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Comparative Biochemistry of Hemoglobins

V. On the N-terminal Structure of the Protein Moiety from Rat Adult Hemoglobin

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In order to study the N-terminal structure of rat adult hemoglobin, its heterogeneity was primarily recognized by agar-gel electrophoresis and cellulose-acetate membrane electrophoresis. Then, the main component of rat adult hemoglobin was isolated and purified by column chromatography on CM-cellulose with the elution by the salt concentration gradient of phosphate buffer, and globin was prepared by removing heme from the component. This rat adult globin, examined by urea dissociation electrophoresis using cellulose-acetate membrane, was recognized to contain two kinds of polypeptide chains. On the other hand, this globin was dinitrophenylated to make DNP-globin. This DNP-globin was then hydrolyzed with 6 N HCl for 1/4, 1, 6, 10 and 20 hr, and from the hydrolysates the DNP-amino acids and DNP-peptides were extracted with ether or ethyl acetate, which were separated, identified and determined quantitatively by silica gel-celite column chromatography and paper chromatography. As the results, it has proved that rat adult globin, just like human adult globin, consists of two polypeptide chains with valylleucyl sequence as the N-terminal structure and two polypeptide chains with valylhistidylleucyl sequence as the N-terminal structure.

INTRODUCTION

The rat may be one of the most convenient experimental materials to study the genetic control mechanism in biosynthesis of mammalian hemoglobin because the animal is easily multiplied and because the animal has different hemoglobin in some strains as stated by Rosa[11]. Generally, the primary structure of protein, or the sequence of amino acids in protein, is strictly controlled by the gene. From this point, therefore, the author has had interest in the primary structure of rat adult hemoglobin.

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Concerning rat hemoglobin, JOPE[6], examining its ultraviolet spectral absorption, has recognized that both adult and fetal hemoglobins from the rat have a tryptophan band at 291.0 mμ. HAUROWITZ et al[5]. suggested the heterogeneity of rat hemoglobin from the result of the experiment by the alkali denaturation method. SYDENSTRICKER et al[13]. and FINE et al[3]., however, perfoming the experiments of paper electrophoresis and agar-gel electrophoresis, have reported that rat hemoglobin is homogeneous. On the other hand, GIRI and PILLAI[4] recognized two minor components besides one major component from rat hemoglobin. The difference between these various reports may be easily supposed to be due to the strain differences, and later, ROSA[11], as stated above, made an interesting report on the heterogeneity of rat hemoglobin owing to the strain difference of the rat. Now, the author carried out the analysis of the N-terminal structure as the first step to the study on the primary structure of hemoglobin from adult rats of the Wistar strain.

MATERIALS AND METHODS

1) *Preparation of Hemoglobin*

Blood was collected from adult rats of the Wistar strain by puncturing the heart. After the addition of 3.8% sodium citrate, the blood was centrifuged at 1,000−2,000 r. p. m. for 10 min at 0−5°C to remove the plasma, and the blood cell layer separated was washed with 0.9% physiological saline solution three times. The preparation of hemoglobin solution was performed by a modified method of DRABKIN's[2]. That is, to the washed red blood cells, one volume of distilled water and 0.4 volume of toluene were added, and after being shaken violently, the mixture was allowed to stand at a room temperature for 30 min. and then centrifuged at 12,000 r. p. m. for 60 min at 0−5°C. In the centrifuge tube, the mixture was separated into three layers, toluene, hemoglobin and strome downwards, and the hemoglobin solution was obtained from the middle layer.

2) *Agar-gel Electrophoresis*

Agar-gel electrophoresis of the hemoglobin solution was performed by a modified method of SHIBATA's[12]. As the buffer, Tris-EDTA-borate buffer was used. The composition of the buffer at pH 7.2 was 18.50 g of Tris (hydroxymethyl) aminomethane, 7.25 g of ethylenediaminetetraacetic acid and 10.00 g of boric acid, and that of the buffer at pH 8.6 was 27.5 g of Tris (hydroxymethyl) aminomethane, 3.5 g of ethylenediaminetetraacetic acid and 4.5 g of boric acid, which were both dissolved in water to make each 500 cc solution. These buffers were diluted to three volumes immediately before use. Bacto agar

(Difco) was added to the buffers at a rate of 0.8w/v. Electrophoresis was made at 0−5°C at 200V for 40 min with the 7.2 buffer and at 100V for 80 min with the 8.6 buffer.

3) *Cellulose-Acetate Membrane Electrophoresis*

The cellulose-acetate membrane electrophoresis was performed by a modified method of KOHN's[7]. The cellulose acetate membrane employed was OXOID (manufactured at OXO Co., England), which was cut into 1×5 cm strips and used. The composition of the buffer at pH 7.2 was 18.50 g of Tris (hydroxymethyl) aminomethane, 7.25 g of ethylenediaminetetraacetic acid and 10.0 g of boric acid which were dissolved in distilled water to make a 500 cc solution, and this buffer was diluted to three volumes immediately before use. The composition of the buffer at pH 8.6 was 6.05 g of Tris (hydroxymethyl) aminomethane, 6.00 g of disodiume thylenediaminetetraacetate, dihydrate, and 4.60 g of boric acid, which were dissolved in distilled water to make a 1,000 cc solution. Electrophoresis was made at 0.4 mA/cm for 60 min at a room temperature, and the hemoglobin was dyed with ponceau 3R.

