The variability encountered in the plasma level profiles of any one individual was not an artifact due to changes in protein binding since, unlike microbiological assay techniques, the present fluorometric method measures total oxytetracycline. Neither was the variation between subjects caused by weight differences in the subjects, since none of the analyses was improved by taking the weight of the subjects into account. It is possible that plasma level profiles could be made more reproducible by stabilizing physiological conditions, *e.g.*, transit times (8). However, such changes could only be of use in bioavailability studies and not in general clinical practice; even then, attempts at such stabilization could be beneficial to one formulation and detrimental to another.

In the present studies the mean peak plasma levels of oxytetracycline (2.14 mcg./ml.) attained after oral administration of a solution of 500 mg. of the hydrochloride are only 40% higher than the serum levels of the reference oxytetracycline (1.55 mcg./ml.) used in the study of Blair *et al.* (9), in which the dose of 250 mg. of the hydrochloride was administered in a capsule. This difference could be unreal, caused by the relatively few subjects used in the present study, but it could equally be the result of different physiological conditions of the GI tract.

The present results illustrate the difficulty in obtaining reproducible plasma level profiles of oxytetracycline, even under fairly carefully controlled conditions. The variability could be much greater after the oral administration of more complicated formulations than that used here. One way around the problem is to study plasma levels achieved after multiple doses, as in normal clinical practice, and the results of such studies will be the basis of further reports.

REFERENCES

(1) "Cylinder Plate Assay for Oxytetracycline in Serum," National Center for Antibiotic and Insulin Analysis, Food and Drug Administration, U. S. Department of Health, Education, and Welfare, Washington, D. C., 1969.

(2) M. E. Caplis, H. S. Ragheb, and E. D. Schall, *J. Pharm. Sci.*, **54**, 694(1965).

(3) L. G. Chatten and S. I. Krause, *ibid.*, **60**, 107(1971).

(4) J. E. Hayes, Jr., and H. G. DuBuy, *Anal. Biochem.*, **7**, 322 (1964).

(5) K. H. Ibsen, R. L. Saunders, and M. R. Urist, *ibid.*, **5**, 505 (1963).

(6) K. W. Kahn, *Anal. Chem.*, **33**, 862(1961).

(7) A. M. Walter and L. Heilmeyer, "Antibiotic-Fibel," 2nd ed., Stuttgart, West Germany, 1965.

(8) G. Levy and B. K. Rao, *J. Pharm. Sci.*, **61**, 279(1972).

(9) D. C. Blair, R. W. Barnes, E. L. Wildner, and W. J. Murray, *J. Amer. Med. Ass.*, **215**, 251(1971).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 20, 1972, from the *Safety of Medicines Department, Imperial Chemical Industries, Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire, England.*

Accepted for publication December 22, 1972.

The authors are indebted to Mr. J. A. Lewis for his advice and his statistical evaluation of the plasma level profiles.

▲ To whom inquiries should be directed.

Specificity of Antibodies Produced by Linear Antigenic Polypeptides of a Known Primary Structure: Synthesis and Use of Poly(L-tyrosyl-γ-glycyl-L-glutamyl-L-alanylglycyl)glycine Methyl Ester

BRIAN J. JOHNSON▲ and CHARLES CHENG

Abstract □ The specificity of antibodies formed in rabbits in response to the antigenic challenge of poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester (I) was studied with respect to the role of the glutamyl residue. For this purpose the polypeptide poly(L-tyrosyl-γ-glycyl-L-glutamyl-L-alanylglycyl)glycine methyl ester (II) was prepared. When using this material, it was found by cross-reaction and absorption studies that the antibodies to the antigen I possess a high affinity for the γ-carboxyl groups of the glutamyl residue.

