Conformation Dependency of Antibodies to Poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C Ethyl Ester

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Abstract □ The synthesis of poly(L-tyrosyl-ε-*N*-L-glutamyl-L-lysyl-L-alanylglycyl)glycine methyl ester (I) is described. This material neither crossreacts with antibodies to poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester nor inhibits the precipitin reaction between these antibodies and the homologous antigen. It was concluded that this lack of crossreaction and inhibitory properties of I is due to the antibodies to poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester being highly dependent on the conformation of their homologous antigen.

Keyphrases □ Poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester—synthesis, conformation dependency of antibodies □ Antigens, polypeptide produced—conformation dependency to poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester □ Polypeptides, linear—synthesis, immunochemical properties

It recently was reported that antibodies to the polypeptide poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester (1–3) are most probably dependent on the conformation of the antigen (4). To investigate this point further, it was necessary to construct a polymer that possessed a similar order of amino acids as the antigen poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester but that differed markedly from it in conformation. This article reports the synthesis, as outlined in Scheme I, and immunochemical properties of poly(L-tyrosyl-ε-*N*-L-glutamyl-L-lysyl-L-alanylglycyl)glycine methyl ester (I).

CHEMISTRY

The polymerizing unit, *O*-benzyl-L-tyrosyl-ε-*N*-benzyloxycarbonyl-L-lysyl-L-alanylglycine pentachlorophenyl ester hydrochloride (V), and the necessary intermediates for its preparation were synthesized as detailed in the *Experimental* section. The polymerization was performed at a reagent concentration of 100 mmoles/l. in the presence of a preformed monomer, since this has been shown to produce linear high molecular weight polypeptides (1, 2, 5–12). Following this established procedure, the insoluble polymer, poly(*O*-benzyl-L-tyrosyl-ε-*N*-benzyloxycarbonyl-L-lysyl-L-alanylglycyl)glycine methyl ester (VI), was prepared, from which the protecting groups were removed by the use of anhydrous hydrogen bromide in trifluoroacetic acid to yield poly(L-tyrosyl-L-lysyl-L-alanylglycyl)glycine methyl ester (VII). After extensive dialysis to remove low molecular weight polymers and cyclic materials, it was lyophilized and coupled to *N*-benzyloxycarbonyl-γ-*tert*-butyl-L-glutamic acid pentachlorophenyl ester (13). The protecting groups were removed from the resulting polymer by the use of anhydrous hydrogen bromide in trifluoroacetic acid to yield poly(L-tyrosyl-ε-*N*-L-glutamyl-L-lysyl-L-alanylglycyl)glycine methyl ester (I). After extensive dialysis, the polymer was purified and fractionated by passage through a calibrated column of Sephadex G-50 (14). By this means, the molecular weight of the polypeptide was found to be 2×10^4.

IMMUNOCHEMISTRY

Incremental amounts of the polypeptide I were added to aliquots of antiserum to poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester. No precipitin reaction was observed when up to 10,000 mcg. of I was used. Inhibition studies were also performed to investigate the possibility that the polypeptide I could be a hapten for the antigen poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester. Incremental amounts of the polypeptide I were added to aliquots of antiserum to poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)-glycine-1-^{14}C ethyl ester containing a known amount of the antigen. No inhibition of the precipitin reaction was observed in the presence of up to 10,000 mcg. of the polypeptide I.

CONCLUSIONS

Previous work indicated that antibodies to poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester are most probably dependent upon the conformation of the antigen. To investigate this point further, poly(L-tyrosyl-ε-*N*-L-glutamyl-L-lysyl-L-alanylglycyl)-glycine methyl ester (I) was synthesized by substituting the glutamyl residue with the ε-*N*-L-glutamyl-L-lysyl residue. Due to this displacement, the spacial orientation of the various residues of I should be considerably different from that of the antigen poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester. It was shown that the polypeptide I does not crossreact with antibodies produced by poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester, nor does it inhibit the precipitin reaction between this antiserum and the homologous antigen. Thus, it was concluded that antibodies produced by poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester are highly dependent on the conformation of the antigen.

