CLXIV. THE ACID-DENATURATION OF HAEMOGLOBIN.

By NORMAN URQUHART MELDRUM¹.

From the Biochemical Laboratory, Cambridge.

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THE change undergone by proteins in aqueous solutions when subjected to the action of various influences, such as those of acid, alkali, heat, light and alcohol, is known as "denaturation." Acids and alkalis produce metaprotein, a product soluble in acids and alkalis, but, in the absence of salts, insoluble at the isoelectric point. Boiling an isoelectric suspension of metaprotein converts it into an insoluble product like that obtained from native isoelectric protein under the influence of heat, alcohol, or shaking.

The coagulation of haemoglobin has been studied by Chick and Martin [1910], who used heat as denaturant: they found denaturation to take place only in the presence of water, and to follow the unimolecular law. They found the reaction to be characterised by the temperature coefficient of 1.3 per 1°, a value confirmed by Hartridge [1912], and by Lewis [1926]. Lewis obtained evidence that not only did H^+ and OH^- ions catalyse the reaction, but that water also entered into it, as a reactant rather than as a catalyst. He confirmed the observation of Chick and Martin that under certain specified conditions salts decrease the rate of denaturation, and his work was extended by Cubin [1929], who found that formaldehyde prevented the flocculation of denatured haemoglobin at p_H values greater than 7.

Wu [1929] distinguishes between "denatured" and "coagulated" protein. He regards them as chemical species distinct from each other. "Coagulation" occurs when protein is heated at the isoelectric point or when it is subjected to dehydrating agents, and involves a decrease in the number of free amino- and carboxyl-groups. Such condensed protein is rather insoluble in acids and alkalis. "Denatured" protein is obtained by the action of acids and alkalis, especially on warming, and denaturation consists, according to Wu, in the splitting of the protein with liberation of free $-NH_2$ and $-COOH$ groups. Such denatured protein is much more soluble in acids and bases than is the condensed material.

The action of alcohol upon methaemoglobin is to give a substance which has lost the power of combining with oxygen [Arnold, 1900]. It was studied by Van Klaveren [1901] under the name of kathaemoglobin, and is now called

¹ Beit Memorial Research Fellow.

parahaematin [Keilin, 1926]. Alkali yields a similar product [Adair, unpublished observation quoted by Keilin, 1926], as do many other reagents, and it is now clear that parahaematin is a compound between denatured, or condensed, protein and oxidised haematin [Keilin, 1926]. Reduction gives the haemochromogen of Bertin-Sans and de Moitessier [1893] and of Anson and Mirsky [1925].

In general the denaturation of proteins has been regarded as irreversible. Though Michaelis and Rona [1910] report the heat-coagulation of electrolyte-free serum-albumin to be reversed by the action of alkalis, an observation confirmed by Spiegel-Adolf [1926], no like case appeared to be known, either with regard to condensed or denatured proteins, until Anson and Mirsky [1925] stated that the acid-denaturation of haemoglobin was essentially reversible. When a solution of haemoglobin was acidified and the mixture neutralised, complete precipitation resulted. By the action of alkalis on this coagulum, especially in the presence of cyanide, native haemoglobin could be isolated, best as the carbon monoxide derivative. It may be urged that some native material is carried down by the denatured protein on flocculating. Anson and Mirsky advanced two arguments against this: the yield of denatured reversed protein was about 70 %—yet when entirely denatured protein was precipitated in the presence of native, the latter was undiminished in concentration. Secondly the temperature of denaturation had no effect on the yield of reversed material obtained.

The problem has a dual nature. In the first place haemoglobin behaves as a typical protein, denatured or native. If its denaturation be reversible then it appears probable that the denaturation of other proteins may be so likewise. In the second place this phenomenon has bearings on the haemoglobin system itself. Anson and Mirsky [1925] regard haemoglobin and haemochromogen as forming an equilibrium, the point of balance being decided by the p_H . At strongly acid p_H values haemochromogen predominates in the equilibrium,

while near neutrality the protein is mainly haemoglobin.

