[Chem. Pharm. Bull. 18(12)2431—2440(1970)]

UDC 547.556.33.09 : 547.758.09 : 615.31.034

Pharmacokinetic Studies of Biliary Excretion. I. Comparison of the Excretion Behavior in Azo Dyes and Indigo Carmine[1]

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(Received May 14, 1970)

The kinetic investigation about the factors affecting the biliary excretion was tried with azo and indigoid dye in rat. Five azo dyes, *i.e.*, Azorubin S, Amaranth, New Coccine, Ponceau R, Ponceau SX, and Indigo Carmine as indigoid dye were used.

(1) All dyes except New Coccine used here began to be excreted within 2—5 min and the peak of the excretion rate appeared between 10 and 20 min after administration. But as for New Coccine which had the very low excretion ratio, the delay of the excretion was observed and the peak of the excretion rate was hardly observed.

(2) The dose dependency of biliary excretion which had been found in riboflavin was also found in dyes, and accordingly, the dependency was considered not to be specific to a biological substance such as riboflavin.

(3) In the excretion rate constant, all dyes which showed high dose type excretion pattern had two rate constants k_1 and k_2 as shown in riboflavin, and the ratios of them were between 1.4 and 3.

(4) It was also found that not only the number of sulfonate groups, but also the position of them had an effect on the biliary excretion ratio.

(5) The biliary excretion pattern of Indigo Carmine which is likely to be excreted in urine was not different essentially from those of azo dyes of which the ratio was high.

The biliary excretion plays one of the most important roles in the transfer processes of drugs in the body. Particularly the enterohepatic circulation, forming with the reabsorption in gut after biliary excretion, gives great infiuence on the toxicity and sustained action of drugs. It has been known[3] that biliary excretion depends on many factors, such as molecular weight, water solubility, chemical structure, number and position of substituents and so on. Schanker[4] reported that all molecules and ions whose size is less than that of protein molecules tend to be excreted in bile. On the other hand, Brauer[5] divided compounds which make their appearance in bile into three classes according to their concentration in bile in relation to their concentration in blood. Furthermore, Williams[6] discussed the relationship with molecular weights, metabolism and toxicity. Although there are many studeis, concerning the biliary excretion and its mechanism as stated above, "The structural factor is not yet clearly defined" as Williams pointed out.[6]

In order to solve some factors affecting the biliary excretion, the authors have intended the kinetic study using water soluble tar dyes which are used as fed dye or for hepatic function test, since they have low toxicity and low absorption in gut. As they have, still more, many derivatives in chemical structure series, such as azo dyes, triphenylmethane dyes, phthalein

1) Partial fulfillment of Doctor of Pharmaceutical Science degree requirement of Tatsuji Iga to the Graduate School, University of Tokyo.
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3) R.L. Smith, *Progr. Drug. Res.*, as a review article, **9**, 299 (1966).
4) L.S. Schanker, *Pharmacol. Rev.*, **14**, 501 (1962).
5) R.W. Brauer, *J. Am. Med. Assoc.*, **169**, 1462 (1969).
6) R.T. Williams, P. Millburn and R.L. Smith, *Ann. N.Y. Acad. Sci.*, **123**, 110 (1965).

dyes and are easily determined quantitatively, and their ratios of biliary excretion are high, therefore they are convenient substrates to study the excretion pattern, mainly respective to the chemical structures. In the present paper, the excretion pattern and excretion ratio and the effects of the position of sulfonate groups have been studied for five azo dyes[7] of which molecular weights are between about 450 and 600. And in addition, the excretion pattern of Indigo Carmine as indigoid dye,[7] one of indigoid dyes has been compared with that of azo dyes. Indigo Carmine which is used as fed dye or for renal function test, has 466 molecular weight and is known to have the low biliary excretion ratio. The reason of the low ratio has been also discussed in the present study.

Experimental

Drug Administration and Samplings——Male albino rats (Donryu) weighing 250—260 g were used. Bile fistula cannulation was operated for the excretion of the drug study. The drug was administered through a femoral vein, and bile samples were taken at given times. Light ether anesthesia was used for the operation and samplings.

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. All other reagents were commercially available and of special grade.

Analytical Methods——After 0.1 ml bile sample was diluted 30 times by de-ionized water, the sample was centrifuged at 0—5° for 10 min, at 12000 rpm of Kubota KRP-6 Cnetrifuge (about $12000 \times g$). The excreted dye was determiend 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.

Table I. Dyes used in This Study

Type	Name	Molecular formula	Molecular weight	Absorption max. (mμ)
Azo dyes	Azorubin S	$C_{20}H_{12}O_7N_2S_2Na_2$	502.45	515
	Amaranth (FD Red No. 2)	$C_{20}H_{11}O_{10}N_2S_3Na_3$	604.50	522
	New Coccine (FD Red No. 102)	$C_{20}H_{11}O_{10}N_2S_3Na_3$	604.50	507
	Ponceau R (FD Red No. 101)	$C_{18}H_{14}O_7S_2Na_2$	452.42	504
	Ponceau SX (FD Red No. 4)	$C_{18}H_{14}O_7S_2Na_2$	452.42	500
Indigoid dye	Indigo Carmine (FD Blue No. 2)	$C_{16}H_8N_2O_8S_2Na_2$	466.37	610

Result and Discussion

In the previous paper[8] the excretion pattern of riboflavin was studied for rather long period, 24 hr, and it was found that the large amount of excretion occurred at the early stage when a high dose (1—10 μmole) was administered. While a low dose (0.1 μmole) was administered, the early excretion was slow and a kind of delay period was found. In the present paper, since the excretion was almost completed until 4 hr except in the case of NC as shown later, the data until the time were used to study the biliary excretion pattern.

