THE DETERMINATION OF REDUCING GROUPS WITH PORPHYRINDIN, WITH SPECIAL REFERENCE TO EGG ALBUMIN*

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Kuhn and Desnuelle (1) have introduced the use of the blue dye, porphyrindin, for the determination of sulfhydryl groups in proteins. This dye has a very high potential (+0.57 volt at pH 7, referred to the normal hydrogen electrode (2)), and oxidizes cysteine stoichiometrically to cystine (1). They determined the —SH groups in heat-denatured egg albumin by adding increasing amounts of porphyrindin at 0° and pH 7.2 to a series of Thunberg tubes containing the same amount of protein, and noted in which the dye was completely decolorized. The —SH groups corresponded to 0.58 per cent cysteine (Greenstein (3) found 0.50 per cent by this method). In repeating Kuhn's experiments, we were not able to obtain a satisfactory end-point. There was a fairly clear demarcation between the colorless tubes and the first incompletely decolorized one, but in the subsequent tubes, more and more dye was reduced.

Although the determination of the —SH groups in heat-denatured egg albumin gave results in agreement with those of Todrick and Walker (4) by 2,6-dichlorophenol indophenol oxidation and those of Mirsky and Anson (5) by oxidation with cystine, Kuhn and Desnuelle recognize that other oxidizable groups may be present in proteins.

Greenstein (3, 6) has extended the use of porphyrindin to the determination of —SH groups liberated in proteins by the action of urea, guanidine, and their derivatives. His technique was

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somewhat different; he first treated the proteins with urea or guanidine hydrochloride at 25°, then titrated with a porphyrindin solution at this temperature. The —SH groups liberated from egg albumin in the presence of urea or of guanidine hydrochloride amounted to 1.00 and 1.24 per cent of cysteine, respectively.

The experiments reported below show that porphyrindin oxidizes not only —SH groups, but under certain conditions tyrosine also. With the technique here described a cysteine content of 1.35 per cent was found for heat-denatured egg albumin. In native egg albumin the phenolic and —SH groups are either absent or inaccessible to oxidation. Heat denaturation is associated with the liberation of —SH groups, while dispersion by guanidine hydrochloride is accompanied by the appearance of reactive —SH groups as well as reactive phenolic groups.

EXPERIMENTAL

Porphyrindin was synthesized as described by Kuhn and Franke (2); the condensation of HCN with acetoxime was carried out according to Porter and Hellerman (7). Recrystallization of porphyrindin[1] from pyridine was unsuccessful.

Standardization of Porphyrindin Solutions—Kuhn establishes the titer of porphyrindin solutions with *pure* cysteine in a series of Thunberg tubes at 0° and pH 7.2. This procedure gives satisfactory results. By Greenstein's method, a standard solution of cysteine hydrochloride, neutralized with ammonia to pH 7, is titrated with porphyrindin at 25°. We find that under these conditions both solutions deteriorate at 25°. However, the direct titration can be accomplished in the following manner. Porphyrindin (5 to 7 mg. per 10 cc.) is dissolved in 0.2 M phosphate buffer, pH 7.2, at 0°, and the solution filtered. 2 cc. of the porphyrindin solution are titrated, in a test-tube kept at 0°, with an aqueous solution of metal-free cysteine hydrochloride (about 50 mg. per 100 cc.) to the disappearance of the blue color.

[1] An absorption maximum at 653 mμ has been reported for the dye (2). Using the Pulfrich photometer, we find that Zeiss Filter S-57 gives the highest extinction coefficient (determined at room temperature with freshly prepared solutions at pH 7.2 and 9). The same result was obtained with a number of pure preparations (one kindly furnished by Dr. J. P. Greenstein).

The concentration of the cysteine hydrochloride solution is established photometrically (8) or iodometrically ((9), *cf.* (10)). 1 mg. of cysteine is equivalent to 1.16 mg. of porphyrindin.

Porphyrindin may also be standardized iodometrically. 2 cc. of cold porphyrindin solution, prepared as described above, are added to 3 cc. of a solution containing 0.3 gm. of KI in 0.5 N HCl, the flask being whirled during the addition.[2] Iodine is immediately liberated and titrated with 0.01 N thiosulfate. 1 cc. of 0.01 N thiosulfate is equivalent to 1.40 mg. of porphyrindin. A standardized porphyrindin solution may be used to establish the titer of cysteine solutions.

