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Comparative Biochemistry of Hemoglobins

I. Comparison of a Few Properties of Various Hemoglobins*

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The problem of the molecular evolution of hemoglobin has been studied by comparing agar-gel electrophoresis, ultraviolet absorption spectra and resistance to alkalis in fifteen varieties of hemoglobins ranging from human hemoglobin to fish hemoglobin. The differences observed in the hemoglobins seem to be due to differences in the structures of these hemoglobins.

INTRODUCTION

PERUTZ et al.[16] presented a model of the tertiary structure of horse hemoglobin based on the results of X-ray analyses, while BRAUNITZER et al.[3] and KONIGSBERG et al.[11] determened the primary structure of the α- and β-polypeptide chains of human hemoglobin. In addition, BRAUNITZER et al.[4] stated that though relatively many differences were observed between the α- and β-polypeptide chains in their primary structures, a more detailed comparison of these chains showed some homologity between them. Later, SCHROEDER et al.[18] examined the primary structure of the γ-polypeptide chain of human fetal hemoglobin, and found that the β- and γ-polypeptide chains consist of the same number of amino acids and that there is larger homologity between their sequences.

On the other hand, BRAUNITZER and MATSUDA[5][13] studied the primary structure of horse hemoglobin, and found that the α-chain of horse hemoglobin is composed of 141 amino acids, as many as that of human hemoglobin, and that their amino acid sequences resemble each other with amino acid exchanges in only 17 positions. According to SMITH[21], the β-chain of horse hemoglobin has been presumed to be composed of 146 amino acids, as many as are present in the ß-chain of human hemoglobin, and to possess an analogous amino acid sequence.

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Moreover, as it well known, the myoglobin in muscles resembles the hemoglobin in red cells in that it possesses a heme as a prosthetic group and is a chromoprotein possessing biological activity similar to that of hemoglobin. Furthermore, KENDREW et al.[10] and PERUTZ et al.[16] have observed a large homologity between the tertiary structures of sperm whale myoglobin and horse hemoglobin.

On the basis of these facts, hemoglobin and myoglobin obtained from various animals are presumed to have evolved from one ancestor molecule. The conception of the evolution in protein molecules which had been initiated by ANFINSEN[1] and others has come to be given attention through the study on hemoglobins. The notion of molecular disease was first obtained from the study of hemoglobinopathy by PAULING et al.[15], and was later developed mainly by ITANO et al.[8] Differences in the primary structures have been studied mainly by INGRAM et al.[7] Recently, ZUCKERKANDL and PAULING [23] stated that the developmental mechanism of moleular disease in hemoglobin was fundamentally quite similar to that of molecular evolution. In order to trace the evolution of the hemoglobin molecule, ZUCKERKANDL, JONES and PAULING[22] compared the fingerprints of the tryptic peptides from various hemoglobins ranging from human hemoglobin to lamprey hemoglobin.

The authors, because of interest in the evolution of the protein molecule, tried to compare hemoglobins from various animals ranging from man to fish by studing a few of their properties in the following way.

MATERIALS AND METHODS

Blood samples were obtained from a human adult (one of the authors), a newborn infant (cold blood), a monkey (*Macaca ehrus*), a dog, a cat, a horse, a cow, a pig, a sheep, a rabbit, a guinea pig, a chicken (*white leghorn*), a pigeon, a toad (*Bufo vulgaris japonicus*) and a yellowtail (*Seriala*).

ISOLATION OF HEMOGLOBINS

The isolation of hemoglobins was performed mainly by DRABKIN's method.[6] The red cells were separated by centrifugation in a refrigerating centrifuge at 1000—2000 r.p.m. for 5 min. to remove the plasma, and then washed three times with a 0.9 % saline solution. The red cells obtained from a yellowtail, however, were washed with a 1.35 % saline solution (NaCl 1.35 g, KCl 0.06 g, $NaHCO_3$ 0.02 g $CaCl_2$ 0.025 g and $MgCl_2$ 0.035 g in 100 cc). The washed red cells were hemolyzed by the addition of 2 volumes of distilled water and 0.4 volumes of toluene. The hemolysate was centrifuged at 15,000 r.p.m. for 1 hour.

