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Biopharmaceutical Study of the Hepato-biliary Transport of Drugs. IV.[1] Development of the Method to Investigate the Process of the Active Secretion of Drugs from the Hepatocytes into the Bile Canaliculi and Its Application to the Biliary Excretion of Organic Anions in Rat

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A new method to investigate the active excretory process of drugs from the hepatocytes into the bile canaliculi has been developed. This method is, briefly speaking, to retrogradely infuse several reagents through a tubing which is cannulated into the common bile duct and the bile-canalicular membrane of the hepatocytes is thought to be mainly solubilized or modified by infusing surfactants, organic solvents, proteases, lipase, metabolic inhibitors and thiol reagents, because both polyacrylamide gel electrophoretical patterns and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoretical patterns of the bile which was obtained following intrabiliary retrograde infusion of Triton X-100 and SDS display the existance of more proteins than that of normal bile. Moreover, the active excretion of organic anionic dyes, bromphenol blue and uranine, from the hepatocytes into bile was almost inhibited by infusing surfactants retrogradely. The active excretory system is thought to have the following characteristics: it could be solubilized by surfactants and organic solvents, ethanol–ether (3: 1) and acetone and it is affected by phospholipase D or thiol reagents ,*p*-chloromercuribenzene sulfonic acid and *N*-ethylmaleimide. A bile-canalicular membrane protein to which the three organic anionic dyes, bromphenol blue, uranine and eosine, are bound has been detected in the Triton X-100 solubilized membrane fraction by polyacrylamide gel electrophoresis and the molecular weight is estimated to be about 80000 by SDS polyacrylamide gel electrophoresis.

In our previous reports,[1,3–5] the basic principles for the hepato-biliary transport of organic anions were investigated using many organic anionic dyes which could be classified into the two categories according to their hepato-biliary transport characteristics, namely:

(i) bile level>liver level>plasma level

(ii) bile level>plasma level≧liver level

Moreover it became clear that the liver cytoplasmic Y and Z proteins have significant roles not only for the hepatic uptake of organic anions but also for their intracellular transport. However, the important problem whether these cytoplasmic binding proteins participate in the process of active excretion of organic anions from the hepatocytes into the bile canaliculi has not been solved. This problem could not be solved easily, unless the following two points are satisfied; a compound which exists in the liver cytoplasm as a free form, namely not bound to the cytoplasmic binding proteins, is discovered and a new method to specifically investigate the excretory process from the hepatocytes into the bile canaliculi is developed. The second point appears to be very difficult, because there detects no report which deals with the excretory process of either endogenous compounds or exogenous compounds from the hepatocytes into

1) K. Takada, O. Narumiya, and S. Muranishi, *Chem. Pharm. Bull.* (Tokyo), 23, 729 (1975).
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3) *a*) K. Takada, Y. Mizobuchi, and S. Muranishi, *Chem. Pharm. Bull.* (Tokyo), 22, 922 (1974); *b*) K. Takada, M. Ueda, M. Ohno, and S. Muranishi, *ibid.*, 22, 1477 (1974).
4) K. Takada, S. Muranishi, and H. Sezaki, *J. Pharmacokinet. Biopharm.*, 2, 495 (1974).
5) K. Takada, H. Nakae, and S. Muranishi, *Proc. Symp. on Drug Metabolism and Action*, 5, 127 (1973).

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the bile canaliculi. Therefore it was attempted to develope a new method by which investigators can study only the excretory process from the hepatocytes into the bile canaliculi. The intrabiliary retrograde infusion method which has been recently developed and has been employed by several investigators to study the reabsorption of iodipamide,[6] phenolphthalein glucuronide,[7] and other compounds[8] from the biliary trees attracted our attention and was applied to investigate the active excretory process of organic anions from the hepatocytes into the bile canaliculi. At first, an attempt to solubilize the active excretory system by the intrabiliary retrograde infusion of surfactants, Triton X-100 and sodium dodecyl sulfate (SDS), which are known as a membrane solubilizer[9] was performed and thereafter the characterization of the system was carried out.

Experimental

Materials——Triton X-100 was obtained from Wako Pure Chemical Co., Ltd. β-Glucuronidase (bovine liver) with a potency of 3000 Fishman units/mg, protein molecular weight markers and digestive enzymes were obtained from Sigma Chemical Co., Ltd. Other reagents were analytical grade products from Nakarai Chemicals Co., Ltd.

Hepato-biliary Transport Experiment of Uranine in Whole Rat——The animal experimental method is precisely described in the previous report.[3a]

Analytical Method: Plasma Level: A 1 ml of rat plasma was adequately diluted with 0.02 M phosphate buffer, pH 7.4, and its fluorescence intensity was measured at an excitation wavelength of 490 nm and an emission wavelength of 510 nm, setting the intensity of fluorescence standard solution to 100 unit. As the fluorescence intensity of the metabolite, uranine glucuronide, is extremely little as compared with that of uranine, this fluorescence intensity depends upon the concentration of uranine itself. Additionally, to a 0.1 ml of plasma, 10 ml of 1N NaOH was added and the resulting mixture was incubated for 30 min in a water bath for hydrolysis of uranine glucuronide. After cooling, the fluorescence intensity was measured with the same method as mentioned above. As the fluorescence intensity depends upon both uranine and its glucuronide, the concentration of the glucuronide was estimated by subtraction.

Liver Level: To 5 ml of 50% liver homogenate, 5 ml of acetone–methanol (1: 1) mixture was added and the resulting mixture was centrifuged for 20 min at 15000 rpm after mechanical shaking for 15 min. Then the supernate was diluted with 0.02M phosphate buffer, pH 7.4, and the fluorescence intensity was measured according to the method described in plasma level.

Bile Level: A 0.05 ml of bile sample was diluted adequately with 0.02M phosphate buffer, pH 7.4, and the fluorescence intensity of uranine was measured. Additionally, to 0.05 ml of bile sample, 5 ml of 1N NaOH was added and the resulting mixture was heated for 30 min in a water bath. After cooling, the fluorescence intensity was measured and the concentration of uranine glucuronide was estimated from this increase of fluorescence intensity.

Determination of the Metabolic Product of Uranine——Thin-layer Chromatography (TLC): Samples of bile were spotted on Silicagel Spotfilm® and developed ascendingly with *n*-butanol–glacial acetic acid–water (4: 1: 1).

Paper Chromatography: Samples of bile were applied to sheets of Toyo filter paper and the chromatograms were developed ascendingly with the solvent system, pyridine–isoamyl alcohol–water (7: 7: 6).

Paper Electrophoresis: Samples of bile were applied to the Whatman chromatograph paper (0.3 mm thick) and electrophoresis was carried out in a Mitsumi multi-purpose electrophoresis chamber. A barbital buffer, pH 8.6, and a current of 3 mA for 5 hours were used. In the three experiments, there detected two yellow spots respectively after electrophoresis and chromatographies.

Uptake of Uranine by the Liver Slices——Mainly according to the method of Schanker, *et al.*,[10] the experiment was carried out. Liver slices, each weighing 5 g, were suspended in 10 ml of Krebs–Ringer phosphate buffered solution, pH 7.4, containing 1 g glucose/liter and 0.01 mM concentration of uranine. As an inhibitory experiment, bromphenol blue (BPB), finally 0.05 mM, was added to the incubation mixture. The mixture was shaken in a metabolic shaker at 37° in an atmosphere of oxygen. At 30 min after incubation, liver slices and 0.5 ml of the incubation medium were removed and the uranine contents were measured spec-

6) J.A. Nelson, L.Z. Benet, and H.I. Goldberg, *J. Pharm. Pharmacol.*, 24, 995 (1972).
7) J.H. Gustafson and L.Z. Benet, *J. Pharm. Pharmacol.*, 26, 937 (1974).
8) *a)* A.G. Clark, P.C. Hirom, P. Millburn, and R.L. Smith, *J. Pharm. Pharmacol.*, 23, 152 (1971); *b)* G. Czok and H.G. Dammann, *J. Pharm. Pharmacol.*, 24, 821 (1972); *c)* R.E. Peterson and J.M. Fujimoto, *J. Pharmacol. Exptl. Therap.*, **185**, 150 (1973).
9) R. Coleman, *Biochem. Biophys. Acta*, **300**, 1 (1973).
10) P.L. Gigon, P.K. Nayak, and L.S. Schanker, *Proc. Soc. Exptl. Biol. Med.*, 132, 1103 (1969).

