Hemoglobin Heterogeneity in the Pekin Duck[1] (34540)

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Four electrophoretically distinct hemoglobins are found in Pekin duck erythrocytes. Two occur in the early embryo (1), two others in the adult (1, 2). One of the embryonic and both adult hemoglobins occur in the late embryo and duckling (1). The hemoglobin heterogeneity exhibited by the duck is a phenomenon probably common to all animal species. In the species examined, differences between hemoglobins reflect differences in amino acid composition of corresponding globins (3). In the present work, two hemoglobins were obtained from erythrocytes of the adult Pekin duck (*Anas domesticus*) by ion-exchange chromatography. The hemoglobins were compared with respect to amino acid composition of the constituent globins, synthesis by erythrocytes *in vitro*, and location within the cells. Incorporation of glycine-2-^{14}C and ^{59}Fe into the hemoglobins was used to estimate synthesis. Comparison of heme exchange between the ferri-forms of the hemoglobins in cells and solution was used as a tentative test for location.

Experimental. The animals used were male, 1–1.5 years old, and weighed about 3 kg. They were obtained from a local farm, kept some weeks in the laboratory before use, and fed a commercial ration plus cracked corn in the laboratory. Venous blood was taken from the animals in heparin as required and promptly cooled in ice before further treatment. Two forms of hemoglobin were obtained from blood as follows: Erythrocytes were collected in the centrifuge, washed with 3 vol of 0.156 *M* potassium chloride, and suspended in 3 vol of the chloride in the cold. The suspension was frozen in liquid nitrogen, thawed, and centrifuged 0.5 hr at 34,000*g*. The cell extract, *i.e.*, the supernatant liquid, was added to a column of Sephadex G-75 in 0.016 *M* sodium barbital (5, 5-diethylbarbiturate) buffer, pH 7.6, at room temperature. Hemoglobin was eluted from the column with the barbital buffer and added to a column of DEAE–cellulose (Selectacel No. 72, obtained from Carl Schleicher and Schuell Co.) in the same buffer. One form of hemoglobin was removed from the DEAE–cellulose by addition of the buffer alone, the other by subsequent addition of 0.2 *M* KCl in the buffer. For present convenience, the hemoglobins eluted from the DEAE–cellulose with the barbital and the barbital-chloride are designated *Hb*'s I and II, respectively. The columns employed varied in size with the amount of hemoglobin to be separated. For 40–50 μmoles, Sephadex and DEAE columns 1.7 cm in diameter and, respectively, 40 and 20 cm in length were used. Hemoglobin in the eluates and cell extracts was converted into cyanmethemoglobin for quantitative determination (4). In the present study, a mole of hemoglobin or globin signifies an amount equivalent to 1 mole of heme.

Separation of erythrocyte hemoglobin as outlined is illustrated in Fig. 1. Since the cell extract used was prepared and subsequent manipulations were made under room air, the pigment separated was mainly oxyhemoglobin. Of the pigment applied to the DEAE–cellulose column, 85% was eluted with the barbital (*Hb* I), 13.6% with the barbital-chloride (*Hb* II). Practically the same yields of *Hb*'s I and II were obtained from equivalent carbon monoxide hemoglobin and from cyanmethemoglobin. Lower yields were obtained from equivalent methemoglobin. The