Stability of Porphyrindin Solutions—The crystalline dye is not entirely stable (1); it is best kept at low temperature. In our experience, the deterioration of porphyrindin solutions at 0° amounted to 3, 5, and 9 per cent in 1, 2, and 4 hours, respectively. At 25° deterioration occurred at the rate of about 0.5 per cent per minute. We have therefore carried out all experiments with porphyrindin at 0°, determining the titer of the porphyrindin solutions before and after each experiment.

Reaction of Amino Acids with Porphyrindin—Sulfhydryl compounds, such as cysteine and glutathione, are stoichiometrically oxidized to the corresponding —S—S— compounds, even when an excess of porphyrindin is used. Guanidine hydrochloride has no effect on the reaction. Cysteine (0.86 mg.) was treated for 15 minutes at 0° in an evacuated[3] Thunberg tube, in 6.5 cc. of solution containing 2 cc. of 0.5 M phosphate buffer of pH 7.2, with 2.08 mg. of porphyrindin.[4] The excess porphyrindin was then titrated with a standardized solution of cysteine hydrochloride; 1.10 mg. of the porphyrindin were reduced (calculated 1.06 mg.). In a parallel experiment with the addition of 3 gm. of guanidine hydrochloride at pH 7.2 the same amount (1.10 mg.) of porphyrindin was reduced. Glutathione (2.80 mg.) was treated under the same conditions with 3.00 mg. of porphyrindin; 1.31

[2] Although porphyrindin is destroyed on short contact with acid, the liberation of iodine from HI is quantitative under the conditions described.

[3] The tubes were alternately evacuated and filled with nitrogen several times, then left evacuated up to the time of titration.

[4] The oxidation of cysteine is complete in 30 to 60 seconds, even if no excess of porphyrindin is added.

mg. of porphyrindin were reduced (calculated 1.28 mg.). In a parallel experiment with 3 gm. of neutral guanidine hydrochloride, 1.29 mg. of porphyrindin were reduced.

The *l* forms of cystine,[5] cysteic acid, tryptophane, hydroxyproline, and histidine, the *dl* forms of methionine, serine, phenylalanine, and threonine did not reduce porphyrindin when approximately 1 to 10 mg. of the amino acid and 1 to 2 mg. of porphyrindin in a total volume of about 3 cc. were allowed to stand at 0° and pH 7.2 for 15 minutes in an evacuated[3] Thunberg tube.

Tyrosine is oxidized by porphyrindin at 0° and pH 7.2 with the formation of a pink[6] oxidation product. It can be seen from Fig. 1 that the oxidation depends on the reaction time (Curve 1) and on the amount of porphyrindin present (Curve 2). The addition of 3 gm. of guanidine hydrochloride at pH 7.2 to the reaction mixture has no effect on the oxidation of tyrosine by porphyrindin (the triangles on Curve 2 represent experiments with added guanidine hydrochloride).

Native Egg Albumin and Porphyrindin—Native egg albumin does not decolorize porphyrindin solutions (1, 5), even in the presence of a large excess of the dye. There is no reaction when 34.3 mg. (*i.e.*, 1 micromole on the basis of a molecular weight of 34, 300 (10, 11)) of native egg albumin, six times recrystallized and then dialyzed, are allowed to stand for 10 minutes at 0° and pH 7.2 in a volume of 3 cc. with 2 mg. of porphyrindin (14 microequivalents). However, if such egg albumin-porphyrindin solutions are shaken continuously for 10 minutes at 0° in evacuated[3] Thunberg tubes, a small amount of porphyrindin is reduced, presumably by surface-denatured egg albumin.

[5] Cystine and cysteic acid are extremely resistant to oxidation by porphyrindin at 0° and pH 7.2. No reduction of porphyrindin occurred when 2.5 microequivalents of these substances were treated in a volume of 2.5 cc. with approximately 25 microequivalents of porphyrindin for 2 hours. On the other hand, the sulfinic acid corresponding to cysteine and the disulfoxide of cystine are oxidized by porphyrindin at 0° and pH 7.2, although at a much slower rate than —SH compounds. Several hours were required for the reactions to be completed, even when a large excess of porphyrindin was used. A more detailed study of these reactions will be presented in a later publication, in which the possible presence of the sulfinic acid in denatured egg albumin will be considered.

[6] With very small amounts of porphyrindin a yellow color is obtained.

