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Effect of Age on the Distribution of Drug in Blood

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The distribution of drugs in blood of rat in relation to age were studied in an *in vivo* experimentation.

The volume of erythrocyte and plasma were reversed at about the age of 60—70 days when the body weights were about 200 g and this evidence was supported in the hematocrit value which exceeded 50% at this stage. It was elucidated that this reversion caused by the relative decrease in the volume of plasma fraction while the volume of erythrocyte fraction was kept almost constant at these stages of the animal.

Reflecting the fluctuation in the volume of these fraction, the distribution ratio (E/P) increased with increasing in the age. Concerning the effect of dose on the E/P ratio, that the ratio increased with increasing the dose of the administered drug was demonstrated and the mechanism of this phenomena was also discussed.

Bioavailability of drug[2] is defined as the rate and the extent of absorption from a dosage form as reflected by the time-concentration curve of the administered drug in the systemic circulation. Recently, attentions have been focused on bioavailability of drug in relation to efficacies and safeties of drug therapy and these inclinations being increase attentions and reconsiderations on drug concentration in the blood. Aside from these recent tendencies, blood level of drug had been utilized in investigations of both fields of clinical medicine and pharmaceutical sciences.

It is well known that blood is not homogeneous liquid but consist of many components and although plasma, red blood cell, white blood cell and platelets are known as main constituents, blood is separated in general into two fractions of solid and liquid. A few literatures had revealed that drug existed not only in the liquid fraction but also in the solid fraction.[3] However, attentions were concentrated only in drug level in the whole blood in most of literatures and investigators ignored the level in which fraction did most of drug exist.

Once Davis[4] and Anton[5] suggested that drug bound to protein which was located in the liquid fraction of blood was inhibited to develop its pharmacological activity. Taking their suggestions into considerations, it would be easily speculated that the drug bound to the solid fraction of blood would also lose chances of development its pharmacological activity. Since binding of drug to some constituents in blood might inhibit the drug to transport to site of action which would locate out of the systemic circulation.

In view of these speculations, drug concentration in whole blood might present little information in relation to therapeutic efficacy of drug in certain cases, and more basic investigations concerning the distribution of administered drug in blood should be undertaken.

Before starting studies, our attentions were focused on the volume of each fraction of solid and liquid in blood, and its change due to animal age, which might affect drug distribution

1) Location: *Kawara-cho Shogoin, Kyoto.*
2) "Drug Bioequivalence, A Report from the Office of Technological Assessment by Drug Bioequivalence Study Panel," U.S. Government Printing Office, Washington D.C. July, 1974.
3) K.W. Lee, D.M. Abelson, and Y.O. Kwon, *Am. J. Clin. Nutr.*, **21**, 223 (1968); B. Duhm, W. Haul, H. Medenwald, K. Patzschke, and L. Wegner, *Arzneim.-Forsh.*, **19**, 858 (1969); S.B. Ross, *J. Pharm. Pharmacol.*, **27**, 322 (1975).
4) B.D. Davis, *J. Clin. Invest.*, **22**, 753 (1943).
5) A.H. Anton, *J. Pharmacol. Exptl. Therap.*, **129**, 282 (1960).

in blood. In this report, the variations in drug distribution in blood of experimental animal in relation to age were studied.

Experimental

Materials——Metoclopramide and sulfadimethoxine were kindly supplied from Fujisawa Pharmaceutical Co. Ltd., and Chugai Seiyaku Co. Ltd., respectively. All other drugs and reagents used in the present study were of analytical grade and used without further purifications.

Animals——All the experiments in this report were performed with male albino rat of Wistar strain and they were taken away from their mothers on the 20th day of age from their births. More than 50 rats of 24 days of age were transported into our laboratory and housed in stainless-steel cages placed in an animal room (Hojyo Seisakusho, Kyoto) maintained at $21 \pm 1°$ for more than 5 months. No restriction on food and water was loaded during these periods of housing. Rats fed commercial laboratory rations and drunk tap water freely. On the day of age of 30, 60, 90, 120, and 150, all the rat were measured their body weights and several rats having averaged body weight were chosen to measure the volume of whole blood and the volume fraction of both of liquid and solid, and those were presented as plasma volume and erythrocyte volume for the sake of convenience in this report respectively.

