¹⁴C]cholesterol-bearing lipoproteins were prepared by centrifugation of rat blood taken by cardiac puncture approx. 12 h after intraperitoneal injection of 5 μ Ci of DL-[2-¹⁴C]mevalonic acid lactone. The [¹⁴C]cholesterol-bearing lipoproteins were added to the perfusate within 0.5 h of collection.

Liver perfusion technique. Livers were perfused by a method similar to that described by Miller, Bly, Watson & Bale (1951). Perfusate was circulated by a roller pump and oxygenated in a glass-walled vessel that maintained a thin perfusate film for gaseous exchange with a humidified, warmed (37°C) gas mixture (O₂ + CO₂, 95:5). The perfusate was filtered, before gaseous exchange, by passage through a fine stainless-steel screen (pore size 0.15 mm). Perfusion of the liver was maintained through the portal vein by means of a fine glass cannula, the perfusate being delivered directly after gaseous exchange. Perfusion pressure was maintained at 15 cm of perfusate (Kunkel & Eisenmenger, 1949). No cannulation of the hepatic vein was found to be necessary, the perfusate being allowed to leave the liver and return directly to a reservoir from which it was recirculated around the system. Bile was collected through a fine nylon cannula (Portland Plastics Ltd., Hythe, Kent, U.K.) tied into the common bile duct and carefully positioned to exclude any possibility of occluding the fine branches of the biliary tree. The liver was supported in a Perspex vessel machined to a shape similar to the rat diaphragm *in vivo*. The liver was perfused with its diaphragmatic surface downwards, the position of individual liver lobes being adjusted so as to avoid torsion of their hila. The whole of the perfusion apparatus was placed in a glass-fibre chamber and the temperature was maintained automatically at 37°C by a series of heavy-duty resistance wires round the inside of the chamber and connected through a rheostat. All of the glassware, apart from the gas-exchange vessel, was coated with silicone.

The perfusate consisted of pooled blood taken from

rats of the same strain as the liver donors. Blood donors were lightly anaesthetized with ether before being bled by clean cardiac puncture. Heparin was added to the perfusate at a concentration of 2000 units/50 ml. Liver donor animals were anaesthetized with ether and the method used for hepatectomy was similar to that described by Brauer, Pessotti & Pizzolato (1951). The portal vein was cannulated with a glass cannula previously filled with perfusate and connected to the perfusion apparatus by a long silicone rubber tube. Immediately after cannulation the flow of perfusate was started; after travelling through the liver the perfusate was allowed to escape through an incision in the inferior vena cava. In this way the flow of perfusate was maintained while the liver was being isolated, the period of circulatory arrest being on average about 30 s and never more than 90 s.

Experiments to assess the chemical composition of the perfusate. These experiments were designed to study changes that might occur in the perfusate as a consequence of the perfusion process. A rise in the plasma haemoglobin concentration indicated a mean haemolysis rate of 2%/h and was accompanied by a 2% increase in the perfusate haematocrit value. Perfusate Na^+ concentration rose from 147 to 154 mequiv./l and K^+ concentration rose from 6.1 to 12.3 mequiv./l. This change in K^+ concentration was due to haemolysis. Perfusate pH values fell from 7.37 to 7.29 and plasma total CO_2 content fell from 16.1 to 14.2 mequiv./l. Perfusate haemoglobin oxygen saturation fell from 97 to 91%.

General metabolic and histological status of the isolated perfused liver. The metabolic status of the isolated perfused rat liver has been examined in several studies by the measurement of perfusate and bile flow rates (Brauer *et al.* 1951; Ostashever, Gray & Graff, 1960; Burton *et al.* 1960; Danielsson *et al.* 1962), by the study of biochemical functions (Kay & Entenman, 1961) and by light microscopy (Brauer *et al.* 1951) and electron microscopy. The following results were obtained in the present study.

