CLXXVI. THE PROTEIN COMPONENT OF THE ERYTHROCYTE MEMBRANE OR STROMA.

By ERIK JORPES.

*From the Physiological Chemistry Department of the
Caroline Institute, Stockholm.*

(Received August 6th, 1932.)

OUR chemical knowledge of the structural moiety of the red blood corpuscles is very scanty, particularly when compared with the thoroughness with which the haemoglobin has been studied. This lack of knowledge contrasts strongly with the keen interest, which has long been directed to the study of haemolysis, agglutination and permeability phenomena, for which the erythrocyte membrane is of the utmost importance.

Concerning the structure of the mammalian erythrocyte the view already advanced by Leeuwenhoek in 1719 viz. "*vesiculam aqua plenam habemus*," seems to be that of the present day also. According to this view the inner haemoglobin sol is surrounded by a kind of membrane, which after the loss of its fluid content forms the ghosts or "Blutschatten." This question is thoroughly dealt with in Weidenreich's monograph [1903]. Since that time the term stroma has generally been used in the sense of membrane, although the name was originally introduced by Rollett in 1871, to designate the trabecular framework in the meshes of which Brücke in 1861 and Rollett supposed that the haemoglobin solution was held. It is beyond the scope of this investigation to discuss more closely the histological details in the structure of the mammalian erythrocyte. The main purpose is to study the protein moiety of the membrane or stroma.

The lipoids of the red blood corpuscles have been thoroughly studied both from the qualitative and the quantitative points of view. It is, however, impossible to find any detailed information in the literature about the protein component. Thus Wooldridge [1881] and Halliburton and Friend [1889] describe only some properties of the stromata, the latter state that they contain a globin similar to that of haemoglobin and that no phospho- or nucleo-proteins are present. No information is gained from the work of Beumer and Bürger [1912], and their preparations contained 50–70 % haemoglobin. A more recent paper by Haurowitz and Sládek [1928] deals with analysis of stroma preparations, containing from 0.07 to 0.16 % iron. This admixture of haemoglobin caused their figures for the content of arginine-nitrogen to vary between 4.2 and 10.4 % of the total nitrogen and those for the histidine-nitrogen from 3.2 to 10.4 %.

For several reasons, however, it is essential to know the nature of the stroma protein. First, it is known that the acid proteins are strong antigens, as is the erythrocyte membrane, whereas haemoglobin and the basic proteins have very weak antigenic properties, if any at all. Furthermore, a most elaborate theory of the permeability of ions through the erythrocyte membrane is founded on the assumption that the membrane contains a basic protein, some kind of globin similar to that of haemoglobin.

The fact that the erythrocyte membrane is non-permeable to cations has been explained by assuming that it contains a basic protein, which with its positive electric charge would prevent the passage of the cations. As has been shown by Michaelis and his co-workers in a series of publications [1925; 1926; Fujita, 1926] the permeability of artificial membranes to ions is largely influenced by the electric charge of the membrane, if the diameter of the pores is small enough. When a membrane with pores of a suitable size separates two solutions containing the same salt in different concentrations, the diffusion of the cations through the membrane is prevented, if the membrane has a positive charge, and that of the anions, if it has a negative charge. Thus the non-permeability of the erythrocyte membrane towards cations could be easily explained, if the membrane contained a layer of a basic protein. Mond [1927] brought forward some evidence in favour of this view. In a slightly alkaline solution of p_H 8.3 the system according to him is reversed and the membrane is permeable to cations but not to anions. The change occurs at the supposed isoelectric point of globin, where the charge is reversed. Hence Mond [1928] makes the following statement: "Danach besteht die Grenzschicht der Erythrocyten aus basischen Eiweissphasen (Globin) und Lipoidphasen, die mosaikartig in ein Stromagerüst eingelagert sind." He admits however that an acid protein may also occur in the membrane contributing to its electric charge, which is known to be negative [Höber, 1904].

THE SEPARATION OF THE MEMBRANES OF THE RED BLOOD CORPUSCLES.

The membranes may be flocculated out after haemolysis either with immune serum or with acid. Precipitation with acid is the oldest and most generally employed method. As early as 1863, in W. Kuhne's text-book of physiological chemistry, a method is given of separating the membrane by passing a stream of carbon dioxide through the blood after haemolysis. Hoppe-Seyler and Wooldridge precipitated the membranes by means of acid potassium sulphate, which they believed to shrink the membranes, but they did not refer to the acid character of the salt. Later the method of introducing carbon dioxide after haemolysis with distilled water and the removal of the leucocytes was used by Abderhalden, Bang, Beumer and Bürger and others. In the present investigation the membranes were sometimes flocculated with immune serum, but this was mainly done by the addition of acid.

Attention was at first given to the possibility that the substance precipitated with the acid might be an artificial product. The haemoglobin might, as soon as it became positively charged on the acid side of p_H 6.8, have been precipitated together with negatively charged lecithin, just as the nucleoproteins are artificial products, *i.e.* salts of negatively charged nucleic acids with positively charged proteins [Hammarsten, 1924]. In that case, the protein component of the membrane would have the qualities and composition of haemoglobin. However, analyses of preparations with even a high haemoglobin content showed that such was not the case.

