Pharmacokinetic Aspects of Biliary Excretion. Dose Dependency of Riboflavin in Rat[1,2]

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In order to know the kinetic aspects of the enterohepatic circulation, the excretion in bile and the elimination from blood of riboflavin were studied in rat. Three riboflavin derivatives, i.e. free riboflavin (FR), flavin-5'-phosphate (FMN), and flavin adenin dinucleotide (FAD) were used.

　(1) Biliary Excretion
　1) Irrespective of the kinds of administered riboflavin derivatives, FR was the major and FAD was the minor excreted in bile, but no FMN was found.
　2) A remarkable dose dependency of biliary excretion was found. When high doses (1—10 μmole) were administered, the rapid excretion occurred instantaneously and excretion was proceeded to 80% within 2 hr, and the excretion time course was described with two rate processes of rate constants k_1 and k_2. On the other hand, when low doses (0.05—0.1 μmole) were administered, the excretion was slow and proceeded to only 50% after 4—5 hr, and the time course was described with one rate process of rate constant k'. It was an interesting result that k' had the similar value to that of k_2.
　3) Some kinetic model for the dose dependency were discussed.
　(2) Elimination from Blood
　1) FMN and FAD were found to be dephosphorylyzed very rapidly in blood.
　2) The elimination of riboflavin from blood after i.v. administered was very fast and obeyed the two compartmental model.
　3) The blood concentration time course of i.v. injected riboflavin was compared between a normal and a cannulated rat. The reabsorption of riboflavin could not be ascertained.

The phenomenon of Enterohepatic circulation of biological substances has been well known for these decades and is now being studied as one of the transfer processes of drugs in a body. In the field of pharmaceutical sciences, the study of enterohepatic circulation plays a great role for the development of the sustaining release preperation and for the establishment of dose schedule and on the other in the field of physiology, it gives an interesting points of view to the regulation mechanism of the biological systems. But studies of the circulation are clinical ones and as far as the authors understand, no quantitative or kinetic study for the mechanism has been given yet. Therefore, the authors intended the pharmacokinetic study of the circulation. Riboflavin which is reported to have the Enterohepatic circulation by Makimura,[4] De Preux,[5] Yagi,[6] Tedeschi,[7] Levy[8] was chosen as the representative objective substance in the present paper, where the excretion in bile and the elimination from blood was studied.

1) This forms Part XV of "Studies on Absorption and Excretion of Drug." by H. Nogami; Part XIV: H. Nogami, M. Hanano, S. Awazu and H.H. Moon, Chem. Pharm. Bull. (Tokyo), 17, 2097 (1969).
2) This work was presented at the 89th Annual Meeting of Pharmaceutical Society of Japan, Nagoya, April 1969, and abstracted in part from the thesis presented by Tatsuji Iga to the Graduate School, the University of Tokyo, in partial fulfillment of Master of Pharmaceutical Science degree requirement.
3) Location: Hongo, Bunkyo-ku, Tokyo.
4) H. Makimura, Acta. Med. Keijo, 12, 147 (1929); through C.A., 24, 4808 (1930).
5) R. De Preux, Z. Ges. Exptl. Med., 110, 12 (1942).
6) K. Yagi, Bitamin, 7, 128 (1954).
7) G.G. Tedeschi, Boll. Soc. Ital. Biol. Sper., 30, 663 (1954); through C.A. 49, 1919 (1955).
8) G. Levy and W.J.J. Jusko, J. Pharm. Sci., 56, 58 (1967).

The remarkable dose dependency of biliary excretion pattern of riboflavin was found and is to be reported here.

Experimental

Drug Administration and Samplings

Male albino rats (Donryu) weighing 245—255 g were used.

Bile fistula and femoral artery cannulation were operated for the excretion of the drug in bile and the elimination from blood study, respectively. The drug was administered through a femoral vein, and bile or blood samples were taken at given times. Ether anesthesia was used for the operation.

Materials

Three riboflavin derivatives were used, i.e. riboflavin-5'-phosphate (FMN), free riboflavin (FR), and flavin adenine dinucleotide (FAD). (Wako pure chemical industries, LTD). All other reagents were commercially available and of special grade.

Analytical Methods

Fluorometrical measurements of riboflavin and its natural derivatives in small quantities of blood reported by Buch, et al.[9] were used after some modification as the following.

