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Electrophoretic Investigations of Blood Serum in Fasted Rats

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Effect of fasting and prosperity or decay of serum proteins of rats were studied with electrophoretic and other techniques.

Total concentration of serum protein decreased progressively during fasting, and at the same time volume of the circulating serum was also observed to decrease. These results reflected in a rise in arterial hematocrit value.

As for compositions of blood serum, about 45% of albumin was lost during three-day-fasting, whereas an amount of globulins lost during the same periods of fasting was about 37%. Among globulins, α_2-, β- and γ-fractions were observed to decrease significantly.

These decays might bring about some alterations in a mode of drug transfer in the body. Using these serums in vitro equilibrium dialysis was performed. Serum protein binding of sulfamethoxazole had a tendency to decrease with increasing periods of fasting, probably due to a fall in the concentration of serum albumin in fasted rats.

Reduction in the amount of proteins of blood serum, particularly albumin, was assumed to affect the drug transfer in body and some discussions about the drug efficacy in fasted rats were made.

Keywords——fasting; rat; electrophoresis; *in vitro* serum binding; serum protein concentration; serum volume in the circulation; hematocrit

Interaction of drugs with serum proteins is thought to be one of the un-negligible factors in attempting to evaluate an efficacy of a drug in therapy because many drugs bind to serum proteins in respective degree and the fraction bound may not exert the pharmacological effect until it is dissociated from the protein complex.[2] In addition to such physico–chemical properties of drugs, physiological conditions of animal may be probable to bring about changes in response to drugs as well. A decrease in the quantity of available serum proteins as a result of disease, for instance, a malnutrition may lead to an increased intensity of drug efficacy because the fraction of unbound drug is increased with a decrease of protein concentration, if the drug is ordinarily bound to serum proteins.

In the course of our studies initiated with the purpose to investigate how the drug absorption from intestine would be modified in the fasted animal, there observed many physiologically interesting evidences; one of which was a fasting-induced increase in hematocrit value.[3] In other words, there occurred a decrease in the volume of blood serum in proportion to the duration of fasting. Blood serum is the common medium through which all exchanges of nutritions and other substances are made in the body and it is serum through which the drug is transported to sites of action, excretion and metabolism. Therefore, in such a physiologically undesirable condition as a decreased volume of blood serum, drug transfer within body may be probable to be largely impaired.

Although serum deficits are one of the predominant characteristics of fasting as is stated below in the present work, how serum is affected by an acute fasting is not always completely elucidated yet. For studies of the effect on serum proteins of diseases and other abnormalities

1) Location: *Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606, Japan.*

2) *a*) R.R. Levine, "Pharmacology, Drug Actions and Reactions," Little, Brown and Company (INC), Boston, U.S.A., 1973, p. 71; *b*) B.B. Brodie, *Proc. Roy. Soc. Med.*, **58**, 946 (1965); *c*) A.H. Anton, *J. Pharmacol. Exp. Ther.*, **129**, 282 (1960); *d*) A. Goldstein, *Pharmacol. Rev.*, **1**, 102 (1949).

3) T. Komuro, S. Kitazawa, and H. Sezaki, *Chem. Pharm. Bull.* (Tokyo), **23**, 909 (1975).

in men and also experimental animals, electrophoretic investigations are now of most value. In the present work, relations between fasting and serum proteins of rats were studied with the electrophoretic and other techniques and some discussions concerning the meaning of fasting-induced changes in characteristics of serum were made, but an effect of alterations of serum proteins produced by fasting on the drug transfer in body was not investigated yet in the present work, although more detailed investigations should be of course necessary in future.

Experimental

Drugs and Chemicals——Sulfamethoxazole, diphenylhydantoin and other chemicals used in the present work were of analytical grade and were obtained from a commercial source (Nakarai Chemical Co., Ltd., Kyoto, Japan) and used without further purification.

Animal——Male albino rats of Wistar strain were used in all the experiments. The rats purchased were housed in stainless-steel cages in an animal room[4] maintained at 23±2° and free access to tap water and a commercial solid food for laboratory rats (Oriental Yeast Industry Co., Ltd., Tokyo, Japan) for periods of at least three days before the experiments in order to acclimatize to laboratory conditions.