(a) Perfusate flow rates were between 1.1 and 2.4 ml/min per g wet wt. of liver after 15 min of perfusion; this rate fell by about 10% during the 4 h period.

(b) Bile flow rates were 0.33 g/h per 10 g wet wt. of liver in the first hour (15 perfusions) and fell to 0.25 g/h per 10 g wet wt. of liver in the fourth hour. Bile flow rates were measured *in vivo* for 48 h after the insertion of a polyethylene cannula into the common bile duct. The mean flow rate was 0.49 g/h in the fourth hour after cannulation and this fell to 0.29 g/h after 16 h. After 36 h a value of 0.49 g/h was reached.

(c) After the addition of 2.5 mg of bromsulphthalein to the perfusate, the biliary excretion of bromsulphthalein was studied. This was repeated after 40 min, 160 min and 280 min perfusion in each of three perfusions. The excretion pattern was similar after each injection, although the delay between the addition of the bromsulphthalein and the peak biliary excretion was somewhat longer after the second and third injections than after the first. The recoveries of bromsulphthalein were 84% after 40 min, 80% after 160 min and 84% after 280 min perfusion.

(d) Samples were taken directly into 0.38% aq. HClO_4 at intervals of 30 min to measure the concentration of glucose in the perfusate. Immediately after the insertion of the liver into the system there was a rise in the glucose

concentration; this was followed by a fall to values that were, on average, 12 mg/100 ml higher than the original concentrations.

(e) The concentration of urea in the perfusate rose throughout the perfusions, the mean hourly increments being 25, 26, 20 and 18 mg/100 ml respectively.

Pieces of several lobes were taken directly into formalin-0.9% NaCl (1:9, v/v) after the 4 h perfusion period and were then sectioned and stained with haematoxylin and eosin. These studies showed that the architecture of the liver was normal despite the 4 h perfusion experiment. Tissue was fixed for electron microscopy by perfusion with a 0.25% solution of glutaraldehyde in Ringer solution (9.0 g of NaCl, 0.4 g of KCl, 0.2 g of CaCl_2 and 0.2 g of NaHCO_3 in 1 litre of water) at a pressure of 15 cm of water; fixation of the tissue appeared to be almost instantaneous, and was followed by treatment with osmium tetroxide before the preparation of sections for electron microscopy. These studies again reinforced the light microscope studies in emphasizing the well-maintained cellular appearance of the tissue.

Animals. Blood and livers were taken from adult female Wistar rats of an inbred strain maintained in the Department of Biochemistry, University of Edinburgh. The body weights of the liver donors were 200–240 g.

RESULTS

Bile acid secretion. Livers from female rats were perfused for 4 h and bile collections were made at 1 h intervals. Hydrolysed bile samples were shown to contain Pettenkofer-positive material which could be separated into two well-defined fractions by column chromatography. The former had a chromatographic mobility similar to that of chenodeoxycholic acid on thin-layer chromatograms; the latter behaved in this respect as cholic acid. A rapid fall in the excretion rate of bile acids (Fig. 1) suggested that, in spite of other satisfactory indications of liver function, the liver might be failing during perfusion. It was also possible that the bile acid excretion in the first part of the perfusions might be the excretion of bile acids present in liver and bile at the start of the perfusion period and not a real indication of synthesis.

Metabolism of DL-[2- ^{14}C]mevalonic acid. The rate of conversion of hepatic cholesterol into bile acids can be followed by the addition of DL-[2- ^{14}C]mevalonic acid to the perfusate and the subsequent study of the conversion of the newly synthesized [^{14}C]cholesterol into ^{14}C -labelled bile acids. DL-[2- ^{14}C]Mevalonic acid (5 μCi) (The Radiochemical Centre, Amersham, Bucks., U.K., specific radioactivity 2–10 $\mu\text{Ci}/\text{mmol}$) was added to the perfusate in 0.1 ml of acetone at the start of the 4 h perfusion period (three perfusions). Samples of perfusate were taken at 1 h intervals for the determination of plasma cholesterol concentration and specific radioactivities. During these perfusions a small increase in plasma cholesterol concentration was found

Fig. 1. Biliary excretion of bile acids in isolated rat liver perfusions. ■, Dihydroxy (chenodeoxycholic) acid; □, trihydroxy (cholic) acid. Liver donors were normal rats.