1) **Biliary Excretion Study**——Bile samples of 0.4 ml was put into a 8 ml polyethylene centrifuge tube and 4 ml of 5% CCl_3COOH was added to remove protein. After kept in dark at 0—5° for 15 min, it was centrifuged at 0—5° for 10 min, at 12000 rpm of Kubota KRP-60 Centrifuge (about $12000 \times g$).

Supernates of 0.8 ml and 2 ml were put into each 25 ml graduated light–resistant test tube. The former was hydrolyzed at 37° for 20 hr in dark and afterwards 0.2 ml of 2.4M K_2HPO_4 was added to give a 1 ml solution (B).

To the latter was added 2.4M K_2HPO_4 of 0.5 ml and after a sufficient shaking the solution was divided into two portions, 1 ml (A) and 1.5 ml. The 1.5 ml portion was put into a 8 ml polyethylene tube. Four ml benzyl alcohol saturated with water previously was added to the tube and FR was extracted to a benzyl alcohol layer with a vigorous shaking. After the upper layer (benzyl alcohol layer) was removed as far as possible, 1.3 ml aqueous phase was put into a 8 ml polyethylene tube, to which 2 ml $CHCl_3$ saturated with water was added to remove the residual benzyl alcohol from the aqueous phase and the solution was centrifuged at 0—5° for 5 min, at 6000 rpm. The clarified aqueous phase of 1 ml was put into a graduated light–resistant test tube (C).

Each 1 ml of A, B and C was diluted adequately by de-ionized water and the relative fluorescence intensity was determined at 522 mμ excited at 359 mμ with Hitach 203 Fluorospectro Meter using 0.002 μmole/ml FR solution as the standard.

2) **Elimination from Blood Study**——After 0.3 ml isotonic NaCl solution was added to 0.1 ml whole blood sample, the solution was treated with the same procedure in the biliary excretion study.

3) **Calculation**——Let the amount of FR, FMN and FAD in sample (μmole) be x', y' and z', respectively and the relative fluorescence intensity of A, B and C be X, Y and Z, respectively. The following simultaneous equations were obtained.

$$X = A_1 x' + A_2 y' + A_3 z'$$
$$Y = B_1 x' + B_2 y' + B_3 z'$$
$$Z = C_1 x' + C_2 y' + C_3 z'$$

where A_1—A_3, B_1—B_3, C_1—C_3 are coefficients of FR, FMN or FAD for three parts A, B and C, respectively. Since the above coefficients were known separately with standard FR, FMN and FAD solution, x', y' and z' were obtained as the roots of the simultaneous equations. And the excreted amounts x, y and z were calculated with the following equations.

$$x = x' \times \frac{V_s}{0.4}$$

$$y = y' \times \frac{V_s}{0.4}$$

$$z = z' \times \frac{V_s}{0.4}$$

where V_s is a total sample volume. Hitach 5020E digital computer was used in the computer center of the University of Tokyo to solve the simultaneous equations.

9) H.B. Buch and O.H. Lowry, *J. Biol. Chem.*, **175**, 457 (1948).

: Endogenious riboflavin could be neglected in the present study where the minimum dose was 0.005 μmole.

Stability of FMN and FAD in Blood

To 0.3 ml whole blood was added 0.1 ml 10 μmole FMN or FAD solution and the solution was incubated at 37°. At a given time the reaction was stopped with the addition of 4 ml 5% CCl_3COOH. The residual FMN or FAD amount was determined as the above.

Results and Discussion

I. Biliary Excretion

Bile Flow——It is a very important factor for the biliary excretion study to obtain a constant and sufficient amount of bile flow. Since long acting anesthetics, such as phenobarbital, urethane *etc.*, tend to decrease body temperature and biological activity and consequently to bring about low bile flow rate, therefore ether anesthesia was adapted for the operation.

After the operation, the rat was put in a restraining cage[10] and bile flow was sampled at a given time under an awaken condition. During the time course of sampling, neither body temperature decrease nor bile flow rate decrease was observed. A typical bile flow rate for 24 hr was showen in Fig. 1.
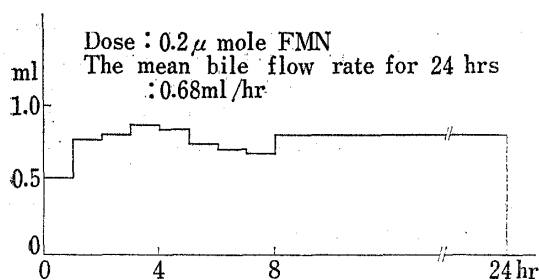

