Reduction of Biological Substances by Water-Soluble Phosphines: Gamma-Globulin (IgG)

WOLFRAM[1] demonstrated that a water-soluble tertiary phosphine, *Tris*-hydroxymethyl phosphine, was capable of reducing the disulfide bonds of keratin. KIRSCHENBAUM, BAKKER, THIEL and PLOTCH[2] have used *Tris*-hydroxymethyl phosphine to activate papain, a sulfhydryl-dependent enzyme. The ability of tertiary phosphines to replace sulfhydryl compounds in these reactions suggested that these phosphines might serve as reagents for the reductive cleavage of immunoglobulins. The present study compares the reductive cleavage of human gamma-globulin (IgG) by 2 tertiary phosphines, *Tris*-hydroxymethyl phosphine and *Tris*-carboxyethyl phosphine, with the cleavage produced by a commonly used sulfhydryl compound, 2-mercaptoethanol.

Reduction of human gamma-globulin (Cohn F II) with various dilutions of mercaptoethanol and fractionation of the alkylated products in propionic acid gel filtration through Sephadex G-75 was carried out as described by FLEISCHMAN, PAIN and PORTER[3]. Reduction with *Tris*-hydroxymethyl phosphine[4] and *Tris*-carboxyethyl phosphine[5] was carried out in a similar manner. The final concentrations of the reaction mixtures were 0.5% in protein and either $0.2\,M$, $5 \times 10^{-2}\,M$, $1.25 \times 10^{-2}\,M$, $3.1 \times 10^{-3}\,M$, $1.5 \times 10^{-3}\,M$, $7.8 \times 10^{-4}\,M$, $3.9 \times 10^{-4}\,M$ or $9 \times 10^{-5}\,M$ of each reducing agent. The solutions were incubated with the reducing agents for 3 h at 37 °C before incubation with iodoacetamide for 20 h at 4 °C. Starch gel electrophoresis in $8\,M$ urea and formate buffer, pH 3.0, for 20 h at 4 °C was carried out as described by EDELMAN and POULIK[6]. A pool of human sera from patients with rheumatoid arthritis and having a latex agglutination titer of greater than 1/10,000 was used to test the effect of the phosphines on the rheumatoid factors. Reaction mixtures were prepared that were 1/10 dilutions of serum and contained either mercaptoethanol or *Tris*-hydroxymethyl phosphine in concentrations ranging from $0.1\,M$ to $1.9 \times 10^{-4}\,M$. After 24 h the contents of each tube were equally divided. One portion was continuously dialyzed for 104 h against pH 7.4 phosphate buffer. The other portion was dialyzed against pH 7.4 phosphate buffer for 2 h, then against 2 changes of 100 volumes of $0.02\,M$ iodoacetamide in phosphate buffer pH 7.4 for 6 h and finally against phosphate buffer alone for 96 h. The sera were diluted 1/10 with glycine buffer, pH 8.0, and tested for rheumatoid factor activity using the Hyland latex slide agglutination test. Agar gel diffusion studies in 1% ionagar were performed as described by OUCHTERLONY[7].

Reduction and alkylation of gamma-globulin resulted in the separation of 2 components from the unreduced gamma-globulin (Figure 1). Variable amounts of unreduced protein migrated as the slowest band. When compared by acid-urea-starch gel electrophoresis the mobilities of the 2 main components and the slowest component were similar for all 3 reducing agents (Figure 1).

Separation of the 2 components of the reduced and alkylated F II was achieved by G-75 sephadex gel filtration in $1\,M$ propionic acid. The products of reduction by mercaptoethanol and phosphines were recovered in comparable elution volumes. Two major peaks, A and B were seen (Figure 2). The optimum yield of the 2 components was achieved when $3.1 \times 10^{-3}\,M$ *Tris*-hydroxymethyl phosphine and $5 \times 10^{-2}\,M$ *Tris*-carboxyethyl phosphine were used. Less fraction B was recovered when $0.2\,M$ 2-mercaptoethanol was used as a reducing agent, but this was the most efficient concentration of all those utilized in the current study. Fraction B, when dialyzed against 0.9% NaCl solution, remained clear. Fraction A, however, consistently precipitated under these conditions, and as noted by FLEISCHMAN, PAIN and PORTER[3] migrated as the slower component on acid-urea-starch gel electrophoresis (Figure 1). In agar gel diffusion studies with rabbit anti-human gamma-globulin lines of complete identity were noted between unreduced