In later experiments they have shown [1930] that the prosthetic group plays no decisive part in affecting reversibility. It is possible to cause the reversion of the denaturation of free globin in the complete absence of haematin. Yet the haematin moiety does play some part in the phenomena observed. Thus Wu and Lin [1927] observed that the amount of reversed protein was greater in the ratio of 1.8 to 1 when the initial protein was reduced haemoglobin in place of oxyhaemoglobin. The haemoglobins of various species appear to behave differently. When a solution of ox haemoglobin is acidified with acid, in the ratio of 4 cc. $N/5$ HCl to 6 cc. 10 % haemoglobin, and neutralised after at least 10 minutes, complete precipitation results, the colour of the coagulum being brown like that of acid haematin. After standing for a few minutes the colour changes to the red of parahaematin, and methaemo-

globin passes into solution. With horse haemoglobin the effects are different. The brown colour of the coagulum changes to the pink of parahaematin, but solution of methaemoglobin does not take place so readily, and the alkaline cyanide reagent of Anson and Mirsky must be used for native material to be obtained in the soluble state. The parahaematin of the haemoglobin of the horse seems to be stable over a wider p_H range than that of the ox (personal communication of Mr R. Hill).

EXPERIMENTAL.

When a solution of oxyhaemoglobin is acidified, changes take place in the system: acid methaemoglobin is formed if the p_H be not too low, and the solution contains free native and free denatured globin, as well as acid haematin. The analysis of such a system is complex; the first phenomenon studied was the formation of acid haematin from acid methaemoglobin.

In the majority of these experiments a solution of methaemoglobin was used. Ox blood corpuscles were washed with isotonic saline four times, laked with an equal volume of distilled water, and the stromata removed with alumina cream. Thereafter the procedure was identical with that advocated by Stadie and Ross [1926] for the electro dialysis of haemoglobin, except that the protein was first converted with 10 % ferricyanide to methaemoglobin.

The buffers employed throughout are those of McIlvaine (0.1 *M* citric acid and 0.2 *M* alkaline phosphate). The buffering power of these buffers is approximately linear throughout the p_H range used here, from p_H 2.2 to 4.8.

A titration curve of each methaemoglobin solution used was constructed by adding HCl in known amount and determining the p_H by the quinhydrone electrode. At the p_H values concerned this does not appear to denature the proteins. Thus the p_H values were not appreciably affected on allowing the solutions to stand with quinhydrone as they would have been had denaturation occurred progressively. A similar titration curve was obtained for gum arabic in 8 % solution. From these curves the amount of acid needed to bring the protein and gum arabic solutions to any p_H could be calculated roughly.

The experiments were carried out as follows. To buffer solution in a standard flask was added the solution of methaemoglobin to be investigated. The buffering power of the protein was overcome by adding acid in the amount calculated from the titration curves referred to. The final p_H was thus that of the buffer. It was found to be a matter of indifference here whether the acid was run into the solution before, or after, addition of the protein solution. The acid solution of protein thus obtained then contained acid methaemoglobin and acid haematin. The relative amounts of these substances were then estimated. This was done by use of the double-wedge trough described by Hill [1929].

In these experiments on acid methaemoglobin and acid haematin, the standard solutions put in the trough, one in each side, consisted of acid

methaemoglobin at p_H about 5.8¹, and acid haematin in 4 % gum arabic solution, dissolved in McIlvaine buffer at the p_H of the acid mixture of methaemoglobin and acid haematin to be analysed. Ten or twenty readings were then taken at the positions where the spectrum of the standards matched in all respects that of the solution analysed. Two sources of error militate against accuracy. The degree of dispersion affects the absorption [Keilin, 1926], and the standard contains no parahaematin. The latter effect can be of small importance, as the absorption of parahaematin is slight, and as it can only be in low concentration. In studying the formation of acid haematin from acid methaemoglobin the author has accepted the view of Keilin that the former indicates a physical state, namely a colloidal suspension of haemin protected by protein or gum arabic, rather than a definite chemical individual.

Fig. 1. The formation of acid haematin from acid methaemoglobin.

Curve 1. 2 cc. (approx.) 5 % solution of electrolysed ox methaemoglobin + 12.5 cc. of McIlvaine buffer + 12.5 cc. water. 0.1 *N* HCl was then added, in amount sufficient to overcome the buffering of the protein and the volume diluted to 50 cc. The standards were acid methaemoglobin at a p_H of 5.5 in water, and a solution of acid haematin in 4 % gum arabic and McIlvaine buffer (12.5 cc. in 50 cc. total volume) at the p_H of the solution studied.

