2842-2848.
Liu, T. Y., and Chang, Y. H. (1972), Communication at American Chemical Society Meeting, Boston, April, 1972.
Moore, P. B. (1971), *J. Mol. Biol. 60,* 169-184.
Pesce, A. J., Rosén, C. G., and Pasby, T. L. (1971), Fluorescence Spectroscopy, New York, N.Y., Marcel Dekker.
Pochon, F., and Ekert, B. (1973), *Eur. J. Biochem. 36,* 311-316.
Pochon, F., Ekert, B., and Perrin, M. (1974), *Eur. J. Biochem. 43,* 115-124.
Schechter, N., Elson, D., and Spitnik-Elson, P. (1975), *FEBS Lett. 57,* 149-152.
Spirin, A. S., Kisselev, N. A., Shukulov, R. S., and Bogdanov, A. A. (1963), *Biokhimiya 28,* 920-930.
Spitnik-Elson, P. (1962), *Biochim. Biophys. Acta 55,* 741-747.
Spitnik-Elson, P. (1965), *Biochem. Biophys. Res. Commun. 18,* 557-562.
Spitnik-Elson, P., and Atsmon, A. (1969), *J. Mol. Biol. 45,* 113-124.
Spitnik-Elson, P., Greenman, B., and Abramovitz, R. (1974), *Eur. J. Biochem. 49,* 87-92.
Steiner, R. F., Lippoldt, R. E., Edelhoch, H., and Frattali, V. (1964), in Quantum Aspects of Polypeptides and Polynucleotides, Weissbluth, M., Ed., New York, N.Y., Interscience, pp 355-366.
Stern, O., and Volmer, M. (1919), *Phys. Z. 20,* 183-188.
Teale, F. W. J. (1960), *Biochem. J. 76,* 381-388.
Teale, F. W. J., and Weber, G. (1957), *Biochem. J. 65,* 476-482.
Traut, R. R., and Haenni, A. L. (1967), *Eur. J. Biochem. 2,* 64-73.
Vogel, Z., Zamir, A., and Elson, D. (1969), *Biochemistry 8,* 5161-5168.
Weltman, J. K., Szaro, R. P., Frackelton, A. R., Dowben, R. M., Bunting, J. R., and Cathou, R. E. (1973), *J. Biol. Chem. 248,* 3173-3177.
Zamir, A., Miskin, R., and Elson, D. (1971), *J. Mol. Biol. 60,* 347-364.
Zamir, A., Miskin, R., Vogel, Z., and Elson, D. (1974), *Methods Enzymol. 30,* 406-426.

Dimers and Trimers of Immunoglobulin G Covalently Cross-Linked with a Bivalent Affinity Label†

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ABSTRACT: A bivalent affinity label, bis(α-bromoacetyl-ε-2,4-dinitrophenyllysylproline)ethylenediamine, has been synthesized. Treatment of anti-2,4-dinitrophenyl antibodies with this compound produces a mixture of covalently and noncovalently cross-linked material. Only specific antibodies are covalently cross-linked, suggesting that covalent attachment occurs in the variable regions. Covalently cross-linked dimers and trimers have been isolated from the reaction mixture in a high state of purity, in yields of about 12 and 4%, respectively. The complexes are stable in solutions containing 10^{-4} M hapten and can therefore be used as sensitive probes of immune effector functions.

The interactions of antibodies with antigens provoke a variety of physiological responses (Metzger, 1974), which, in the case of immunoglobulin G (IgG),[1] include activation of the complement system (Müller-Eberhard, 1975; Reid and Porter, 1975), direct binding of antigen–antibody complexes to lymphocytes (Dickler, 1976), and clearance of immune complexes from the circulation (Dorrington and Painter, 1974; Mannik et al., 1971; Waldmann et al., 1971). Antigenic recognition occurs at the N-terminal end of the immunoglobulin molecule (Davies et al., 1975), while the interaction with lymphocytes (Dickler, 1976), macrophages (Unkeless and Eisen, 1975), and components of the complement system (Reid and Porter, 1975) is thought to take place in the Fc portion, some 80 Å away. Two general mechanisms have been proposed (Metzger, 1974) to explain how antigen binding can exert an effect at this distance. According to one proposal, an antigen-induced conformational change within the immunoglobulin molecule would alter the structure of the Fc portion in a way which would cause it to activate the immune effector systems. An alternate mechanism for triggering requires that two or more immunoglobulin molecules be brought in close proximity, as occurs when antibodies of the IgG class interact with multivalent antigens. In cases where the receptors for immunoglobulin are multivalent (e.g., Clq binds greater than 10 IgG molecules (Schumaker et al., 1976) and some cells bind 10^5 to 10^6 immunoglobulin molecules (Unkeless and Eisen, 1975; Kulczycki and Metzger, 1974)), the overall binding constant for a complex containing *n* immunoglobulin molecules would approximate the binding constant for one molecule to the *n*th power. In this way, the affinity of antibody for these receptors could be increased tremendously, even in relatively small complexes.