Measurement of Blood Volume of Rats——Although there have been several methods concerning measurement of blood volume in an experimental animal,[6] Evans blue dye dilution method[7] with modifications was utilized in the present experiment.

After an anaesthetization with intraperitoneal administration of 5% sodium pentobarbital solution in dose of 50 mg/kg for head, the rat was fastened on an operating plate and heparine solution containing 1000 unit per ml was injected into the left femoral vein in dose of 0.02 ml per 200 g of body weight to prevent coagulation during the course of experimentation. The right femoral artery was cannulated with an ATOM polyethylene tubing having 2 French scale. Fifty microliter of blood was obtained in a heparinized capillary tubes through the tubing and hematocrit was determined by centrifugation at 12000 rpm for 5 min using Kubota Hematocrit KH-120.

Approximate one-tenth gram of a solution of 1% Evans blue in an isotonic saline was administered as quick as possible intravenously in the right femoral vein. To obtain the dose accurately the syringe used was weighted before and after the injection. An aliquot of 50 microliter of blood was transferred into the heparinized capillary tube through the polyethylene tubing at each time of 20, 30, 45, and 60 minutes after the injection. After the measurements of hematocrit of these blood samples, 20 microliter of the plasma fractions were transferred to a test tube, and the plasma fraction was diluted by addition of 4 ml of 0.1 N HCl and blue color was determined spectrophotometrically at a wavelength of 620 nm with Hitachi Spectrophotometer model 124. The concentration of Evans blue in plasma fraction was calculated from the prepared calibration curve of the dye solution. Since full logarithmic plots of time-concentration relationship of the dye in the plasma was found linear in the period of observation. An extraporation seemed to reasonable to obtain the initial concentration of the dye at time 0 which corresponded the time of dye administration.

Volumes of blood, plasma and erythrocyte were calculated by the following equation;

$$B.V. = P.V./(1-Ht/100) \quad (1)$$

$$P.V. = D/ICE \quad (2)$$

$$E.V. = B.V.-P.V. \quad (3)$$

where $P.V.$=plasma volume (ml), D=dose of Evans blue (mg), ICE=initial concentration of Evans blue (mg/ml), $B.V.$=blood volume (ml), $E.V.$=erythrocyte volume (ml), Ht=hematocrit (%).

The experiment was conducted more than five times with rats having the same body weight and the same age in day, and the results were averaged.

Measurement of Drug Distribution in Blood——Several rats having age of 30, 60—70 and 120 in day were employed in this experiment. Rats was anaesthetized in the same manner as was stated previously. Isotonic saline solution containing drug in a given concentration was administered through the left femoral vein. After elapsing 5 minutes, an aliquot of 50 microliter of arterial blood was collected and hematocrit was determined.

Simultaneously, approximate 0.5 ml of blood was withdrawn into a heparinized small test tube, and 0.3 ml of the blood were used to separate plasma and erythrocyte fraction by centrifugation at 2500 rpm for 30 min. One-tenth ml of both plasma and the intact blood were transferred to glass stoppered test tubes respectively, and the following procedures for determination of the drug in respective sample were conducted.

6) G.F. Cartland and F.C. Koch, *Amer. J. Physiol.*, **85**, 540 (1928); J.Q. Griffith and R. Canpbell, *Proc. Soc. Exp. Biol., N.Y.*, **36**, 38 (1937); A.J. Gramt, R.T. Pels, and E.B. Reeve, *J. Physiol.*, **116**, 59 (1952); D.T. Overbey, J.C. Moore, O.W. Shadle, and H.C. Lawson, *Am. J. Physiol.*, **151**, 290 (1974).

7) J.G. Gibson and W.A. Evans, *J. Clin. Invest.*, **16**, 301 (1937).

An aliquot of 3 ml of distilled water was added to hemolysis and 2 ml of 10% trichloroacetic acid were added to remove protein. After vigorous shaking, both solutions were centrifuged at 2500 rpm for 20 min and 3 ml of the supernatants were placed in another respective test tubes. Regular Bratton-Marshall procedure[8] was applied and developed color was estimated spectrophotometrically. Absorption maxima used in the determination of sulfadimethoxine was 550 nm and of metoclopramide was 538 nm, and the concentrations were obtained by consulting respective calibration curves.

