Immunochemical Characterization of the Antigenic Determinant of a Synthetic Polypeptide*

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ABSTRACT: Synthetic poly-Glu^{42}-D-Lys^{28}Ala^{30} (GLA^{30} (LDL)) has a molecular weight of approximately 26,000 and is immunogenic in several species (sheep, rabbit, guinea pig). The antibody elicited in 2 sheep shows restricted cross-reactivity, giving 50% and 90% reaction with one isomeric polymer, GLA^{30} (LLL), but less than 10% reaction with several others GLA^{30} (DDD), (DLL), (LLD). After digestion with chymotrypsin, pronase, papain, or pepsin, the GLA^{30} (LDL) polymer becomes more than 80% dialyzable. The digests no longer precipitate with homologous sheep and rabbit antisera (containing 50–200 μg of antibody N per ml), but are excellent inhibitors in the quantitative precipitin reaction. Approximately 25 μg of digest N per ml of antiserum produces 50% inhibition. The dialyzable fraction of the chymotryptic digest was chromatographed on Sephadex (G-50 and DEAE) and a peptide was isolated which was electrophoretically homogeneous and retained full serological activity. This peptide has a molecular weight of approximately 2300, determined by analytical ultracentrifugation, a sedimentation constant of 0.5 S, an intrinsic viscosity ([η]) of 0.056 dl/g and an amino acid ratio, glutamic:lysine:alanine of 6:8:6. Evaluation of the viscosity–molecular weight data with the Flory–Fox relationship (Flory, P. J., and Fox, T. G. (1951), *J. Amer. Chem. Soc. 73*, 1904) yields an approximate end-to-end distance of 40 Å. When the physical constants were analyzed by the Scheraga–Mandelkern equation (Scheraga, H. A., and Mandelkern, L. (1953), *J. Amer. Chem. Soc. 75*, 179) a β value of 1.94×10^6 was obtained, which indicates internal consistency of this data. From the above it is concluded that the 20 amino acid peptide may have conformationally restricted regions and contains most of the antigenic determinant(s) of the parent polymer.

For a number of years synthetic α-amino acid polymers have been employed as antigens to investigate the nature of the immune response (Maurer, 1964; Sela, 1966). Considerable insight into the size of peptide antigenic determinants has been gained through studies of the specificity of the antibodies elicited with polypeptidyl proteins. Thus Arnon *et al.* (1965) demonstrated that rabbit antibodies to poly-L-lysyl rabbit serum albumin were most effectively inhibited by oligolysines composed of 5–6 residues. In another study Sage *et al.* (1964) found penta-L-alanine to be the best inhibitor of anti-poly-L-alanyl bovine serum albumin antibodies. Similar results have been obtained with the anti-poly-D-alanyl human serum albumin system (Schechter and Sela, 1965a). In subsequent reports (Schechter and Sela, 1965b, 1967) the specificity of the immunoglobulin G fraction as well as whole antisera to poly-DL-alanyl proteins was demonstrated to be preferentially directed toward D-alanyl residues. In this system tetraalanine was an excellent inhibitor, but only if it was of the D configuration. Recently this work has been extended to show that both IgG and IgM antibodies recognize the same size unit—*i.e.*, a pentapeptide (Haimovich *et al.*, 1969).

In contrast, the antigenic determinants of linear co- and terpolymers of α-amino acids have received little attention, although these materials are generally quite potent immunogens (Maurer, 1964). Inhibition studies with individual amino acids and related compounds have not proven of much value in these systems (Gill *et al.*, 1963). However earlier reports (Gill *et al.*, 1968, 1965) have indicated that it may be feasible to define the determinants of polypeptides through a study of the peptides obtained by enzymatic hydrolysis. We have therefore begun the isolation and characterization of immunologically active fragments prepared by degradative methods. The results obtained with one such polypeptide, G^{42}L^{28}A^{30} (LDL) (Table I), provide the basis of this report.

Materials and Methods

Polypeptides. The synthetic polymers used in this study (Table I), obtained from the Pilot Chemical Company, Watertown, Mass., were synthesized by the *N*-carboxyanhydride method previously described (Katchalski and Sela, 1958) using an anhydride:initiator ratio of 200–300:1. Aqueous solutions of the polymers were adjusted to pH 7.0 and dialyzed against deionized water before use. Oligopeptides (hexaglutamic acid and pentaalanine) were purchased from Yeda, Rehovot, Israel, and used without further purification.

Enzymes. The enzymes employed, along with suppliers, were as follows: α-chymotrypsin, 3 X crystallized, Worthington Biochemicals (Lot 700-706); pepsin, 3 X crystallized, Pentex, Inc. (Lot EZ2462); papain, 2 X crystallized, Worthington Biochemicals (Lot 5577); pronase, C grade, Calbiochem (Lot 34062).

Antisera. Adult sheep received 8–10 foreleg injections of 20 mg of the GLA^{30} (LDL) polymer in complete Freund's adjuvant over a 9-month period. Periodic bleedings (100–300 ml) were taken and when antibody levels of 0.5–1.0 mg per ml were reached the aminals were exsanguinated. Individual sera from the same sheep which contained similar amounts of precipitating antibody were pooled—provided the bleedings were not too widely spaced in time. Thus, the sequence of letters or

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TABLE I: Synthetic Polymers of α-Amino Acids Used in This Study.

Polymer[a]	Prepn No.	Abbreviation	Approximate Mol Wt
Poly-Glu^{60}-Ala^{40}	M-56	G^{60}A^{40} (LL)	40,000[b]
Poly-Glu^{42}Lys^{28}Ala^{30}	M-21-F	GLA^{30} (LLL)	50,000[b]
Poly-Glu^{42}Lys^{28}-D-Ala^{30}	M-52	GLA^{30} (LLD)	30,000[b]
Poly-Glu^{42}-D-Lys^{28}-Ala^{30}	M-53-C	GLA^{30} (LDL)	26,000[c]
Poly-D-Glu^{42}Lys^{28}-Ala^{30}	M-50	GLA^{30} (DLL)	21,000[b]
Poly-D-Glu^{42}-D-Lys^{28}-D-Ala^{30}	M-54	GLA^{30} (DDD)	70,000[b]

[a] Polypeptide nomenclature based upon recommendation of IUPAC-IUB Commission on Biochemical Nomenclature, 1967. [b] From Maurer (1965a). [c] Determined in this report

numerals following any animal number represent the sequence of bleedings, with Exs representing exsanguination. The sera were routinely decomplemented with specific precipitates of bovine serum albumin rabbit anti-bovine serum albumin (Maurer *et al.*, 1964), filtered, centrifuged at 10,000 rpm for 4 hr, and stored frozen in 5-ml aliquots. The other sheep antisera used in this study, *i.e.*, anti-GLA^{30} (LLL), anti-G^{60}A^{40}, anti-dinitrophenyl (DNP) bovine serum albumin were prepared in a similar fashion.