Fig. 2. Specific radioactivity of perfusate plasma total cholesterol in isolated rat liver perfusions. DL-[2-¹⁴C]-Mevalonic acid (5 μ Ci) was added to the perfusate at the start of each perfusion (3).

Fig. 3. Extinction of Pettenkofer reaction (510nm) and recovery of radioactivity from individual fractions of liquid/liquid partition column of biliary bile acids from second hour of perfusion experiment. Each fraction was made up to a constant volume and a sample was removed for liquid-scintillation counting; the remainder was used for the Pettenkofer reaction. ----, Pettenkofer reaction; O, radioactivity.

[mean value 69.5mg/100ml (range 59.9–73.2) at the start of the perfusions, 78.5mg/100ml (range 71.6–82.3) at the end of the fourth hour]. The specific radioactivity of the plasma cholesterol (Fig. 2) rose throughout the 4h perfusion period. At the end of these experiments the whole of the perfusate and a major lobe of liver were taken.

The liver tissue was cut finely and both perfusate and liver were extracted with ethanol under reflux before silicic acid chromatography (see the Methods section). Liebermann-Burchardt-positive material was eluted in a fashion that corresponded to the elution pattern when a mixture of cholesterol ester and unesterified cholesterol was examined. Radioactivity eluted from the column corresponded exactly to the Liebermann-Burchardt-positive material; no additional radioactivity was detected.

Bile was collected in hourly samples from these perfusions. After hydrolysis and liquid/liquid partition chromatography (Fig. 3) of a sample from the second hour of a perfusion Pettenkofer-positive material was found in tubes 4–7 and 9–12. Radioactivity was recovered in the same tubes and the extinction of the Pettenkofer reaction corresponded with the recoveries of radioactivity. Material from each of these groups of tubes was run on thin-layer chromatograms and scanned for radioactivity

Fig. 4. Biliary excretion of bile acids in rat liver perfusions with addition of $5\mu\text{Ci}$ of DL-[2- ^{14}C]mevalonic acid to the perfusate (■, chenodeoxycholic acid; □, cholic acid). Incorporation of radioactivity (○) into total bile acids is shown.

before being sprayed with ethanolic phosphomolybdic acid. In each case there was a single, symmetrical peak of radioactivity corresponding to the mobility of a pure sample of bile acid. Material was taken from each group of tubes and added to pure chenodeoxycholic acid (tubes 4–7) and pure cholic acid (tubes 9–12); recrystallization to constant specific radioactivity was obtained without a significant decrease in the initial specific radioactivity. The bile acid excretion rates (Fig. 4) decreased in the same manner as in the first group of perfusions (Fig. 1). However, the total radioactivity incorporated into biliary bile acids did not fall in the same way.

Samples of bile from the same group of perfusions were extracted with boiling ethanol and the unhydrolysed extracts were run on thin-layer chromatograms with butanol–acetic acid–water (10:1:1, by vol.) as developing solvent system (Ganshirt, Koss & Morianz, 1960). The majority of the radioactivity was found to have a mobility corresponding to

Fig. 5. Incorporation of radioactivity into chenodeoxycholic acid (○) and cholic acid (Δ) excreted by isolated rat liver perfusion preparation. DL-[2- ^{14}C]mevalonic acid ($5\mu\text{Ci}$) was added to the perfusate at the start of each perfusion.

authentic taurocholic acid whereas smaller amounts corresponded to taurochenodeoxycholic acid and glycocholic acid. Radioactivity was incorporated into chenodeoxycholic acid and cholic acid (Fig. 5) in a ratio equal to the ratio of their masses, there being no evidence to suggest preferential incorporation at an early stage in the perfusions.