Distribution of drug in both fractions of erythrocyte and plasma was expressed in the present report by E/P which signified a ratio of drug concentration in the erythrocyte fraction over that of the plasma fraction, and calculation was made by the following equation:

$$E/P = \{(A-B)/EV\}\{PV/B\} \quad (4)$$

where EV=erythrocyte volume (ml), PV=plasma volume (ml), A=amount of drug in blood (μg), B=amount of drug in plasma (μg), $(A-B)/EV$= drug concentration in 1 ml of erythrocyte, B/PV=drug concentration in 1 ml of plasma.

The value of EV and PV were substituted by the result obtained in the experiment described in the previous paragraph.

Results

Body Weight and Blood Volume

The relationship between body weight and blood volume of rats in the function of age are illustrated in Fig. 1. Body weight increased following the normal growth curve of this animal during the period of observations,[9] that is, from 30 to 150 days in age. Parallel relationship was found in increase of these two parameters of animal size, that is, blood volume increased with increase in body weight.

As illustrated in Fig. 1, the increasing rate of these parameters seemed to decrease when the age of the animal reached 120 days, and conjecturing from these curvatures, body weight and blood volume seemed to reach a certain plateau just after the age of 150 days. However,

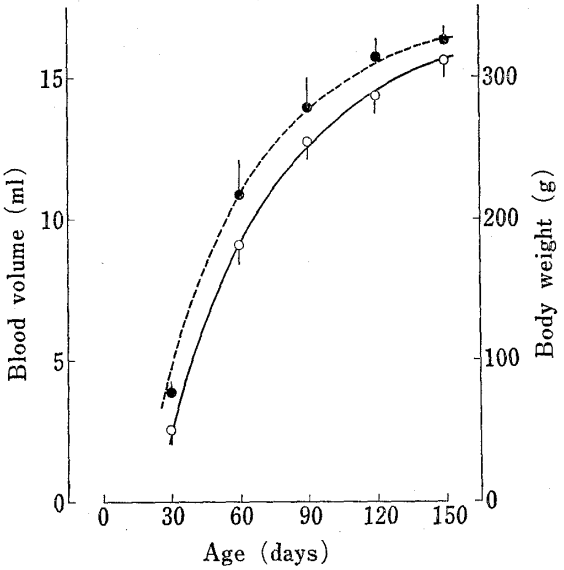

Fig. 1. A Relationship between Body Weight and Blood Volume in a Function of Age of Wistar Strain Male Albino Rat

—○—: body weight, ---●---: blood volume
Each value represents an average of at least five determinations.

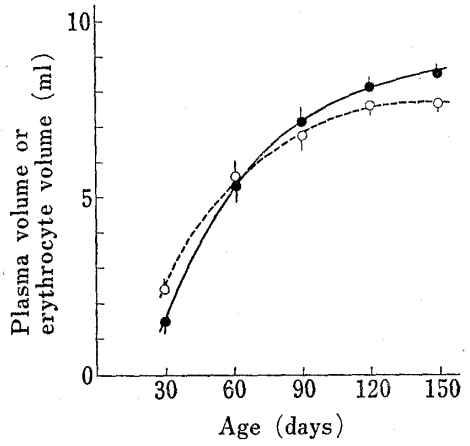

Fig. 2. Plasma and Erythrocyte Volume in a Function of Age of Wistar Strain Male Albino Rat

—●—: erythrocyte volume, ---○---: plasma volume
Each value represents an average of at least five determinations.

8) A.C. Bratton, E.K. Marshall, Jr., *J. Biol. Chem.*, **128**, 537 (1939).
9) G. Zbinden, "Experimental and Clinical Aspects of Drug Toxicity," in Vol. 2 of "Advances in Pharmacology," ed. by S. Grattini and P.A. Shore, Academic Press, New York, 1963, p. 26.

in the case when the rat is employed as an experimental animal in the field of pharmaceutical sciences, the rat having body weight between 150 and 300 g was preferred to choose and it is important to estimate the blood volume of the rat in the range of our observations. Data obtained so far, the ratio of the blood volume to the body weight of this animal was found almost constant to 0.06 especially in the final period of the observations which corresponds 90—150 days in age, which is almost similar to that of adult man.[10]

Plasma and Erythrocyte Volume

As the result of pursuing growth in volume of both plasma and erythrocyte Fig. 2 was obtained. Fig. 2 shows that the plasma volume exceeded that of erythrocyte at an early stage, however, this relationship was reversed at 150 days in age of this animal and the reversion was occurred at about 60—70 days in age. In other words, large amount of plasma fraction in comparison to erythrocyte fraction existed in blood at 30 days, and the increasing rate of the plasma fraction was relatively lower than that of the erythrocyte fraction and on the days of 60—70 days in age, the volume of both erythrocyte and plasma became almost balanced and the erythrocyte fraction exceeded in volume thereafter. These phenomena were also supported in the result of measuring hematocrit of blood of the animal.

