stages of stress, consumption of serum glucose and FFA was increased because of continuation of struggle and shivering and of maintenance of body temperature, glycogenolysis and lipolysis were accelerated, liver glycogen content was virtually exhausted, serum glucose levels decreased and FFA levels increased. It seems that an augmentation in sympathetic nerve activity, an increase in a release of catecholamines from the adrenals as well as an increase in adrenocorticotropic hormone (ACTH) secretion, and other factors participated in such a variation in these body components. As stress progressed, gluconeogenesis began to be accelerated, which probably resulted in an increase in serum glucose levels together with a decrease in FFA levels 12 hr after stress and in a further increase in serum glucose concentration accompanied with a further decrease in FFA concentration and with accumulation in liver glycogen content 18 hr after stress. Glucocorticoids might play a role in these changes. However, problem of suppression of glycogenolysis remained in this case. Gastric erosions were generated 1 hr after stress and EI progressively developed.

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In Vitro Binding of Sulfobromophthalein to Cytoplasmic Protein from Liver, Kidney, and Small Intestinal Mucosa of Rat and Rabbit[1)]

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Sephadex G-75 gel filtration of cytoplasmic protein with BSP *in vitro* yielded elution patterns characteristic of the protein sources as liver, kidney, and small intestinal mucosa of rat and rabbit. In the both species, binding of sulfobromophthalein (BSP) with Y protein was observed to be predominant in the liver, and weak or not recognized in the kidney and small intestinal mucosa, as was to be expected. On the other hand, the BSP elution with Z protein fraction was appreciable in the rabbit kidney and the rat small intestinal mucosa, and it appeared that the role of Z protein on the fate of the organic anion in the body was not so simple. Two unknown fractions, Y′ and Y″ protein, were recognized in the BSP elution pattern with cytoplasmic protein of the rat kidney, and Y′ protein was recognized in that of the rabbit kidney and small intestinal mucosa.

Sulfobromophthalein (BSP) is used clinically in the testing of hepatic function because the normal liver excretes most of the dye within a short time. To elucidate the mechanism of the hepato-biliary excretion, the binding of BSP to the rat liver has been studied by several research groups in recent years. Arias, *et al.*[3–7)] studied the roles of the rat liver cytoplasmic

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organic anion binding protein Y and Z on the hepatic uptake of BSP. About Y and Z protein, it was suggested that they bound various organic anions *in vivo* and *in vitro*, and they were considered to be important determinants in the transfer of anions from plasma into parenchymal liver cells. On the other hand, Muranishi, *et al.*[8] emphasized the main role of Y protein on the hepato-biliary transport. Further, Arias, *et al.*[7] demonstrated by means of immunodiffusion analysis with monospecific antiserum against rat liver Y protein that an immunologically similar protein was contained in the rat kidney and small intestinal mucosa, and very recently they studied[9] the characteristics of Y protein of the rat kidney by immunodiffusion analysis, amino acid analysis, electrophoresis, and electrofocussing *etc.* However, BSP binding pattern of Y and Z protein in the rabbit has not been shown by the use of gel filtration yet.

The purpose of this report is to obtain well-defined BSP binding pattern by cytoplasmic protein in the kidney and small intestinal mucosa as well as in the liver of rat and rabbit through the gel filtration, and to disclose the difference in the BSP binding characteristics of the proteins among the source tissues and species.

Experimental

Reagents——Sulfobromophthalein sodium tetrahydrate (BSP) was purchased from Aldrich Chemical Company Inc. Sephadex G-75 was purchased from Pharmacia Fine Chemicals Co. Bovine serum albumin used was Fraction V, Armour Laboratories Co.