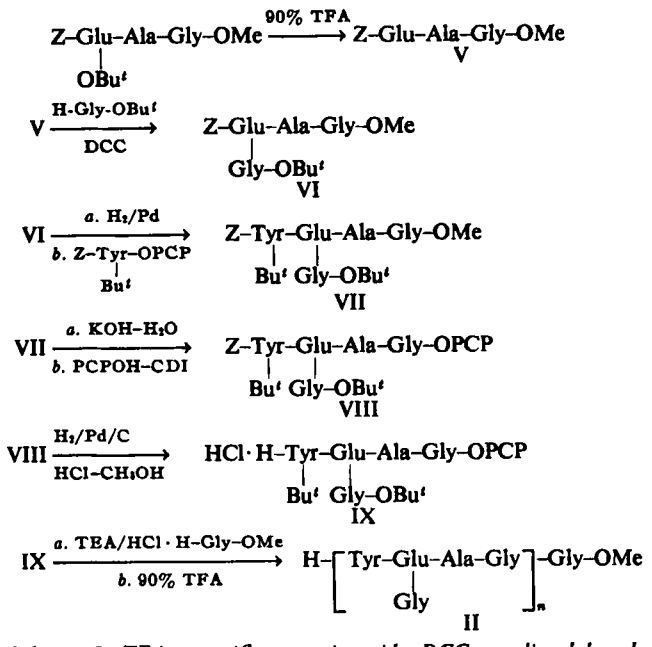
Keyphrases □ Polypeptides, linear—synthesis, immunochemical properties □ Antibodies, polypeptide produced—specificity □ Poly(L-tyrosyl-γ-glycyl-L-glutamyl-L-alanylglycyl)glycine methyl ester—synthesis, used to study antibody specificity

It has previously been reported that the antibodies produced by rabbits against the polypeptide poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester (I) (1–3) are most probably dependent upon the conformation of the antigen (4–7). These antibodies also have been shown to possess a specificity for the phenolic hydroxyl groups and the aromatic moiety of the tyrosyl residue (8). However, no such specificity has been shown for the alanyl residue (9). The next phase of this work has been to study the specificity of these antibodies pertaining to the role of the glutamyl residue. This paper reports the characterization of the specificity of antiserum produced by rabbits against the antigen I as studied by cross-reactions and absorption studies. For this purpose the following polymer was synthesized and used: poly(L-tyrosyl-γ-glycyl-L-glutamyl-L-alanylglycyl)-glycine methyl ester (II).

DISCUSSION[1]

The polypeptide II was prepared from the polymerizing unit, L-tyrosyl-γ-(*tert*-butyl-glycyl)-L-glutamyl-L-alanylglycine pentachlorophenyl ester hydrochloride (IX), which was synthesized as outlined in Scheme I and as detailed in the *Experimental* section. The polymerization of IX was performed by the procedure that has been shown to produce linear high molecular weight polypeptides (1, 2, 4–8). After removal of the protecting *tert*-butyl groups, the

[1] All amino acids are of the L-variety.



Scheme I—TFA = trifluoroacetic acid, DCC = dicyclohexylcarbodiimide, PCP = pentachlorophenyl, CDI = 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate, and TEA = triethylamine

crude polypeptide II was purified by dialysis and then fractionated by successive diafiltrations through membranes (Diaflo) into four different molecular weight fractions: $>5 \times 10^4$, $2\text{–}5 \times 10^4$, $1\text{–}2 \times 10^4$, and $<1 \times 10^4$.

Eight rabbits were immunized against I using the previously reported protocol (3). It was found that each serum gave a positive precipitin reaction with the homologous polymer I. The serum from each animal was pooled, because it was assumed that each rabbit had responded to the same antigenic determinants in this time interval (10). Incremental amounts of the polypeptide II were added to 1-ml. aliquots of this pooled antiserum and a cross-reaction occurred. The relative precipitin curves for the polypeptides I and II are shown in Fig. 1. To quantitate the amount of antibody not precipitated by polymer II, a separate experiment was performed. A quantity equal to the equivalence point amount of the heterologous polypeptide II was reacted with the pooled serum. After removal of the precipitate, 30 mcg. of the homologous polypeptide I was added to the resulting supernatant liquid. Further precipitation was obtained and quantitated by analysis for nitrogen (Kjeldahl) as shown in Table I.

It has been found that the heterologous polypeptide II cross-reacts with anti-I-serum; however, it precipitates less antibody than the homologous antigen I. The amount of the heterologous polymer II required to attain the equivalence point is slightly larger than that necessary for the homologous material. It would appear from these cross-reactions that the conformation of polymer II is similar to that of the homologous antigen I. The lower precipitating ability of polymer II indicates a decreased affinity of the antibody-combining site for this material. By using the rationale that the determinants of the heterologous polypeptide II are in the same orientation as those of the antigen I, it is suggested that the observed differences in the binding ability of the heterologous polypeptide II is due only to the modification of the glutamyl residue. Thus, it would appear that changes in the distance of the side-chain carboxyl group from the backbone polypeptide cause a large decrease in precipitating ability. It was, therefore, concluded that the antibody-combining sites have a high affinity for the γ-carboxyl groups of the glutamyl residue.