The bile flow rate, under light ether anesthesia tended to fluctuate somewhat until 1 hr and after that it became rather constant, while the body temperature decreased about 2.5—3.5° at initial 30 min and attained the relatively constant temperature during the experiment. But the effect of fluctuation on the excreted amount of a dye was found to be neglected. Being shown in Fig. 1 as the typical example, the bile flow rate was rather different each other, but the similar excretion patterns were obtained.

7) The symbols for dyes in the present study are as follows: Azorubin S=AS, Amaranth=AM, New Coccine=NC, Ponceau R=PR, Ponceau SX=PX, Indigo Carmine=IC.

8) H. Nogami, M. Hanano, S. Awazu and T. Iga, *Chem. Pharm. Bull.* (Tokyo), **18**, 228 (1970).

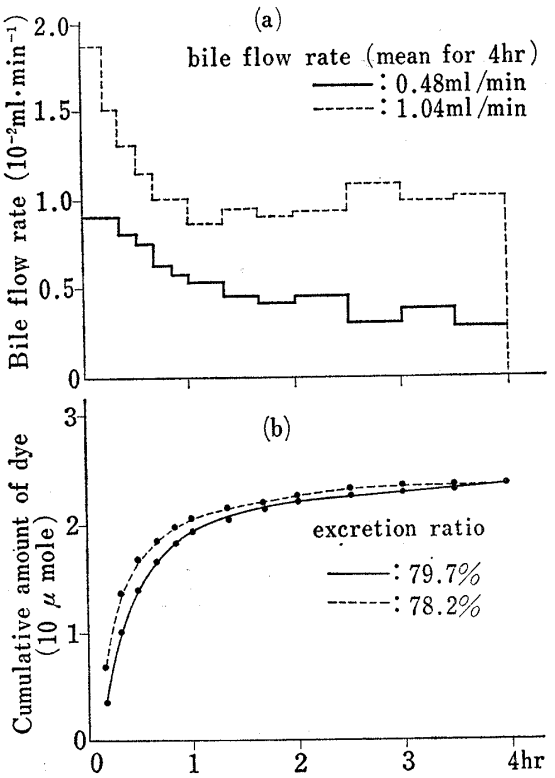


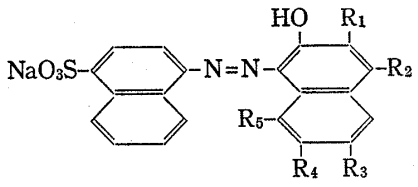
Fig. 1. (a) The Mean Bile Flow rate for 4 hr
(b) Cumulative Dye Excretion Curves in Bile

Thirty micro mole of Amaranth (AM) was administered.

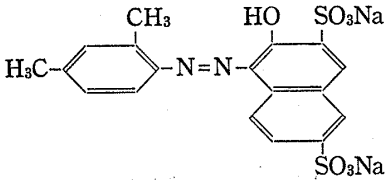
Azo Dyes

Azo dyes have been used as the most popular fed dyes, and the metabolism and excretion of them have been extensively studied by many workers[9–12] up to this time. Among azo dyes used here, AS is generally used for hepatic function test as well as bromosulfophthalein, and the other four, AM, NC, PR[13] and PX[13] have been used frequently as popular fed dyes for a long time. Their molecular weights are between about 450 and 600. As shown in Chart 1 AS, AM and NC have two naphthalene structures linked with azo linkage and are differentiated only with position of sulfonate groups. PR and PX have one naphthalene structure and are different each other in the position of azo benzene and substituents.

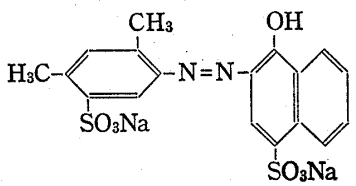
As for the relation between biliary excretion and chemical structures, Ryan and Wright[11] reported that carboxyl groups of Red 10 BS, Granine 2-GS and Tartrazine have effects on the excretion. Radomsky and Mellinger[10] reported about the metabolism of azo dyes that the orally administered AM, PX and sunset yellow in rats are reduced with azo reductase in gut and afterwards the metabolites are excreted in urine.



	R_1	R_2	R_3	R_4	R_5
Azorubin S (AS)	H	SO_3Na	H	H	H
Amaranth (AM)	SO_3Na	H	SO_3Na	H	H
New Coccine (NC)	H	H	SO_3Na	H	SO_3Na



Ponceau R (PR)



Ponceau SX (PX)

Chart 1. Chemical Structure of Azo Dyes used in This Study

(1) Excretion Ratio——Since some azo dyes used here showed dose dependency on the biliary excretion as written in later, the excretion ratios of 15 and 30 μ mole doses were listed in Table II.

9) J.W. Daniel, *Toxicol. Appl. Pharmacol.*, **4**, 572 (1962).
10) J.L. Radomsky and T.J. Mellinger, *J. Pharmacol. Expt. Therap.*, **136**, 259 (1962).
11) A.J. Ryan and S.E. Wright, *Nature* (London), **195**, 1009 (1962).
12) M. Ishidate, Z. Tamura, T. Nakajima and K. Samejima, *Chem. Pharm. Bull.* (Tokyo), **10**, 75 (1962).
13) In recent years, PR and PX have been inhibited to use as fed dyes in our country.