Preparation of Cytoplasmic Protein——Supernatant fractions were prepared from the homogenates of the liver, kidney, and small intestinal mucosa of adult male Donryu rats and male rabbits in the following manner. Rats and rabbits were anesthetized with ether and intraperitoneal injection of pentobarbital sodium, respectively. The liver was perfused with ice-cold 0.25 M sucrose through the portal vein to remove the blood. The kidney was perfused through hilar vessels with ice-cold 0.25 M sucrose to remove as much blood as possible. The small intestine was removed from duodenum to terminal ileum, washed with ice-cold 0.25 M sucrose, and opened lengthwise, and the mucosa was scraped with a glass slide. Various tissues were rapidly removed, and perfused with ice-cold 0.25 M sucrose, and a 25% homogenate was prepared in 0.25 sucrose–0.01 M Na-phosphate buffer (pH 7.4) using a moter-driven teflon pestle homogenizer. The homogenate was filtered through cheesecloth, and centrifuged at 105000 ***g*** for 120 min with a Hitachi 65P ultracentrifuge at 2°. After the surface lipid was removed, the supernatant fraction was carefully collected, and used for the gel filtration studies. Unless immediately used, the supernates were stored at −20°.

***In Vitro* Gel Filtration with Sephadex G-75**——Various concentration of BSP (0.1—30 μmole) was added to the supernatant fraction, and the mixtures were incubated at 2° for 2 hr. After that the supernatant-BSP mixtures were placed on a Sephadex G-75 column equilibrated with 0.01 M phosphate buffer (pH 7.4) at 4°. The size of column was 3.2 × 92 cm or 3.5 × 90 cm. Elution was performed using the same buffer solution and the flow rate was 23—25 ml/hr. Each tube in the collector contained 4.0 ml. The elution patterns were monitored using a Hitachi 139 UV spectrophotometer: protein at 280 nm and BSP at 580 nm (after alkalization with 2N-NaOH). Further, protein was determined by the method of Lowry, *et al.*[10] using bovine serum albumin as a reference.

Results and Discussion

Binding Pattern of BSP with Rat Liver, Kidney, and Small Intestinal Mucosa Cytoplasmic Protein

Sephadex G-75 gel filtration of rat liver, kidney, and small intestinal mucosa cytoplasmic protein with BSP added *in vitro* yielded an elution pattern shown in Fig. 1. The binding of albumin fraction, Y protein, and Z protein with BSP was clearly noticed (Fig. 1a).

On the other hand, in the case of rat kidney protein, the binding of BSP to Y and Z protein was not observed distinctly even with 1.0 μmole BSP perhaps owing to their poor contents. However, two unknown peaks were recognized in the region of lower molecular weight than that of Y protein (Fig. 1b). The peaks appeared on repeated runs with all the

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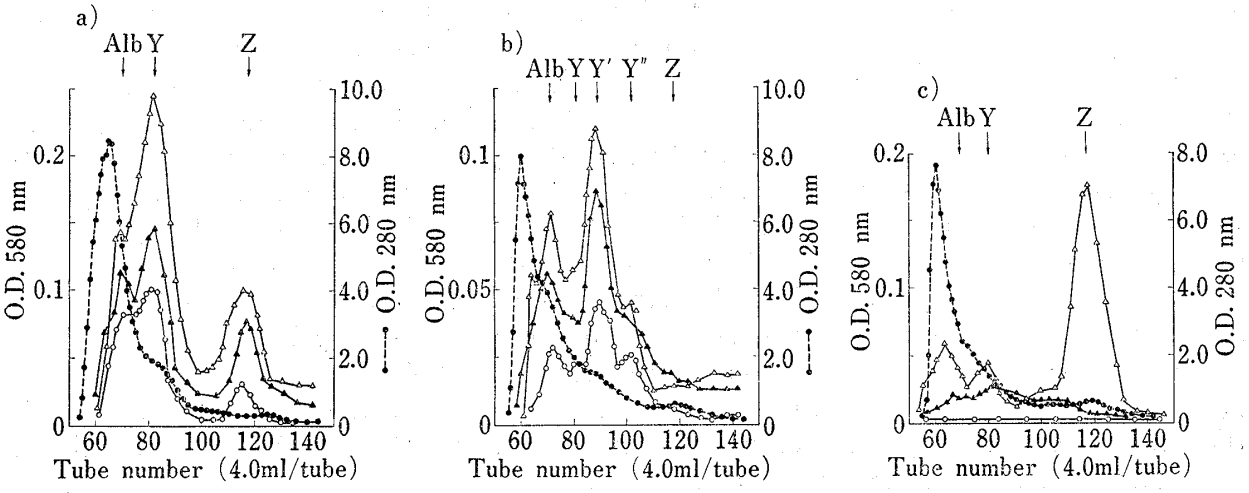