The hemolysate was then divided into 3 layers in a centrifuge tube. The middle layer, which contained hemoglobin, was taken out and dialyzed against distilled water.

AGAR-GEL ELECTROPHORESIS

For agar-gel electrophoresis, the method of SHIBATA et al[19]. was used. Using an apparatus as illustrated Fig. 1, electrophoresis was performed in a refrigerator. The hemoglobin solution was placed at the end of the agar-gel plate (D) in Fig. 1. As buffers, tris-EDTA-Borate buffers[20] (pH 8.6 and pH 7.2) were used. When pH 8.6 buffer was used, the electrophoresis was done at 100 V for 80 min, and when pH 7.2 buffer, at 200 V for 40 min. The tris-EDTA-Borate buffer compositions are shown in Table 1. After performing the electrophoresis,

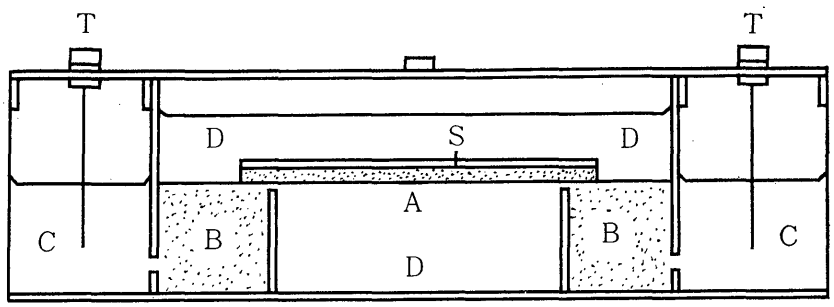

Fig. 1. Agar-gel electrophoresis apparatus
A : Ager-gel Plate.
B : Accessory compartment filled with agar.
C : Electrode vessel filled with hexane.
D : Cooling space filled with hexane.
S : Slide glass.
T : Electric terminal.

Table 1.

Compositions of Tris-EDTA-Borate Buffers for Agar-Gel Electrophoresis

	Grams required to make 1000 cc of buffer	
	pH 8.6	pH 7.2
Tris	55.0	37.0
EDTA	7.0	14.5
Boric acid	9.0	20.0

These buffers are diluted to 3 velumes with distilled water immediately before using.