Table II. Averaged Biliary Excretion Ratio (%)[a]

Dye	Dose (μmole) 15	30
Azorubin S	57.1 (2)[b]	53.8 (3)[b]
Amaranth	81.5 (3)	82.8 (3)
New Coccine	12.3 (2)	10.9 (3)
Ponceau R	41.9 (3)	42.0 (3)
Ponceau SX	66.4 (2)	71.3 (3)
Indigo Carmine	7.3 (2)	10.3 (3)

a) ratio $= \frac{\text{excreted amount in 4 hr}}{\text{dose}} \times 100$ (%)

b) number of experiments

In respect of relation between chemical structure and excretion, it was found that AM was the most excretable among AS, AM and NC. These three have the common basic structure and only difference is that AS has two sulfonate groups but the other have three. In comparison between AS and AM, the former had about 50% excretion ratio but the latter had almost 80% and it was suggested that the number of sulfonates has a remarkable effect on their excretion in bile. This agrees well with the results of Radowsky and Mellinger[10] that AM is more excretable than Ponceau SX and sunset yellow which have two sulfonate groups. But on the other hand, NC which has three sulfonate groups had 10—13% excretion ratio until 4 hr and after that it was still excreted for a rather long time. The difference between the most excretable AM and the least excretable NC is that the former has a sulfonate group at R_1 and the latter has at R_5. This was considered to show that the position of sulfonate group has an effect on the excretion ratio as well as the number of the groups. As for PR and PX which are not in the series of the above dyes, it was found that the excretion ratio of PR was about 40% and on the other the ratio of PX was almost 70%, although they have the same molecular weights and the same number of sulfonate groups. Since the former has the groups in the side of azo linkage, while the other in the both side of the linkage as shown in Chart 1, the difference in the excretion ratios was also considered to suggest that the position of sulfonate group was one of the factors which affect the biliary excretion ratio. The position effect has been found also in the studies with triphenylmethane dyes in the authors' laboratory, and will be reported in the next paper.

(2) Excretion Pattern——All dyes except NC used here began to be excreted within 2—5 min and the peak of the excretion rate appered between 10 and 20 min after administration. But as for NC which had the very low excretion ratio, the delay of the excretion was observed and the peak of the excretion rate was hardly observed in 4 hr. The delay period tended to increase as the dose increased. These excretion patterns were shown in Fig. 2—14. And moreover, Nelson plots[14] were obtained from the cumulative curve of the excretion.

In the previous paper,[7] as shown in Fig. 15, Nelson plots of riboflavin were found to be divided into high dose type and low dose type which are termed type A and type C in this series of study, respectively. Type A was composed of two straight lines which gave two rate constants k_1 and k_2. Type C gave one straight line after the early lag period. Since the process of the straight line was considered to be the same with that of the rate constant of k_2, the rate constant of type C was designated to k_2 in the previous paper. As illustrated in Fig. 3—14, azo dyes used here also showed the similar patterns. The values of k_1 and k_2 obtained from Nelson plots for each dye were listed in Table III.

14) E. Nelson, *J. Pharm. Sci.*, **132**, 181 (1961).

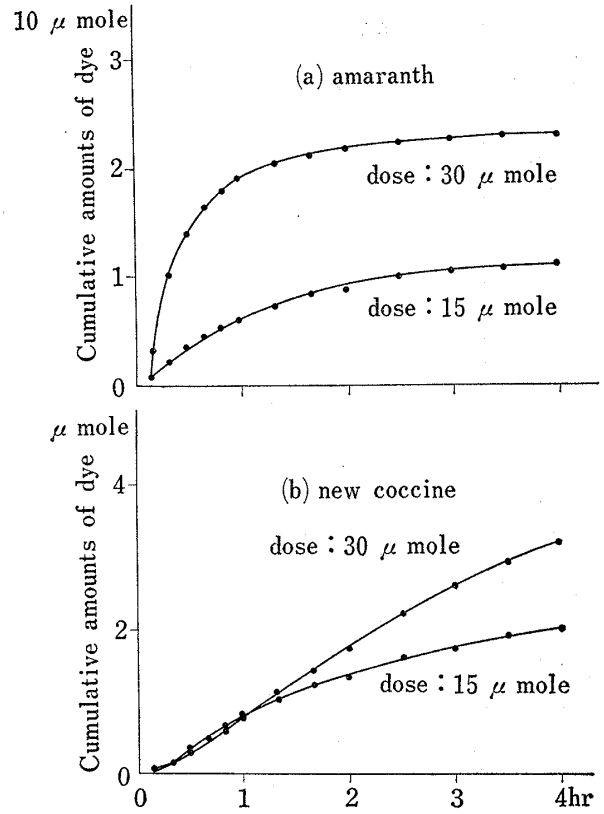


Fig. 2. (a) Cumulative Amaranth (AM) Excretion Curves in Bile
(b) Cumulative New Coccine (NC) Excretion Curves in Bile