New Zealand white rabbits were immunized in the footpads, with a total of 12 mg of the GLA^{30} (LDL) polymer per animal (in complete Freund's adjuvant) over a 4-month period. Serum pools were prepared and stored as described above.

Immunological Assays. Precipitin tests were performed on undiluted sera, as previously described (Maurer *et al.*, 1964), except that antibody content was estimated with the Folin–Ciocalteau reagent from a calibration curve prepared with a polymer–antipolymer specific precipitate.

Quantitative precipitin inhibition tests were carried out by adding varying quantities of inhibitors, dissolved in 0.15 M phosphate–saline, pH 7.2 (PBS) (Kabat and Mayer, 1961) to a constant volume of undiluted serum. After 45 min at 37° each tube received an amount of precipitinogen designed to give maximal precipitation. After an additional 30-min incubation at 37°, the tests were stored overnight at 4° and then centrifuged, washed, and analyzed as in the precipitin assay. Controls included: (1) serum with precipitinogen but with no inhibitor and (2) serum with inhibitor but with no precipitinogen. The per cent inhibition was calculated from eq 1

$$\% \text{ inhibition} = (1 - X/Y)(100) \quad (1)$$

where X = amount of antibody precipitated in the presence of inhibitor and Y = amount of antibody precipitated in the absence of inhibitor.

Passive cutaneous anaphylaxis (PCA) tests were performed in guinea pigs as previously described (Ovary, 1958; Maurer, 1965a).

The passive hemagglutination assay was essentially that described by Maurer *et al.* (1963) except that a polymer solution of 50 μg/ml was used to coat the tannic acid treated erythrocytes. Results are expressed as the highest dilution of serum producing complete agglutination.

Analytical Procedures. A Beckman Model DU spectrophotometer was used for both ultraviolet and visible absorbancy measurements. Total nitrogen content was measured by the Markham (1942) modification of the micro-Kjeldahl technique. Amino-terminal amino acids were identified with 1,4-dinitrofluorobenzene as described by Jarvis and Strominger (1967). Ether-soluble DNP-amino acids were identified by thin-layer chromatography on Brinkman silica gel glass plates (F-254) in the following solvent systems: (1) chloroform–methanol–glacial acetic acid (95:5:1), (2) benzene–pyridine–glacial acetic acid (80:20:2), (3) solvent 2 followed by solvent 1. The individual spots were quantitated, after chromatography in solvent 1, by elution of the gel with ethanol and determining the absorbancy of the eluates at 360 nm, assuming ϵ_M 1.72×10^4 for DNP-alanine and 1.74×10^4 for DNP-glutamic acid (Rauen, 1956). The acid phase of the extraction mixture was chromatographed in 1-butanol–pyridine–glacial acetic acid–water (68:40:14:25). The only product identifiable was ε-DNP-lysine. It was also quantitated spectrophotometrically at 360 nm using an ϵ_M 1.75×10^4 (Rauen, 1956). Qualitative N-terminal analysis was done by a modification of the phenyl isothiocyanate reaction of Edman (1953; Piggot and Press, 1967; Margoliash, 1962).

The enzyme digests were also chromatographed on silica gel plates, and peptides revealed with 0.25% ninhydrin in acetone.

Amino Acid Analysis. Samples (2.5 mg/ml of 6 N HCl) were hydrolyzed in sealed tubes under nitrogen for 22 hr at 105°. Amino acids were then determined by the method of Spackman *et al.* (1958) with a Phoenix Model K-500 automatic analyzer on a 60 cm Piez-Morris column at 50°.

High-Voltage Paper Electrophoresis. This was done on Whatman 3MM paper in a Savant flat plate apparatus (pH 3.6 and 6.0) at 1000–2000 V for 1–2 hr, as well as in a tank-type assembly (pH 3.6) at 2200 V, 45 min. The buffers were mixtures of pyridine and acetic acid. Peptides were detected with 0.25% ninhydrin in acetone.

Analytical Ultracentrifugation. All sedimentation analyses were done in a Spinco Model E ultracentrifuge at 20°. Molecular weights were determined by equilibrium sedimentation, using the low speed technique (Richards and Schachman, 1959) for the peptide and the high speed technique (Yphantis, 1964) for the polymer. A Yphantis six-chambered cell was used with diluent serving as reference solvent. The equilibrium distribution of the samples were determined with Rayleigh optics, and the molecular weights evaluated from

$$M = \frac{2RT}{\omega^2(1 - \bar{v}\rho)(r_b^2 - r_a^2)} \times \frac{J_{eq}}{J_{sb}} \quad (2)$$

where R is the gas constant, T is the absolute temperature, ω is the rotor angular velocity, r_a and r_b are the distances from the center of rotation to upper and lower column miniscl, J_{eq} is the concentration difference across the cell (measured in fringes), and J_{sb}, measured from a synthetic boundary experiment is the fringe count proportional to the initial concentration of polymer. $\bar{v}$ is the partial specific volume of the solute calculated from the amino acid composition and ρ the solution density.

The molecular weight of the peptide isolated from a chymotryptic digest (BPI) was also evaluated graphically from a plot

of ln c (concentration) *vs.* r^2 according to the equation

$$M = \frac{2RT}{\omega^2(1 - \bar{v}\rho)} \times \frac{\mathrm{d} \ln c}{\mathrm{d}r^2} \quad (3)$$

after calculation of the meniscus concentration (c_m).

Sedimentation velocity experiments were performed in double sector synthetic boundary cells, as well as single sector cells, with schlieren optics. Rotor speeds were 41,000–56,000 rpm. A Nikon comparator was used to analyze all photographic plates.

