CLXVI. THE PREPARATION AND SOME PROPERTIES OF THE GLOBIN OF OXYHAEMOGLOBIN.

BY ROBERT HILL
AND HENRY FRANCIS HOLDEN (*Beit Memorial Research Fellow*).

From the Biochemical Laboratory, Cambridge.

(Received November 1st, 1926.)

I. INTRODUCTION.

It has long been known that haemoglobin consists of two portions, the iron-containing pigment, reduced haematin, combined with a protein, globin. The physical properties of the compound have been widely studied, and the protein obtained after removal of the pigment has been subjected to extensive chemical analysis. The globin, however, isolated by one or other of the modifications of the original method discovered by Schulz [1898] differs widely in solubility from the original haemoglobin. It is, rather, a metaprotein, similar to those obtained by the brief action of dilute acids on albumins or globulins. Thus, while soluble in either dilute acids or alkalis, it is insoluble at its isoelectric point, which according to Osato [1922] lies slightly on the alkaline side of the neutral point. Again, on treating its alkaline carbonate solution with a solution of haemin in alkaline carbonate and a reducing agent, the absorption spectrum of the solution is not that of haemoglobin. The spectrum is that of a haemochromogen and the substance is similar to other compounds of this class in that it is oxidised to haematin by the air. True globin under these conditions might be expected to yield haemoglobin and oxyhaemoglobin.

The protein to be described in this paper yields methaemoglobin on treatment with haemin dissolved in alkaline carbonate. Typical haemoglobin and oxyhaemoglobin can thence be obtained. A survey of the literature reveals scattered claims to have resynthesised oxyhaemoglobin in spectroscopically observable amounts from the products of its partial disruption. Two such claims are those of Bertin-Sans and de Moitessier [1892] and of Anson and Mirsky [1925; cf. Barcroft, 1925]. The evidence on which these claims are based will be discussed later, but it seems doubtful if any of these workers prepared true globin in sufficient quantities to admit of extensive investigations, or reasonably free from the products of its own denaturation.

The experiments described in this paper fall into three main groups. A method is given for preparing globin in quantities sufficient for many experiments. A preliminary account of some of the properties of this protein is given. Details are given of a method of preparation of protoporphyrin [Fischer and Putzer, 1926]—the porphyrin of haemoglobin—which seems more

practicable than the methods described in the literature. The other experiments deal with the reactions of globin with haemin, porphyrins and some metallic derivatives.

II. THE PREPARATION OF GLOBIN.

Anson and Mirsky's [1925] view, that acid haematin obtained directly from oxyhaemoglobin by the action of dilute acid is a compound of globin and haematin, has been shown by Keilin [1926] to be unnecessary for the explanation of its properties. The apparent solubility of the pigment is due to the protective action of the protein. In the Schulz method of preparing denatured globin this protective action is inhibited by the use of alcohol which simultaneously denatures the globin. Various attempts have been made to remove the haematin from such a solution without the use of alcohol, by great dilution and the use of charcoal or other adsorbing agents. These methods are, however, quite unsuitable for the preparation of any reasonable quantity of globin.

It seemed probable that the best conditions would be obtained by the use of the minimum amount of acid necessary to decompose all the oxyhaemoglobin at the lowest temperature at which ice was not deposited. After a few trials it was observed that the stroma proteins, particularly when swollen with ether, have a great power of absorbing haematin from an acid solution of haematin protected by globin. The addition of carefully purified kieselguhr rendered filtration easy. The following technique, while it has been modified to permit of the preparation of larger quantities of globin, yields a solution suitable for spectroscopic experiments. A scrupulous attention to detail is essential if a reasonable yield of about 40 %, calculated on the amount of oxyhaemoglobin originally present, is to be obtained. Higher yields have been obtained; variations in samples of blood appreciably affect the yield. All the apparatus must be boiled in chromic acid before each experiment, and it is advisable to use it solely for the purpose of preparing globin. The centrifuge must be kept carefully cleaned and it was found advisable to keep one instrument for the sole purpose of centrifuging the blood from which the globin was to be prepared. The air of the room must be free from any traces of volatile organic substances or acids. The most suitable room is a chamber in a refrigerating plant. If the work is carried out in an ordinary laboratory a cool day is absolutely essential and the preparation should be conducted in a small room in which no other chemical operations are being carried out. Most of the work has been done with ox blood but the method has been found to hold good for the blood of other mammals.