In order to study the relationship between antigenic recognition and effector system triggering, it would be desirable to

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[1] Abbreviations used: IgG, immunoglobulin G; Z, benzyloxycarbonyl; NHS, *N*-hydroxysuccinimide; DCC, dicyclohexylcarbodiimide; FDNB, 1-fluoro-2,4-dinitrobenzene; Dnp, 2,4-dinitrophenyl; Tnp, 2,4,6-trinitrophenyl; BADL, α-bromoacetyl-ε-Dnp-lysine; Me_2F, dimethylformamide; Me_2SO, dimethyl sulfoxide; $(BADL\text{-}Pro)_2$-EDA, bis(α-bromoacetyl-ε-Dnp-Lys-Pro)ethylenediamine.

have a variety of stable, well-defined antibody–antigen complexes. We have recently been approaching this problem through the use of bivalent affinity labels.[2] Affinity labels are compounds which bind to specific antibodies by virtue of an antigenic determinant, but which are also able to form covalent bonds subsequent to binding. Bivalent affinity labels would thus be expected to link two binding sites together irreversibly, either within the same antibody molecule, or between two different molecules, depending upon the length of the spacer joining the two affinity labels. In these studies, bromoacetyl-Dnp-lysine (BADL) (Weinstein et al., 1969) is used as the affinity label. This compound attaches covalently to anti-Dnp antibodies via lysine or tyrosine residues close to or in the antigen binding site (Givol, 1974) and has the advantages of being relatively stable, easy to synthesize, and containing a carboxyl group, for forming the bivalent product. In this paper we will describe the use of a short bivalent affinity label in the preparation of stable dimeric and trimeric immunoglobulin complexes.

Materials and Methods

Materials. *N*-Benzyloxycarbonyl(Z)proline (Z-Pro) and α-Z-Lys were obtained from the Sigma Chemical Co. *N*-Hydroxysuccinimide (NHS), ε-aminocaproic acid and ethylenediamine were purchased from Aldrich Biochemicals, and dicyclohexylcarbodiimide (DCC) and 1-fluoro-2,4-dinitrobenzene (FDNB) were obtained from the Eastman Organic Chemical Co. *N*-ε-2,4,6-Trinitrophenyllysine (Tnp-Lys) and keyhole limpet hemocyanin were obtained from Schwarz/Mann Biochemicals. Sepharose and Sephadex were purchased from Pharmacia Fine Chemicals, Inc., and Ultrogel from LKB. ^{125}I was purchased from NEN as the "low pH solution". It was diluted to a concentration of 50 mCi/ml with water before using. Complete Freund's adjuvant was purchased from Difco Laboratories and AG50W-X8 cation-exchange resin was obtained from Bio-Rad Laboratories. Nonimmune rabbit IgG was purchased from Miles Laboratories and was fractionated on Sepharose 4B before using.

Z-Pro-NHS was prepared by coupling NHS to Z-Pro in dioxane with DCC.

(Z-Pro-NH-CH$_2$-)$_2$. Z-Pro-NHS (6.9 g) was reacted with 0.66 ml of ethylenediamine in dioxane. The product partially crystallized from dioxane. Upon addition of ether, 4.0 g of crude material was obtained.

(HCl-Pro-NH-CH$_2$-)$_2$. Four grams of (Z-Pro-NH-CH$_2$-)$_2$ was dissolved in 10 ml of acetic acid. HBr (15 ml) in acetic acid was then added, and the reaction was allowed to proceed under anhydrous conditions for 2 h at room temperature. Upon addition of ether, a crystalline product was obtained. The product was dissolved in water and fractionated on a 1.5 × 90 cm Sephadex G-10 column in 0.1 M HCl. The major peak was pooled and the solvent evaporated.

α-Z-ε-Dnp-Lys. α-Z-Lys (5.6 g) and $NaHCO_3$ (4.0 g) were dissolved in water and 7.6 g of FDNB in ethanol was added with stirring. After reacting 1 h at room temperature, ethanol was removed by evaporation. The compound was taken up in additional water and extracted twice with ether. The pH was then brought to 2.5–3.0 with H_3PO_4 and the compound extracted into ethyl acetate. After drying with Na_2SO_4, the solvent was removed by evaporation and the oily precipitate washed with petroleum ether. A nonsolid product (7.1 g) was obtained.

α-Z-ε-Dnp-Lys-NHS. α-Z-ε-Dnp-Lys (7.1 g) and NHS (1.9 g) were coupled using DCC in dioxane. After filtering, the solvent was removed by evaporation. The product was dissolved in $CHCl_3$ and crystallized from this solvent on standing. After addition of ether, 5.1 g of compound was obtained. Anal. Calcd for: C, 53.04%; H, 4.60%; N, 12.89%. Found: C, 53.04%; H, 4.68%; N, 12.67%.

(α-Z-ε-Dnp-Lys-Pro-NH-CH$_2$-)$_2$. α-Z-ε-Dnp-Lys-NHS (2.2 g) in dimethylformamide (Me_2F) was added to 0.6 g of (HCL-Pro-NH-CH$_2$-)$_2$ in 25 ml of 0.5 M $NaHCO_3$. Additional Me_2F was added to give a clear solution. After reacting 2 h at room temperature, the solvent was removed and the compound was dissolved in $CHCl_3$. After extracting once each with 0.5 M $NaHCO_3$, 1 M HCl, and H_2O, the organic phase was dried with Na_2SO_4, and the solvent removed by evaporation. The yield was 0.7 g of a gummy product. Anal. Calcd for: C, 55.45%; H, 5.36%; N, 15.53%. Found: C, 55.69%; H, 5.57%; N, 15.24%.