Each different type of point $\times$, Δ , and $\bullet$, refers to separate experiments on the protein.

Curve 2 was carried out with a different sample of protein.

Points $+$ were obtained with 40 cc. of McIlvaine buffer in 50 cc. aqueous solution, $\circ$ points with 20 cc. buffer in 50 cc. total volume. A third sample of protein has been studied. Its behaviour is shown by points $\odot$. The experiments were carried out in McIlvaine buffer undiluted.

Curves 1 and 2 (Fig. 1) show formation of acid haematin from acid methaemoglobin as a function of p_H . Two different protein solutions have been studied. The results are similar but the curves do not coincide. On the

¹ It is not possible to have the methaemoglobin standard at the p_H of that to be analysed, for under such conditions it would dissociate to acid haematin and become denatured.

other hand, separate estimations on any one protein preparation agreed admirably, as can be seen from Fig. 1. Solutions of methaemoglobin brought to a p_H of 3.3 or 3.5 in McIlvaine buffer precipitate completely, as though fully denatured, when made isoelectric.

The protein solutions studied in curves 1 and 2 were treated with the alkaline cyanide reagent of Anson and Mirsky, and allowed to stand at a p_H of about 7.8 for 3 hours. Then the solutions were half saturated with ammonium sulphate and the precipitated protein filtered off. On reducing with sodium hydrosulphite and passing in carbon monoxide, the spectrum of carboxyhaemoglobin became visible. The whole was filtered after standing overnight. The total amount of the "reversed" protein was then estimated in the double-wedge trough. The standard solutions were carboxyhaemoglobin in one wedge and water in the other. The yields of native reversed protein obtained from the protein solutions which gave Fig. 1 are plotted in Fig. 2. It seems, that though there appears to be some acid methaemoglobin in

Fig. 2. Amount of carboxyhaemoglobin obtained from acid protein after the treatment with alkaline cyanide advocated by Anson and Mirsky.

The points have the same meaning as in Fig. 1.

solutions which precipitate entirely on neutralisation, yet this acid methaemoglobin is insufficient in amount to account for the yield of "reversed" protein obtained. This discrepancy is tabulated in Table I. It is perhaps due to the dissociation to acid haematin and free native globin. As the properties of protein solutions change rapidly with time, it is a matter of some difficulty to obtain sufficient data in the course of a few days from one solution of protein to provide a comparison of the amounts of acid methaemoglobin present and reversed protein obtained.

The behaviour of acid protein on neutralisation is worthy of closer examination. When a solution of methaemoglobin is brought to p_H 2.5, say, and then neutralised, all the protein is precipitated, and on redissolving the coagulum in the minimum amount of alkali and neutralising the alkaline solution as rapidly as possible, all the protein is again reprecipitated, and on filtering the coagulum only a small fraction of the total protein comes with the filtrate.

Table I.

p_H	Acid methaemoglobin estimated in double-wedge trough			Native protein estimated in double-wedge trough as carboxyhaemoglobin		Value of R	
	Exp. 1	Exp. 2	Exp. 3	Exp. 2	Exp. 3	Exp. 2	Exp. 3
	%	%	%	%	%	%	%
Protein preparation No. 1							
4.57	91.5	93.8	92	86.7 (?)	—	0.0	0.0 (?)
4.03	85	84	86	82	88.3	4.1	14
3.81	86	85.7	71.9	88	88.3	29.7	29.1
3.66	65.3	30.4 (?)	56.9	83.4	89.1	44.9	34.9
3.56	43.2	38.5	35	73.1	86.6	53.2	60
3.32	12.2	12.6	11.4	73	79.8	83.5	84.5
3.19	0	0	0	64.8	77.2	—	—
2.94	0	0	0	64.2	76.3	—	—
2.76	0	0	0	60.7	—	—	—
2.60	0	0	0	64.6	74.9	—	—
2.43	0	0	0	66	76.6	—	—
2.21	0	0	0	66	77.2	—	—
Protein preparation No. 2							
4.62	88.6	100.0	—	100	—	Mean values of R given here	
4.24	71.7	69.1	—	95	—	0.0 %	
4.01	17 (?)	50.8	—	95	—	26.4	
3.66	—	3.3	—	73.5	—	47.4	
3.56	9.2	0	—	54.9	—	92.0	
3.46	0	0	—	66	—	—	
3.30	0	0	—	63.3	—	—	
3.10	0	0	—	68	—	—	
2.61	0	0	—	—	—	—	

Here R is given by the ratio

$$\frac{(\text{Total native HbCO} - \text{acid methaemoglobin}) \times 100}{\text{Total native HbCO}}$$

The yield of "reversed".HbCO is plotted in Fig. 2; the amount of acid methaemoglobin in Fig. 1, curves 1 and 2.