Fig. 3 shows hematocrit changes in the rat in function of age. As illustrated in Fig. 3, the value was apparently less than 50% on the day of 30, however, the value became more than that on the day of between 60—70 in age which corresponded to the evidence found in the previous experiment.

It is of importance to realize that fluctuations in volume of these fractions in blood are occurred in the weight range which investigators often employed the animal in their experiments. To study these phenomena more in detail, attentions were turned to reveal a relation of these growths to their body weights.

Fig. 4 shows changes of volumes of both of the fractions per 100 g body weight in function of the age. As illustrated in Fig. 4, the volume of erythrocyte fraction per 100 g of body weight was not affected so much through the period of the observations. While the relative volume of plasma fraction decreased with increasing the age of the animal at the initial period

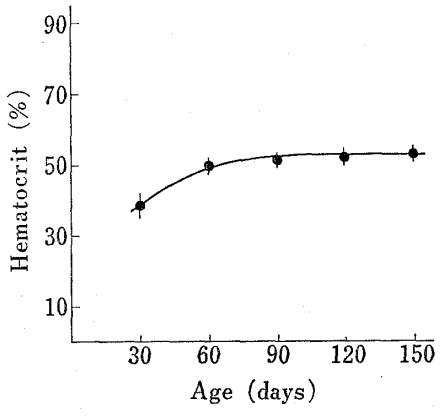

Fig. 3. Hematocrit of Wistar Strain Male Albino Rat Having Different Age

Each value represents an average of at least five determinations.

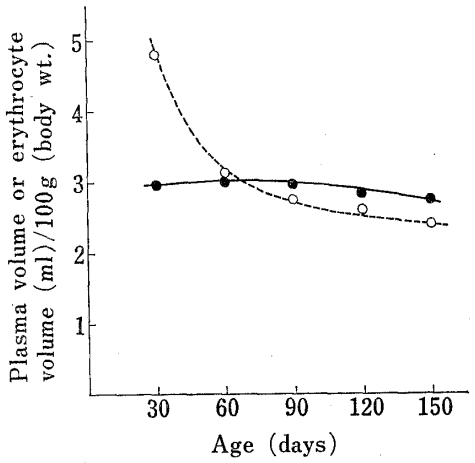

Fig. 4. Plasma or Erythrocyte Volume per 100 g of Rat Body Weithgt in a Function of Age

—●—: erythrocyte volume, ---○---: plasma volume

Each value represents an average of at least five determinations.

10) D.C. Darrow, H.C. Soule, and T.E. Buckman, *J. Clin. Invest.*, **5**, 243 (1928); M. Morse, D.E. Cassls, and F.W. Schlutz, *Am. J. Physiol.*, **151**, 448 (1947).

of the observations and finally reached a plateau just below to that of the another fraction. These two lines in Fig. 4 crossed each other at about the age of 60—70 days. This evidence also supported the above finding that hematocrit reached 50% at the same age of the rat.

However, the data depicted in Fig. 4 indicated that the fluctuation in hematocrit was not due to an expansion in volume of the erythrocyte fraction, since the volume was kept almost constant, which indicated that the practical volume increased in proportion with increasing the body weight, but due to a relative decrease of the plasma fraction.

Distribution of Metoclopramide in Erythrocyte and Plasma Fraction in Blood

Ratios of E/P were plotted as a function of dose of metoclopramide administered in the rat and Fig. 5 was obtained. These lines in Fig. 5 represent the results obtained from the rat having age of 30, 60—70 and 120 days, respectively. The ratio increased in logalithmic scale in the dose, and a regression line of these plots was expressed as a straight line. This relation was observed in all cases in which the experiment was conducted with other rat having different ages.

From the fact that the distribution ratio was not kept constant indifferent to the dose of the drug administered, but increased with increasing the dose, the affinity of the drugs to the erythrocyte fraction might increase with increasing the dose. However, these phenomena required more effort to be investigated more in detail.