Fig. 1. Elution Pattern of BSP added to Rat Liver, Kidney, and Small Intestinal Mucosa Cytoplasmic Protein

a) 0.1 (○), 0.4 (▲), and 1.0 μmole (△) BSP with liver protein (180.0 mg)
b) 0.1 (○), 0.4 (▲), and 1.0 μmole (△) BSP with kidney protein (180.0 mg)
c) 1.0 (○), 2.5 (▲), and 5.0 μmole (△) BSP with small intestinal mucosa protein (151.7 mg)
The mixture was incubated for 2 hr at 2° and eluted from a column (3.5×90 cm) at flow rate 23—25 ml/hr. Optical density at 280 nm indicates protein and that at 580 nm (after alkalization) indicates BSP.

amounts (0.1—1.0 μmole) of BSP used. The fractions corresponding to the peaks are called Y′ and Y″ protein in this report.

The binding of BSP (1.0 and 5.0 μmole) to rat small intestinal mucosa cytoplasmic protein was also examined (Fig. 1c). Unlike the liver and kidney protein, BSP binding could not be detected with 1.0 μmole BSP. In the case of 2.5 μmole BSP, it was observed that BSP was bound to albumin fraction and Y protein but not to Z protein. However, with high amount of BSP such as 5.0 μmole, the binding to Z protein was the most prominent. This may indicate that Y and Z protein contents are much lower in the small intestinal mucosa than in the liver, and that Z protein is lower in the affinity to BSP but higher in the BSP binding capacity than Y protein.

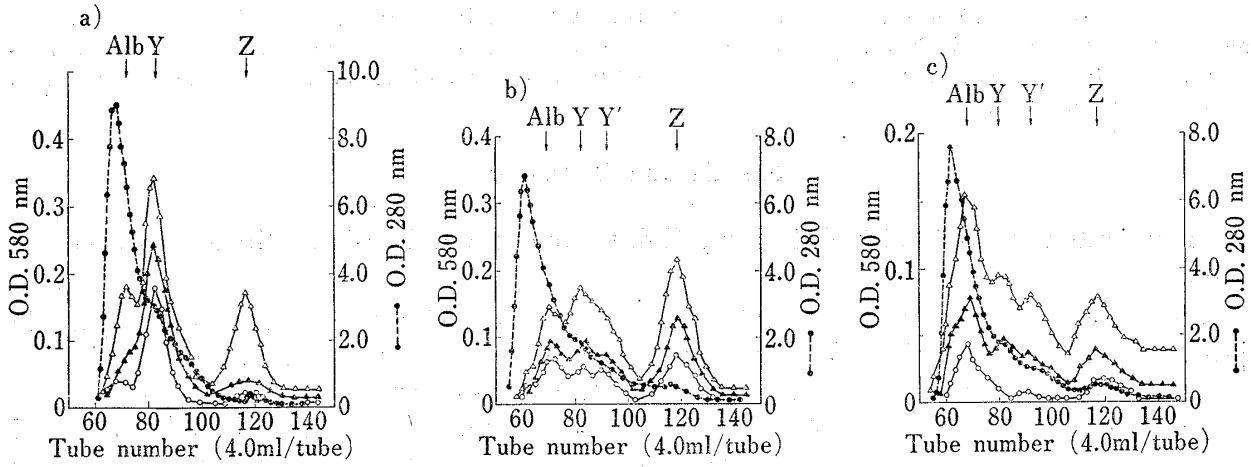

Fig. 2. Elution Pattern of BSP Added to Rabbit Liver, Kidney, and Small Intestinal Mucosa Cytoplasmic Protein