If, on the other hand, the alkaline solution of protein is allowed to stand for some minutes and then neutralised with acid and filtered, a large amount is now found to be soluble. In other words the protein has "reversed" in the alkaline solution. When the protein solution is reduced with sodium hydro-sulphite and treated as above, the haemoglobin is found to "reverse" more rapidly than when no reduction occurs. The following data illustrate the behaviour of methaemoglobin.

Into three 25 cc. graduated flasks were run 6.25 cc. McIlvaine buffer, p_H 2.9, and an equal volume of water, 0.5 cc. of protein solution, and 0.87 cc. of 0.1N HCl. Each flask was allowed to stand for some time at the acid p_H , flasks 1 and 2 for 8 minutes, flask 3 for 25 minutes. (Time is a non-variant in such a system, and does not affect the final result.) At the end of the times concerned the protein solutions were neutralised with NaOH and the precipitate redissolved in the minimum amount of alkali and immediately neutralised with acid and filtered. The protein in the filtrate was then estimated as alkaline methaemoglobin. The solution in flask 3 was allowed to stand for 10 minutes in alkali before neutralisation with acid and filtration. Then the

dissolved material was estimated as with flasks 1 and 2. The results are set out below.

Flask No.	...	1	2	3
Time kept alkaline		0 mins.	0 mins.	10 mins.
Alkaline methaemoglobin		10 % (?)	0 % (?)	64 %

It is thus clear that for "reversion" to take place the protein must stand for some time in alkaline solution. At the end of this time soluble protein is obtained which shows a spectrum apparently identical with that of alkaline methaemoglobin. When the system is studied spectroscopically the spectrum of alkaline methaemoglobin is visible from the beginning. A solution of methaemoglobin treated exactly as before shows, on spectroscopic examination when in alkaline solution, the spectrum of alkaline methaemoglobin, even though it can be entirely precipitated on adding acid. This spectrum seems to exist initially at the same intensity as when the protein is entirely reversed. Thus the change in solubility occurring when the protein stands in alkali is not paralleled by any corresponding change in spectrum. If spectroscopic evidence be accepted as a criterion of denaturation, then native protein exists as alkaline methaemoglobin, even though this can be completely precipitated on neutralisation. Similarly the spectrum of reduced haemoglobin can be seen in such solutions and also that of carboxyhaemoglobin.

That no evident change in spectrum occurs on standing is shown by the following data.

In each of two 50 cc. graduated flasks were put 12.5 cc. of McIlvaine buffer p_H 2.21 and 2.47 respectively, 3.8 and 2.8 cc. of 0.1 *N* HCl and water to make the final volume 25 cc. Then 1 cc. of methaemoglobin solution was run in, and the protein allowed to stand for 30 minutes in acid solution. After this the solution was neutralised, the coagulum dissolved in the minimum alkali required, and the spectrum studied.

A series of 10 readings was then taken over a short range of time, the final reading being about 6 minutes after the first, and the composition of the protein mixture calculated from the mean of each adjacent two. The standards in the double-wedge trough were alkaline methaemoglobin in one side and an alkaline solution of parahaematin in the other. The results are tabulated below.

Reading No.	...	1	2	3	4	5
				p_H 2.21		
% alkaline methaemoglobin		64	62	66	66	67
				p_H 2.47		
% alkaline methaemoglobin		67	64	64	65	67

These values are, however, precisely those which would be obtained on "reversion" of the protein according to the method of Anson and Mirsky.

When the protein solution is reduced the spectrum of reduced haemoglobin can be seen, and, like alkaline methaemoglobin, reduced haemoglobin appears

to exist under those conditions in a solution which is entirely precipitated on bringing to the isoelectric point. The same holds for carboxyhaemoglobin.