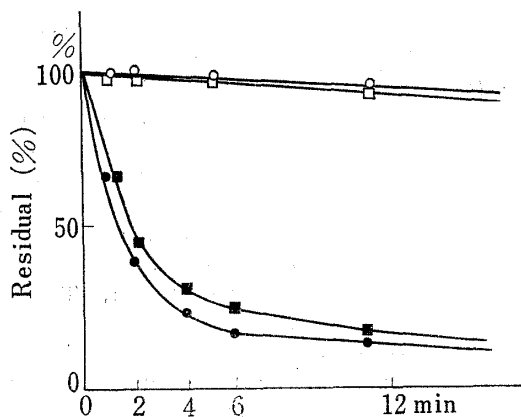Fig. 1. The Mean Bile Flow Rate for 24 hr

Fig. 2. Dephosphorylation of FMN and FAD to FR in Blood at 37°

—O—: FMN in 0.9% NaCl as the control
—●—: FMN in whole blood: 0.9% NaCl (3:1)
—□—: FAD in 0.9% NaCl as the control
—■—: FAD in whole blood: 0.9% NaCl (3:1)
all substrates concentrations are 0.25 μmole/ml
FMN: riboflavin-5'-phosphate
FAD : flavin adenin dinucleotide
FR : free riboflavin

TABLE I. The Mean Bile Flow Rates at Various Doses for 24 hr

Dose	FMN[a]			FAD[b]			FR[c]		
0.05	0.65[d]—0.82[e]	0.68[f]	(3)[g]	0.68[d]—0.69[e]	0.69[f]	(3)[g]			
0.1	0.61 —0.66	0.63	(5)	0.56 —0.60	0.57	(2)	0.64[d]—0.78[e]	0.71[f]	(3)[g]
0.2	0.67 —0.78	0.74	(7)						
1.0	0.65 —0.85	0.72	(4)	0.75 —0.81	0.78	(2)			
10.0	0.64 —0.85	0.67	(5)	0.76		0.76	(1)		
Control[h]	0.60 —0.86	0.69	(5)						

a) riboflavin-5'-phosphate b) flavin adenin dinucleotide c) free riboflavin
d) minimum rate (ml/hr) e) maximum rate (ml/hr) f) averaged rate (ml/hr)
g) number of experiments h) no drug was administered

10) J.L. Bollman and J.C. Cain, *J. Lab. and Clin. Med.*, **33**, 1348 (1948).

The slight differences of the bile flow rates were observed for individuals and time, but significant difference of the rates for drugs and doses were not observed, as shown in Table I.

Excretion of Riboflavin in Bile——Irrespective of the types of riboflavin derivatives, free riboflavin (FR) was mainly excreted in bile. Whenever 0.1 μ mole of FR, FMN or FAD was administered, nearly same amount of FR as the major excrete and slight amount of FAD were found in bile, but FMN was not observed. This can be explained with the results of Fig. 2 which showed that FMN and FAD were readily hydrolyzed in blood to give FR.

The Effects of Doses on the Biliary Excretion Pattern——FMN which have the highest solubility amoung the three riboflavin derivatives was used.

When 10 μ mole of FMN was given, the large amount of biliary excretion of FR began within a few minutes and the fluorescence of FR was observed even with bare eyes.

But on the other hand 0.1 μ mole of FMN did not show such a rapid excretion. Cumulative excretion curves of Fig. 3 show this remarkable difference clearly.
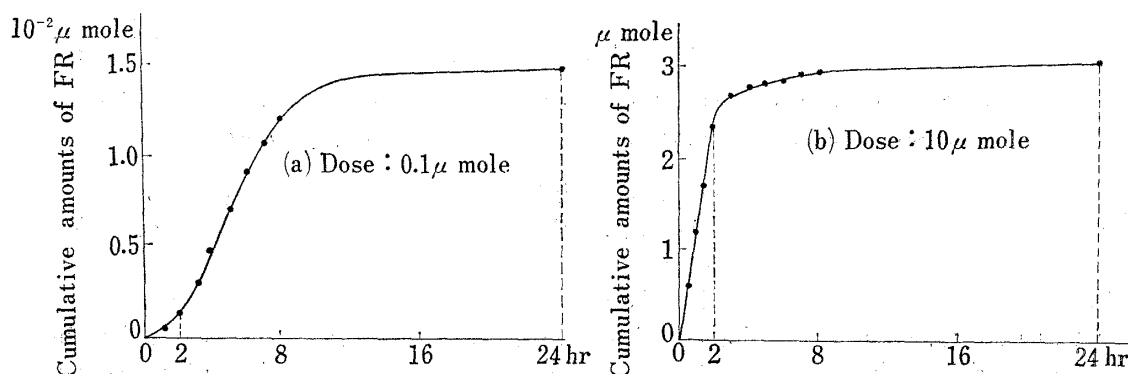
Fig. 3. Cumulative FR Excretion Curves in Bile Riboflavin-5'-phosphate (FMN) was administered.

