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EVALUATION OF HEPATOBILIARY FUNCTION IN THE RAT BY THE SEGMENTED RETROGRADE INTRABILIARY INJECTION TECHNIQUE

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Abstract—The mode of transfer of solutes across the biliary tree epithelium in the reabsorptive direction was studied by the segmented retrograde intrabiliary injection (SRII) technique. The principle of the SRII is to deliver an initial "segment" of solution containing a radioactive marker compound into the biliary system through the bile duct cannula and to wash this marker in with 0.9% saline solution in excess of the biliary capacity. Immediately following the SRII, bile flow is restarted and bile is collected to determine the quantity of the marker recovered. The marker compound which was recovered in bile represented that fraction of the solute which was trapped by the biliary epithelium (canaliculus), thus leading to recovery of the compound in bile with the peak concentration located at a position corresponding to the distended biliary tree capacity. With the compounds used, little re-excretion of the systemically absorbed fraction occurred. The filtration process was found to be dependent on the molecular weight of the marker compound, with larger compounds being recovered in bile (retained in the biliary tree) to a greater extent than smaller ones. The equivalent pore radius for this process appeared to be the same as that reported by others for biliary excretion in the orthograde direction. Additional factors such as the volume of the saline wash and the SRII rate were also shown to influence the retention of the marker compound in the biliary system. Another mode of transfer of solutes also took place in the SRII, because even for large molecular weight compounds over 50 per cent was lost from the biliary tree. There was no evidence of any process for excluding this fraction by the biliary tree epithelium. A further study of glucose transport in the biliary tree was suggested.

Even though biliary excretion is an important pathway for the excretion of many drugs and xenobiotics [1–3], the mechanisms involved in the transfer of compounds from liver to bile are poorly understood. For instance, filtration, secretion and reabsorption are well defined processes in the kidney, while relatively little is known about such processes which may exist in the liver. This lack of information, in part, arises from the lack of appropriate techniques to apply to the liver.

In a series of earlier papers, Forker *et al.* [4–7] reported that certain metabolically inert solutes, such as mannitol and erythritol, are excreted in bile by osmotic filtration produced by the osmotic effect of bile salts. Furthermore, Forker [6] suggested that the osmotic filtration which occurs at the canalicular membrane takes place across pores with equivalent radii of 5–10 Å. In this paper, we describe a method called the segmented retrograde intrabiliary injection (SRII) technique, which we believe can be applied to study several modes of transfer of drugs across the biliary tree epithelium in the reabsorptive direction. We will present evidence that, in the SRII technique, molecular sieving plays a part in the transfer process.

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MATERIALS AND METHODS

Chemicals. The various compounds administered by SRII were: [1-^{3}H]-D-mannitol (24 Ci/mmole), [*methyl*-^{3}H]-3-*O*-methyl-D-glucose (3.62 Ci/mmole), [1,2-^{3}H]polyethylene glycol (PEG, 0.7 mCi/g), [^{3}H]inulin (100 mCi/g), [^{125}I]bovine serum albumin (1.12 mCi/mg) (New England Nuclear Corp., Boston, Ma); [U-^{14}C]erythritol (18.1 mCi/mmole), [6,6'-(n)-^{3}H]sucrose (2.0 Ci/mmole), [^{3}H]water (5 mCi/ml), [^{3}H]dextran (33.6 mCi/g) (Amersham/Searle Corp., Arlington Heights, IL); and [U-^{14}C]-D-glucose (10.98 mCi/mmole) (ICN Pharmaceuticals, Inc., Irvine, CA). All compounds were administered in 0.9% saline.

Animal preparation. Male Sprague–Dawley rats (ARS Sprague–Dawley, Madison, WI), weighing 295–414 g, were anesthetized with pentobarbital sodium (45 mg/kg, i.p.). The femoral vein was cannulated with PE50 tubing and, following laparotomy, the common bile duct was cannulated with PE20 polyethylene tubing. The proximal end of the bile duct cannula, 10 cm long with a 27 gauge steel tube at the distal end, was positioned just below the bifurcation of the common bile duct near the liver hilus [8]. After surgery, a thermistor probe was inserted into the rectum and temperature was monitored with a Tele-Thermometer (Yellow Springs Instrument Co., Yellow Springs, OH). The rats were then placed near incandescent lamps to maintain body temperature at 37 ± 0.5°.