Rats of about 170 g in body weight were randomly separated into two groups, one group was deprived of food for a given periods of time but allowed free access to tap water (the fasted group), and the other group of animals provided with both food and water freely (the non-fasted control group). Details are described in the other paper.[5]

Electrophoresis——Electrophoretic analyses were performed according to the standard operation method.[6] An aliquot of serum samples was applied on the buffer-impregnated strip made of a cellulose acetate membrane (Separacs®, Johko Sangyo Co. Ltd., Tokyo, Japan). The strip was attached to the electrophoretic tank. Separations on the serum samples were carried out for 25 min at 0.7 mA. in veronal buffer solution, pH 8.6, ionic strength 0.06 on the strip. After the run was completed, the strip was dried and then dyed with ponceau-3R solution. The relative amounts of proteins in the fraction were determined by using the integrating photometric scanner (Model OZ 801, Asuka MFG Co., Ltd., Tokyo, Japan). Absolute concentrations were estimated by reference to total serum protein levels.

Protein Concentration of Serum——Total protein concentration of serum obtained from rats under various stages of fasting was measured, using Atago Refractometer Model II (Atago Seisakusho Co., Ltd., Osaka, Japan).

Volume of Blood Serum——To determine the volume of blood serum, Evan's Blue dye dilution method[7] with modifications was utilized in this experiment. Approximate 100 mg of Evan's Blue dye solution of 1.0% was introduced as quick as possible into a right femoral vein. At a given interval of time, an aliquot of 50 μl of blood samples was drawn out into a glass capillary tube through a polyethylene catheter which was placed in a femoral artery and after centrifugation blue color of separated serum was determined spectrophotometrically at wave length of 615 nm using Hitachi Double Beam Spectrophotometer Model 124. From the time course observations of serum concentration of Evan's Blue dye, circulating serum volume was estimated in the regular manner.[8]

Hematocrit Value——Arterial blood samples were drawn out into a glass capillary tube through a polyethylene catheter placed in a femoral artery and after centrifugation for five minutes at 12000 rpm, the percentage of blood made up of red blood cells was measured, which is called the "hematocrit." Details are described in the other paper.[5]

Equilibrium Dialysis——In the present work, sulfamethoxazole was employed for the binding experiment. Determinations of serum protein binding of the drug were performed by suspending sacs made of Visking dialysis tubings which contained 2 ml of a 1: 6 diluted rat serum in 4 ml of 1/15M phosphate buffer solution, pH 7.4 with the drug to be tested. Dialysis was carried out for 4 days at 4°. At the end of the dialysis, an aliquot of buffer solution outside the sac was pipetted to be tested. Concentration of the drug used here was 0.5 mM.

Displacement experiments of sulfamethoxazole (SM) bound to proteins by diphenylhydantoin (DPH) were also carried out in a similar manner except that both drugs were placed together in the phosphate buffer

4) S. Kitazawa and T. Komuro, *Igaku no Ayumi*, "in press."
5) S. Kitazawa and T. Komuro, *Chem. Pharm Bull.* (Tokyo), "in press."
6) I. Kanai and M. Kanai, "Rinsho Kensaho Teiyo," Kanahara Publisher Co. Ltd., Tokyo, 1975, pp. VIII-20—VIII-25.
7) M.I. Gregersen and R.A. Rawson, *Physiol. Rev.*, **39**, 307 (1959).
8) H. Funaki, "Drug Transfer in Biological Systems," ed. by M. Nakagaki, Nankodo Co., Ltd., Tokyo, 1968, pp. 119—181.

solution outside the sac and a percent displacement was calculated as follows: [percent bound of SM (alone)-percent bound of SM (with DPH)] ÷ percent bound of SM (alone) × 100.

Sulfamethoxazole was determined by the colorimetric method as described earlier.[9]

Results

Electrophoretic Analysis of Serum Proteins

The results of electrophoretic analyses of serum compositions of rats before and during a certain period of fasting and other hematologic data are summarized in Table I. Concentration of total serum proteins which was a mean value of 6.52% at the pre-fasting stage was progressively decreased with an increasing period of fasting and reached to a mean concentration of 6.08% after the fasting of three days. Volume of circulating serum was also reduced from 6.60 ml to 4.27 ml during the same periods of fasting. This indicated that more than 35% of the initial volume of serum was reduced during the course of three days fasting. This result clearly reflected in a value of hematocrit which changed from an average value of 42.9% at the beginning of the fasting to 54.2% after the three days fasting. The decrease in volume of circulating serum was not limited only in the volume but accompanied in a concentration of components existed in blood serum.