EXPERIMENTAL[1]

***N*-*tert*-Butoxycarbonyl-*O*-benzyl-L-tyrosyl-ε-*N*-benzyloxycarbonyl-L-lysyl-L-alanylglycine Methyl Ester (II)**—To a solution of 13.3 g. (0.029 mole) of ε-*N*-benzyloxycarbonyl-L-lysyl-L-alanylglycine methyl ester hydrochloride (15) in 200 ml. of methylene chloride was added 17.8 g. (0.029 mole) of *N*-butoxycarbonyl-*O*-benzyl-L-tyrosine pentachlorophenyl ester (16) and 3.03 g. (0.03 mole) of triethylamine. The reaction mixture was stirred overnight at room temperature and then concentrated under reduced pressure to yield a solid. This material was dissolved in ethyl acetate, washed with a 10% citric acid solution and water, and then dried. The solution was concentrated under reduced pressure to yield a solid, which was chromatographed on a column of ScilicAR CC-7 using chloroform–methanol (1:1) as the eluent to yield 15.5 g. (69%), m.p. 158–159°; $[\alpha]_D^{29}$ −3.8° (c 2.4 in dimethylformamide).

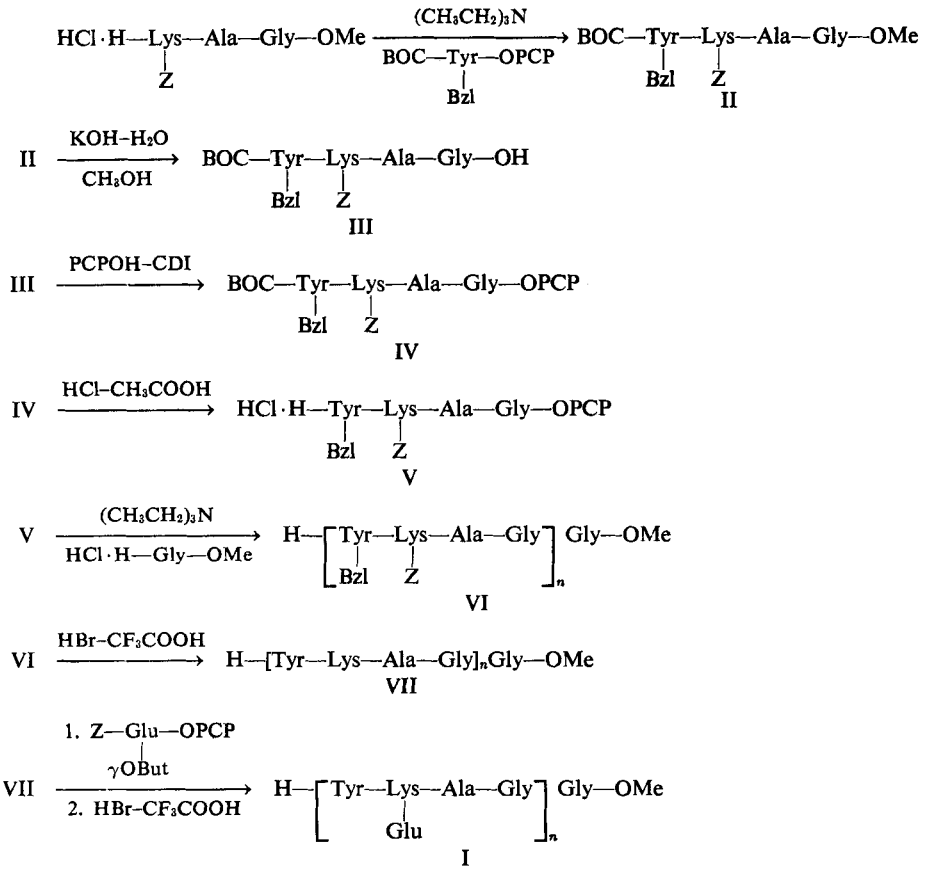
Anal.—Calc. for $C_{41}H_{53}N_5O_{10}$: C, 63.5; H, 6.9; N, 9.0. Found: C, 63.2; H, 7.0; N, 8.8.

***N*-*tert*-Butoxycarbonyl-*O*-benzyl-L-tyrosyl-ε-*N*-benzyloxycarbonyl-L-lysyl-L-alanylglycine (III)**—To a solution of 15 g. (0.0194 mole) of the tetrapeptide II in 200 ml. of methanol was added 20 ml. of 1 *N* KOH. The mixture was stirred at room temperature for 90 min. and then was evaporated under reduced pressure to give an oil. This material was flooded with a 10% citric acid solution, extracted into ethyl acetate, and then washed with water and dried (sodium sulfate). This solution was concentrated under reduced pressure to give a solid, which was crystallized from ethyl acetate–hexane to yield the tetrapeptide free acid, 14.1 g. (94%), m.p. 185–186°; $[\alpha]_D^{29}$ −7.9° (c 2.1 in dimethylformamide).