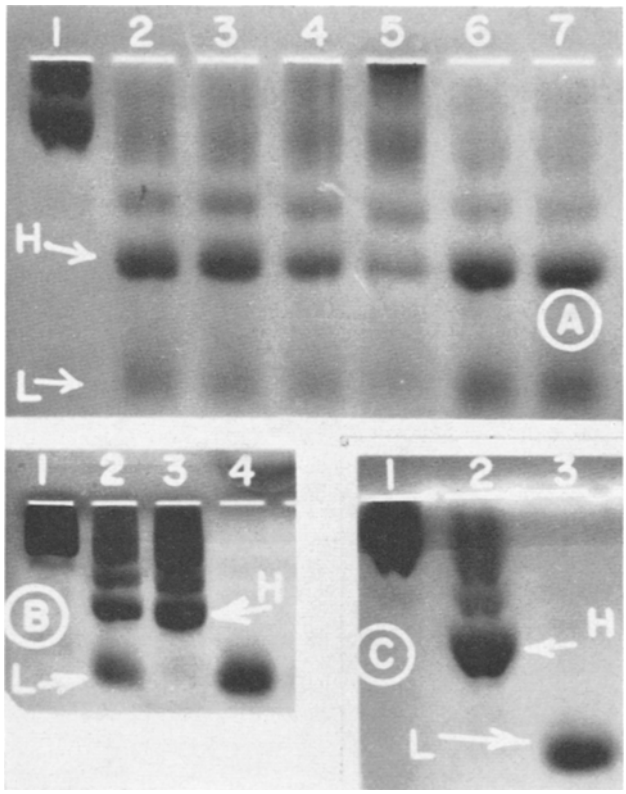

Fig. 1. Acid-urea-starch gel electrophoresis of Cohn FII and its reduction products. (A) Slots filled with Cohn FII which had been subjected to the following treatment: (1) None, (2) $0.2\,M$ mercaptoethanol for 3 h, (3) $0.2\,M$ mercaptoethanol for 5 h, (4) and (5) $1.25 \times 10^{-2}\,M$ *Tris*-hydroxymethyl phosphine for 3 and 5 h respectively, (6) and (7) $1.56 \times 10^{-3}\,M$ *Tris*-carboxyethyl phosphine for 3 and 5 h respectively. (B) Cohn FII (1), Cohn FII reduced with $3.1 \times 10^{-3}\,M$ *Tris*-hydroxymethyl phosphine, (2) and fraction A, (3) and B, (4) obtained from a gel filtration with Sephadex G 75 of the products of this reduction. (C) Cohn FII (1), fraction A (2) and fraction B (3) from a Sephadex gel filtration of FII reduced by $5 \times 10^{-2}\,M$ *Tris*-carboxyethyl phosphine. L and H refer to light and heavy chains throughout.

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2 D. M. KIRSCHENBAUM, E. BAKKER, L. THIEL and S. PLOTCH, Fedn Proc. Fedn Am. Socs. exp. Biol. *26*, 837 (1967).

3 J. B. FLEISCHMAN, R. H. PAIN and R. R. PORTER, Archs Biochem. Biophys., Suppl. *1*, 174 (1962).

4 *Tris*-hydroxymethyl phosphine was obtained as a gift from Dr. L. J. WOLFRAM of Harris Research Labs., Washington, D.C. and as a gift from Hooker Chemical Co., Niagara Falls, New York.

5 *Tris*-carboxyethyl phosphine was obtained as a gift from Dr. M. GRAYSON, American Cyanamid Co., Stamford, Connecticut.

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7 O. OUCHTERLONY, Progr. Allergy *5*, 1 (1958).