Reflecting the above evidence that the relative plasma volume decreased with increasing the age, the ratio increased with increasing the age of the animal. These results suggested that the distribution ratio varied depending on the age of the animal and the variation might be due to the difference in volume of the fraction of erythrocyte and plasma which had been mentioned above.

Distribution of Sulfadimethoxine in Erythrocyte and Plasma Fraction in Blood

Essentially similar pattern of curves which were presented in Fig. 5, were obtained in the plot of E/P *vs.* dose, as illustrated in Fig. 6, in the case when sulfadimethoxine was employed

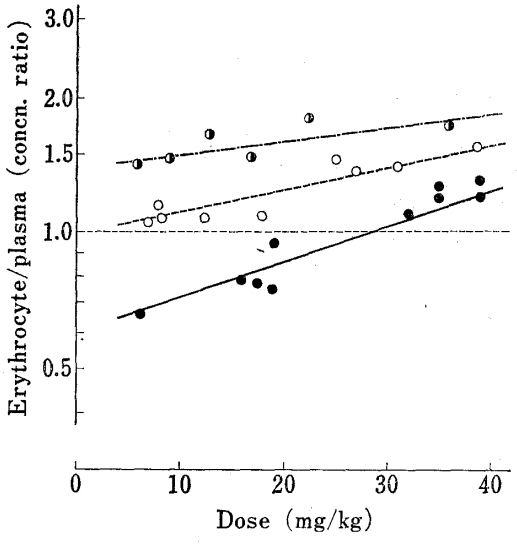

Fig. 5. Effect of Age on the Relationship of E/P Concentration Ratio and the Dose of Metoclopramide Administered to Rat

—●—: 30 days: log $y=0.079x-0.22$: $r=0.97$
---○---: 60—70 days: log $y=0.046x+0.00001$: $r=0.88$
—◑—: 120 days: log $y=0.039x+0.14$: $r=0.90$

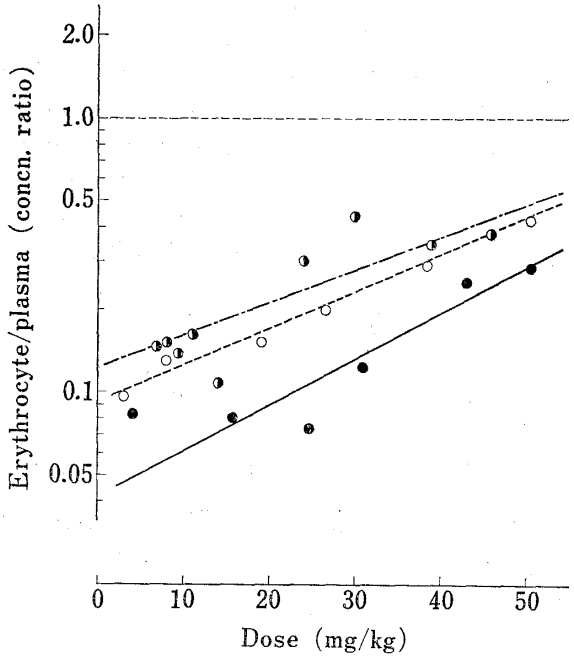

Fig. 6. Effect of Age on the Relationship of E/P Concentration Ratio and the Dose of Sulfadimethoxine Administered to Rat

—●—: 30 days: log $y=0.017x-1.377$: $r=0.89$
---○---: 60—70 days: log $y=0.013x-1.013$: $r=0.99$
—◑—: 120 days: log $y=0.012x-0.092$: $r=0.88$

in place of metoclopramide. The regression lines obtained from the same age rat moved upward with increasing in the age of the animal, which demonstrated that the distribution of the drug in erythrocyte fraction increased with increasing in the age.

However, significant difference was observed in the E/P ratio of sulfadimethoxine to that of metoclopramide. In the cases of metoclopramide, the plots scattered about 1.0 of the E/P ratio, but the plots concerning sulfadimethoxine scattered about 0.1 of the E/P ratio. These evidences suggested that distribution in the erythrocyte and plasma of these two drugs were quite different in nature, and judging from the results, sulfadimethoxine was apt to distribute in the plasma fraction, while metoclopramide distributed in both fractions of blood in the same degree.