SRII technique. The principle of the SRII is to deliver an initial "segment" of solution containing a radioactive marker compound into the biliary system through the bile duct cannula and wash this marker in with excess 0.9% saline solution. Figure 1A shows a rat set up for an SRII. The bile duct cannula is connected to the infusion apparatus. The latter consists of a segment of polyethylene tubing (PE20) filled with 40 μl of the marker solution and attached to a syringe (5 ml) on a Harvard infusion pump (Harvard Apparatus Co., Dover, MA). Once the bile duct cannula is connected to the infusion apparatus, the infusion pump is turned on to deliver a 40-μl segment of solution containing the radioactive marker (5–10 μCi/ml 0.9% saline), followed by a wash in with 110 μl of 0.9% saline. Immediately following the SRII, the apparatus was detached from the bile duct cannula (taking less than 5 sec), and bile drops 1 to 10 and even-numbered drops 12 to 20 were collected serially in separate liquid scintillation vials containing 5 ml of scintillation medium. The radioactive content of each bile drop was expressed as the percentage of the total counts administered by SRII, and the data were plotted as a function of the cumulative volume of bile recollected (Fig. 1B). The area under the curve in Fig. 1B thus measures the percent recovery of the marker compound in bile. All animals received a total SRII volume of 150 μl (40 μl radiochemical + 110 μl saline) with the exception of one experiment, in which a group of animals received eight different SRII volumes. (A 10-min interval was allowed between each SRII procedure in a given animal.)

Analytical procedures. The radioactive content of bile samples for ^{3}H and ^{14}C was estimated by counting in a liquid scintillation spectrometer (model 3310, Packard Instruments, La Grange, IL), as described previously [9]. The scintillation medium consisted of 4 g of 2,5-diphenyloxazole (PPO) and 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) dissolved in 1.0 liter toluene and 0.5 liter Triton X-100. Bile samples containing ^{125}I were assayed in an automatic gamma counting system (model 1195, Searle Analytic Inc., Des Plaines, IL).

Statistical analysis. A one-way analysis of variance, followed by Dunnett's test [10], was used to evaluate the results in Fig. 4. Other results were evaluated by the Student's unpaired *t*-test [11]. For all statistical analysis, significance was set at $P < 0.05$.

RESULTS

The results shown in Fig. 1B serve as an example to illustrate the SRII approach. The marker, [^{3}H]mannitol, was given by SRII at a rate of 11.3 μl/sec. The mean ($N = 6$) content of [^{3}H]mannitol in each bile drop was expressed as a percentage of the total dose of radioactivity administered. The first few drops collected contained little [^{3}H]mannitol. The content then increased to a maximum in drop numbers 5 to 8 and fell off rapidly. Thus, a curve of distribution develops with the peak concentration of [^{3}H]mannitol corresponding to a recollected bile volume of about 45 μl. Since the total volume of the SRII was 150 μl, the first 40 μl of the SRII solution containing the [^{3}H]mannitol