Anal.—Calc. for $C_{40}H_{51}N_5O_{10}$: C, 63.1; H, 6.75; N, 9.2. Found: C, 62.9; H, 6.8; N, 9.4.

***N*-*tert*-Butoxycarbonyl-*O*-benzyl-L-tyrosyl-ε-*N*-benzyloxycarbonyl-L-lysyl-L-alanylglycine Pentachlorophenyl Ester (IV)**—

[1] Melting points were taken on a Mel-temp apparatus and are uncorrected.



Scheme I

To a solution of 14.0 g. (0.0184 mole) of the tetrapeptide free acid III in 500 ml. of methylene chloride were added 5.85 g. (0.022 mole) of pentachlorophenol (PCPOH) and 9.33 g. (0.022 mole) of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate (CDI). The mixture was stirred overnight and concentrated under reduced pressure to give a solid. This material was suspended in ethyl acetate and washed with water. The organic layer was dried (sodium sulfate) and concentrated under reduced pressure to give a solid, which was crystallized from methanol to yield the tetrapeptide activated ester, 12.1 g. (66.7%), m.p. 240°; $[\alpha]_D^{29}$ − 13.9° (c 0.9 in dimethylformamide).

Anal.—Calc. for $C_{40}H_{50}Cl_5N_5O_{10}$: C, 54.7; H, 5.0; N, 6.9. Found: C, 54.9; H, 5.3; N, 6.8.

***O*-Benzyl-L-tyrosyl-ε-*N*-benzyloxycarbonyl-L-lysyl-L-alanylglycine Pentachlorophenyl Ester Hydrochloride (V)**—To 12.0 g. (0.0119 mole) of the tetrapeptide pentachlorophenyl ester IV was added 44 ml. of 1 *N* hydrogen chloride in glacial acetic acid. The reaction mixture was stirred at room temperature for 30 min. and then concentrated under reduced pressure to give the hydrochloride. This material was crystallized from methanol–ether to yield 8.2 g. (73%), m.p. 250°; $[\alpha]_D^{29}$ − 12.7° (c 1.2 in dimethylformamide).

Anal.—Calc. for $C_{41}H_{43}Cl_6N_5O_8$: C, 52.0; H, 4.6; N, 7.4. Found: C, 52.3; H, 4.7; N, 7.2.

Poly(L-tyrosyl-L-lysyl-L-alanylglycyl)glycine Methyl Ester (VII)—To a solution of 2 mg. of glycine methyl ester hydrochloride in 5 ml. of dimethyl sulfoxide containing 2.5 g. (0.0247 mole) of triethylamine was added a solution of 7.5 g. (0.0079 mole) of the polymerizing unit V in 74 ml. of dimethyl sulfoxide. The reaction mixture was shaken for 1 week, after which the precipitated polymer was collected by centrifugation. This material was washed with three 35-ml. portions of water, three 35-ml. portions of methanol, and three 35-ml. portions of ether and then dried to give 2.9 g. (51%) of the fully protected polymer. This material was treated with 200 ml. of trifluoroacetic acid through which was bubbled dry hydrogen bromide gas for 2 hr. The solution was concentrated under reduced pressure to yield a solid, which was washed thoroughly with ether. This material was dissolved in 50 ml. of 2 *N* HCl, dialyzed against 20 l. of distilled water over 2 days, and then lyophilized to yield 1.0 g. (27.8%) of the free polypeptide I. Amino acid ratios found in an acid hydrolysate were: $Tyr_{1.0}Lys_{1.0}Ala_{1.0}Gly_{1.0}$.

Poly(L-tyrosyl-ε-*N*-glutamyl-L-lysyl-L-alanylglycyl)glycine Methyl Ester (I)—To a suspension of 0.6 g. (0.00132 mole) of the polypeptide VII in 100 ml. of dimethylformamide was added 3 g. (0.0051 mole) of *N*-benzyloxycarbonyl-γ-*tert*-butyl-L-glutamic acid pentachlorophenyl ester (13). The mixture was shaken for 1 week, and the crude polypeptide was precipitated by the addition of 500 ml. of water; this was collected, washed with methanol and ether, and dried under vacuum. This material was suspended in 200 ml. of trifluoroacetic acid, and dry hydrogen bromide was bubbled through the solution for 2 hr. The solution was concentrated under pressure to yield a solid. The solid was washed thoroughly with ether, dissolved in water by the addition of 4 *N* NaOH to pH 7.5, dialyzed against 15 l. of distilled water, and then lyophilized to yield the polypeptide I as its sodium salt, 0.5 g. (68%). Amino acid analyses of an acid hydrolysate showed: $Tyr_{1.1}Lys_{1.0}Glu_{1.0}Ala_{1.0}Gly_{1.0}$.

