Chem. Pharm. Bull. 19(12)2609—2616(1971)

UDC 615.31.011.4.034.076.9

Pharmacokinetic Studies of Biliary Excretion. IV. The Relationship between the Biliary Excretion Behavior and the Elimination from Plasma of Azo Dyes and Triphenylmethane Dyes in Rat[1,2]

Tatsuji Iga, Shoji Awazu, Manabu Hanano and Hisashi Nogami

Faculty of Pharmaceutical Sciences, University of Tokyo[3]

(Received June 30, 1971)

The relaionship between the biliary excretion behavior and the elimination from plasma was studied using the same series of azo and triphenylmethane dyes in the previous papers in rat. Amaranth (AM) and new coccine (NC) in azo dyes, and Brilliant blue FCF (BB) and light green SF (LG) in triphenylmethane dyes, were used for the comparison.

In azo dyes, the plasma elimination constants of NC, $0.034\times10^{-1}\text{min}^{-1}$ was almost three times samller than that of AM, $1.19\times10^{-1}\text{min}^{-1}$, but the elimination curves were considered similar with each other. And the difference of the binding ratio with plasma protein was only 10%. It was suggested that the remarkable difference of the biliary excretion behaviour between AM and NC, did not depend on the first step in the blood and that there were some other factors such as uptaking into liver celles, urinary excretion, metabolism and so on.

As for triphenylmethane dyes, the plasma elimination of BB was remarkably rapid and it's half life was about 2.8 min. And it was of interest that the bile to plasma concentration ratio reached more than 500 at 15 min after intravenous injection. The elimination of LG was very slow and it's half life was about 40 min and 15 times lager than that of BB. And the binding ratio of LG was 2.5 times larger than that of BB. It was concluded that the remarkable difference of biliary excretion between BB and LG depended mainly upon the difference of binding ratio with plasma protein and slow disappearance from blood of LG.

It has been known[4] that the biliary excretion depends on many factors, such as molecular weight, water solubility, chemical structure, uptake into liver cells and so on. In the previous papers,[5-7] the authors intended to try to elucidate the effect of sulfonate and halogen groups in the kinetic study using five series of water soluble tar dyes.[8] It was concluded that sulfonate group beyond other groups had a remarkable effect on the biliary excretion ratio and pattern, and that the position of a sulfonate group as well as the number was important to determine its effect.

On the other hand, it was also found that the halogen group had great influence on the biliary excretion as well as the sulfonate group, and was suggested that the effect of halogen group depended on the total electro-negativity of substituents.

It is said,[4] in general, that the biliary excretion has three steps in large clasification, *i.e.* the first step is the transfer form blood to liver, the second is some phenomena in the

1) Part III: T. Iga, S. Awazu and H. Nogami, *Chem. Pharm. Bull.* (Tokyo), **19**, 297 (1971).
2) Partial fulfilment of Doctor of Pharmaceutical Science degree requirement of Tatsuji Iga to the Graduate School, University of Tokyo.
3) Location: *Hongo, Bunkyo-ku, Tokyo.*
4) R.L. Smith, *Progr. Drug. Res.*, **9**, 229 (1966).
5) T. Iga, S. Awazu, M. Hanano and H. Nogami, *Chem. Pharm. Bull.* (Tokyo), **18**, 2431 (1970).
6) T. Iga, S. Awazu and H. Nogami, *Chem. Pharm. Bull.* (Tokyo), **19**, 273 (1971).
7) T. Iga, S. Awazu and H. Nogami, *Chem. Pharm. Bull.* (Tokyo), **19**, 297 (1971).
8) Azo dyes, triphenylmethane dyes, xanthene dyes, indigoid dye and sulfophthalene dye.

liver, for example uptaking into liver cells or metabolism, and the third is the transfer from liver into bile.

There are many factors affecting the biliary excretion in these three steps. In this paper the authors intended to study the above first step both *in vivo* and *in vitro*, and to compare the relationship between plasma elimination and biliary excretion behavior, using the same series of water soluble tar dyes in the previous papers.[5,6]

Experimental

Materials——Dyes used in this study were shown in Table I. All dyes were purchased from Wako Pure Chemical Industries, Ltd. and Tokyo Chemical Industries, Co., Ltd. The bovine serum albumin (BSA) was also purchased from Wako Pure Chemical Industries (powder, fraction V). And all other reagents were commercially available and of special grade.

