PREPARATION OF ANTIBODY-FERRITIN CONJUGATES FOR IMMUNO-ELECTRON MICROSCOPY

J. SRI RAM, SAROJ S. TAWDE, G. B. PIERCE, JR., and A. R. MIDGLEY, JR. From the Department of Pathology, The University of Michigan, Ann Arbor

We have previously reported (1) that the bifunctional reagent, *p,p'* difluoro-*m,m'* dinitrodiphenyl sulfone (FNPS)[1] can be used for conjugation of antibodies to ferritin in a one-step procedure, under conditions causing little loss of precipitating capacity of the antibody. In this brief report are discussed some details of these studies, the characterization of the antibody-ferritin conjugates, and the advantage of this procedure over the previous methods (2). The biologic problem in which these techniques were applied was discussed in an earlier paper (3).

In the preliminary studies, anti-bovine serum albumin (BSA) globulins were reacted with ferritin (Fe) in the presence of FNPS, and the formation of an immunologically active globulin-ferritin conjugate was established by paper electrophoresis as well as precipitin studies. Treatment of FNPS with an amino acid such as lysine, or with hot sodium carbonate which causes its hydrolysis with the formation of dihydroxydinitrodiphenyl sulfone (4), prior to addition to antibody-ferritin mixture, prevented the formation of any conjugate. This suggests that the two proteins were covalently linked and that the reactive fluorines of FNPS were involved in the reaction.

Optimal conditions for the conjugation have now been established (Table I), and the procedure described below has been employed in our immunoelectron microscope studies (3). To a mixture of 160 mg of rabbit globulin (RGG) and 460 mg of ferritin, dissolved in sufficient cold 2 per cent aqueous sodium carbonate to constitute a 4 per cent protein solution, was added 1 ml of chilled acetone containing 5 mg of FNPS. After stirring in the cold (2–4°C) for 24 hours, the reaction mixture was dialyzed exhaustively against normal saline and centrifuged to remove a small precipitate.

To assess the extent of loss in the precipitating activity of anti-BSA following conjugation with ferritin under the reaction conditions mentioned above, precipitin studies (5) were made with untreated anti-BSA, crude conjugate, and a control preparation (antibody-ferritin mixture treated as noted above without FNPS). These results are presented in Fig. 1. Curve *1*, the precipitin curve obtained with the control preparation, was slightly lower than the homologous curve (Curve *2*) with a shift in the equivalence point from 34 μg N for curve *2*, to 26 μg N for curve *1*. This represents a loss of about 25 per cent of precipitable antibody since 25 per cent less antigen was required to precipitate all of the antibody. Curve *3*, the precipitin curve of the crude conjugate, was higher than the homologous curve. This was in accordance with the expected contribution to the protein of the specific precipitates by the ferritin part of the conjugate. The equivalence point of this precipitin curve was brought still lower (21 μg N), and complete precipitation of the antibody occurred with 40 per cent less antigen than with the untreated antibody. After the conjugation reaction the crude conjugate thus had 40 per cent less antibody activity. Iron analysis (6) of the specific precipitate obtained with the crude conjugate at the equivalence point gave a value of 202 μg. This corresponds to 1.0 mg of ferritin. The anti-

[1] FNPS can now be purchased from General Biochemicals, Chagrin Falls, Ohio.

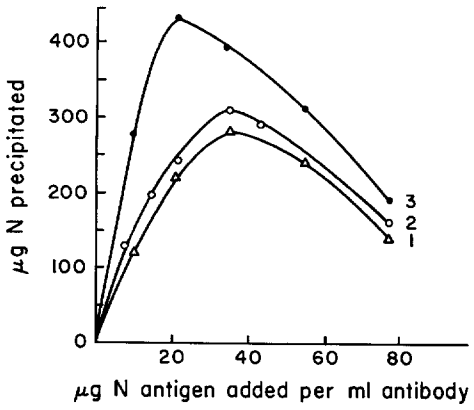

FIGURE 1

Precipitin behavior of anti-BSA globulins after the conjugation reaction. Curve *1*. Precipitin reaction of anti-BSA plus ferritin. Control preparation. Curve *2*. Precipitin reaction of untreated anti-BSA. Curve *3*. Precipitin reaction of anti-BSA after the conjugation reaction ("crude conjugate"). Initial antibody concentrations in the three preparations were identical.

body content (conjugated plus unconjugated) was calculated to be 1.75 mg (Curve *3*). Since the purified conjugate appears to be an equimolar compound (see below), the amount of RGG in combination with 1 mg of Fe would be 0.35 mg, or one-fifth of the total precipitated. It would thus appear that about 20 per cent of the total precipitable antibody was in chemical combination with ferritin.

The crude conjugate was purified by electrophoresis in agar medium under the conditions described by Das and Giri (7). This involved electrophoresis at pH 8.6, cutting out the agar band containing the pure conjugate, and freezing and thawing the agar which results in separation of the conjugate from the agar granules. Borek and Silverstein (8) employed continuous flow paper electrophoresis with equally effective results.