Tris : Tris-(hydroxymethl) amino methane
EDTA : Ethylenediamine-tetraacetic acid

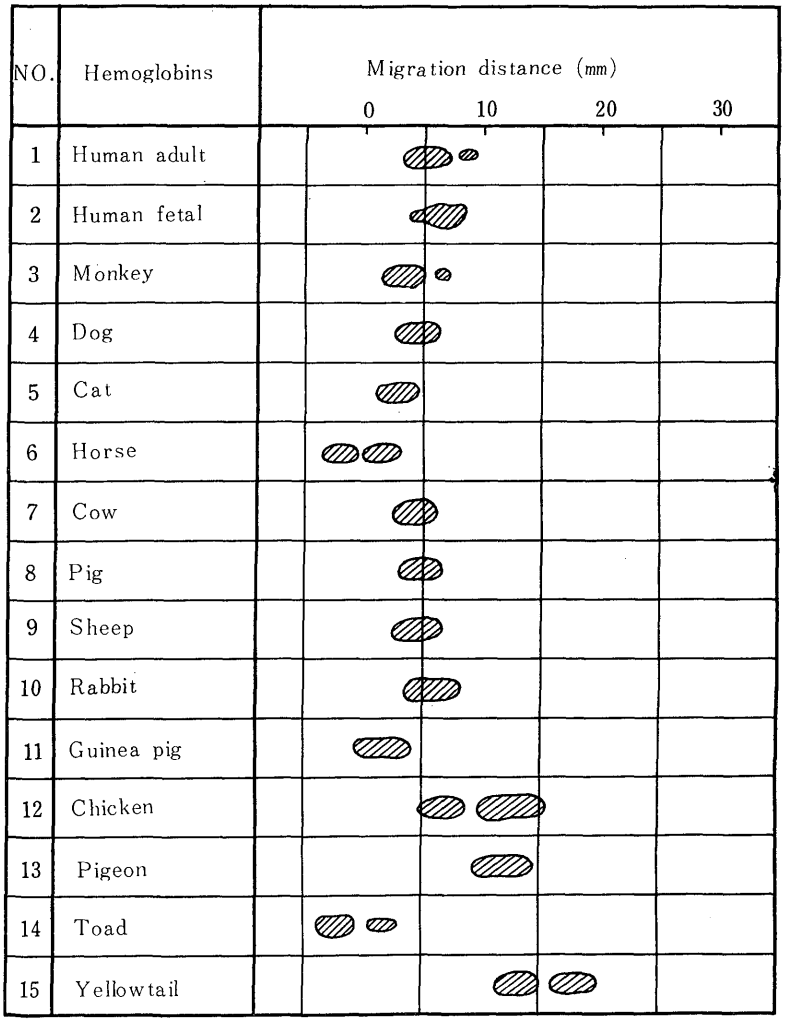

Fig. 2. Agar-Gel Electrophoresis of Various Hemoglobins
Tris-EDTA-Borate Buffer (pH 8.6), 200 V, 40 min.

the agar-gel plate was taken out together with the slide glass, and the migration distance (mm) of the hemoglobin was measured.

ULTRAVIOLET ABSORPTION SPECTRA

In the measurement of the ultraviolet absorption spectra, the hemoglobin solution was first dialyzed against phosphate buffer at pH 7.0, and the absorption spectra at 260 mμ to 300 mμ was measured by the use of a recording spectrophotometer (Type HITACHI ETS-2).

ALKALI DENATURATION METHOD

The measurement of alkali denaturation was performed by the

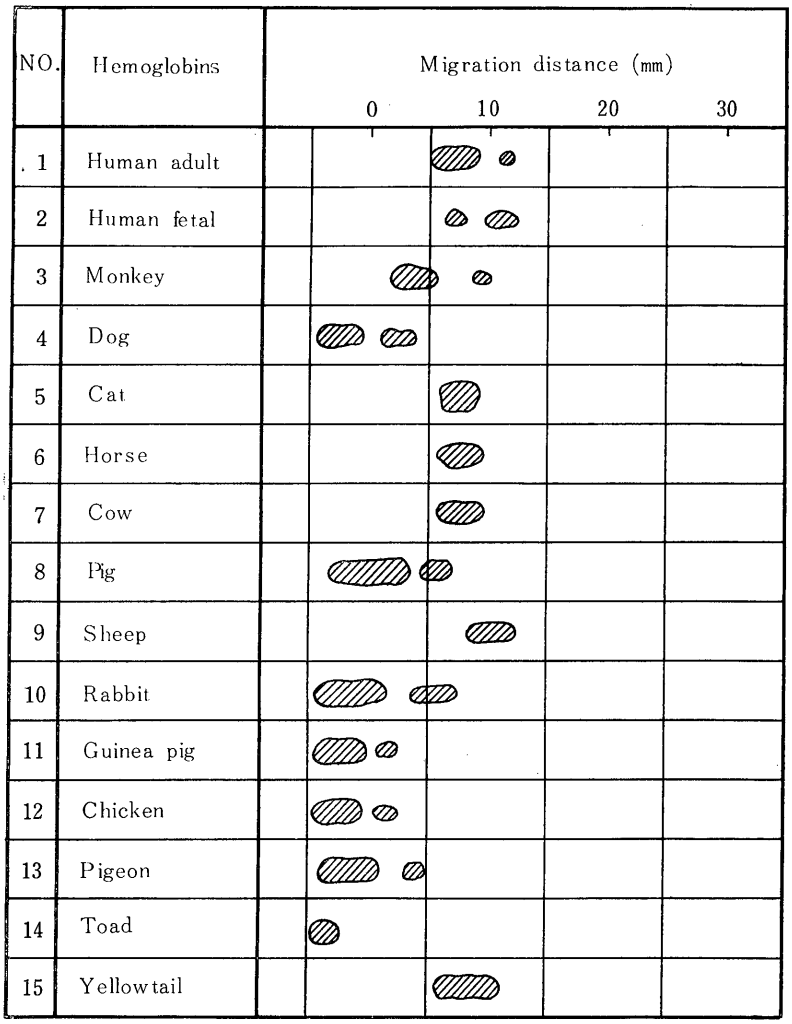

Fig. 3. Agar-Gel Electrophoresis of Various Hemoglobins
Tris-EDTA-Borate Buffer (pH 7.2), 100 V, 80 min.