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trofluorometrically. The analytical method for the uranine contents in the liver slices is the same described in liver level analytical method.

DEAE-Cellulose Ion-Exchange Chromatography of the Liver 100000 *g* Supernate——At 10 min after uranine, 10 μmole/300 g body weight, was administered intravenously to rats, the liver which had been perfused with 0.25M sucrose in 0.02M Tris–HCl buffered solution, pH 7.4, to remove the entrapped blood was removed and was homogenized to a 25% homogenate with the same medium infused in a Teflon-glass motor driven homogenizer. The homogenate was centrifuged at 4° in a Hitachi 55P ultracentrifuge at 100000*g* (av.) for 120 min. The supernatant fraction was separated from the pellet and surface lipids. A 20 ml of the 100000*g* supernate was then applied onto a diethylaminoethyl (DEAE)-cellulose column (2 × 10 cm) equilibrated with 0.02M Tris–HCl–0.01M NaCl, pH 7.4. The column was washed with about 800 ml of the buffer, a linear gradient of 0.01M—2.0M NaCl in 0.02M Tris–HCl, pH 7.4, was applied and the eluate was collected (10 ml/tube). The flow rate was 100 ml/hr. The following elution patterns were monitored using a Hitachi spectrophotometer: protein at 280 nm, nucleic acid at 260 nm, uranine and uranine glucuronide at 450 nm and using a Hitachi spectrofluorometer: uranine at an excitation wavelength of 490 nm and an emission one at 510 nm. The experimental method of the gel–chromatography using columns of Sephadex G-75 and G-25 were precisely described in the previous report.[3b)]

Intrabiliary Retrograde Infusion Experiment——Male Wistar rats, 230—280 g, were anesthetized by an intraperitoneal injection of urethan, 0.9 g/kg. After laparotomy, the common bile duct was cannulated with a polyethylene tubing (0.45 mm in the inside diameter and 0.75 mm in the outside diameter) of which length was 5 cm. The bile duct cannula was positioned in the proximal third of the common bile duct so as to prevent the contamination of bile with pancreatic juice. To this tubing, a joint tubing (0.75 mm i.d.) of which length was 2 cm was attached as shown in Fig. 1. To retrogradely infuse reagent solution, at first, the infusion tubing (0.45 mm i.d., 0.75 mm o.d.) which was attached to a syringe filled with air was filled with a reagent solution by sucking through the syringe before connected with joint tubing. In this case, the length of the infusion tubing was previously calibrated so that the infusion tubing can contain 50 μl aliquot of the reagent solution. The interface between the in fused reagent solution and the air indicates the end of the solution. Then the infusion tubing was connected with previously cannulated tubing through a joint tubing and the reagent solution was infused for 30 seconds by pushing the handle of the syringe. After that, the handle was held on and the infusion was ceased after 10 min. After the infusion tubing was pulled off from the joint tubing, a bile-collecting tubing (0.45 mm i.d., 0.75 mm o.d.) of which length was 10 cm was connected with the cannulated tubing through the joint tubing and the test compound was administered intravenously to rat, 10 μmole/300 g body weight. After injection, bile samples were collected for three 10 min periods into tared bottles and the volume was considered to be equivalent to the weight of the bile. At 30 min after the bile collection was started, a blood sample was taken *via* the aorta into a heparinized syringe and the liver was removed immediately. The organic anionic dye contents in these samples were measured according to the analytical method described above or somewhere else.[3a,b)] Used reagents were SDS, 2 mM and 50 mM; Triton X-100, 4%; pronase, 1%; trypsin, 0.25%; papain, 4%; phospholipase D, 1%; dinitrophenol (DNP), 5 mM; ouabain, 10 mM; *p*-chloromercuribenzene sulfonic acid (PCMBS), 50 mM; N-ethylmaleimide (NEM), 25 mM. These reagents were dissolved in 0.13M NaCl in 20 mM Tris–HCl buffered solution, pH 7.4. As a sham operation experiment, a 50 μl aliquot of the above isotonic buffered solution was infused. As organic solvents, ethanol–ether (3: 1) and acetone were employed.

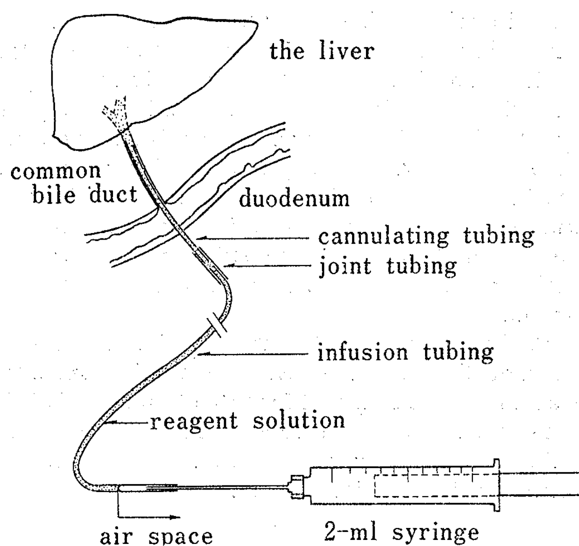

Fig. 1. Schematic Representation of Intrabiliary Retrograde Infusion Method

This device is composed of four parts as illustrated above. The cannulating tubing which has been cannulated through the opening where the bile flows into duodenum is connected with infusion tubing through the joint tubing. Both the cannulated tubing and infusion tubing have the same size and the inside diameter of the joint tubing is equal to the outside diameter of both cannulating and infusion tubings. The air space is used as a index of the end of the retrogradely infused reagent solution.

SDS Polyacrylamide Gel Electrophoresis——Electrophoresis was carried out by the method of Fairbanks[11)] in 6% acrylamide gels. The sample treatment was performed as follows. After the intrabiliary retrograde treatment with 50 μl aliquots of SDS (50 mM), Triton X-100 (4%), ethanol–ether (3: 1), acetone, pronase (1%), papain (4%), and trypsin (0.25%), bile samples, 0.2 ml, were collected. Besides, normal bile

11) G. Fairbanks, T.L. Steck, and D.F.H. Wallach, *Biochemistry*, 13, 2606 (1971).

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and the liver 100000g supernate were obtained. Though all thus obtained bile samples could be directly employed for polyacrylamide gel electrophoresis without SDS, these samples must be treated in the case of SDS polyacrylamide gel electrophoresis by adding the following (to the stated final concentration): 1% SDS, 10% sucrose, 10 mM Tris–HCl (pH 8), 1 mM ethylenediaminetetraacetic acid (EDTA) and 1% 2-mercaptoethanol, and were incubated at 37° for 60 min to promote reduction of disulfide bonds by 2-mercaptoethanol. Molecular weight markers, catalase, bovine serum albumin (BSA), oval albumin and cytochrome c, were treated in the same medium with the same condition. Each sample, 15 μl, was taken up in a micro syringe and discharged gently beneath the tracking dye solution, coomassie blue (20 μg/ml) in 5% sucrose-1 mM EDTA-10 mM Tris–HCl (pH 7.4) onto the top of the gel of which size was 0.7×8.0 cm. Electrophoresis was performed for 5 hours at 5 mA/gel at 4°. After electrophoresis, the dye front was marked by insertion of a small piece of stainless steel wire. According to the method of Fairbanks,[11] gel staining, coomassie blue staining for proteins and periodic acid–Schiff (PAS) staining for carbohydrate, was carried out.