a) 0.1 (○), 0.4 (▲), and 1.0 μmole (△) BSP with liver protein (180.0 mg)
b) 0.1 (○), 0.4 (▲), and 1.0 μmole (△) BSP with kidney protein (180,0 mg)
c) 0.4 (○), 5.0 (▲), and 10.0 μmole (△) BSP with small intestinal mucosa protein (151.7 mg)
The mixture was incubated for 2 hr at 2° and eluted from a column (3.5×90 cm) at flow rate 23—25 ml/hr. Optical density at 280 nm indicates protein and that at 580 nm (after alkalization) indicates BSP.

Binding of BSP Added to Rabbit Liver, Kidney, and Small Intestinal Mucosa Cytoplasmic Protein

The elution pattern of liver, kidney, and small intestinal mucosa cytoplasmic protein from rabbit with BSP is shown in Fig. 2. The binding to Y protein was the highest in the liver with all over the amount of BSP used, but an increase in the binding to Z protein was remarkable with the increasing amount of BSP from 0.4 to 1.0 μmole (Fig. 2a). BSP was preferably bound to Y protein until the saturation was attained, after which BSP was progressively bound to Z protein.

In the rabbit kidney, binding to Z protein was higher than that to Y protein with 0.1—1.0 μmole BSP. However, binding to Y protein as well as to Z protein remarkably increased with an increase in the amount of BSP. Binding to Y′ protein was also detected in the kidney protein.

Binding to albumin fraction was the highest in the small intestinal mucosa with various amount of BSP, but with a higher amount of BSP (5.0 and 10.0 μmole) the binding to Y, Y′, and Z protein remarkably increased. However, the binding with BSP was generally lower than that in the other tissues (Fig. 2c).

Comparison of BSP Binding Patterns by Liver, Kidney, and Small Intestinal Mucosa Protein of Rat with Those of Rabbit

The binding of BSP to albumin fraction, Y and Z protein of rabbit liver was somewhat higher than that of rat (Fig. 3a). In the both species, BSP elution peak with Y protein was the highest in the liver among the three kinds of source tissues. This may support the important role[3-8] of Y protein on the hepato-biliary transport of BSP.