method which was described by MATSUDA et al[14]. The hemoglobin solutiom was primarily diluted with distilled water so as to give about 0.5 absorbancy at 415 mμ. A 2.0 cc portion of this diluted hemoglobin solution was put into a 1 cm quartz spectrophotometer cell and then mixed with a 1.0 cc portion of phosphate buffer (134 g of $Na_2HPO_4 \cdot H_2O$ per liter adjusted with NaOH to pH 13.0). The decrease in absorbancy at 415 mμ was then measured every minute for 15 min by means of a spectrophotometer. Finally, the reaction was accomplished by heating the solution at 37°C for 15 min, and the final value was measured. The percentage of the denatured hemoglobin was obtained and given as a function of time by the application of the following equation:

$$P = \frac{E_0 - E}{E_0 - Ee}$$

P : Denatured hemoglobin (%)
E_0 : First absorbancy
E : Measurement every minute
Ee : Measurement after heating

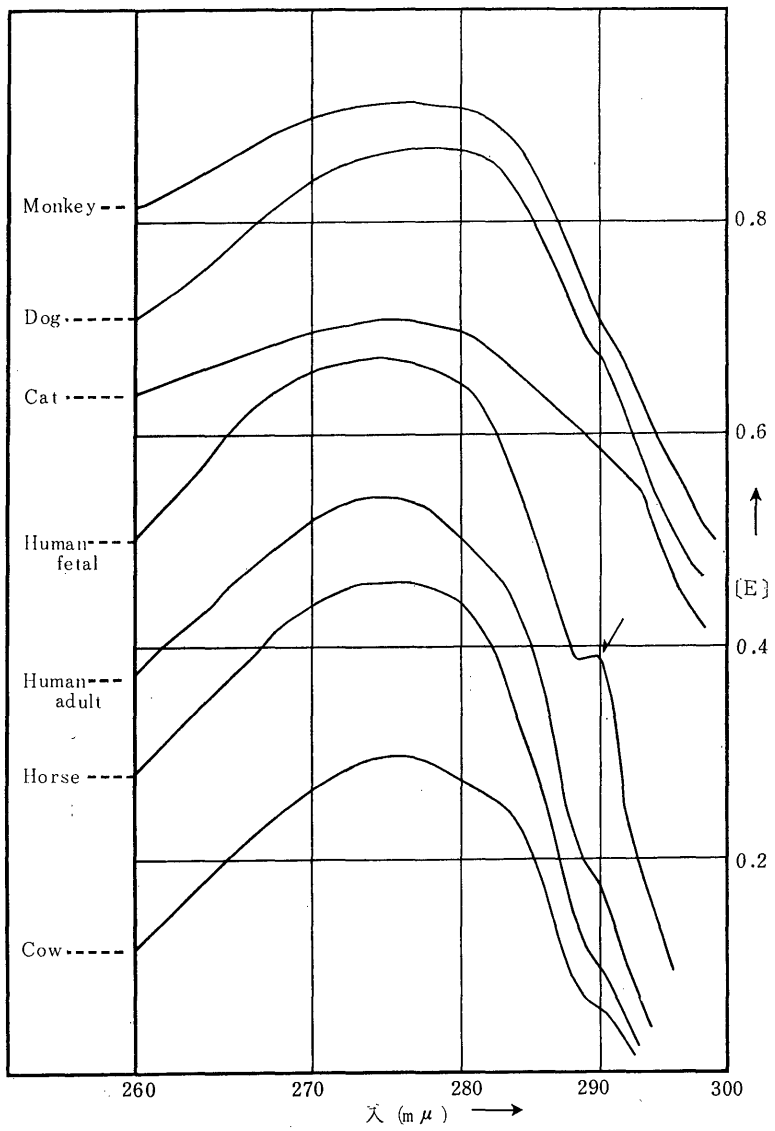

Fig. 4. Ultraviolet Absorption Spectra of Various Hemoglobins. The arrow indicates a tryptophan notch.