EXPERIMENTAL[1]

N-Benzyloxycarbonyl-L-glutamyl-L-alanylglycine Methyl Ester (V)—To 20 g. (0.0417 mole) of N-benzyloxycarbonyl-γ-*tert*-butyl-L-glutamyl-L-alanylglycine methyl ester (1) was added 90 ml. of 90% trifluoroacetic acid. The solution was stirred at room temperature for 50 min. and then concentrated under reduced pressure to yield a solid. This material was crystallized from ethyl acetate to yield 14.5 g. (82%) of the product, m.p. 178–180°; $[\alpha]_D^{24}$ −7.2° (c 4.2 in dimethylformamide).

Anal.—Calc. for $C_{19}H_{25}N_3O_8$: C, 53.90; H, 5.95; N, 9.93. Found: C, 53.75; H, 5.94; N, 9.70.

N-Benzyloxycarbonyl-γ-(*tert*-butyl-glycyl)-L-glutamyl-L-alanylglycine Methyl Ester (VI)—To a cooled solution of 14.5 g. (0.0343 mole) of N-benzyloxycarboxyl-L-glutamyl-L-alanylglycine methyl ester (V) and 5.0 g. (0.038 mole) of glycine *tert*-butyl ester in 200 ml. of methylene chloride was added 7.8 g. (0.0378 mole) of dicyclohexylcarbodiimide. This mixture was stirred overnight at room temperature and then concentrated under reduced pressure to yield a solid. This material was taken up in ethyl acetate, and the insoluble urea filtered off. The filtrate was washed successively with 1 *N* HCl, water, 10% sodium bicarbonate solution, and water and then dried (sodium sulfate). This solution was concentrated under reduced pressure to yield a solid, which was chromatographed on a column of Silicar CC-7 using chloroform–ethyl acetate (1:1) as the eluent to give 7.2 g. (40%) of the tetrapeptide, m.p. 141°; $[\alpha]_D^{24}$ −8.6° (c 3.2 in dimethylformamide).

Anal.—Calc. for $C_{25}H_{36}N_4O_9$: C, 55.95; H, 6.76; N, 10.44. Found: C, 55.91; H, 6.65; N, 10.37.

N-Benzyloxycarbonyl-*O-tert*-butyl-L-tyrosyl-γ-(*tert*-butyl-glycyl)-L-glutamyl-L-alanylglycine Methyl Ester (VII)—A fine suspension of 7.2 g. (0.0135 mole) of VI and 0.6 g. of 10% palladium-on-charcoal in 100 ml. of methanol was treated with 0.492 g. (0.0135 mole) of dry hydrochloric acid in methanol, and the suspension was hydrogenated for 2 hr. The reaction mixture was filtered and the filtrate was concentrated under reduced pressure to give an oil. To this material, dissolved in 200 ml. of methylene chloride and 1.36 g. (0.00135 mole) of triethylamine, was added 9.2 g. (0.00148 mole) of N-benzyloxycarbonyl-*O-tert*-butyl-L-tyrosine pentachlorophenyl ester (1). This mixture was stirred overnight at room temperature and then concentrated under reduced pressure to give a solid; the solid was suspended in ethyl acetate, washed successively with 1 *N* HCl and water, and dried (sodium sulfate). Concentration of this solution produced a solid, which was chromatographed on a column of Silicar CC-7 using chloroform–ethyl acetate (1:1) as eluent. The major fraction obtained was crystallized from ethyl acetate–hexane to yield 5.8 g. (59%) of the heptapeptide, m.p. 204.5–206°; $[\alpha]_D^{24}$ −15.1° (c 4.3 in dimethylformamide).

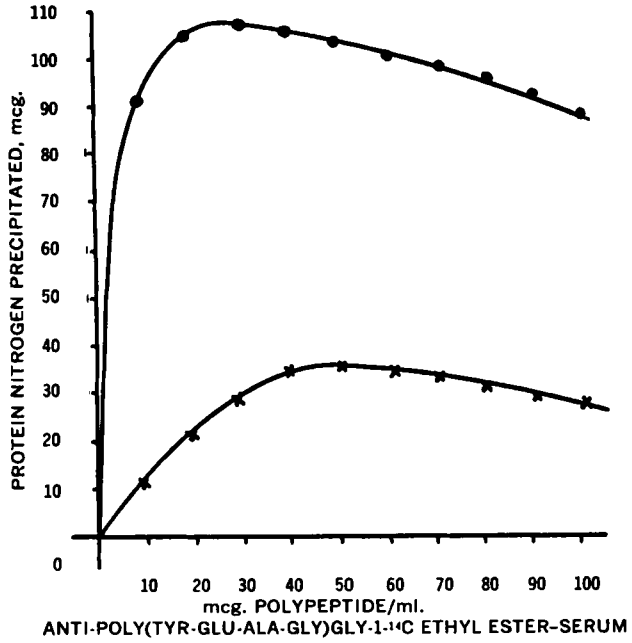


Figure 1—*Precipitin curves. Key:* ●, *Poly(Tyr-Glu-Ala-Gly)$_n$Gly; and* ×, *Poly(Tyr-Glu-Ala-Gly)$_n$Gly.*
Gly

[1] Melting points were taken with a Mel-Temp apparatus and are uncorrected.

