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ACTION OF *N*-BROMOSUCCINIMIDE ON HUMAN HEMOGLOBIN AND ITS POSSIBLE BEARING ON HEME–GLOBIN LINKAGE

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SUMMARY

A reaction of *N*-bromosuccinimide with the heme groups of hemoglobin has been studied spectrophotometrically. The reaction brings about the disappearance of characteristic absorption peaks of hemoglobin and is accompanied by the release of inorganic iron from the heme groups. Urea is obligatory for the reaction to take place at pH 4.0, while it can occur in the absence of urea at pH 7.0.

The spectrum of hemoglobin which does not show any peak in the Soret region at pH 4.0 is "normalized" in the presence of urea or sucrose at the same pH. The effect of "normalization" in 8 M urea is apparent over the pH range 3.0–4.5.

From the obligatory requirement of urea and sucrose for "normalization" of spectrum and the dependence of the release of inorganic iron on the concentration of urea, it is suggested that heme groups are "buried" within the globin at pH 4.0 and not dissociated from globin as supposed before.

INTRODUCTION

NBS, used in recent years for the modification of indole to oxindole residues and for the specific cleavage of *C*-tryptophyl peptide bonds, has found successful application in the study of proteins[1–4], viruses[5] and in the implication of indole residues in the enzymic activity of trypsin[6], chymotrypsin[7] and lysozyme[8]. In the course of a study of species differences in some mammalian hemoglobins it was observed that NBS, under conditions employed to cleave *C*-tryptophyl peptide bonds, attacked the heme groups also. The results concerning the NBS–heme reaction together with some spectral data obtained using urea and sucrose media are presented in this paper as they appear to have a bearing on the problem of heme–globin linkage. Results pertaining to species differences in *C*-tryptophyl peptide bonds will be communicated later.

MATERIALS AND METHODS

Human HbCO was crystallised according to DRABKIN[9]. The crystals were stored in cold in the mother liquor, dialysed and used when needed. The concentrations of

Abbreviations: NBS, *N*-bromosuccinimide; HbCO, carboxyhemoglobin.

HbCO solutions were measured spectrophotometrically as cyanmethemoglobin at 540 mμ using $\varepsilon_M = 46 \cdot 10^3$ for four heme groups.

NBS was prepared according to VOGEL[10]. It was twice recrystallised from water (m.p. 173–175°, decomp.) and assayed 97 % for active halogen by iodimetric titration. NBS solutions were made in water fresh before use.

Buffers

Urea–acetate buffer: Urea (Merck) was dissolved in water with sodium acetate and its pH adjusted to required value with glacial acetic acid using a Beckman model-G pH meter. The pH of such solutions, when stored, was always checked before use.

Sucrose–acetate buffer: Sucrose (British Drug Houses, Analar) was dissolved with sodium acetate and its pH was adjusted as above.

Standard iron solution was prepared to contain 0.1 mg ferric iron/ml according to HAWK *et al.*[11].

αα'-Dipyridyl

A solution of αα'-dipyridyl (British Drug Houses) was made in water (5 mg/ml).

Ascorbic acid

It was a Merck product. 0.5 M solutions were made in water fresh before use.

Spectral measurements were made using a Beckman model-DU spectrophotometer.

Estimation of tryptophan

To 3 ml solution of hemoglobin* (absorbancy 0.5–0.8 at 280 mμ in 8 M urea–0.1 M sodium acetate or sodium acetate buffer, pH 4.0). NBS (0.01 M) was added in graded increments of 10 μl. The decrease in absorbancy at 280 mμ was instantaneous. The maximal decrease in absorbancy was multiplied by the empirical factor 1.31 and the tryptophan content was calculated using 5500 as the molar extinction coefficient for tryptophan[1,2]. Corrections in absorbancies for dilution caused by the addition of NBS were made.

Spectral study of NBS–heme reaction at pH 7.0 and 4.0

To 3 ml of hemoglobin in 0.1 M sodium acetate or 8 M urea–0.1 M sodium acetate buffer (pH 4.0), NBS (0.005 M) was added in graded increments of 20 μl and the solutions were left at room temperature (24–26°) for 4 h. As will be seen later, hemoglobin exists as methemoglobin at pH 4.0 and has its absorption maximum at 400 mμ under these conditions. Hence absorbancies at this wavelength were recorded to follow the NBS–heme reaction at pH 4.0.