Table I. Dyes used in This Study

Dye	Name		Molecular formula	Molecular weight	Absorption max (mμ)
Azo dyes	amaranth	(FD red No. 2)	$C_{20}H_{11}O_{10}N_2S_3Na_3$	604.5	522
	new coccine	(FD red No. 102)	$C_{20}H_{11}O_{10}N_2S_3Na_3$	604.5	507
Triphenylmethane dyes	brilliant blue FCF	(FD blue No. 1)	$C_{37}H_{34}O_9N_2S_3Na_2$	792.9	618
	light green SF	(FD green No. 2)	$C_{37}H_{34}O_9N_2S_3Na_2$	792.9	632

Drug Administration and Samplings——Male albino rats (Donryu) weighing 250—260 g were used. Bile fistula and femoral artery cannulation were operated for the excretion of the dye in bile and the elimination from plasma study. Thirty μmole of dye in 0.3 ml isotonic buffer solution were administered through a femoral vein, and bile and blood samples were taken at given times. Light ether anesthesia was used for the operation and samplings.

Binding with Bovine Serum Albumin (BSA) and Plasma Protein——The equilibrium dialysis methods were used. Three ml of BSA solution (concn. 10^{-4}M/liter) or rat plasma in Visking tube (Type 20/32) was put into a light resistant glass stoppered tube containing 30 ml isotonic pH 7.3 buffer solution of 1μ mole/ml dye concentration and dialyzed at 4° for 120 hr. The dye concentration was selected to correspond to approximately to the plasma concentration when the dye was administered intravenously.

Analytical Methods——1) Biliary Excretion Study: The procedure was carried out in the same way as described in the previous paper.[5] The excreted dye was determined as the equivalent amount to the authentic dye from the optical density at the wave length for each dye listed in Table I, using Hitachi 124 spectro-photometer.

2) Elimination from Blood Study: Light Green SF (LG): After adding 0.1 ml of 1/10 N *p*-toluenesulfonic acid to the 0.1 ml of plasma sample, the sample soution was diluted with 0.8 ml of de-ionized water and was measured the optical density at 640 mμ in microcell using Hitachi 124 spectro-photometer.

Other dyes: One tenth ml of plasma sample was diluted 4 or 5 times with de-ionized water and the optical density was measured at the wave length listed in Table I in microcell using Hitachi 124 spectrophotometer.

Result and Discussion

I. Elimination from Plasma

The elimination from plasma (blood) is the important first step in the biliary excretion or the enterohepatic circulation. There have been many reports[9–16] about the elimination

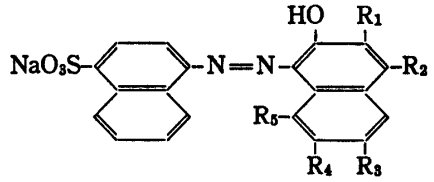
9) G.D. Delpart, Jr., *Arch. intern. Med.*, **32**, 401 (1923).
10) G.V. Taplin, O.M. Meredith and H. Kade, *J. Lab. clin. Med.*, **45**, 665 (1955).
11) W. Glaser, W.D. Gibbs and G.A. Andrews, *J. Lab. clin. Med.*, **54**, 556 (1959).
12) F.J. Ingelfinger, S.E. Bradley, A.I. Mendeloff and P. Kramer, *Gastroenterology*, **11**, 646 (1948).
13) A.I. Mendeloff, P. Kramer, F.J. Ingelfinger and S.E. Bradley, *Gastroentrology*, **13**, 222 (1949).
14) R.W. Brauer and R.L. Pessotti, *J. Pharmacol. Exptl. Therap.*, **97**, 359 (1949).
15) B. Combes, H.O. Wheeler, A.W. Childs and S.E. Bradley, *Trans. Assn. Amer. Physicianc.*, **69**, 276 (1956).
16) T.G. Richards, V,R, Tindall and A. Young, *J. Physiol.*, London, **138**, 38p (1957).

