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Studies on the mechanisms of biliary excretion of circulating glycoproteins

The carcinoembryonic antigen

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The transport of carcinoembryonic antigen from the circulation to the bile has been studied in the rat. Biliary excretion was directly proportional to the intravenously administered dose. Approximately 1–1.5% of the injected dose is excreted in the bile. Asialo-(carcinoembryonic antigen), asialo-fetuin and asialo-(α_1-acid glycoprotein) behaved similarly. The transit time through the liver for both native- and asialo-(carcinoembryonic antigen) was calculated as 47 min. Sialic acid content was not affected during biliary excretion. Glycoproteins that entered the liver cell lysosomes were not excreted in the bile. A mechanism by which circulating proteins may by-pass the hepatocytes before being excreted in bile is suggested. Alternative mechanisms for protein entry into bile are discussed.

The protein and glycoprotein components of bile have received little attention and their origin is still not understood. Studies of electrophoretic patterns of bile have shown that about 80% of the bile proteins are also found in serum and the remainder comprise the so-called 'bile specific' fraction (Dive, 1971; Dive & Heremans, 1974). Evans *et al.* (1976) and Kakis & Yousef (1978) have shown the presence of at least 14 protein components in rat using SDS/polyacrylamide-gel electrophoresis, and Mullock & Hinton (1978) showed again the presence of at least 14 protein components using crossed immunoelectrophoresis against anti-(rat) serum and anti-(rat liver) plasma membrane. The 'bile specific proteins' therefore may comprise, at least in part, membrane proteins which have been removed from the liver cells by the detergent action of the bile (Evans *et al.*, 1976; Mullock *et al.*, 1977).

Dive and his co-workers have investigated the origin of proteins in the bile in both man and the dog (Dive & Heremans, 1974; Dive *et al.*, 1974). They concluded that the majority of biliary proteins arise from the circulation and not by direct liver synthesis.

Abbreviations used: AcNeu, *N*-acetylneuraminic acid; butyl-PBD, 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole; CMP-[^{14}C]Sia, cytidine 5'-monophospho[^{14}C]-sialic acid; SDS, sodium dodecyl sulphate.

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The mechanism by which the proteins are transferred from the circulation into the bile is still not clearly understood. Various suggestions have been made including (*a*) the mistaken discharge of granules containing serum proteins into the bile by the hepatocytes, (*b*) the discharge of serum proteins taken up by lysosomes (see Mullock & Hinton, 1978), (*c*) simple diffusion (Dive, 1971) and (*d*) receptor mediated transport such as that proposed for IgA (Mullock & Hinton, 1979). It is significant, however, that although many of the serum proteins are found in the bile the pattern obtained by electrophoresis is substantially different from that found in serum (Hinton & Mullock, 1977; P. Thomas, unpublished work) suggesting a differential uptake of these proteins from the serum.

During studies on the metabolic fate of carcinoembryonic antigen with the perfused rat liver it was observed that about 10% of the administered dose appeared in the bile during the course of the perfusion (Thomas & Hems, 1975; Thomas *et al.*, 1976). Experiments *in vivo* have also shown that intravenously administered carcinoembryonic antigen or its asialo derivative appear in the bile of the rat in what seems to be an undegraded form (Thomas & Summers, 1978). A related glycoprotein, the so-called non-specific cross-reacting antigen, also behaves in this way (Thomas *et al.*, 1979). This paper therefore deals with the biliary excretion of carcinoembryonic antigen and its asialo form by the rat, in an attempt to ascertain the molecular properties necessary for biliary excretion

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of circulating glycoproteins and the mechanism of transference from blood to bile.

Materials and methods

Chemicals

Carcinoembryonic antigen was isolated from perchloric acid extracts of human colonic carcinoma metastatic to the liver by the method described by Krupey *et al.* (1972). Asialo-(carcinoembryonic antigen) was prepared by treatment of carcinoembryonic antigen with neuraminidase (*Vibrio cholerae*) (Westwood *et al.*, 1974). Fetuin was purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. and its asialo derivative prepared as for carcinoembryonic antigen. Human α_1-acid glycoprotein was prepared from plasma as described by Whitehead & Sammons (1966) and asialo-(α_1-acid glycoprotein) prepared as above. Radioiodinations of carcinoembryonic antigen and asialo-(carcinoembryonic antigen) were carried out by the chloramine-T method (Greenwood *et al.*, 1963) and α_1-acid glycoprotein, fetuin and their respective asialo derivatives were iodinated by using solid-supported lactoperoxidase (David & Reisfeld, 1974). Asialo-(carcinoembryonic antigen) with terminal [^{3}H]galactose was prepared by treatment of the glycoprotein with galactose oxidase followed by reduction with NaB^3H_4 as described by Westwood *et al.* (1974).