Table I—Absorption Data

Polypeptide	Protein Nitrogen (Kjeldahl) Precipitated at Equivalence Point[a], mcg.	Protein Nitrogen (Kjeldahl) Precipitated by I after Absorption[a], mcg.	Protein Nitrogen (Kjeldahl) Precipitated by Polypeptide[a], %
(Tyr–Glu–Ala–Gly)$_n$Gly (I)	106	0	100
(Tyr–Glu–Ala–Gly)$_n$Gly (II) Gly	33	71	32

[a] Per milliliter of anti-poly(Tyr-Glu-Ala-Gly)Gly-1-^{14}C ethyl ester-serum.

Anal.—Calc. for $C_{38}H_{53}N_5O_{11}$: C, 60.40; H, 7.06; N, 9.26. Found: C, 60.18; H, 6.99; N, 9.16.

***N*-Benzyloxycarbonyl-*O*-*tert*-butyl-L-tyrosyl-γ-(*tert*-butyl-glycyl)-L-glutamyl-L-alanylglycine Pentachlorophenyl Ester (VIII)**—To a solution of 7.4 g. (0.0098 mole) of VII in 350 ml. of methanol was added 9.8 ml. of 1 *N* KOH. The solution was stirred for 1 hr. at room temperature and then concentrated under reduced pressure. The residue was flooded with water, acidified with 10% citric acid solution, and extracted into ethyl acetate. This solution was dried (sodium sulfate) and concentrated under reduced pressure to give a solid. To this material dissolved in 150 ml. of methylene chloride were added 2.88 g. (0.0108 mole) of pentachlorophenol and 4.6 g. (0.0108 mole) of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide *metho-p*-toluenesulfonate. The mixture was shaken at room temperature for 2 days and then concentrated under reduced pressure to give a solid, which was washed with water and crystallized from methanol to yield 5.2 g. (49%) of the activated ester, m.p. 203.5–205.5°; $[\alpha]_D^{24}$ −15.0° (c 2.66 in dimethylformamide).

Anal.—Calc. for $C_{43}H_{50}Cl_5N_5O_{11}$: C, 52.15; H, 5.09; N, 7.08. Found: C, 51.90; H, 5.00; N, 6.94.

***O*-*tert*-Butyl-L-tyrosyl-γ-(*tert*-butyl)-L-glutamyl-L-alanylglycine Pentachlorophenyl Ester Hydrochloride (IX)**—A suspension of 5.1 g. (0.00515 mole) of the pentapeptide activated ester VIII and 0.7 g. of 10% palladium-on-charcoal in 300 ml. of methanol was treated with 0.188 g. (0.00515 mole) of dry hydrochloric acid in methanol, and the suspension was hydrogenated for 2 hr. The mixture was filtered and the filtrate was concentrated under reduced pressure to give a solid, which was crystallized from methanol–ether to yield 2.3 g. (50%) of the polymerizing unit IX, m.p. 186°; $[\alpha]_D^{24}$ −4° (c 2.50 in dimethylformamide).

Anal.—Calc. for $C_{35}H_{45}Cl_6N_5O_9$: C, 47.10; H, 5.08; N, 7.85. Found: C, 46.85; H, 5.07; N, 8.12.

Poly-(L-tyrosyl-γ-glycyl-L-glutamyl-L-alanylglycyl)glycine Methyl Ester (II)—To a solution of 0.82 g. (0.00815 mole) of triethylamine and 0.585 mg. of glycine methyl ester hydrochloride in 5 ml. of dimethyl sulfoxide was added a solution of 2.1 g. (0.00233 mole) of the polymerizing unit IX in 18 ml. of dimethyl sulfoxide. The mixture was shaken for 1 week and then centrifuged to yield the fully protected polymer. This polymer was washed with three 35-ml. portions of water, three 35-ml. portions of methanol, and three 35-ml. portions of ether and dried to give the fully blocked polymer. This material was treated with 30 ml. of 90% trifluoroacetic acid, stirred for 50 min. at room temperature, and then concentrated under reduced pressure to yield the crude polypeptide II. This material was suspended in 20 ml. of water and dissolved by the addition of 1 *N* NaOH to pH 7.5. The solution was filtered and the filtrate was dialyzed against distilled water overnight, acidified with 6 *N* HCl to pH 2.5, and dialyzed against distilled water for a day. The precipitated polypeptide II was collected by centrifugation and then lyophilized to yield 0.3 g. (27%); the amino acid ratio of an acid hydrolysate was: $Tyr_{0.9}$ $Glu_{1.0}$ $Ala_{1.0}$ $Gly_{2.1}$.