4) *Column Chromatography*

Chromatography was carried out using CM-cellulose as the adsorbent by the elution with the salt concentration gradient of phosphate buffer. First, CM-cellulose (Brown, 0.65 meq/g) was activated by washing with 1 N HCl, distilled water, acetone, distilled water, 1 N ammonia, distilled water, 1 N HCl and distilled water, successively. Then the CM-cellulose, after equilibrated with M/100 pH 6.8 phosphate buffer (containing 0.01% KCN) was packed into a 1×60 cm column to a height of 50 cm, and equilibrated again thoroughly. A 2.5 cc portion of hemoglobin solution containing approximately 150 mg of hemoglobin was put in a visking tube and dialyzed against M/100 phosphate buffer (containing 0.01% KCN) at 0−5°C overnight. Then this hemoglobin solution was put on to the column and eluted by the concentration gradient from M/100 to M/5 with M/100 pH 6.8 phosphate buffer (containing 0.01% KCN) in the mixing bottle, and with M/5 pH 6.8 phosphate buffer in the supplying bottle. The eluate was collected in 5 cc fractions and the extinction of the eluate was measured at 415 mμ by the Spectronic 20 (Baush and Lomb Co.).

5) *Cellulose-Acetate Membrane Electrophoresis Accompanied with Urea Dissociation*

Cellulose-acetate membrane electrophoresis accompanied with urea dissociation of globin was carried out according to the following method : The cellulose-acetate membrane used was OXOID (manufactured at OXO Co., England), which was cut into 1×5 cm strips. The buffer used was a veronal buffer of ionic strength 0.045. The

composition of the veronal buffer was 2.21 g of sodium diethylbarbiturate, 1.17 g of sodium acetate, anhydrous and 20.2 cc of N/10 acetic acid, which were dissolved in distilled water to make a 500 cc solution. The pH of the buffer was 8.6. From this veronal buffer, four kinds of buffer were prepared by adding acetic acid to adjust their pH to 6.5, 7.0, 8.0 and 8.5, respectively. To these buffers, urea was added so that the concentration was 6 M. Preincubation was carried out by putting the globin in 8 M urea solution at 16−18°C for 30−40 min. Electrophoresis was performed at 0.4 mA/cm for 40 min.

6) *Analysis and Estimation of N-terminal Amino Acids and Peptides*

a) Dinitrophenylation of rat adult hemoglobin

Dinitrophenylation of rat adult hemoglobin was performed according to themethod of RHINESMITH et al[10]. That is, 40 cc of 2.5% hemoglobin solution was adjusted to pH 9.0 by adding 0.1 N NaOH. To this solution, 500 mg of Na_2CO_3 and 1.0 cc of 2, 4-dinitrofluorobenzene(DNFB) were added and the mixture was stirred at 40°C for 3 hr. When the mixture was adjusted to pH 2.0 with 0.1 N HCl, yellow-green DNP-hemoglobin was precipitated.

b) Preparation of DNP-globin

To remove heme of DNP-hemoglobin, the method of ANSON and MIRSKY[1] was employed. That is, the DNP-hemoglobin was suspended in 40 cc of 0.1 N HCl and the suspension was put drop by into a mixture of 500 cc of acetone and 15 cc of concentrated HCl, cooled at -20°C with methanol and dry ice, while the mixture was being stirred. Then the mixture was strirred for 20 min more. The yellow DNP-globin obtained in this way was washed with three portions of acetone and subsequently with three portions of ether and dried.

c) Hydrolysis of DNP-globin and extraction of DNP-amino acids and DNP-peptides from the hydrolysate

One hundred milligram portions of the dried DNP-globin were suspended in 6 N HCl, and hydrolyzed at 105°C using a reflux condenser for 1/4, 1, 6 and 10 hr, respectively. Each of these hydrolysates of the DNP-globin was extracted with four 25 cc portions of ether. Then the ether extract was washed with four 10 cc portions of distilled water (containing 1−2 drops of 6 N HCl). Subsequently, the 40 cc of distilled water used for washing were extracted with a 25 cc portion of ether, and the ether layer was added to the previous ether extract. The remnant water layer was added to the water layer of the first hydrolysate and extracted with ten 10 cc portions of ethyl acetate. Then, this ethyl acetate layer was extracted with ten 5 cc portion and five 10 cc portions and one 15 cc portion of distilled water (containing 1−2

drops of 6 N HCl). Thus, the ether extract and the ethyl acetate extract of the DNP-amino acids and DNP-peptides were obtained from the hydrolysate of the DNP-globin. These extracts, after evaporation to dryness, were used for the following experiments.