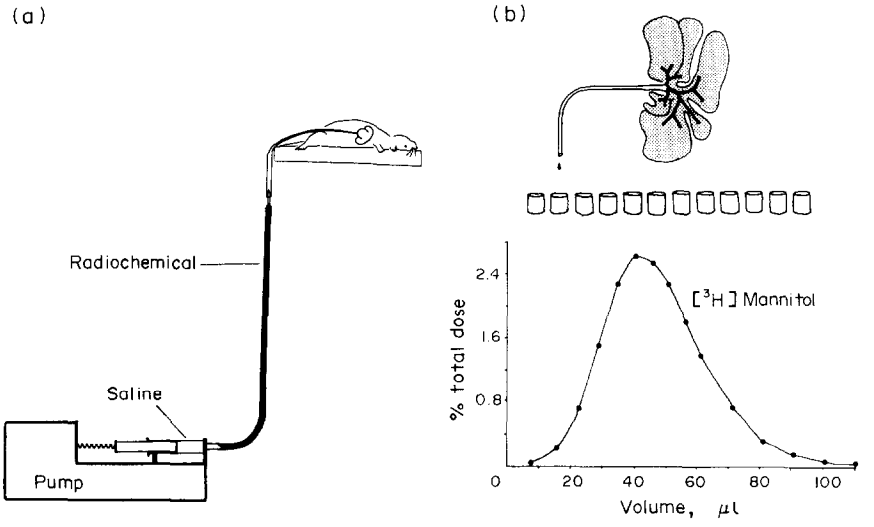

Fig. 1. Illustration of the segmented retrograde intrabiliary injection (SRII) method for the rat. Panel A: the injection apparatus consists of an infusion pump connected to tubing which is filled with 40 μl of solution containing a radioactive marker compound ([^{3}H]mannitol). The injection apparatus is shown attached to the bile duct cannula of the rat. An SRII is given by the infusion pump which delivers the 40 μl segment of radioactive solution and 110 μl of 0.9% saline wash into the bile duct of the rat. Panel B: within 5 sec following SRII, the injection apparatus is detached from the bile duct cannula and individual drops of bile are collected in scintillation vials. The illustration shows bile drops falling from the bile duct cannula of the rat liver. The content of [^{3}H]mannitol in each bile drop was expressed as the percentage of the total counts administered by SRII, and the data are shown plotted as a function of the cumulative volume of bile recollected. The resulting SRII recollection curve for [^{3}H]mannitol was the mean of six rats.

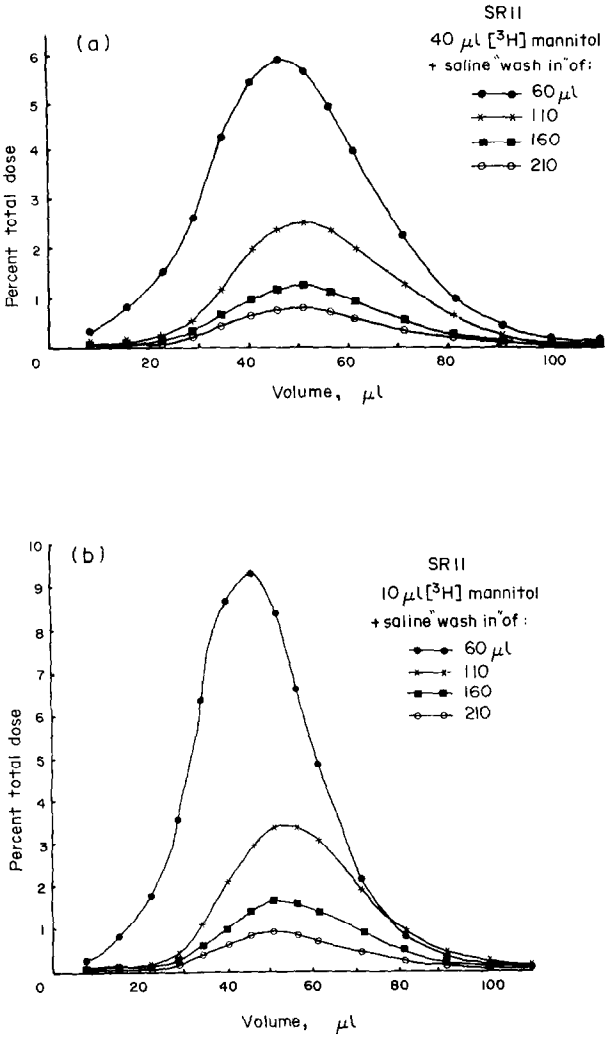

Fig. 2. Mean SRII bile recollection curves obtained through varying the saline wash in volume from 60 to 210 μl. In this experiment, each of six rats received eight different SRIIs at a rate of 11.3 μl/sec. In panel A, the initial segment of the SRII consisted of the standard 40-μl solution of [^{3}H]mannitol, while in panel B the segment contained 10 μl of [^{3}H]mannitol solution.

should have gone deep into the liver as a result of the 110 μl saline wash in; however, the peak content of mannitol in bile occurred at a cumulative volume of about 45 μl. Subtracting the bile duct cannula dead space of 11.3 μl from this value gave a volume of 34 μl which corresponded to the mean distended biliary tree capacity of the rat [12]. It is deduced from the results that mannitol recollected in bile must have been retained at the border lining the biliary tree (possibly including the canalicular membrane), and that mannitol which crossed this border entered the systemic circulation and was not re-excreted effectively into bile [13].