Polyacrylamide Gel Electrophoresis——Used disc electrophoretical system has the following composition; lower gel (pH 8.9): 6% acrylamide, 0.4% N,N′-methylenebisacrylamide, 0.12M HCl, 9.15% Tris, 0.1% Triton X-100, upper gel (pH 6.7): 3.75% acrylamide, 0.31% N,N′-methylenebisacrylamide, 0.06M HCl, 0.75% Tris, 0.1% Triton X-100. The used buffer in both electrodes compartments was Tris–glycine buffer, 0.6% Tris and 2.88% glycine, containing 0.1% Triton X-100. Both gels were photomerized with the aid of the following catalysis: 0.005% riboflavin for upper gel and 0.115% N,N,N′,N′-tetramethylethylenediamine for lower gel. A 15 μl aliquot of the bile samples was discharged gently onto the top of the upper gel, where the size of the gels was as follows; 0.7×1.0 cm for upper gel and 0.7×6.0 cm for the lower gel. Electrophoresis was carried out towards the cathode at 5 mA/tube for 5 hours.

Organic Anion Binding Membrane Protein——At 15 min after organic anionic dyes, BPB, uranine and eosine, (10 μmole/300 g body weight), were administered to rats, 230—280 g, intrabiliary retrograde infusion of a 50 μl aliquot of Triton X-100 (4%) was performed for 10 min by the same way mentioned before. After the infusion was ceased, bile samples were collected. To a 200 μl aliquot of the bile samples, a 50 μl of 50% sucrose in Tris–glycine buffer (0.6% Tris and 2.88% glycine) containing 0.1% Triton X-100 was added and the resulting mixture was discharged to twelve gels; 20 μl/gel for ten gels which were employed to prepare the organic anion binding membrane protein and 10 μl/gel for two gels which were employed for staining, both coomassie blue staining and PAS staining. Polyacrylamide gel electrophoresis was performed according to the method described above. After electrophoresis, ten gels were cut into 3-mm slices and the same slices were put together. The organic anionic dyes were extracted as follows; 6 ml of 0.1N NaOH was added in the case of uranine and eosine, 6 ml of 0.1M phosphate buffer, pH 7.4, was added in the case of BPB and the resulting mixture was shaken during a night. After centrifuged for 20 min at 2000 rpm, uranine and eosine were measured spectrofluorometrically at excitation wavelengths of 490 nm and 513 nm respectively and at emission wavelengths of 510 nm and 535 nm respectively. BPB was measured spectrophotometrically at 600 nm.

Reabsorption of BPB from the Biliary Trees——The experiment was performed mainly according to the method of Clark, *et al.*[8a] After intrabiliary retrograde administration of BPB, 5 μmole/300 g body weight, bile samples were collected for three 10 min periods into tared bottles. At 15 min after the administration, a blood sample was taken through a tubing which had been previously cannulated into the carotid artery. At 30 min, then, the liver was removed. BPB contents of these samples were measured according to the method described in the previous report.[3a]

Results

Hepato-biliary Transport Characteristic of Uranine

The hepato-biliary transport characteristic of uranine is represented in Table I. Not only uranine itself but also its metabolite was excreted into rat bile after uranine was administered intravenously. Although it was found in the report of Webb, *et al.*[12] that the metabolite of uranine is uranine glucuronide, experiment was performed again to confirm this result in the rats used in present study. Paper chromatograms, thin–layer chromatograms and paper electrophoresis of the bile from rats to which uranine had been administered revealed the presence of two yellow spots. One of them had fluorescence and indicated an *Rf* value identical with that of authentic uranine sample in each of the analytical systems. The other spot which had no fluorescence showed a different *Rf* value in all the systems. Evidence that the conjugated form of uranine is a uranine glucuronide was obtained on treating the bile from injected animals with β-glucuronidase. Samples were incubated with the enzyme (1 mg/ml

12) J.M. Webb, M. Fonda, and E.A. Bouwer, *J. Pharmacol. Exptl. Therap.*, 137, 141 (1962).

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Table I. Hepato-biliary Transport Characteristic of Uranine and the Effect of BPB

	Plasma[a] level (μg/ml)	Liver[a] level (μg/g w.w.)	Bile[b] level (μg/ml)	B/P ratio[c]	L/P ratio[d]	B/L ratio[e]	% recovery in bile for 20 min
Uranine	63.7±1.8	20.8±1.4	1442±198	22.7±3.6	0.33±0.02	70.0±13.2	14.6±1.2
Uranine glucuronide	1.3±0.7	—	677±192	—	—	—	5.4±1.5
Pre-administration of BPB[f]							
Uranine	90.7±9.4	26.1±1.9	205±79	2.37±1.07	0.29±0.05	7.70±2.60	1.30±0.26
Uranine glucuronide	126±3.0	—	719±256	—	—	—	3.23±0.49

a) Blood and the liver were removed at 20 min after the intravenous administration of uranine to rats, 10 μmole/030 g body weight, and the dye contents were measured.
Liver level is expressed per gram liver wet weight.
b) Bile was collected for two 10 min periods after the administration of uranine and this bile level represents the second period.
c) bile/plasma concentration ratio
d) liver/plasma concentration ratio
e) bile/liver concentration ratio
f) Uranine, 10 μmole/300 g body weight, was administered following BPB, 50 μmole/300 g body weight, and the dye contents in plasma, liver and bile were also measured.
Each value is the mean±S.E. for 3—5 animals.

in acetate buffer, pH 4.5) at 38° for 24 hr. After incubation, the enzyme protein was precipitated with 10% trichloroacetic acid solution and aliquots of the supernatant fluid were employed for the thin-layer chromatograms, paper chromatograms and paper electrophoresis. Enzymatic treatment resulted in complete disappearance of the conjugated spot. Therefore, it was certainly confirmed that the metabolite of uranine is its glucuronide. The concentration of unchanged form, however, was higher about two times than the metabolite in the bile.

As the liver level of uranine is lower than the plasma level (liver/plasma ratio=0.33), it is thought that uranine belongs to the type (ii) compounds.

To examine whether uranine shares the same hepato-biliary transport system with BPB which has been employed in our series studies as a representative model of organic anions actively excreted into rat bile, BPB, 50 μmole/300 g body weight, was administered intravenously at 2 min prior to the administration of uranine, 10 μmole/300 g body weight. In this case, both the bile level and percentage recovery in bile of the dye were extremely decreased as shown in Table I. But it is found that the liver level as well as L/P ratio of uranine was not so affected by the pre-administration of BPB as compared with bile level and B/L ratio.

The phenomena that the bile level of uranine glucuronide was increased a little and its percentage recovery into bile was considerably decreased by the pre-administration of BPB are thought to be due to the decreased bile flow. Therefore it may be mentioned that the biliary excretion of uranine glucuronide is not so influenced as that of uranine itself by the preadministration of BPB.

To investigate the uptake process of uranine from the blood into the liver, uptake experiment using liver slices was performed and the results are represented in Table II. As shown in this Table, the uptake of uranine by the liver slices was not depressed when BPB was five times more added to the incubation medium than uranine. Therefore it is suggested that uranine is not entrapped into the liver by the same particular transport system which takes part in the hepatic uptake of BPB.

Then the binding of uranine to the liver 100000 ***g*** supernate, was determined by equilibrium dialysis method and the binding percentage figure of uranine to 100000 ***g*** supernate was found not to be so large. But the possibility of the existence of the binding of uranine to the macromolecules which is contained in the liver 100000 ***g*** supernate can not be denied (Table II).

Moreover, the binding of uranine to the liver cytoplasmic organic anion binding proteins, X, Y and Z, was examined using a column of Sephadex G-75 (Fig. 2). In this figure, the

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Table II. Uptake of Uranine by Liver Slices and the Binding of Uranine to the Liver 100000 *g* Supernate

	S/M ratio[a)]	% bound to the liver[b)] 100000 *g* supernate
Uranine	0.98±0.26[c)]	15.4±2.2
BPB Uranine	1.05±0.11[d)]	—

a) Rat liver slices were incubated for 30 min at 37° in Krebs-Ringer phosphate solution. Uranine concentration in slice water (S) and in incubation medium (M) is expressed as a S/M ratio. Each value is the mean ±S.E. for slices from 3 animals.
b) Using cells for equilibrium dialysis, the binding of uranine to rat liver 100000 *g* supernate was measured after the cells were shaken during two days at 4°. Each value is the mean±S.E. for 3—5 experiments.
c) Initial uranine concentration was 0.01 mM.
d) Initial concentrations of uranine and BPB were 0.01 mM and 0.05 mM respectively.