of dyes from plasma into bile up to this time. Particularly, in the clinical function test for liver, the disapperance of bromsulphthalein (BSP) has been extensively studied by many workers.[12-16] On the other hand, in the study of the biliary excretion mechanism, many organic compounds were often discussed with the bile to plasma concentration ratio.[4,17-19] Furthermore, in the physiological field, the uptaking into liver cells from blood or plasma has been well studied mainly about bromsulphthalein (BSP).[20-25]

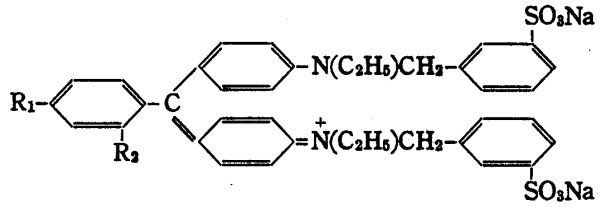
In this paper, the authors intended to try the kinetic study of the elimination from plasma using amaranth (AM) and new coccine (NC) in azo dyes, brillinat blue FCF (BB) and light green SF (LG) in triphenylmethane dyes. And the relationship between the plasma elimination pattern and the biliary excretion behavior reported in the previous papers,[5,6] was dis cussed.

Azo Dyes

AM and NC were selected in this series of dyes, since these two dyes have the same molecular weight and the numbers of sulfonate substituent on the common chemical structure as shown in Chart 1, but showed the remarkably different biliary excretion behaviors. As shown in the previous paper,[5] in the excretion ratio in 4 hr, AM was excreted into bile about 80%, but NC was only about 10%. And furthermore, in the excretion pattern, AM showed clearly the dose dependency in this dose range studied (3—30 μ mole) and typical high dose type (Type A)[26] in 30 μ mole doses. On the other hand, as for NC, the excretion was very slow and showed only low dose type (Type B)[26] in the same dose range. The biliary excretion behavior in the previous paper,[5] was shown in Fig. 1—3.



	R_1	R_2	R_3	R_4	R_5
amaranth (AM)	SO_3Na	H	SO_3Na	H	H
new coccine (NC)	H	H	SO_3Na	H	SO_3Na



	R_1	R_2
brilliant blue FCF (BB)	H	SO_3Na
light green SF (LG)	SO_3Na	H

Chart 1. Chemical Structure of Azo and Triphenylmethane dyes used in This Study

The elimination curves from plasma were shown in Fig. 4. It was found that the elimination followed the two compartment open system, and the pharmacokinetic constants were

17) L.S. Schanker, *Pharmacol. Rev.*, **14**, 501 (1962).
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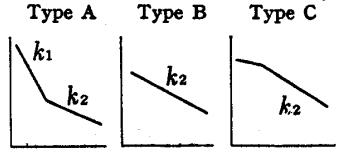
19) P.K. Nayak and L.S. Schanker, *Am. J. Physiol.*, **217**, 1639 (1969).
20) G.L. Robinson and E.S. Snell, *J. Clin. Invest.*, **39**, 236 (1960).
21) H.O Wheeler, J.I. Meltzer and S.E. Bradley, *J. Clin. Invest.*, **39**, 1131 (1960).
22) C.D. Klaassen and G.L. Plaa., *J. Appl. Physiol.*, **22**, 1151 (1967).
23) C.A. Goresky, *Am. J. Physiol.*, **207**, 13 (1964).
24) R.H. Adams, J. Gordon and B. Combes, *Gastroenterology*, **51**, 373 (1966).
25) C.D. Klaassen and G.L. Plaa., *Am. J. Physiol.*, **215**, 971 (1968).
26) Type A Type B Type C



see ref. 5 for detail.

