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The Formation of Complexes between Haemoglobins and Plasma Proteins in a Variety of Animals

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Heilmeyer (1933), who investigated by spectrophotometry the effect of adding alkaline haematin to serum, showed that there was a definite combination of the haematin and some protein constituents. Later Fairley & Bromfield (1934) and Fairley (1941*a*, *b*) identified methaemalbumin formed from the combination of haemoglobin released by the intravascular breakdown of erythrocytes and the albumin fraction of the plasma in man and monkey. This complex was not found in other animals. Clark, Harris & Rosenberg (1949) investigated the combination of mesohaem with a number of proteins. They found that a variety of proteins formed haemochromogens, including human serum albumin, and showed that methaemalbumin is not a haemochromogen. Rosenfield & Surgenor (1949) have shown that haematin and serum albumin combine in the molar ratio 1:1 to form methaemalbumin, but found no spectrophotometric evidence for the combination of haematin with other plasma proteins. Haem combines with denatured proteins in the molar ratio 1:2 to form haemochromogens, but does not combine with native proteins (Anson & Mirsky, 1928, 1934; Holden & Freeman, 1929). Haem combines with native globin to form a complex which resembles native haemoglobin in many respects (Anson & Mirsky, 1930, 1934; Jope, 1949). It differs from native haemoglobin in its spectral absorption in the near-ultraviolet region (Jope, 1949; Jope & O'Brien, 1949). Very little is known of complex formation between haemoglobins and other fractions of serum proteins in other vertebrate animals.

Changes in electrophoretic mobility of proteins have been used widely to investigate their interactions with a number of large molecules, including other proteins. This paper describes the use of paper electrophoresis, supplemented by spectrophotometry, to reveal the formation of a family of complexes between haemoglobin and its derivatives and plasma proteins in a variety of animals: man, horse, ox, dog, pig, goat, rabbit, guinea pig, duck, turtle and frog.

METHODS

Blood was collected by venepuncture or heart puncture into an oxalated tube and centrifuged at 2500 rev./min. for 20 min. to separate the plasma. Haemoglobin was prepared from the sedimented erythrocytes by the method of Drabkin (1946) and diluted to a concentration of 40 mg./ml. with barbitone buffer (I 0·075; pH 8·6). From this solution, the following were prepared: (*a*) oxyhaemoglobin, by saturation with air; (*b*) reduced haemoglobin, by addition of a trace of sodium dithionite and subsequent storage under CO_2; (*c*) carbon monoxy haemoglobin, by saturation with coal gas; (*d*) methaemoglobin, by addition of a minimal amount of potassium ferricyanide and subsequent dialysis; (*e*) alkaline haematin, from purified ox haemin by the method of Fischer (1941).

Paper electrophoresis was conducted under kerosene (b.p. 200–300°), with broad sheets of Whatman no. 1 filter paper and barbitone buffer (I 0·075; pH 8·6). The potential gradient was 5–7 v/cm. A graded series of buffered mixtures of plasma and haemoglobin was prepared as shown in Table 1. Immediately after mixing each sample was examined spectrophotometrically to confirm the exclusive presence of the appropriate haem–protein. Spots (0·005 ml.) of a series of dilutions of the mixture were applied at 15 mm.

Table 1. *Scheme of mixing dilution series of haemoglobin–plasma–buffer*

	Parts (by vol.)										
Plasma	10	10	10	10	10	10	10	10	10	10	10
Haemoglobin (40 mg./ml.)	0	1	2	3	4	5	6	7	8	9	10
Barbitone buffer, pH 8·6; *I* 0·075	10	9	8	7	6	5	4	3	2	1	0
Resultant haemoglobin concn. (mg./ml.)	0	2	4	6	8	10	12	14	16	18	20

intervals along a pencilled line of origin on the paper. Equivalent volumes of untreated plasma were also applied to this line. Electrophoresis was conducted for 5 hr. at 24 and 200 v.

Since they are not distinguished by the stains commonly used the plasma proteins and haemoglobin were estimated separately. The strip containing plasma alone was cut off and developed in bromophenol blue; the remaining strips were stained with benzidine reagent to reveal the haemoglobin-containing spots on the paper strip.