When a study is made of the spectrum shown by haemoglobin solution brought originally to an acid p_H and then to p_H 7.8 to 8, it is found that the spectrum varies according to the p_H to which the protein was brought initially. Fig. 3 shows the relationship between the amount of alkaline methaemoglobin estimated spectroscopically and the initial p_H of the solution before making alkaline. The abscissae refer, not to the p_H of the protein solution during the time of estimation, but to the p_H of the McIlvaine buffer at which denaturation took place. As has been before mentioned the amount of native protein as estimated in this way seems independent of the solubility of the protein at the isoelectric point, *i.e.* of the length of time the protein has stood in alkali at p_H about 8. The details of these experiments are as follows. After having made the protein solution acid in precisely the manner described for the experiments with acid methaemoglobin and acid haematin, alkali was added in amount just sufficient to redissolve the precipitated protein. A clear solution was obtained which showed the spectrum of alkaline methaemoglobin. This was placed in the cell of the double-wedge trough and estimated against standards of alkaline methaemoglobin and an alkaline solution of parahaematin. Table II gives some numerical data obtained in this way.

Table II.

p_H	Alkaline methaemoglobin %	Carboxyhaemoglobin in alkaline solution %	Carboxyhaemoglobin in acid solution %
4.57	—	—	104
4.03	—	—	—
3.81	—	88.3	105
3.66	—	89.1	87.5
3.56	—	86.6	85.7
3.32	—	79.8	74
3.19	—	77.2	77.9
2.94	—	76.3	77.5
2.76	—	79	73
2.60	—	74.9	(?)
2.43	—	76.6	(?)
2.21	—	77.2	(?)
4.62	100	100	—
4.24	—	95	—
4.01	65.3	—	—
3.66	53.5	73.5	—
3.56	51.5	54.9	—
3.46	57.2	66	—
3.30	—	63.3	—
3.10	55.2	68	—
2.61	47.5	—	—
4.20	90.1	—	—
4.05	74	—	—
3.78	72	—	—
3.56	76	—	—
3.33	64.2	—	—
3.12	63.2	—	—
2.90	64	—	—

Similar curves have been obtained by bringing solutions of methaemoglobin to a series of definite p_H values in McIlvaine buffer and then "reversing" the solutions according to the method of Anson and Mirsky. This involves the treatment of the solution with alkaline cyanide reagent for a few hours, the p_H being about 8, followed by the separation of insoluble protein with half saturated ammonium sulphate, reduction of the filtered solution with sodium hydrosulphite and saturation with carbon monoxide. The reversed protein was estimated as carboxyhaemoglobin in the double-wedge trough, the standards being carboxyhaemoglobin and water. Table II shows the data obtained thus, and Fig. 3 illustrates the similarity between

Fig. 3. A comparison of protein estimated as alkaline methaemoglobin after having been made acid in buffer, with that obtained as carboxyhaemoglobin after the cyanide treatment of Anson and Mirsky.

In order to obtain the solution of alkaline parahaematin the solution of haemoglobin was brought to a p_H of about 3 in phthalate buffer (Clark), and about 2.5 cc. *N* HCl added per 5 cc. phthalate buffer; the whole was warmed to about 60° and allowed to stand for about $\frac{1}{2}$ hour, after which it was neutralised and made slightly alkaline. Points +, ● show the behaviour of protein so treated, while points O show the native material obtained after "reversion."

the amount of "reversed" protein and that estimated as alkaline methaemoglobin in the manner previously described. It is clear that the two curves coincide closely.

The problem may be attacked in a slightly different manner. When an acid solution of protein, such as has been studied above, is reduced with sodium hydrosulphite and aerated, the spectrum of oxyhaemoglobin can be seen. The bands rapidly disappear owing to the instability of the molecule at acid p_H . Partial neutralisation makes for greater stability, but it was never found possible to estimate the oxyhaemoglobin. When an acid solution of protein is reduced with sodium hydrosulphite in the presence of CO a solution is obtained which can be entirely precipitated on neutralisation. The solution shows a spectrum apparently identical with that of carboxyhaemoglobin, provided the p_H of the acid solution is not too low, when the spectrum of CO-reduced haematin appears; and on estimating the substance which resembles carboxyhaemoglobin against a standard of carboxyhaemoglobin in the double-wedge trough, a curve is obtained which coincides between p_H 4 and 5 with those obtained by reversion and as alkaline methaemoglobin. The spectrum of carbon monoxide-haemochromogen is very similar to that of

carboxyhaemoglobin and cannot be distinguished from it by the optical methods here used. Were, however, the protein entirely denatured and the substance estimated entirely CO-haemochromogen, it is hard to see how the amount of haemochromogen could vary (see Fig. 4) as no acid CO-reduced haematin could be observed at any p_H less than about 2.8. Before estimating the native protein the solutions were aerated thoroughly in each case in order to oxidise any haemochromogen present.