Viscosity. A Cannon–Ubbelohde suspended level, semimicro dilution viscometer was used for these studies. The flow time at 25.00° for solvent (0.1 M NH_4HCO_3) and solution was determined to within 0.01 sec. Due to the long efflux times (over 200 sec) and low molecular weights, kinetic energy, and shear corrections were neglected. Data are reported in terms of intrinsic viscosity ([η]), obtained from a plot of concentration (c) *vs.* η_{sp}/c to infinite dilution, where

$$\eta_{sp} = \frac{\text{outflow time solution}}{\text{outflow time solvent}} - 1 \quad (4)$$

Enzymatic Hydrolyses. All digestions were carried out with the aid of a Radiometer TTIC pH-Stat apparatus. In each case the polymer was dissolved in water (5–10 mg/ml) and adjusted to the pH recommended by the enzyme supplier with HCl or NaOH. In the experiment with papain the polymer solution was made 0.005 M in dithiothreitol. After addition of enzyme (final concentration 0.05–0.1 mg/ml), the reaction was allowed to proceed at room temperature for 24–48 hr. The digests were then neutralized and lyophilized.

Column Chromatography. Gel filtration of the enzyme digests were done on Sephadex G-50 superfine, which had been swollen in 0.01 M NH_4HCO_3 solution. DEAE-Sephadex A-50 was used for ion-exchange chromatography, after conversion to the bicarbonate form.

Column effluents were monitored for peptide content with a Vanguard 1056 OD analyzer at 220 nm.

"*Discontinuous Dialysis.*" In one experiment the chymotryptic digest of GLA^{30} (LDL) was dialyzed against small volumes of water (one-third the digest volume) and the dialysate was removed and replaced with fresh water at 0.5, 1, 2, 5, 12, 24, 72, and 172 hr. Each dialysate was lyophilized, dissolved in water to identical concentrations, and examined by tlc and precipitin inhibition tests.

Results

Antibody Response to GLA^{30} (LDL). Both sheep (*i.e.*, 474 and 486) as well as 3 out of 6 rabbits responded with precipitating antibody to this polymer. The remaining 3 rabbits, though negative in precipitin tests, gave positive PCA reactions with GLA^{30} (LDL). We have been unable under normal test conditions (Maurer, 1965a), to obtain PCA reactions in guinea pigs with any sheep antiserum. The precipitin curves obtained with exsanguination bleedings of the 2 sheep as well as rabbit 987 are shown in Figure 1. The curves obtained with other bleedings did not differ significantly from those shown. In no serum could a "prozone" (inhibition of precipitation) be detected at low antigen concentrations. These sheep sera did not precipitate with the optically variant polymers GLA^{30} (LLD), (DLL), (DDD) or with the G^{60}A^{40} (LL) polymer. However, the GLA^{30} (LLL) polymer precipitated 21 μg of antibody N/ml from serum 474 exs and 180 μg of antibody N/ml from serum 486 exs. This amounts to 30% of the homologous reaction in the former serum and 90% in the latter serum.

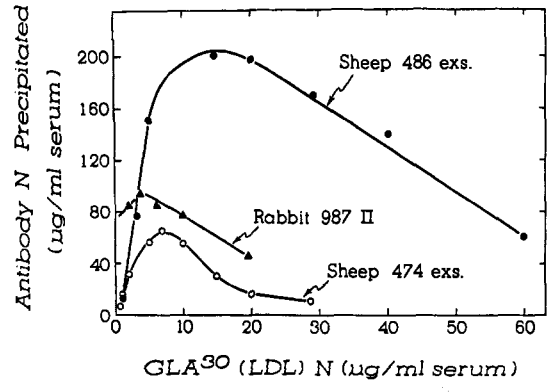

FIGURE 1: Quantitative precipitin analysis of sheep and rabbit anti-GLA^{30} (LDL) sera with the homologous polymer. Varying quantities of GLA^{30} (LDL) were added to a constant amount of antiserum, incubated at 37° for 30 min and then at 4° for 2–5 days. The samples were centrifuged and the precipitates analyzed for protein with the Folin–Ciocalteau reagent.

The GLA^{30} (LDL) polymer was tested by passive hemagglutination with 4 sheep anti-GLA^{30} (LLL) sera. Three of these sera gave a positive reaction (titers of 1:16–1:64); one (628) was completely negative, although this serum contained more than 1 mg per ml of antibody precipitable by the homologous antigen.

Enzymatic Degradation of GLA^{30} (LDL). All of the enzymes used degraded this polymer on the basis of NaOH or HCl uptake in the pH-Stat and the appearance of free amino acids and peptides by tlc. The extent of degradation was measured only in the chymotryptic digest, where in 24 hr approximately 20–25 amino groups were liberated per mole of intact polymer. This was an upper limit of degradation as evidenced by the fact that no further increase in ninhydrin color was obtained at 48 hr. All of the digests (*i.e.*, chymotrypsin, papain, pepsin, pronase) were more than 80% dialyzable, indicating significant cleavage in each case.

When 500 mg of the digests were applied to Sephadex G-50 columns (2.5 × 97 cm) each showed the same elution pattern, demonstrated in the upper frame of Figure 2 for only the chymotryptic digest, which was composed of two distinct peaks (B and C). The elution profiles for each enzyme digest were superimposable with the exception of the pronase products in which case no peak C could be found.

Immunological Properties of the Digests. None of the enzymatic digests were able to precipitate antibody from the anti-GLA^{30} (LDL) sera. However, each could inhibit precipitation (>85%). The fractions (0.5 → 172 hr) from "discontinuous dialysis" gave identical inhibition curves and tlc spots. The major peaks (*i.e.*, those eluted at the same point as chymotryptic P-B [Figure 2]) from the G-50 columns were very efficient inhibitors of precipitation. In each instance, 50% inhibition of serum 486 exs was obtained with 20–25 μg of peptide nitrogen per ml of serum. In contrast, the minor peaks (P–C) were rather poor inhibitors where 3 mg/ml of serum produced a maximum inhibition of 14% with chymotrypsin P–C, 17% with papain P–C, and 20% with pepsin P–C.