Oxidation of Heat-Denatured Egg Albumin by Porphyrindin—Crystalline egg albumin (34.3 mg. in 3 cc. of phosphate buffer of pH 7.2, 0.5 M) was denatured by heating in an evacuated[3] Thunberg tube for 10 minutes in a boiling water bath with vigorous shaking[7] (1). After being cooled to 0°, the tube was filled with nitrogen and a definite amount of porphyrindin was added, either as solid or dissolved in phosphate buffer. The volume was adjusted with buffer to about 5 cc.; the tube was evacuated[3] again and shaken for 10 minutes in ice. The excess porphyrindin was then titrated at 0° with cysteine hydrochloride solution.

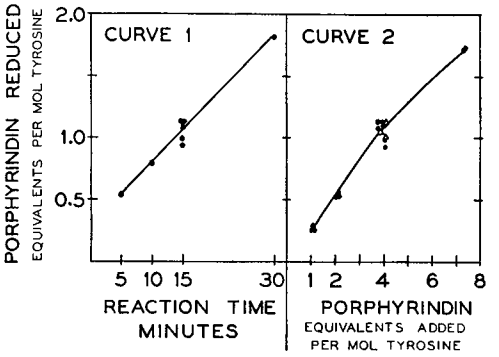

FIG. 1. Reaction of tyrosine with porphyrindin. Curve 1, reaction of 0.5 mg. of tyrosine with 1.54 mg. of porphyrindin (4 equivalents per mole), volume 6.5 cc., 0.2 M phosphate buffer, pH 7.2, 0°; Curve 2, reaction of 0.5 mg. of tyrosine with varying amounts of porphyrindin, reaction time 15 minutes, volume 6.5 cc., 0.2 M phosphate buffer, pH 7.2, 0°; the triangles represent experiments with 3 gm. of guanidine hydrochloride added.

In all the experiments with heat-denatured egg albumin (Fig. 2), even with the largest amount of porphyrindin, there was no pink or yellow color in the protein suspensions after the excess of the dye had been decolorized by cysteine. This indicates that no tyrosine was oxidized. The —SH groups determined from the amount of porphyrindin which was completely decolorized (Kuhn's "end-point") correspond to 0.43 per cent cysteine or to a transfer of 1.2 atoms of hydrogen per mole of protein. As the amount of porphyrindin in the reaction mixture is increased, more is reduced. When 10 to 27 microequivalents of porphyrindin per micromole of egg albumin are added, the apparent cysteine

[7] Reliable results are obtained only with fairly uniform protein suspensions.

content is approximately constant at 1.35 per cent; *i.e.*, a transfer of about 4 hydrogen atoms per mole.

When 34.3 mg. of egg albumin preparation, which had been heat-denatured, dried, and kept without special precautions (Preparation C; *cf.* (10, 12)), were suspended in 3 cc. of phosphate buffer, pH 7.2, and shaken[3] for 15 minutes at 0° with 39 microequivalents of porphyrindin, no porphyrindin was reduced, although the preparation yielded cysteine on HCl hydrolysis.[8] This negative result may perhaps be attributed to autoxidation of the denatured protein.

Oxidation of Egg Albumin by Porphyrindin in Presence of Guanidine Hydrochloride—Greenstein (3) treated native egg al-

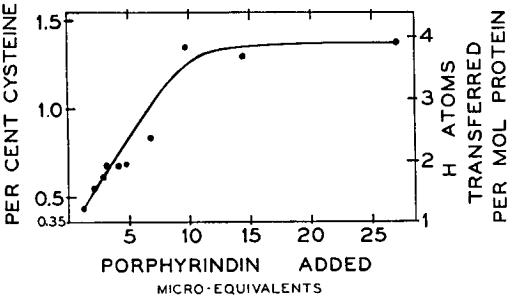

FIG. 2. Reaction of heat-denatured egg albumin with varying amounts of porphyrindin. 34.3 mg. of egg albumin (1 micromole) in 4 to 5 cc. of 0.2 M phosphate buffer, pH 7.2, 10 minutes at 0°.

bumin solutions in open tubes at 25° and pH 7 with guanidine hydrochloride for 45 minutes, then titrated with a porphyrindin solution to the appearance of the blue color. A maximum of titratable groups, corresponding to 1.28 per cent cysteine, was reached after treatment with 0.8 gm. of guanidine hydrochloride per 2 cc. of protein solution.