(HBr-ε-Dnp-Lys-Pro-NH-CH$_2$-)$_2$. (α-Z-ε-Dnp-Lys-Pro-NH-CH$_2$-)$_2$ (0.4 g) was dissolved in 4 ml of acetic acid and 8 ml of HBr in acetic acid was added. After 2 h at room temperature, the product was precipitated by addition of ether, giving 200 mg of a sticky compound.

Bromoacetic acid–NHS was prepared from bromoacetic acid and NHS in dioxane with DCC. The product crystallized from ether.

BADL was prepared by the method of Weinstein et al. (1969), except that the reaction was terminated after 30 s by addition of HCl (M. Wilchek, personal communication).

(BADL-Pro)$_2$-EDA. (HBr-ε-Dnp-Lys-Pro-NH-CH$_2$-)$_2$ (200 mg) was dissolved in 2 ml of Me_2F and added to 180 mg of bromoacetic acid–NHS in 2 ml of Me_2F. Immediately after addition of 0.1 ml of triethylamine, the mixture was placed on a 2.5 × 100 cm Sephadex G-10 column and eluted in Me_2F–1 mM aqueous HCl (9:1). Fronting material was pooled and the solvent removed by evaporation. The compound was dissolved in acetone and crystallized on addition of ether. The yield was 112 mg. Anal. Calcd for: C, 44.28%; H, 4.80%; N, 15.50%; Br, 14.76%. Found: C, 43.21%; H, 4.74%; N, 15.67%; Br, 15.16%.

The most difficult part of the synthesis is the introduction of the bromoacetyl group. This step often involves side reactions leading to impure compounds (M. Wilchek, personal communication). Although the analysis of (BADL-Pro)$_2$-EDA reported here is acceptable, we have obtained several preparations in which the Br content is several percent low.

Dnp-ethylenediamine-HCl was prepared by reacting FDNB with a large excess of ethylenediamine in acetone. The product crystallized when added to aqueous 1 M HCl.

Dnp-ε-aminocaproic acid was prepared by reacting FDNB with ε-aminocaproic acid. The product crystallized from aqueous solution upon acidification.

Dnp-Keyhole Limpet Hemocyanin. Keyhole limpet hemocyanin (50 mg) was dissolved in 1 ml of 1 M sodium borate, pH 9.2, and 0.1 ml of 1 M FDNB in ethanol was added. The precipitate thus obtained was dialyzed exhaustively vs. phosphate-buffered saline and resuspended in 30 ml of the same buffer.

Tnp-lysine-Sepharose was prepared by the method of March et al. (1974) using 24 mg of Tnp-Lys and 100 g of activated Sepharose 4B.

Anti-Dnp Anitbodies. Five randomly bred albino rabbits were injected each with 2 mg of Dnp keyhole limpet hemocyanin in complete Freund's adjuvant. After 20 days the rabbits were boosted with the same antigen, and immunization was

[2] We have recently learned that similar studies, using a different bivalent affinity label, are currently being carried out by Plotz (1976).

continued at 3-week intervals. Bleeding was begun after 1 month and was continued on a biweekly basis. Antisera were pooled after each bleeding and stored at −60 °C.

Anti-Dnp antibodies were isolated by passing 60 ml of antiserum over a 1.6 × 25 cm Tnp-lysine-Sepharose 4B column, with 3 cm of AG 50W-X8 (200–400 mesh), sodium form, at the bottom. After washing extensively with 0.15 M NaCl–0.01 M sodium acetate, pH 5.5, the antibodies were eluted with 40 ml of 0.01 M Dnp-ethylenediamine in the above buffer. The protein thus obtained was concentrated by ultrafiltration and passed over a 2.5 × 100 cm Sepharose 4B column (and more recently over an Ultrogel AcA 22 column of the same size) in 0.15 M NaCl–5 mM Veronal, pH 7.4. The IgG peak (which included most of the protein) was pooled and concentrated. The yield was usually about 3 mg of anti-Dnp antibody from each ml of serum used. The average affinity of the antibodies for Dnp-lysine was about 3×10^6 L/M as determined by equilibrium dialysis.

Sheep antibodies were prepared in essentially the same way, except that the antiserum was obtained 10 days after injecting a sheep with 5 mg of Dnp-keyhole limpet hemocyanin.

Cross-Linking Reaction. Anti-Dnp antibodies at a concentration of approximately 10 mg/ml were dialyzed against 0.15 M NaCl–0.02 M sodium borate, pH 8.7. An equimolar amount of $(BADL\text{-}Pro)_2$-EDA in dimethyl sulfoxide (Me_2SO) was added (final Me_2SO concentration less than 5%) and the reaction was allowed to proceed 24 h at 30 °C. The reaction mixture was then centrifuged and the supernatant fractionated, as described below.