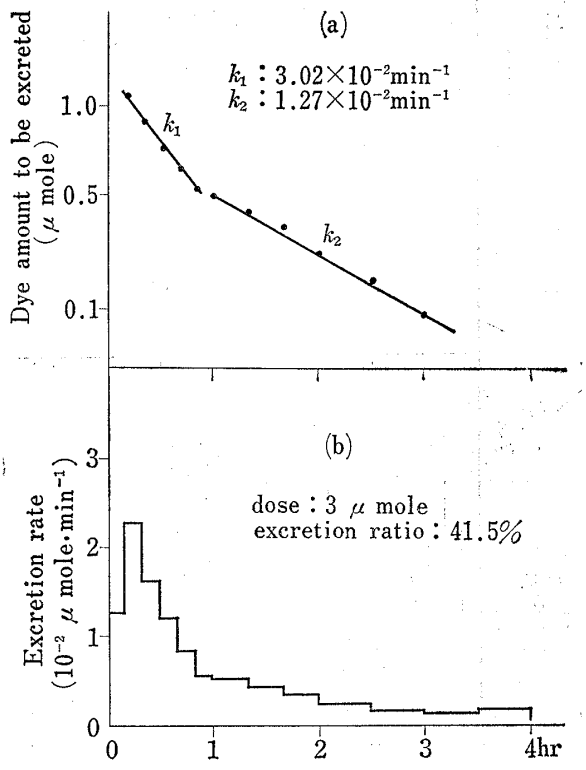


Fig. 3. (a) Semilogarithmic Plots of Azorubin S (AS) in the Body to be Excreted in Bile
(b) Averaged Excretion Rate of Azorubin S (AS)

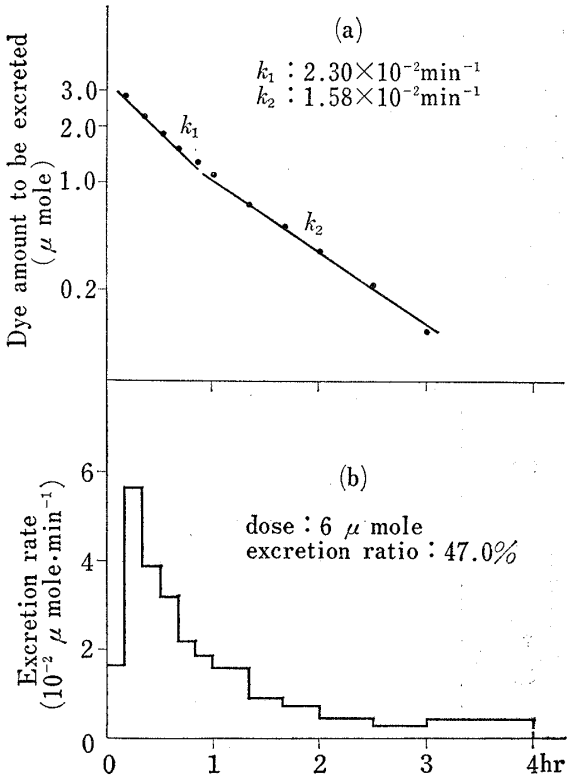


Fig. 4. (a) Semilogarithmic Plots of Azorubin S (AS) in the Body to be excreted in Bile (b) Averaged Excretion Rate of Azorubin S (AS)

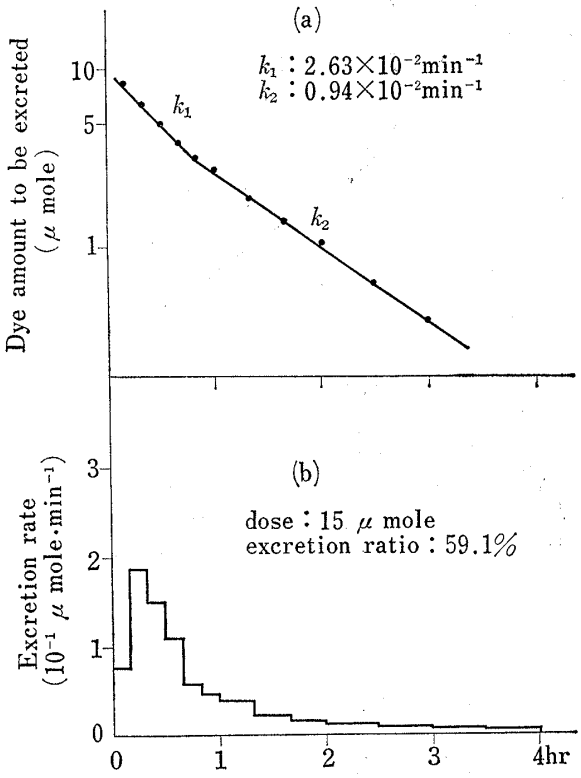


Fig. 5. (a) Semilogarithmic Plots of Azorubin S (AS) in the Body to be excreted in Bile (b) Averaged Excretion Rate of Azorubin S (AS)

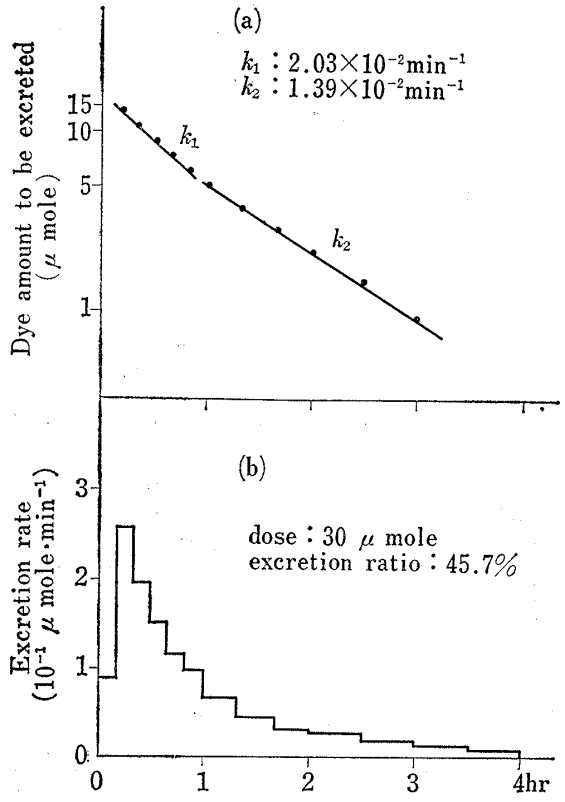


Fig. 6. (a) Semilogarithmic Plots of Azorubin S (AS) in the Body to be excreted in Bile
(b) Averaged Excretion Rate of Azorubin S (AS)