Benzidine reagent. This was prepared from the following two solutions: (i) 1 g. of benzidine dissolved in 10 ml. of acetic acid and made up to 100 ml. with 95% ethanol. This may be stored in a dark glass bottle for not longer than 1 month; (ii) 2 ml. of H_2O_2 (30 vol.) was diluted with water to 100 ml. This deteriorated after 2 or 3 days. One volume of (i) was mixed with 6 vol. of (ii) immediately before use.

The paper strip was dipped into the reagent for 20 sec., then washed in tap water acidified with a few drops of acetic acid for 20 sec. It was then pressed between sheets of blotting-paper and dried at room temperature. Blue-green spots changed to greyish blue after 1–2 hr. and then the colour began to fade. Estimates were therefore made within 1–2 hr.

Semi-quantitative estimation of the haemoglobin spots was made either by scanning the strip with a densitometer or by elution of the spot with the buffer used for electrophoresis and measurement of the absorption at 410 mμ.

The unused portions of oxyhaemoglobin–plasma mixture were placed in stoppered tubes with a few drops of toluene and incubated at 37°. From time to time the pH and absorption spectrum (700–320 mμ) of these portions were noted. Paper electrophoresis was carried out with specimens after incubation for both 4 and 24 hr.

The following mixtures were examined in this way: (1) plasma plus haemoglobin of the same species; (2) plasma plus haemoglobin of different species; (3) plasma plus oxyhaemoglobin, reduced haemoglobin, carbon monoxy haemoglobin or methaemoglobin; (4) plasma plus haem or haematin.

RESULTS

Electrophoresis

Since all patterns were obtained under identical conditions of voltage, ionic strength, pH and temperature, the mobility of all components may be compared directly by inspection of the distance of migration of the spots from the line of origin. Haemoglobin spots may be referred to the accompanying strip of the plasma pattern.

Fig. 1 shows that in all cases free haemoglobin appears as a single spot with almost the same mobility as β-globulin or fibrinogen, but when it is mixed with plasma from the same species the number of benzidine-staining spots and their mobilities are characteristic of the species, as shown in Table 2.

The binding capacity of oxyhaemoglobin to the plasma components has been estimated by eluting each spot (a presumed spot) found by electrophoresis of a dilution series and estimating the amount of free and bound haemoglobin. The results, set out graphically in Fig. 2, show that a maximum amount of haemoglobin moves with each plasma protein, the amount varying with the particular protein and the species of animal.

When haemoglobin is mixed with plasma from a different species, the pattern is characteristic of the animal from which the plasma is taken, differing only in that the free haemoglobin retains its own typical mobility.

Mixtures of plasma with reduced haemoglobin, carbon monoxy haemoglobin or methaemoglobin show exactly the same patterns as oxyhaemoglobin, except that with methaemoglobin a greater proportion of it moves with the plasma components (see below).

In the course of incubation experiments, the pH has been found to remain fairly constant. No haem or haemochromogen has been detected, but the quantity of methaemoglobin increases with time, and the electrophoretic patterns after 4 and 24 hr. approximate to that of methaemoglobin.

When plasma is mixed with haem or haematin, a highly diffuse electrophoretic pattern without isolated spots is obtained and the free haem or haematin becomes adsorbed on to the paper. Only after long incubation does the typical haemochromogen spectrum begin to appear.