Fig. 4. Comparison of carboxyhaemoglobin obtained after treatment with alkaline cyanide with that estimated at acid p_H values down to p_H 2.75.

Points $\circ$ refer to values obtained by making acid in McIlvaine buffer (12.5 cc. buffer in 50 cc. total volume), neutralising with $N/5$ NaOH and dissolving in about 5 cc. of cyanide solution, adding an equal volume of sat. $(NH_4)_2SO_4$ and filtering before converting to HbCO.

Points $+$ were obtained by comparing the acid solution of protein with standards of carboxyhaemoglobin. The abscissae have different meanings for the two curves. For curve A the abscissae refer to the p_H to which the protein was brought in buffer, and for curve B to the p_H at which the estimation was carried out.

Table III gives data obtained in a series of preliminary experiments. The protein was about 5 % in concentration. To 1 cc. of this protein was added $N/5$ HCl in the amounts tabulated. Different volumes of $N/5$ NaOH were then added to this acid solution, reduction effected by adding $Na_2S_2O_4$, and then CO was passed in. The reduced solutions were then studied in the double-wedge trough, the standard solutions being carboxyhaemoglobin and water.

Table III.

cc. $N/5$ HCl added per 1 cc. protein	cc. $N/5$ NaOH added to acid mixture	% HbCO	cc. $N/5$ NaOH added	% HbCO
10	8.5	71.7	10	85
10	9.5	—	8.5	81
10	9.5	—	9.5	73.4
10	15.0	76.4	—	—
10	10.0	75.5	12.0	—
10	10.0	79.0	12.0	78.0
5	5.0	75.0	4.75	70.0
5	4.5	64.0	4.75	62.0

Where excess alkali was added the reduction was carried out by means of Stokes's reagent (alkaline reduction in 10 % sodium tartrate and dilute ferrous sulphate). The existence of HbCO at strongly acid p_H having been shown to be likely, the next series of experiments consisted in measuring the amount of HbCO obtainable between p_H 2.2 and 4.7. The curve thus obtained (Fig. 4) coincides closely with that obtained on reversion of the acid protein. Table II

compares the yield of native protein, obtained after reversion by the method of Anson and Mirsky, with the amounts estimated in the double-wedge trough at acid p_H as HbCO, and with the protein estimated as alkaline methaemoglobin.

Peculiarities in the behaviour of haemoglobin.

Chick and Martin found the temperature coefficient of the denaturation of haemoglobin to be 1.3 per 1°. Temperature, however, has little effect on the yield of native protein by reversion.

10 cc. $N/5$ HCl was added to 6.5 cc. 10 % oxyhaemoglobin. After 3 minutes the whole was neutralised with $N/5$ NaOH. The coagulum was washed with water and dissolved in KCN-buffer. The system was then reduced with $Na_2S_2O_4$ and CO bubbled through. Estimation of the amount of native protein showed that the effect of temperature is very small, the yield being about 43 % when the denaturation was carried out at 0°, and 32 % when at 40°.

The length of time the protein is kept at an acid p_H has no effect on the percentage recovery after the first minute when the temperature is 40°. This absence of effect of time is shown whatever the p_H ; thus the amount of denatured protein does not increase on standing at, say, p_H 3.5 to the value obtained rapidly for p_H 2.8. There is no progressive increase on standing at acid reactions. The method used was as follows.

4 % ox methaemoglobin was acidified as in the previous experiment at a temperature of 40°. After definite intervals of time $N/5$ ammonia was run in. The precipitate was then dissolved in 2 % ammonia and reduced with Stokes's reagent. Aeration produced oxyhaemoglobin, which was estimated spectrophotometrically as in the case of other derivatives of haemoglobin. Fig. 5 illustrates the results obtained. Ox, sheep, and horse haemoglobins behaved

Fig. 5. Time-denaturation curve.