After a rapid dephosphorylation in blood (See Fig. 2.), free riboflavin (FR) was excreted in bile.

Ten μ mole of FMN gave a linear excretion from an initial stage, while the other gave a sigmoid like excretion. Further the excreted amount for every one hour was shown in Fig. 4.
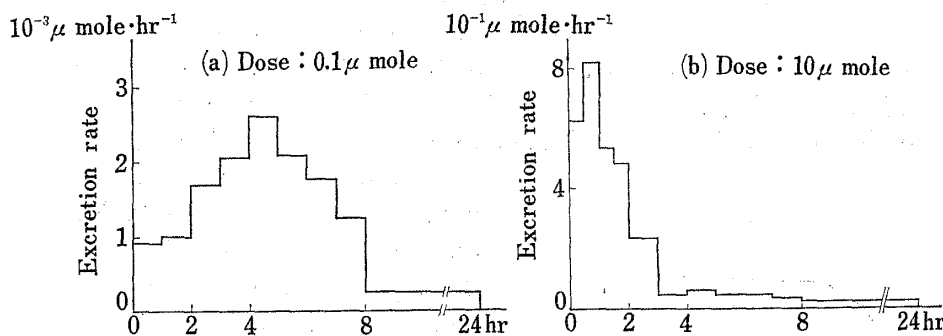
Fig. 4. Averaged Excretion Rate of Free Riboflavin

(Data in Fig. 3 was used.)

As seen easily, 10 μ mole of FMN gave a rapid excretion and 80% of the total excreted amount was found excreted within 2 hr. On the other hand, 0.1 μ mole of FMN showed a slow excretion pattern and the highest rate was found between 4 and 5 hr, until which only 50% of the total excreted amount was excreted. In order to describe this difference quantita tively, the semi–logarithmic amount of FR in the body to be excreted was plotted against time as shown in Fig. 5 according to Nelson.[11]
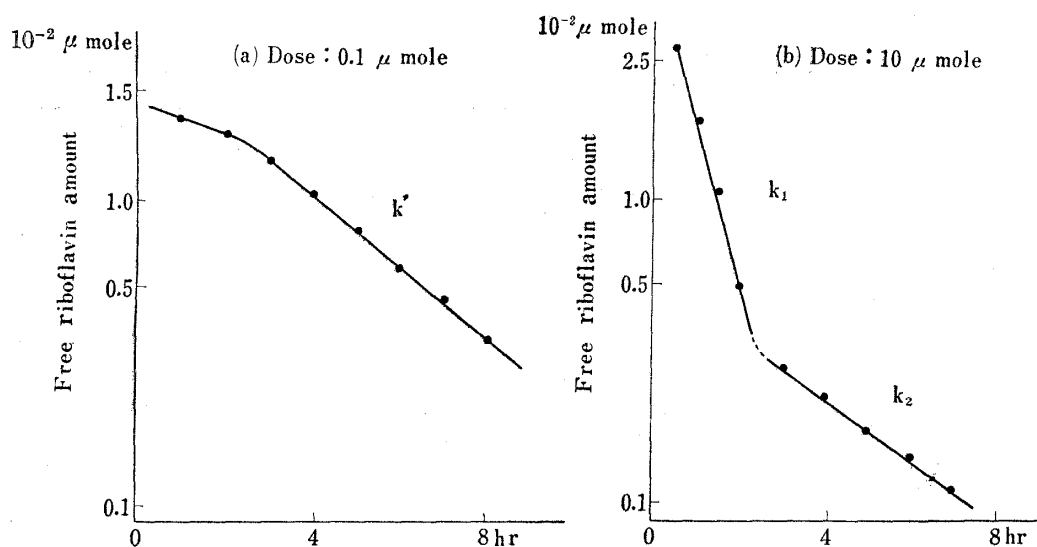
11) E. Nelson, *J. Pharm. Sci.*, **50**, 181 (1961).