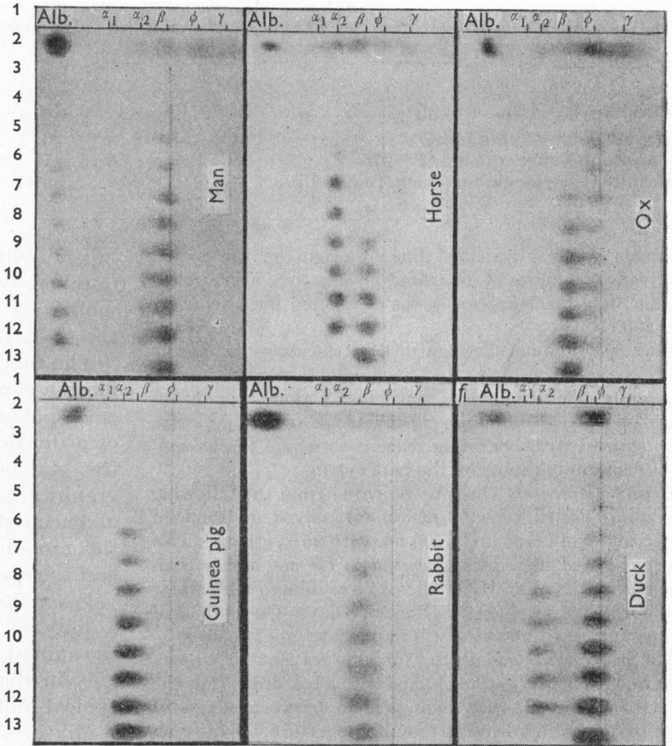
Although the electrophoretic pattern of methaemoglobin–plasma mixtures is almost the same as that of haemoglobin–plasma mixtures, there is some indication that methaemoglobin is carried with the plasma proteins in more definite proportions. An attempt has therefore been made to determine the molar ratios of methaemoglobin and specific plasma proteins for a variety of animals under the conditions described above. Plasma and methaemoglobin solution were mixed in the ratio 2:1 (a considerable excess of methaemoglobin), and subjected to electrophoresis. At the conclusion of electrophoresis, the quantity of protein in each

Key

1 Name of electrophoretic fractions of plasma
2 Electrophoretic pattern of plasma (developed in BPB)
3 Plasma
4 Plasma–Hb mixture (10:1)
5 Plasma–Hb mixture (10:2)
6 Plasma–Hb mixture (10:3)
7 Plasma–Hb mixture (10:4)
8 Plasma–Hb mixture (10:5)
9 Plasma–Hb mixture (10:6)
10 Plasma–Hb mixture (10:7)
11 Plasma–Hb mixture (10:8)
12 Plasma–Hb mixture (10:9)
13 Haemoglobin

(Developed in benzidine reagent)

(*b*)



(*a*)

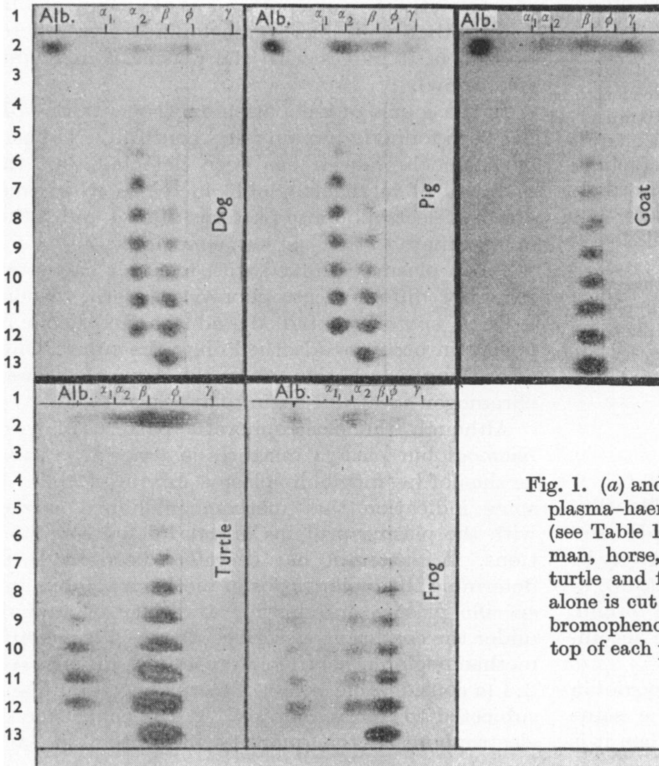


Fig. 1. (*a*) and (*b*), Electrophoretic patterns of plasma protein, plasma–haemoglobin mixtures arranged in serial dilutions (see Table 1) and of haemoglobin in a variety of animals: man, horse, ox, guinea pig, rabbit, duck, dog, pig, goat, turtle and frog. The strip of paper containing the plasma alone is cut from the sheet after electrophoresis, stained in bromophenol blue and replaced as a protein marker on the top of each plate. See Key for meaning of nos. 1–13.