TABLE I. General and Hematologic Data in Fasted Rats

		Non-fasted	1-Day-fasted	2-Day-fasted	3-Day-fasted
Total serum protein (g%)		6.52±0.13	6.37±0.11	6.32±0.09[a]	6.08±0.18[b]
Albumin (g%)		2.14±0.07	1.97±0.03[b]	1.90±0.02[b]	1.82±0.02[b]
Globulin (g%)	α_1	1.42±0.04	1.72±0.06[b]	1.76±0.02[b]	1.84±0.06[b]
	α_2	1.23±0.06	0.99±0.08[b]	0.93±0.08[b]	0.78±0.03[b]
	β	1.26±0.06	1.24±0.04	1.27±0.06	1.16±0.03[a]
	γ	0.47±0.04	0.45±0.05	0.46±0.04	0.48±0.03
Serum A/G ratio		0.49±0.03	0.45±0.01[a]	0.43±0.01[b]	0.43±0.01[b]
Serum Volume (ml)		6.60±0.10	6.02±0.39	5.34±0.15[b]	4.27±0.80[b]
Arterial hematocrit (%)		42.9±3.5	46.3±1.6[b]	48.9±3.5[b]	54.2±3.6[b]
Body weight (g)		171.7±1.4	152.0±3.1[b]	138.8±1.7[b]	130.5±1.9[b]

Figures were means and standard deviations of values in four or more rats.
a) significantly different from the corresponding non-fasted control value, $p < 0.05$
b) significantly different from the corresponding non-fasted control value, $p < 0.01$

As for each fraction of serum proteins, the concentration of albumin fraction in serum was progressively reduced and the serum A/G ratio was observed to be decreased, where the ratio was referred to a ratio of amount of albumin fraction to that of globulin fraction. Among globulin fraction, the concentration of α_1-globulin was increased, whereas that of the α_2-globulin was decreased. The β- and γ-globulins changed in a small extent and some fluctuations were observed in each level during fasting.

Based upon the volume of circulating serum and its fraction's concentration shown in Table I, fasting-induced changes in the total amount of

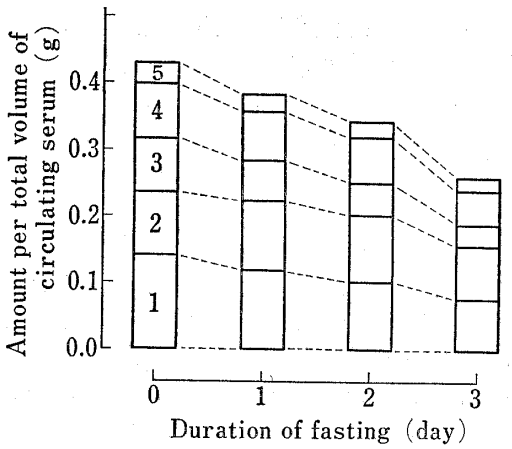

Fig. 1. Effect of Fasting on Each Serum Fraction of Rats

1: albumin, 2: α_1-globulin, 3: α_2-globulin, 4: β-globulin, 5: γ-globulin

9) K. Kakemi, H. Sezaki, T. Komuro, K. Ikeda, and H. Kishi, *Chem. Pharm. Bull.* (Tokyo), **18**, 2386 (1970).

each composition of circulating serum were estimated and the results calculated were graphically represented in Fig. 1. Three-day-fasting caused a significant decrease in the albumin fraction, α_2-, β- and γ-globulin fractions. Concerning the albumin fraction, about 45% of the initial amount was lost through the period of fasting, whereas total globulins were lost only by about 37% through the same period. This indicated that albumin fraction rather than globulins was more severely affected by the fasting and this was clearly supported by changes of the serum A/G ratio given in Table I.

Serum Binding of Salfamethoxazole

An effect of fasting-induced changes in the compositions of circulating serum proteins as described above was investigated with attention to protein binding of a drug. Binding studies were made at sulfamethoxazole level of 0.5 mM with an *in vitro* equilibrium dialysis method. The results expressed in terms of percent drug bound, are presented in Table II. Percentages of sulfamethoxazole bound showed a tendency to decrease slightly with increasing periods of fasting, probably due to a decrease in the concentration of serum albumin.