Metabolism of [^{14}C]cholesterol-bearing lipoproteins. The equilibration of [^{14}C]cholesterol-containing lipoproteins added to the perfusate with the liver cholesterol, and its subsequent conversion into bile acids (three perfusions), was studied. Perfusate plasma samples were taken at the start of the perfusion (30s after the addition of radioactive plasma to the perfusate) and thereafter at 1h intervals. The perfusate plasma cholesterol concentration rose from a mean of 68mg/100ml at the start of the perfusions to 86mg/100ml after 4h. The total radioactivity in the perfusate (Fig. 6) fell rapidly during the first hour and more slowly thereafter. Approx. 50% of the total radioactivity added had been removed from the perfusate after the 4h perfusion period.

Bile acid excretion rates (Fig. 7) again showed a decrease, for both cholic and chenodeoxycholic acids, at the end of the first hour but the rates were fairly well maintained thereafter. The dissociation between the radioactivity incorporated into bile acids and the mass of bile acids excreted was again evident in these experiments.

Fig. 6. Perfusate total cholesterol specific radioactivities after addition of $[^{14}\text{C}]$ cholesterol-bearing rat lipoprotein at the start of each perfusion.

Effects of biliary drainage on cholesterol and bile acids. (a) Perfusions with addition of DL-[2- ^{14}C]mevalonic acid. Livers taken from rats immediately after and 40 h after bile duct cannulation and biliary drainage were perfused (three livers in each group). Approx. 10 min after the start of each perfusion 4 μCi of DL-[2- ^{14}C]mevalonic acid was added to the perfusate reservoir and mixed by swirling. Samples of perfusate and bile were collected at 1 h intervals throughout the 4 h perfusion period. Both cholic acid and chenodeoxycholic acid excretion rates decreased when the perfusions were carried out immediately after bile duct cannulation, whereas the excretion rates for these primary bile acids were well maintained for 4 h if the livers were taken from rats that had been subjected to biliary drainage for 40 h. At least twice as much radioactivity was incorporated into total bile acids during each hour in the perfusion of livers removed 40 h after the onset of bile drainage (Fig. 8). This high rate of incorporation occurred both into chenodeoxycholic acid and into cholic acid throughout the period of perfusion (Fig. 9). The concentration of total cholesterol in the perfusate did not change during the perfusions (Fig. 10) and the appearance of radioactivity in perfusate plasma total cholesterol showed that the specific radioactivity rose in both groups of perfusions. The specific radioactivity of the liver cholesterol in the livers removed from animals immediately after the start of biliary drainage was less than 10 c.p.m./ μg after the first

Fig. 7. Biliary excretion of bile acids in rat liver perfusions with addition of $[^{14}\text{C}]$ cholesterol-bearing rat lipoprotein added at the start of each perfusion (■, chenodeoxycholic acid; □, cholic acid). Incorporation of radioactivity (○) into total bile acids is shown.

hour but greater than 20 c.p.m./ μg in two of the group following 40 h drainage.

(b) Perfusions with addition of $[^{14}\text{C}]$ cholesterol-bearing lipoprotein. In these perfusion experiments the $[^{14}\text{C}]$ cholesterol-bearing lipoproteins were added to the perfusate approx. 10 min after the start of each perfusion. Samples of perfusate and bile were collected at 1 h intervals. The patterns of excretion of cholic and chenodeoxycholic acids were similar to those described above. Cholesterol specific radioactivities in the perfusate decreased in both series of perfusions to about 50% of the starting values and there was no difference in the rate of fall (Fig. 11). The recoveries of radioactivity showed that both cholic acid and chenodeoxycholic acid became labelled within the first hour, there being a higher rate of conversion of added $[^{14}\text{C}]$ cholesterol both into $[^{14}\text{C}]$ cholic acid and into $[^{14}\text{C}]$ chenodeoxycholic acid in the perfusions of livers studied 40 h after bile duct cannulation (Fig. 12).