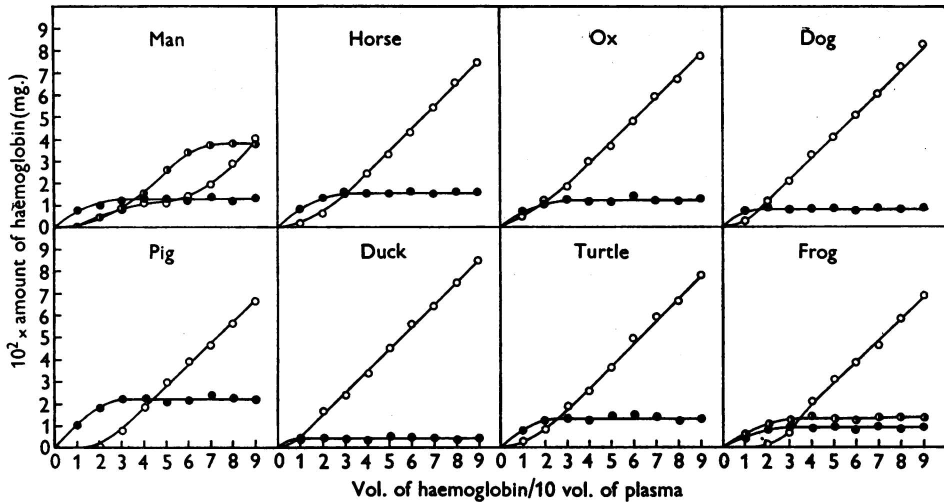


Fig. 2. Plots of free and bound haemoglobin eluted from the electrophoretic patterns of the serial plasma–haemoglobin mixtures in a variety of animals. Each point on the curves represents the amount of haemoglobin eluted from a spot on an electrophoretic pattern as in Fig. 1. ○, Free haemoglobin; ●, haemoglobin bound to α_2-globulin (or plasma fibrinogen of ox); ◑, haemoglobin bound to albumin.

Table 2. *Relative mobilities of benzidine-staining spots after electrophoresis of homologous haemoglobin–plasma mixtures*

		Mobility of spot equivalent to			
Animal	No. of spots	Haemoglobin	Fibrinogen	α_2-Globulin	Albumin
Goat, rabbit, guinea pig	1	+	.	.	.
Ox	2	+	+	.	.
Horse, dog, pig, duck	2	+	.	+	.
Turtle	2	+	.	.	+
Man, frog	3	+	.	+	+

fraction was estimated by a micro-Kjeldahl method (Keys, 1940) and the maximum amount of methaemoglobin to combine with each fraction was estimated by measuring the absorption at 410 mμ. Assuming the molecular weight of ferrous and ferric haemoglobins to be 68 000, albumin 70 000, fibrinogen 330 000 (Shulman, 1953), and α_2-globulin 150 000 (discussed below), the combining molar ratios of the complexes approximated to 1:1 in many cases (Table 3).

Spectrophotometry

The nature of the combination between the haemoglobins and plasma proteins has been investigated by examination of the absorption spectrum of the substances eluted from regions corresponding to the benzidine-staining spots, after electrophoresis of mixtures of plasma and haemoglobins. A mixture containing excess of haemoglobin was applied as a strip to the line of origin of a wide sheet of filter paper. An identical amount of plasma (without haemoglobin) was applied to the line of origin of a similar sheet. Both sheets were subjected to electrophoresis as described previously. At the end of electrophoresis, a longitudinal strip was cut from each sheet and developed to show the positions of the haemoglobin and plasma proteins. Cross strips were cut from each sheet to include each identified band, and these were eluted in the buffer used for electrophoresis. The continuous absorption curves of each specimen so prepared were traced over the range 700–320 mμ (with a Beckman spectrophotometer, Model DU).

Mixtures of haemoglobin and plasma, referred to above, which were incubated for 4 or 24 hr. before electrophoresis, were similarly treated and examined.

The absorption maxima in the visible region and the peak wavelengths in the near-ultraviolet region are listed in Table 4. It is found that the free haemoglobin spot has its own typical absorption bands in each region but that the absorption maxima of the complexes fall into two ranges in the near ultraviolet. With oxyhaemoglobin, reduced haemoglobin or carbon monoxy haemoglobin bound to either α_2-globulin or fibrinogen, the absorption

bands in the visible region are the same as for these haemoglobins in the free state. However, the Soret band is shifted to 410 mμ, being similar to that of recombined haemoglobin, where the shift has been taken to indicate partial denaturation due to standing at room temperature (Jope, 1949). It is not known whether such denaturation could weaken electrovalent links and hydrogen bonds so as to make possible the anchoring of specific plasma proteins.