In view of the desirability of precipitating the membranes at the reaction of the blood, antisera were prepared by the injection of blood corpuscles of horse into goat and rabbit. When immune serum was added to the extent of 1–5 % to the haemolysed horse blood, even without the addition of sodium chloride, a precipitate was obtained which could be removed by centrifuging. Alternate washing in distilled water and physiological salt solution produced a considerable fading, but thorough separation of the haemoglobin was achieved only occasionally.

The old method of using carbon dioxide after dilution with several volumes of distilled water was also not found to be advantageous. The precipitate was not easy to filter through a cloth, owing to the fact that carbon dioxide does not give a sufficiently high degree of acidity. In four different preparations after passing carbon dioxide through the solutions for half an hour, p_H values of 6.03, 6.15, 6.10 and 6.09 (quinhydrone) were obtained. Even if a flocculation took place in haemolysed horse blood at p_H 6.20 (possibly 6.40) the precipitate was easiest to separate from the mother-liquor at p_H 5.6–5.1. Furthermore, as the carbon dioxide escaped, the precipitate became more difficult to handle. Since bisulphite solution easily gave rise to the development of methaemoglobin, precipitation by mineral acid was selected as the most suitable method. If 0.1 to 0.01 *N* HCl is added with rapid stirring, there is no danger, owing to the strong buffering effect of the proteins, that the reaction will be too acid. The flocculation of the membranes in horse blood is optimum at a p_H of about 5.5. If the preparation is carried out at a low temperature, there is very little risk that the haemoglobin will be denatured. According to Booth [1930] haemoglobin is denatured at 0° instantaneously at p_H 3, and in 10 minutes to 50 % at p_H 3.5. At p_H 5.5, the risk of denaturation therefore should be insignificant.

As far as haematin development is concerned, Svedberg and Nichols [1927] maintain that this does not take place in carbon monoxide haemoglobin when the p_H is between 6.0 and 9.05. When acid is added to haemolysed horse blood, no change in colour is seen until the p_H is 4.3. When it is 4.0, the change is more distinct. No haematin formation need therefore be apprehended when the range of p_H is between 6.0 and 5.5.

On haemolysis by dilution with distilled water, there is the risk that any globulin present in the corpuscles might be precipitated and mixed with the

corpuscle membrane. Dilution with distilled water and adjustment of the p_H to between 5 and 6 give all the pre-requisites for precipitation of the globulins. It was therefore necessary to prove that the precipitate obtained on the addition of acid to blood haemolysed with distilled water belongs to the membrane.

A strong argument in favour of this supposition is the very fact that no further precipitation is obtained with acid after removal of the precipitate obtained with immune serum. Presumably the immune serum acts on and flocculates the membranes.

Another argument is the fact that the membrane can be removed by passing the liquid through a Seitz filter. Blood haemolysed with distilled water and not entirely transparent becomes quite clear when passed through a Seitz filter and gives no precipitate on the addition of acid to the filtrate. Possibly existing globulins would in no case have been quantitatively precipitated at the reaction of the blood and after passage through the filter a turbidity would appear when the p_H was adjusted to 5.4 or 5.6. The conclusive proof, however, is given by certain experiments with saponin haemolysis. If the paste of corpuscles is diluted with saline instead of with distilled water and haemolysis is produced with saponin, no globulins should be precipitated. Here, too, the liquid is not quite clear, but becomes so after passage through the Seitz filter. Before filtration, the addition of acid to the blood, haemolysed with saponin, results in the characteristic precipitation, but after it there is no clouding or opalescence. It is thus clear that the precipitate herein regarded as the protein component of the erythrocyte membrane, obtained by the addition of acid to the haemolysed blood, does actually originate from the membrane and not from any protein present in solution within the blood corpuscles.

When precipitation had been obtained with acid, it was necessary to wash the membranes free from haemoglobin. With this object in view, the precipitate was chilled and filtered through silk. When it was again suspended in cold water the precipitate could usually be more easily filtered. Continued washing with distilled water in a centrifuge caused only a slight removal of haemoglobin from the precipitate. Though the last washing might be quite colourless the precipitate was usually still red and preparations made from it contained between 30 and 60 % haemoglobin. The treatment employed by some authors with a dilute carbonate solution, followed by reprecipitation with acid, had a certain purifying effect in some cases, but also easily caused methaemoglobin formation.