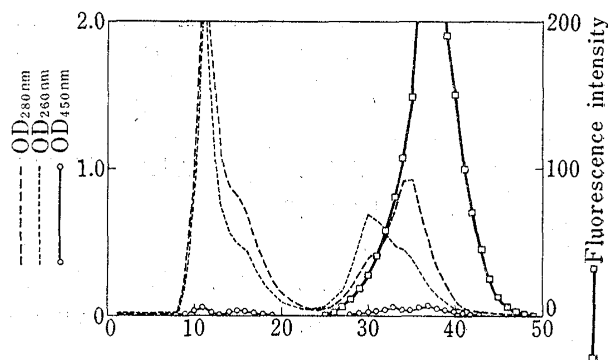

Fig. 2. Elution Pattern of Protein (OD_{280nm}), Nucleic Acid (OD_{260nm}) and Yellow Dyes (OD_{450nm} and fluorescence intensity) from the 100000 *g* Supernate from Rat Liver

5 ml of the 100000 supernate was applied onto a column of Sephadex G-75 (2.5×50 cm) and was eluted with 0.01M phosphate buffer, pH 7.4. Collection was performed, 7 ml/tube, and the flow rate was 30—50 ml/hr.

two peaks of OD_{450nm} at the tube numbers 11 and 15 do not represent the existence of uranine and uranine glucuronide because of the contamination of proteins *etc.*, but the peaks of uranine and uranine glucuronide are detected at tube numbers 33 and 37 which differ from the fraction of X, Y and Z binding proteins. From this experiment, it may be mentioned that both uranine and uranine glucuronide are not bound to the organic anion binding proteins.

To examine whether uranine is bound to the macromolecules other than X, Y and Z binding proteins, DEAE-cellulose ion-exchange chromatography and gel-filtration using a column of Sephadex G-25 were performed (Fig. 3 and 4). In these figures, although only the elution patterns which were obtained after the liver 100000 *g* supernate was applied onto these columns are represented, the same elution patterns were obtained when dyes, uranine and uranine glucuronide, were applied by themselves onto the same columns. Namely, uranine was eluted at tube numbers 30—35 in a DEAE-cellulose column and at tube numbers 43—48 in a Sephadex G-25 column. On the other hand, uranine glucuronide was eluted at tube numbers 13—17 in a DEAE-cellulose column and at tube numbers 22—26 in a Sephadex G-25 column. It is shown from Fig. 4 that the peak of uranine is clearly separated from that of proteins or nucleic acids. By taking these points into consideration, the two peaks corresponding to uranine and uranine glucuronide in Fig. 3 and 4 are thought to be unbound dyes.

Attempt to modify the Excretion of Organic Anionic Dyes by Intrabiliary Retrograde Infusion with Several Reagents

As the hepatic uptake of uranine from the blood stream is thought to depend on a simple diffusion mechanism, it may be mentioned that the excretory process from the liver into bile is the rate-determining step for the total hepato-biliary transport of uranine. Consequently it is necessary to examine whether the excretory process from the liver cells into the bile canaliculi could be affected by the intrabiliary retrograde infusion of some reagents, especially surfactants which have been employed as a solubilizer of biomembrane.

After intrabiliary retrograde infusion with a 50 μl aliquot of 4% Triton X-100 solution was performed for 10 min, uranine was immediately administered intravenously to rats, 10 μmole/300 g body weight, and the plasma, liver and bile levels of uranine were examined (Table III). In this case, not only the bile level but also the percentage recovery of uranine into bile was considerably decreased by the treatment with Triton X-100, and especially, the active excretion of uranine glucuronide was almost completely inhibited in the same animals.

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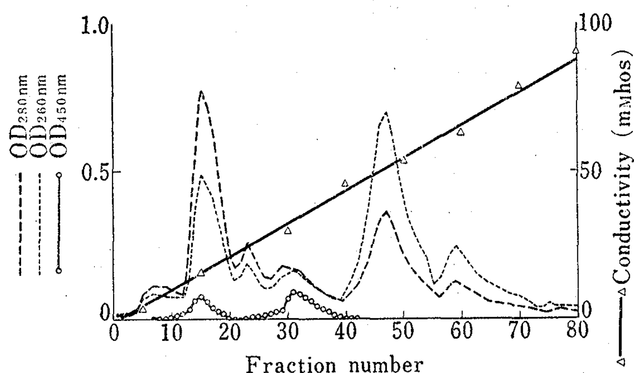

Fig. 3. Elution Pattern of Protein (OD_{280nm}), Nucleic Acid (OD_{260nm}) and Yellow Dyes (OD_{450nm}) from the 100000 ***g*** supernate from Rat Liver through a DEAE-cellulose Column

20 ml of the 100000 ***g*** supernate was placed onto a DEAE-cellulose column (2×10 cm). After washing with 0.01M NaCl, elution was performed with a gradient of 0.01M—0.2M NaCl (pH 7.4). The 0.02M NaCl wash eluate and the beginning of the gradient are not shown on the figure. Collection was performed, 10 ml/tube, and the flow rate was 100 ml/hr.

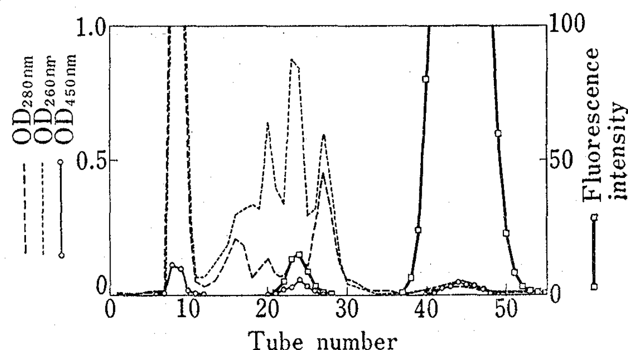

Fig. 4. Elution Pattern of Protein (OD_{280nm}), Nucleic Acid (OD_{260nm}) and Yellow Dyes (OD_{450nm} and fluorescence intensity) from the 100000 ***g*** Supernate from Rat Liver

5 ml of the 100000 ***g*** supernate was applied onto a column of Sephadex G-25 (3×30 cm) and was eluted with 0.01M phosphate buffer, pH 7.4. Collection was performed, 7 ml/tube, and the flow rate was 30—50 ml/hr.

Table III. Effect of Retrogradely Infused Triton X-100 on the Hepatobiliary Transport of Uranine

	Plasma level (μg/ml)	Liver level (μg/g w.w.)	Bile level (μg/ml)	B/P ratio	L/P ratio	B/L ratio	% recovery in bile for 20 min
Uranine	142±17	39.3±2.6	538±84	3.87±1.12	0.21±0.04	14.3±4.7	4.9±2.0
Uranine glucuronide	227±4.4	—	177±50	—	—	—	0.5±0.3

After the intrabiliary retrograde infusion with a 50 μl aliquot of 4% Triton X-100 was performed for 10 min, uranine, 10 μmole/300 g body weight, was administered intravenously to rats. Blood and the liver were removed at 20 min after the administration of uranine and the bile was collected for two 10 min periods.
The bile level represents the second period.
Each value is the mean±S.E. for 3—5 animals.