Where haemoglobins are bound to albumin, there is an absorption maximum at 625 mμ and a Soret band at 400·3 mμ. After electrophoresis of mixtures of methaemalbumin and plasma, the free haemoglobin has the typical absorption maxima at 631 and 406 mμ, but the α_2-globulin-bound and fibrinogen-bound complexes have their Soret bands around 410 mμ, indicating that such binding promotes a change of the ferric haemoglobin to partially denatured ferrous haemoglobin. The albumin-bound complexes show absorption at 625 and 400·3 mμ, which is characteristic of ferrihaemalbumin.

DISCUSSION

All of the complexes which have been examined in the course of this investigation have the same absorption maxima in the visible region and thus cannot be detected by ordinary spectroscopy.

A feature of particular interest is that both ferrous and ferric haemoglobins have been found to be capable of forming complexes with some components of the plasma protein. The origin of the haemoglobin is unimportant since the specificity of the complexes resides in the proteins present in the plasma. Combination is made possible by partial denaturation of the haemoglobins or by complete oxidation to the ferric state, owing to the length of time during which the solutions remain at room temperature. Free-hanging-paper electrophoresis has confirmed that the combination does not depend upon contact with the immersion fluid (kerosene).

The fact that each plasma protein has a definite capacity for binding haemoglobin suggests a chemical attachment of the haemoglobin to that protein. Where an attempt has been made to determine the molar ratio of combination the result has not always been 1:1 but, apart from errors in determinations, this may be due to inexact estimation of molecular weight and to inhomogeneity of the protein fractions. As for α_2-globulin fractions, they embody a mixture of proteins, largely unidentified. This mixture could differ between species. But the α_2-globulins generally appear quite rich in carbohydrate. They are glycoproteins and mucoproteins with molecular weights from 200 000 to 300 000. The α_2-globulin fraction is also rich in

Table 3. *Molar ratios of combination between plasma proteins and methaemoglobin*

The molarity of methaemoglobin is taken as unity.

Species	Total plasma protein (g./100 ml.)	Fractions expressed as % of total plasma protein			Amount (mg./0·005 ml. of plasma)			Maximum combined methaemoglobin (mg.)	Molar ratio: Protein/methaemoglobin
		Albumin	α_2-Globulin	Fibrinogen	Albumin	α_2-Globulin	Fibrinogen		
Man	7·22	59·6	.	.	0·2150	.	.	0·2050	1·05
Man	7·22	.	9·2	.	.	0·0332	.	0·1280	1·18
Horse	7·30	.	12·0	.	.	0·0439	.	0·0198	1·01
Ox	5·12	.	.	16·8	.	.	0·0430	0·0778	1·14
Dog	5·50	.	8·3	.	.	0·0229	.	0·0102	0·99
Pig	6·85	.	16·2	.	.	0·0555	.	0·0241	1·06
Duck	3·51	.	5·8	.	.	0·0102	.	0·0043	1·05
Turtle	3·80	26·0	.	.	0·0493	.	.	0·0428	1·12
Frog	2·84	34·0	.	.	0·0483	.	.	0·0393	1·20
Frog	2·84	.	21·8	.	.	0·0309	.	0·0112	1·25

Table 4. *Absorption maxima and Soret bands (in mμ) of haemoglobin, haemoglobin–plasma mixtures and eluates of bound haemoglobins*