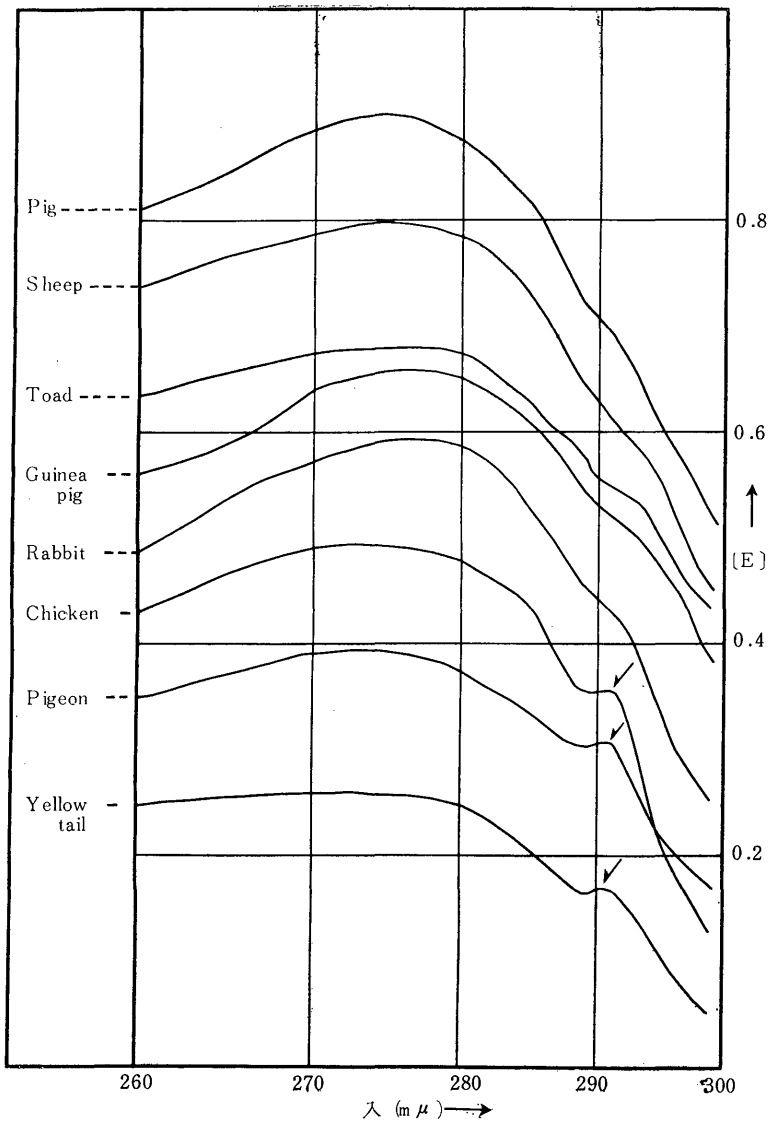

Fig. 5. Ultraviolet Absorption Spectra of Various Hemoglobins. The arrows indicate trytophan notches.

RESULTS AND DISCUSSION

Figs. 2 and 3 illustrate the results of agar-gel electrophoreses with pH 8.6 and pH 7.2 tris-EDTA-Borate buffers, respectively. As shown in these figures, hemoglobins obtained from various animals give individually different migrations. In Fig. 2, horse hemoglobin is divided into two approximately equivalent components, which is consistent with the results of BANGHAM et al[2]. and PERUTZ et al[17]. Chicken hemoglobin is also divided into two components, which is also shown