d) Silica gel-celite column chromatography

In order to separate, identify and determine quantitatively the DNP-amino acids and DNP-peptides from the hydrolysate of DNP-globin, column chromatography was performed using silica gel-celite as the adsorbent, according to the method of MTSUDA et al[8]. The adsorbent used in the experiment was a mixture of silica gel of 100 mesh (manufactured for chromatographic analysis at Mallinckrodt Chemical Work, U. S. A.) and celite 545 (Wako Chemical Industry Ltd., Japan) at a rate of 2 : 1. The adsorbent was packed in a column 0.9 cm in diameter to a height of 15 cm and after the top was made even, the adsorbent was washed with 5 cc of ether, 10 cc of a mixture of ether and acetone in equal volumes, 2 cc of ether, and 7 cc of ligroin successively and finally equilibrated with 7 cc of the mixture which is to be used as the developer. The ether-extracted DNP-compounds, dissolved in 3 cc of a solvent 2AA10AL, were put on to the pretreated column and successively developed with a developer 8AA4AL. And the migration of the yellow bands were recorded at every 7 cc of the developer. The band of a low mobility was taken out out of the column, eluted with an ether-ethanol mixture (4 : 1), evaporated to dryness and dissolved in 3 cc of a solvent 2AA10AL. The solution was applied to the column like the previous treatment, and developed with a developer 2F8EL, and the migration of the band was measured at every 7 cc of the developer. The ethyl acetate-extracted DNP-components, dissolved in 3 cc of a solvent 20AAB, put on to the column and developed with developers, 8AA4AL, 3AA15AL and 4AA20AL, successively. The migration of the band was recorded at every 7cc of the developer. All these treatments were in a dark room with organic solvents purified as perfectly as possible.

(NOTE) 2AA10AL is a ligroin solvent containing 2 cc of acetic acid and 10 cc of acetone in the whole volume of 100 cc, 8AA4AL a ligroin solvent containing 8 cc of acetic acid and 4 cc of acetone in 100 cc, 2F8EL a ligroin solvent containing 2 cc of formic acid and 8 cc of ethyl acetate in 100 cc, 20AAB a benzene solvent containing 20 cc of acetic acid in 100 cc, 3AA15AL a ligroin solvent containing 3 cc of acetic acid and 15 cc of acetone in 100 cc, and 4AA20AL a solvent containing 4 cc of acetic acid and 20 cc of acetone in 100 cc.

e) Estimation of DNP-amino acids and DNP-peptides

The DNP-amino acids and DNP-peptides separated by silica gel-celite column chromatography were dissolved in glacial acetic acid and had their extinction measured at the wavelength of 340 mμ using a spectrophotometer (HITACHI EPU-2). And the μ mole numbers of these DNP-components were calculated by multiplying by 6.2 their extinctions at 340 mμ when they were dissolved in 100 cc of glacial acetic acid. Then the mole numbers of the DNP-amino acids and DNP-peptides were calculated as the values per mole of hemoglobin assuming the molecular weight of rat adult hemoglobin as 66,000.

f) Paper chromatography of DNP-amino acids and DNP-peptides

For another identification of the DNP-amino acids and DNP-peptides which had been separated, identified and determined quantitatively, two-dimensional paper chromatography was carried out using 1.5 M phosphate buffer (pH 6.0) and 2N NH_3-BuOH solution. The 1.5 M phosphate buffer was prepared by dissolving 1 mole of NaH_2PO_4 and 0.5 mole of Na_2HPO_4 in distilled water so that the whole volume was 1 L. The 2N NH_3-BuOH solution was prepared by mixing butanol with 2 N ammonia solution of an equal volume, allowing the mixture to stand for some time, and taking out the butanol layer.

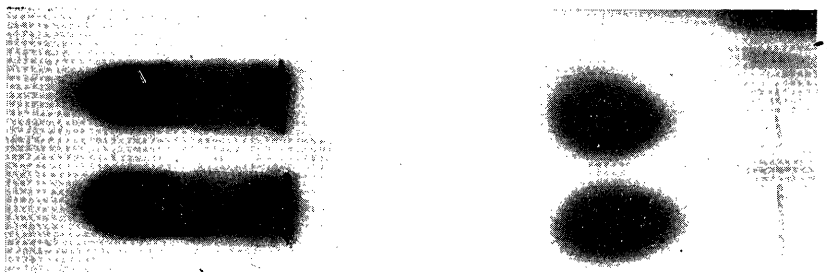
g) Identification of amino acid by the automatic amino acid analyzer

The amino acid except N-termini in the DNP-peptides were obtained by hydrolyzing the DNP-peptides once more at 105°C for 20 hr. The amino acids obtained were identified by the automatic amino-acid analyzer (HITACHI KLA-2).