	Animal										
Conditions of treatment	Man	Horse	Ox	Dog	Pig	Goat	Rabbit	Guinea pig	Duck	Turtle	Frog
Oxyhaemoglobin, freshly prepared	577·8	576·7	578·0	578·3	577·8	577·6	578·2	576·5	577·9	576·6	576·8
	540·3	542·0	540·0	540·8	539·8	540·0	541·3	543·5	542·2	540·8	540·2
	414·5	414·5	414·5	414·5	414·5	414·5	414·5	414·5	414·5	414·5	414·5
Oxyhaemoglobin, incubated 4 hr.	577·8	576·7	478·0	578·3	577·8	577·6	578·2	576·5	577·9	576·6	576·8
	540·3	542·0	540·0	540·8	539·8	540·0	541·3	543·5	542·2	540·8	540·2
	412·5	412·5	412·5	412·5	412·4	413·8	413·4	414·0	412·3	411·8	412·5
Oxyhaemoglobin, incubated 24 hr.	631·0	630·0	631·0	630·8	631·0	630·0	630·0	631·2	631·3	630·8	631·0
	565·0	556·0	565·0	565·0	563·0	564·6	558·0	558·8	563·2	564·0	563·0
	406·0	406·0	405·6	406·0	406·0	405·5	405·6	406·0	406·6	405·8	406·0
Oxyhaemoglobin–plasma mixture,	631·0	630·0	631·0	630·8	631·0	630·0	630·0	631·2	631·3	630·8	631·0
incubated 4 hr.	577·8	576·7	578·0	578·3	577·8	577·6	578·2	576·5	577·9	576·6	576·8
	540·3	542·0	540·0	540·8	539·8	540·0	541·3	543·5	542·2	540·8	540·2
	410·0	410·0	409·7	410·2	410·0	413·8	413·4	414·0	409·5	410·3	410·0
Oxyhaemoglobin–plasma mixture,	625·0	630·0	631·0	630·8	631·0	630·0	630·0	631·2	631·3	625·0	625·0
incubated 24 hr.	400·3	410·0	409·7	410·2	410·0	405·5	405·6	406·0	409·7	400·1	400·3
Oxyhaemoglobin, free oxyhaemo-	631·0	630·0	631·0	630·8	631·0	630·0	630·0	631·2	631·3	630·8	631·0
globin spot, eluted	577·8	576·7	578·0	578·3	577·8	577·6	578·2	576·5	577·9	576·6	576·8
	540·3	542·0	540·0	540·8	539·8	540·0	541·3	543·5	542·2	540·8	540·2
	412·5	412·9	412·5	412·8	412·3	413·8	413·4	414·0	412·5	412·3	412·5
Oxyhaemoglobin–albumin complex,	625·0	.	.	.	.	.	.	.	.	625·0	625·0
eluted	400·3	.	.	.	.	.	.	.	.	400·1	400·3
Oxyhaemoglobin–α_2-globulin	577·8	576·7	.	578·3	577·8	.	.	.	577·9	.	576·8
complex, eluted	540·3	542·0	.	540·8	539·8	.	.	.	542·2	.	540·2
	410·0	410·0	.	410·2	410·0	.	.	.	409·7	.	410·0
Oxyhaemoglobin–fibrinogen	.	.	578·0	.	.	.	.	.	.	.	.
complex, eluted	.	.	540·0	.	.	.	.	.	.	.	.
	.	.	409·7	.	.	.	.	.	.	.	.
Haemoglobin, freshly prepared	565·0	556·0	565·0	565·0	563·0	564·6	558·0	558·0	563·2	564·0	563·0
	425·0	423·8	424·5	425·0	423·5	423·5	424·0	424·8	423·8	424·5	424·5
Haemoglobin, free haemoglobin	631·0	630·0	631·0	630·8	631·0	630·0	630·0	631·2	631·3	630·8	631·0
spot, eluted	406·0	406·0	405·6	406·0	406·0	405·5	405·6	406·0	406·6	405·8	406·0
Haemoglobin–albumin complex,	625·0	.	.	.	.	.	.	.	.	625·0	625·0
eluted	400·3	.	.	.	.	.	.	.	.	400·1	400·3
Haemoglobin–α_2-globulin complex,	631·0	630·0	.	630·8	631·0	.	.	.	631·3	.	631·0
eluted	565·0	556·0	.	565·0	563·0	.	.	.	563·2	.	563·0
	410·0	410·0	.	410·2	410·0	.	.	.	409·7	.	410·0
Haemoglobin–fibrinogen complex,	.	.	631·0	.	.	.	.	.	.	.	.
eluted	.	.	565·0	.	.	.	.	.	.	.	.
	.	.	409·7	.	.	.	.	.	.	.	.
Carbon monoxy haemoglobin,	570·5	570·3	570·0	570·4	570·0	570·5	570·2	570·0	570·0	571·5	571·7
freshly prepared	535·0	535·1	534·5	535·7	535·0	535·2	535·0	535·2	535·0	535·8	535·5
	419·0	419·5	419·5	420·0	419·8	419·2	420·0	419·8	419·5	420·2	420·0
Carbon monoxy haemoglobin–albumin	625·0	.	.	.	.	.	.	.	.	625·0	625·0
complex, eluted	400·3	.	.	.	.	.	.	.	.	400·1	400·3
Carbon monoxy haemoglobin–α_2-	631·0	630·0	.	630·8	631·0	.	.	.	631·0	.	631·0
globulin complex, eluted	565·0	556·0	.	410·2	410·0	.	.	.	409·7	.	410·0
Carbon monoxy haemoglobin–	.	.	631·0	.	.	.	.	.	.	.	.
fibrinogen complex, eluted	.	.	565·0	.	.	.	.	.	.	.	.
	.	.	409·7	.	.	.	.	.	.	.	.
Methaemoglobin, freshly prepared	631·0	630·0	631·0	630·8	631·0	630·0	630·0	631·2	631·3	630·8	631·0
	406·0	406·0	405·6	406·0	406·0	405·5	405·6	406·0	406·6	405·8	406·0
Methaemoglobin–albumin complex,	625·0	.	.	.	.	.	.	.	.	625·0	625·0
eluted	400·3	.	.	.	.	.	.	.	.	400·1	400·3
Methaemoglobin–α_2-globulin	631·0	630·0	.	630·8	631·0	.	.	.	631·3	.	631·0
complex, eluted	410·0	410·0	.	410·2	410·0	.	.	.	409·7	.	410·0
Methaemoglobin–fibrinogen	.	.	631·0	.	.	.	.	.	.	.	.
complex, eluted	.	.	409·7	.	.	.	.	.	.	.	.