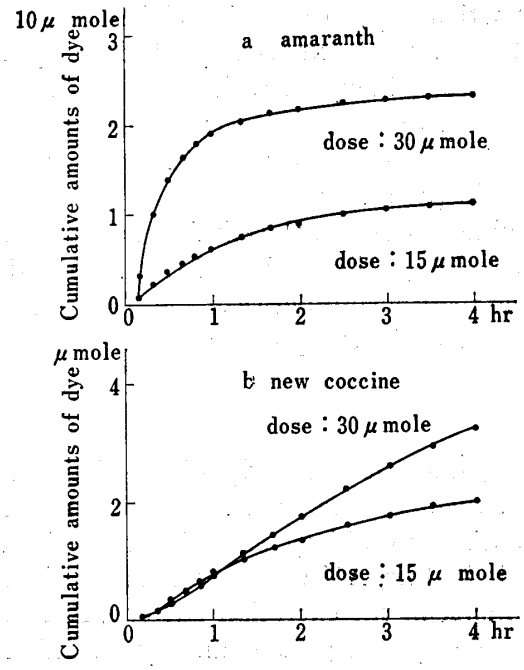


Fig. 1. (a) Cumulative Amarnath (AM) Excretion Curves in Bile. (b) Cumulative New Coccine (NC) Excretion Curves in Bile

data from the previous paper[5]

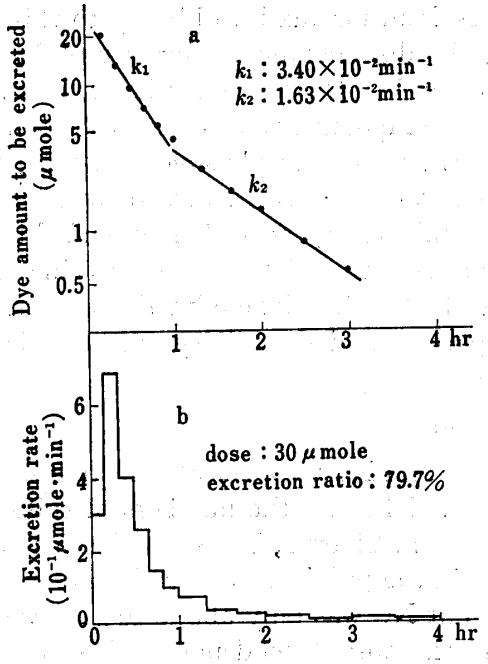


Fig. 2. (a) Semilogarithmic Plots of Amaranth (AM) in the Body to be Excreted in Bile. (b) Averaged Excretion Rate of Amaranth (AM).

data from the previous paper[5]

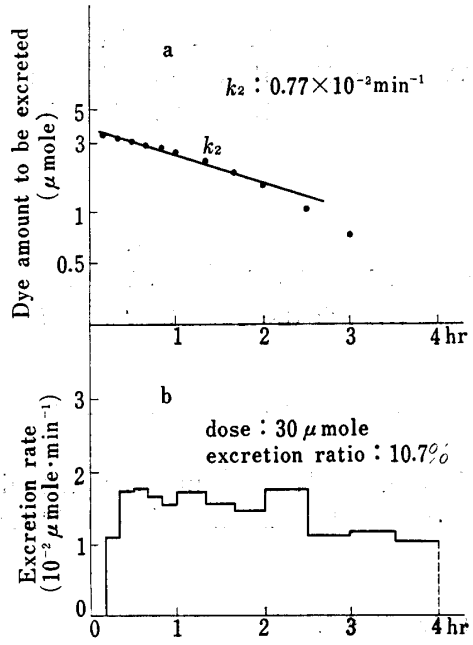


Fig. 3. (a) Semilogarithmic Plots of New Coccine (NC) in the Body to be Excreted in Bile. (b) Averaged Excretion Rate of New Coccine (NC).

data from the previous paper[5]

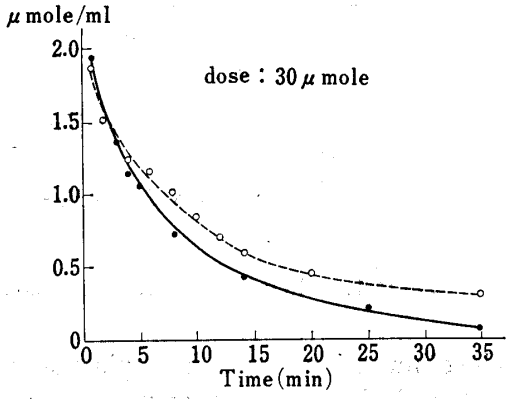


Fig. 4. Plasma Elimination Curves of Amaranth (AM) and New Coccine (NC).