Another measurement was the area under the SRII recollection curve, a value which indicated the percentage of the administered dose of mannitol recovered in bile. In Fig. 1B, the area under the curve corresponded to a recovery in bile of 18.8 ± 2.2 per cent (mean ± S.E.) of the administered [^{3}H]mannitol. In other words, 18.8 per cent of the mannitol was retained in the biliary system, while 81.2 per cent of the administered dose escaped into the liver parenchymal tissue and the sinusoidal circulation.

Figure 2 shows what effect the volume of the SRII solution has on the per cent recovery of the radioactive marker compound (area under SRII curve) and the position of the peak of the SRII curve. In this experiment each of six rats received eight different SRIIs at a rate of 11.3 μl/sec. Figure 2 gives the mean SRII curves obtained through varying the saline wash in volume from 60 to 210 μl. In panel A, with the segment containing 40 μl of [^{3}H]mannitol solution, recoveries of 45.3 ± 2.1, 19.1 ± 2.4, 8.9 ± 1.3 and 5.5 ± 1.0 per cent (mean ± S.E.) were obtained with saline wash in volumes of 60, 110, 160 and 210 μl respectively. In panel B, the segment contained 10 μl of [^{3}H]mannitol solution, and recoveries of 60.2 ± 3.1, 25.7 ± 3.2, 12.4 ± 1.5 and 6.9 ± 0.9 per cent were obtained with saline wash in volumes of 60, 110, 160 and 210 μl respectively. At each wash in volume larger than 60 μl, the peak

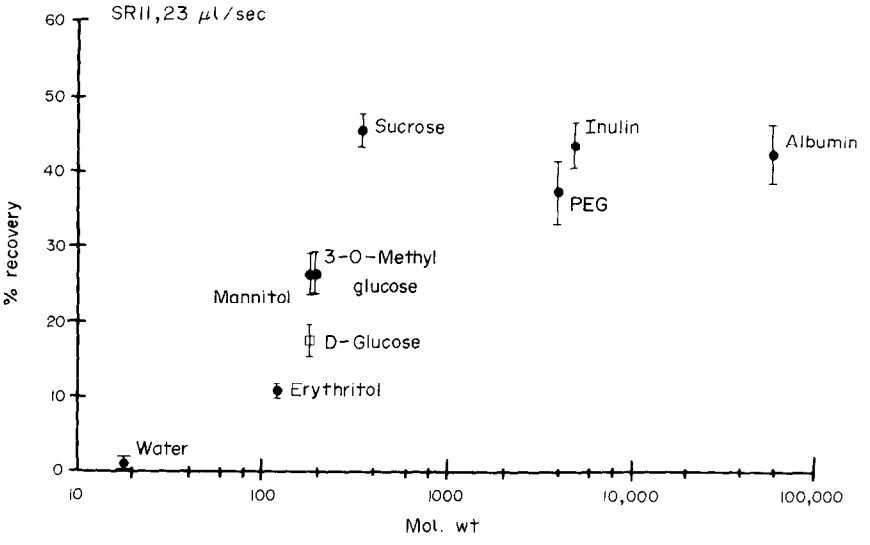

Fig. 3. Relation of molecular weight of the marker compound administered by SRII to its recovery in bile (area under SRII recollection curve). Each point on the graph represents the per cent recovery (mean ± S.E.) obtained from separate groups of at least six rats each. The standard SRII procedure consisting of 40 μl of a radioisotope solution followed by a 110 μl saline wash in was used in all cases.