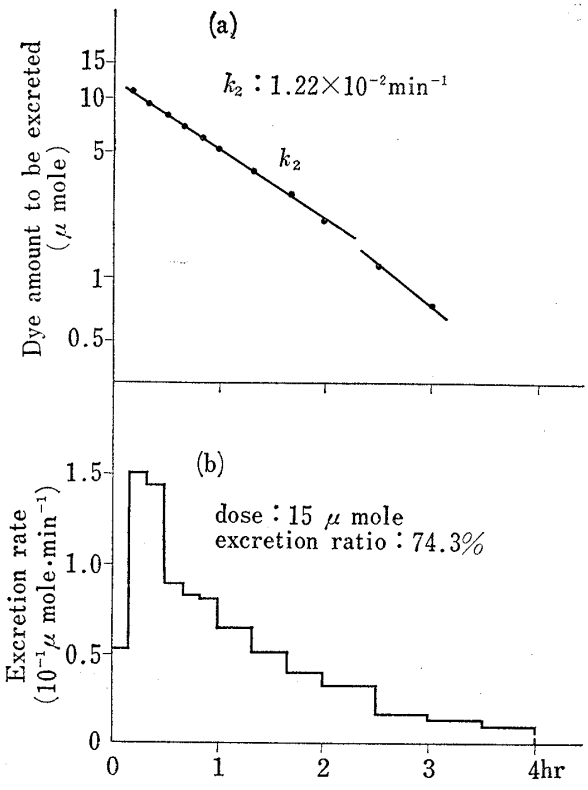


Fig. 7. (a) Semilogarithmic Plots of Amaranth (AM) in the Body to be excreted in Bile
(b) Averaged Excretion Rate of Amaranth (AM)

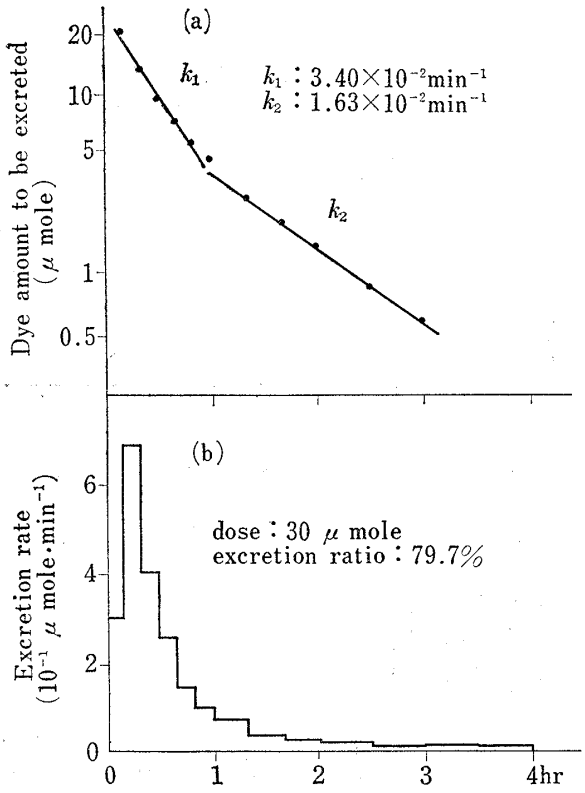


Fig. 8. (a) Semilogarithmic Plots of Amaranth (AM) in the Body to be excreted in Bile
(b) Averaged Excretion Rate of Amaranth (AM)

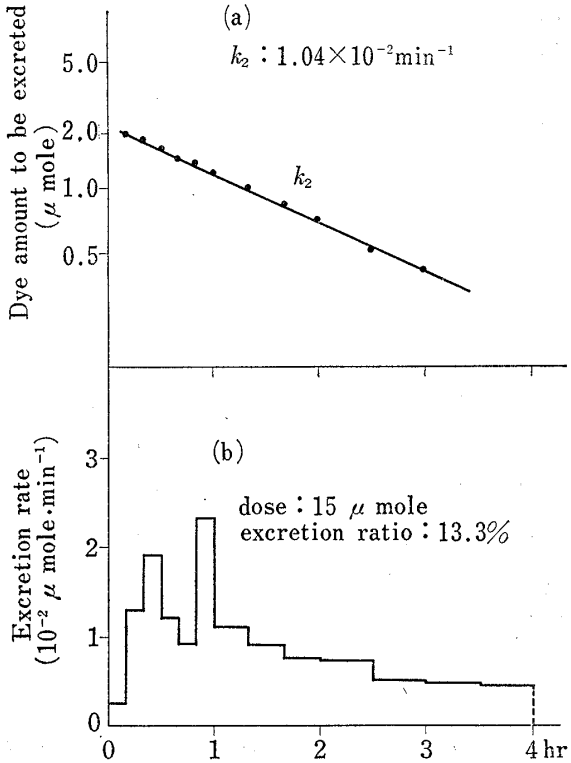


Fig. 9. (a) Semilogarithmic Plots of New Coccine (NC) in the Body to be excreted in Bile
(b) Averaged Excretion Rate of New Coccine (NC)