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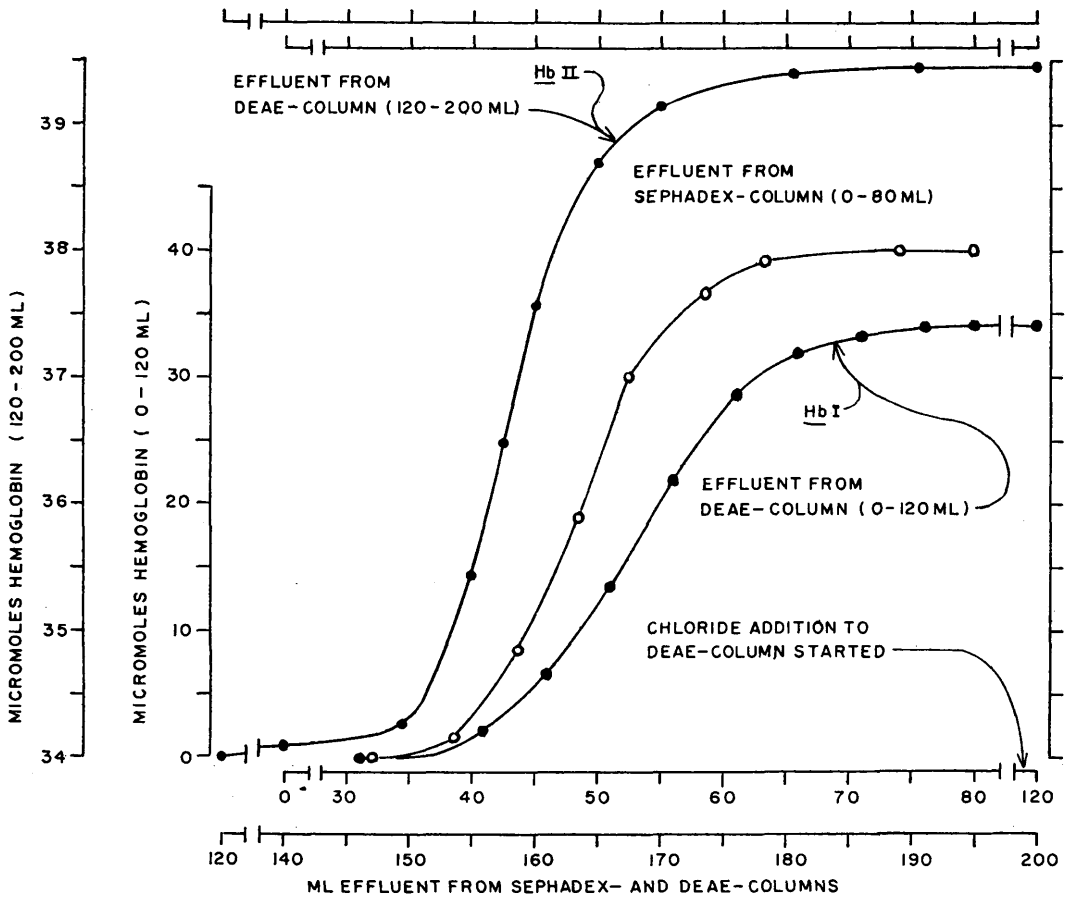

FIG. 1. Separation of duck erythrocyte hemoglobins. Ten milliliters of erythrocyte extract prepared as outlined in the text and containing 40.8 μmoles of total hemoglobin were added to a column of Sephadex G-75 in 0.016 *M* sodium barbital, pH 7.6. The hemoglobin was eluted from the column with the barbital buffer and added to a column of DEAE-cellulose in the buffer. A portion of the hemoglobin, arbitrarily designated *Hb* I, was eluted from the DEAE with the buffer, another, designated *Hb* II, with 0.2 *M* potassium chloride.

methemoglobin retained by the DEAE column after the elution with the barbital-KCl was in a form that resisted removal with 0.1 *N* alkali. No further pigment separation was obtained when preparations of *Hb*'s I and II from the ferrohemoglobins and from cyanmethemoglobin were chromatographed as outlined, when *Hb* II was eluted from the DEAE-cellulose with a gradient of KCl in the barbital buffer, or when *Hb* I preparations were chromatographed on CM–cellulose. In tests for separation of *Hb* I on CM–cellulose, the pigment was added to a column of the cellulose in 0.016 *M* barbital buffer, pH 8.0, and eluted with a 0.2 *M* solution or a gradient of sodium barbital. In the tests outlined below, the separations of the hemoglobin in cell extracts differed somewhat from that of Fig. 1. In these tests, the hemoglobin was added to the DEAE-cellulose column as it emerged from the Sephadex column and the effluent from the DEAE-cellulose column ccorresponding to that between 80 and 140 ml in Fig. 1 was discarded.

For amino acid assay of the globin components of the *Hb*'s I and II, initial pigment preparations were rechromatographed, dialyzed overnight against 500 vol of water, and filtered. The hemoglobin concentrations in the filtrates were determined. Globin corre-

sponding to a known amount of hemoglobin was precipitated from each filtrate by addition of 12 vol of 0.02 *M* hydrochloric acid in acetone (5). The globin preparations were quantitatively collected in the centrifuge, dissolved in water, and reprecipitated with the acid acetone. For spectrophotometric estimation of tryptophane (6), the reprecipitated material was washed with 0.01 *M* ammonium hydroxide in methanol to remove acetone and dissolved in 0.1 *N* sodium hydroxide. For determination of half-cystine and methionine, the reprecipitated material was dried, oxidized with performic acid, and hydrolyzed 22 hr in 6 *N* hydrochloric acid (7). For assay of other amino acids, the material was dried and hydrolyzed 22-96 hr in the hydrochloric acid. All hydrolyzates, after removal of volatile acid, were dissolved in buffer and applied to an amino acid analyzer (8). Corrections for loss of serine and threonine during the 22- to 96-hr hydrolyses were made by linear extrapolation of observed results to zero time. The observed results for other amino acids were independent of hydrolysis time. The weights of the reprecipitated globins dried at 90° were determined.