RESULTS AND DISCUSSION

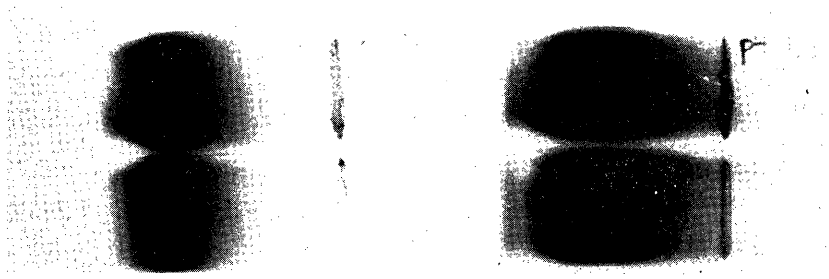
Rat adult hemoglobin is very crystallizable as is noticed in the experiments, and its crystallization in the preparation often made the experiments hard. The microscopical examination of the crystals reveal red fine crystallines. It has been already reported by PONDER[9] that this phenomenon of crystallization is observed remarkable especially in deoxygenated hemoglobin.

The results of agar-gel electrophoresis and cellulose-acetate membrane electrophoresis of rat adult hemoglobin were shown in Fig. 1. The agar-gel electrophoresis gave one main component and two following minor components with the pH 7.2 buffer, but no separated minor components with the pH 8.6 buffer. The cellulose-acetate membrane electrophoresis, though not giving any minor components observable with the pH 7.2 buffer, gave one main component and one preceding



pH 7.2, 200V, 40 min. pH 8.6, 100V, 80 min.

AGAR-GEL ELECTRPHORESIS



pH 7.2, 0.4 mA/cm, 60 min. pH 8.6, 0.4mA/cm, 60 min

CELLULOSE-ACETATE MEMBRANE ELECTROPHORESIS

Fig. 1. Agar-gel and cellulose-acetate membrane electrophoresis of rat adult hemoglobin

minor component with the pH 8.6 buffer. From these results, it was suggested that the hemoglobin from the adult rats of the Wistar strain contains one main component and at least two minor components. This fact agrees with the report of GIRI and PILLAI[4]. Therefore, to examine the N-terminal structure of the main component, the main component must be purified by removing the minor components present together with it. In the present experiment, the main component was isolated and purified by column chromatography using CM-cellulose as the adsorbent. Fig.2 represents a chromatogram obtained. As shown in this figure, two minor components were eluted preceding the main component. This fact agrees with the result of agar-gel electrophoresis which is above-mentioned. Then, excepting these minor components, the eluate containing the main component was collected, dialyzed for 48 hr and lyophilized. Before examining the N-terminal structure of the globin moiety of the main component separated and purified, the author made an investigation into the varieties of polypeptide chain which the globin moiety contains. That is, this main component was dissolved in distilled water and the globin was prepared according to the method of ANSON and MIRSKY[1], just like the treatment of preparation of DNP globin from DNP-hemoglobin which was described previously. This