Homogeneity of the purified conjugate was always confirmed by the appearance of a single band in electrophoretic studies. Four prepara-

TABLE I

Effect of Various Reaction Conditions on the Extent of Conjugation

Reaction conditon varied	Conjugation	Other remarks
	*Per cent**	
A. pH		
9.7 (0.5 per cent carbonate)	18	RGG (160 mg) + Fe (460 mg); 4 per cent total protein conc.; 5 mg FNPS in 1 ml acetone; reaction for 24 hours. There was excessive precipitation after dialysis of the pH 11 mixture.
10.2 (1 per cent carbonate)	43	
10.5 (2 per cent carbonate)	53	
11.0 (1 per cent carbonate + NaOH)	—	
B. Reaction time (hrs.)		
1	21	Reaction carried out in 1 per cent carbonate (other conditions as in A).
6	35	
10	43	
24	49	
C. Protein concentration (gm per cent)		
1	34	Reaction in 1 per cent carbonate (other conditions as in A). The concentration of Fe limited the study to 8 per cent.
4	49	
8	52	
D. FNPS (mg in 1 ml acetone)		
2	32	Reaction in 2 per cent carbonate (other conditions as in A). Excessive precipitation of the preparation with 10 mg FNPS prevented accurate estition.
5	51	
10	—	

* Estimated by cellulose acetate paper electrophoresis. The figures represent the per cent of total dye eluted.

tions of the purified conjugate were characterized by determining the total iron and protein content (9). From these values the ratio of ferritin to globulin was calculated and was found to range between 1 to 1.1 and 1 to 1.3. It would appear that the conjugate was predominantly an equimolar compound as was the one obtained with the diisocyanates (8). When the purified conjugate obtained with anti-BSA was reacted with BSA, anti-RGG, and anti-Fe, respectively, in Ouchterlony gel diffusion technique (10), single precipitin bands were obtained in all cases, indicating that the native structures of both the proteins were left essentially intact after the conjugation and purification procedures.

FNPS appears to offer several advantages over the diisocyanates as a reagent for conjugation of proteins. Its reaction with proteins is both stoichiometric and mild (4, 11, 12) and is, therefore, predictable. On the other hand, the chemistry of the reaction of the diisocyanates with proteins appears poorly understood, as the amount of reagent bound is unaccountably high (13). The reagent appears to polymerize on the protein surface (13). Tolylene-2,4-diisocyanate was recently shown to polymerize under certain conditions (14). These secondary reactions may explain the loss of antibody activity on direct reaction with this reagent (2). Even during the two-step conjugation reaction, the precipitating activity of the antibody was reported to be lost in the case of anti-ovalbumin antibodies (8), whereas no such loss was reported in the case of some others (2).

These studies were supported by grants from the United States Public Health Service (E-4401) and the American Cancer Society (Institutional Research Grant No. IN-40D).

Dr. Tawde was a visiting investigator from the Indian Institute of Science, Bangalore, India.

Dr. Pierce is a Markle Scholar in Medical Sciences.

Received for publication, November 30, 1962.

BIBLIOGRAPHY

1. TAWDE, S. S., and SRI RAM, J., *Arch. Biochem. and Biophysics*, 1962, **97**, 430.
2. SINGER, S. J., *Nature*, 1959, **183**, 1523.
   SINGER, S. J., and SCHICK, A. F., *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 519.
3. PIERCE, G. B., JR., MIDGLEY, A. R., JR., and SRI RAM, J., *J. Exp. Med.*, 1963, **117**, 339.
4. WOLD, F., *J. Biol. Chem.*, 1961, **236**, 106.
5. KABAT, E. A., and MAYER, M. M., Experimental Immunochemistry, Springfield, Illinois, C. C Thomas, 2nd edition, 1961.
6. SCHALES, O., *in* Standard Methods of Clinical Chemistry, (D. Seligson, editor), New York, Academic Press, Inc., **2**, 1958.
7. DAS, B. R., and GIRI, K. V., *J. Indian Inst. Sc.*, 1959, **41**, 67.
   DAS, B. R., *Biochim. et Biophysica Acta*, 1962, **58**, 52.
8. BOREK, F., and SILVERSTEIN, A. M., *J. Immunol.*, 1961, **87**, 555.
9. MARKHAM, R., *Biochem. J.*, 1942, **36**, 790.
10. OUCHTERLONY, O., *Progr. Allergy*, 1958, **5**, 1.
11. TAWDE, S. S., SRI RAM, J., and IYENGAR, M. R., *Arch. Biochem. and Biophysics*, 1963, **100**, 270.
12. WOLD, F., *Biochim. et Biophysica Acta*, 1961, **54**, 604.
13. SCHICK, A. F., and SINGER, S. J., *J. Biol. Chem.*, 1961, **236**, 2477.
14. BEACHELL, H. C., and NGOC SON, C, P,, *Polymer Letters*, 1963, **1**, 25.