the copper-containing protein caeruloplasmin, with a molecular weight of 150 000. There is also some overlapping of the β_1-globulin due to diffusion from the β_1-globulin fraction, especially of the metal-binding protein with a molecular weight of 90 000. For comparison, an arbitrary value of 150 000 was chosen as the figure for the α_2-globulins, being a value about mid-way between the proteins of high and low molecular weights.

The fact that haemoglobins of very different origins have the same capacity for complex formation makes it likely that it is the haem prosthetic group and not the protein moiety which is involved in the combination. The suggestion that the whole haemoglobin molecule, and not only the haem, becomes attached to the plasma fraction receives some support from the fact that haem or haematin alone forms no such complexes.

Unlike Keilin (1944), who found the instant formation of haemochromogen upon mixing haem and albumin, I found no haem or haemochromogen, even after incubation. It therefore seems unlikely that haem is set free and later combines with the plasma protein. Nor has there occurred the complete denaturation of plasma proteins which is usually required before haem can form haemochromogen. It appears possible, then, that there is a binding, through the prosthetic group, of a partially denatured haemoglobin to some or all of the α_2-globulin and fibrinogen of the plasma. O'Hagen (1955) found that in human ferrihaem-albumin the attachment to native serum albumin is through the haematin propionic acid groups and this may also be so for haemoglobin bound to turtle or frog albumin, since the Soret bands are the same. However, if whole haemoglobin is, in fact, bound to plasma protein, it remains to be explained why the electrophoretic mobility of the latter is unaltered. Much more work is needed to clear up these questions and to investigate a wider range or vertebrates.

Results obtained raise the further question of a possible role of plasma proteins in the disposal of lysed haemoglobin in the blood of man and other vertebrates. Kench, Gardikas & Wilkinson (1950) have shown that methaemoglobin and methaem-albumin were as effective in forming bile pigment as was haemoglobin, and that all were more effective than was haematin, which is suggestive evidence that such complexes may play more than an accidental part in the metabolism of haemoglobin.

SUMMARY

1. The formation of complexes between partially denatured ferrous and ferric haemoglobins and plasma proteins of a variety of vertebrate animals has been studied by immersed-paper electrophoresis and visible and ultraviolet spectrophotometry.

2. Haemoglobin, irrespective of its origin, forms complexes with α_2-globulin of horse, dog, pig and duck plasma; with fibrinogen of ox plasma; with albumin of turtle plasma; and with α_2-globulin and albumin of human and frog plasma. No such complexes are formed with the plasma proteins of goat, rabbit or guinea pig.

3. These complexes are not haemochromogens but may be partially denatured haemoglobins bound by the prosthetic group to specific plasma proteins which act as vehicles.

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