The defibrinated blood was centrifuged as rapidly as possible and the corpuscles washed four times with 0.9 % sodium chloride solution. An equal volume of water was added to the mass of corpuscles and the solution of oxyhaemoglobin so obtained could be stored for three or four days at 0° when so desired. In a bath at - 2° were placed the following: a test-tube containing

a few cc. of 2*N* hydrochloric acid and a 1 cc. pipette graduated in hundredths of a cc.; a test-tube with rods standing in distilled water; several test-tubes each containing 5 cc. of the cooled oxyhaemoglobin solution; a dry 70 cc. flask with a rubber stopper; a flask containing three times as much distilled water as the total volume of the oxyhaemoglobin solution; another flask containing some ether which had been well washed with water to remove alcohol and peroxides. In an ice-chest were a measuring cylinder and some small conical flasks and in each a funnel with a fluted filter-paper. A Büchner funnel in an ice-chest was used for dealing with larger quantities of globin. All the apparatus and solutions were thoroughly cooled before the experiment was started.

To 5 cc. of the cooled oxyhaemoglobin solution was added enough 2*N* hydrochloric acid to convert it completely to acid haematin in three or four minutes; about 0.68 cc. was required. The right amount was found by trial, excess being avoided. Immediately on addition of the acid the mixture was stirred with a cooled rod and a hand spectroscope was used to see that no oxyhaemoglobin remained. 15 cc. of the ice-cold water were placed in the cooled cylinder and 5 cc. added to the viscid mass of acid haematin. On stirring it dissolved and was poured into the 70 cc. flask, the test-tube being washed out with the remaining 10 cc. of water into the same flask. About 7 cc. of the ether and 1 g. of purified kieselguhr were added. The flask was closed with the stopper, removed from the bath, wrapped in a cloth and shaken vigorously for thirty seconds, cooled and shaken again for thirty seconds. After standing in the bath for a minute it was filtered in the ice-chest.

During these operations every care must be taken to prevent the temperature rising above 0°. It has been found that under favourable conditions the amount of denaturation due to the ether is small. Most of the loss occurs during the decomposition of the oxyhaemoglobin. The diluted solution of acid haematin has been kept over night at 0° without much further denaturation occurring. After filtration *N*/2 ammonia was immediately added until the maximum precipitate was obtained and the metaprotein filtered off in the ice-chest. The solution was placed in an open dish in a vacuum desiccator and the ether removed by a vacuum pump. After removal of the ether the globin solution is stable at room temperature. After dialysing away the salts at 0° a sample was dried at 0° over sulphuric acid in the vacuum of a mercury pump. It remained undenatured in the solid state at room temperature for several weeks.

The ox globin thus obtained contained up to about 2 % of methaemoglobin. It was freely soluble in distilled water over the range p_H 5–10. It is easily denatured by acids, strong alkalis or organic solvents. It is not precipitated by saturating its solution with sodium chloride but is partially precipitated by half-saturation with ammonium sulphate and completely by 60 % saturation. If this is carried out at 0° most of the globin can be recovered undenatured from the precipitate.

This globin reacts with a solution of haemin in sodium carbonate to give methaemoglobin, thus realising the scheme proposed by Küster [1910]. In 1 % sodium carbonate solution it does not yield a haemochromogen with haematin and ferrous sodium tartrate. It appears to combine with porphyrins, producing a change in the spectrum of a solution of haematoporphyrin in alkaline carbonate which can be readily observed with a direct vision spectro-scope. Globin is rapidly denatured in solution by sunlight in the presence of small amounts of haematoporphyrin. Such a solution after exposure to light is incapable of yielding methaemoglobin on treatment with haematin.