Polyacrylamide gel electrophoresis was carried out in 0.2% sodium dodecyl sulfate–Tris-acetate buffer at pH 7.4 using Pharmacia PAA 4/30 gradient slab gels and a Pharmacia GE-4 electrophoresis apparatus. Samples were denatured in 1% sodium dodecyl sulfate–0.01 M Tris-Cl–10% glycerol, pH 8.0, at 100 °C for 3 min prior to electrophoresis. Electrophoresis was allowed to proceed at 20 °C for about 0.5 h after bromphenol blue markers passed through the gel. Slabs were then stained with 0.05% Coomassie blue in 25% 2-propanol–10% acetic acid–65% water and destained using a Pharmacia GD-4 destainer.

Fractionation of Cross-Linked Material. Two parallel 1.5 × 90 cm Pharmacia columns were coupled so that the first drained directly into the second. The first column contained Sephadex G-200 and the second Ultrogel AcA 22. The columns were preequilibrated with 1 mM Dnp-ε-aminocaproic acid in Veronal-buffered saline, and 5 ml or less of sample was applied to the first column. Elution was maintained at 3–3.5 ml/h, using a Marriott flask to keep the pressure constant. Fractions (3 ml) were collected, and protein concentration was determined by adding 0.75 ml from each fraction to 0.5 ml of a thick AG1-X8 (200–400 mesh) slurry and mixing. The tubes were centrifuged and their OD determined at 280 and 365 nm. The OD at 280 nm was corrected for the absorbance of Dnp-ε-aminocaproic acid by subtracting 0.32 × OD at 365 nm.

Iodination of Products. Iodination was carried out by the procedure of Sonoda and Schlamowitz (1970) as modified by Unkeless and Eisen (1975). Briefly, proteins were brought to 2 mg/ml concentration and dialyzed vs. 0.05 M sodium phosphate, pH 7.0, containing 1 mM Dnp-ε-aminocaproic acid. To 50 μl of protein, 0.5 mCi of ^{125}I in 10 μl was added. The solution was chilled to 0 °C in an ice bath, and a 15-fold molar excess of Chloramine-T in the 0.05 M phosphate was added. After reacting 0.5 h at 0 °C, 100 μl of 0.04% tyrosine was added, followed by 50 μl of 0.1 M KI. The solution was then passed over a Sephadex G-25 coarse column (packed in a 12-ml disposable syringe) in phosphate-buffered saline, and fronting material pooled. Protein iodinated in this way usually had a specific activity in the range of 10^9 cpm/mg.

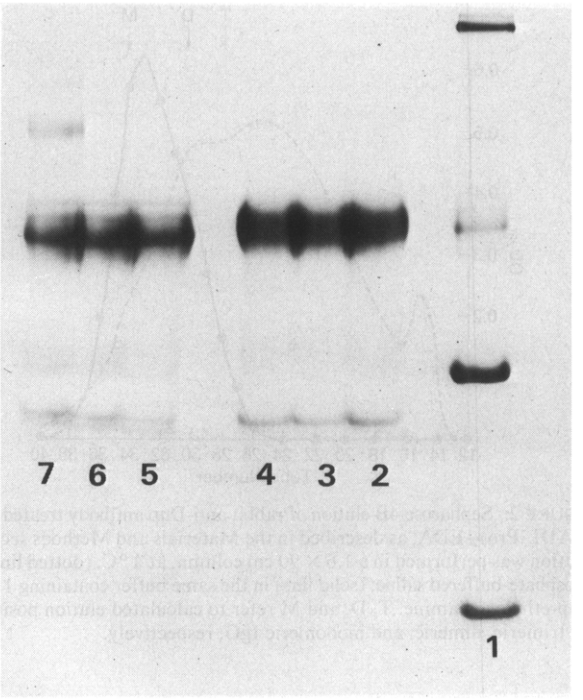

FIGURE 1: Gradient sodium dodecyl sulfate slab gel of IgG treated in 0.15 M NaCl–0.02 M sodium borate (pH 8.7) for 24 h at 30 °C with various reagents. Gel is purposely overloaded to show reaction products. (1) Markers, IgM, IgG, bovine serum albumin, ribonuclease. (2–4) Nonimmune rabbit IgG treated with (2), 4% Me_2SO, (3) BADL and 4% Me_2SO, and (4) $(BADL\text{-}Pro)_2$-EDA and 4% Me_2SO. (5–7) Rabbit anti-Dnp antibody treated with (5) 4% Me_2SO, (6) BADL and 4% Me_2SO, (7) $(BADL\text{-}Pro)_2$-EDA and 4% Me_2SO. IgG was incubated with equimolar amounts of BADL and $(BADL\text{-}Pro)_2$-EDA.