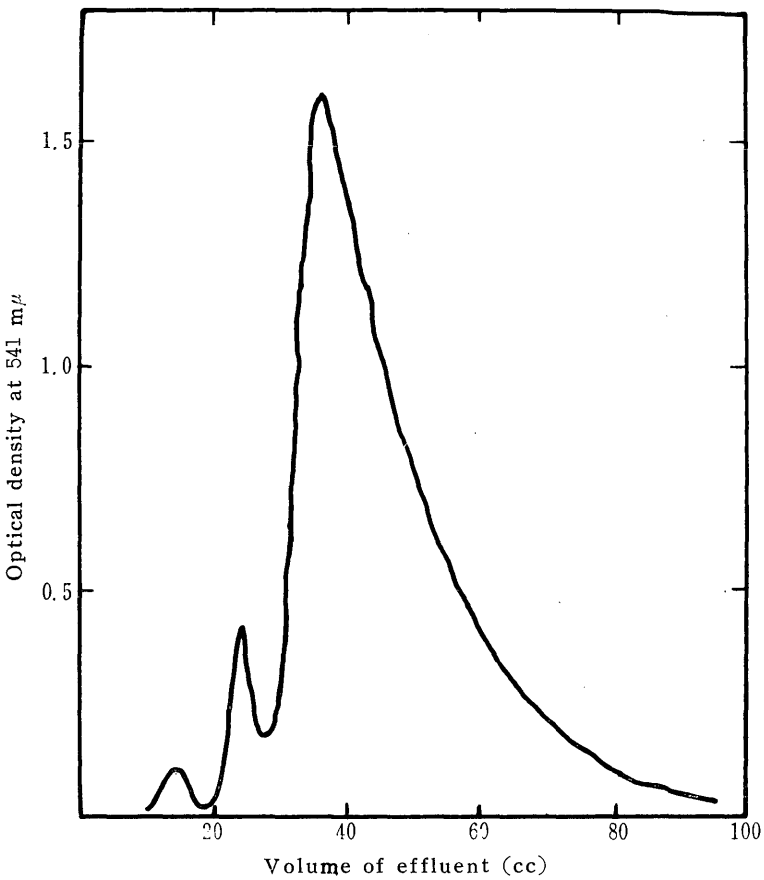


Fig. 2. Column chromatogram of rat adult hemoglobin on CM-cellulose

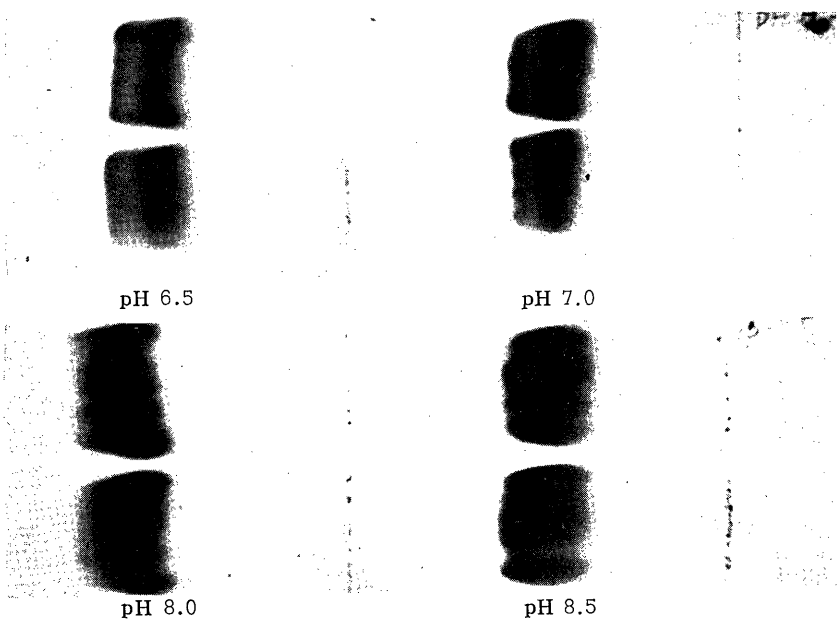


Fig. 3. Cellulose-acetate membrane electrophoresis with urea of rat adult globin (0.4 m/cm, 40 min)

globin was examined by cellulose-acetate membrane electrophoresis accompanied with urea dissociation. Fig. 3 gave the results obtained. As this figure shows, although the pH 8.0 buffer and the pH 8.5 buffer gave only one polypeptide chain, the pH 6.5 buffer and the pH 7.0 buffer presented two bands clearly distinguishable. This fact indicates that the main component of rat adult hemoglobin is composed of two kinds of polypeptide chains.

The analysis of the N-terminal structure of the globin moiety from the main component in rat adult hemoglobin was carried out mainly by the DNP-method. That is, DNP-hemoglobin was prepared from the main component according to the method mentioned above, and DNP-globin was prepared from it by removing the heme. The DNP-globin

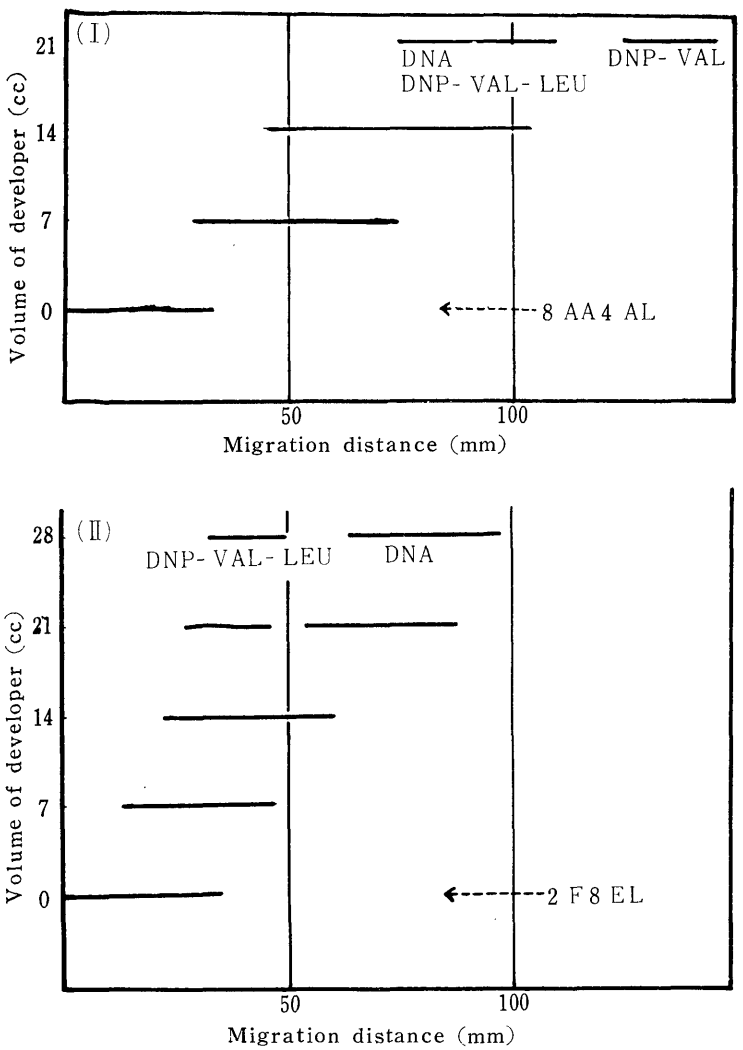


Fig. 4. Silica gel-celite chromatography of ether-extracted DNP-compounds in partial hydrolysate of DNP-globin from rat adult hemoglobin