Molecular Weight Determination—A calibrated column of Sephadex G-50 (2.5 × 38.0 cm.) was employed for the molecular weight determination. With 0.15 *M* NaCl as eluent, 4 mg. of the sodium salt of the polypeptide I was passed through it and the polypeptide was eluted in a volume equivalent to that corresponding to a molecular weight of 2×10^4.

Immunochemical Procedures—To 1-ml. aliquots of rabbit antiserum to poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester were added incremental amounts of up to 10,000 mcg. of the polypeptide I. No precipitin reaction was observed.

To 1-ml. aliquots of the rabbit serum to poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester were added incremental

amounts of up to 10,000 mcg. of the polypeptide I. To each tube was added the equivalent point amount of the homologous antigen (30 mcg.), and the tubes were then incubated at 37° for 1 hr. After standing at 4° for 48 hr., the precipitates were collected, washed twice with buffer (0.05 M K_2HPO_4–NaOH, pH 7.0), and collected by centrifugation. The total amount of protein precipitated was estimated by nitrogen analysis (Kjeldahl). No inhibition of the precipitin reaction was observed using the polypeptide I.

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Correlations between *In Vitro* Transport in a Three-Phase Model Cell and *In Vivo* Absorption of a Series of Sulfanilamides

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Abstract □ A three-phase model cell, employing a liquid lipid barrier, was used to establish correlations between reported *in vivo* absorption data and experimentally determined *in vitro* transport rates for a series of N^1-substituted heterocyclic sulfanilamides. These studies, using isopentyl acetate as the lipid phase, involved the effect of pH on the rate constants for three derivatives and the effect of the N^1-substituent on the overall transport for unionized species. Correlations were made between the *in vitro* data and *in vivo* gastric, intestinal, and rectal absorption data, indicating the utility of this model to simulate passive drug absorption. Transport rates and partition coefficients were also found to be related.

Keyphrases □ Sulfanilamides—correlations between *in vitro* transport in three-phase model cell and *in vivo* absorption □ Cell, three-phase model—*in vitro* transport–*in vivo* absorption correlations, sulfanilamides □ Absorption, *in vivo*—correlated to *in vitro* transport, three-phase model cell, sulfanilamides, liquid lipid barrier

There are several types of three-compartment kinetic models in use to simulate the transport of drugs across biological membranes, especially the GI tract. These systems were classified and reviewed recently by Herzog and Swarbrick (1). Those *in vitro* systems utilizing a solid membrane have an obvious advantage in that they more closely resemble the *in vivo* situation. However, one advantage of using a liquid lipid to form the membrane phase is the ease with which the composition of the latter can be varied. This readily permits investigation of such factors as lipid polarity (2) and solubility parameter (3). Three-compartment model cells employing a liquid lipid membrane phase have been used in several attempts to establish correlations between *in vitro* and *in vivo* transport processes. Agostini and Schulman (4) used the so-called "Schulman cell" to investigate ion flux and water migration across biological membranes. Perrin (5) and Augustine and Swarbrick (2) reported using this cell in studies designed to simulate the GI absorption of drugs. In addition to the lipid phase composition, it was shown that stirring rate, the surface to volume ratio of the phases, and temperature also affect the transport rate constants obtained (2). To date, however, no work has been reported using this cell to study a homologous series of compounds.

The present study reports the *in vitro* transport rates of a series of N^1-substituted heterocyclic sulfonamides determined using a Schulman-type liquid lipid transport model. Correlations between the *in vitro* transport rates, partition coefficients, and *in vivo* absorption data, taken from the literature for the same compounds, are described.

EXPERIMENTAL

Materials—The following chemicals were used as received (the figures in parentheses give the determined melting points which, in all cases, corresponded to literature values): sulfanilamide (164.5–166.5°), sulfathiazole (200–204°), sulfamerazine (234–238°), sulfamethazine (198–199°), sulfapyridine (191–193°), and sulfisoxazole (194°). The buffer ingredients were reagent grade and were used as