Results

Cross-Linking Conditions. In preliminary experiments it was determined that maximal cross-linking occurred when $(BADL\text{-}Pro)_2$-EDA and anti-Dnp antibodies were incubated together in equimolar amounts at pH 8.5–9.0. Under these conditions, cross-linking has been observed to occur to equivalent extents at protein concentrations ranging from 6 to 25 mg/ml. The sodium dodecyl sulfate slab gel in Figure 1 shows the results of treating nonimmune rabbit IgG and rabbit anti-Dnp IgG antibodies 24 h at 30 °C with either Me_2SO alone, BADL in Me_2SO, or $(BADL\text{-}Pro)_2$-EDA in Me_2SO. Covalent cross-linking is observed only when the bivalent affinity label reacts with the rabbit anti-Dnp antibodies. Similar results were also obtained with sheep anti-Dnp IgG. Since nonimmune IgG failed to show cross-linking, we may assume that the covalent points of attachment are in the antigen-binding (i.e., variable) regions of the immunoglobulins.

An obvious feature of Figure 1 is the relatively low yields of covalently linked products. This probably arises in part from the presence of antibody molecules within the total anti-Dnp preparation which will bind the Dnp group, but which will not form a covalent attachment with the bromoacetyl moiety (Weinstein et al., 1969). In our preparations, approximately 60–70% of the combining sites are capable of interacting with $(BADL\text{-}Pro)_2$-EDA covalently. Thus, at best we might expect about 40% (0.63^2) dimer and 25% (0.63^3) trimer formation.

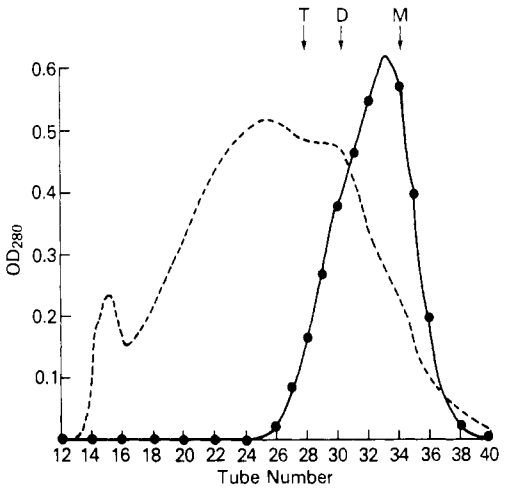

FIGURE 2: Sepharose 4B elution of rabbit anti-Dnp antibody treated with $(BADL\text{-}Pro)_2$-EDA, as described in the Materials and Methods section. Elution was performed in a 1.6 × 90 cm column, at 4 °C; (dotted line) in phosphate-buffered saline; (solid line) in the same buffer containing 1 mM Dnp-ethylenediamine. T, D, and M refer to calculated elution positions for trimeric, dimeric, and monomeric IgG, respectively.

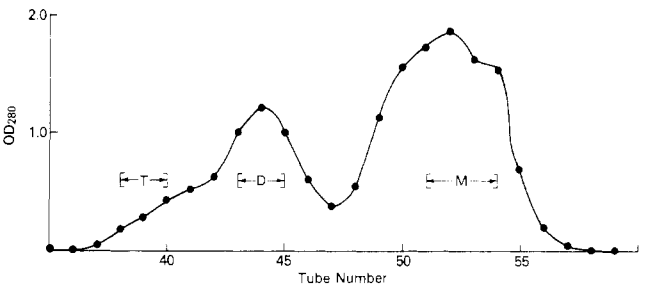

FIGURE 3: Elution of rabbit anti-Dnp antibodies treated with $(BADL\text{-}Pro)_2$-EDA on parallel Sephadex G-200–Ultrogel AcA22 columns, as described in the Materials and Methods section. Fractions designated as M (monomer), D (dimer), and T (trimer) were pooled and concentrated. The optical density observed in the monomer peak is out of the linear range of the spectrophotometer and, thus, appears artificially low in this figure.

In fact, the actual amount of polymer observed is appreciably lower than these estimates, indicating lower efficiency in covalent binding, and perhaps the occurrence of nonproductive side reactions of the bromoacetyl group. In gels which have been overloaded with monomer to a greater extent than in Figure 1, covalent aggregates as large as pentamer can be observed, but as expected the relative amounts appear to fall geometrically with increasing size.

In addition to intact immunoglobulin, a variety of smaller fragments are also apparent. These arise during the overnight incubation at 30 °C and are probably proteolytic digestion products (Connell and Painter, 1966).

Isolation of Covalently Linked Products. Under nondenaturing conditions, the antibodies form both covalent and noncovalent bonds with the bivalent affinity label leading to the production of high-molecular-weight complexes (similar to previous observations of Hyslop et al. (1970) using a bivalent hapten). This is shown in Figure 2 (dotted line) where a cross-linked reaction mixture was eluted on Sepharose 4B in phosphate-buffered saline. Polyacrylamide gel electrophoretic analysis in sodium dodecyl sulfate indicates that the interactions giving rise to these complexes are largely noncovalent (Figure 1). In order to dissociate the noncovalent bonds in these complexes, a portion of the same reaction mixture was eluted on the same column, but in the presence of a large molar excess of monovalent hapten (1 mM Dnp-ethylenediamine in phosphate-buffered saline). The elution profile is shown in Figure 2 (solid line). On Sepharose 4B, the dissociated material migrates as a single peak, somewhat in front of the IgG monomer. This experiment demonstrates that noncovalent aggregates dissociate in the presence of monomeric hapten, but that the resolving power of Sepharose 4B is not sufficient to separate monomeric immunoglobulin from dimers and trimers.