Faced with the need of finding some other means, I tried, after washing with distilled water, to dissolve the haemoglobin in an acid buffer solution. The assumption was made that the protein of the membrane itself must have its isoelectric range on the acid side of the neutral point, more exactly at a p_H between 5 and 6 for horse blood. Its passage into a more coarsely dispersed phase and its flocculation on the addition of acid must evidently be

due to a diminished electrolytic dissociation. This is also corroborated by the fact that the precipitate disappears again on the acid side of the given p_H range. If this were the case, the risk of loss through washing in a buffer solution of a suitable acidity would be slight, while the haemoglobin at a p_H of, for instance, 5.5 would be more extensively dissociated than in distilled water. A phosphate buffer with the acidity given above was chosen and the effect was striking. After the membrane precipitate had been suspended in this buffer solution, vigorously shaken with pieces of ice and glass beads and then centrifuged, the mother-liquor turned very red and the membrane precipitate was pale. This washing was repeated a few times. It was important to change the concentration of the phosphate in the washing fluid occasionally, though without changing the p_H . In this way it was possible to get almost colourless preparations. In the first preparation made in this way, the haemoglobin content, ascertained by iron analysis, fell through a single washing with the buffer solution from 55 to about 15 % of the dry substance. The precipitate used in this case had not given up any further haemoglobin to distilled water. This shows plainly that the iron in such carefully treated preparations is contained in the haemoglobin and is not present to any significant degree in any other form, as Beumer and Bürger implied.

The method of isolation employed.

Two litres of fresh, defibrinated horse blood are filtered through gauze and the corpuscles washed with cold physiological salt solution at least 4 times in a centrifuge taking 2 litres. After washing, the tubes are left standing overnight in the refrigerator. Next morning, 6 volumes of cold distilled water are added, after which the leucocytes are centrifuged away at a speed of 2400 revolutions per min. for 20 minutes. This amount of water is, according to Ponder [1925], required for complete haemolysis. According to Abderhalden and Deetjen [1907], the leucocytes are removed from the plasma when centrifuged for 15 minutes at 1500 revolutions per min. They remain in a sticky layer at the bottom of the centrifuge-tubes, from which the liquid can be decanted off. The smallest amount of acid is then found, which gives the optimum of flocculation. For the whole quantity, 80 to 100 cc. N HCl are usually required. When the liquid has been chilled with ice, it is poured into 25 litres of cold distilled water. The acid is diluted with 2 litres of distilled water in which ice is placed and slowly added by vigorous stirring to the 25 litres. The whole is then poured into large glass cylinders in the cold room, where the membranes settle. The clear liquid is removed and the precipitate collected on pieces of silk. The latter process takes about 2 hours. The voluminous precipitate is suspended in 10 litres of distilled water and the liquid this time runs easily through the silk. The precipitate should be allowed to drain thoroughly. It is suspended in 1.5 litres of distilled water together with pieces of ice and glass beads and is pressed through a tightly stretched filter-cloth (No. 40) by scrubbing with a hard brush. This is a very important process.

Without such mechanical treatment the washing will not be effective. After this the precipitate is centrifuged for 15 minutes. The clear liquid can be poured off. The precipitate is suspended in 1.5 litres of buffer solution (prepared from 15 cc. stock solution to 1.5 litres distilled water) and shaken vigorously with pieces of ice and glass beads. After centrifuging the washing is repeated in the same way as long as haemoglobin is removed. It is important that the later washings should be made with buffer solutions alternating with 15 cc. and 30 cc. stock solution to every 1.5 litres cold water. If the preparation is successful, the membrane protein is almost colourless. If too long a time has been spent on the preparation the brown colour of methaemoglobin often appears. The preparation after haemolysis should be completed in the course of 4 or 5 hours. In order to remove some impurities introduced with the ice, the precipitate is suspended at a low temperature in cold distilled water and 0.1 *N* sodium hydroxide added until the reaction is slightly alkaline to litmus but still acid to phenolphthalein. The impurities are centrifuged off together with some denatured protein. An addition of the same amount of 0.1 *N* hydrochloric acid re-precipitates the membrane protein. It is allowed to settle, after which it is centrifuged off, and dried with cold alcohol and ether.

The yield in the different preparations varied considerably, *viz.* between 1.0 and 2.5 g.

The stock buffer solution is prepared by the addition of a little secondary sodium phosphate to an almost saturated solution of primary potassium phosphate, after which the p_H is adjusted colorimetrically by the addition of suitable amounts of acid or alkali.

ANALYSIS OF THE MEMBRANE PREPARATIONS.

As stated above the preparations were washed with alcohol and ether. In order to avoid haematin formation no washing with hot alcohol was made. In spite of this the lipoids were practically completely removed as found on analysis of one of the largest preparations.

The iron and nitrogen contents of some of the stroma preparations are given in Table I, calculated on dry substance (115° *in vacuo*).

Table I.

Preparation...	1	2	3	4	5	6	7	8
	Horse	Horse	Horse	Sheep	Sheep	Horse	Horse	Horse
Ash (%)	0.45	0.89	—	—	—	—	—	—
Nitrogen (%)	14.83	14.16	14.01	14.75	14.71	14.64	15.40	15.29
Fe (%)	0.047	0.049	0.048	0.058	0.064	0.069	0.091	0.136

Purified samples of some other proteins of the blood were also analysed. The oxyhaemoglobin was recrystallised twice. Its nitrogen content calculated on dry substance was 16.80 %. The figure given by Mitchell and Hamilton [1929] is 16.86. Fibrin from ox blood was washed free of haemoglobin, defatted and dried with alcohol and ether. Its nitrogen content was 16.82 %

calculated on ash-free dry substance (16.91 Mitchell and Hamilton). Serum-globulin from horse blood was reprecipitated with ammonium sulphate twice and dialysed, first against tap water and then in the electro-dialyser of Pauli. Its nitrogen content was 15.46 % calculated on dry ash-free substance.