DISCUSSION

The experiments reported here indicate that the isolated perfused rat liver preparation, with whole heparinized rat blood as perfusate, is a suitable medium for study of the conversion of cholesterol into bile acids. The perfusate flow rates reported

Fig. 8. Biliary excretion of bile acids from perfused livers. Livers were removed from donors immediately after the formation of biliary fistulae (a) and 40 h after the formation of biliary fistulae (b). DL-[2-¹⁴C]Mevalonic acid was added to the perfusate at the start of the liver perfusion. ■, Chenodeoxycholic acid; □, cholic acid. Radioactivity (○) represents incorporation into total bile acids.

Fig. 9. Recoveries of radioactivity in chenodeoxycholic acid (○) and in cholic acid (Δ) secreted during perfusions of livers taken from rats immediately after (a) and 40 h after (b) formation of biliary fistulae. DL-[2-¹⁴C]Mevalonic acid was added to the perfusate at the start of each perfusion experiment.

here were similar to those described for whole blood by Burton *et al.* (1960) and our finding of well-maintained bile flow rates indicates that perfusion rates were adequate to support this function of the liver.

Bile flow rates fell during the 4 h perfusion by about 25%. Bile salts are known to have a powerful choleretic effect in the rat (Sperber, 1965). Bile salts are constantly being reabsorbed from the intestine *in vivo* and the liver is subjected to a

Fig. 10. Total cholesterol concentrations (a and b) and specific radioactivity (c and d) in plasma from whole blood perfusate of liver perfusions. DL-[2- 14 C]Mevalonic acid was added to the perfusate after the start of the liver perfusion. Livers were removed from rats immediately after (a and c) and 40 h after (b and d) formation of biliary fistulae.

continuous stimulus to bile secretion. However, in the isolated perfused liver preparation this stimulus is absent after the first hour of perfusion and hence the decrease in bile flow rates is not surprising. Bile flow rates were measured in fistula rats and the mean rate in the fourth hour was 0.49 g/h falling to 0.28 g/h after 16 h. The flow rates after 16 h biliary drainage are equivalent (in a physiological sense) to the isolated liver flow rates since the liver is secreting only small quantities of bile salts at that time. The mean bile flow rates in the isolated perfused preparation (0.24 g/h) and the

rate in the biliary fistula preparation (0.28 g/h) compare well. Ostashever *et al.* (1960) suggested that bile production may not be a critical index of viability of the perfused liver, since they could find no correlation between the metabolic activity of perfused livers from hyperthyroid rats and their bile flow rates. Danielsson *et al.* (1962) however, considered that bile production was a major criterion of viability of the perfused liver and stopped their perfusions when bile flow rate had fallen to 0.21 g/h.

In this study it was impossible to distinguish

Fig. 11. Specific radioactivity of total cholesterol in plasma from whole blood perfusate of isolated liver perfusions. [^{14}C]Cholesterol-bearing lipoproteins were added to the perfusate approx. 10 min after the start of each perfusion. Livers were removed from rats immediately after (a) and 40 h after (b) formation of biliary fistulae.

Fig. 12. Biliary excretion of bile acids in rat liver perfusions with addition of [^{14}C]cholesterol-bearing rat lipoprotein at the start of each perfusion (■, chenodeoxycholic acid; □, cholic acid). Incorporation of radioactivity (○) into total bile acids is shown. Livers were removed from rats immediately after (a) and 40 h after (b) formation of biliary fistulae.

histologically between sections from perfused livers and normal livers apart from the presence of occasional necrotic cells. The appearances with the electron microscope showed that the architecture of the cells was well preserved and the intracellular structure appeared normal.