In another set of experiments, HbCO was dissolved in 0.05 M sodium phosphate buffer (pH 7.0) (without urea) and NBS was added as above. Absorbancies at 418 mμ were recorded as before after 4 h.

* Throughout this paper, the term "hemoglobin" is used as a general term and when distinctions are made, they are referred to in the appropriate places as the particular derivative of the protein in question.

Estimation of inorganic iron released by the action of NBS

0.1 ml of HbCO was mixed with 1 ml of urea–acetate buffer (pH 4.0) and varying amounts of NBS, up to 0.2 ml were added to each tube. The final volume in each tube was 1.4 ml. The concentration of urea was 8 M, and that of hemoglobin was $9 \cdot 10^{-5}$ M. At the highest level of NBS, the molar ratio of NBS to hemoglobin was 160. After 4 h of incubation at 25°, trichloroacetic acid was added to a final concentration of 10 % (w/v). The total volume was 3.2 ml. The contents were centrifuged. The inorganic iron in the supernatant was estimated essentially according to the method of KITZES *et al.*[12] and the details are as follows. To 1-ml aliquot of the supernatant, 0.5 ml ascorbic acid, 1 ml of 3 M sodium acetate buffer (pH 4.7), 0.5 ml $\alpha\alpha'$-dipyridyl were added in that order and the total volume was made to 5 ml with water. The colour was read in a Klett–Summerson colorimeter using a 500 filter. A blank for iron in the reagents was made by substituting water for hemoglobin. Since the supernatants had a brown colour and showed considerable absorption at 500 mμ, 1-ml aliquot of each supernatant was treated as in colour development but distilled water was substituted for dipyridyl. Its absorption was deducted from that of the corresponding test solution. The colour values were converted to iron content using a standard curve.

In experiments relating the release of iron to urea concentration at constant level of NBS (120 moles of NBS per mole of hemoglobin), each test solution had a corresponding blank to which no NBS had been added. This blank and the reagent blank were used for the correction of colour values.

Absorption spectra in varying urea concentrations

To a constant amount of hemoglobin, 8 M urea–0.1 M sodium acetate buffer (pH 4.0) was added in varying quantities and the solutions were made up to a known volume with 0.1 M sodium acetate buffer (pH 4.0) so that the solutions were around $1.6 \cdot 10^{-6}$ M in hemoglobin and 2 M, 4 M, 6 M or 8 M in urea. They were equilibrated for at least 2 h before spectral measurements. No change in absorbancy was noticed when stored for 20 h at 24–26°.

Absorption spectra in sucrose

As in the above experiment, a concentrated solution of sucrose in 0.1 M sodium acetate (pH 4.0) was added to hemoglobin and diluted with 0.1 M sodium acetate buffer of the same pH to the required volume. Sucrose solutions were either 50 or 70 %.

Spectral studies in urea medium at various pH values

Hemoglobin was dissolved in a solution of urea in potassium phthalate or sodium acetate and its pH adjusted by the addition of concentrated HCl or glacial acetic acid using a glass electrode to the required value. The volumes were adjusted to give a final concentration of 8 M urea in 0.1 M potassium phthalate or 0.1 M sodium acetate. The solutions were equilibrated for 20 h before recording the absorption at 400 mμ.

All absorbancies are expressed on a molar basis.

RESULTS

The addition of NBS to hemoglobin did not cause any decrease in absorbancy at 280 mμ until about 18 moles of NBS per mole of HbCO were added and thereafter

the extinction decreased in a linear fashion (Fig. 1), consuming about 15 moles of NBS. The maximal decrease in absorbancy corresponds to 6.2 moles of tryptophan which is in good agreement with the value calculated from the data of HILL AND CRAIG[13]. The molecular weight of hemoglobin was assumed to be 66000 in these calculations. The NBS consumed (about 18 moles) before any change in absorbancy at 280 mμ was noted in hemoglobin, and the amount of NBS consumed for the oxidation of one mole of tryptophan (2.5 moles) are in fair agreement with the reported values[3].

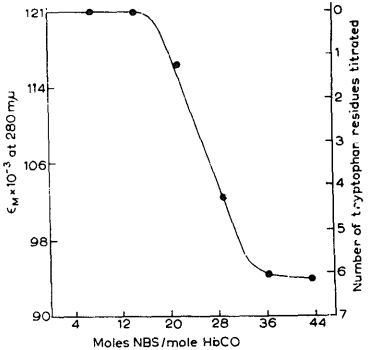

Fig. 1. "Titration" of tryptophan residues in human hemoglobin with NBS at pH 4.0 in 8 M urea–0.1 M sodium acetate medium.