Adequate resolution was achieved using the Sephadex G-200–Ultrogel AcA 22 parallel columns, as described in the Materials and Methods section. Sephadex G-200 was used since good separation between monomer and higher aggregates was obtained on this gel, while Ultrogel AcA 22 (exclusion limit 10^6) was required for separation of dimer and trimer (mol wt 300 000 and 450 000). The elution profile of the reaction mixture in buffer containing Dnp-ϵ-aminocaproic acid is shown in Figure 3. Fractions indicated in the figure were pooled and concentrated by vacuum dialysis against the elution buffer. Examination of the products by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 4) shows the fractions to be highly purified, covalently linked, dimers and trimers. No contaminants can be observed in the dimer fraction, but traces of higher aggregates are visible in the trimer. From 75 mg of starting material, 8.8 mg (12%) of dimer and 3.0 mg (4%) of trimer were obtained.

If the various fractions were concentrated using Amicon ultrafiltration cells or if the Dnp-ϵ-aminocaproic acid was removed by dialysis, insoluble material formed from either the dimer or trimer fractions. This is presumably due to the formation of higher molecular weight complexes, suggesting that the purified preparations contain molecules with free binding sites and with unbound halves of the bivalent affinity label. Dimer and trimer samples were therefore stored and used in buffers containing 10^{-3} to 10^{-4} M Dnp-ϵ-aminocaproic acid.

Characterization of the Products. Molecular weight analysis of the products shown in Figure 4, using IgM, bovine serum albumin, and ribonuclease as markers, yields values of 221 000, 442 000, and 604 000 for the monomer, dimer, and trimer, respectively. These molecular weights are somewhat greater than the expected values (mol wt of IgG = 150 000) probably as a result of using unreduced proteins and unreduced markers (Reid et al., 1972). However, the ratio of molecular weights is 1:2:3, indicating that the products are, when denatured, monomer, dimer, and trimer.

Dimer and trimer fractions were iodinated with ^{125}I (Materials and Methods) and eluted on the G-200–AcA 22 parallel columns in buffer containing 10^{-4} M Dnp-ϵ-aminocaproic acid. The results are shown in Figure 5, where it will be seen that both products migrate as a single peak. Based on the elution positions of IgG and bovine serum albumin, the dimer and trimer migrate as proteins with molecular weights of 300 000 and 450 000, respectively. These estimates are very approximate since they are based on only two markers, and since gel filtration under nondenaturing conditions can give erroneous molecular weight values (Ackers, 1970).

In Figure 5, 93% of the radioactive material in the dimer sample was contained in tubes 42–49 and 86% of the trimer in tubes 37–45 (corresponding roughly to twice the width at one-half maximum height). In both cases, most of the radioactivity not included in the major peak was of lower molecular weight (6.0% for the dimer and 12.7% for trimer). Only 1.2 and 1.1% of the dimer and trimer fractions, respectively, migrated

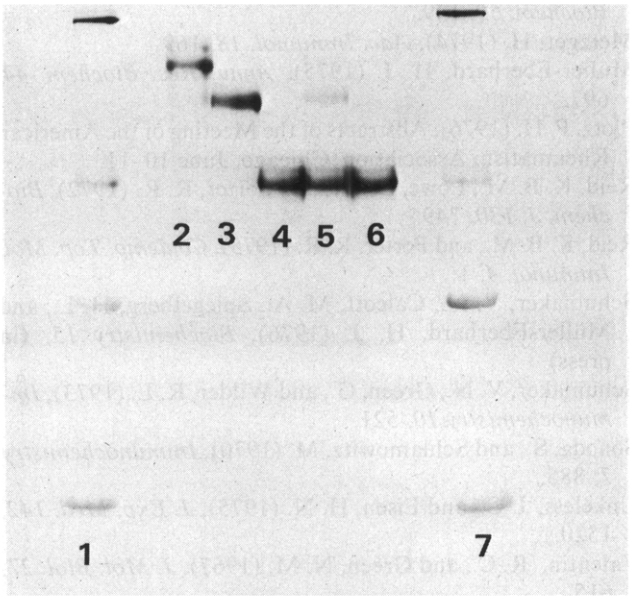

FIGURE 4: Gradient sodium dodecyl sulfate slab gel of purified fractions (see Figure 3) of cross-linked anti-Dnp antibodies. Positions 1 and 7, markers (IgM, IgG, bovine serum albumin, ribonuclease); 2, trimer fraction; 3, dimer fraction; 4, monomer fraction; 5, reaction mixture prior to fractionation; 6, control antibody, treated with 4% Me_2SO.