Fig. 5. Semilogarithmic Plots of Free Riboflavin in the Body to be Excreted in Bile

biliary excretion rate constants: $k'=1.80 \times 10^{-1} hr^{-1}$, $k_1=7.55 \times 10^{-1} hr^{-1}$, $k_2=1.49 \times 10^{-1} hr^{-1}$

Ten μ mole of FMN dose showed a very interesting feature which was composed of two straight lines. The first straight line gave the biliary excretion rate constant (k_1) of 7.55 $\times 10^{-1} hr^{-1}$ and the second gave the other rate constant (k_2) of $1.49 \times 10^{-1} hr^{-1}$.

When 0.1 μ mole of FMN was administered on the other, the biliary excretion rate followed the first order after some lag periods of about 3 hr. The lag period was assumed to be due to the uptake of riboflavin by liver cells or the life cycle of the cells. The biliary excretion rate constant (k') was $1.80 \times 10^{-1} hr^{-1}$. It was an interesting result that k' had the simialr value to that of k_2.

These results are explained as the following. There are two mechanisms of high and low rate in a biliary excretion, and when a high dose is administered, a large amount of the drug is excreted rapidly at an early period with the rate constant k_1 to a certain level in body, and then the drug is excreted slowly with the rate constant of k_2.

On the other hand, when a low dose is administered, the drug is excreted slowly with the rate constant of k_2 after the early lag period. This may be attributed to that the biliary excretion is one of the regulation mechanisms to maintain the substance on a certain level in the body. And it is interesting that even vitamin which is not a foreign compound is excreted rapidly with such a mechanism of the biliary excretion when the high dose is administered.
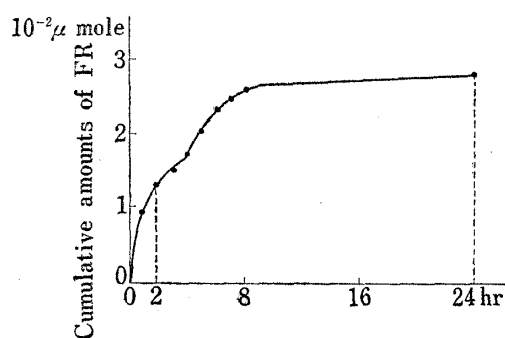
In order to know the excretion pattern dependency on the dose, 0.05 μmole up to 10 μ mole doses were administered. The low dose pattern as in Fig. 3 (a) appeared below 0.1 μ mole and the high dose one as in Fig. 3 (b) appeared above about 1 μmole. But the critical concentration where the low or high dose pattern appeared was not clear due to the individual differences of the rats. When the intermediate dose was administered, the early lag period disappeared, but the excretion was rather slow. The typical figure is shown in Fig. 6.

And the half life of the excretion decreased when the dose amount increased. The quantitative analysis on the pattern dependency on the dose are now being undertaken in our laboratory.

Fig. 6. Cumulative FR Excretion Curves in Bile: Intermediate Excretion Pattern between High and Low Dose

Dose: 0.2 μmole reboflavin-5'-phosphate (FMN)

FR : feree reboflavin

II. Elimination of FR from Blood

When FMN was administered, it was hydrolysed very rapidly to FR as shown in Fig. 2 from blood.

Since Fig. 7 gave straight line in the latter period and the secondary plots of the usual method gave also a straight line, the two compartmental model was expected to this case. And the data were fitted to the two compartmental model with an interactive least suquare method programmed as the routine in our laboratory.[12] The good fittness (Fig. 8) and the pharmacokinetic constants with their standered errores were obtained in Table II.
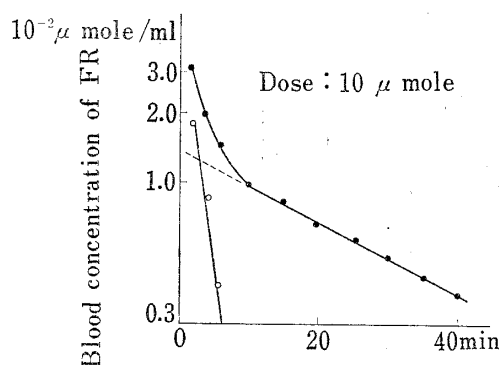
Fig. 7. Semilogarithmic Plots of Blood
Concentration of Free Riboflavin (FR)