CMP-[^{14}C]Sia (ammonium salt; 216mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Other chemicals were of analytical reagent grade whenever possible.

Collection of bile

The bile ducts of male August rats (approx. 250g) were cannulated with 0.5mm bore Teflon tubing. Rats were anaesthetized throughout the duration of the experiments with 'Sagatal' (sodium pentabarbitone, May and Baker Ltd., Dagenham, Essex, U.K.) administered intraperitoneally. Bile was collected for periods up to 9h, and if not analysed immediately was stored at −20°C. The protein content of the bile samples was determined essentially by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. (See Evans *et al.*, 1976.)

Polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out as described by Evans *et al.* (1976).

Monosaccharide analysis

Monosaccharide analysis was carried out by the method described by Clamp *et al.* (1971), with a Perkin–Elmer F-30 gas chromatograph. (See Westwood *et al.*, 1974.)

Isolation of carcinoembryonic antigen from bile samples

To 2.5ml of rat bile containing approx. 50μg of carcinoembryonic antigen an equal volume of 2M-perchloric acid was added at 4°C. After standing for $\frac{1}{2}$h the precipitate was removed by centrifugation and the supernatant diluted to 20ml with 0.1M-sodium phosphate buffer, pH7.2, containing 0.9% NaCl. This solution was then applied to a column (5ml) of Sepharose 4B to which anti-(carcinoembryonic antigen) antibody had been bound as described by Westwood (1978). The column was washed with the phosphate buffer and carcinoembryonic antigen eluted with 2M-KCNS (Westwood, 1978). Samples of carcinoembryonic antigen were dissolved in rat bile and treated as above, as control materials for the glycoproteins that had passed into the bile from the circulation of the rat. These materials isolated from the bile had an immunoreactivity per mg of protein 60–70% of that of the original materials. Immunoreactivities were calculated by radioimmunoassay as described by Laurence *et al.* (1972). The samples were analysed for their carbohydrate content by the method of Clamp *et al.* (1971). (See Westwood *et al.*, 1974.) The results of the analyses are given in Table 1.

Table 1. *Carbohydrate contents of carcinoembryonic antigen isolated from rat bile after intravenous injection*
Specific activity is the radioimmunoassay value per mg of compound compared with the original injected material. Figures in parentheses are for the control samples (see the Materials and methods section).

	Carcinoembryonic antigen	Asialo-(carcinoembryonic antigen)
Sugar (mol %)		
Fucose	21.4 (21.0)	22.1 (22.0)
Mannose	12.1 (11.8)	12.4 (12.8)
Galactose	28.0 (29.2)	28.6 (30.2)
N-Acetylglucosamine	32.5 (31.9)	36.9 (35.0)
Sialic acid	6.0 (6.1)	— —
Specific activity (immunoreactivity)	0.63 (0.70)	(0.72) (0.69)

Preparation of [^{14}C]sialic acid-(carcinoembryonic antigen)

Sialyltransferase (EC 2.4.99.1) from rat liver was prepared and its activity determined with asialofetuin as acceptor as described by Hickman *et al.* (1970). CMP-[^{14}C]Sia (3μCi), sialyltransferase (150units) and asialo-(carcinoembryonic antigen) (4mg) in 0.05M-Tris/HCl buffer, pH7.5, containing 0.005M-$MgCl_2$ and 0.01M-EDTA (0.5ml), were incubated together at 37°C for 3h. The mixture was dialysed against 0.1M-phosphate buffer, pH7.4, containing 0.9% NaCl (two changes of 2 litres).

The dialysis residue was chromatographed on a column of Sephadex G-200 (1.6cm × 100cm) and the position of the [^{14}C]AcNeu-(carcinoembryonic antigen) determined by both radioimmunoassay and scintillation counting. Aqueous samples (0.05ml) were added to a scintillation fluid (10ml) comprising toluene (3.8ml), *p*-dioxane (3.8ml), methanol (2.3ml), butyl-PBD (0.07g) and naphthalene (0.8g). The active fractions were dialysed against water (five changes of 2 litres) and freeze-dried. The sample of carcinoembryonic antigen (2.8mg) retained 100% activity in the radioimmunoassay with a specific radioactivity (^{14}C) of 3.5 × 10^4d.p.m./mg. This gave a value of 3.8mol of [^{14}C]AcNeu per mol of carcinoembryonic antigen. Assays with the thiobarbituric acid method (Warren, 1959) showed a total sialic acid content of 4.5mol per mol. The original preparation of carcinoembryonic antigen contained 11mol of sialic acid per mol thus giving an incorporation of approx. 40%. This material contained approx. six terminal residues of galactose and thus behaved as asialo-(carcinoembryonic antigen), being taken up directly by the hepatocytes without prior Kupffer cell involvement (Thomas *et al.*, 1977).

