A modified method for the purification of erythrocuprein

A copper-containing protein named haemocuprein was isolated from the erythrocytes of different mammals by MANN AND KEILIN[1]. The preparation from ox-blood contained 0.34 % Cu, and there was evidence that the protein was obtained in a highly purified state. The authors also tried to purify a corresponding protein from human erythrocytes and obtained a preparation with a copper content of 0.21 %. A copper protein of human erythrocytes was recently highly purified and characterized[2,3] and it was shown to have the same copper content and probably the same molecular weight as ox-blood haemocuprein. The authors[2] suggested the name erythrocuprein for the human-erythrocyte protein, and they showed that erythrocuprein seems to account for all or nearly all of the copper in the erythrocytes.

The preparation method for erythrocuprein described by MARKOWITZ *et al.*[2] is to a great part identical with that used by MANN AND KEILIN[1]. KIMMEL *et al.*[3] simplified this method by replacing some of the later purification steps by an electrophoretic fractionation. As in the procedure of KIMMEL *et al.*[3], the present method of purification retains steps 1–3 in the method of MARKOWITZ *et al.*[2] but the further purification is achieved by chromatography on the chloride form of DEAE-cellulose[4]. The erythrocuprein prepared in this manner shows a slightly higher copper content than the one obtained previously and appears homogeneous in the ultracentrifuge and in free electrophoresis.

The starting material consists of human blood stored for 1–4 weeks at 4° in acid citrate–dextrose solution. Typical runs were performed with 6 l blood. All buffers and salt solutions were made from reagent-grade chemicals in deionized water.

After removal of the hemoglobin by treatment with the chloroform–ethanol mixture, the remaining protein solution was treated with aq. lead acetate, and the precipitate was extracted with 0.33 *M* phosphate buffer, pH 6.0 (for details see MARKOWITZ *et al.*[2]). The phosphate solution was dialyzed against deionized water at 4° until free from phosphate. To the dialyzed solution, Tris–HCl buffer (pH 7.4) was then added to a final concentration of 0.005 *M*. The column used was jacketed and cooled with tap water (8°). It was packed with DEAE-cellulose (from Distillation Products Industries, Rochester 3, N.Y., U.S.A.) and equilibrated with 0.005 *M* Tris–HCl buffer (pH 7.4). The protein solution was then filtered through the column. Approx. 65 % (varying between 50 and 85 %) of the protein material ran through the column. Erythrocuprein is adsorbed to the column and forms an easily detectable light greenish zone. At the top of the column a yellowish-brown zone is usually formed. After washing the column with 200–300 ml 0.005 *M* Tris–HCl (pH 7.4) the elution is performed by means of a concentration gradient (for details, see the caption to Fig. 1). In the elution pattern (Fig. 1) erythrocuprein was localized through its color. The peak behind the erythrocuprein is due to a yellowish-brown protein. It shows a very high H_2O_2-decomposing activity and seems, at least partly, to consist of catalase.

The pooled fractions of erythrocuprein from the chromatographic experiment was extensively dialyzed against deionized water and lyophilized. Usually several different chromatographic preparations of erythrocuprein were pooled and further purified by rechromatography (column dimensions, 1.3 × 18 cm; mixing chamber,

Abbreviations: DEAE-, diethylaminoethyl-; Tris, tris(hydroxymethyl)aminomethane.