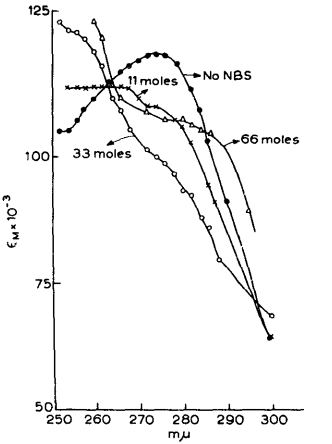

Fig. 2. Spectral changes accompanying the addition of NBS to hemoglobin at pH 4.0 in 8 M urea–0.1 M sodium acetate (ultraviolet range).

Changes in the absorption spectrum of hemoglobin after the addition of NBS

The colour of hemoglobin in 8 M urea solution appeared to be bleached when more than 33 moles of NBS were added. Spectral measurements showed interesting changes (Figs. 2, 3 and 4). The changes in the ultraviolet range are similar to those seen in other proteins[3,4] (Fig. 2) and are due to the oxidation of the indole nucleus. The spectrum of the protein with the absorption maxima at 500 and 640 mμ (Figs. 3 and 4) is similar to that of methemoglobin when no NBS is added, but the Soret maximum is at 400 mμ instead of 405–408 mμ[14]. Hence this shows that HbCO is converted to methemoglobin under these conditions and it is this form of the protein that reacts with NBS at pH 4.0. Addition of 11 and 33 moles of NBS showed a slight change in intensity but the shapes of the spectral curves remained similar to that of untreated methemoglobin. When 66 moles of NBS were added, the spectrum changed totally both intensity and characteristic shape in the Soret and the 500–700-mμ region, suggesting a total alteration in the heme structure. Addition of NBS in the absence of urea resulted in the turbidity of solutions.

"Titration" of heme-moiety with NBS

Fig. 5 shows the results of a study where the absorbancy is expressed as a function of NBS added.

At pH 4.0, in 8 M urea medium, there is no decrease in absorbancy at 400 mμ until about 30 moles are added and thereafter the drop is rapid. The reaction of heme

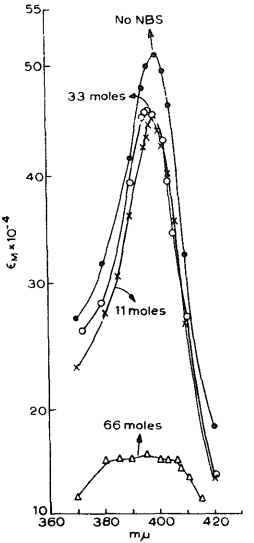

Fig. 3. Spectral changes in the Soret region accompanying the addition of NBS to hemoglobin. For conditions of experiments see Fig. 2.

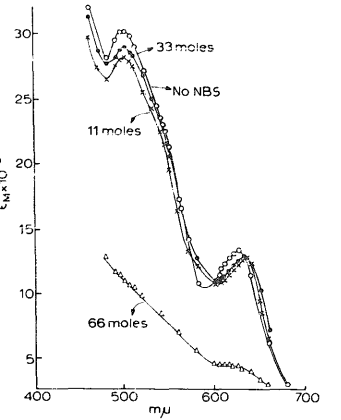

Fig. 4. Spectral changes in the 500–700-mμ region accompanying the addition of NBS to hemoglobin. Conditions as in Fig. 2.

with NBS is essentially complete when about 95 moles of NBS are added. About 60 moles of NBS are needed for a 50 % reduction in heme absorption. Omitting the (approx.) 30 moles of NBS needed before any change in the heme absorption is produced, about 30 moles of NBS per mole of hemoglobin or 7.5 moles per mole of heme group are needed for 50 % reduction in absorption.

At pH 7.0, NBS can react with heme groups in the absence of urea. There is no lag before any change in heme absorption is encountered. For 50 % reduction in heme absorption, about 160 moles of NBS per mole of hemoglobin or 40 moles of NBS per heme group are needed.

Release of inorganic iron after the addition of NBS

Addition of NBS caused inorganic iron to be released (Fig. 6). There is a corre-

lation between the amount of NBS added and the iron released. After about 108 moles of NBS are added, the iron released was about 90 % of the theoretical value; omission of urea in the buffers did not result in significant release of iron, but the solutions became turbid.