Discussion

Volume of Blood, Erythrocyte and Plasma

Our studies started with measuring the volume of blood, erythrocyte and plasma into which drug might distribute. Since to understand these volumes and their variations in relation to age was considered essential thing to do first in the study. In the course of our studies, it was revealed that the volume of erythrocyte and plasma were reversed at about the age of 60—70 days with the body weight was about 200 g, and this evidence was reflected in the hematocrit value or packed red cell volume which exceeded 50% at the same age of this animal. This evidence had been often encountered in our laboratory when larger rat was employed in experimentations.

Once Watson[11] presented variation of hematocrit value in the course of growth of man, and demonstrated that the hematocrit value was about 56.6% at birth, but this value decreased with increasing the age not more than 1 year and minimum value was about 35.2% in man at 1 year old and thereafter the value increased as increasing in age. However, the value did not exceed 50% and the average value of hematocrit in man indicated 46.2%. These evidences in the variation of hematocrit value in man were also supported by the experiment conducted by Singer[12] and his coworkers.

Aperia and Herin[13] measured the value of Sprague-Dawley strain of male rat between the age of 17—60 days. Although they did not measured the value on the age of our argumentations, judging from the figure presented in the literature in which the value reached a plateau from the day of 30 days old, the hematocrit value would be 45% in average and might not exceed 50% at most so far as in normal physiological conditions. Taking these evidences into considerations, the findings reported in the present paper might be restricted only in male rat of Wistar strain, and the strain difference might exist in the hematocrit value in the rat.[14]

Reversion in the volume of these fractions in blood was brought about by relative decreasing of the plasma fraction to increasing the body weight. While the erythrocyte fraction increased in parallel with increasing the body weight. Although of course, the reason of this relative decreasing of the plasma fraction could not revealed in this report, this evidence might affect in our studies hereafter in more or less degree and efforts should be accumlated to elucidate the effect of this relative decreasing of the plasma fraction in future.

The Method Determining E/P Ratio of Drug in Blood

There had been a few works in literature attempting to determine the distribution ratio of drug in blood using *in vitro* or *in vivo* experimentations. Most of the studies applying

11) C. Watson, *J. Arch. Intern. Med.*, **86**, 797 (1950).
12) R.B. Singer, J. Shohl, and D.D. Bluemle, *Clin. Chem.*, **1**, 287 (1955).
13) A. Aperia and P. Herin, *Am. J. Physiol.*, **228**, 1319 (1975).
14) J. Metcoff and C.B. Favour, *Am. J. Physiol.*, **141**, 695 (1944); C.F. Wang and D.M. Hegsted, *Am. J. Physiol.*, **156**, 218 (1949); M.I. Gregersen, *Am. Rev. Physiol.*, **13**, 397 (1951); E.H. Belcher and E.B. Harriss, *J. Physiol.*, **139**, 64 (1957).

in vitro techniques purposed to elucidate a relation of chemical properties of drug used in experiment to affinity to the erythrocyte fraction or erythrocyte itself.

On the other hand, in studies using *in vivo* technologies, labelled compounds were administered and their distribution ratios were determined directly by counting radiations in both of the fractions in blood.[15] However, such technology is not always applicable in all of the experimentations.

Recently, Evans[16] determined distribution of propranolol in blood and, although they tried to reveal time course changes of the ratio by intensive sampling of blood, hematocrit value of the animal was assumed constant during the period of the investigation. Ignorance of the hematocrit change was made in other reports.[17]

It is well known that the hematocrit is changed with intensity and volume of blood sampling and the change will be amplified in such a small animal as rat. Taking the evidence into consideration, the most suitable method of determining E/P ratio of drug was tried to obtain and equation 4 was derived with substitution of hematocrit value obtained every time of the determination. The method was find out to be applicable to studies intending to reveal time-course changes of the ratio in such conditions as mass volume and intensive sampling of animal blood.

The Distribution of Drug in Blood

That only portion of administered drug, that is, the drug which is not bound to any components in blood, does develop its pharmacologic activity is known and this evidence has been supported by many reports in literature.[18] From view point of drug efficacy, studies concerning the distribution of drug in blood should be attracted attentions as well as drug binding to protein in the plasma. Along with these tendencies, this study has been undertaken and revealed that the age of animal which influences volume of fractions in blood affected the ratio in an extent which the investigators might not neglect. Moreover, these fluctuations in the ratio occurred in rat with ages of 50—150 days corresponding to body weight of 90—300 g which are often used as experimental animals in medical and pharmaceutical investigations.