As this kind of success to inhibit the active excretion of organic anions in the *in vivo* whole animal level has never been reported, the application of the intrabiliary retrograde infusion method to inhibitory experiment is conceivable to be very useful and to be contributive to the study of biliary excretion experiments. The excretory process for organic anions from the liver cells into the bile was more precisely studied using BPB which has been employing in our series experiments as a most suitable model compound because of its non-metabolizing in the liver and the results are represented in Table IV. In sham-operated animals, a 50 μl aliquot of 0.13M Tris-HCl buffer solution isotonized with NaCl was infused for 10 min. Thereafter, BPB was administered intravenously, 10 μmole/300 g body weight, and bile collection was performed for three 10 min periods. The results are expressed in the form of the indices of the hepatobiliary transport of BPB, namely, plasma, liver and bile levels, percentage recovery in bile for 30 min, B/P, L/P and B/L ratios. With respect to all of the indices of the hepato-biliary transport of BPB, there detects no obvious difference between sham-operated experiment and the control experiment. Then the effect of infusion volume and of concentration of SDS on the biliary excretion BPB was checked. Even when the infusion volume was increased from 50 μl to 100 μl where the concentration of SDS was 50 mM, there is not so great difference between the two experiments with respect to the bile level of BPB and it may be mentioned that more significant inhibitory effect of SDS on the biliary excretion

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TABLE IV. Inhibition of the Biliary Excretion of BPB by Intrabiliary Retrograde Infusion of Several Reagents

	Plasma level (μg/ml)	Liver level (μg/g w.w.)	Bile level (μg/ml) 0—10 min	10—20 min	20—30 min	% recovery in bile for 30 min	L/P ratio	B/L ratio	B/P ratio	Bile flow (ml/10 min)
No treatment[a]	43.9± 18.7	127± 31.5	3821± 976	6921± 438	6002± 801	44.8± 11.8	5.0± 2.7	41.7± 4.7	254± 153	0.15± 0.04
Sham operation[b]	37.5± 10.9	107± 15.3	2773± 917	7512± 1152	7349± 1150	41.1± 5.3	3.7± 1.7	68.0± 21.8	229± 93	0.13± 0.02
SDS 50 mM 100 μl[c]	107± 29.3	147± 18.5	227± 58	814± 169	854± 126	4.8± 1.8	1.4± 0.3	5.9± 1.1	8.2± 1.5	0.13± 0.03
SDS 50 mM	99.3± 3.1	122± 3.7	198± 35	781± 114	776± 62	7.1± 1.3	1.2± 0.01	6.4± 0.9	7.8± 1.1	0.20± 0.009
SDS 2 mM	36.4± 19.4	155± 5.7	2421± 243	7118± 763	6733± 522	33.0± 1.3	5.6± 3.7	43.2± 2.8	234± 143	0.11± 0.008
Triton X-100 4%	141± 26.5	185± 15.9	368± 125	815± 442	748± 300	3.3± 0.9	1.4± 0.3	4.0± 1.7	5.1± 1.5	0.12± 0.05
Pronase 1%	49.7± 22.5	98.7± 6.3	2563± 514	6385± 722	5700± 82	35.8± 10.2	2.4± 1.1	58.3± 4.0	141± 72	0.13± 0.02
Trypsin 0.25%	47.5± 23.8	132± 18.2	2253± 645	6449± 627	6141± 414	34.7± 6.9	3.4± 1.4	47.7± 11.0	171± 104	0.12± 0.01
Papain 4%	29.9± 10.8	84.9± 14.5	2778± 1044	6663± 372	5680± 754	40.0± 1.7	3.1± 1.4	71.3± 20.6	209± 54	0.14± 0.02
Phospholipase D 1%	33.0± 8.0	83.1± 6.9	1769± 422	7240± 256	7374± 825	33.2± 2.1	2.6± 0.6	89.1± 10.6	228± 27	0.11± 0.01
Phospholipase D 1%+Pronase 1%	82.7± 26.6	98.7± 14.4	2438± 848	6114± 453	5666± 478	25.9± 5.3	1.3± 0.3	61.1± 7.4	72.8± 20.1	0.11± 0.01
DNP 5 mM	28.9± 11.5	91.1± 18.4	2183± 736	5032± 2790	5894± 489	41.2± 11.1	3.4± 1.1	45.8± 34.6	228± 91	0.13± 0.02
Ouabain 10 mM	18.8± 4.4	82.5± 12.1	3401± 1053	8192± 1201	6885± 760	47.4± 5.4	4.7± 1.9	84.1± 10.5	382± 106	0.14± 0.01
PCMBS 50 mM	43.8± 14.6	128± 22.7	2094± 1067	6075± 1478	5562± 1324	29.9± 2.4	3.0± 0.6	43.2± 3.7	130± 14	0.13± 0.02
NEM 25 mM	51.2± 25.1	97.2± 13.2	1737± 357	5672± 711	4063± 1010	32.7± 5.4	2.5± 0.9	41.6± 16.5	113± 70.5	0.14± 0.02
PCMBS 50 mM +SDS 50 mM	127± 18.6	122± 10.1	478± 105	1496± 228	1597± 305	9.4± 2.2	1.0± 0.07	13.1± 2.6	12.8± 3.1	0.16± 0.03
Ethanol–Ether (3:1)	146± 66.2	134± 31.8	209± 61	575± 225	635± 213	4.5± 3.3	1.1± 0.6	5.1± 2.4	5.9± 5.3	0.17± 0.06
Acetone	109± 30.6	131± 27.8	275± ±52	878± 519	927± 594	7.0± 3.1	1.3± 0.4	7.2± 4.6	9.9± 9.1	0.18± 0.03

After the intrabiliary retrograde infusion was performed for 10 min, BPB, 10 μmole/300 g body weight, was administered intravenously to rats. The blood and liver were removed after 30 min and bile was collected for three 10 min periods. BPB contents in these samples were measured spectrophotometrically.

a) Hepato-biliary transport experiment was performed without intrabiliary retrograde infusion treatment.

b) Hepato-biliary transport experiment was performed in the rats which had been infused with a 50 μl aliquot of 20 mM Tris–HCl buffer solution, pH 7.4, containing 0.13M NaCl.

c) The infusion volume was 100 μl only in this experiment. In all of the other experiments, the infusion volume was 50 μl.
Each value is the mean±S.E. for 3—8 animals.

of BPB is not detected by increasing infusion volume. Accordingly, the intrabiliary retrograde infusion volume was adjusted to be 50 μl in all the following experiments. When the concentration of SDS infused was decreased to 2 mM, the inhibitory effect of SDS on the biliary excretion of BPB was decreased. When intrabiliary retrograde treatment with 4% Triton X-100 was performed, the biliary excretion of BPB was also significantly inhibited. In the treatment with both surfactants, the B/P ratio of BPB was dramatically decreased and this decrease is thought to largely depend on the reduction of the B/L ratio of BPB, because the reduction of B/L ratio is far larger than that of L/P ratio. As the B/L ratio is recognized to represent

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the active excretory process from the liver into the bile canaliculi, the inhibitory effect of these surfactants is suggested to occur at the active excretory process.

Then to investigate precisely the property of the active excretory system which participates in the biliary excretion of BPB, the retrograde treatment was performed with the following reagents; digestive enzymes, metabolic inhibitors, thiol reagents and organic solvents. Although, in the three protease treatments, 1% pronase, 0.25% trypsin and 4% papain, no inhibitory effect was detected on the biliary excretion of BPB, a little inhibition appeared in 1% phospholipase D treatment experiment. Moreover, the two metabolic inhibitors, DNP and ouabain, showed no effect on the biliary excretion of BPB. When the concentration of ouabain was increased to 50 mM, the biliary excretion of BPB was considerably inhibited, but the rats were not survived within 15 min. On the other hand, when thiol reagents, 50 mM PCMBS or 25 mM NEM, were infused, the percentage recovery figures of BPB into bile for 30 min were 29.9(%) and 32.7(%) respectively, and the bile levels of the three 10 min periods were lower than that of the sham-operated experiment. This results suggest that thiol-reagents inhibit the biliary excretion of BPB. In organic solvents treatments, ethanol–ether (3: 1) or acetone, the biliary excretion of BPB was extremely inhibited as large as surfactants.

Polyacrylamide Gel Electrophoresis

To check whether the inhibitory effect of the retrogradely infused surfactants and organic solvents was caused by solubilizing the bile-canalicular liver plasma membrane or not, SDS polyacrylamide gel electrophoresis was performed about the bile samples obtained after the intrabiliary retrograde infusion of several reagents. The results are represented in Fig. 5.