obtained was hydrolyzed and the DNP-compounds in the hydrolysate were extracted with ether and with ethyl acetate. Fig. 4 shows the result by silica gel-celite column chromatography of the ether extract. The upper illustration of Fig. 4 represents the development with 8AA-4AL separating into two bands. The faster band is consistent with the synthesized DNP-valine and the slower band with the synthesized DNP-dinitroaniline in mobility in the column chromatography. Then the slower band was eluted out of the silica gel-celite column, and developed again with 2F8EL, and the result was shown in the lower illustration of Fig.4. Two bands were observed in this case, too, and the faster band was also consistent with the synthesized dinitroaniline. The slower band was again eluted out and evaporated into dryness in vacuum, and after 20 hours' rehydrolyzation, the DNP-compound in it was extracted with ether. This DNP-compound was subjected to silica gel-celite column chromatography with a developer 8AA4AL and its mobility was observed to be the same as that of the synthesized DNP-valine. On the other hand, when the watersoluble portion which was not extracted with ether was examined by two-dimensional paper chromatography, leucine was detected in it. Leucine was also proved to be present by the automatic amino-acid analyzer which is mentioned later. From the results of these experiments, it is presumed that the DNP-compounds which were extracted with ether from the HCl hydrolysate of DNP-globin may be DNP-valine, DNP-valylleucine and dinitroaniline, respectively. Among them,dinitroaniline is known to yield as a by-product of the DNP-method and is thought not to relate the N-terminal structure. The N-terminal structure of valylleucine is known in the α-polypeptide chain of human hemoglobin. Therefore, DNP-valylleucine was obtained from the HCl hydrolysate of dinitrophenylated human adult hemoglobin and was compared in the mobility in the silica gel-celite column chromatography with the DNP-compound from rat adult hemoglobin which is presumed to be DNP-valylleucine. The result showed that the two DNP-compounds from human and rat hemoglobin were almost the same. Fig. 5 shows the result by silica gel-celite column chromatography of the DNP-compounds extracted with ethyl acetate. As shown in the figure, when the development was performed with three verieties of developer 8AA4AL, 3AA15AL and 4AA20AL, three bands were separated. The most slowly-moving bands is thought to be ε-mono-DNP-lysine having no relation with the N-terminal structure. The other two bands, which were both presumed to be DNP-peptides, were eluted out of tne silica gel-celite column, and hydrolyzed for 20 hr. Thus, DNP-valine was found in the ether extract of rehydrolysate of the fastest band by silica gel-celite column chromatography, and leucine and imidazole-mono-DNP-histidine were found in the remnant water-soluble portion by paper chromatog-