55 cc. 4 % ox methaemoglobin were acidified with 6.5 cc. $N/5$ HCl and $N/5$ ammonia was run in after various intervals.

alike in showing no progressive increase in denaturation on standing for various lengths of time at acid reaction at 0°, 20°, and 40°.

The influence exerted by foreign proteins seems to be slight. To 0.06 g.

methaemoglobin was added 0.06 g. crystalline egg-albumin; the control lacked the egg-albumin. The solutions were acidified with $N/5$ HCl to p_H 3. The undenatured protein was estimated as alkaline methaemoglobin in the spectrophotometer. The amounts were 70 % in the experiment and 68 % in the control. A similar result was obtained for laked blood.

Salts have but a small effect on the recovery of undenatured protein. To varying concentrations of NaCl in a constant volume of aqueous solution (14 cc.) 1 cc. of 5 % methaemoglobin solution was run in. 5 cc. of $N/5$ HCl were added to the solution of salt and methaemoglobin, and after 10 minutes 5 cc. $N/5$ NaOH. Then the protein was treated with alkaline cyanide, ammonium sulphate ($\frac{1}{2}$ sat.), and estimated as HbCO. The results of two experiments are shown in Fig. 6.

Fig. 6. Effect of increasing concentrations of NaCl on the yield of native protein.

The behaviour of the protein when subjected to repeated denaturation is of considerable interest. To protein in standard flasks was added HCl and after 5 minutes alkali in amount insufficient to neutralise the acid. For 5 cc. HCl were added 4.5 cc. $N/5$ NaOH. This was done once, twice, and three times. Then the protein was treated with cyanide, and estimated as HbCO. The yield of native material was never found to decrease on repetition of denaturation (Table IV).

Table IV.

No. of times acidified ...	1	2	3
	Percentage of native protein		
1	62.8	68	56
2	80	73	73
3	55	58	56

When the process is repeated but the protein made alkaline between each acidification, the yield of undenatured protein is still unaffected unless the denatured protein is separated from the native material, when the native residues denatured in the normal manner [Anson and Mirsky, 1929]. This observation has been confirmed by the author.

DISCUSSION.

The experimental results described above present some remarkable features which it may be well to emphasise. The yields of "reversed" protein

seem largely independent of the temperature at which denaturation takes place; above a p_H of about 2.6 the extent of denaturation is completely independent of the p_H , and of the time of standing in acid solution. The steady state reached after a few minutes may be due to an equilibrium along the lines suggested by Anson and Mirsky, or to the whole of the protein being altered. It has been found that 100 % yield of native protein is never obtained, *i.e.* that some "irreversible" denaturation always takes place. (Wu and Lin state that they obtained complete "reversion" in one case.) It is very hard to conceive how the yield of "reversed" product can be independent of time and of repetition of acidification unless some alteration takes place in the whole of the protein. This change may be due to some denaturation in the classical sense of the word, followed by stabilisation of the remainder by the denatured product. If only part of the protein were denatured it might be expected that the residue of native material would become denatured as the protein stood in acid solution, and indeed the more so the longer it stood.

It is evident that there are two alternative views:

- (a) the equilibrium view of Anson and Mirsky;
- (b) the view that part of the protein is denatured and that this protects the remainder from further denaturation.

Now it has been shown that at acid p_H the spectrum of acid methaemoglobin can be detected, and, on reduction and aeration, that of oxyhaemoglobin. Some undenatured protein remains. Yet the amount of native material thus observed is always much smaller than that obtained by reversion. Some reversion may take place. But when the solution is made alkaline and the solution examined for alkaline methaemoglobin, the substance is seen in amount which accounts for the total native protein obtained, even though the whole protein is quantitatively precipitated on making isoelectric. Here we have direct evidence for the existence of some complex such as has been conceived in the second of the two suppositions. It may well be that in the acid solutions of protein studied the haematin group is entirely split off from the protein fraction, and that part of the free protein has become denatured, and has combined with the rest of the globin to form a complex stable to further treatment. On making alkaline it may be that the haematin fraction recombines with the globin and yields methaemoglobin, this being a rapid reaction. The alkali may further attack the complex between native and denatured protein, this being a slow reaction. Thus, if it be assumed that the complex between native and denatured material is insoluble at the isoelectric point, whether the protein is combined with haematin or not, we have a complete explanation of all the observed facts. For the alkaline methaemoglobin formed initially would thus be precipitated on neutralising at once; but after the slow dissociation had taken place between the native protein and the denatured, neutralisation would precipitate only the denatured fraction.