Table II. *In Vitro* Binding of Sulfamethoxazole (SM) to Serum Protein obtained from the Fasted Rats and its Displacement by Diphenylhydantoin (DPH)

	% bound of SM		%displaced
	SM alone	SM+DPH	
Non-fasted	20.4±1.3	17.8±1.8	12.7
1-day-fasted	19.2±1.7	16.5±1.9	14.1
2-day-fasted	19.5±2.0	16.2±2.7	16.9
3-day-fasted	18.9±0.9[a)]	15.9±2.0	15.9

Figures were means and standard deviations of values in four rats.
a) significantly different from the corresponding non-fasted control value, $p < 0.05$

Serum binding of the drug in the presence of another drug, diphenylhydratoin which is known to bind to a great extent to serum proteins[10] and seems to serve as a displacing drug, appeared to be similar to that of the drug alone, that is, the fraction of sulfamethoxazole bound was decreased with an increased time of fasting. However, a displacement of sulfamethoxazole by the same concentration of diphenylhydantoin increased progressively from a mean value of 12.7 to 15.9%. These results suggested that there might bring about a change in the capacity of serum proteins to sulfamethoxazole because of the co-existence of diphenylhydantoin with a strong binding affinity to proteins and/or that the capacity might be decreased gradually with an increased periods of fasting in rats.

Discussion

Electrophoretic inspections of serum proteins obtained from a patient are of most value in clinical medicine because a certain kind of electrophoretic pattern often leads to a certain disease.[11] Besides, electrophoretic data obtained from a subject are sometimes useful to estimate a change of his condition. In laboratory experiments as well, electrophoretic technique is considered to be efficient to have a knowledge about hematological condition of a subjected animal. In the course of our experiments, much attentions were being denoted to investigating a relation between fasting and drug transfer in body with experimental animals, and so electro-

10) *a*) B.S.D. Kurata and G.R. Wilkinson, *Clin. Pharmacol. Ther.*, 16, 355 (1974); *b*) L. Lund, A. Berlin, and P.K.M. Lunde, *ibid.*, 13, 196 (1972).
11) T. Kawai, "Plasma Proteins," Igaku Shoin Co., Ltd., Tokyo, 1969, part 4, p. 341.

phoretic investigations of serum proteins of fasted rats were carefully undertaken in the present work.

Malnutrition is said to result a fall of serum albumin since circulating serum albumin is in general consumed as an energy source.[12] Fasting may be considered as an extreme case of a malnutrition, that is, a condition deprived of any nutritional substance from the outside of body. Therefore, a similar phenomena might be expected in fasted rats, too. In Table I and also in Fig. 1 are shown our results obtained. In Table I, a reduced concentration of serum albumin induced by fasting is presented to be apparently dependent on the duration of the fasting and further, in Fig. 1, total amount of albumin in the circulation is obvious to decrease progressively with increasing periods of fasting.

On the other hand, a serum A/G ratio is clinically known to be sensitive indicator to estimate a nutritional state and in general the ratio is reported to be in a low level in malnutritional patients.[13] Concerning the fasted rats, our results indicated the ratio being lowered by the fasting. The serum A/G ratio is, however, dependent on relative amounts of both albumin and globulin fractions and so attentions must be given to the fact that either fraction was more severely affected by the fasting. In the present case, albumin fraction rather than globulins was observed to be more labile to fasting because percent decrease of total amount of albumin in the circulation, 44.9%, was larger than that of globulins, 37.0%. This phenomenon was fundamentally coincident with results reported by other investigators in the experimental animals.[3,14]

It goes without saying that albumin is one of the most essential compositions for the organisms to maintain its internal fluid environment in an appropriate condition. Now, there might occur an attractive question about a fasting that a fall in the concentration of albumin in blood serum was clearly observed in fasted rats. Judging from an evidence that about seventy-five or more percent of serum oncotic pressure is due to the albumin composition of blood serum,[15] a fall in the concentration might be associated with a reduction in the serum oncotic pressure and therefore might be extremely unfavorable for keeping the body fluid environment in the required constant condition. However, osmotic pressure of the extravascular fluids is said to be severely regulated within body and it should be true in fasted rats, too. In this point, an attention was concentrated on an evidence that α_1-globulin level in blood serum increased progressively with an increasing period of fasting, in contrast to a decreased concentration of albumin. As α_1-globulin can be thought to play a part in the maintenance of serum oncotic pressure, probably owing to a comparatively low molecular weight,[16] a fall in the serum oncotic pressure due to a decreased albumin might be effectively compensated by a rise in the fraction of α_1-globulin in fasted rats.