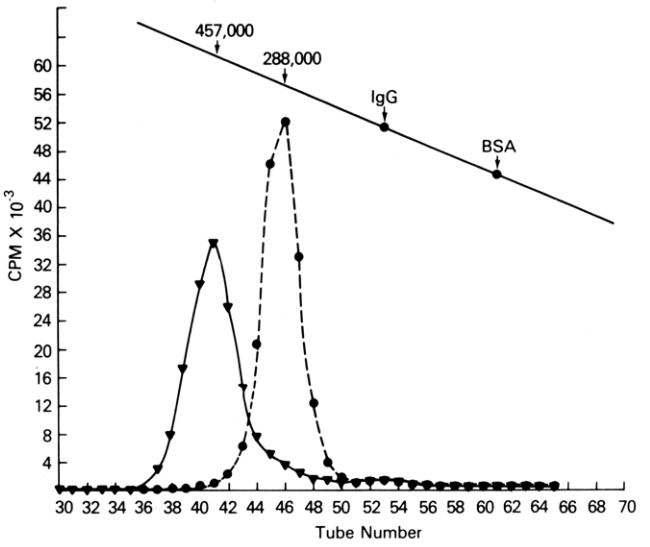

FIGURE 5: Elution of ^{125}I-labeled trimer (—▼—) and dimer (- -●- -) on the Sephadex G-200–Ultrogel AcA22 double columns, in Veronal-buffered saline containing 10^{-4} M Dnp-ϵ-aminocaproic acid. These are results of two separate elutions.

in front of the main peak. The higher molecular weight complexes visible in the trimer fraction (Figure 4) are probably not resolved from the main peak. If we were to consider that all the counts in the dimer preparation which eluted at the peak position of the trimer were in fact trimer, then contamination of dimer with trimer would be at most 2.8%, and the reverse situation, trimer contamination with dimer, would be about 3%. These numbers represent maximum estimates, and the true amounts of cross contamination are probably lower.

Discussion

The purpose of our current studies is to investigate the interaction of antibody with immune effector systems subsequent to the binding of antigen. In this paper we describe the preparation of IgG derivatives which we are currently using as models for antigen–antibody complexes to probe the mechanisms of interaction of complement with antibody, and the binding of immunoglobulin to lymphoid cells bearing receptors for IgG. The compounds mimic antigen–antibody complexes in that attachment occurs at the antibody binding site. Unlike normal complexes, however, the attachment is covalent, and therefore irreversible. This allows us to isolate stable complexes of known size, and to be certain that the size remains constant during experimentation (provided experiments are performed in the presence of excess (10^{-4} M) hapten). In previous studies (see review by Metzger, 1974), immunoglobulin aggregates, which were less well defined with respect to size and nature of cross-linking, were used to probe effector functions. In general, these have included antigen–antibody complexes, heat-induced aggregates, or aggregates formed by nonspecific chemical cross-linking. The complexes and aggregates thus formed have been, at best, restricted in heterogeneity with respect to molecular weight, and the results obtained from experiments using these preparations have been qualitative. With the covalent dimers and trimers, it should not only be possible to define with more precision the size of the complex needed to elicit a given response, but in some cases it should also be possible to measure binding constants of complexes to receptors.

Since the interaction of the dimers and trimers with effector systems is expected to increase greatly with size, it is important that the larger molecular weight contaminants in particular be kept to a minimum. As shown in Figure 4, these oligomers do appear to be quite pure under denaturing conditions, except for trace contaminants of the trimer with tetramer and perhaps pentamer. In other preparations we have reduced this latter contamination with some loss in yield by taking a narrower trimer fraction. Under nondenaturing conditions the possibility of reaggregation exists, as suggested by the appearance of insoluble material when monovalent hapten is removed from dimer and trimer at concentrations greater than 1 mg/ml. In the presence of hapten, however, noncovalent aggregation has not been observed (Figures 2, 3, and 5). Experiments using the affinity cross-linked oligomers, therefore, should employ buffers containing 10^{-3} to 10^{-4} M hapten.

The bivalent affinity label used in these studies contains a relatively short spacer. Assuming about 3 Å for the ethylenediamine group, 3 Å for each prolyl residue, and 5 Å from the prolyl N to the acetylmethylene group of BADL, we would estimate that the distance between points of attachment could reach 19 Å. The Fab fragments are about 40 × 50 Å in cross section so that, unless the point of covalent attachment lies near the circumference of an Fab, bridging of two Fab's within the same molecule would be sterically unfavorable. (BADL-Pro)$_2$-EDA is therefore being used to link binding sites from different molecules. We have not looked for internal cross-linking with (BADL-Pro)$_2$-EDA, but plan to do so with homologous compounds containing more than two prolyl residues.

Although the size of the immunoglobulin complexes as well as the maximum length of the covalent bridges are known, it has not been determined to what degree, if any, the products are cyclic. It has previously been shown by Valentine and Green (1967) and by Hyslop et al. (1970) that the interaction of anti-Dnp antibodies with bivalent haptens yields largely cyclic dimers and trimers, in agreement with theory (Crothers and Metzger, 1972; Schumaker et al., 1973). It might be expected, therefore, that the covalent products described here are also largely cyclic.