Results

Protein content of rat bile

Bile was sampled every ½h for up to 5h after initial cannulation of the bile duct and was examined for its total protein content by the method described by Evans *et al.* (1976) and also by SDS/polyacrylamide-gel electrophoresis. As reported by Evans *et al.* (1976) and Kakis & Yousef (1978) little change in the electrophoretic pattern of the bile was seen over this time period with 13–15 bands visible on the gels. Total protein and protein concentration also did not show a great deal of variation and the results are expressed in Fig. 1(*a*,*b*).

Biliary excretion of intravenously administered glycoproteins

Fig. 2 shows the pattern of biliary excretion obtained after the intravenous injection of ^{125}I-labelled carcinoembryonic antigen. The liver of the rat still contains large amounts of labelled glycoproteins at the time of the second injection of the unlabelled glycoprotein (Thomas & Summers, 1978). This label is associated with secondary lysosomes (Thomas *et al.*, 1977). However, the injection of unlabelled material did not displace the labelled material from the liver so causing it to appear in the bile. Results from the converse experiment, where unlabelled carcinoembryonic antigen is first administered followed by the ^{125}I-labelled material, confirmed the conclusion that once carcinoembryonic antigen enters the hepatocyte and

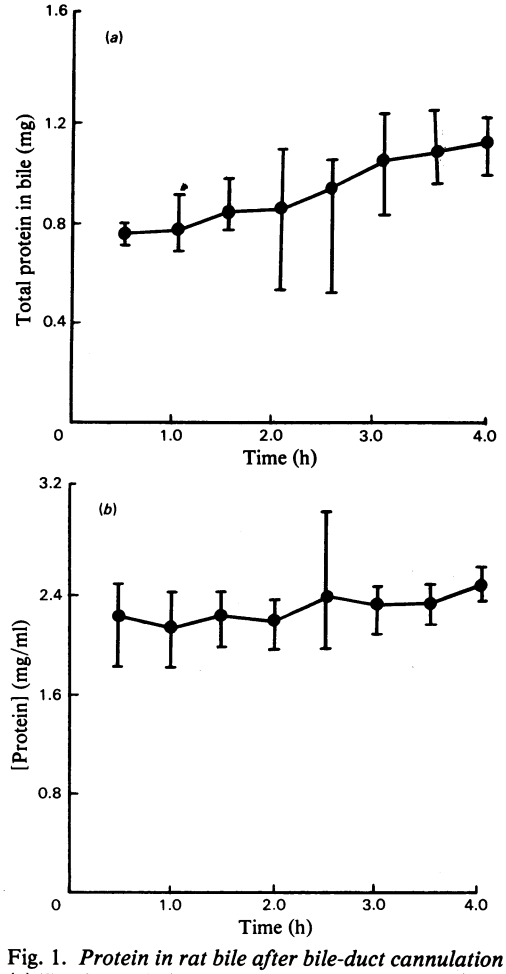

Fig. 1. *Protein in rat bile after bile-duct cannulation*
(*a*) Total protein in rat bile collected at 0.5h intervals as a function of time after bile-duct cannulation. Points are mean values for 20 rats with the ranges shown by error bars. (*b*) Concentration of protein in rat bile (mg/ml) from a 0.5h collection as a function of time after bile-duct cannulation. Points are mean values for 20 rats with the ranges shown by error bars.