Purification of the Chymotryptic Fragments of GLA^{30} (LDL). Previous kinetic analyses of the degradation of GLA^{30} (LDL) had indicated that chymotrypsin treatment would provide fragments of a size amenable to both chemical and serological

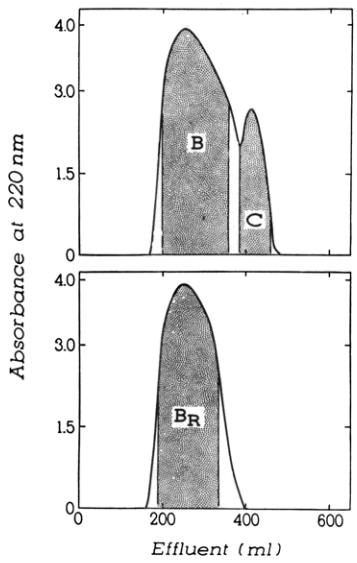

FIGURE 2: Sephadex G-50 fractionation of chymotryptic digest of GLA^{30} (LDL). Column dimensions 2.5 cm × 97 cm. Upper pattern, approximately 500 mg of digest applied in 0.01 M NH_4HCO_3; lower pattern, refractionation of peak B on same column. Eluent was 0.01 M NH_4HCO_3 in each case.

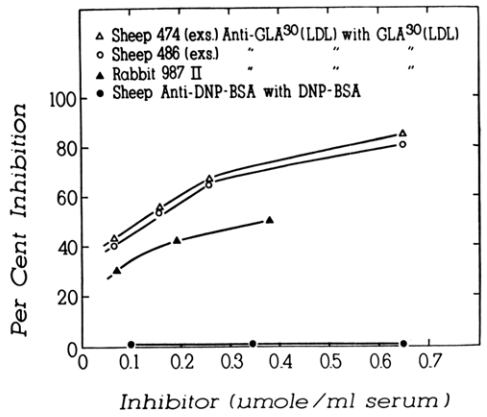

FIGURE 3: Inhibition of precipitation by chymotryptic peptide (BPI). Varying quantities of inhibitor were added to a constant volume of antiserum. After 45 min at 37°, precipitinogen (homologous antigen) was added, reincubation followed at 37° for 30 min and overnight at 4°. The precipitates were analyzed as described in the text.

analysis. Therefore, attempts were made to further purify the products of this enzymatic treatment. As mentioned above, the chymotryptic digest was first fractionated on Sephadex G-50. The major peak (B) was then rechromatographed on the same column (Figure 2, bottom frame), and the upper 50% of the peak (B_R) pooled and lyophilized. This material was dissolved in 0.01 M NH_4HCO_3 at a concentration of 60 mg/ml and applied to a column of DEAE-Sephadex A-50 and eluted stepwise with increasing concentrations of NH_4HCO_3. Seven column volumes of the starting buffer eluted 85–90% of the applied material in a broad peak. Increasing the salt concentration to 0.5 M eluted all of the remaining peptide. Thin-layer chromatography demonstrated that only the first eluate (0.01 M) was free from smaller peptides. Although preliminary experiments indicated that the entire 0.01 M fraction was immunologically and electrophoretically similar, only the first column volume of eluate (BPI) was pooled for further analysis. This procedure led to a yield of only 30–35% of the applied material but was expected to reduce heterogeneity.

Physicochemical Properties of GLA^{30} (LDL) and Peptide BPI. Only one ninhydrin-positive spot was detectable after high-voltage electrophoresis for each material. Likewise, only one peak was evident in each case during sedimentation equilibrium experiments. The sedimentation coefficients of the peptide BPI and intact GLA^{30} (LDL) polymer were 0.45 S and 1.40 S, respectively. No concentration dependence was apparent.

The molecular weight of the polymer, calculated from eq 2, was 26,000. That of the peptide, calculated from the ln *c vs.* r^2 plot, was 2343. This figure represents an average obtained from substituting the slope of the line drawn through the experimental points into eq 3. However, the molecular weights did not differ significantly at either extreme, *i.e.*, 2300 at the column meniscus and 2393 at the column bottom. The uniformity of values obtained throughout the column (fitting a straight line in a ln *c vs.* r^2 plot) indicates a solute of homogeneous molecular size.

Extrapolated intrinsic viscosity values ([η]) were 0.162 dl/g for GLA^{30} (LDL) and 0.056 dl/g for peptide BPI. Neither substance demonstrated any significant concentration dependence under these conditions.

Quantitative amino acid analysis of BPI revealed glutamic acid, alanine, and lysine in the molar ratio of 6.2:8.3:5.8 (average of 3 determinations). Assuming limited heterogeneity in the peptide chain length and a reasonable accuracy in the ultracentrifugal molecular weight, the determined molar ratios would best reflect an overall composition of 6 glutamic acid, 8 lysine, and 6 alanine residues per chain of peptide. The molecular weight calculated from such a composition, and used in these studies, is 2224. On a nitrogen basis, 95% of the applied sample could be accounted for in the three amino acids. The combined yield of N-terminal amino acids, determined spectrophotometrically after FDNB treatment, was only 70% of that expected for BPI on the basis of a molecular weight of 2224. This figure has been corrected for the loss of DNP-amino acids due to hydrolysis. Only DNP-alanine and DNP-glutamic acid (in the ratio of 77:23) could be detected in the ether phase of the reaction mixture. No di-DNP-lysine was present. The ε-DNP-lysine recovered from the aqueous phase accounted for 78–88% of that expected from a $G^6L^8A^6$ composition. After one cycle of Edman degradation, only the phenylthiohydantoin derivatives of glutamic acid and alanine could be identified, with the latter compound qualitatively predominant. It can be concluded, therefore, that lysine is not an amino-terminal residue in this peptide.

Immunological Characterization of the Chymotryptic Fragment BPI. This peptide was an effective inhibitor of both sheep and rabbit anti-GLA^{30} (LDL) sera (Figure 3). The specificity of the inhibitor was ascertained by examining its effect on the precipitation of the unrelated system, DNP-bovine serum albumin sheep anti-DNP-bovine serum albumin (Figure 3) where quantities of BPI up to 0.65 μmole were completely without effect. Inhibition tests with P–C (*i.e.*, the undefined peptide mixture from Sephadex G-50) in the presence of BPI produced curves 5–10% above those given by peptide BPI alone, except at the higher levels of BPI where no enhancement of the inhibition could be effected with ratios of P–C: BPI of 2. This would seem to indicate contamination of P–C with BPI.