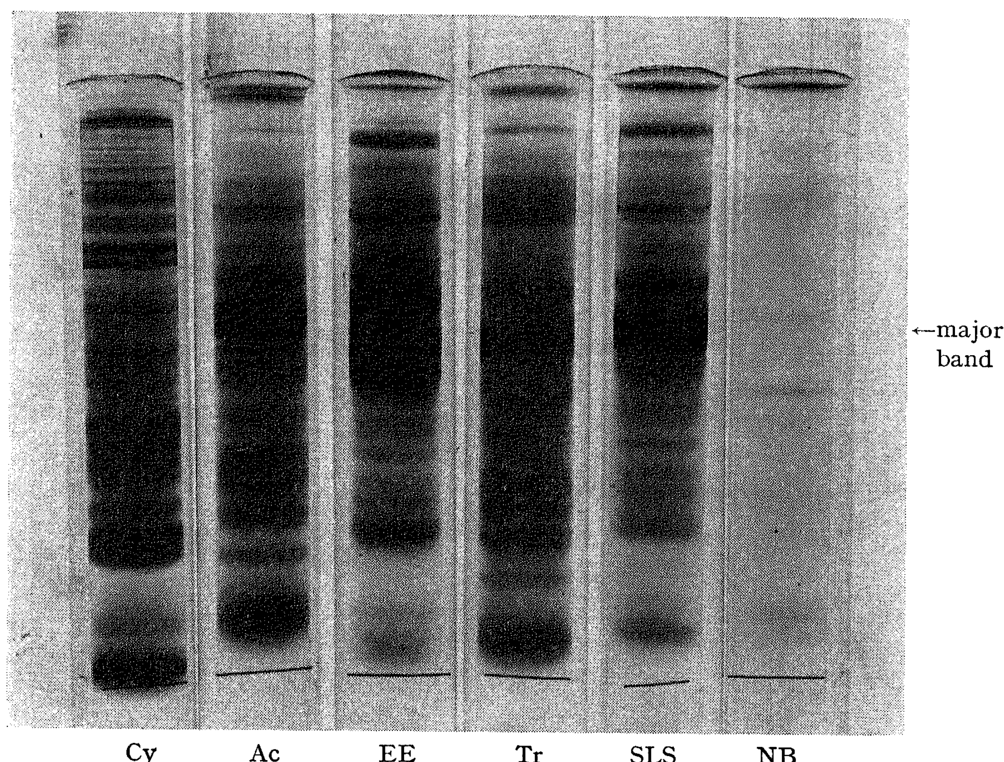

Fig. 5. SDS Polyacrylamide Gel Electrophoretical Patterns of Bile Samples and Liver 10000 *g* Supernate

Details of sample preparation and staining are given in Method. After electrophoresis on 6% acrylamide gel, staining was performed for protein with coomassie blue. Stainless steel wire inserted before fixation and staining shows the final position of the tracking dye in each gel.
NB = normal bile
SLS = bile samples obtained after intrabiliary retrograde infusion with SLS (50 mM)
Tr = bile samples obtained after intrabiliary retrograde infusion with Triton X-100 (4%)
EE = bile samples obtained after intrabiliary retrograde infusion with ethanol–ether (3: 1)
Ac = bile samples obtained after intrabiliary retrograde infusion with acetone
Cy = the liver 100000 *g* supernate

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The SDS polyacrylamide gel electrophoretical patterns of the gels for the bile treated with proteases which are not represented in this figure, did not so differ from that of normal bile sample, gel (NB). With respect to the other gels, many protein bands which are stained with coomassie blue are detected and especially the "major band" which is shown with arrow in

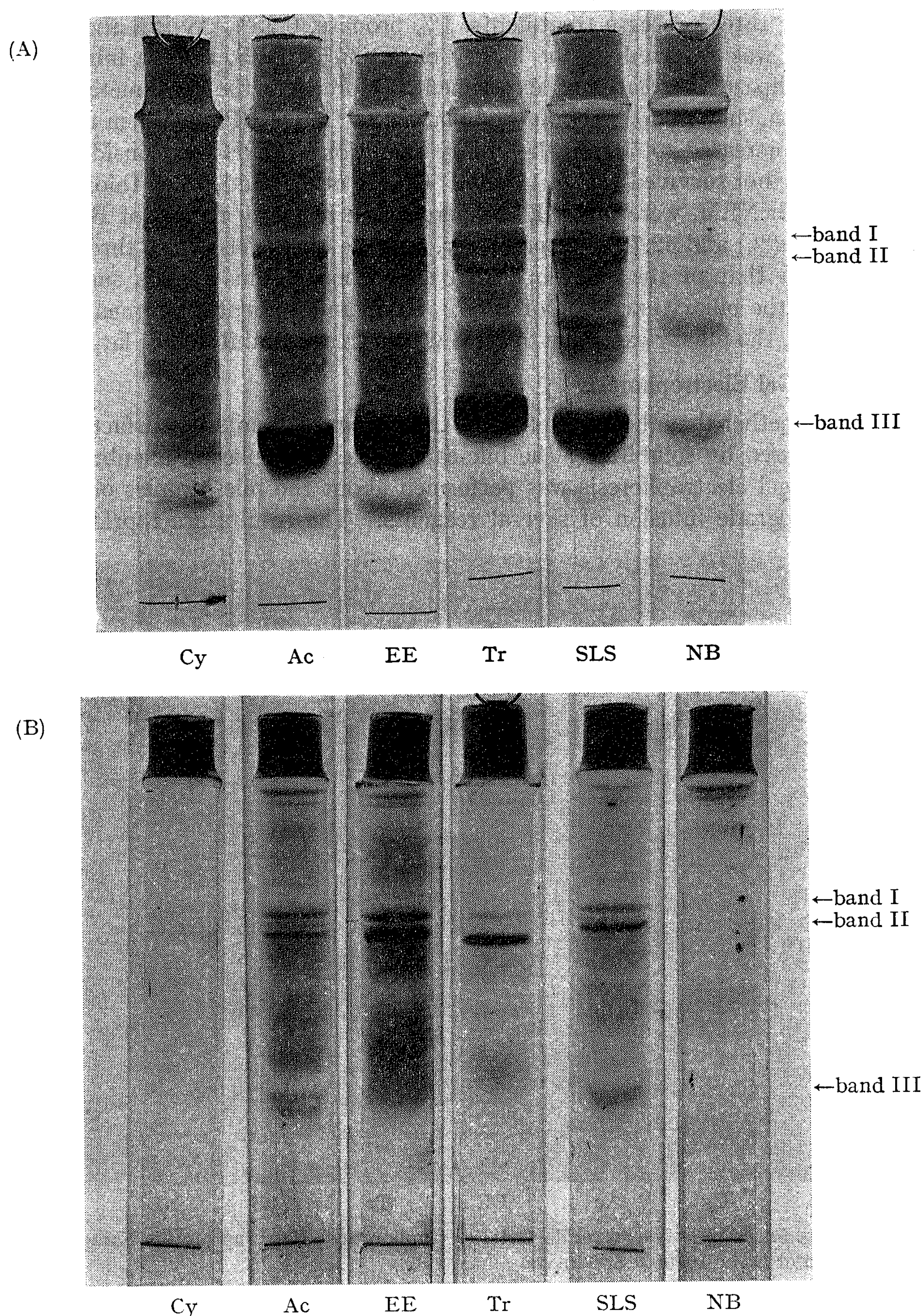

Fig. 6. Polyacrylamide Gel Electrophoretical Patterns of Bile Samples and the Liver 100000 g Supernate

Details of sample preparation and stainings are given in Method. After electrophoresis on 3.75% (upper gel)—6.0% (lower gel) acrylamide gel, staining was performed for protein with coomassie blue (A) and for carbohydrate by PAS procedure (B).

The abbreviations show the gels are the same as in Fig. 5.

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this figure is detected in common. The protein patterns of the gels corresponding to surfactants treatments, gel (SDS) and gel (Tr), and organic solvents treatments, gel (EE) and (Ac), respectively, differ extremely from that of the gels which correspond to the normal bile, gel (NB), and to the liver cytoplasmic fraction, gel (Cy). From this electrophoretical patterns, it is suggested that the membrane proteins, especially, the "major protein," are solubilized by the intrabiliary retrograde treatment with both surfactants and organic solvents.

Moreover, polyacrylamide gel electrophoresis without SDS was carried out with the same bile samples and the results are represented in Fig. 6. In Fig. 6 (A), all the gels were stained with coomassie blue and three protein bands, band I, II and III, are revealed in common in the gels (SLS, Tr, EE and Ac). As these protein bands are not revealed in both gels (NB) and (Cy), it is thought that these proteins are not detected obviously in both the liver cytoplasm and bile samples and that they are derived from the bile-canalicular plasma membrane. When PAS staining was performed about the same gels (Fig. 6 (B)), the band III was also stained, though the other two bands, band I and II, were stained more clearly. Therefore, the "band III protein" is thought to have hydrocarbon chains.