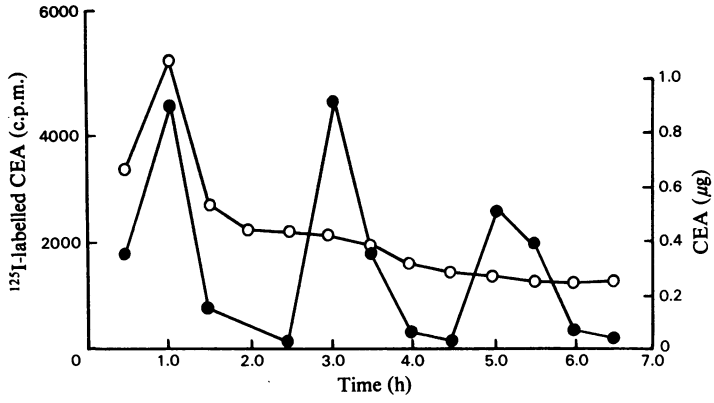

Fig. 2. *Biliary excretion of carcinoembryonic antigen (CEA)*
Time course showing the effect of subsequent injections of unlabelled glycoprotein after injection of the ^{125}I-labelled glycoprotein. O——O, ^{125}I-labelled glycoprotein (c.p.m.). ●——●, Amount of glycoprotein determined by radioimmunoassay. Injections were given at time 0, 2 and 4 h. 200 μg of carcinoembryonic antigen were given intravenously at each time period. The injection at time 0 contained 1 μg of ^{125}I-labelled carcinoembryonic antigen (1.25×10^6 d.p.m.).

is incorporated into the secondary lysosomes then it cannot be chased into the bile.

Effect of dose on the biliary excretion of carcinoembryonic antigen

The effect of dose on the biliary excretion of carcinoembryonic antigen is seen in Fig. 3(*a*) where the amount of glycoprotein in the bile increases in a fairly linear fashion with increasing dose. However, Fig. 3(*b*) expresses the same results as a percentage of the dose found in the bile against the dose administered. It can be seen that the percentage excretion remains relatively constant over the dose range. Transport of carcinoembryonic antigen into bile was not saturated at doses of up to 1 mg. Similar results were obtained when asialo-(carcinoembryonic antigen), asialo-fetuin and asialo-(α_1-acid glycoprotein) were administered. With all these glycoproteins biliary excretion was at a maximum within 1 h of the intravenous injection and these were virtually undetectable after 2 h. Between 1 and 2% of the dose was excreted into the bile. Grant *et al.* (1980) have found similar results for the excretion of enterokinase in the bile.

Determination of transit time for carcinoembryonic antigen

The transit time is taken to be the time between the injection of the glycoprotein and its peak concentration in the bile minus the estimated transit time across the biliary tree and through the catheter. Transit times were calculated as described by Daniel & Henderson (1970). Mean bile flow for the experiments described was 0.7 ml/h and the dead space for the biliary tree was taken as 0.005 ml/g of liver for the rat (Barber-Riley, 1963). These estimations along with a total catheter volume of 0.04 ml gave a transit time for carcinoembryonic antigen of 47 min when the peak concentration was found to occur at 54 mins after intravenous injection. A similar figure was calculated for asialo-(carcinoembryonic antigen). The transit time for both carcinoembryonic antigen and asialo-(carcinoembryonic antigen) is rapid and there is no evidence of a continued leakage into the bile.

Biliary excretion of [^{14}C]sialic acid-(carcinoembryonic antigen)

Owing to the inability to completely resialate the asialo-(carcinoembryonic antigen) this material was removed from the circulation by the mechanism established for asialo-(carcinoembryonic antigen), i.e. hepatocyte and not Kupffer cell uptake predominates (Thomas *et al.*, 1977). Fig. 4 shows the biliary excretion of the ^{14}C-labelled glycoprotein determined both by radioimmunoassay and scintillation counting for ^{14}C. These results showed that 0.97% of the injected material appeared in the bile as determined by scintillation counting for ^{14}C whereas a value of 1.29% was calculated from the radioimmunoassay results. Furthermore an identical time course was observed for both determinations, the specific activities (ratio of ^{14}C radioactivity scintillation counts to the radioimmunoassay value) at any time point being virtually identical. Thus it appears that at least 75% of the labelled sialic acid is still retained on the molecule during its passage from blood to bile. Little if any of the sialic acid is