The activity of peptide BPI as an inhibitor of precipitation is presented in the curves of Figure 3; 0.10–0.15 μmole gives

TABLE II: Quantitative Inhibition Data for Peptide BPI in the Sheep and Rabbit GLA^{30} (LDL) Systems.

Serum[a]	I_r 25%	I_r 50%	K_i (× 10^6)
Sheep 486A	6.6	23.5	54,800
B	4.4	15.4	81,300
C	3.5	10.4	93,800
Exs	3.3	13.1	112,500
474	8.0	22.2	38,800
B	2.4	8.3	150,000
Exs	9.4	34.4	40,000
Rabbit 982 II	1.3	3.8	250,000
985 III	2.5	7.1	133,000
987 II	11.2	95.6	40,000

[a] Letters and numerals indicate the temporal sequence of bleedings. The abbreviation exs means exsanguination.

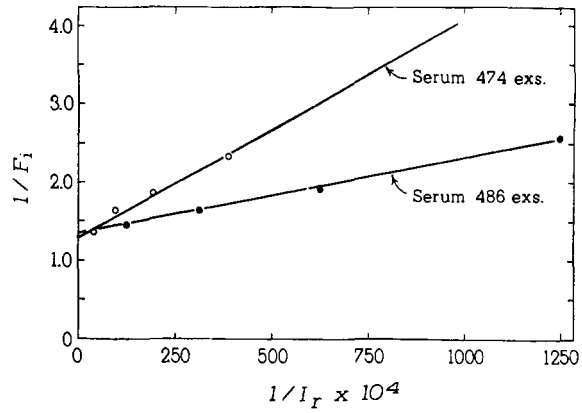

FIGURE 4: Double reciprocal plot of inhibition data with peptide inhibitor (BPI). The sera are sheep anti-GLA^{30} (LDL) pools. F_i is the fractional inhibition and I_r is the molar ratio of inhibitor to antibody.

50% inhibition of the sheep antisera, and 0.38 μmole gives 50% inhibition of rabbit 987 serum. With each serum almost complete inhibition (*i.e.*, 85%) could be obtained at higher inhibitor concentrations.

It appeared, however, that the molar concentration of inhibitor necessary to inhibit individual sera, either partially or completely, was somewhat misleading since the various sera differed considerably in the amount of precipitable antibody contained in a milliliter. A suitable solution to this problem was found empirically by plotting the reciprocal of inhibition *vs.* reciprocal of inhibitor to antibody ratio. This is essentially the same type of analysis used previously by Gill *et al.* (1963). The basic equation describing the inhibition data (using Gill's terminology) is as follows:

$$\frac{1}{F_i} = \frac{1}{K_i}\frac{1}{I_r} + A \qquad (5)$$

where F_i = fractional inhibition given by an inhibitor (*i.e.*, the per cent inhibition divided by 100), I_r = moles of inhibitor added per mole of precipitable antibody, K_i = "inhibition constant"—a dimensionless constant indicative of the absolute efficiency of inhibition, and A—a constant related to the maximum inhibition displayed, but not defined in this treatment (H. J. Callahan, P. H. Maurer, and P. A. Liberti, 1971, unpublished data). According to this equation a plot of $1/F_i$ *vs.* $1/I_r$ should yield a straight line with a slope equal to $1/K_i$. Since the inhibition constant thus obtained reflects not only the degree of inhibition obtained but also the quantity of precipitable antibody being examined in a unit volume of antiserum, it is a sensitive indicator of inhibition activity.

All of the inhibition tests performed with the purified inhibitor (BPI) were analyzed by this method (both sheep and rabbit antisera). A typical plot obtained with 2 sheep antisera (474 exs and 486 exs) is presented in Figure 4. The fact that the experimental points fall on a straight line over a wide range of concentrations indicates that the linear relationship of eq 5 is valid.

It should be realized that in addition to determining an inhibition constant for each serum, this type of mathematical analysis reveals the ratios of inhibitor to antibody necessary to produce a given degree of inhibition, by virtue of the fact that $1/F_i$ is directly proportional to $1/I_r$. The slope of this line, when substituted into eq 5, provides an I_r commensurate with any chosen value of F_i. Table II lists these values (for 25% and 50% inhibition) along with the respective K_i's of the sera. The K_i of each serum is denoted as X 10^6 only to simplify comparisons with the calculations of Gill *et al.* (1965), and should not be interpreted as being equivalent to a thermodynamic association constant (*i.e.*, l./mole). The table shows that the sera do vary in their inhibition constants, with about a fivefold difference evident between highest and lowest values both inter- and intraspecies. As would be expected an increase in I_r is observed with decreasing K_i.

The commercially prepared oligopeptides, hexaglutamic acid and pentaalanine, were also included in precipitin inhibition tests. Neither one produced any significant inhibition at concentrations of 5 mg/ml of antiserum. Oligomers of D-lysine have not yet been tested.

Discussion

Previous reports from this laboratory have shown that GLA^{30} (LDL) is immunogenic in rabbits and guinea pigs (Maurer, 1965a) but not in humans (Maurer, 1965b). We have now shown that this polymer is also a good immunogen in sheep, producing up to 200 μg of precipitable antibody N per ml of serum. The pattern of cross reactions in passive cutaneous anaphylaxis tests reported for rabbit antisera differs somewhat from those found in this study (by quantitative precipitin tests) with the sheep sera. It has been reported that rabbit anti-GLA^{30} (LDL) sera gave positive PCA reactions with the homologous antigen as well as with GLA^{30} (LLL) and GLA^{30} (LLD), and a weak reaction with GLA^{30} (DLL) and G^{60}A^{40} (LL). No cross-reaction with GLA^{30}(DDD) was found. In contrast, we find that neither sheep serum pool gave a significant precipitate with GLA^{30} (LLD), GLA^{30} (DLL), GLA^{30} (DDD), or G^{60}A^{40} (LL). It may well be, therefore, that the sheep antibody is more restricted in its specificity than the rabbit. This cannot be concluded at present since two different assays were used, which do not have the same sensitivity.

The significant cross-reaction of the sheep anti-GLA^{30} (LDL) sera with GLA^{30} (LLL) polypeptide raised the question of whether the GLA^{30} (LDL) polymer used here may be contaminated with GLA^{30} (LLL). This possibility is unlikely in

view of the inability of the GLA[30] (LDL) to precipitate any antibody from the control sheep anti-GLA[30] (LLL) (628). This serum contained 200 μg of precipitable antibody N per ml when tested with its homologous polymer, but no antibody reactive with the GLA[30] (LDL) polymer.