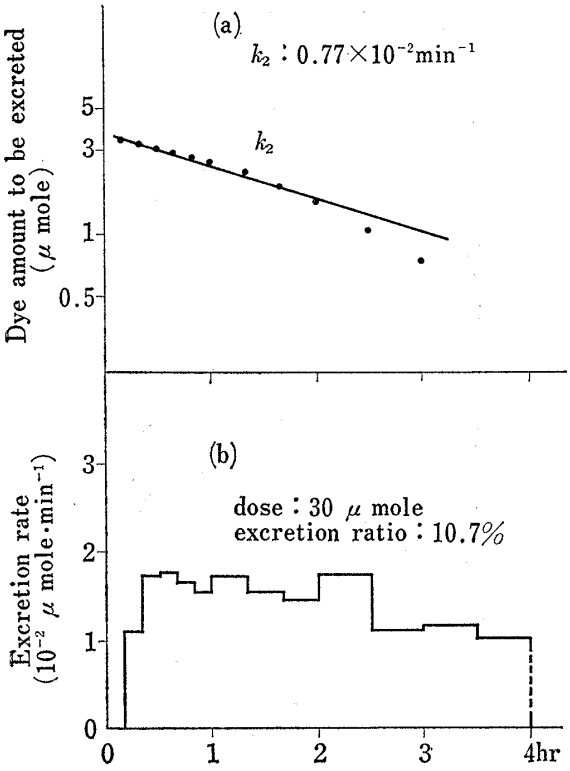


Fig. 10. (a) Semilogarithmic Plots of New Coccine (NC) in the Body to be excreted in Bile (b) Averaged Excretion Rate of New Coccine (NC)

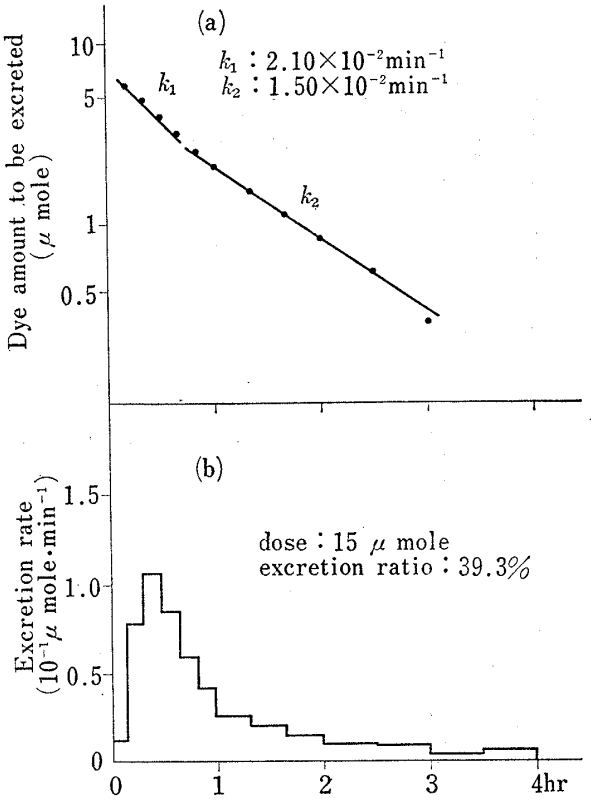


Fig. 11. (a) Semilogarithmic Plots of Ponceau R (PR) in the Body to be excreted in Bile (b) Averaged Excretion Rate of Ponceau R (PR)

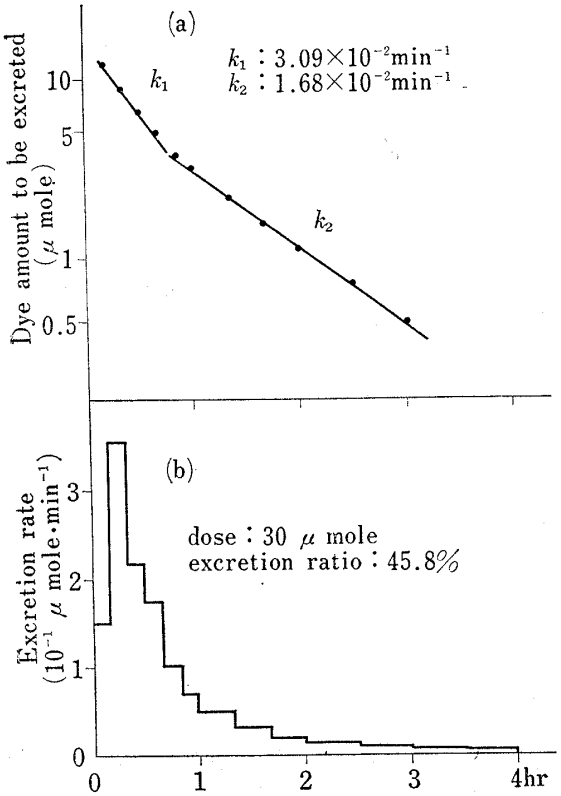


Fig. 12. (a) Semilogarithmic Plots of Ponceau R (PR) in the Body to be excreted in Bile (b) Averaged Excretion Rate of Ponceau R (PR)