THE NITROGEN DISTRIBUTION ACCORDING TO VAN SLYKE.

The procedure of Van Slyke was performed in the usual way. For the sake of comparison the other blood-proteins except serum-albumin were analysed with the same technique.

5 g. air-dry substance were hydrolysed by boiling in 200 cc. 20 % HCl for 48 hours. After removal of the excess of HCl the hydrolysate was diluted to 200 cc. and its nitrogen content determined. The solution was divided into two parts, one of 100 cc. and one of 92 cc. (88 cc. in one case), which were analysed separately for amide-N and humin-N and precipitated with phosphotungstic acid. The arginine analysis was made according to the modification of Plimmer and Rosedale [1925]. A special apparatus was used. The boiling flask was fitted to the cooler directly by a ground connection and washed air was taken in by suction according to the modification of Koehler [1920]

Table II. *Nitrogen distribution according to Van Slyke in some blood-proteins.*

	The stroma protein	Fibrin	Serum-globulin		Oxyhaemoglobin	
g. air-dry substance	2.5	2.5	5		5	
Volume after hydrolysis cc.	100	100	200		200	
Nitrogen in 4 cc., mg. (Kjeldahl)	12.85					
	12.80	14.27	13.78		15.16	
	13.00	14.31	13.81		15.16	
Av.	12.88	14.29	13.80		15.16	
Total nitrogen, mg.	322	357.3	690		758	
Volume taken to analysis, cc.	88	92	100	92	100	92
Amide-N, mg.	26.37	32.77	33.22	30.92	22.14	20.59
Amide-N % of total N	9.30	9.97	9.63	9.75	5.84	5.91
Humin-N, mg.	8.89	9.97	6.27	5.90	11.74	10.89
Humin-N % of total N	3.15	2.17	1.82	1.86	3.10	3.12
Monoamino-N in 25 cc., mg.	16.95					
	16.90	19.24	21.42	20.08	21.85	19.71
	17.00	19.29	21.57	20.18	21.90	19.58
Av.	16.95	19.27	21.50	20.13	21.88	19.65
Monoamino-N % of total N	59.81	58.62	62.32	63.42	57.73	56.36
Diamino-N in 10 cc., mg.	7.52	9.42	8.73	7.99	11.88	11.03
	7.62	9.43	8.88	7.91	11.90	10.96
Av.	7.57	9.43	8.81	7.95	11.89	11.00
Diamino-N % of total N	26.71	28.69	25.54	25.05	31.37	31.55
Nitrogen recovered, %	98.97	99.45	99.31	100.0	98.04	96.94
Arginine analysis on 30 cc. mg. N	6.19	7.70	6.35	6.19	4.64	4.41
	5.91	7.70	6.37	6.19	—	4.34
Av.	6.05	7.70	6.36	6.19	—	4.38
Arginine-N % of total N	14.23	15.58	12.29	13.00	8.16	8.37
Arginine in 100 cc., mg.	125.4	159.2	131.8	128.2	96.1	90.8
Arginine calculated on dry substance, %	6.13	7.94	5.87	6.22	4.21	4.32

through a glass tube fused into the upper part of the flask. The duplicates agreed very well as is seen from Table II. The figures found for haemoglobin are probably too high. The determinations of the amino-nitrogen were made in an all-glass micro-apparatus as recommended by Folley [1930]. This apparatus is a real improvement compared with the ordinary Van Slyke apparatus with its rubber connections.

The details and the results are given in Table II. The stroma protein analysed here is preparation No. 2 in Table I.

It is evident that the stroma protein shows no similarity to haemoglobin in its quantitative composition. The nitrogen distribution according to Van Slyke as well as the low nitrogen content indicates that it is an acid protein. There remained however an uncertainty in the analysis given in Table II, *viz.* the content of the mono-basic amino-acids. On calculating the histidine and lysine contents in the usual way from the figures found for the total N, amino-N and the arginine-(+ cystine-)N no concordant results were obtained. Hence these were left out and instead the arginine and histidine contents of the solution containing the basic amino-acids in 100 cc. were determined by means of the colour reactions of Sakaguchi and Pauly. 1 cc. of the solution was diluted to 75 or 150 cc. and analyses were performed as described in the next two papers. The results were as follows (see Table III):

Table III.

	Arginine found by the ordinary alkaline hydrolysis mg.	Arginine found with the Sakaguchi reaction mg.	% calcu- lated on dry substance	Histidine found with the Pauly reaction mg.	% calcu- lated on dry substance
Stroma protein	125.4	118.6	5.80	53.9	2.63
Fibrin	159.2	159.6	7.97	44.6	2.23
Globulin	131.8	130.0	5.82	47.9	2.14
	128.2	110.4	5.36	41.3	2.00
Oxyhaemoglobin	96.1	76.5	3.35	181.0	7.93
	90.7	69.0	3.29	160.0	7.62
				178.0	7.81
				160.0	7.62

The figures given for oxyhaemoglobin by Vickery and Leavenworth [1928], as found by the isolation method, were 3.32 % arginine and 7.64 % histidine, which are exactly the same as found here by the two colorimetric methods mentioned.