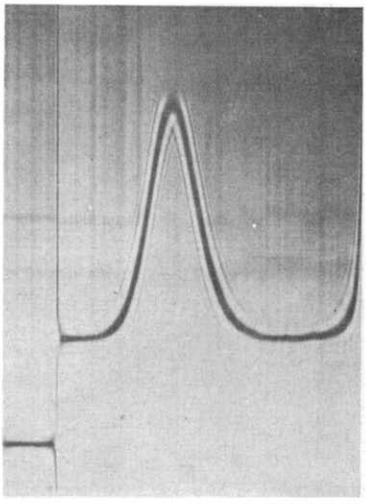

Fig. 2

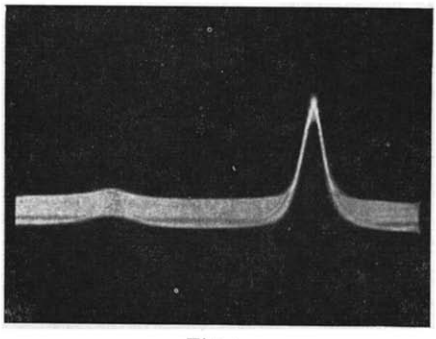

Fig. 3

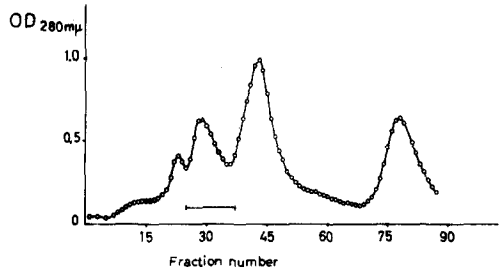

Fig. 1

Fig. 1. Typical elution pattern from DEAE-chromatography for preparation of erythrocuprein. The protein concentration (OD_{280} $m\mu$) is expressed as the absorbancy at 280 mμ in a 1-cm cuvette. Column dimensions, 2.0 × 20 cm; concentration gradient (0.005 *M* Tris–HCl, pH 7.4) ⟶ (0.05 *M* Tris–HCl, pH 7.4 + 0.166 *M* NaCl); mixing chamber, 1000 ml; fraction volume, 8.5 ml. Fractions number 25–37 were pooled and purified further by rechromatography.

Fig. 2. Sedimentation pattern of erythrocuprein. Buffer, sodium phosphate, pH 6.5, I 0.05, containing also 0.10 *M* NaCl. The exposure was taken 106 min after reaching 59,780 rev./min.

Fig. 3. Free electrophoresis of erythrocuprein in sodium phosphate buffer, pH 7.0, I 0.10. Ascending pattern after 184 min. Potential gradient, 7.74 V/cm. Protein concentration, 0.5 %. The small peak to the left in the figure is a salt gradient.

400 ml). Erythrocuprein prepared in this way was used for the homogeneity tests (Figs. 2 and 3) and for the determination of elementary composition.

Some experiments with purification by zone electrophoresis in cellulose columns[5,6] (sodium phosphate buffer, pH 6.5, I 0.05) were not successful, since this procedure seems to cause partial denaturation of the protein.

The visible absorption spectrum of the protein was investigated by means of an automatic recording spectrophotometer (Zeiss PRQ 20A). The spectrum was very similar to that given by MARKOWITZ *et al.*[2]. The minimum in the absorption curve is situated at 530 ± 5 mμ, which seems to be the same as obtained previously. The maximum (670 ± 5 mμ), however, differs somewhat from the value given by MARKOWITZ *et al.*[2]. The difference may be due to difficulties in the exact determination of the point of maximum absorption in the broad and flat absorption peak. This is supported by the fact that the erythrocuprein here has been found to give the same electron-spin-resonance spectrum[7] as that prepared by KIMMEL *et al.*[3].

The protein which was to be analyzed was dissolved in deionized water and precipitated with cold absolute ethanol (—30°). The precipitate was washed with absolute ethanol, acetone and ether and air-dried at room temperature. A sample of

erythrocuprein prepared in this manner contained 16.88 % N and 1.13 % S (corrected for moisture content in the sample). The copper content was determined by the dithizone method[8] on an aqueous solution of the protein from which the copper was released and the protein precipitated by trichloroacetic acid[9]. The protein concentration was determined by micro Kjeldahl analysis using the nitrogen content of 16.88 %. The copper content was found to be 0.38 (0.376) %.

The elementary composition of erythrocuprein prepared in the described manner seems to be in rather good agreement with the values given previously[2,3]. The molecular weight calculated from the copper content (two copper atoms per protein molecule) is 33,800. This is in good agreement with the value calculated from the amino acid composition[3].

On the basis of the comparisons made above there seems to be no doubts that the protein prepared here is identical to that prepared earlier[2,3] but the present procedure may be somewhat more convenient and it also results in a product showing a higher degree of homogeneity.

This investigation has been supported by grants from the Swedish Medical Research Council, the Swedish Natural Science Research Council and the Division of General Medical Sciences, U.S. Public Health Service (RG-6542).

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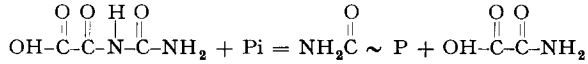
Received August 30th, 1960

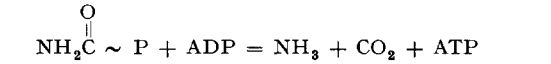
Biochim. Biophys. Acta, 45 (1960) 387–389

Phosphorolysis of carbamyl oxamic acid

Decomposition of the ureido group of carbamyl oxamate by *Streptococcus allantoicus*[1] has been found to be an energy-yielding reaction leading to the formation of ATP. The proposed mechanism for this transformation is as follows:



(1)



(2)

Abbreviations: Pi, inorganic phosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; Tris, tris(hydroxymethyl)aminomethane.