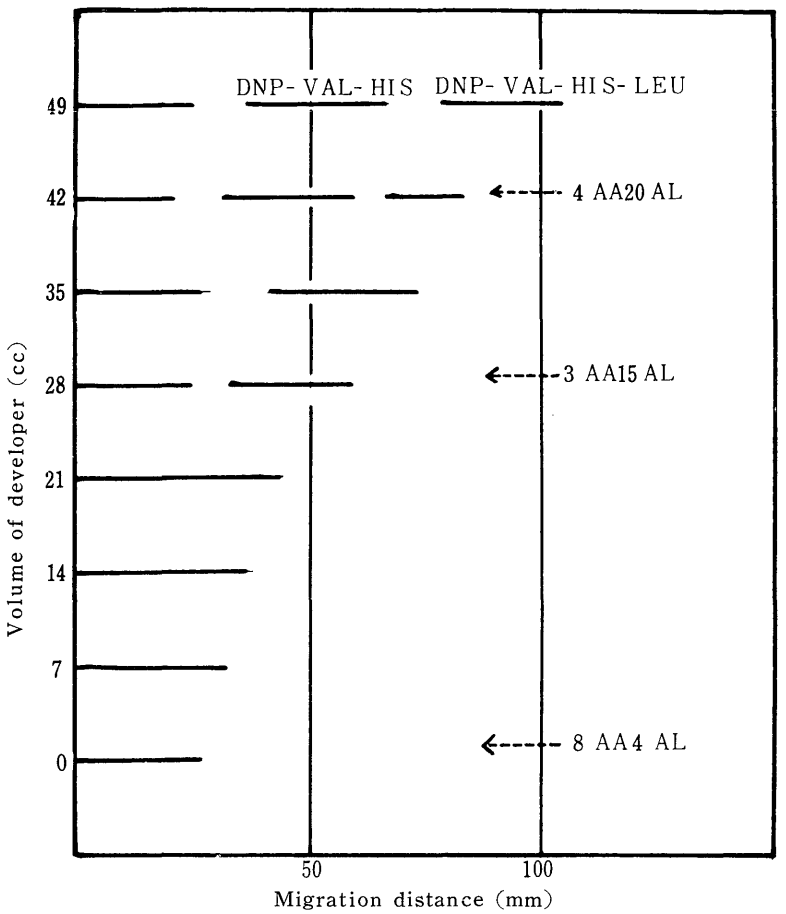
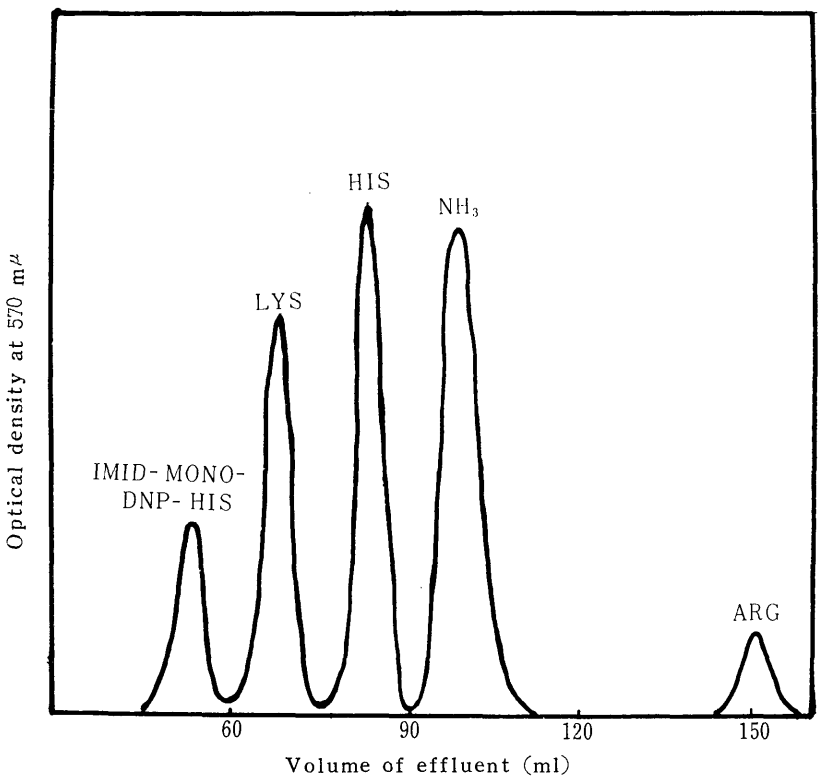


Fig. 5. Silica gel-celite chromatography of ethylacetate extracted DNP-compounds in partial hydrolysate of DNP-globin from rat hemoglobin

raphy. From the ether extract of the middle band was observed DNP-valine, and from the remnant water-soluble portion imidazole-mono-DNP-histidine likewise. Then the amino acids which were not extracted with ether from the rehydrolysate of these two bands were also analyzed by the automatic amino acid analyzer; the column for the analysis of acidic and neutral amino acids gave only leucine coming from the fastest band, and the column for the analysis of basic amino acids, as shown in Fig.6, gave a peak of the presumed imidazole-mono-DNP-histidine, which came from both the bands, at an effluent volume of 45 cc, earlier than lysine. This peak is quite consistent with the peak of the imidazole-mono-DNP-histidine from human adult globin. In this way, the fastest band of the DNP-components in the ethyl acetate extract which is shown in Fig.5 was presumed to be DNP-valylhistidylleucine and the middle band DNP-valylhistidine. As the N-terminal structure of this valylhistidylleucine is present in human adult hemoglo-



Fia. 6. Chromatogram of imidazole mono-DNP-histidine using automatic amino acid analyzer column 0.9×15cm
buffer 0.2 N sodium citrate buffer pH 5.28

bin, DNP-valylhistidine and DNP-valylhistidylleucine were prepared from DNP-human adult globin and examined by column chromatography. They were quite consistent in mobility with the compounds from the present rat adult hemoglobin which were presumed to be DNP-valylhistidylleucine and DNP-valylhistidine, respectively. To make this fact sure, the presumed DNP-valylhistidine and DNP-valylhistidylleucine from rat adult hemoglobin were subjected to paper chromatography as shown in Fig.7, and the result was compared with the results of paper chromatography of the synthesized DNP-valine and the DNP-valylleucine, DNP-valylhistidine and DNP-valylhistidylleucine from human adult hemoglobin with all the same results. From the results of all these experiments performed, it is considered to be doubtless that rat adult hemoglobin, like human adult hemoglobin, consists of two kinds of polypeptide chains, one having a valylleucyl N-terminal structure and the other having a valylhistidylleucyl N-terminal structure. Following to these qualitative studies, a quantitative analysis was carried out on the N-terminal DNP-amino acids and DNP-peptides of rat adult hemoglobin to examine the numbers of each of these two