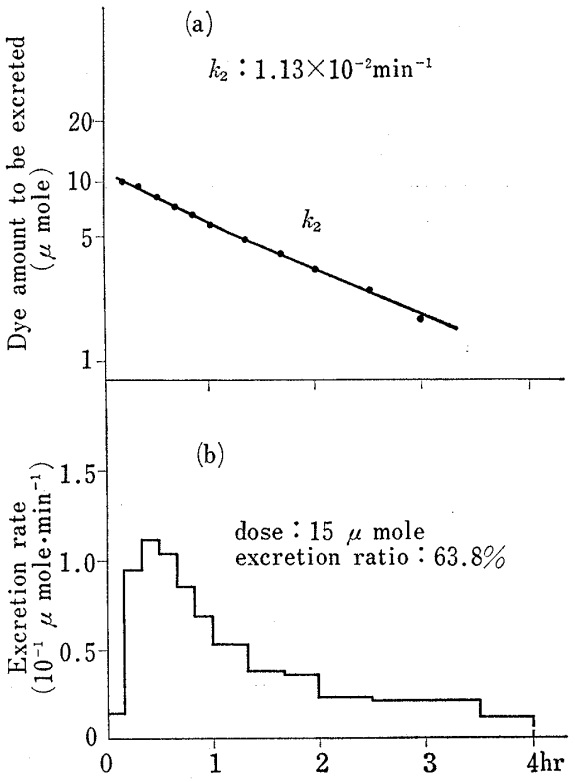


Fig. 13. (a) Semilogarithmic Plots of Ponceau SX (PX) in the Body to be excreted in Bile (b) Averaged Excretion Rate of Ponceau SX (PX)

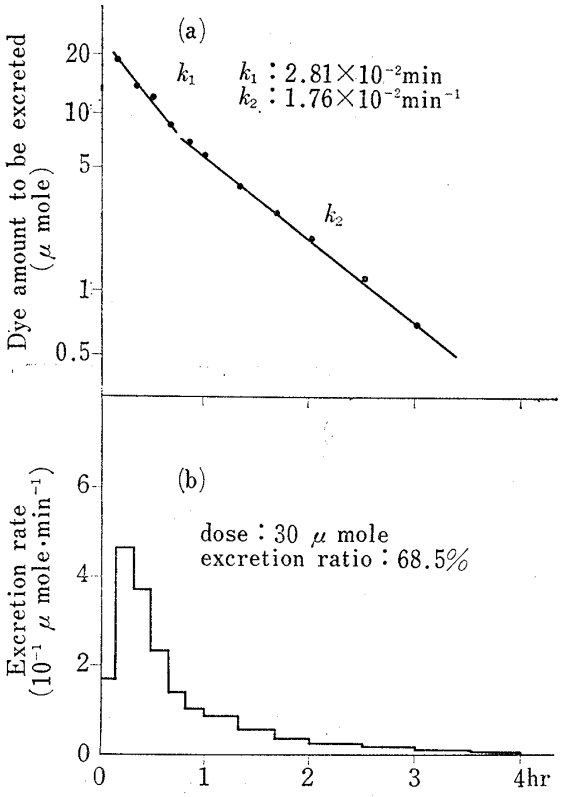


Fig. 14. (a) Semilogarithmic Plots of Ponceau SX (PX) in the Body to be excreted in Bile (b) Averaged Excretion Rate of Ponceau SX (PX)

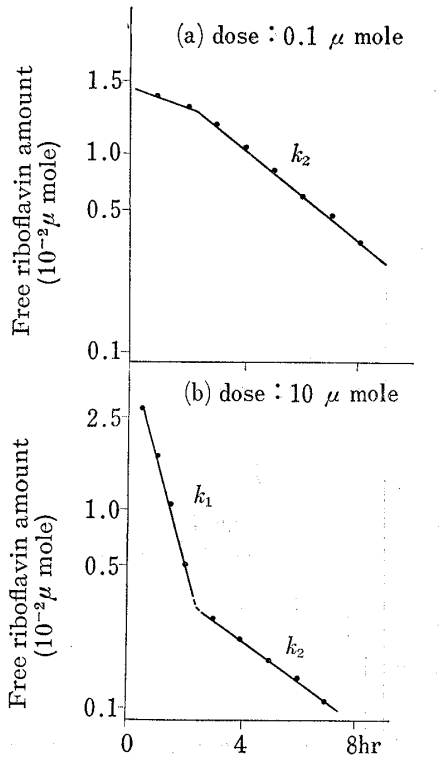


Fig. 15. Semilogarithmic Plots of Free Riboflavin in the Body to be excreted in Bile

biliary excretion rate constants: (a) $k_2=1.80\times10^{-1}$ hr^{-1} (b) $k_1=7.55\times10^{-1}$ hr^{-1}, $k_2=1.49\times10^{-1}$ hr^{-1} (data from the previous paper[8])

Table III. Biliary Excretion Rate Constant

Dye	Dose (μmole)	k_1 (10^{-2} min^{-1})	k_2 (10^{-2} min^{-1})	Type
Azorubin S	30	2.03	1.39	A
	15	2.63	0.94	A
	6	2.30	1.58	A
	3	3.02	1.27	A
Amarath	30	3.40	1.63	A
	15		1.22	B
New Coccine	30		0.77	B
	15		1.04	B
Ponceau R	30	3.09	1.68	A
	15	2.10	1.50	A
Ponceau SX	30	2.81	1.76	A
	15		1.13	B
Indigo Carmine	30	2.27	1.35	A
	15	3.41	1.24	A

As showed type A excretion pattern in the dose range studied here between 3 and 30 μmole, while AM and PR showed different patterns depending on dose. At 15 μmole dose, they showed a kind of low dose type (termed type B in the present paper) where an early lag period was not clear. It was interesting that type B was considered as an intermediate type between type A and C. NC showed type B at both doses of 15 and 30 μmole. The ratios of k_1 to k_2

were between 1.4 and 3 for all dyes used here. They were smaller than the ratio of riboflavin which was about 5.