Chymotryptic treatment of the GLA[30] (LDL) polymer resulted in a completely nonprecipitating digest, which retained practically all of the antigenic determinants of the macromolecular polypeptide, insofar as the precipitating antibody reflects the specificity of any nonprecipitating antibody. Apparently, the active fragments are not grossly different in size since no difference could be detected in the fractions obtained by "discontinuous dialysis" with immunological or physical (*i.e.*, tlc) tests. Each dialysate could completely (*i.e.*, at least 85%) inhibit precipitation of GLA[30] (LDL) by homologous sheep antiserum, and a mixture of each fraction was no more efficient an inhibitor than the individual fractions alone. These results imply that the polypeptide might be composed of a number of similar (or identical) determinants which are separated by the action of chymotrypsin. This suggestion is supported by examining the yields of active fragment which are obtained during fractionation procedures. The active fraction obtained from Sephadex gel filtration (B_R) constitutes more than 75% of the digest. Further "purification" on DE-AE-Sephadex only removes ~25% by weight of the applied sample. The somewhat lower yields found in the large-scale preparation are the result of deliberate attempts to obtain more homogeneous material, rahter than clear-cut indications of distinct fractions. Even by the most conservative estimate the amount of "homogeneous" inhibitor obtained accounts for more than 25% of the intact polymer. Thus it appears likely that the antigenic areas of GLA[30] (LDL) constitute a major portion of the entire polypeptide chain.

It is possible to suggest a model which incorporates the above postulations by a theoretical analysis of the available physical data of the purified inhibitor as well as that of the intact macromolecule. These analyses are based on theories, which have been experimentally refined, relating size and shape to molecular weight, intrinsic viscosity, and sedimentation coefficient of a molecule. If the intrinsic viscosity (0.056 dl/g) of the purified inhibitor (BPI) is fitted to the Flory–Fox relationship (1950, 1951)

$$h^3 = \frac{MA}{2.1 \times 10^{21}} \tag{6}$$

where h is the end-to-end distance, M is the molecular weight, and A is the intrinsic viscosity, a value of $h = 40$ Å is calculated.

If the viscosity–molecular weight data are analyzed by use of the Simha model (Simha, 1940), somewhat different dimensions are obtained. Because the inhibitor is composed of 20 amino acids, it is reasonable to expect that the prolate ellipsoid would be a closer approximation of the polypeptide than the oblate. For prolate ellipsoids the Simha relation is

$$A = \frac{\bar{v}}{100} + \frac{f^2}{15(\ln 2f - 1.5)} + \frac{f^2}{5(\ln 2f - 0.5)} + \frac{14}{15} \tag{7}$$

where $\bar{v}$ is the partial specific volume of the polymer and f is the ratio of the semimajor to the semiminor axis (a/b) of the assumed model. The terms within the bracket are the Simha factor. The f of peptide BPI was calculated using a value of 0.72 cm^3/g for the partial specific volume. The calculated value of f can be converted to the Simha end-to-end distance, h_s, by equating the volume of the molecule, V_m, as determined from v, to the volume of an equivalent prolate ellipsoid, V_{pe}, thus

$$V_m = V_{pe} = \frac{\bar{v}M}{6.02 \times 10^{23}} = \frac{4}{3}fb^3 \tag{8}$$

from eq 8, h_s can be calculated as $h_s = 2a = 2fb$.

For peptide BPI h_s is 60 Å and $2b$ is 9 Å. These are the dimensions the inhibitor would have to have assuming it is a prolate ellipsoid impenetrable to solvent molecules. The length of this prolate ellipsoid is somewhat greater than the end-to-end distance obtained by the Flory–Fox theory (40 Å). In view of the fact that the Flory–Fox equation reflects a more realistic model of a polymer molecule in solution, and since it has been found that for extended macromolecules the Simha treatment tends to overestimate the length (Strauss and Smith, 1953; Liberti and Stivala, 1967), we have assumed that the 40 Å length is a more reasonable value. If the above calculations are made for an oblate ellipsoid, the major and minor axes are 60 Å and 6 Å, respectively. For a polypeptide which could be maximally extended to only 73 Å, this model seems less probable.

Even though we have considered the Flory–Fox end-to-end distance more acceptable than that obtained with the Simha relation, this latter determination is still important in that it places a theoretical maximum on values of h and thus M. In other words, an $[\eta]$ of 0.056 dl/g could not represent a molecular weight, for this peptide, of much more than 2200 by virtue of the Simha calculation.

The Flory–Fox end-to-end distance is significantly lower than what one would expect for an extended peptide composed of 20 amino acids. According to accepted interatomic distances such a peptide should measure 73 Å end-to-end (Pauling *et al.*, 1951). These results imply some conformational restrictions on the peptide inhibitor which are as yet unsubstantiated by other measurements. Assuming for the present that these values are correct, it could be argued that the intact molecule of GLA[30] (LDL) is made up of discrete, ordered regions interspersed with unordered segments. It might be that the conformationally restricted regions represent antigenic determinants, while the so-called "randomly ordered" sequences are nonantigenic but are the sites of chymotrypsin action. Such postulation is not without some precedent since Miller and Monroe (1968) have found that the nonhelical regions of polyglutamic acid and polylysine are probably the sites of papain attack.

Additional data obtained from physical measurements of the intact GLA[30] (LDL) polymer could be interpreted as supporting these conclusions. When the intrinsic viscosity (0.162 dl/g), sedimentation coefficient (1.4 S), and molecular weight of the GLA[30] (LDL) are substituted into the Scheraga–Mendelkern (1953) relationship

$$\beta = \frac{Ns_{20,w}[\eta]^{1/3}\eta_0}{M^{2/3}(1 - \bar{v}(\rho_0))} \tag{9}$$

a value of β equal to 1.94×10^6 is obtained. According to Noelken *et al.* (1965) these data would best fit a model analogous to a "rigid string of beads." For the GLA[30] (LDL) this might be interpreted as compact segments (40 Å) joined by extended (unordered) peptide chains. Although this hypothesis is only speculative, it is consistent with the data available

so far, and provides at least some working model for future studies with synthetic polypeptides of this type.