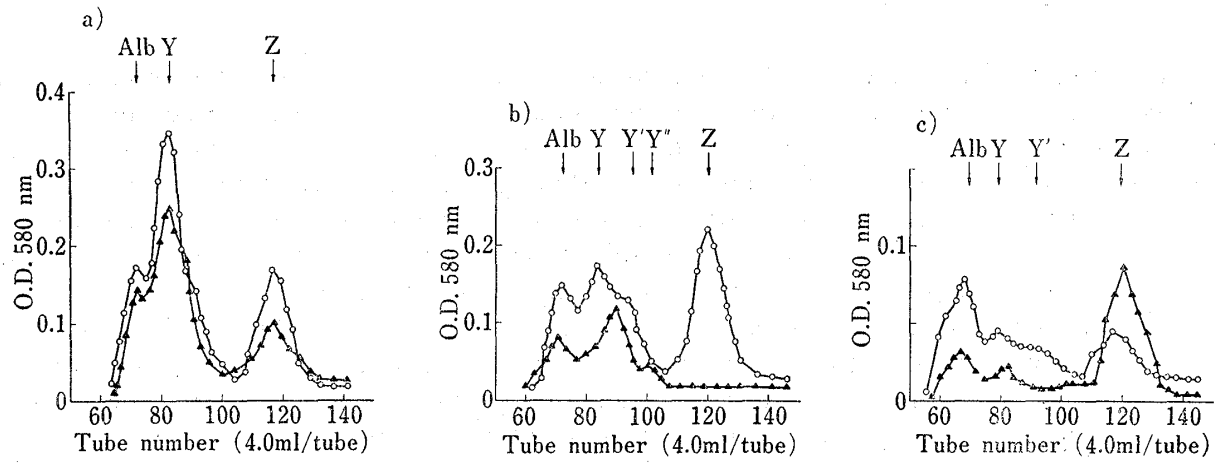

Fig. 3. Comparison of BSP Elution Patterns of Rat with Those of Rabbit

a) 1.0 μmole BSP with liver cytoplasmic protein (180.0 mg)
b) 1.0 μmole BSP with kidney cytoplasmic protein (180,0 mg)
c) 5.0 μmole BSP with small intestinal mucosa cytoplasmic protein (151.7 mg)
Optical density at 280 nm indicates protein and that at 580 nm (after alkalization) indicates BSP.
(▲): rat (○): rabbit

With kidney proteins, BSP elution pattern was different between rat and rabbit: Y and Z proteins were lacked in rat, and Y″ protein was not recognized in rabbit with 1.0 μmole BSP (Fig. 3b). It is noteworthy that kidney Z protein exhibited higher elution peak of BSP than liver Z protein in rabbit. It appears that the role of Z protein on the fate of organic anions in the body is not so simple as that suggested by Arias, *et al.*[3-7]

BSP binding of Y protein of rabbit intestinal mucosa was greater than that of rat, whereas the elution peak with Z protein in rabbit was higher in rat. The binding with Y′ protein was observed in cytoplasmic protein of rabbit mucosa.

The detailed properties of Y′ and Y″ proteins are now under investigation, and will be reported later.

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Physical Properties of Pyrimidine and Purine Antimetabolites. I. The Effects of Salts and Temperature on the Solubility of 5-Fluorouracil, 1-(2-Tetrahydrofuryl)-5-fluorouracil, 6-Mercaptopurine, and Thioinosine

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The effects of sodium chloride, sodium sulfate, and sodium iodide on the solubility of 5-fluorouracil, 1-(2-tetrahydrofuryl)-5-fluorouracil, 6-mercaptopurine, and thioinosine were studied. Sodium chloride and sulfate salted-out these antimetabolites while sodium iodide salted-in them. The solubility of these antimetabolites at various temperatures was also measured. There was no indication of the transition in the stable form in the temperature range studied.

We have earlier observed that alkylxanthines (caffeine, theophylline, theobromine, and caffeine derivatives) and uracil exhibited anomalous salting behaviors.[2)] Since pyrimidine and purine antimetabolites are also highly polar compounds, their physical properties in aqueous solutions can be different from those of slightly polar nonelectrolytes in water. Probably because of the highly polar nature of these antimetabolites, they are not solubilized by surfactants such as polysorbate 80 and sodium lauryl sulfate and not included into the interior cavity of the β-cyclodextrin molecule to any significant extent.[3)]

Since activity rather than concentration is important in partition (which applies to extraction from biological fluids into organic solvents prior to assay) and permeation (which applies to the controlled delivery), the salting behaviors of 4 pyrimidine and purine antimetabolites were examined. The effects of temperature were also determined in order to examine if there is any transition in the stable form with the change in temperature as was observed in ampicillin,[4)] cyclacillin,[5)] and sulfanilamide.[6)]

Experimental

Materials——5-Fluorouracil, 1-(2-tetrahydrofuryl)-5-fluorouracil (THFFU), and thioinosine were gifts from Kyowa Hakko Co., Taiho Pharmaceutical Co., and Morishita Seiyaku Co., respectively, 6-Mercaptopurine hydrate was purchased from Sigma Chemical Co. Sodium sulfate (anhydrous), sodium chloride, sodium iodide, and sodium thiosulfate (pentahydrate) were all of reagent grade and purchased from Wako Pure Chemical Industries.

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