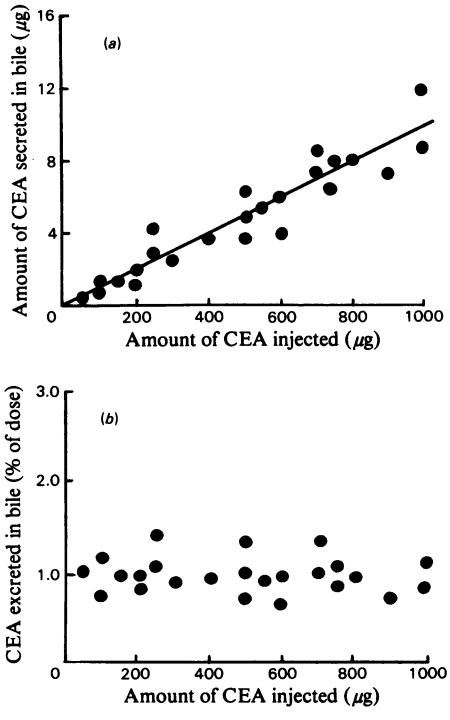

Fig. 3. *Carcinoembryonic antigen excreted in bile*
(*a*) Total amount of carcinoembryonic antigen excreted in bile over a 4 h period in comparison with the amount of glycoprotein injected. (*b*) Results as shown in (*a*) showing the percentage of the dose of carcinoembryonic antigen (CEA) excreted in bile compared with the injected dose. Carcinoembryonic antigen concentrations in bile were determined by radioimmunoassay.

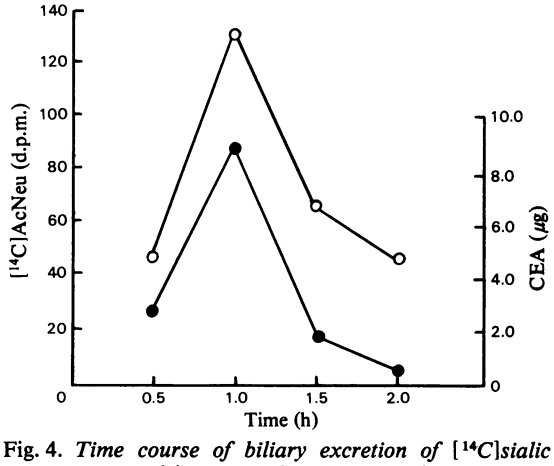

Fig. 4. *Time course of biliary excretion of* [^{14}*C*]*sialic acid-(carcinoembryonic antigen)*
O——O, ^{14}C radioactivity determined by scintillation counting, ●——●, Carcinoembryonic antigen (CEA) determined by radioimmunoassay.

removed in transit from the blood to the bile, supporting the previous findings that little degradation of carcinoembryonic antigen accompanies biliary excretion (Thomas & Summers, 1978). Extraction of the liver after the completion of these experiments and isolation of the ^{14}C-labelled carcinoembryonic antigen by immunoabsorbent chromatography resulted in a preparation which retained 63% of the ^{14}C-labelled sialic acid. When unlabelled carcinoembryonic antigen was injected, the rat killed 2 h later and the glycoprotein extracted and isolated as above, this material contained 58.8 nmol of sialic acid/mg as determined by the gas-chromatographic method of Clamp *et al.* (1971) compared with 72.0 nmol/mg for the original material. Thus in this case 80% of the sialic acid is retained.

Discussion

The results presented in this paper were obtained with the aim of clarifying the mechanism by which proteins are transported from the circulation to the bile. Of the proteins found in bile the majority are serum proteins. Dive *et al.* (1974) and more recently Thomas & Summers (1978) have shown that proteins injected intravenously into the rat will appear in the bile. The idea that proteins may appear in the bile due to the mistaken discharge of secretion granules at the bile canalicular-face rather than at the sinusoidal face of the hepatocyte would therefore not be the only mechanism for protein transport into bile. This would be particularly true for the immunoglobulins, although found in bile they are not synthesized by the liver. Another mechanism which could account for the presence of serum proteins in bile would involve the capture of asialo-glycoproteins by hepatocytes after binding to a galactose-recognizing receptor on the cell membrane as described by Ashwell & Morell (1974). These captured glycoproteins would then pass into secondary lysosomes where they would be digested or, as suggested by de Duve & Wattiaux (1966), they may be discharged into the bile canaliculi.

The results described here for the passage of carcinoembryonic antigen from the circulation to the bile in rats are not consistent with either of these mechanisms. After intravenous injection the peak concentration of both native- and asialo-(carcinoembryonic antigen) appeared in the bile at 54 min (transit time 47 min) and about 1 h later the amount present in bile was zero. At this time, however, (approx. 2 h after intravenous injection) about 70% of the dose injected is still present in the liver (see Thomas & Summers 1978) and can be seen to be associated with secondary lysosomes (Thomas *et al.*, 1977). Thus if discharge of lysosomes at the canalicular surface of the hepatocyte was the

mechanism involved in the transport of carcinoembryonic antigen in bile, the glycoprotein should be detectable in bile for at least 4–6 h and the peak activity would be expected much later than 1 h. Subsequent injections of carcinoembryonic antigen 2 and 4 h after the primary injection were found not to displace the glycoprotein already present in the liver (see Fig. 2). Injection of labelled glycoprotein 2 h after the injection of unlabelled glycoprotein showed that carcinoembryonic antigen appearing in the bile was of the same specific activity as the material injected and was not diluted by the carcinoembryonic antigen already present in the liver. Thomas & Jones (1978) have also shown that damage to lysosomes by Triton WR1339 does not adversely affect the biliary excretion of carcinoembryonic antigen.