For comparison of the synthesis of *Hb*'s I and II *in vitro*, blood was incubated with about 1 μCi of glycine-2-^{14}C and 10^{-2} μCi of $^{59}FeCl_3$ per ml. The hemoglobins were then separated as outlined above and assayed for heme- and globin-^{14}C and heme-^{59}Fe (9–11).

Results and Discussion. The mean and standard deviation of the ratios of amount of *Hb* I to amount of *Hb* II for 12 samples of blood taken at weekly intervals from one animal were 6.00 and 0.12, respectively. The mean and deviation for initial samples from 13 animals were 6.08 and 0.35. The average ratio for blood from nine animals based on electrophoretic separation of the hemoglobins was 5.7 (2). Evidently, the ratio is established by factors common to the species. Two electrophoretically distinct hemoglobins are found in erythrocytes of the mallard and the pochard duck (12). The ratio of amounts of the hemoglobins is 1.63 in the mallard and 3.76 in the pochard. These ratios differ notably from those observed in the Pekin duck.

TABLE I. Amino Acid Composition of Duck Globin.[a]

Amino acid	No. of amino acid residues per 2 equivalents of heme		
	Globin I	Globin II	Globin II-I
Alanine	40.5	40.6	0.1
Arginine	9.36	10.7	1.3
Aspartic acid	24.6	27.2	2.6
Half-cystine	5.02	5.02	0.0
Glutamic acid	20.9	31.6	10.7
Glycine	17.9	18.0	0.1
Histidine	18.1	15.6	—2.5
Isoleucine	12.7	9.14	—3.6
Leucine	31.0	38.8	7.8
Lycine	23.2	23.2	0.0
Methionine	2.86	4.70	1.8
Phenylalanine	16.7	17.9	1.4
Proline	12.0	12.8	0.8
Serine	11.6	14.0	2.4
Threonine	13.2	13.0	—0.2
Tryptophane	4.00	5.70	1.7
Tyrosine	6.39	7.59	1.2
Valine	25.0	23.1	—1.9
Total	295	319	23.7

[a] The data given are means of results for three animals. The standard deviations of the results did not exceed 3% of the corresponding means. The globin weights per 2 equivalents of heme calculated from the weights of the amino acid residues are 32,400 and 35,500 for globins I and II, respectively.

Amino acid assays of globins from *Hb*'s I and II are summarized in Table I. Globin II, that from *Hb* II, contained more amino acid residues than equivalent globin I. The marked difference in number of aspartyl plus glutamyl residues probably accounts for the differences in eletrophoretic and chromatographic behavior of the parent hemoglobins. The mean and standard deviation of the dry weights for globin I were 36,200 and 700 g per 2 moles of heme, respectively. Corresponding values for globin II were 40,200 and 800. The observed globin weights probably included that of hydrochloric acid associated with basic amino acid residues. Correction of the means indicated based on the assumption that the basic residues were associated with equivalent acid yield values of 34,400 and 39,300 for globins I and II, respectively. The corrected values agree roughly with the cor-

TABLE II. Synthesis of Duck Hemoglobins *in vitro*.[a]

Test	Hemoglobin	(cpm/μmole) Heme-^{14}C	Globin-^{14}C	Heme-^{59}Fe
1a	I	390	426	38
	II	530	702	66
b	I	710	799	80
	II	991	1400	125
c	I	1460	1570	164
	II	2020	2720	262
2a	I	1870	1780	258
	II	3000	3400	414
b	I	1160	1160	284
	II	1790	2210	468
3	I	1460	1520	496
	II	4260	2730	865
4	I	1130	1360	234
	II	1570	2160	376
5	I	1230	1340	120
	II	1360	2020	146
Specific activity for II/specific activity for I				
Mean and standard deviations of the ratios				
1–5		1.7 ± 0.6	1.7 ± 0.2	1.6 ± 0.2

[a] Blood was incubated with glycine-2-^{14}C and ^{59}Fe for 0.5, 1, and 2 hr at a, b, and c, respectively, in Test 1, and for 2 hr in the other tests. *Hb*'s I and II were then separated and assayed for heme- and globin-^{14}C and for heme-^{59}Fe. Further details are given in the text. A different animal was used for each test. The results at a and b in Test 2 are for blood samples taken on different days. The ratios of corresponding amounts of *Hb*'s I and II ranged from 5.7 to 6.5.

responding sums of the weights of amino acid residues, 32,400 and 35,500.

The two hemoglobins found in the "duck" differ in amino acid composition, notably in number of glutamyl residues (12). The minor hemoglobin bears the greater number of acid residues. It is not clear whether "duck" signifies the mallard, the pochard, a "domestic" variety, or perhaps all three. In any case, the comparable hemoglobins of the "duck" and the Pekin duck under present study differ in amino acid composition.