○: observed values of Amaranth
●: observed values of New Coccine
———: calculation curve of Amaranth
- - - - - - -: calculation curve of New Coccine

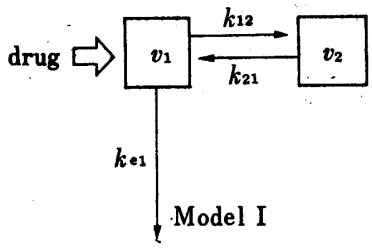
calculated with the iterative least square method programed as the routine in our laboratory.[27] The results of the calculation were shown in Table II.

27) H. Nogami, M. Hanano, S, Awazu and H.H. Moon, *Chem. Pharm. Bull.* (Tokyo), **17**, 2097 (1969).

TABLE II. Pharmacokinetic Constants for the Two Compartment Model[a)]

Dye	k_{12}[a,b)]	k_{12}[a,b)]	k_{e1}[a,b)]	$T_{1/2}$[c)]	V_1[a,d)]	V_2[a,d)]
Amaranth (AM)	0.097	0.190	0.119	5.8	12.6	6.4
New coccine (NC)	0.068	0.034	0.034	20.5	15.4	31.3
Brilliant blue FCF (BB)	0.266	0.330	0.252	2.8	11.6	9.3
Light green SF (LG)	0.065	0.085	0.017	40.8	12.5	9.6

a) calculation was carried out with the least square iteration method programed in our laboratory (Model I.)[27]
b) min^{-1}
c) $T_{1/2}$ (min) $= 0.693/k_{e1}$
d) ml



Although the elimination constant of NC, 0.34×10^{-1} min^{-1} was about three times smaller than that of AM, 1.19×10^{-1} min^{-1}, the elimination curves were considered similar with each other. Since plasma elimination curve could not explain the different pattern of biliary excretion of these dyes, it was suggested that there were some other factors affecting the biliary excretion, such as the difference of uptaking into liver cells, metabolism, affinity to the tissue proteins, urinary excretion and so on. As for the binding to the plasma protein and bovine serum albumin, it is discussed in the latter section.

Triphenylmethane Dyes

In this series of dyes, brilliant blue FCF (BB) and light green SF (LG) were studied, since these two dyes, as well as AM and NC in the former section, have the same molecular

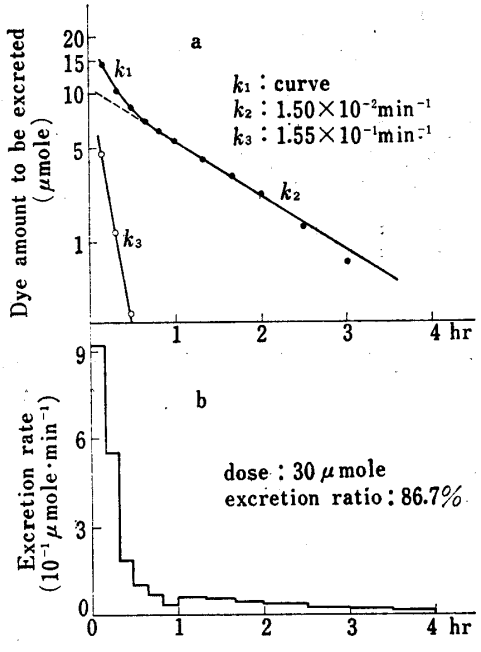


Fig. 5. (a) Semilogarithmic Plots of Brilliant Blue FCF (BB) in the Body to be Excreted in Bile. (b) Averaged Excretion Rate of Brilliant Blue (BB)

—●—: observed values
—○—: secondary plots
data from the previous paper[6)]