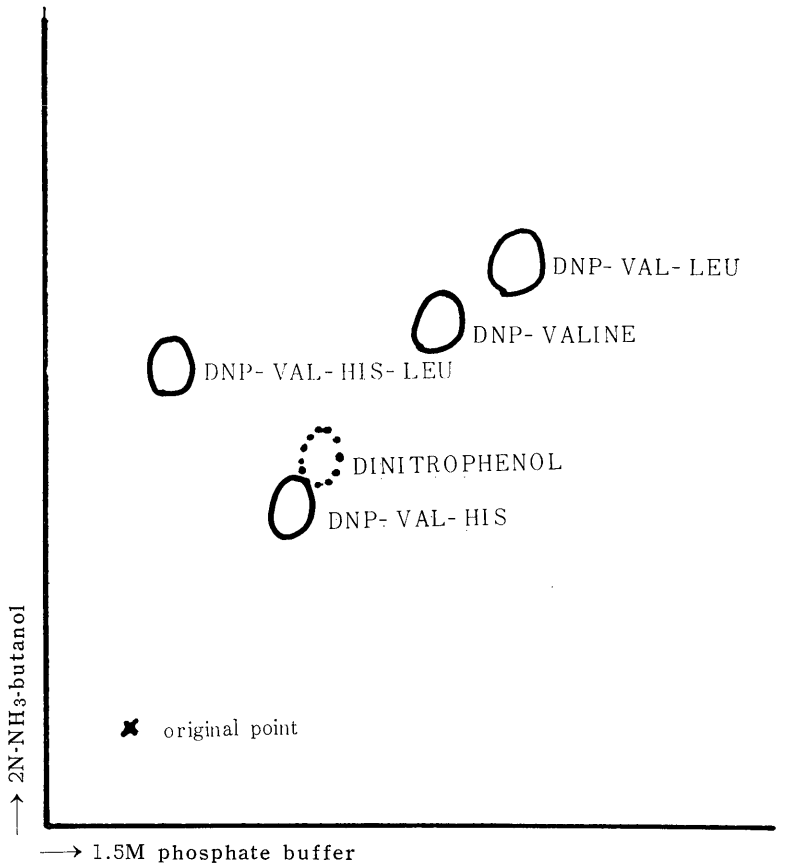


Fig. 7. Two-dimensional paper chromatogram of DNP-amino acids and peptides in partial hydrolysate of DNP-globin from rat adult hemoglobin

Table 1.

Number of N-Terminal residues in the main component of rat adult hemoglobin

hydrolysis time (hr)	DNP-valine		DNP-val-leu		DNP-val-his		DNP-val-his -leu		total
¼	0.14 0.14	0.14	1.41 1.37	1.39	0.59 0.75	0.62	0.59 0.44	0.52	2.72
1	0.55 0.61	0.58	1.01 1.11	1.05	0.81 0.79	0.80	0.41 0.52	0.47	2.90
6	2.00 1.94	1.97	0.67 0.69	0.68	0.66 0.64	0.65	0.18 0.16	0.17	3.47
10	2.60 2.64	2.62	0.44 0.45	0.45	0.36 0.42	0.49	0.08 0.60	0.07	3.52
20	3.52 3.73	3.63	0.04 0.03	0.04	0.28 0.27	0.27	0 0	0	3.96

kinds of polypeptide chains which rat adult hemoglobin contains. The result is shown in Table I. These values have been calculated as mole number per mole of hemoglobin concerning DNP-valine, DNP-valylleucine, DNP-valylhistidine and DNP-valylhistidylleucine which were extracted from the hydrolysates after 1/4, 1, 6, 10 and 20 hours' hydrolysis of DNP-globin from rat adult hemoglobin and separated, purified by silica gel-celite column chromatography and determined quantitatively. From this result, rat adult hemoglobin is presumed to consist of two polypeptide chains with the N-termini of valylleucine and two polypeptide chains with the N-termini of valylhistidylleucine per mole. These N-terminal structures are quite similar to those of human adult hemoglobin. It is of interest in the problem on the evolution of the hemoglobin molecule that rat adult hemoglobin has the same structure as human adult hemoglobin being different from the hemoglobins of the bovine, horse, swine, sheep etc. But this similarity may remain but in its N-terminal structure. For the study on the problem of the molecular evolution, the internal structure of hemoglobin must be examined in future.

CONCLUSION

The main component of hemoglobin of the adult rats of the Wistar strain is considered to consist of two polypeptide chains with a valylleucyl N-terminal structure and two polypeptide chains with a valylhistidylleucyl N-terminal structure.

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FOOT NOTE

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