III. THE REACTION BETWEEN GLOBIN AND HAEMATIN.

In addition to ordinary haemin, the iron derivatives of haematoporphyrin and mesoporphyrin were used. A solution of any one of the three in dilute sodium carbonate reacted with globin to give the corresponding methaemoglobin. On reduction with alkaline ferrous tartrate or ammonium sulphide the corresponding haemoglobin was obtained, which, on shaking with air, formed the corresponding oxyhaemoglobin. The position of the α band of the oxyhaemoglobin obtained from haemin was within 5 Å.U. of that of the oxyhaemoglobin from which the globin was prepared. In the absence of any reducing agent these oxyhaemoglobins yield up their oxygen to vacuum and reabsorb it from the air. Carbon monoxide, in the absence of a reducing agent, displaces the oxygen forming the corresponding CO-haemoglobin. Potassium ferricyanide oxidises each to the corresponding methaemoglobin.

The best indication of the purity of a solution of oxyhaemoglobin is its colour. Artificial oxyhaemoglobin prepared from a solution of haemin in carbonate is apt to be slightly brownish. This was found to be due, not to the globin, but to the fact that in alkaline solution haematin rapidly suffers slight decomposition. If haemin crystals be added to a faintly alkaline solution of globin, avoiding an excess of pigment, the oxyhaemoglobin thence obtained has a colour similar to that of diluted blood. For the conversion of methaemoglobin to oxyhaemoglobin on the alkaline side of p_H 7 care is necessary in the selection of a suitable reducing agent. Sodium hydrosulphite is the chief reducer in common use which is free from nitrogenous substances capable of yielding haemochromogens. In its presence an alkaline solution of haemoglobin does not yield oxyhaemoglobin on shaking with air. After several trials it was decided to use throughout a solution of sodium ferrous tartrate in sodium carbonate, being a nitrogen-free modification of the reagent of Stokes. Fig. 1 and Table I show the positions of bands of derivatives of the artificial haemoglobins as measured by the Hartridge reversion spectro-scope. It has been frequently found that the bands of the artificial oxyhaemoglobin may lie up to four Ångstrom units to the violet of those of the original oxyhaemoglobin. It has not yet been found possible to determine whether any significance is to be attached to this fact.

Fig. 1. Influence of porphyrin on spectra of haemochromogens and oxyhaemoglobins.

Table I. Wave-lengths of bands of derivatives of different porphyrins in Ångstrom units.

			Diluted ox blood for comparison			Haemin (Protoporphyrin)			
Porphyrin-globin	...	...	—	—	—	6224	5673	5426	5079
Methaemoglobin	...	...	6046	5766	5390	6037	5758	5395	
Oxyhaemoglobin	...	...		5768	5387		5765	5385	
CO-haemoglobin	...	...		5699	5357		5693	5352	
Denat. globin haemochromogen				5578	5283		5578	5281	
			Haematoporphyrin				Mesoporphyrin		
Porphyrin-globin	...	...	6126	5590	5348	5001	6120	5586	5341
Methaemoglobin	...	...	5980	5682	5341		5960	5662	5341
Oxyhaemoglobin	...	...		5678	5324			5672	5317
CO-haemoglobin	...	...		5623	5286			5603	5271
Denat. globin haemochromogen				5480	5178			5470	5180

The combination of globin with haematin to form methaemoglobin can occur over a wide range of hydrogen ion concentration. On the acid side of p_H 5 combination is less than 50 %. On the alkaline side the limit has not been ascertained, combination being unaffected by p_H 10.

Fig. 1 shows that the differences in constitution between the three haematis are not sufficient to render any one of them incapable of reacting with globin. The strong tendency to combine with haematin possessed by globin is well shown by the fact that a suspension of the insoluble calcium salt of haematin in a faintly alkaline solution of globin slowly forms methaemoglobin on keeping at 0°.