Even if the figures given here for the arginine and histidine content of the stroma protein are not to be considered as final and are to be confirmed by further analysis it is evident already that there is a striking difference between the stroma protein and haemoglobin.

The nitrogen distribution and the contents of arginine and histidine thus separate the stroma protein from the haemoglobin, but do not give any certain information about its relation to fibrin and globulin. On analysing the

different blood-proteins for tyrosine and tryptophan, the stroma protein is found to be different from all the other blood-proteins. Its content of these two amino-acids differs strikingly from that of fibrin and globulin as is seen in Table V. The main protein of the erythrocyte membrane or stroma is thus, if homogeneous, a protein *sui generis*, which for the sake of convenience could be called stromatin.

Table IV. *Tyrosine and tryptophan contents of the stroma protein as found by the Folin-Marenzi [1929] method.*

Calculated as % of dry substance.						
Preparation No. in Table I ...	5 Sheep	4 Sheep	6 Horse	2 Horse	7 Horse	3 Horse
Iron content, % of dry substance	0.064	0.106	0.0687	0.049	0.091	0.048
Haemoglobin content: Fe % $\times 100/0.345$	18.6	30.7	20	14.2	26.5	14
Tyrosine	3.17					
	3.08		3.10		2.91	
	3.08		3.11	2.90	3.02	
	3.08	2.78	3.15	3.02	3.00	2.90
	2.93	2.70	3.25	3.19	2.83	2.94
	Av. 3.09	2.74	3.15	3.04	2.94	2.92
Tryptophan	1.29					
	1.53					
	1.48					
	1.48			1.43		
	1.45	1.46	1.47	1.52	1.45	
	1.45	1.49	1.47	1.48	1.41	1.50
	1.39	1.51	1.46	1.44	1.47	1.56
	Av. 1.44	1.49	1.47	1.47	1.44	1.53

Table V. *Tyrosine and tryptophan contents of the blood-proteins as found by the Folin-Marenzi method.*

Calculated as % of dry substance.					The stroma protein (horse and sheep)
	Fibrin (ox)	Serum- globulin (horse)	Serum- albumin (horse)	Oxyhaemo- globin (horse)	
Tyrosine	5.60	5.26			
	5.50	5.36			
	5.45	5.22			
	5.69	5.32	4.10		
	5.39	5.22	4.21	2.65	
	5.45	5.04	4.33	2.56	
	5.57	5.09	4.39	3.02	
	5.69	4.89	4.38	3.12	3.01
	Av. 5.54	5.18	4.28	2.84	
Tryptophan	4.28	2.12			
	4.18	2.26			
	4.21	2.18			
	4.32	2.24	1.03	1.28	
	4.04	2.29	0.99	1.25	
	4.11	2.15	0.95	1.29	
	4.01	1.95	1.08	1.24	
	4.35	2.46	1.05	1.25	1.46
Av.	4.19	2.21	1.02	1.26	

THE AMOUNT OF THE STROMA PROTEIN PRESENT IN THE ERYTHROCYTES.

An attempt was made to determine approximately the amount of stroma protein present in the red blood corpuscles. Owing to the difficulty of removing the haemoglobin only indirect methods were tried. The membranes were either filtered off by passing the haemolysed blood through a Seitz asbestos filter or they were flocculated by means of immune serum or with acid. In each case nitrogen analyses were made of the haemolysed blood and of the fluid after removal of the membranes, due attention being paid to the dilution and to the nitrogen content of the immune serum. The loss of nitrogen per volume haemolysed blood was calculated as % of the total N content. The accuracy of the results depends largely on the nitrogen analyses. The error of the Kjeldahl method is not to be neglected, but it was found that other factors, which could not be controlled, exerted a much greater influence. Some typical experiments are given here.

Exp. 1. Corpuscles from horse blood, washed 9 times with saline, were haemolysed with 5 volumes of distilled water. The leucocytes were removed by centrifuging.

mg. nitrogen per 3 cc. of the haemolysed blood (Kjeldahl)

25.22, 25.17 and 25.19. Mean 25.19.

The liquid was pressed through the Seitz filter thus avoiding concentration through evaporation.

mg. nitrogen per 3 cc. after passage through the filter

24.40, 24.65, 24.50, 24.42. Mean 24.49.

Loss 0.70 mg. N per 3 cc. or 2.78 % of the total N before filtration.

Flocculation with acid:

cc. 0.1 N HCl added to 50 cc. of the haemolysed blood	7	8	9	9
p _H (quinhydrone)	5.86	—	5.27	5.30
mg. nitrogen per 3 cc. after removal of the precipitate	21.10	20.80	20.39	20.59
	21.25	20.84	20.44	20.49
Av.	21.18	20.82	20.42	20.54
mg. nitrogen per 3 cc. calculated on the original solution	24.15	24.17	24.10	24.24
Loss of nitrogen in % of total N	4.13	4.05	4.33	3.77

Exp. 2. This experiment was performed on human blood in the same way as in Exp. 1. The amount of nitrogen removed by filtration was 2.37 % of the total N content. On adding acid to the haemolysed blood the following percentages of nitrogen were removed with the precipitate:

At p _H	6.39	6.20	5.72	4.98
%	4.02	4.57	4.64	3.87

Exp. 3. On adding acid to haemolysed horse blood as described in Exp. 1, 4.41 and 4.31 % of the total N were removed.