All of our experiments were carried out in Thunberg tubes in an inert atmosphere.[3] In the first series of experiments (Fig. 3, experiments represented by triangles) 34.3 mg. of egg albumin in 1 cc. of phosphate buffer of pH 7.2 were treated with 3 gm. of guanidine hydrochloride for 1 hour at 25°. The tubes were

[8] Hydrolysis of this egg albumin preparation with 6 N HCl yielded 0.25 per cent cysteine (12). A new method of hydrolysis, with HCl containing large amounts of urea, yielded 0.5 per cent cysteine (unpublished experiments of Brand and Kassell).

cooled to 0°, varying amounts of porphyrindin in 1 to 2 cc. of solution were added, and after 10 minutes at 0° the excess porphyrindin was titrated with cysteine hydrochloride. In the experiments represented by dots in Fig. 3, the same amount of egg albumin and guanidine hydrochloride in 2 to 3 cc. of buffer was treated at once with graded amounts of porphyrindin at 0° for 15 minutes; then the excess porphyrindin was titrated with cysteine hydrochloride.

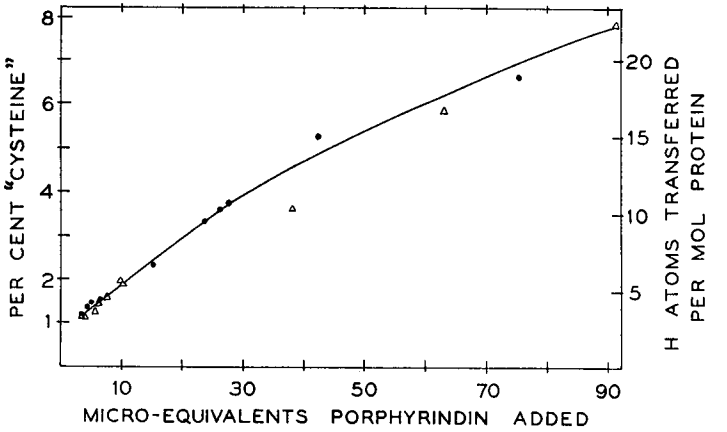

FIG. 3. Reaction of egg albumin with porphyrindin in the presence of guanidine hydrochloride. Dots, reaction of 34.3 mg. of native egg albumin (1 micromole) plus 3 gm. of (neutralized) guanidine hydrochloride with varying amounts of porphyrindin, volume 4 to 5 cc., 0.2 M phosphate buffer, pH 7.2, 15 minutes at 0°; triangles, 34.3 mg. of native egg albumin in 1 cc. of 0.5 M phosphate buffer, pH 7.2, first treated for 1 hour at 25° in an inert atmosphere with 3 gm. of (neutralized) guanidine hydrochloride. After cooling to 0° varying amounts of porphyrindin in 1 to 2 cc. of diluted buffer were added and allowed to react 10 minutes at 0°.

In both experiments the shape of the curve indicates that the reaction was not complete, even when the amount of porphyrindin reduced corresponded to a "cysteine" content of about 8 per cent, or to a transfer of 22 atoms of hydrogen per mole of egg albumin. However, when titration was completed, all the tubes which had been treated with more than 10 microequivalents of porphyrindin showed the pink color[9] characteristic of the oxidation product of tyrosine.

[9] Balls and Lineweaver (13) noted the production of a red color when a large quantity of porphyrindin was added to papain.

The heat-denatured, oxidized egg albumin (Preparation C), which did not react with porphyrindin (*cf.* above), was shaken at 0° and pH 7.2 for 15 minutes with 3 gm. of guanidine hydrochloride and varying amounts of porphyrindin. Under these conditions 1 micromole of the egg albumin reacted with porphyrindin equivalent to the transfer of 5 and 7 hydrogen atoms per mole when 16 and 30 microequivalents of porphyrindin, respectively, were added. The protein did not seem to dissolve, and after titration of the excess porphyrindin, the protein particles were distinctly yellow.[6] It therefore seems likely that part of the porphyrindin was reduced by tyrosine.

SUMMARY

1. Porphyrindin solutions are relatively unstable above 0°.

2. The oxidation of cysteine by porphyrindin does not go beyond the —S—S— stage at 0° and pH 7.2.

3. Tyrosine is oxidized by porphyrindin at 0° and pH 7.2 with the formation of a pink oxidation product.

4. Native egg albumin is stable towards porphyrindin. In heat-denatured egg albumin, —SH groups are oxidized by porphyrindin. In egg albumin dispersed by guanidine hydrochloride, —SH groups as well as phenolic groups are oxidized by porphyrindin.

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