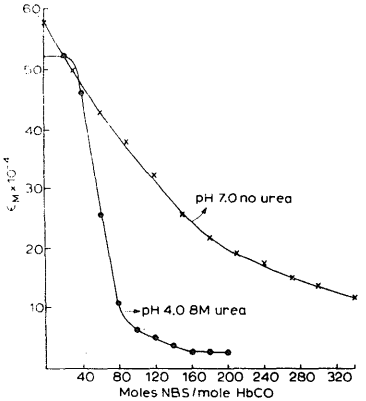

Fig. 5.

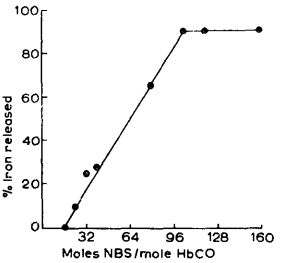

Fig. 6.

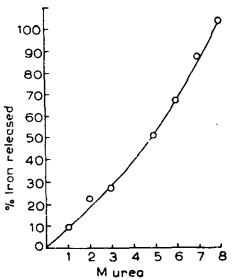

Fig. 7.

Fig. 5. Reaction of heme groups of hemoglobin with NBS.

Fig. 6. Relationship between the NBS added and inorganic iron released from hemoglobin; 8 M urea–0.1 M sodium acetate medium (pH 4.0).

Fig. 7. Release of inorganic iron from hemoglobin after the addition of NBS, as a function of concentration of urea.

Relation of urea concentration to inorganic iron released

The relationship of urea concentration to inorganic iron released is given in Fig. 7. Before the addition of NBS, the hemoglobin was equilibrated for 4 h at the required urea concentration at pH 4.0. NBS corresponding to 120 moles per mole hemoglobin was added carefully with vigorous mixing.

At pH 4.0, in the presence of buffer alone, the iron released was not significant. It increased gradually to the theoretical value at 8 M urea.

Spectral studies in urea solutions at pH 4.0

The hemoglobin solutions had low absorbancy at pH 4.0 in the absence of urea. The absorption spectrum in the Soret region was recorded at different concentrations of urea. For comparison, the spectrum of the same sample of HbCO at pH 7.0 in 0.05 M sodium phosphate buffer is shown (Fig. 8). The spectrum progressively becomes normal both in shape and intensity (ε_M for methemoglobin = $54 \cdot 10^3$ at 400 mμ in 8 M urea) as the urea concentration is raised. This observation is in good agreement

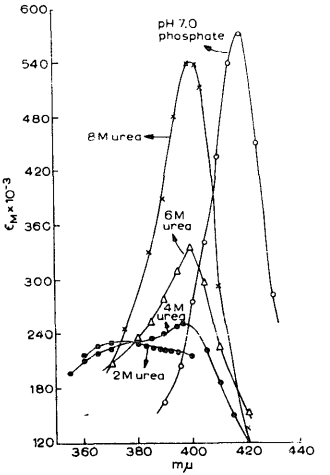

Fig. 8. Absorption spectrum of hemoglobin in the Soret region. Whereever urea was added, the solutions were maintained at pH 4.0. For details see text.

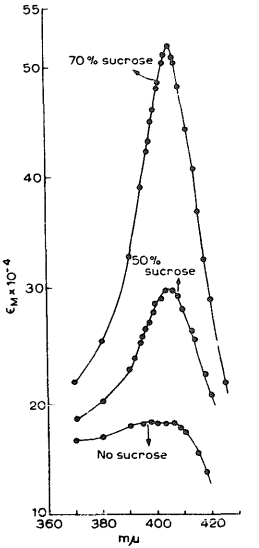

Fig. 9. Effect of sucrose on the absorption spectrum of hemoglobin at pH 4.0.

with the reported lower values[14] (ε_M for methemoglobin = $53.6 \cdot 10^3$ for four hemes) and very near to the absorption of HbCO at neutral pH ($\varepsilon_M = 62 \cdot 10^3$ at 418 mμ). One notable point is that the absorption maximum did not shift significantly between 4 and 8 M urea.

Absorption spectrum in sucrose at pH 4.0

The absorption spectrum of hemoglobin equilibrated with 50 and 70 % sucrose for 8 h at pH 4.0 is shown in Fig. 9. Here also, an effect of "normalization" of the spectrum, which is dependent on sucrose concentration, is evident. In contrast to urea, the absorption maximum did not shift significantly from 406 mμ.