Molecular Weight Determination—The polypeptide II (0.3 g.) was dissolved in 50 ml. of water by the addition of 1 *N* NaOH to pH 7.4. This solution was separated into four different molecular weight ranges by successive diafiltrations through membranes[1]. Each fraction was acidified with 6 *N* HCl, dialyzed, and lyophilized to yield 0.10 g. mol. wt. >50,000, 0.02 g. mol. wt. 20,000–50,000, 0.03 g. mol. wt. 10,000–20,000, and 0.14 g. mol. wt. <10,000.

Immunochemical Procedures—Eight rabbits were treated with I at weekly intervals, using the immunization schedule previously described (3); 25 days after the last injection, all rabbits were bled using the standard heart puncture technique. Serum from each rabbit was tested for the precipitin reaction with the homologous antigen I, and serum from each animal gave a positive precipitin reaction. The serum from each animal was pooled, and this combined serum was used for the following experiments. It was assumed that the antibodies produced by each rabbit after the same time interval was directed against the same antigenic determinants of the antigen I.

Quantitative Precipitin Reactions—To 1-ml. aliquots of the pooled rabbit serum were added incremental amounts of the polypeptide II. Each tube was made up to a total of 2 ml. with buffer (0.1 *M* NaCl–0.05 *M* $NaHCO_3$), incubated for 1 hr. at 37°, and then kept at 4° for 48 hr. The tubes were centrifuged in the cold and the precipitates were washed twice with 1 ml. of buffer (0.05 *M* K_2HPO_4–NaOH), pH 7.0. The total amount of protein that precipitated was estimated by analysis for nitrogen (Kjeldahl). An identical procedure was used for the homologous polymer I, which was run simultaneously with that used for the polypeptide II. The comparative precipitin curves are shown in Fig. 1.

Absorption Studies—The pooled rabbit serum was treated with the equivalence point amount of the heterologous polypeptide II as already described. The precipitate was centrifuged out and the supernatant liquor was poured off into a separate tube. To this was added 30 mcg. of the homologous antigen I. The mixture was incubated at 37° for 1 hr. and then stored at 4° for 48 hr. The precipitate was collected by centrifugation and washed twice with 1 ml. of buffer solution (0.05 *M* K_2HPO_4–NaOH), pH 7.0. The amount of precipitate obtained using this procedure is shown in Table I. A control in which the serum was first absorbed with the homologous antigen I ascertained that the homologous antigen precipitated all of the antibody, since the supernatant liquor gave no further precipitin reaction when 30 mcg. of I was added.

REFERENCES

(1) B. J. Johnson and E. G. Trask, *J. Chem. Soc., Ser. C*, **1969**, 2644.
(2) B. J. Johnson, *J. Pharm. Sci.*, **59**, 1849(1970).
(3) B. J. Johnson and E. G. Trask, *ibid.*, **59**, 724(1970).
(4) B. J. Johnson, *J. Med. Chem.*, **15**, 423(1972).
(5) B. J. Johnson, C. Cheng, and N. Tsang, *ibid.*, **15**, 95(1972).
(6) B. J. Johnson, *ibid.*, **14**, 488(1971).
(7) B. J. Johnson and E. G. Trask, *ibid.*, **14**, 251(1971).
(8) B. J. Johnson and N. Tsang, *ibid.*, **15**, 488(1972).
(9) B. J. Johnson, *J. Pharm. Sci.*, **60**, 332(1971).
(10) E. Maron, R. Arnon, and B. Bonavida, *Eur. J. Immunol.*, **1**, 181(1971).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 10, 1972, from the *Laboratory of Molecular Biology, Department of Biochemistry and Department of Microbiology, Division of Clinical Immunology, University of Alabama Medical School, Birmingham, AL 35233*

Accepted for publication December 21, 1972.

Supported by the Hartford Foundation and the State of Alabama.

▲ To whom inquiries should be directed.

[1] Diaflo OM 50, UM 20E, and UM 10, Amicon Corp., Lexington, Mass.