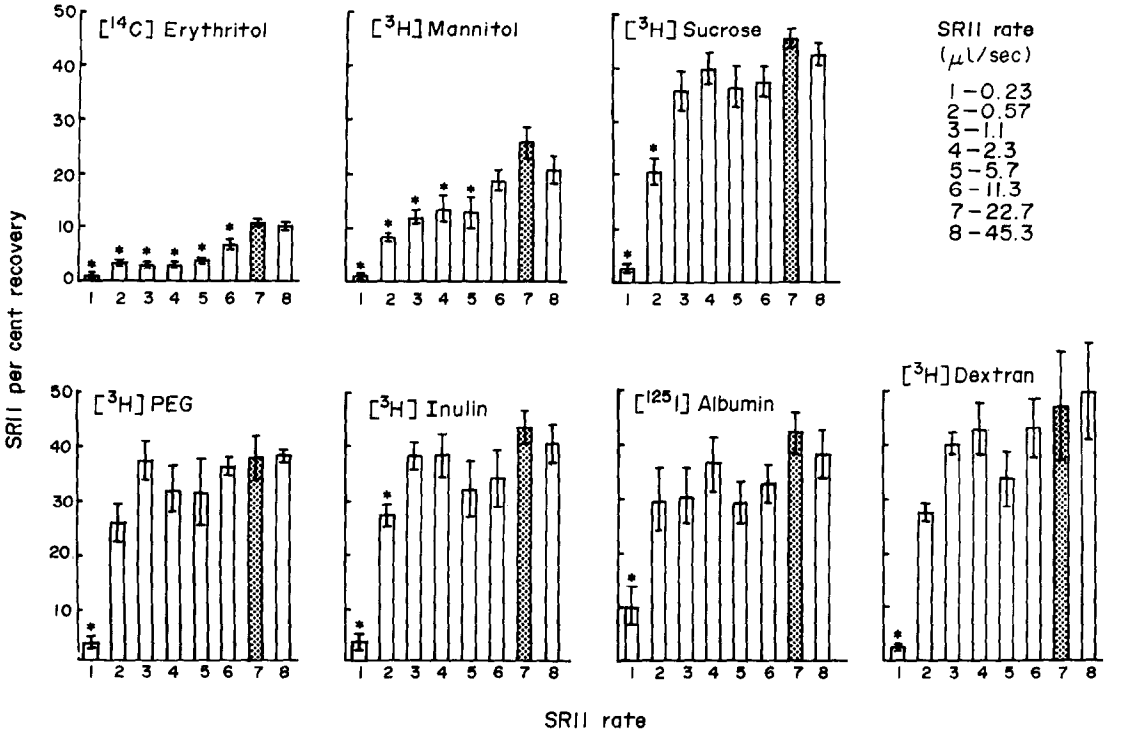

Fig. 4. Effect of SRII rate on the per cent recovery (area under SRII recollection curve) of seven different marker compounds. In each graph, one of the marker compounds was given to a group of at least four rats at the eight different SRII rates shown. The bars represent the mean ± S.E. per cent recovery of the marker in bile, following its administration by the standard SRII procedure (150 μl total volume). For each marker compound, the asterisk indicates those rates which gave significantly different recoveries from that obtained at an SRII rate of 22.7 μl/sec (stippled bars).

position of the SRII recollection curve was similar. Thus, increasing the saline wash in volume decreased the per cent recovery in bile or biliary retention of the marker compound without affecting peak position of the curves, although the recoveries in panel B did tend to be slightly larger than the corresponding values in panel A.

The relation of the molecular weight of the marker compound administered by SRII to its recovery in bile is shown in Fig. 3. Each point on the graph represents the area under the SRII curve, or the per cent recovery (mean ± S.E.) for different marker compounds, obtained from separate groups of at least six rats each. The standard SRII procedure consisting of an initial 40 μl containing the radioactive marker followed by 110 μl saline wash in was given at 11.3 μl/sec. [^{3}H]Water has a very low recovery in bile following SRII, indicating that most of the dose passed readily out of the biliary system, leaving very little remaining in the biliary tree. As the molecular weight of each compound increased, there was an increase in the per cent recovery in bile, indicating that more was retained in the biliary tree. Interestingly, the recovery values did not seem to increase for compounds larger than [^{3}H]sucrose. With the exception of [^{3}H]water, each of the marker compounds in Fig. 3 produced an SRII recollection curve having peak positions similar to the curves shown in Fig. 1 and 2. The results in Fig. 3 demonstrate the direct relation of per cent recovery to the molecular weight of the marker compound. [^{3}H]Mannitol and [^{3}H]-3-*O*-methyl glucose gave very similar per cent recoveries. A significantly lower per cent recovery was found for D-glucose, even though its molecular weight was in the same range as those of mannitol and 3-*O*-methyl glucose. This result indicates that D-glucose was taken up from the hepatobiliary system more rapidly than the other two marker compounds. A possible explanation for this finding and a resulting application for the SRII method will be presented in the Discussion.