IV. THE EQUILIBRIUM BETWEEN METHAEMOGLOBIN AND ACID HAEMATIN.

With a view to obtaining the best conditions for the preparation of globin some preliminary studies were carried out on the above equilibrium. An acid solution of globin, denatured globin, and haematin was prepared at 0° as described in the preparation of globin. Varying amounts of 0.2N ammonia were added to 10 cc. samples and the haematin removed at 0° with ether and kieselguhr. On the filtrates two determinations were performed.

(1) A colorimetric estimation of the proportion of haematin still remaining.

(2) The amount of undenatured globin (both free and combined as methaemoglobin), estimated as oxyhaemoglobin by adding excess of haemin in alkali, reducing and reoxidising.

The results are shown in Fig. 2.

Fig. 2.

The haematin values are somewhat high owing to the inclusion of small amounts of other pigments. A certain amount of recombination to methaemoglobin occurs when the solution is definitely acid. It is noticeable at p_H 4. Although at p_H 5 methaemoglobin begins to dissociate into haematin and globin, the degree of dissociation only gradually increases with increase of hydrogen ion concentration and is substantially complete between p_H 3 and 4. Some experiments on the decomposition of oxyhaemoglobin at 0° in buffer solutions showed that decomposition was complete in about three minutes at p_H 1.9, while at p_H 2.3 ten minutes were required. Near the neutral point practically all the globin, denatured globin and pigment are precipitated together as a bright red mass of "parahaematin" [Keilin, 1926]. On the alkaline side both haematin and globin appear in the filtrate as alkaline methaemoglobin. Over the range covered by these experiments a certain proportion of the globin is still uncombined owing to the removal of some of the haematin by the treatment with ether.

V. THE PREPARATION OF PROTOPORPHYRIN.

1900 cc. of fresh defibrinated sheep's blood were inoculated with a culture of *B. coli*, and allowed to stand three days at 20° . To the completely reduced blood thus obtained 880 cc. concentrated hydrochloric acid were added from a separating funnel fitted with a glass jet introduced beneath the surface of the reduced blood. The bottle was thus filled to the top of the neck and a greased stopper was inserted to exclude all air. It was violently shaken and after two hours the contents were squeezed in strong linen cloth. The solid

residue contained most of the porphyrin as its sparingly soluble hydrochloride. The small amount of porphyrin in the expressed acid was neglected. 1.5 l. of 97 % alcohol were used for extracting the porphyrin hydrochloride from the precipitated proteins and the liquid was filtered. The solution (1450 cc.) containing the porphyrin hydrochloride was poured with stirring into a mixture of 700 cc. 20 % sodium hydroxide solution and 30 cc. saturated sodium tartrate solution. The temperature rose to 40° and the sodium salt of the porphyrin separated as a fine micro-crystalline precipitate. After standing over night the supernatant fluid was decanted. The precipitate was filtered off and washed thoroughly with 50 % alcohol in which the sodium salt is practically insoluble. The porphyrin thus obtained formed a nearly black mass with a conchoidal fracture, and, after drying over calcium chloride in a vacuum, weighed 3.5 g. The preparation was somewhat hygroscopic and readily dissolved in distilled water giving an opalescent solution. It was immediately precipitated by the addition of alkali, salts or dilute acids. In 30 % sulphuric acid a fluorescent purple-red solution was formed, greenish in thin layers. The porphyrin is only sparingly soluble in hydrochloric acid. From its solutions in acids it is precipitated on dilution with water.

The mesoporphyrin hydrochloride was prepared from haemin by heating with formic acid and colloidal palladium as described by Fischer and Putzer [1926].

Haematoporphyrin hydrochloride was prepared from haemin by a slight modification of Nencki's method as described by Hill [1925].

The haemin was prepared by a method similar to that of Schälpejew [1885] employing, however, corpuscles laked with a small amount of ether in place of whole blood, in order to economise in acetic acid.

The chemical relationships between these porphyrins have been elucidated by Fischer and Müller [1925].