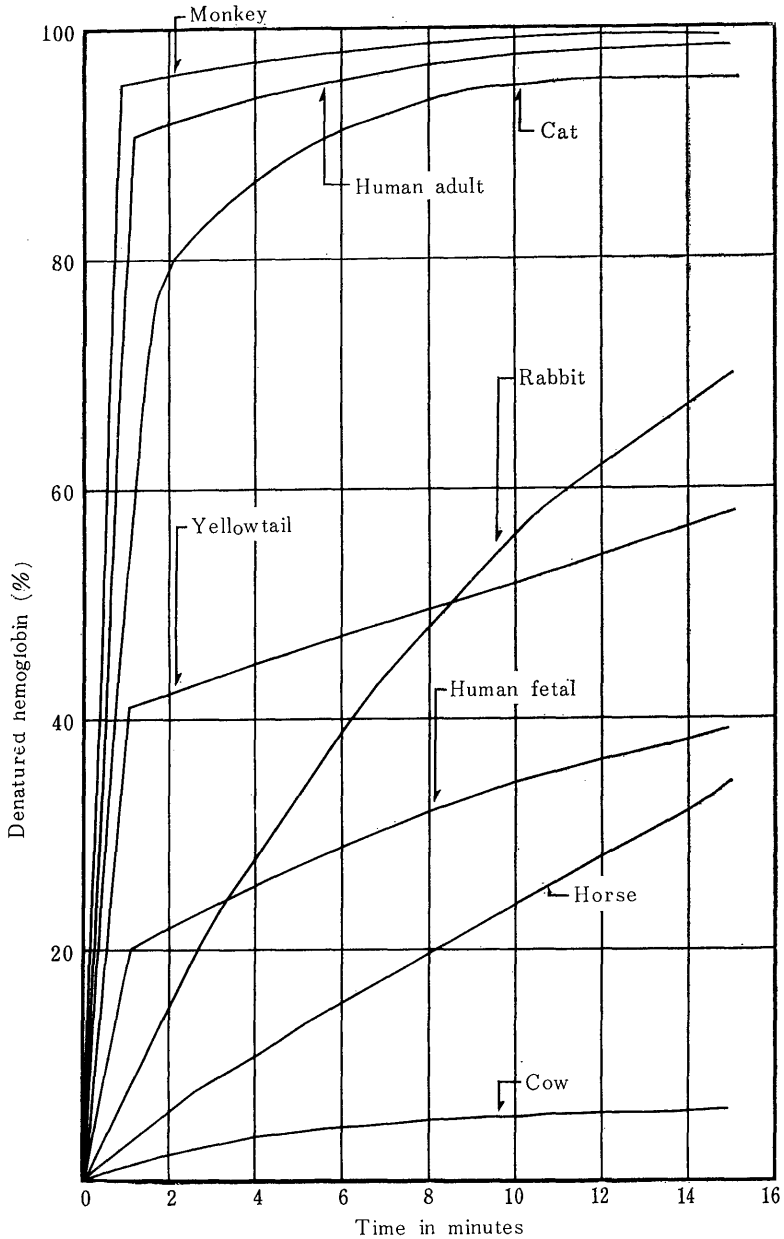

Fig. 6. Alkali Denaturation of Various Hemoglodins
Abscissa : Percentage of denatured hemoglobin.
Ordinate : Time in minutes.

by the results obtained from column chromatography using CM-cellulose. Toad and yellowtail hemoglobins are also divided into two components. In Figs. 2 and 3, the minor component preceding the major one in human adult hemoglobin might correspond to human adult hemoglobin