Whether sialic acid performs any role in the transport of glycoproteins from the circulation to bile is still not clear but it seems unlikely that it does. The results reported here show some loss of [^{14}C]sialic acid when carcinoembryonic antigen is transported from blood to bile (about 25%). This loss may have occurred, however, after entry into the bile and furthermore the glycoprotein was not fully sialiated. Analysis by gas chromatography, however (see Table 1), showed little or no loss of sialic acid in the material isolated from bile and showed consistent analyses for the other sugars compared with the control samples. These results confirm the original observations (Thomas & Summers, 1978) that little, if any, degradation of the molecule has taken place. Similarly, the material retained in the liver lost relatively little sialic acid and from these experiments it cannot be deduced if this loss occurs during the initial uptake by the Kupffer cells or after entry into the hepatocytes. However, if sufficient sialic acid (i.e. 2 mol/mol of glycoprotein) was removed by the Kupffer cells it could be postulated that the subsequent transfer to the hepatocytes may be mediated by the galactose receptor (Ashwell & Morell, 1974).

It is likely, therefore, that the mechanism by which some proteins from the circulation enter the bile is similar to that proposed for much smaller molecules (Sternlieb, 1972). These plasma proteins would, therefore, by-pass the hepatocytes and pass through the fenestrated endothelia of the peribiliary capillaries and enter the periductular spaces where they would be transported across the epithelial cells either by a pinocytotic mechanism (Sternlieb, 1972) or by diffusion through the intercellular spaces, which are not endowed with tight junctions between the basal cytoplasmic projections. The experimental evidence seen in Fig. 2 that the amount of carcinoembryonic antigen present in bile is directly proportional to the amount injected is consistent with both these mechanisms.

Thus there are at least three mechanisms by which proteins may enter mammalian bile. (1) By the detergent action of bile salts on the membranes of the canalicular face of the liver cell. The enzyme 5′-nucleotidase (EC 3.1.3.5) probably appears in bile by this mechanism (Holdsworth & Coleman, 1975). (2) By a specific receptor-mediated transport across the hepatocyte with discharge into the bile at the hepatocyte canalicular surface. This mechanism appears to operate in the transport of polymeric IgA from the circulation into the bile (Jackson *et al.*, 1978; Orlans *et al.*, 1978; Fisher *et al.*, 1979). Mullock & Hinton (1979) have suggested that other glycoproteins may be transported by this mechanism. (3) By a non-specific mechanism either involving pinocytosis or diffusion as described in the present paper for carcinoembryonic antigen. This mechanism may also apply to other serum proteins such as α_1-acid glycoprotein and in particular albumin, where no carbohydrate-recognizing receptor could be involved.

Mechanisms other than the three outlined above may also operate and allow protein access from the circulation to the bile. Of the three mechanisms only mechanism (2) seems to have any obvious biological significance, the other two mechanisms being non-specific. However, it may be that both these mechanisms may make a significant contribution to the turnover of hepatocyte membrane proteins in the case of (1) and to the turnover of the serum proteins in the case of (3).

The quantitative differences in excretion between carcinoembryonic antigen and IgA [25% of injected IgA may be found in bile (Orlans *et al.*, 1978)] may be explained by the fact that little IgA appears in secondary lysosomes (Birbeck *et al.*, 1979; Mullock *et al.*, 1979). These results would suggest that lysosomal uptake prevents biliary excretion.

Experiments with Rhesus monkeys have indicated that the biliary excretion of carcinoembryonic antigen may be more important in primates than is seen here for the rat. Up to 9% of the injected dose was found in the gall bladder of the Rhesus monkey 1.5 h after intravenous injection. (P. Thomas, A. E. Rogers, J. G. Fox & N. Zamcheck, unpublished work). In man extrahepatic biliary duct obstruction (due, e.g., to gall stones) can result in plasma elevations of carcinoembryonic antigen. These elevations will return to normal on relief of the obstruction (Lurie *et al.*, 1975).

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