VI. THE REACTION OF GLOBIN WITH PORPHYRINS.

When a solution of haematoporphyrin or mesoporphyrin in dilute sodium carbonate is added to a solution of globin the colour of the solution undergoes an immediate change from orange to pink. The absorption bands, previously ill-defined, become exceedingly sharp and intense (see Fig. 3 and Table I). The porphyrin is no longer removed by adding calcium carbonate and filtering. Denatured globin does not show these effects. It seems probable that a definite compound of porphyrin and globin is formed. Attempts to introduce iron directly into this compound without denaturing the globin were unsuccessful.

By using a series of buffer solutions and a neutral solution of haematoporphyrin protected by gum arabic it was found that this reaction did not occur in solutions more acid than p_H 5.

On adding haemin in sodium carbonate to a solution of haematoporphyrin-globin methaemoglobin is formed. The ordinary spectrum of porphyrin in

alkali can also be seen, indicating a displacement of the porphyrin by the haematin.

In the case of protoporphyrin the reaction between its sodium salt and globin is exceedingly slow, and several weeks would be necessary in order to reach a state of equilibrium. It can be expedited by preparing a solution of protoporphyrin in 30 % sulphuric acid with the addition of denatured globin as a protective colloid with which it does not combine. If such a solution be made alkaline with sodium carbonate and immediately poured into a

Fig. 3.

solution of globin combination occurs. Ammonia cannot be used instead of sodium carbonate. If to the globin solution a considerable excess of sodium carbonate be added the acid solution of protoporphyrin may be added directly to the globin without denaturation occurring. The colour of such a solution is at first orange and after four or five seconds turns pink.

With the help of a spectrophotometer a quantitative study of this reaction was carried out. Various amounts of protoporphyrin were added to equal quantities of the alkaline globin solution, and the difference between the intensity of absorption at 6220 Å.U. and at 6400 Å.U. was measured in each case. The same globin solution was titrated against haemin by a similar means, the difference in absorption between the α band of oxyhaemoglobin (5780 Å.U.) and the band of alkaline haematin (6200 Å.U.) being measured. It was to be expected that each of these curves would show a discontinuity when all the globin had entered into combination, provided that both substances were undissociated. Within the limits of experimental error it was found that in the protoporphyrin-globin compound each molecule of globin combines with

the same number of molecules of porphyrin as it does with haematin in the form of oxyhaemoglobin.

The details of the method are as follows:

0.0314 g. of the sodium salt of protoporphyrin was dissolved in a mixture of 5 cc. water, 1.5 cc. pure sulphuric acid and 9 cc. glacial acetic acid. When completely dissolved the solution was made up with water to 25 cc. This mixture of acids was found by trial to be the minimum concentration of total acid that would readily dissolve the porphyrin.

To 2 cc. samples of globin solution, of approximately 0.9 % as determined by titration with haemin, were added 14.5 cc. water and 6.5 cc. of 15 % sodium carbonate solution. Varying amounts of protoporphyrin solution were dropped in from a micro-burette with brisk stirring. After addition the solution was made up with distilled water to 50 cc. The intensity of absorption was immediately measured in a 1 dm. tube.

For the titration with haemin, 0.0337 g. of crystalline haemin was dissolved in a little water containing 1 cc. of 15 % sodium carbonate solution and made up to 250 cc.

Fig. 4. At the point A the slope of the curve decreases by one half.

To 1 cc. samples of the globin solution were added varying amounts of haematin from a micro-burette and a constant sufficient amount of alkaline ferrous tartrate solution, great excess of the latter being avoided. After reduction the solution was gently aerated to oxidise both the haemoglobin

and the remaining reducing agent. It was made up to 50 cc. and immediately read in a 1 dm. tube. From the accompanying figure, Fig. 4, the experimental evidence appears to favour the conclusion stated above.