It should be noted that the Scheraga–Mandelkern relationship also serves as a test for the internal consistency of the physical constants obtained (*i.e.*, s, $[\eta]$, mol wt). Noelken *et al.* (1965) have found that the β function is a highly constant figure and varies only from 1.9×10^6 to 2.7×10^6 for proteins of grossly different hydrodynamic properties. Thus, a β showing significant deviation from this range implies some discrepancy in at least one of the measurements. Since the figures calculated for both the intact polymer ($\beta = 1.94 \times 10^6$) and the purified peptide inhibitor ($\beta = 2.16 \times 10^6$) fall within the expected range, an additional degree of confidence can be placed in the validity of these measurements. That the Scheraga–Mandelkern relationship holds for low molecular weight materials has been established in a recent report by Polson (1967).

Although the immunogenicity of linear synthetic polypeptides has been extensively studied, little information is available concerning the isolation and immunochemical characterization of low molecular weight inhibitors from such polymers. Gill *et al.* (1965) have prepared a fragment of approximately 20 amino acid residues which was obtained from the tryptic hydrolysate of poly-Glu^{52}Lys^{33}Tyr^{15} (internally cross-linked by a 2,4-dinitrophenylene bridge), and in a subsequent report (Gill *et al.*, 1968) described several peptides of chain length 13 to 19 amino acids, also isolated from tryptic digests of GLT and its derivatives, which were immunologically active.

As noted earlier, several investigators have elucidated the determinant regions of polypeptidyl proteins and branched synthetic polypeptides (Arnon *et al.*, 1965; Sage *et al.*, 1964; Schechter and Sela, 1965a,b; Haimovich *et al.*, 1969). These studies have shown that in every case a peptide of from four to six amino acid residues most closely approximates the antigenic determinant. In the present study, however, the inhibitor BPI contains about 20 amino acids. Although it cannot yet be said that this fragment represents the minimum size of the determinant in GLA^{30} (LDL), it would be reasonable to assume that the size is larger than that found for determinants in polypeptidyl protein, *i.e.*, four to six amino acids. This assumption is supported by the observation that hexaglutamic acid and pentaalanine as well as the lower molecular weight peptides separated from the chymotryptic digest are incapable of significantly inhibiting the homologous sheep antisera. On the other hand, it may be that the determinant involves a unique sequence of L-Glu, D-Lys, and L-Ala, or that sheep antibodies are different in specificity than rabbit. These possibilities are at present under investigation.

Several reports have shown the determinant regions of proteins to contain approximately 6–8 amino acid residues (Mitchell *et al.*, 1970; Goodman *et al.*, 1968), however, it would be misleading to consider only the number of amino acids present in the fragment. A better evaluation must include the absolute size of the fragments, as determined experimentally. Unfortunately, little of such information is available. This study is apparently the first attempt in this direction. The end-to-end distance of 40 Å calculated for BPI, would be in general agreement with the size estimated by Cebra (1961) for the peptides he isolated from silk fibroin (29–44 Å), if his assumption of random structure in the fragments is valid. The active peptides prepared from globular proteins have often been larger than hexapeptides, *e.g.*, 14–24 residues from ribonuclease (Brown, 1962), 15–19 residues from myoglobin (Crumpton and Wilkinson, 1965), 23 residues from carboxymethylated lysozyme (Gerwing and Thompson, 1968), 18 residues from TMVP (Benjamini *et al.*, 1964, 1965). It is possible, however, that the conformational restrictions known to be present in each of these proteins may influence the antigenic areas so that they are not linear peptides. In any case, it can be said that as an inhibitor the peptide BPI, both in size and number of amino acids, is similar to many of the protein determinants so far studied.

TABLE III: Quantitative Inhibition Data for Several Immune Systems.[a]

System and Inhibitor	I_R (25%)	$K_i \times 10^6$
Silk fibroin, dodecapeptides	1380	238
Polylysine, hexalysine	256	1,900
decalysine	33	4,500
Dextran, isomaltohexaose	34	10,200
Human serum albumin, 60 residue fragment	2.0	238,000
DNP, N^{ϵ}-DNP lysine	0.7	420,000
DPE (4) G^{52}L^{32}T^{15}, 20 residue fragment	1.3	300,000
GLA^{31} (LDL), 20 residue fragment	2.4[b]	150,000
	1.3[c]	250,000

[a] As summarized by Gill *et al.* (1965). [b] Sheep serum 474B. [c] Rabbit serum 982 II.

In comparing the immunological efficiency of peptide BPI with inhibitors obtained for other immune systems, a double reciprocal plot, *i.e.*, $1/F$ *vs.* $1/I_r$, has been used. This analysis has been employed by previous investigators (Gill *et al.*, 1963) with the assertion that it is a variation of a Langmuir adsorption isotherm (Klotz, 1953) describing the adsorption of small molecules onto polyelectrolytes. Although the form of this equation is related to the isotherm, we have been unable to prove a strict mathematical relationship and would thus prefer to consider our analysis as merely an expedient form of data analysis. In addition to "normalizing" the serological results in terms of the amount of precipitable antibody contained in a serum, and thereby permitting more exact comparisons between the potency of different inhibitors with various antisera, this form of analysis also yields the maximum degree of inhibition obtainable with a particular inhibitor in a given system. This value is easily determined from the double reciprocal plot by extrapolating the straight line to the ordinate intercept which represents infinite inhibitor concentration. This extrapolation has proven valid for a number of the sera used in this study and is being evaluated in several others (H. J. Callahan, P. H. Maurer, and P. A. Liberti 1971, unpublished data).

This type of immunochemical analysis does not yield an inhibition constant having any defined units, and further can provide only inhibitor:antibody ratios necessary to effect a given degree of inhibition, but not actual binding ratios. These ratios, as well as ratios of inhibitor:precipitinogen can only be accurately assessed using labeled peptides and polymers. Nonetheless, it is a useful method of comparison, as Table III shows. The data in this table, apart from the GLA^{30} (LDL) system, have been transposed from Gill *et al.* (1965). Both inhibitor:antibody ratios (I_r) and inhibition constants (K_i) must be considered within any system. The best inhibitor

listed is ε-DNP lysine with rabbit antiserum raised against DNP-bovine γ-globulin (Eisen and Siskind, 1964). However, this is a uniquely haptenic system and probably not comparable to the other polysaccharide or protein systems listed.