The relation of per cent recovery of various marker compounds to the rate at which the SRII was given is shown in Fig. 4. The standard SRII procedure (150 μl total volume) was performed on seven groups of at least four rats each. Each rat in a given group received eight different SRIIs of the same radioactive marker compound, administered at infusion rates from 0.23 to 45.3 μl/sec. Within each group, the mean ± S.E. per cent recovery of the marker compound was shown at each of the eight different SRII rates. For each of the marker compounds, Dunnett's test was then used in each group to compare the per cent recovery obtained at a rate of 22.7 μl/sec with the recoveries measured at each of the other rates. An asterisk marks those rates in each group which gave significantly different recoveries from that obtained at a rate of 22.7 μl/sec. With this analysis it was evident that significantly lower recoveries were obtained for each marker compound at an SRII rate of 0.23 μl/sec. Additional changes in recovery at SRII rates from 0.57 to 45.3 μl/sec were obtained with [^{14}C]erythritol and [^{3}H]mannitol, while the remaining compounds showed little change in recovery values over this range of rates.

A final experiment was performed to assess whether damage might be produced by the SRII procedure. From the results given in Table 1, it was seen that the percentage of administered dose of sulfobromophthalein (BSP) recovered in the bile was the same before and after the SRII by either the paired or unpaired *t*-test. Furthermore, the shape of the BSP biliary excretion curve was the same before and after the SRII (data not given). We could obtain no evidence of damage, at least by this test.

Table 1. Biliary excretion of sulfobromophthalein in rats before and after five SRIIs with 0.9% sodium chloride solution*

Animal	% BSP recovered		Change
	Before SRII	After SRII	
1	88.9	86.6	−2.3
2	100.1	92.3	−7.8
3	92.4	91.2	−1.2
4	88.3	92.0	+3.7
5	85.8	93.8	+8.0
6	91.9	94.3	+2.4
Mean ± S.E.	91.2 ± 2.0	91.7 ± 1.1	

* Sulfobromophthalein (BSP) sodium (0.5 mg) was given into the femoral vein. With a Gilson fraction collector, 2 drops of bile were collected per tube up to 100 drops. Then, a series of five SRIIs of 150 μl each of 0.9% sodium chloride solution was given to each rat over a 2.5 hr period. Within 15 min after the fifth SRII, the BSP administration was repeated and the bile collected. Each tube contained 3.5 ml of alkaline buffer, and the optical densities were read at 580 nm on a Gilford spectrophotometer. The amount of BSP in each tube was calculated from the standard curve.

DISCUSSION

Previously the SRII procedure was presented as one of the three methods of estimating the capacity of the distended biliary tree of the rat [12]. There, sucrose was used as the marker compound. The idea was developed that, if part of the labeled sucrose was retained at the canalicular membrane and the volume of the saline wash exceeded the distended biliary tree volume, then the peak content of [^{3}H]sucrose in the bile recollection curve should mark the limit (canaliculus) of the biliary tree volume. Thus, in the SRII method, the [^{3}H]sucrose was administered in a 40 μl volume, followed by a saline wash in volume well in excess of the anticipated distended biliary tree capacity. The rationale for the use of sucrose in the SRII capacity determination was that sucrose should, in part, be retained at the canalicular membrane [6, 14]. Therefore, with this limited excretion of sucrose in bile, it was not surprising that a similar restriction was imposed for the passage of sucrose from bile to sinusoidal blood in the SRII procedure. In the present experiment with [^{3}H]mannitol as the marker compound, the SRII recollection curve in Fig. 1B gave a peak point at about 45 μl. When the bile duct cannula dead space of 11.3 μl was subtracted from the 45 μl, a volume

of about 34 μl was obtained. This value corresponded to our previous estimates of the distended biliary tree capacity by the sucrose SRII method [12]. As we studied the SRII procedure more, it became evident that it could be used for other purposes. One of the findings in the present work was that the biliary tree, and by inference the canalicular membrane, appeared to partially retain mannitol and restrict its absorption. This latter conclusion is based on the finding that the mannitol content in the recollected bile always peaked at what we considered the distended biliary tree capacity, for all saline wash in volumes in excess of 60 μl (Fig. 2). The bile duct cannula dead space plus the distended biliary tree volume was about 50 μl (Fig. 2, peaks); thus the wash in volume of 60 μl was not large enough to push the mannitol to its limiting filtration site within the biliary tree. Even though wash in volumes of 110 μl or more of saline did not affect the location of the peak, the area under the curve, i.e. the per cent recovery, fell when the volume of saline was increased from 110 to 210 μl. This per cent recovery represented in our thinking the portion of the marker compound which was held at the site where the filtration was occurring, and the decrease in recovery represented the decrease in the amount of mannitol retained at this site as the wash in volume was increased.