Changes in extinction at different pH values in 8 M urea medium

Fig. 10 shows a plot of intensity at 400 mμ against pH. All solutions were in 8 M urea. The maximal intensity is produced at pH 3.8 and the curve falls steeply after pH 4.0. This effect is discussed later.

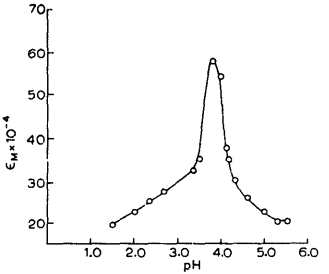

Fig. 10. Absorption at 400 mμ as a function of pH in 8 M urea medium.

DISCUSSION

The present studies are concerned with the behaviour of hemoglobin at pH 4.0, acid to its isoelectric point of pH 6.8, and where the absorption spectrum does not exhibit its normal features with respect to shape and intensity.

The NBS–hemoglobin reaction can be divided into three stages: (a) reaction with up to 18 moles of NBS, probably involving –SH groups[3], and not producing a change in extinction at 280 mμ (Fig. 1); (b) the NBS–tryptophan reaction with the further addition of about 15 moles of NBS, resulting in the "titration" of all the six tryptophan residues; and (c) the NBS–heme reaction, which causes a total change in the spectrum of the heme group, but which occurs only in the presence of urea (Figs. 3 and 4) when excess to 33 moles of NBS are added.

Addition of around 11 and 33 moles of NBS per mole of hemoglobin at pH 4.0, corresponding respectively to completion of stage (a) above in the oxidation of –SH groups and stage (b) at which both all –SH and tryptophan residues are oxidised, do not cause any change in the shape of the spectrum in the visible region, but cause only a slight change in intensity. It would appear that there is no drastic change in the absorption spectrum and hence, the structure of heme groups would seem almost unaltered at these levels of NBS. Hence it is clear that under these conditions, the heme groups cannot be attacked before the reactive groups in the protein moiety are attacked. In fact, by a proper choice of reaction conditions, particularly by using dilute NBS solutions, it is possible to keep the heme groups unaltered as judged by the spectrum and completely oxidize the tryptophans. Further, the linear drop in intensity at 280 mμ (Fig. 1) and the lag of about 30 moles of NBS before any decrease in absorbancy at 400 mμ is encountered (Fig. 5) support the above contention. But, the nature of the spectrum after the addition of about 66 moles of NBS indicated a total alteration in the structure of heme groups.

The chelation of the iron atom is due to the highly conjugated porphyrin system;

so is the characteristic Soret absorption. Hence a change in the Soret absorption must indicate a change in these specialised ring systems. As expected, the observed change in the spectrum and of structure is accompanied by the release of inorganic iron. The release of inorganic iron was due to the action of NBS as shown by the progressive increase of iron released on the addition of increasing amounts of NBS (Fig. 6) and which is essentially complete in 8 M urea (Fig. 7). The heme reaction essentially required a total of about 95 moles of NBS (Fig. 5) and the release of iron required a total of about 108 moles (Fig. 6). Although no appreciable alterations in the visible spectrum of hemoglobin oxidized with up to 33 moles of NBS are to be observed, release of about 15 % of inorganic iron could be demonstrated under these conditions. This seems contrary to the earlier observations, *i e*, the heme groups are not attacked until about 33 moles of NBS are added. The release of iron at this level of NBS may be due to the relatively high concentration of NBS (0.1 M) used in contrast to the low concentration of NBS—0.01 M and 0.005 M—employed in spectral studies (Figs. 3 and 4) and NBS–heme "titration" (Fig. 5). Thus, while the decrease in extinction at 400 mμ and release of iron may not be synchromous, it is possible, however, that the different experimental conditions, particularly the large difference in concentrations of NBS solutions employed and the small volume (1.4 ml) of the reaction mixture employed in iron release studies account for the difference between the values of 95 and 108 moles of NBS needed, respectively, to cause complete alteration in the visible spectrum and for complete release of iron.