VII. THE REACTION OF GLOBIN WITH METALLO-PORPHYRINS.

Mesoporphyrin was chosen as the porphyrin most suitable for the demonstration of this characteristic of globin, owing to the ease with which its metallic derivatives can be isolated in a crystalline condition. The method of their preparation was based on that described by Fischer and Putzer [1926] for the metallic derivatives of protoporphyrin. After washing with 10 % acetic acid and with water, the metallic derivative was suspended in 1 % sodium carbonate solution and its absorption spectrum measured in the presence and in the absence of globin. Before measurement the solutions containing globin were shaken with a little calcium carbonate and filtered. This removed any pigment not in combination with the globin. The time necessary for combination apparently depended on the degree of solubility of the pigment in the dilute sodium carbonate. The tin derivative combined at once; the copper incompletely even after 12 hours. No other colloidal substance could be found, *e.g.* denatured globin, serum protein, capable of producing a similar effect. It can however be simulated by the addition to the pigment of excess of a suitable organic solvent, *e.g.* pyridine. In the latter case the absorption bands, though sharp compared with those of the suspension, differ in wavelength from those of the globin compound. The quality of the change in the absorption spectrum is very similar to that observed in the case of the free porphyrins.

The metallo-porphyrins of manganese and cobalt [Laidlaw, 1904] can be reduced to a form reoxidisable by the air. Their complete reduction occurs only under conditions which denature globin. Consequently the attempt which was made to obtain information concerning the effect of globin on their oxidation failed. Metallic derivatives of haemato- and proto-porphyrin have been observed to yield similar globin compounds. We have been unable to observe this effect with uroporphyrin or its iron and copper derivatives. Dr Allott kindly supplied a specimen of this porphyrin. The zinc derivatives of haemato- and of meso-porphyrin, when combined with globin, and in the absence of any removable pigment, possess a spectrum in which the α band has two well-defined maxima. In ether the α band of the free zinc porphyrin, though sharp, appears single.

The changes in the absorption spectra of some metallic derivatives of mesoporphyrin as the result of combination with globin are shown in Table II. Denatured globin can take up in solution some metallo-porphyrins, but the appearance and position of the bands are different from those of the metallo-porphyrin-globin compounds.

Table II. *The effect of globin on the spectra of metallic derivatives of mesoporphyrin.*

Metal	Medium	Wave-length of bands, Å.v.		State of pigment without globin	Appearance of bands
		α	β		
Mn	Carbonate	5924	5537	Solution	Indefinite
	Globin	5803	5469		More definite
Co	Carbonate	5600	5260	Suspension	Indefinite
	Globin	5587	5252		Sharp
Ni	Carbonate	5627	5261	Suspension	Indefinite
	Globin	5490	5119		Very sharp
Cu	Carbonate	5779	5409	Suspension	Indefinite
	Globin	5577	5215		Very sharp
Zn	Carbonate	5735	5362	Suspension	Indefinite
	Globin	{5837} {5724}	5418		Sharp
Sn	Carbonate	5714	5356	Solution	Sharp
	Globin	5740	5338		Very sharp

DISCUSSION.

Any consideration of the various claims existing in the literature to have resynthesised oxyhaemoglobin from a pigment and a protein involve an enquiry into the value to be attached to spectroscopic evidence, with regard to the particular experimental conditions under which it is obtained. In dealing with the absorption spectra of solutions containing more than one light-absorbing substance, not only the position but the relative intensity of the bands must be borne in mind. An experiment [cf. Keilin, 1925] will illustrate the necessity for the utmost reserve in interpreting such evidence. If in a test-tube 2 cc. of ox blood that has been diluted with 100 volumes of water be treated with 0.5 cc. of 10 % sodium hydroxide the protein is completely denatured in a few minutes. 2 cc. of the same diluted blood are then added. If the solution be reduced with ferrous tartrate and sodium carbonate, without the addition of any more sodium hydroxide, the spectrum of the reduced haemoglobin is practically invisible and on reoxidation the spectroscopic evidence might easily lead to the conclusion that haemochromogen had been oxidised to oxyhaemoglobin. If, instead of oxidising the solution, the hydroxyl ion concentration be gradually decreased, the haemochromogen band becomes less intense and shifts up to 10 Å.v. towards the red. The band of reduced haemoglobin gradually becomes more noticeable. Parahaematin, described by Keilin [1926], has a red colour and its absorption spectrum bears some resemblance to that of oxyhaemoglobin. The bands however are ill-defined and lie somewhat to the violet of the corresponding bands of oxyhaemoglobin. The possibility of parahaematin formation near the neutral point, and the low selectivity of the absorption of both haematin and methaemoglobin as compared with oxyhaemoglobin, may have led some workers to assume the resynthesis of oxyhaemoglobin on an insufficient experimental basis.