The plasma cholesterol consists of a heterogeneous pool of lipoproteins containing cholesterol in equilibrium with cholesterol in many tissues including the liver (Avigan, Steinberg & Berman, 1962). The establishment of equilibrium between liver and plasma is very rapid *in vivo* (Popják &

Beeckmans, 1950; Hotta & Chaikoff, 1955). In the present experiments the plasma total cholesterol concentration rose slightly. The significance of this increase is complicated by the fact that there was evidence suggesting slight haemoconcentration during the perfusions. This rise may not represent a true net shift of cholesterol into the perfusate but is similar to that observed by Kay & Entenman (1961) and Roheim, Haft, Gidez, White & Eder (1963). The rise in plasma total cholesterol specific radioactivity following addition of DL-[2- 14 C]-mevalonate (Fig. 10) to the perfusate demonstrates the transfer of [14 C]cholesterol from the hepatic synthetic site to the plasma. The specific radioactivities found tended towards a plateau after 2 h (Fig. 11). It can be inferred from the rising cholesterol specific radioactivities and relatively constant concentration that a transfer of cholesterol into the liver occurred at the same time. This is confirmed by the decrease in specific radioactivity of the total cholesterol in the perfusate after the addition of [14 C]cholesterol as lipoprotein. The rate of fall in this case also supported the evidence for the rapid plasma/liver cholesterol equilibration *in vivo*.

The total cholesterol in the perfusate in these experiments was approximately 15 mg and was roughly equal to the total hepatic cholesterol. The disappearance from the perfusate of about 50% of the radioactivity added in the form of [14 C]cholesterol (Fig. 11) indicates that equilibrium between perfusate and liver was complete. The form in which cholesterol is added to the perfusate of an isolated liver preparation may affect the way in which equilibration occurs with the liver. Thus, after a 4 h perfusion, liver cholesterol (Danielsson *et al.* 1962) was found to have a higher specific radioactivity than plasma cholesterol when an emulsion of [14 C]cholesterol was added, whereas after addition of blood containing [14 C]cholesterol in the form of lipoprotein only 8% of the total radioactivity had been taken up by the liver and the average specific radioactivity of liver cholesterol was less than half that of plasma. The rapid uptake of the emulsified form was attributed to the preferential uptake of the emulsion by liver. Whether cholesterol taken up by the liver in the form of an emulsion or as lipoprotein is metabolized in the same fashion is not known, but the relationship of the uptake of these forms and cholesterol newly synthesized in the liver will affect the interpretation of data regarding bile acid synthesis.

Harold, Felts & Chaikoff (1955) studied the fate of [4- 14 C]cholesterol added to the perfusate of a rat liver perfusion preparation as a suspension in 0.9% sodium chloride and found that radioactivity was excreted mainly as taurochenodeoxycholic acid with smaller amounts of taurocholic acid and a third

more polar unidentified compound. In the perfusion experiments reported here radioactivity was incorporated into both taurochenodeoxycholic acid and taurocholic acid (Fig. 5), the specific radioactivities being approximately equal throughout. There was no evidence of incorporation of radioactivity into a more polar material. Chenodeoxycholic acid is not considered to be a precursor of cholic acid (Bergstrom & Sjoval, 1954) and the parallel incorporation of radioactivity in these perfusion experiments, as in experiments *in vivo*, indicates a common precursor cholesterol pool.

The high excretion rates (Figs. 4, 8a and 12a) of both bile acids found during the first hour rapidly fell to low, reasonably constant values for the remaining 3 h. A relatively constant rate of incorporation of [14 C]cholesterol-containing lipoprotein into bile acids (Fig. 12a) indicated that bile acid synthesis rates were not changing during the perfusions and that study of the mass of bile acids excreted did not give a true measure of synthesis rates even where the liver was studied in isolation from the entero-hepatic circulation. When [2- 14 C]-mevalonate was added to the perfusate, recoveries of radioactivity in bile acid showed a maximum in the second hour, followed by a steady fall.