The problem what process is concerned with k_1 and k_2 in the biliary excretion is still remained for a future study.

Indigoid Dye

Only Indigo Carmine (IC) was studied in this series. IC is used clinically for renal function test or as fed dye and is known to be excreted almost in urine and scarcely in bile. Although the biliary excretion ratio of the dye is very low, it was studied for the comparison with the dyes which had the high excretion ratios of as stated in the previous section. It has been reported that the biliary excretion ratio of IC increases in the case of renal failure. The molecular weight of it is 466.4 and it has the similar value to those of azo dyes (Table I) and the structure is shown in Chart 2. The biliary excretion ratio of it was 7—13% in the present study as listed in Table II. It may be ascribed to >NH< or >C=C< moiety that it has the low ratio though it has two sulfonate groups. The excretion patterns of IC showed type A at both doses of 15 and 30 μmole, while the excretion ratio of it was similar to that of NC which was excreted very gradually in type C. The ratios of k_1 to k_2 were between 2 and 3, and were similar to those of the above azo dyes which were more excretable. Accordingly it was considered that the low biliary excretion ratio was due to the larger uptake by kidney than that by liver, and that the mechanism is not essentially different from that of azo dyes.

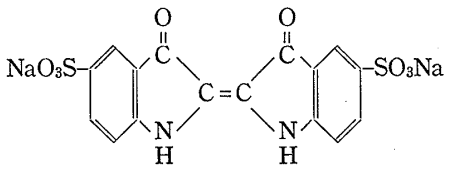


Chart 2. Chemical Structure of Indigo Carmine (IC)

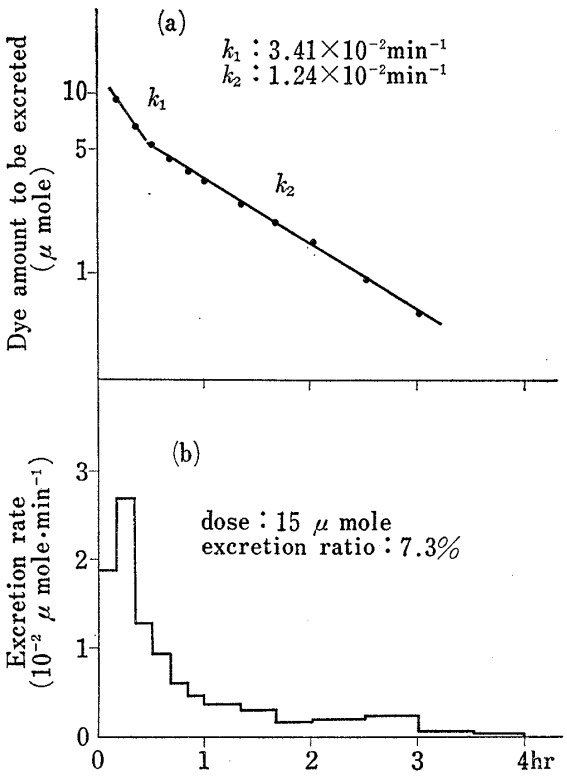


Fig. 16. (a) Semilogarithmic Plots of Indigo Carmine (IC) in the Body to be excreted in Bile
(b) Averaged Excretion Rate of Indigo Carmine (IC)

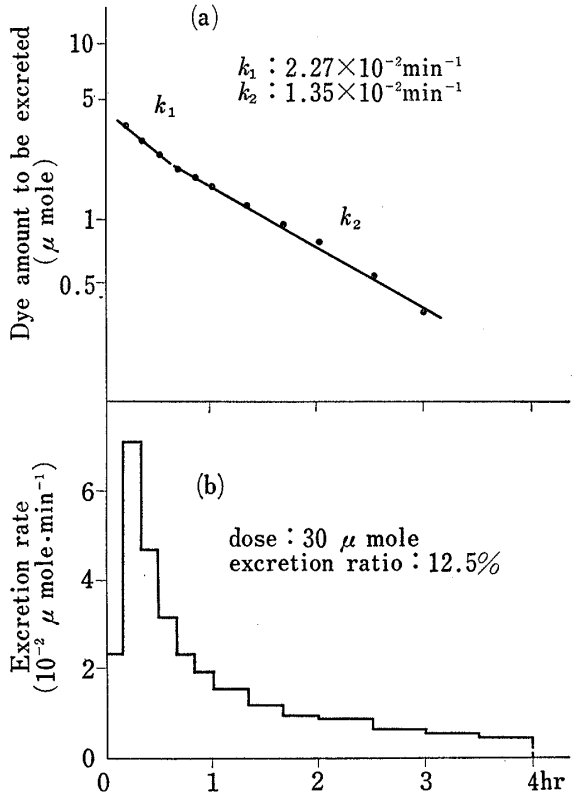


Fig. 17. (a) Semilogarithmic Plots of Indigo Carmine (IC) in the Body to be excreted in Bile
(b) Averaged Excretion Rate of Indigo Carmine (IC)

Summarizing the above results, the dose dependency of biliary excretion which had been found in riboflavin was also found in dyes, and accordingly, the dependency was considered not to be specific to a biological substance such as riboflavin.

It was also found that the position of sulfonate groups and the number of them had an effect on the biliary excretion ratio. It was the interesting results that the excretion pattern of a dye which is likely to be excreted in urine (IC) was not different essentially from those of azo dyes of which the ratio was high.