Bertin-Sans and de Moitessier [1892] claimed to have separated from oxyhaemoglobin a globin from which the original oxyhaemoglobin could be synthesised by neutralising very slowly an alkaline solution containing this globin together with added haematin. The ox blood was coagulated with ether and treated with a boiling solution of 10 % tartaric acid in ethyl alcohol. They record no measurements of the position of the bands of the resynthesised oxyhaemoglobin. It is difficult to see how any could have been detected in the presence of the relatively very large amounts of parahaematin which must have been formed by their procedure. It is very difficult to remove the last traces of ammonium sulphide from solutions; and since they used this reducing agent their synthetic CO-haemoglobin might have been CO-haemochromogen, owing its formation to the denatured globin present and a small amount of ammonium sulphide which had escaped oxidation. Again no data of the position of the bands are recorded and the spectra are of a similar pattern and the corresponding bands differ very slightly in wave length. The spectrum of a mixture of parahaematin and haematin simulates that of alkaline methaemoglobin.

If denatured globin be precipitated between p_H 6 and 7 by salts or alcohol, instead of at its isoelectric point, p_H 8, it will redissolve in distilled water after washing. It is reprecipitated at p_H 8. This probably explains the solubility in distilled water of the protein prepared by these workers. Such a neutral solution of denatured globin was prepared during the course of the experiments described in this paper and tested for freedom from (undenatured) globin. On treatment with a nearly neutral solution of haemin in alkali it yielded a clear red solution of denatured globin parahaematin. Neutralisation and subsequent oxidation of a solution of denatured globin haemochromogen convert it into parahaematin. No experiment could be found recorded in which, in any solution of globin, the methaemoglobin present was estimated by reduction and reoxidation without the addition of haematin, and the increased yield of oxyhaemoglobin due to the addition of haemin in alkaline carbonate to this solution determined. This test is illustrated in Fig. 4. Unless the greatest precautions are observed denatured globin prepared by the Schulz method may well contain, as does the globin prepared by the method of this paper, traces of methaemoglobin. This essential precaution was unfortunately neglected by even so careful a worker as Menzies [1894]. Perhaps it is significant that, with the exception of Menzies, all these workers have claimed to obtain a resynthesis of oxyhaemoglobin only under the conditions essential to the formation of parahaematin.

The globin prepared as described in this paper may be distinguished from that prepared by any modification of Schulz's process by the following tests. It is soluble over the range p_H 5–10. It is precipitated from a neutral solution by 60 % saturation with ammonium sulphate and provided that the precipitation is carried out at a low temperature the precipitate is readily soluble in solutions containing less than this amount of the salt. In a pure state it

does not yield a haemochromogen in a solution made alkaline with sodium carbonate. Under these conditions denatured globin readily yields its haemochromogen.

Experiments dealing with the conversion of haemochromogen to haemoglobin by decrease in the hydroxyl ion concentration of the solution do not appear to have excluded the possibility that in the solutions two proteins were present simultaneously. It was found that globin could remove the pigment from denatured globin haemochromogen and so produce haemoglobin. In a solution containing globin, denatured globin and haematin, the pigment may well combine with either protein according to the degree of alkalinity.