The definition of the distribution of drug in erythrocyte fraction was remained rather obscure in a sence in the present report. It should be assumed that the amount distributed in the fraction included an amount of drug penetrated through erythrocyte membrance and reached into the erythrocyte, and that of bounded on surface of erythrocyte membrance. However, discriminations of these two fractions in drug behaviour needed more fine and different techniques. Although accurate discrimination of these finer behaviours of drug in the erythrocyte fraction could not achieved in the present report, general drug distribution which might suggest the amount of drug freed from the erythrocyte fraction could achieve in an extent.

An interesting evidence was find out in the present study concerning the amount of drug distributed in the erythrocyte fraction. As was shown in Fig. 5 and Fig. 6, E/P ratio had a tendency of increase as increasing in the dose of the drug. To explain this evidence, we should prepare another concept that could explain a partition phenomenon of certain compound between two unmixible solvents. Since the partition ratio was in most cases constant

15) B. Duhm, W. Maul, H. Medenwald, K. Datzschke, L.A. Wegner, and K. Schlossmann, *Z. Naturforsch.*, **22**, 70 (1967).
16) G.H. Evans, A.S. Nies, and D.G. Shand, *J. Pharmacol. Exptl. Therap.*, **186**, 114 (1973).
17) D. Kurata and G.R. Wilkenson, *Clin. Pharmacol. Therap.*, **16**, 355 (1974); U. Klotz, G.R. Avant, H.S. Schenber, and G.R. Wilkinson, *J. Clin. Invest.*, **55**, 347 (1975); R.H. Cotham and D. Shand, *Clin. Pharmacol. Therap.*, **18**, 535 (1975).
18) W.F. Bousquet, "Role of Drug Disposition in Modifying Drug Response," in Chap.4 of "Current Concepts in the Pharmaceutical Sciences; Biopharmaceutics," ed. by J. Swarbrick, Lea and Febiger, Philadelphia, 1970, pp. 143—202.

indifferent to concentrations of the compound, the concept of partition coefficient could be established. This fact suggested us that the drug distribution in erythrocyte fraction might not explain by such a simple concept as drug partition in two solvents, but more complicated phenomena should be underlying. Our attentions was turned to look at the phenomena concerning drug protein interaction. Many investigators had proven in their *in vitro* experiments that the protein binding of drug decreased as the concentration of the drug increased.[19] Supposing that these evidences also occurred *in vivo*, that is, actually in blood of the animal, both phenomena of the protein binding and the distribution into the erythrocyte fraction would behave quite reverse direction in the function of the concentration of drug. These behaviours in both of the phenomena made us possible to speculate the dose dependency of the distribution ratio in most simple way in relation to protein binding as follow that the protein binding decreased with increasing the concentration of the drug and the drug unbound to protein would transfer into the erythrocyte fraction, and as the result of this transference, the amount distributed to the fraction should increased with increasing the concentration of the drug.

As the matter of fact, the distribution ratio of metoclopramide was demonstrated larger than that of sulfadimethoxine as mentioned above. The percent bound to protein of these two drugs demonstrated *in vitro* experiments 28.6% in metoclopramide[20] and 95.7% in sulfadimethoxine,[21] respectively. Quite reverse relationship was find out between these two drugs used in the present study. These speculations and evidences suggested strongly that the distribution of drug in the erythrocyte fraction should not be considered withoutful ful the protein binding characteristics of the drug in blood.

Accepting the relation of both of drug trapping phenomena, authors are still left in doubtful that the distribution of drug in the erythrocyte fraction might not be explained in such a simple as speculated above. As indicated before, the amount of the drug distributed in the erythrocyte fraction would be consist of drug bound to the surface of erythrocyte and that of transferred into erythrocyte. Taking these two processes into account, it would be considerably difficult to analyse which process would play dominant when the concentration of drug increase. More efforts should be undertaken to elucidate more definitely the distribution of drug in blood in future.

19) I.M. Klotz, F.M. Walker, and R.B. Pivan, *J. Am. Chem. Soc.*, **68**, 1486 (1946).
20) H. Sezaki, Personal Communication.
21) M. Yamazaki, M. Aoki, A. Kamada, and N. Yata, *Yakuzaigaku*, **27**, 40 (1967).