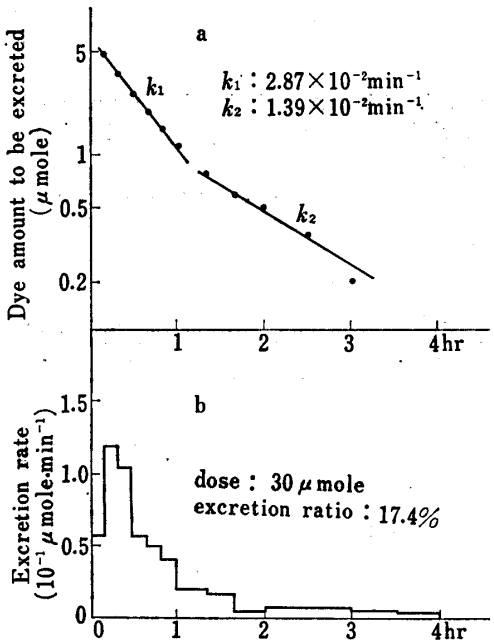


Fig. 6. (a) Semilogarithmic Plots of Light Green SF (LG) in the Body to be Excreted in Bile. (b) Averaged Excretion Rate of Light Green SF (LG).

data from the previous paper[6)]

weight and numbers of sulfonate substituent on the common structure as shown in Chart 1. As reported in the previous paper,[6] the biliary excretion ratio in 4 hr of BB was above 90%, but that of LG was only about 20%. On the other hand, the excretion patterns were not so different from each other, though in the Nelson plot, BB showed curve line in the period corresponding to k_1 of high dose type (Type A) as shown in Fig. 5, 6.

The elimination curves from plasma of these two dyes, were shown in Fig. 7. It was found that the elimination also followed the two compartment open model as well as AM and NC, and the data were calculated with the same program. The pharmacokinetic constants were listed in Table II.

From these results, it was found that BB eliminated from plasma very rapidly after intravenous injection and the half life of it's plasma elimination ($T_{1/2}$) was about 2.8 min. And this value was the shortest one amonng dyes of this series. Furthermore, the fact that the large amount (more than 80%) of the dye was excreated in the early period (0—30 min), meant the poor uptaking into liver cells or binding to the liver tissue proteins. These results coincided well with that BB was excreted in bile concentrically and rapidly in short time. The plasma elimination curve and the cummulative excretion curve in bile until 60 min after 30 μ mole doses were shown in Fig. 8.

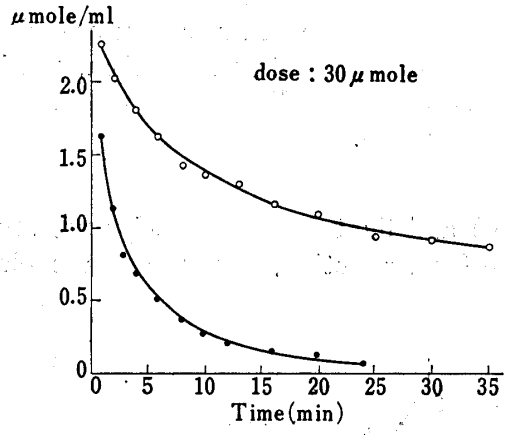


Fig. 7. Plasma Elimination Curves of Brilliant Blue FCF (BB) and Light Green SF (LG)

● : observed values of Brilliant Blue FCF
○ : observed values of Light Green SF
——: calculation curves

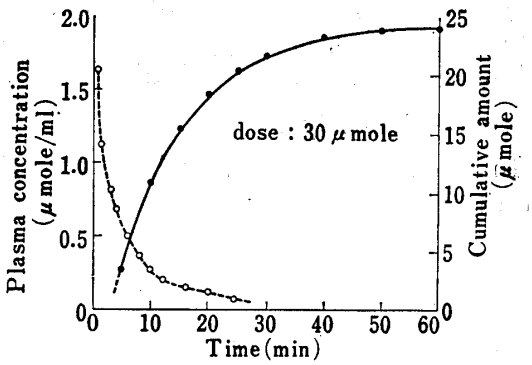


Fig. 8. Cumulative Excretion Curve in Bile and Plasma Concentration Time Course of Brilliant Blue FCF (BB).