—●—: observed values
—○—: secondary plots for the two
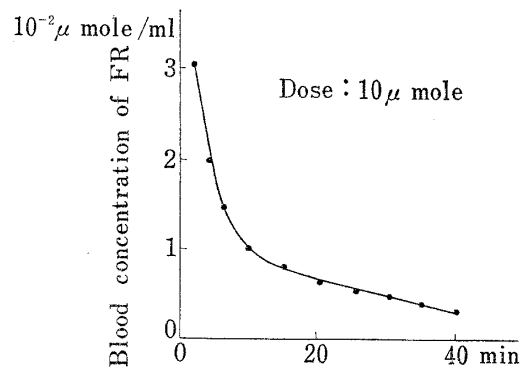   compartmental model

Fig. 8. Blood Concentration Time Course
of Free Riboflavin (FR)

●　: observed values
———: calculated curve with iterative
          least square method

Since the blood volume of the 250 g body weight rat can be assumed about 20 ml, the large volume of the compartment I means the rapid distribution of riboflavin in the body. This is consistent with the report[13] that riboflavin was observed in tissues soon after the administration.

TABLE II.　Parameters for the Two Compartmental Model[a] Calculated
by the Least Square Iteration Method

Dose: 10 μ mole[b]	S.E.[e]	Dose: 10 μ mole[b]	S.E.[e]
k_{12}[c]	0.196	0.031	V_1[d]	179	14
k_{21}[c]	0.118	0.022	V_2[d]	298	77
k_{el}[c]	0.120	0.013			

a)
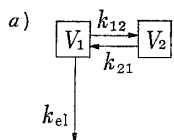
b)　riboflavin-5'-phosphate (FMN)
c)　μmole/ml
d)　ml
e)　standard error

III. Enterohepatic Circulation

Since Levy[8] suggested the enterohepatic circulation of riboflavin from the data of the urinary excretion in man, the authors intended to ascertain the reabsorption of riboflavin excreted from bile duct directly with the blood concentration data.　The blood concentration time course of $i.v.$ injected riboflavin was compared between a normal rat and a canulated rat. But no significant difference was observed, and the reabsorption could not be ascertained.

12)　H. Nogami, M. Hanano, S. Awazu and H.H. Moon, $Chem.$ $Pharm.$ $Bull.$ (Tokyo), **17**, 2097 (1969).
13)　A. Hirt and K. Wimmer, $Klin.$ $Wochschr.$, **18**, 733 (1939).

The probable reason is that the disappearence from blood was so rapid that the blood concentration decreased too low for the determination after 1 hr, and that the total sample volume of blood was limited and many samplings for a long period was impossible. The case of a higher dose administration will be checked later for the enterohepatic circulation.

IV. Pharmacokinetic Models

Some pharmacokinetic models were discussed to explain the biliary excretion and blood concentration time course of riboflavin.
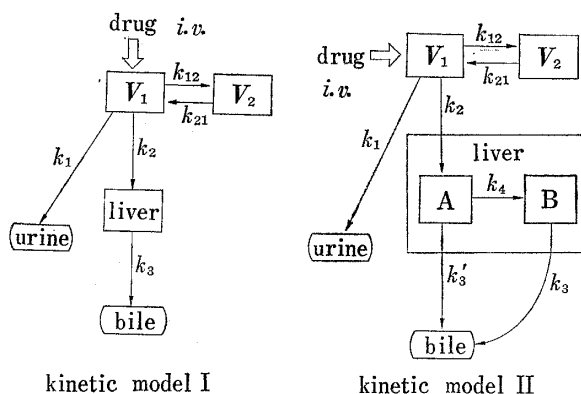

kinetic model I   kinetic model II

Fig. 9.   Kinetic Models for Biliary Excretion

V: Distribution volume of compartment
k : Rate constants

Model I of Fig. 9 was assumed to have liver as the new compartment in addition to the usual two compartmental model. This model could not explain the long lasting biliary excretion which succeeded to the rapid disappearance from blood and the remarkable dose dependency of the excretion pattern.

In Model II of Fig. 9, Liver was divided further into two compartments A and B. It was assumed that when the dose above a certain level was administered the rapid excretion process of k_3 would occure beyond the slow excretion process of A→B→Bile. The data fitting to the model is in progress using Model II as the working hypothetical model. From the above results, it is probable that the biliary excretion mechanism is complicated and different from the urinary excretion and that the further study in other drugs is necessary.

Since a rat has no gallbladder, the excreted amount of riboflavin from bile duct could be assumed as the amount excreted directly from liver with bile flow. Therefore it was not necessary to pay any consideration of the interferance of the drug deposit in gallbladder.

And riboflavin is essentially non–toxic and has a very soluble derivative (FMN), therefore it was possible to administer such a high dose in the present report. These are the probable reason why the remarkable dose dependency was found and analyzed kinetically in the present report.