An important point that emerged from the above experiments is that urea is obligatory for both the reactions, *i.e.*, for altering the spectrum and for the release of iron. Another notable point is that the fall in extinction at 400 mμ is observed when the first two stages of the reaction are complete. The conditions for this experiment are the mildest (Fig. 5) and hence, there is no change in absorbancy at 400 mμ before about 30 moles of NBS are added. These observations suggest that heme is "buried" within the globin at pH 4.0 and NBS can react with it only when the protein is "denatured" by urea.

NBS can react with heme groups at pH 7.0 also, but the presence of urea is not needed. Under these conditions there is no lag as NBS–tryptophan reactions take place instantaneously at pH 4.0, while the reaction occurs only slowly at higher pH values. PERUTZ[15] has shown that heme groups of oxyhemoglobin at pH 7.0 lie on the surface of the peptide chains. It is also known that small molecules like nitrosobenzene[16] readily react with heme groups in the absence of agents like urea at neutral pH. These studies serve to show a distinct difference in the availability of heme groups to NBS at pH 4.0 from that encountered at pH 7.0. The heme groups which are unreactive towards NBS at pH 4.0 become "normalized" and fully reactive at the same pH when 8 M urea is also present.

Preliminary experiments on the reaction of crystalline hemin in 50% methanol–sodium acetate buffer (pH 4.0) with NBS showed that the fall in extinction at 400 mμ was linear with the amount of NBS added. The colourless product obtained had no characteristic absorption maximum in the ultraviolet range and did not give any colour reaction with *p*-dimethylaminobenzaldehyde. Hence, it is unlikely that the reaction product is a porphyrin or a pyrrole in nature.

The low absorbancy of hemoglobin at acid pH, below 5.3, is interpreted as being due to the dissociation of the heme from globin[17] and that a group of pK 4.7 is involved

in heme binding. The absorbancy of hemoglobin at pH 4.0 was high ($\varepsilon_M = 54 \cdot 10^4$) in the presence of 8 M urea, close to that of HbCO at pH 7.0. This appeared to be the effect of urea and indeed increasing concentrations of urea (2–8 M) progressively "normalized" the spectrum, so that at pH 4.0 in 8 M urea, both the shape and extinction approached very closely those found for HbCO (Fig. 8). This may not be due to any specific effect of urea, since sucrose, known to produce "molecular perturbations"[18] also "normalized" the spectrum. The effects of sucrose and urea are not quite identical as urea produces a shift in λ_{max} while sucrose does not. The normal intensity at the Soret region is often taken as the criterion for the intactness of the heme–globin linkage and if this is so, it may be inferred that heme is combined to globin in a more or less normal manner, also at pH 4.0 in presence of 8 M urea or 70 % sucrose.

Though urea is necessary for the "normalization" of spectrum, the heme–globin linkage seems to be intact even in the absence of urea at pH 4.0 as inorganic iron is not released unless urea is present in the medium.

STEINHARDT AND ZAISER[19] have pointed out that heme may be combined with globin at low pH, but in a way different from that obtained at neutral pH. The present studies provide experimental evidence for their statement. Heme–globin linkage appears to be intact, but it is not identical to that obtained at neutral pH, since the presence of urea or sucrose is obligatory for the linkage to exhibit its normal spectral properties and in the case of urea to exhibit the normal reactivity of the heme towards NBS. Evidence in the literature favours the participation of a histidine residue in heme–globin linkage[15,20,21] but WYMAN[20] mentions that other types of linkages may be operative in denatured hemoglobin.

The low absorbancy at pH 4.0, attributed to the dissociation of iron–protein linkage[17], may also be due to the change in conformation around the heme moiety brought about by the breakdown of hemoglobin into its subunits[22]. It is conceivable that such a change in conformation may also render the heme group unavailable for attack by NBS in the absence of urea.

The "normalization" of heme absorption in the presence of urea is apparent over the pH range 3.0–4.5, pH 3.8 being effective for complete "normalization" (Fig. 10). It was possible to detect this effect as NBS-tryptophan reactions are generally carried out around pH 4.0. This "normalization" perhaps arises because the conformation of the globin under these conditions is of such a nature which permits the iron atom and the protein ligands to cooperate to form a linkage which may be much the same as in hemoglobin at neutral pH.

It is well known that peptide chains of globin show separation at pH 4.0 (see ref. 22) and it may be that 8 M urea causes reassociation of the chains forming a globin in which heme is combined in the normal manner. In this connection, it would be of interest to examine the molecular weight of hemoglobin at pH 4.0 in presence of 8 M urea and this is currently under investigation.

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