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References

Ackers, G. K. (1970), *Adv. Protein Chem. 24,* 343.
Connell, G. E., and Painter, R. H. (1966), *Can. J. Biochem. 44,* 371.
Crothers, D. M., and Metzger, H. (1972), *Immunochemistry 9,* 341.
Davies, D. R., Padlan, E. A., and Segal, D. M. (1975), *Contemp. Top. Mol. Immunol. 4,* 127.
Dickler, H. B. (1976), *Adv. Immunol.* (in press).
Dorrington, K. J., and Painter, R. H. (1974), *Prog. Immunol., Int. Congr. Immunol., 2nd 1,* 75.
Givol, D. (1974), *Essays Biochem. 10,* 73.
Hyslop, N. E., Dourmashkin, R. R., Green, N. M., and Porter, R. R. (1970), *J. Exp. Med. 131,* 783.
Kulczycki, A., and Metzger, H. (1974), *J. Exp. Med. 140,* 1676.
Mannik, M., Arend, W. P., Hall, A. P., and Gilliland, B. C. (1971), *J. Exp. Med. 133,* 713.
March, S. C., Parikh, I., and Cuatrecasas, P. (1974), *Anal. Biochem. 60,* 149.
Metzger, H. (1974), *Adv. Immunol. 18,* 169.
Müller-Eberhard, H. J. (1975), *Annu. Rev. Biochem. 44,* 697.
Plotz, P. H. (1976), Abstracts of the Meeting of the American Rheumatism Association, Chicago, June 10–11.
Reid, K. B. M., Lowe, D. M., and Porter, R. R. (1972), *Biochem. J. 130,* 749.
Reid, K. B. M., and Porter, R. R. (1975), *Contemp. Top. Mol. Immunol. 4,* 1.
Schumaker, V. N., Calcott, M. A., Spiegelberg, H. L., and Müller-Eberhard, H. J. (1976), *Biochemistry 15,* (in press).
Schumaker, V. N., Green, G., and Wilder, R. L. (1973), *Immunochemistry 10,* 521.
Sonoda, S., and Schlamowitz, M. (1970), *Immunochemistry 7,* 885.
Unkeless, J. C., and Eisen, H. N. (1975), *J. Exp. Med. 142,* 1520.
Valentin, R. C., and Green, N. M. (1967), *J. Mol. Biol. 27,* 615.
Waldmann, T. A., Strober, W., and Blaese, R. M. (1971), *Prog. Immunol. 1,* 891.
Weinstein, Y., Wilcheck, M., and Givol, D. (1969), *Biochem. Biophys. Res. Commun. 35,* 694.

Abundance of tRNA^{Phe} Lacking the Peroxy Y-Base in Mouse Neuroblastoma†

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ABSTRACT: Affinity chromatography on anti-Y (Y is a tricyclic imidazopurine to which is attached a complex four-carbon side chain) antibody immobilized to Sepharose was used to determine the proportion of rat liver tRNA^{Phe} species containing the peroxy Y-nucleoside. Unfractionated mammalian tRNA was aminoacylated with labeled phenylalanine. The phenylalanyl-tRNA was then chemically acetylated to yield *N*-acetylphenylalanyl-tRNA. When this preparation was applied to the antibody column, between 6–10% of the radioactivity was not bound to the column, indicating a deficiency of peroxy Y-nucleoside in a minor isoaccepting tRNA^{Phe} species. In contrast to normal tissues (including embryonic tissue), about 85% of the tRNA^{Phe} from mouse neuroblastoma C-1300 or N-18 tumors lack the peroxy Y-base, a property which is not affected by tumor age. Rat liver labeled *N*-acetylphenylalanyl-tRNA preparations were resolved on Plaskon chromatography (RPC-5) into two minor peaks closely followed by a major component. A high proportion of the two minor tRNA^{Phe} species was unable to bind to anti-Y antibodies. Upon mild acid treatment, the minor and major tRNA^{Phe} species eluted simultaneously from Plaskon columns, at a much reduced salt concentration. These results would indicate that the two minor tRNA^{Phe} species from rat liver as well as the major component contain a tricyclic imidazopurine base that differs from each other in its side chain. About 85% of the *N*-acetylphenylalanyl-tRNA from neuroblastoma was resolved by Plaskon chromatography as an early eluting peak. The position of this major neuroblastoma tRNA^{Phe} species was not altered by mild acid treatment, and its elution position from the column almost coincides with that of acid-treated normal rat liver tRNA^{Phe}. The latter results would suggest that most of the tRNA^{Phe} chains from neuroblastoma lack the tricyclic imidazopurine of normal rat liver tRNA^{Phe}, but are very close if not identical in primary nucleotide sequence.

Mammalian tRNA^{Phe} contains a highly modified peroxy Y[1]-nucleoside located next to the 3′ end of the anticodon. The mammalian peroxy Y-nucleoside differs from the yeast Y-nucleoside by the presence of a hydroxy peroxide group on the β carbon of the side chain (Blobstein et al., 1973). It was previously shown that goat antibodies against yeast tRNA^{Phe} are specific to the Y-nucleoside (Fuchs et al., 1974) and cross-react with the peroxy Y-nucleoside from rat liver tRNA^{Phe} (Salomon et al., 1975). These antibodies when immobilized on Sepharose enabled us to determine the proportion of rat liver

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[1] Abbreviations used are: Y, tricyclic imidazopurine to which is attached a complex four-carbon side chain; Na_2EDTA, disodium ethylenediaminetetraacetate.