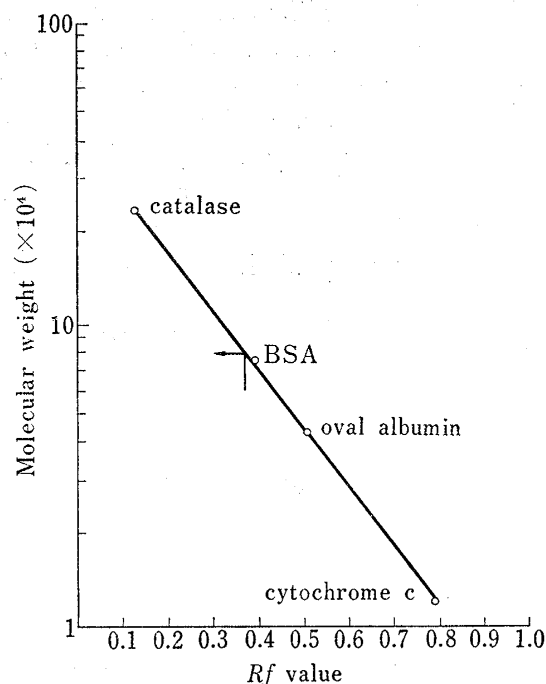

Fig. 7. Molecular Weight Estimation of the "Band III Protein" by 6% Polyacrylamide-gel Electrophoresis in SDS

A plot of the logarithms of the molecular weights *vs* the migration distances of the marker proteins are shown and this calibration curve permits an estimation of the molecular weight of the "band III protein."

When this "band III protein" was extracted with distilated water and the extract was applied to the gels for SDS disc electrophoresis after treated with SDS and 2-mercaptoethanol. After electrophoresis, coomassie blue staining was performed and the electrophoretical pattern of this extracted protein, namely "band III protein" was compared with that of Fig. 5. It was confirmed that the "band III protein" is equivalent to the "major protein".

By means of the calibration curve which was made of four molecular weight markers, its molecular weight was estimated to be about 80000 (Fig. 7).

Moreover, it was found that the intravenously administered organic anionic dyes, uranine, eosine and BPB, were partly bound to this "band III protein" (Fig. 8). Although only a peak, unbound dye's peak, was detected with respect to the control experiment (A-1, B-1 and C-1), two peaks were detected for all the dyes in this figure; the highest peak, fraction no. 20—25 for uranine, 24—26 for eosine and 21—24 for BPB respectively, is due to unbound dye and the higher peak, fraction no. 16 for uranine, 16 and 17 for eosine and 17 and 18 for BPB respectively, is due to the dye bound to the "band III protein". The protein bound dye's peak was visual during the electrophoresis in the case of eosine and BPB.

Reabsorption of BPB from the Biliary Trees

The effect of the retrogradely infused Triton X-100 on the reabsorption of BPB from the biliary trees was examined to clarify how retrogradely infused surfactant affects the reverse transfer from bile. As a control experiment, the recovery of BPB into the bile was estimated after 5 μmole of BPB was retrogradely administered to rats. By subtracting this recovered amount of BPB from the administered amount, an apparent reabsorption percentage figure was calculated. The results are represented in Table V. To ascertain the reabsorption

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of BPB from the biliary trees, plasma samples were removed at 5 min after the administration and their BPB contents were determined. As shown in this table, the reabsorption of BPB is apparent from the fact that the apparent reabsorption calculated from bile level was about 48% and there was sufficiently detectable amount of BPB in the plasma.

When intrabiliary retrograde infusion with Triton X-100 was performed, both the apparent reabsorption percentage figure and the plasma level of BPB were extremely increased. Therefore, the reabsorption of BPB from the biliary trees indicated to be accelerated by the previous intrabiliary retrograde infusion with a surfactant.

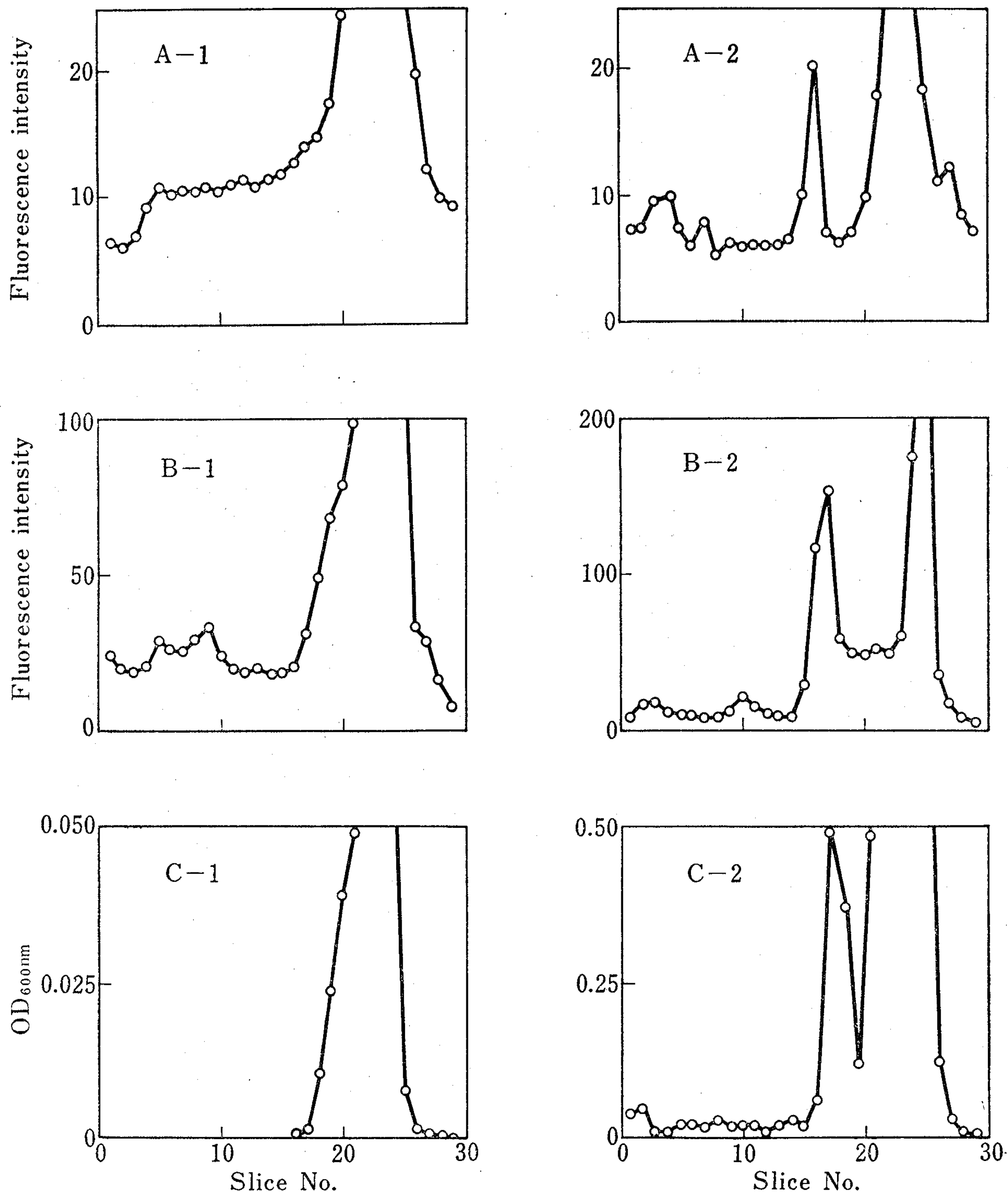

Fig. 8. Binding of Organic Anionic Dyes to the "Band III Protein"

At 10 min after organic anionic dyes were administered intravenously to rat respectively, 10 μmole/300 g body weight, the intrabiliary retrograde infusion with Triton X-100 was performed for 10 min and bile collection was performed. After 6% polyacrylamide gel electrophoresis was carried out in 0.1% Triton X-100 containing buffers using thus obtained bile samples, the gels were sliced for extracting the dyes and the dye contents in each slices was determined as described under "Experimental Procedure."