To 100 cc. of the haemolysed well washed blood corpuscles 7 cc. of anti-horse immune serum from the goat were added and the precipitate was

removed by centrifuging in stoppered tubes. The nitrogen content of the supernatant liquid was determined. After correction for the dilution and the nitrogen content of the immune serum it was found that 4.31 % of the original amount of nitrogen had been removed with the precipitate.

To the solution of the haemolysed corpuscles in this experiment sodium chloride was added to 0.9 %. After centrifuging in stoppered test-tubes nitrogen was determined on the clear liquid. 7.92 % of the total N had been removed with the precipitate. On adding immune serum and sodium chloride at the same time to the haemolysed blood 11.33 % of the nitrogen content was removed, which corresponds to the summarised effects of the immune serum and of the sodium chloride alone. The effect of sodium chloride is taken as an expression of a "reversible haemolysis" in the sense of Brinkman and Szent-Györgyi [1923].

A series of experiments of this kind was made with human, horse and cow bloods. The results were very often less convincing than in the experiments quoted above. Sometimes the same percentage of nitrogen was removed by filtration through a Seitz filter as by precipitation with acid. In the two experiments given here however more nitrogen was flocculated out by means of acid (4.4-6 %) than was removed by filtration (2.4-2.8 %). In other cases there was never a full agreement between the results obtained after adding immune serum or acid to the haemolysed blood. The figures obtained with acid in one and the same blood also varied considerably, owing to circumstances which could not be explained.

It seems probable that the stroma protein in normal human and horse blood should amount to not more than 4-4.5 % of the total protein content of the erythrocytes. Furthermore the experiments tend to show that the stroma protein obtained by haemolysis of human or horse blood with 5 volumes of distilled water flocculates quantitatively on adding acid to the liquid to a p_H of about 5.5.

DISCUSSION.

The stroma protein has a characteristic composition differing from that of the other blood-proteins. Its physical properties also are very characteristic. Its voluminous consistency, its elasticity and shrinking tendency resemble those of fibrin. Owing to its acid nature it certainly contributes to the negative electric charge of the membrane as is shown by its de-ionisation and flocculation on the addition of acid to a p_H of about 5.5. Concerning its colloidal structure it is difficult to make any statements.

Together with the lipoids it forms the membrane which according to Weidenreich [1903], Gough [1924] and others surrounds the haemoglobin sol. The lipoids are found by different authors to be slightly more than 1 % of the dry substance of the erythrocytes. If the stroma protein is about 4 % of the dry substance, as suggested above, then the lipoids are about 20 % of the dry substances of the membrane. The same order of magnitude 16-24 %

was also found by Beumer and Bürger [1912], who showed that the lipoids are to be found quantitatively in the membrane of the erythrocytes. The view that the stroma protein together with the lipoids form a definite structural unity is furthermore supported by the recent findings of Iwatzura [1926], Grigant, Debray and Furstner [1925] and Vahlquist [1931] that the erythrocytes have a definite content of lipoids in spite of the changes occurring in serum in connection with the resorption of food.

As to the question of the occurrence of a basic protein in the erythrocyte membrane it will be very difficult to get any direct evidence supporting such a view. It is questionable whether there are other proteins in the membrane than that analysed here.

The fact remains that the erythrocyte membrane is non-permeable to cations. Possibly the haemoglobin, even although its isoelectric point is on the acid side of the p_H of the blood exerts a certain influence in the direction supposed by Michaelis and Mond or that globin with its isoelectric point at p_H 7.5 [Roché, 1930] acts in the same way. Taking into account the strongly acid character of the stroma protein it must however be admitted that the permeability or non-permeability of the erythrocyte membrane to ions is still far from being fully explained.

On the other hand, seeing that the acid proteins have strong antigenic properties, the acid characters of the stroma protein would be consistent with the suggestion that some of the immunising properties of the red blood corpuscles were located in it.

METHODS.

The determination of tyrosine and tryptophan.

Amongst the methods recommended for the determination of tyrosine, that of Folin and Ciocalteau [1927], as modified by Folin and Marenzi [1929], is probably the most reliable. For reasons which will be stated later, however, some alterations of their technique can be made with advantage. Thus, the method can be very much simplified if the silicic acid, dissolved by alkali from the test-tubes during hydrolysis, is not filtered off. It will be removed together with the tryptophan-mercury compound, and does not interfere with the tryptophan analysis. It is not even necessary to remove the brownish colour by means of kaolin if the readings are made in a photometer. A second filtration is thus avoided, and the time for which the sulphuric acid acts upon the tryptophan is shortened. In order to avoid humin formation, the alkaline solution can furthermore be diluted by 6 cc. water before 3 cc. 7N H_2SO_4 are added. These alterations are not essential.