Excluding ε-DNP-lysine, the most potent inhibitors are enzymatically prepared fragments of synthetic polypeptides. The K_i of peptide BPI is 1000-fold greater than that of the dodecapeptide mixture of Cebra (1961), 50-fold higher than decalysine (Arnon *et al.*, 1965), 25-fold higher than isomaltohexaose (Kabat, 1956), and about equal to that of the 60 residue fragments obtained from human serum albumin (Press and Porter, 1962). Although the data shown for BPI in Table III were obtained with the sera best inhibited by this peptide, the results with the other serum pools were also striking. As Table II shows the inhibition constants in homologous systems ranged down to 39,000 (serum 474A), but the ratios for 25% inhibition were always at least three times lower than decalysine, isomaltohexaose, or the fibroin peptides in their respective systems. This is a clear-cut indication of greater efficiency of inhibition in BPI than most other systems.

Further studies on this unique peptide, such as its antigenicity in rabbits, guinea pigs, and sheep as well as binding tests and sequential enzymatic degradation have been initiated. It is anticipated that these investigations may elucidate the underlying causes of its unusual immunological potency.

References

Arnon, R., Sela, M., Yaron, A., and Sober, H. A. (1965), *Biochemistry 4*, 948.

Benjamini, E., Young, J. D., Peterson, W. J., Leung, C. Y., and Shimizu, M. (1965), *Biochemistry 4*, 2081.

Benjamini, E., Young, J. D., Shimizu, M., and Leung, C. (1964), *Biochemistry 3*, 1115.

Brown, R. K. (1962), *J. Biol. Chem. 237*, 1162.

Callahan, H. J., Maurer, P. H., and Liberti, P. A. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 736.

Cebra, J. J. (1961), *J. Immunol. 86*, 190.

Crumpton, M. J., and Wilkinson, J. M. (1965), *Biochem. J. 94*, 545.

Edman, P. (1953), *Acta Chem. Scand. 7*, 700.

Eisen, H. N., and Siskind, G. W. (1964), *Biochemistry 3*, 996.

Flory, P. J., and Fox, T. G. (1950), *J. Polym. Sci. 5*, 745.

Flory, P. J., and Fox, T. G. (1951), *J. Amer. Chem. Soc. 73*, 1904.

Gerwing, J., and Thompson, K. (1968), *Biochemistry 7*, 3888.

Gill, T. J., III, Kunz, H. W., Friedman, E., and Doty, P. (1963), *J. Biol. Chem. 238*, 108.

Gill, T. J., III, Kunz, H. W., and Marfey, P. S. (1965), *J. Biol. Chem. 240*, PC2337.

Gill, T. J., Papermaster, D. S., Kunz, H. W., and Marfey, P. S. (1968), *J. Biol. Chem. 243*, 287.

Goodman, J. W., Nitecki, D. E., and Stoltenberg, I. M. (1968), *Biochemistry 7*, 706.

Haimovich, J., Schechter, I., and Sela, M. (1969), *Eur. J. Biochem. 7*, 537.

Jarvis, D., and Strominger, J. L. (1967), *Biochemistry 6*, 2591.

Kabat, E. A. (1956), *J. Immunol. 77*, 377.

Kabat, E. A., and Mayer, M. M. (1961), Experimental Immunochemistry, C. C Thomas, Springfield, Ill., p 120.

Katchalski, E., and Sela, M. (1958), *Advan. Protein Chem. 13*, 243.

Klotz, I. M. (1953), *Proteins 1*, 727.

Liberti, P. A., and Stivala, S. S. (1967), *Arch. Biochem. Biophys. 119*, 510.

Margoliash, E. (1962), *J. Biol. Chem. 237*, 2161.

Markham, R. (1942), *Biochem. J. 36*, 790.

Maurer, P. H. (1964), *Progr. Allergy 8*, 1.

Maurer, P. H. (1965a), *J. Exp. Med. 121*, 339.

Maurer, P. H. (1965b), *J. Immunol. 95*, 1095.

Maurer, P. H., Gerulat, B. F., and Pinchuck, P. (1963), *J. Immunol. 90*, 381.

Maurer, P. H., Gerulat, B. F., and Pinchuck, P. (1964), *J. Biol. Chem. 239*, 922.

Miller, W. G., and Monroe, J. (1968), *Biochemistry 7*, 253.

Mitchell, B., Levy, J. G., and Nitz, R. M. (1970), *Biochemistry 9*, 1839.

Noelken, M. E., Nelson, C. A., Buckley, C. E., III, and Tanford, C. (1965), *J. Biol. Chem. 240*, 218.

Ovary, Z. (1958), *Progr. Allergy 5*, 459.

Pauling, L., Corey, R. B., and Branson, H. R. (1951), *Proc. Nat. Acad. Sci. U. S. 37*, 205.

Piggot, P. J., and Press, E. M. (1967), *Biochem. J. 104*, 616.

Polson, A. (1967), *Biochim. Biophys. Acta 140*, 197.

Press, E. M., and Porter, R. R. (1962), *Biochem. J. 83*, 172.

Rauen, H. M. (1956), Biochemisches Taschenbuch, Springer Verlag, Berlin, p 190.

Richards, E. G., and Schachman, H. K. (1959), *J. Phys. Chem. 63*, 1578.

Sage, H. J., Deutsch, G. F., Fasman, G., and Levine, C. (1964), *Immunochemistry 1*, 133.

Schechter, I., and Sela, M. (1965a), *Biochim. Biophys. Acta 104*, 298.

Schechter, I., and Sela, M. (1965b), *Biochim. Biophys. Acta 104*, 301.

Schechter, I., and Sela, M. (1967), *Biochemistry 6*, 897.

Scheraga, H. A., and Mandelkern, L. (1953), *J. Amer. Chem. Soc. 73*, 179.

Sela, M. (1966), *Advan. Immunol. 5*, 29.

Simha, R. (1940), *J. Phys. Chem. 44*, 25.

Spackman, D. H., and Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Strauss, U. P., and Smith, E. H. (1953), *J. Amer. Chem. Soc. 75*, 6186.

Yphantis, D. A. (1964), *Biochemistry 3*, 297.