These studies extend the observations of Kay & Entenman (1961) and Klevay & Hegsted (1968) and show that one effect of chronic biliary drainage in the rat is to increase the rate of synthesis of both chenodeoxycholic acid and cholic acid in the isolated liver preparation. When [2- 14 C]mevalonic acid was added to the perfusate, radioactivity appeared rapidly both in the cholesterol in the perfusate itself, and in the bile acids secreted by the preparation. The total recovery of radioactivity in bile acid was higher where livers were taken from donors subjected to 40 h biliary drainage; this increased rate of incorporation was seen both for cholic acid and for chenodeoxycholic acid. The specific radioactivities of both of these acids were equal throughout suggesting that the synthesis of these acids was from a common cholesterol precursor pool.

A similar excretion pattern was seen when [14 C]cholesterol was added to the perfusate as lipoprotein. Approx. 50% of the total amount of radioactivity added to the perfusate disappeared from it during the 4 h perfusion, and radioactivity appeared rapidly in bile acids secreted in bile. Again, the specific radioactivities of the bile acids were equal throughout each perfusion, suggesting a common precursor cholesterol pool.

The mechanisms controlling the rates of bile acid synthesis are not clear. The rate of cholesterol synthesis may increase in advance of the rise in bile acid synthesis rates (Myant & Eder, 1961). However, the present studies show that both newly

synthesized cholesterol (i.e. synthesized from [2-¹⁴C]mevalonic acid in these perfusions) and cholesterol added as lipoprotein act as precursors for the synthesis of bile acids and that, where bile acid synthesis rates are high, increased amounts of both these forms of cholesterol serve as precursors at an increased rate. There was no indication that the rate of transfer of lipoprotein cholesterol into the liver was higher when livers from rats with bile fistulae were perfused.

The present work shows that there is a dissociation between the rate of bile acid excretion and the recovery of radioactivity. Thus, whereas the mass excreted remained constant (Fig. 1) the radioactivity rose and then fell. The specific radioactivity of bile acids secreted at a point in time reflects the specific radioactivity of the precursor cholesterol and the way in which the specific radioactivities of the bile acids changed therefore points to similar changes in cholesterol specific radioactivity during synthesis from [2-¹⁴C]mevalonic acid. The subsequent fall in specific radioactivity is probably associated with distribution of this cholesterol both in the perfusate plasma and throughout hepatic cholesterol.

These studies have established that when rat livers were perfused under physiological conditions both chenodeoxycholic acid and cholic acid were secreted and that radioactivity was incorporated into both of these compounds after the addition of [¹⁴C]cholesterol-bearing lipoprotein or DL-[2-¹⁴C]-mevalonic acid to the perfusate. The majority of these radioactive bile acids were secreted in the form of taurine conjugates. The rates of incorporation of radioactivity from [¹⁴C]cholesterol-bearing lipoprotein confirmed that bile acid synthesis was taking place at a constant rate. The specific radioactivities of the bile acids were equal during the perfusion period suggesting that there was a common (precursor) cholesterol pool. The dissociation between the rate of incorporation of radioactivity in bile acids and their rate of excretion demonstrated that conclusions regarding synthesis rates could not be drawn from excretion rates. Incorporation of radioactivity into both cholic acid and chenodeoxycholic acid was shown to be increased after 48h biliary drainage *in vivo*. This was demonstrated after the addition of either [¹⁴C]-cholesterol-bearing lipoprotein or of DL-[2-¹⁴C]-mevalonic acid to the perfusate, showing that as a consequence of biliary drainage both newly synthesized cholesterol and plasma cholesterol were utilized as substrate for bile acid synthesis at increased rates.

This work was financed in part by a Medical Research Council Grant. I.W. P.-R. was supported by an Edinburgh University Faculty of Medicine Post-Graduate Scholarship.

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