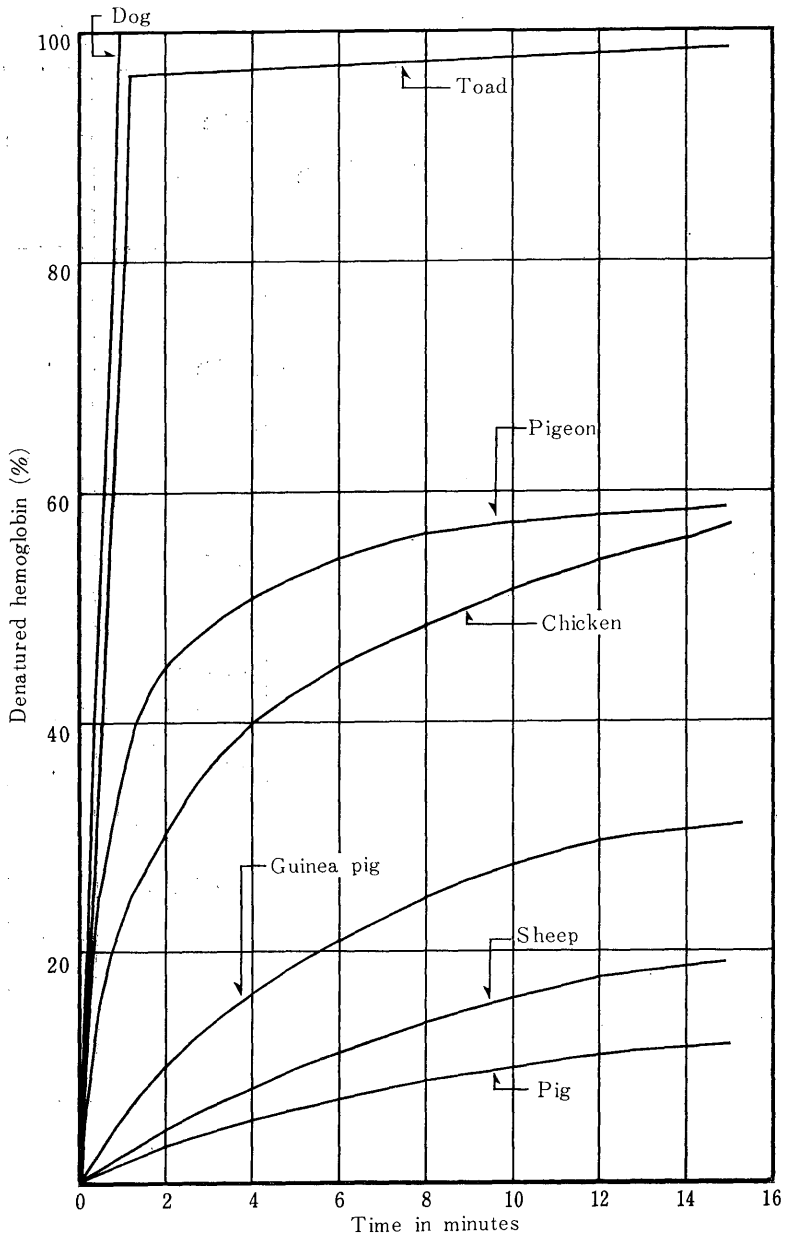

Fig. 7. Alkali Denaturation of Various Hemoglobins
Abscissa : Percentage of denatured hemoglobin.
Ordinate : Time in minetes.

A_2 (KUNKEL'S[12] HbA_2), and the minor component following the major one in human fetal hemoglobin might be consistent with human adult hemoglobin present in cord blood. It is of interest that monkey hemoglobin, like human hemoglobin, has a minor component preceding a

major component. Fig. 3 shows that dog, pig and rabbit hemoglobins are composed of two individual components, and that guinea pig and pigeon hemoglobins, differing from the results in Fig. 2, and chicken hemoglobin, as is also seen in Fig. 2, have their minor components. Yellowtail hemoglobin shows only one main component in Fig. 3. In the case of cat, cow and sheep hemoglobins, on the other hand, only one component was observed both at pH 8.6 and pH 7.2, but it cannot be concluded from only these experiments that these three kinds of hemoglobins are composed of only one component. Fig. 4 and 5 illustrate the ultraviolet absorption spectra of hemoglobins from various species of animals. The differences in the ultraviolet absorption spectra of human adult and fetal hemoglobins were first discovered by JOPE.[9] Human fetal hemoglobin differs from the adult type in the presence of a remarkable tryptophan notch at 289.8 mμ. In Fig. 4 in this work, human fetal hemoglobin is the only one which has a remarkable tryptophan notch. However, as is shown in Fig. 5, the authors found that the hemoglobins of such birds as chickens or pigeons possess a remarkable tryptophan notch like that of human fetal hemoglobin. It is left to future experiments to determine whether or not this fact is common to all birds. A remarkable tryptophan notch was observed in yellowtail hemoglobin, as well as in human fetal hemoglobin or bird hemoglobin.

In Figs. 6 and 7, the alkali denaturation velocities of various hemoglobins are compared. The main components of human adult, monkey, dog, toad and cat hemoglobins are very unstable in alkalis. The alkali denaturation curves of human adult, monkey and toad hemoglobins suggest that these hemoglobins each possess a major component which is less resistant in alkalis, and a minor component which is more resistant in alkalis. Cow, pig, sheep, guinea pig and human fetal hemoglobins are very resistant in alkalis. Rabbit, pigeon and chicken hemoglobins have a resistance between those of the above two groups. The alkali denaturation of yellowtail hemoglobin is of interest in that it suggests that the hemoglobin may be composed of two approximately equivalent main components which are quite different in their resistance in alkalis.

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FOOT NOTE

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