A more important question is that of the turbidity which tends to appear after boiling the tyrosine solution containing mercuric sulphate and sulphuric acid. Folin and Ciocalteau recommended boiling for 15 minutes, but, in order to avoid any turbidity, Folin and Marenzi shortened the time to 5 minutes. This however is not sufficient. The colour developing after the addition of

nitrite will not be maximum after such brief boiling, and the agreement between the figures is not nearly so good as when the boiling is continued for 10 or 15 minutes. In the former case, a difference of 4–10 % between the duplicates was often obtained, while in the latter the difference seldom exceeded 1 %. The longer time of boiling is therefore preferable. The turbidity which appears can be filtered off before adding the nitrite solution. After boiling, the flasks are cooled and left standing for several hours. Water is then added to 100 cc. and, after mixing, the solution is filtered into another 100 cc. flask. To this, 1 cc. of the 2 % nitrite solution is added. The error thus introduced in the volume is negligible if the controls are analysed in the same way.

The Folin-Marenzi procedure is to be corrected at least in one detail. They state that the reading should be made "without undue delay." By measuring the colour intensity in the Zeiss photometer, it will be found that the colour of the Millon reaction does not reach its maximum until 8–10 minutes after adding the nitrite solution. After 3 minutes, the colour intensity is 88 % of the final, after 4 minutes 95 % and after 5 minutes 97 %. Since fading sets in 20–25 minutes after the addition of the nitrite, the reading is to be made from 10 to 18 minutes. The reading is made with filtrum S. 50 of the photometer.

In spite of Folin and Ciocalteau's statements to the contrary, no true proportion could be found between the amount of tyrosine present and the intensity of the colour.

This is evident from the following table which contains the results of duplicate analyses of three different concentrations of pure tyrosine.

Amount of tyrosine in 100 cc., mg.	0.5	1.0	2.0
Scale readings (S. 50; $d=2.006$)	65.1	44.5	20.8
	65.1	44.2	21.2
ϵ or $-\log_{10} \frac{I_p}{I_a}$	0.186	0.351	0.682
	0.186	0.354	0.674
ϵ for 1 mg. tyrosine ($d=2.00$)	0.372	0.351	0.341
	0.372	0.354	0.337

This reaction can be used for colorimetric purposes, only if the standard approaches the unknown very nearly.

For the photometer, a table is made, in which the non-logarithmic abscissa expresses the amounts of tyrosine used, and the numbers on the scale are given on the logarithmic ordinates. The points fall very nearly on a straight line.

The accuracy of the method was checked by subjecting known amounts of tyrosine (0.5–2.5 mg.) to hydrolysis together with 70 mg. of gelatin. From 91 to 102 % were recovered, the mean figure being 95.8 ± 0.94 % (17 analyses).

The determination of tryptophan did not give nearly such good results as that of tyrosine. When pure tryptophan mixed with tyrosine and gelatin was subjected to hydrolysis and subsequent analysis, according to the micro-method of Folin and Marenzi, only 71–98 % of the calculated amount of

tryptophan was recovered. The mean figure was $82.4 \pm 2.18\%$ (17 analyses). The greatest accuracy in the duplicates was obtained by using pure tryptophan solutions directly, without precipitating with mercuric sulphate. However, the true proportionality in colour which Folin and his co-workers found, was not obtained even here. Thus, using tyrosine as standard, the following figures were obtained for ϵ , re-calculated for 1 mg. tyrosine in 100 cc. (S. 72).

When 0.2 mg. tyrosine was taken in 100 cc.	0.935
„ 0.4 „ „	0.873
„ 0.8 „ „	0.832
„ 1.5 „ „	0.783
„ 2.0 „ „	0.754

By plotting the figures for the amounts of tryptophan (or tyrosine) on semilogarithmic paper, as stated above, a straight line was obtained, and the reaction is thus to be considered as quite reliable for pure tryptophan solutions. The cause of the difficulties met with in the ordinary analysis, might have been incomplete precipitation of tryptophan, or an interference of mercury salts with the colour reaction. The latter seems to be the more probable since it was found that even small amounts of mercuric salts, added to pure tryptophan solutions, impaired the results, even if the boiling with hydrochloric acid were performed in the usual way. This uncertainty could not be avoided and therefore a correction for the loss was made in calculating the tryptophan content.

By the technique used the figures found for the tyrosine and tryptophan contents of oxyhaemoglobin are about the same as found by Folin and Ciocalteu, *viz.* 2.84 % tyrosine and 1.26 % tryptophan against 3.15 and 1.28 % as found by these workers.

The determination of iron.

The volumetric method with permanganate as elaborated by Murray [1924] was found to give good results down to 1 or possibly 0.5 mg. Fe. It was necessary however to determine smaller quantities. For that purpose the method recommended by Lorber [1927] was tried. After some difficulties with the ashing of the material reproducible results were obtained. The intensity of the yellow colour developed in the alkaline solution containing ferric iron and sulphosalicylic acid was measured in the Zeiss photometer in filter S. 47. No true proportionality between the amount of iron present and the colour intensity was obtained, the smaller amount giving the relatively stronger colour. On plotting the figures found with solutions of ferric chloride, or ferric alum on semilogarithmic paper as described above, the points, however, fall nearly on a straight line. Hence such a table is used in the ordinary work. The colour is very stable and is not influenced by moderate changes in the concentration of ammonia or salts.