A-1, B-1, C-1: bile samples obtained after *i.v.* administration of uranine, eosine and BPB respectively

A-2, B-2, C-2: Triton X-100 treated bile samples obtained after *i.v.* administration of uranine, eosine and BPB respectively

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Table V. Effect of the Retrogradely Infused Triton X-100 on the Reabsorption of BPB from the Biliary Trees

	Plasma level (μg/ml)	Liver level (μg/g w.w.)	Bile level (μg/ml)			Apparent[a)] reabsorption percentage
			0—10 min	10—20 min	20—30 min	
Control[b)]	1.04±0.12	52.9±6.8	4613±954	2562±132	1482±386	47.5±5.3
Triton X-100[c)] pre-treatment	22.7±9.0	101±2.3	1033±293	476±107	449±75	88.9±3.2

a) By subtracting the recovered amount of BPB into bile from the administered amount of BPB, the apparent reabsorption percentage figure was calculated.
Each value is the mean±S.E. for 3—5 animals.
b) 5 μmole of BPB was retrogradely administered to rats and bile samples were collected for three 10 min periods. The blood and liver were removed at 5 min and 30 min after the administration respectively and BPB contents in these samples were measured.
c) 5 μmole of BPB was retrogradely administered to rats after the intrabiliary retrograde infusion with a 50 μl aliquot of 4% Triton X-100 was performed for 10 min and the bile samples were collected for three 10 min periods. The blood and liver were removed after 5 min and 30 min respectively

Discussion

In our previous reports,[1,3,5] it became clear that the liver cytoplasmic organic anion binding proteins have important roles on both the hepatic uptake process from the blood stream and the intracellular transport of organic anions which are transported into bile by an active transport system. These binding proteins are also thought to take part in the metabolic process in the liver,[13,14] being suggested that ligandin, namely organic anion binding Y protein, is one of the metabolic enzymes by which glutathion conjugation is catalysed.

However, it is uncertain whether these binding proteins participate in the subsequently occurred process, namely the active excretion of organic anions from the liver cells into the bile canaliculi. In order to solve this important problem, first, it was tried to find a model of organic anions which do not bind to the liver cytoplasmic binding proteins and then uranine was selected as a candidate. Uranine is metabolized in the liver and both uranine itself and its metabolite, uranine glucuronide, are excreted into bile together, but it was able to carry out the analysis of only uranine separated from uranine glucuronide. In the whole animal experiments, it became clear that uranine belongs to the type (ii) compounds because of its low L/P ratio, less than one. It also became apparent that uranine is transported from the blood stream into bile by the same active transport system that excretes BPB (Table I). As the L/P ratio of uranine was not significantly affected by the pre-loading of BPB, the uptake process was more precisely examined using liver slices. In this *in vitro* system, the uptake process of uranine by the liver slices was not also inhibited by BPB. Therefore it is thought that uranine and BPB do not share the same transport system when they enter into the liver.

The probability that uranine is bound to the liver cytoplasmic organic anion binding proteins, X, Y and Z, and to the other macromolecules in the liver cytoplasm was examined by means of gel-chromatography using a Sephadex G-75 and G-25 columns and of ion-exchange chromatography using a DEAE-cellulose column, but it was suggested that uranine which is actively excreted into bile exists in the liver cytoplasm as an unbound form, namely uranine is not bound to the macromolecules in the liver cytoplasm (Fig. 2, 3, and 4). Therefore, it may be mentioned that the liver cytoplasmic organic anion binding proteins do not have important roles on the excretory process from the liver cells into the bile for organic anions. However, this excretory process is thought to be the rate-limiting step for the total hepatobiliary transport of uranine.

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Only a few report which deal with the excretory process from the liver cells into the bile for both endogenous and exogenous compounds have been published. Among them, report by Gregory, *et al.*[15] serves us as a good reference. Their study is based on employing the differential centrifugation method, but it takes too much time to prepare the active excretory system after test compounds are administered to rats. Therefore, the intrabiliary retrograde infusion method was introduced to the present *in vivo* study because of its simplicity and rapidity. As a preliminary experiment, a surfactant was infused to solubilize the active excretory system, of which location in the liver has not been precisely decided. After the infusion of surfactant, the biliary excretion of the two models of organic anions, uranine and BPB, were extremely decreased (Table III and IV). and it appears that we have succeeded in inhibiting the active excretion of both organic anions in the *in vivo* whole animal experiment.

It was next tried to make clear the nature of the active excretory system using BPB as a model of organic anions because of its non-metabolizing in the liver. Although three proteases did not affect the biliary excretion of BPB, the following either explanation is thought to be applicable, namely these proteases were inactivated by the presence of bile, or the hydrocarbons which are thought to be located on the external side of the bile-canalicular plasma membrane[16] prevents the action of proteases. On the other hand, thiol reagents as well as phospholipase D inhibited the biliary excretion of BPB to some extent. Therefore, it is thought that the active excretory system has supposedly a moieties like SH-enzymes in itself.

The inhibitory effect of organic solvents and surfactants, however, is far stronger than that of thiol reagents and phospholipase D. The reason for these difference is supposed that the effect of both organic solvents and surfactants must be essential and that one of the components of the active excretory system may be solubilized by them, while thiol reagents merely modified one part of the active excretory system. This is because a number of protein bands caused from the bile-canalicular plasma membrane were detected in the bile samples which were obtained after the treatment with organic solvents and surfactants (Fig. 5 and 6), and because the electrophoretical patterns of the bile samples obtained following the treatment with proteases were almost the same as that of normal bile. As the metabolic inhibitor, ouabain, has been demonstrated to have no effect on the active transport of organic acids or bases because of its being actively transported by the liver into bile,[17,18,19] and as it is reported that DNP does not decrease the active biliary excretion of bile acids,[20] it is also likely that these two metabolic inhibitors did not affect the active biliary excretion of BPB in this intrabiliary retrograde infusion treatment. Considering the effect of phospholipase D, it may be able to digest the active excretory system itself but only some of its moieties may have been broken or damaged by the digestive action of the enzyme.

Spreading out this conception, the accelerative effect of the intrabiliary retrograde treatment with Triton X-100 on the reabsorption of BPB from the biliary trees will be reasonable, that is, not only the active excretory system but also a barrier for the reabsorption of organic anions have been partly removed and consequently the reabsorption of BPB from the biliary trees was accelerated. Although only a little has been known about this barrier up to date, we have obtained the clue to the active excretory system, because three organic anionic dyes which are actively excreted into rat bile were bound to the "band III protein" detected in the solubilized fraction by both surfactants and organic solvents. As this "band III protein" was stained with PAS procedure, this protein is thought to be a glycoprotein and its estimated molecular weight is about 80000 (Fig. 7). However, the problem whether this protein is a

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carrier protein of the active excretory system or not has not been solved yet and more detailed experiments will be needed about this point.

As mentioned above, the study concerning the biliary excretory process of organic anions was performed by means of intrabiliary retrograde infusion method. But, here, one more problem whether only the excretory process from the liver cells into bile canaliculi can be investigated by this method or not, should be solved. As shown in Table IV, the liver level of BPB was increased and the decrease in B/L ratios is far larger than that of L/P ratios by the intrabiliary retrograde infusion with SDS. In addition, many electrophoretically detected proteins of the bile samples which were obtained by the treatment with both organic solvents and surfactants have a property of glycoprotein which are thought to be located on the external side of the plasma membrane.[16] By comparing the electrophoretical patterns of the bile samples obtained after treated using surfactants or organic solvents with that of the liver cytoplasmic fraction, it becomes clear that their proteins do not have their origins in the liver cytoplasm. Therefore, it may be concluded that the biliary excretory process from the liver cells into the bile can be specifically examined by the intrabiliary retrograde infusion method.

This infusion method is thought to be applicable to the fields other than biopharmaceutics in the study of biliary system too. In fundamental fields, this method could be employed to obtain the bile-canalicular plasma membrane of the hepatocytes, because the electrophoretical pattern of thus obtained bile samples do not so differ from that of Neville, *et al.*[21]

Acknowledgement We wish to thank Professor Hitoshi Sezaki for his helpfull discussions.

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