In the preparation of globin, the action of the acid on oxyhaemoglobin always denatures a certain proportion of the protein. This denatured globin can be readily freed from globin. It is then insoluble at its isoelectric point. Its alkaline solution yields on treatment with haemin in sodium carbonate solution haemochromogen but no haemoglobin. Its neutral solution as mentioned above yields parahaematin and no methaemoglobin. If denaturation of globin be defined either in terms of the alteration in solubility at the isoelectric point or in terms of its reaction with haemin in alkaline carbonate, the fact of its preparation from oxyhaemoglobin shows that separation of the pigment does not necessarily involve denaturation.

The combination of globin with compounds not containing iron, *i.e.* porphyrins and their other metallic derivatives, indicates that the porphyrin residue plays a part in the formation of haemoglobin independently of the iron. The following evidence may suggest an explanation of the mechanism of the action of globin on such compounds. The spectrum of a solution of a porphyrin in the presence of globin is similar in the sharpness of the bands to that of the same porphyrin in true solution in an organic solvent [cf. Keilin, 1926]. Adair [1925] has shown that each molecule of globin is combined with four molecules of pigment in oxyhaemoglobin. Hartridge and Roughton [1923] have shown that the reduction of oxyhaemoglobin is a monomolecular reaction. It would therefore seem probable that in a molecule of any globin-pigment compound the pigment molecules are separated from one another by distances large compared with their own molecular dimensions. The quantitative relationships between protoporphyrin globin and oxyhaemoglobin are in agreement with this conception. Haematin displaces haematoporphyrin rapidly and protoporphyrin slowly from their globin compounds. These facts taken in conjunction may suggest that protoporphyrin globin is haemoglobin without iron.

SUMMARY.

1. From the oxyhaemoglobin of ox blood has been prepared a protein which is considered to be the true globin of this conjugated protein.
2. This globin is soluble in the presence or absence of salts over the range p_H 5–10.

3. Spectroscopic evidence is presented of the identity of the reconstituted oxyhaemoglobin and the original oxyhaemoglobin from which the globin of the former was prepared.

4. Globin reacts with haematin over the range p_H 5–10 to give methaemoglobin. This equilibrium has been investigated on the acid side.

5. Evidence is given of the occurrence of compounds of globin with certain porphyrins and their metallic derivatives.

6. Oxyhaemoglobins have been prepared from the iron compounds of haematoporphyrin and mesoporphyrin.

The authors desire to record their gratitude to Sir F. G. Hopkins for his continual interest and encouragement, to the Government Grant Committee of the Royal Society for a grant covering the cost of certain apparatus, and to the Cambridge University Medical Grant Committee for the gift of a spectrophotometer.

One of them (R. H.) is indebted to the Medical Research Council for a grant.

REFERENCES.

- Adair (1925). *Proc. Roy. Soc. Lond. A*, **108**, 627.
 Anson and Mirsky (1925). *J. Physiol.* **60**, 50.
 Barcroft (1925). *Physiol. Reviews*, **5**, 596.
 Bertin-Sans and de Moitessier (1892). *Compt. Rend. Acad. Sci.* **114**, 923.
 Fischer and Müller (1925). *Z. physiol. Chem.* **142**, 155.
 Fischer and Putzer (1926). *Z. physiol. Chem.* **154**, 50.
 Hartridge and Roughton (1923). *Proc. Roy. Soc. Lond. A*, **104**, 416.
 Hill (1925). *Biochem. J.* **19**, 341.
 Keilin (1925). *Proc. Roy. Soc. Lond. B*, **98**, 331.
 — (1926). *Proc. Roy. Soc. Lond. B*, **100**, 129.
 Küster (1910). *Z. physiol. Chem.* **66**, 249.
 Laidlaw (1904). *J. Physiol.* **31**, 464.
 Menzies (1894). *J. Physiol.* **17**, 415.
 Osato (1922). *Biochem. Z.* **132**, 485.
 Schälpejew (1885). *Ber. deutsch. chem. Ges.* **18**, Ref. 232.
 Schulz (1898). *Z. physiol. Chem.* **24**, 449.