If filtration were the process responsible for the retention of mannitol, there should be a relation between the molecular weight of the marker compound and the degree of retention or molecular sieving which occurred. Such a relation was suggested by the results in Fig. 3. The recovery in bile (retention) increased as the molecular weight of the marker compound increased. However, this statement requires further clarification. There was a portion of the solution which appeared to pass into the liver by hydraulic flow without filtration. This fraction is exemplified in Fig. 3 by the solutes with molecular weights above sucrose. Since a plateau was reached in recovery (near 50 per cent) and the increase in molecular weight of the solutes did not increase the recovery further, it was evident that this fraction which was not recovered in bile was lost from the biliary tree through large pores (or through a process such as pinocytosis) which did not exclude the solutes. As indicated earlier, this fraction was not trapped in the liver or biliary tree since that portion of the solute which was not recovered in bile was recovered quickly and quantitatively in the perfusate of the *in situ* isolated perfused rat liver [13]. On the other hand, those portions that were recovered in bile depended on the molecular weight of the solute.

In the SRII system, the following description for transfer of water and solutes crossing from the biliary tree into the liver seems appropriate:

$$\text{Water flow} = \text{hydraulic flow} + \text{osmotic flow}$$
$$\text{Solute flow} = \text{ultrafiltration} + \text{diffusion}.$$

These equations are from House [15]. During an SRII, loss of water from the biliary tree was most likely due to hydraulic flow generated by the SRII; net osmotic flow was probably nil. Solute was ultrafiltered by the hydraulic water flow and diffusion of solute would probably be relatively small during the SRII. If hydraulic flow with ultrafiltration were the main driving force for the solute transfer, this transfer should be restricted by molecular sieving. Forker developed the idea that molecular sieving of the solute occurred through 5–10 Å pores in the canalicular membrane. Even though flow through the canalicular membrane would be in a retrograde direction in our SRII experiments, the relation between the per cent recovery in bile and the molecular weight of the solute suggested to us that, in the SRII, molecular sieving occurred through the same size pores as designated by Forker [6]. That is, the change in per cent recovery was possibly an indication of the reflection coefficient [15] for the solutes. The critical change in reflection coefficient occurred in the molecular weight range between erythritol and sucrose, the same range as involved in Forker's experiment. Our results in Fig. 4 also support this concept. At the higher rates (1.1 to 45.3 μl/sec) of SRII of sucrose, polyethylene glycol, inulin, albumin and dextran, the per cent recovery was not sensitive to increased rates of infusion. Over this same range of rate of infusion, the per cent recoveries of erythritol and mannitol were dependent on the rate of infusion. This dependency, we feel, is analogous to the situation where Forker took the mean rate of change of clearance which occurred with change in bile flow and equated it to the coefficient of molecular sieving. Put in another way, we would expect that the occurrence of molecular sieving would be detected as a change in per cent recovery when the infusion rate was changed. In support of this argument, we have taken the results at infusion rates faster than 1.1 μl/sec. At slower infusion rates, we believe that hydraulic flow was slow so that diffusion of the solute (rather than solvent drag) appeared to become a significant factor. This belief was based on our previous finding [16] that solutes diffused from the biliary tree into the liver when left in contact with the biliary tree for several minutes under conditions where net hydraulic fluid flow was zero (by occlusion of the bile duct).

Finally, we wish to point out an important observation which relates to glucose transport. In Fig. 3 the per cent recoveries of [^{3}H]mannitol and [^{3}H]-3-*O*-methyl glucose were found to be similar. However, [^{14}C]-D-glucose which had a molecular weight near that of mannitol and 3-*O*-methyl glucose gave a recovery which was significantly less than that of the latter two compounds. These data indicate that D-glucose was taken up from the biliary tree more rapidly than the other two compounds. This observation served as the basis of a subsequent study [17] in which a transport system for glucose was demonstrated. Also, a preliminary study on the use of the SRII technique to demonstrate an amino acid uptake system in the biliary tree was reported [18].

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