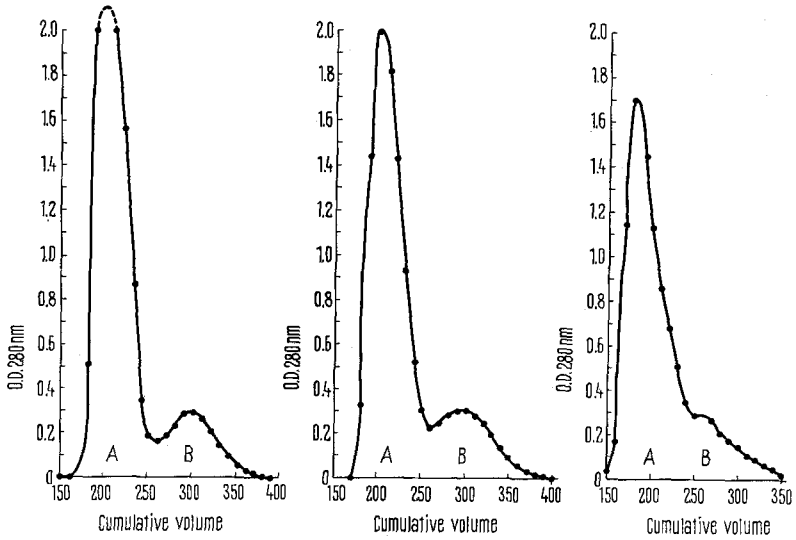

Fig. 2. Sephadex G 75 gel filtration patterns of Cohn FII by $3.1 \times 10^{-3} M$ *Tris*-hydroxymethyl phosphine (left) and $5 \times 10^{-2} M$ *Tris*-carboxyethyl phosphine (center). The solvent used was $1 M$ propionic acid. These patterns are similar to those obtained by FLEISCHMAN, PAIN and PORTER using $0.75 M$ 2-mercaptoethanol[3]. The yield of fraction B using $0.2 M$ 2-mercaptoethanol (right) was less than that obtained with the tertiary phosphines.

A comparison of the capacities of mercaptoethanol (ME) and *Tris*-hydroxymethyl phosphine (THP) to inactivate rheumatoid factor. The serum in a final dilution of 1/100 was tested with a latex slide agglutination test and was graded as positive or negative[a]

Final concentration of reducing agent	ME + iodo-acetamide	ME + buffer	THP + iodo-acetamide	THP + buffer
$0.1 M$	—	—	—	
$5 \times 10^{-2} M$	—	—	—	
$2.5 \times 10^{-2} M$	—	—	—	—
$1.25 \times 10^{-2} M$	—	—	—	—
$6.3 \times 10^{-3} M$	—	—	—	—
$3.7 \times 10^{-3} M$	—	+	—	—
$1.6 \times 10^{-3} M$	—	+	—	+
$8.0 \times 10^{-4} M$	—	+	—	+
$4.0 \times 10^{-4} M$	+	+	—	+
$2.0 \times 10^{-4} M$	+	+	—	+

[a] (—), no latex agglutination, i.e. reduction has occurred.

human F II and human F II reduced by any of the agents used.

Rheumatoid factor was inactivated by concentrations of *Tris*-hydroxymethyl phosphine of $4 \times 10^{-4} M$ (Table). At least partial reversibility of this phenomenon was demonstrated by the return of activity in nonalkylated solution dialyzed free of reducing agents. Alkylation with iodoacetamide prevented the return of activity. Iodoacetamide may be added simultaneously with the tertiary phosphine to the protein solution, combining the reduction and alkylation process, without interferring with the reduction reaction. Mercaptoethanol and *Tris*-hydroxymethyl phosphine have disagreeable odors while *Tris*-carboxyethyl phosphine is a colorless, water-soluble solid without detectable odor which allows for pleasant use.

In the present study the reduction of human serum protein Fraction II (IgG) with mercaptoethanol, *Tris*-hydroxymethyl phosphine and *Tris*-carboxyethyl phosphine gave similar products, as determined by their electrophoretic mobilities, their Sephadex gel filtration properties and their antigenic activity to anti-Fraction II on an OUCHTERLONY plate. These results indicate that water-soluble tertiary phosphines function as effective and convenient reducing agents for human gamma-globulin and yield protein products similar to those obtained with mercaptoethanol.

In addition the tertiary phosphines are effective agents in the inactivation of rheumatoid factor, a phenomenon which is at least partially reversible[8].

Résumé. Deux composés polypeptides sont formés par la réduction de la gamma-globuline par des phosphines tertiaires hydrosolubles; réduction qui s'opère à de plus faibles concentrations des phosphines que du 2-mercapto-éthanol. Avec ces 2 méthodes, les produits de la réaction sont comparables. De plus, les phosphines peuvent inactiver le facteur rheumatoïde, tel que le révèle l'agglutination au latex.

M. E. LEVISON, A. S. JOSEPHSON and D. M. KIRSCHENBAUM

Department of Medicine and Department of Biochemistry, State University of New York, Downstate Medical Center, Brooklyn (New York 11203, USA), 25 June 1968.

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