Estimates of glycine-2-^{14}C and ^{59}Fe incorporation into *Hb*'s I and II in blood *in vitro* are summarized in Table II. In each test, the heme and globin specific activities were greater for *Hb* II than for *Hb* I, while the incorporations, *i.e.*, specific activities × amount of hemoglobin, were greater for I than for II. If the immediate sources of the materials incorporated into the hemoglobins were the same for both, the greater incorporations for *Hb* I indicate a higher rate of synthesis for I than for II. However, the greater specific activities for *Hb* II suggest that the erythrocyte may complete synthesis of II before I.

Exchange of heme between certain human methemoglobins occurs in solution (13). Results of tests for such exchange between the duck hemoglobins within erythrocytes and in solution are given in Table III. In the cells, the nitrite used to oxidize hemoglobin to the ferri-form affected globin synthesis slightly, but depressed heme synthesis notably (Test A, cf. *1* with *3a*. The nitrite had little effect on the distribution of labeled heme between *Hb*'s I and II within the cells (Test A, cf. *1–3*). The oxidant induced change in the distribution of labeled heme between the hemoglobins in solution (Test A, cf. *4* and *5* with *1–3*; Test B, cf. *1* and *4* with *2* and *3*). The direction of the change, *i.e.*, increase and decrease in the proportion of the labeled heme associated with *Hb*'s I and II, respectively, indicates an exchange of heme between the ferri-homoglobins. The lack of such exchange within the erythrocytes suggests segregation of the hemoglobins, perhaps within different cells. In man, multiple forms of hemoglobin have been found within the same erythrocyte (14, 15) and segregated within different cells (15, 16).

Summary. Two hemoglobins were obtained from erythrocytes of the adult, male Pekin duck by use of ion-exchange chromatography. The hemoglobins were found to differ in amount within the cells by about 6-fold and in amino acid composition of the constituent globins. The globin corresponding to the minor hemoglobin contains the greater number of amino acid residues, notably those of aspartic plus glutamic acid. The difference between the globins in acid residues may

TABLE III. Heme Exchange Between Duck Methemoglobins.[a]

	Test	Heme specific activity (cpm/μmole)			Globin specific activity (cpm/μmole)		
		Hb I	*Hb* II	II/I	*Hb* I	*Hb* II	II/I
A 1	Blood + glycine-2-^{14}C, 2-hr incubation	979	2250	2.3	896	2300	2.6
2	Extract of cells from *1*, 2-hr incubation	960	2310	2.4	841	2190	2.6
3	Blood + glycine-2-^{14}C + nitrite						
	a 2-hr incubation	389	959	2.5	756	2180	2.9
	b 4-hr incubation	702	1690	2.4	1300	3510	2.7
4	Broken cells from *3a*, 2-hr incubation	425	736	1.7	702	2110	3.0
5	Extract of cells from *3a*, 2-hr incubation	435	705	1.6	689	1970	2.9
B 1	Blood + glycine-2-^{14}C, 2-hr incubation	1300	2580	2.0	1500	2880	1.9
2	Extract of cells from *1*, 2-hr incubation	1240	2410	1.9	1400	2630	1.9
3	Extract of cells from *1* + nitrite, 2-hr incubation	1370	1980	1.4	1560	3140	2.0
4	Cells from *1* + unlabeled plasma + nitrite, 2-hr incubation	1520	3040	2.0	1940	4000	2.1

[a] Preparation of the broken cells and cell extract was as indicated under Experimental. Nitrite was added in amounts equivalent to five times the hemoglobin in the blood and other materials. An 0.156 *M* solution of sodium nitrite was added to blood, the solid salt to the other materials. Conversion of hemoglobin to methemoglobin was about 95% complete 0.7 hr after addition of the oxidant and complete at 1 hr. At the end of the incubations, potassium cyanide was added to the text mixtures in amounts equivalent to the hemoglobin present plus 1 μmole/ml of mixture. *Hb*'s I and II were then separated and assayed for heme- and globin-^{14}C as outlined under Experimental.

account for the difference in chromatographic behavior of the parent hemoglobins. The relationship between the incorporation of glycine-2-^{14}C and ^{59}Fe into the hemoglobins by erythrocytes *in vitro* suggest a higher rate but later completion of synthesis for the major hemoglobin than for the other. The ferriforms of the hemoglobins exchange heme in solution but not within the erythrocytes; thus, the hemoglobins may be confined within different cells.

Amino acid assay of the globins was carried out by Roslyn Shapanka with the advice and consent of Dr. Lewis J. Greene.

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