In the work here described the estimation of native protein falls ultimately

in every case on the formation of a conjugated protein. The validity of this procedure may be discussed with advantage. The question at issue is whether the formation of derivatives of globin, having spectra apparently identical with those of native protein, is a sufficient criterion for the existence of native globin. When a protein undergoes some secondary change, in every case it yields haemochromogen when coupled with reduced haematin [Anson and Mirsky, 1925]. Globin behaves as a typical protein in this respect. The action of acid, alkali, heat, light, shaking, urea, all give substances which have the haemochromogen spectrum. The least alteration in the protein appears to destroy its power of forming haemoglobin. This being the case it seems not improbable that were the protein concerned in these experiments altered in any fundamental way, the spectra of acid and alkaline methaemoglobin, of reduced and oxyhaemoglobin, and of carboxyhaemoglobin would not have been observed in solutions which could be entirely precipitated.

It has been suggested by Wu and Lin [1927] and by Anson and Mirsky [1929] that the sensitivity of oxyhaemoglobin to acids is greater than is that of reduced haemoglobin and they have suggested that the phenomena observed by Anson and Mirsky are due to the conjugation of the globin with the haematin. Anson and Mirsky have shown that similar effects may be obtained on acidifying solutions of globin, *i.e.* the prosthetic group may play some part in the phenomena, though not a decisive one. There is no evidence that like phenomena can be observed with other protein solutions.

SUMMARY.

1. The formation of acid haematin from acid methaemoglobin has been studied as a function of p_H .

2. It has been found that some acid methaemoglobin appears to exist at p_H values at which the protein is entirely precipitable, and that hence reversion cannot account for all the native protein obtained after this procedure.

3. Curves have been obtained for the amount of soluble native protein obtained from methaemoglobin solutions brought to different p_H values.

4. On making an acid solution of protein neutral and then dissolving in the minimum of excess alkali, a solution is obtained which is entirely reprecipitated on neutralising with acid. If the solution be allowed to stand at an alkaline p_H the solubility increases, and finally a solution is obtained which is only partially precipitable on bringing to the isoelectric point.

5. A solution of protein made alkaline after having been acid shows the spectrum of alkaline methaemoglobin irrespective of whether the protein is soluble or insoluble at the isoelectric point, *i.e.* of the time through which the solution has stood alkaline. Estimation of the alkaline methaemoglobin in such solutions yields curves which coincide with those obtained after "reversion" by Anson and Mirsky's method.

6. Besides alkaline methaemoglobin, which precipitates entirely at the isoelectric point, solutions of reduced haemoglobin, oxyhaemoglobin, and

carboxyhaemoglobin can be made, which behave in a similar manner. The properties of all these solutions alter on standing, and the dissolved substances become only partially precipitable on neutralisation.

7. It is possible to obtain solutions at acid p_H which show spectra identical with that of carboxyhaemoglobin but which precipitate entirely on neutralisation. Estimation of the amount of native protein, measured by the intensity of this spectrum, yields curves similar to those obtained when the natural protein is estimated as alkaline methaemoglobin, or when it is subjected to the reversion procedure of Anson and Mirsky.

8. The methaemoglobin solution having been brought to a p_H of, say, 2.5, it has on no occasion been found possible to obtain all the material in the soluble native state, by any process whatever.

9. The author has confirmed the findings of Anson and Mirsky that the temperature at which denaturation takes place has little effect on the yield of native material after treatment with alkali, and that the extent of denaturation is unaffected by length of treatment with acid, after the first few minutes.

10. Denatured proteins combine with haematin to yield haemochromogen when the haematin is reduced, and parahaematin when it is oxidised. Yet the insoluble protein studied shows the spectrum of native derivatives of haemoglobin, reduced and oxy-haemoglobin, carboxyhaemoglobin, and alkaline methaemoglobin, in amount sufficient to account for all the native material obtained after treatment with alkaline cyanide. This phenomenon may be explained if it be assumed that there is formed a complex between the denatured fraction and the undenatured, this complex resembling denatured protein in being insoluble at the isoelectric point, and native protein in showing the typical spectrum of undenatured derivatives of haemoglobin.

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