The following procedure was found satisfactory. The substance was ashed in a 1.5×3 cm. platinum dish in an electric oven for an hour or two. Potassium nitrate was then added (about 50 mg.) and the dish heated in the oven for 10 minutes. After cooling 0.5 cc. concentrated sulphuric acid was added and the dish heated over a free flame. The contents of the dish were then transferred to a 25 cc. flask with 15 cc. of water. After cooling 20 % KOH solution was added and 0.5 cc. of a 20 % solution of sulphosalicylic acid. More of the KOH solution was added until the colour became violet. The solution was neutralised with a few drops of 20 % ammonia solution and then 0.5 cc. more of the ammonia solution was added. After dilution to 25 cc. and filtering, if the solution were not quite clear, the reading was made, preferably in filter S. 47.

0.02–0.05 mg. Fe was found a convenient quantity for a single analysis.

The accuracy obtained was not particularly good. Often single analyses fall out of the series. Generally there was a deviation of 2–3 % in the duplicates and an error of 5 % was frequent. For the purpose of this investigation however the method is to be considered as satisfactory provided a sufficient number of analyses be made.

SUMMARY.

In order to study the protein of the erythrocyte membrane or stroma, membranes were isolated from horse and sheep bloods. After haemolysis with distilled water the "ghosts" were flocculated by adjusting the reaction with hydrochloric acid to a p_H of about 5.5. They were washed relatively free of haemoglobin with distilled water and a slightly acid dilute phosphate solution.

The membrane preparations contained 14–30 % haemoglobin according to the iron analyses.

The low nitrogen content, 14–15 %, and the nitrogen distribution according to Van Slyke correspond to those of the acid proteins. Herein and through its content of arginine and histidine the membrane protein differs from haemoglobin and globin. The figures for arginine and histidine found were 5.80 and 2.63 %, whereas those of haemoglobin are 3.32 and 7.75 %.

The membrane protein does not show any resemblance to fibrin or globulin. It differs from these through its content of tyrosine and tryptophan. Hence the membrane protein, if homogeneous, is a protein *sui generis*. The name stromatin is suggested for it.

The protein of the membrane or stroma amounts to roughly 4 % of the total protein content of the normal erythrocytes.

The bearing of these findings upon the permeability of the erythrocyte membrane towards ions is discussed.

The author's thanks are due to "Stiftelsen Therese och Johan Anderssons minne" for several grants and to Mr Otto Bjurling for facilities offered.

REFERENCES.

- Abderhalden and Deetjen (1907). *Z. physiol. Chem.* **53**, 280.
Beumer and Bürger (1912). *Arch. exp. Path. Pharm.* **71**, 310.
Booth (1930). *Biochem. J.* **24**, 158.
Brinkman and Szent-Györgyi (1923). *J. Physiol.* **58**, 204.
Folin and Ciocalteu (1927). *J. Biol. Chem.* **73**, 627.
— and Marenzi (1929). *J. Biol. Chem.* **83**, 89.
Folley (1930). *Biochem. J.* **24**, 961.
Fujita (1926). *Biochem. Z.* **162**, 245.
Gough (1924). *Biochem. J.* **18**, 202.
Grigant, Debray and Furstner (1925). *Kongressen-Zentralbl. inn. Med.* **40**, 649.
Halliburton and Friend (1889). *J. Physiol.* **10**, 532.
Hammarsten (1924). *Biochem. Z.* **144**, 383.
Haurowitz and Sládek (1928). *Z. physiol. Chem.* **173**, 268.
Höber (1904). *Pflüger's Arch.* **101**, 607; **102**, 196.
Iwatzuru (1926). *Pflüger's Arch.* **214**, 295.
Koehler (1920). *J. Biol. Chem.* **42**, 267.
Lorber (1927). *Biochem. Z.* **181**, 391.
Michaelis and Fujita (1925). *Biochem. Z.* **158**, 11 and 28; **159**, 370; **161**, 47; **164**, 32.
— and Dokan (1926). *Biochem. Z.* **162**, 258.
Mitchell and Hamilton (1929). *The biochemistry of the amino-acids.* (Chemical Catalog Co., New York.)
Mond (1927). *Pflüger's Arch.* **217**, 618.
— (1928). *Pflüger's Arch.* **220**, 69.
Murray (1924). *Biochem. J.* **18**, 852.
Plimmer and Rosedale (1925). *Biochem. J.* **19**, 1004.
Ponder (1925). *Biochem. J.* **19**, 552.
Roché (1930). *Meddel. Carlsb. Lab.* **8**, 51.
Svedberg and Nichols (1927). *J. Amer. Chem. Soc.* **49**, 2920.
Vahlquist (1931). *Biochem. J.* **25**, 1628.
Vickery and Leavenworth (1928). *J. Biol. Chem.* **79**, 377.
Weidenreich (1903). *Erg. Anat. Entwicklungsgesch.* **13**, 1.
Wooldridge (1881). *Arch. Anat. Physiol.* **5**, 387.