—●—: cumulative excretion curve in bile
---○---: plasma concentration time course

The bile to plasma concentration ratio of every five minuts till 25 min after intravenous injection of BB in the same experiment in Fig. 8, were shown in Table III. It is of interest

Table III. The Bile to Plasma Concentration Ratio[a]

Time (min)	Concentration (μ mole/ml)			Bile/Plasma Concentration ratio	
	Bile	Plasma			
5	55.51[b]	0.50[c]	0.48[d]	111[c]	116[d]
10	110.40	0.27	0.29	409	381
15	92.21	0.16	0.15	576	615
20	54.10	0.13	0.09	276	601
25	35.90	0.06	0.07	400	513

a) the same experiment in Fig. 8
b) average concentration for 5 min
c) observed value
d) calculated value

that these ratios showed remarkable great values and that at 15 min the biliary concentration reached more than 500 times of plasma concentration.

On the other hand, as for LG, the elimination from plasma was much slower than that of BB, and the concentration in plasma was maintained high level even at 30 min after intravenous injection (Fig. 7). The half life of this dye was about 40 min and 15 times larger than that of BB. It was suggested that the binding ratio of LG to the plasma protein was very high and that the concentration was kept in plasma for relatively long time, and this would cause to delay its biliary excretion. As for the binding to the rat plasma protein and BSA, it is discussed in the latter section.

In the comparison of the pharmacokinetic constants, BB showed larger values than LG except for distribution volumes. For example, the elimination rate constant (k_{el}) of BB was $2.52\times10^{-1}\,min^{-1}$, but that of LG was $0.17\times10^{-1}\,min^{-1}$, and the former was 20 times larger than the latter as shown in Table II.

II. Binding with Plasma Protein and Bovine Serum Alubumin (BSA)

It is well known that a large number of drugs are carried in the body in the bound form with plasma proteins such as serum albumin. Therefore, in the study of the elimination from plasma or in the estimation of the plasma concentration, the binding with the plasma protein has a great importance, and often gives much influence to the effectivity of the drug.

In this present study, the influence of the binding with rat plasma protein and bovine serum albumin (BSA) on the biliary excretion, were studied *in vitro* using equilibrium dialysis method. The results after dialysis at 4° for 120 hr were listed in Table IV.

Table IV. The Relationship between the Binding Ratio and the Biliary Excretion Ratio

Dye	Binding Ratio (%)[a]		Excretion Ratio in 4 hr (%)[b,c]
	BSA	Rat Plasma Protein	
Amaranth (AM)	36.0	57.9	82.8
New coccine (NC)	41.3	68.7	10.9
Brilliant blue FCF (BB)	16.2	39.7	93.7
Light green SF (LG)	86.9	91.2	21.6

a) Visking tube contained three ml BSA (Bovine Serum Albumin) solution (concn. 10^{-4}M/ml) or rat plasma (mixture of 30 animals) and the external phase contained 30 ml isotonic pH 7.3 buffer solution. The initial dye concentration was 1 μ mole/ml in the external phase. The results were obtained after dialysis for 120 hr at 4°.
b) data from the previous papers[4,6]
c) The value was the average of 8—9 animals.

In the comparison between AM and NC in azo dyes, the binding ratio with rat plasma of these dyes were 57.9% and 68.7%, respectively, and the difference was only about 10%. On the other hand, the binding ratio with BSA (concn. 10^{-4} M/liter) of AM was 36.0% and that of NC was 41.3%. It was well elucidated from these results that the pharmacokinetic constants listed in Table II did not show great differences for each other. It was, furthermore, suggested that the remarkable difference of the biliary excretion behavior between AM and NC, did not depend on the first step in the blood and that there were some other factors affecting the biliary excretion behaviors of these dyes. A future detailed study will be necessary for these dyes.

As for BB and LG, the authors reported in the previous paper[6] that the binding ratios with plasma protein after dialysis at 37°, were 65.3% and 94.7%, respectively. Since the dialysis of plasma at 37° for more than one hundred hours as in the previous paper was considered to cause denaturation of plasma protein, the dialysis temperature was kept at 4° in the

present study. As shown in Table III, the results at 4° showed the similar tendency of those at 37°.

From these results, it was concluded that the remarkable difference of biliary excretion between BB and LG depended mainly upon the difference of binding ratio with plasma protein and slow disapperance from blood of LG.

Acknowledgement This work was supported by the Grant-in Aid for Scientific Research provided by the Ministry of Eduaction to which the authors are grateful. We are also grateful to Dr. Tohru Fuwa for his kind advice.