In the transfer of drugs in body, serum proteins, particularly albumin is most important because of its binding property. Sulfonamides are known to bind mainly to an albumin fraction.[17] In this work, albumin fraction was decreased progressively by fasting as presented in Table I, and this suggested that a binding capacity of albumin fraction might be lowered in fasted rats. In fact, this was made sure of in the present investigations of changes in the extent of protein-bound-sulfamethoxazole in fasted rats. Protein binding of sulfamethoxazole was assumed to be mainly dependent on the albumin concentration. However, considering

12) J.B. Allison, *Physiol. Rev.*, **35**, 664 (1955).
13) T. Kawai, "Plasma Proteins," Igaku Shoin Co., Ltd., Tokyo, 1969, p. 398.
14) *a*) B.F. Chow, J.B. Allison, W.H. Cole, and R.D. Seeley, *Proc. Soc. Exp. Biol. Med.*, **60**, 14 (1945); *b*) H. Nakamura and S. Suzuki, *Seibutsu Butsuri Kagaku*, **8**, 5 (1962).
15) N. Toida and K. Uchizono (eds), "Textbook of Modern Physiology," Vol. II, 3rd ed., IgakuShoin Co., Ltd., Tokyo, 1971, p. 184.
16) T. Kawai, "Plasma Proteins," Igaku Shoin Co. Ltd., Tokyo, 1969, p. 135.
17) *a*) F.J. Di Carlo, *Toxicol. Appl. Pharmacol.*, **5**, 61 (1963); *b*) J. Clausen, *J. Pharmacol. Exp. Ther.*, **153**, 167 (1965).

the results shown in Table II that percent displacement of sulfamethoxazole by diphenylhydantoin tended to increase with an increasing time of fasting, it was suggested that to a certain degree sulfamethoxazole might bind to other proteins besides albumin, and/or that some changes in properties of serum proteins might be brought about in the state of fasting. In fact, a certain kind of sulfonamide was reported to bind not only to albumin but also to other proteins[17b] and thyroxine is known to bind to a large extent to a prealbumin and a globulin in analbuminemic patients[18] and on the other hand, binding affinity of testosterone to serum proteins was reported to be varied, and dependent on pathological and physiological conditions of patients.[19] Accordingly, it was assumed that the binding affinity of sulfamethoxazole might be altered by fasting. But details are not clear and further studies will be required to investigate in details the connection between the drug and other protein besides albumin in fasted rats.

In attempting to grasp a true effect of a drug, it has been often stated in literature and also in drug therapy that attentions must be payed to changes in both pathological and physiological conditions of patients and experimental animals as well. As for the case of fasting mentioned in the present work, fasting lowered, for example, a concentration of serum protein in rats, and this means that a concentration of unbound drug may rise gradually. In general, binding to protein, by decreasing the concentration of free drug in the circulation, lowers the concentration gradient driving the drug out of the circulation and retards its rate of transfer across the capillary to its site of action. In the state of fasting, an opposite phenomena will be expected to occur, that is, by a rise in the concentration of free drug in the circulation, effective drug concentration in its site of action should rise gradually. This may be true because, in the intestinal drug absorption experiments reported previously,[3] blood concentration of a drug was observed to be higher in the fasted rat than the non-fasted control although percent absorbed of the drug from intestine was rather lower as compared to the non-fasted. Accordingly, it might be assumed that free drug concentration should have a tendency to rise in the fasted rats. These suggest that less amount of drug introduced ordinarily into blood might be enough to be effective therapeutically in the state of fasting. In future, investigations about the drug transfer in body under a physiologically abnormal state as the result of a certain disease will be of value to look for a more effective dosage schedule.

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18) C.S. Hollander, G. Bernstein, and J.H. Oppenheimer, *J. Clin. Endocrinol. Metab.*, **28**, 1064 (1968).
19) C. Pohlman, M.C. Hallberg, E.M. Zorn, A. Guevara, and R.G. Wieland, *Am. J. Med. Sci.*, **258**, 270 (1969).