EFFICIENCY OF GLUTAMYL PEPTIDE POLYMERS AS PLASMA VOLUME EXTENDERS

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(Received for publication, January 10, 1956)

In previous work we have indicated the potential use of polymers of polyglutamic acid as plasma volume expanders, calling attention to the unique advantages offered by these polyelectrolytes for this purpose (1, 2). Particularly important in this connection are (*a*) the enhanced oncotic potency conferred on these compounds by the Donnan effects associated with the highly ionized molecule, and (*b*) the opportunity of altering the size and shape of the molecule to fit the requirements of blood stream retention, without qualitatively changing its composition. The necessity for exploiting the latter property became apparent when it was demonstrated that linear polyglutamyl peptide of 15,000 molecular weight, with molecular length approximately equal to that of serum albumin but narrower in diameter, and even linear peptides of molecular weight up to 120,000 were rapidly excreted in human beings *via* the kidney (3). Methods of polymerizing these peptides in such fashion as to increase their diameter were developed, and the products formed were demonstrated to be characterized by increased blood stream retention in human beings (2).

Further work has been directed toward the development of a molecule of dimensions that would function as a plasma extender with a half-life of 15 to 20 hours in humans. Many polymers have been made and tested for this purpose. Finally, a group which are in the proper range has been produced. The results of the clinical tests with this group, and pertinent data on some of their physicochemical properties relating to their oncotic efficiency, form the subject of this report.

Materials and Methods

The starting peptide[1] was prepared from a deep culture filtrate of *Bacillus subtilis*, and purified by previously described methods (4, 2).

Preparation of Backbone Peptide.—The number average molecular weight of different lots of this starting peptide usually ranged from 30,000 to 100,000. By suitable fractionation of

[1] We are indebted to Merck and Company, Inc., of Rahway, New Jersey, for providing this starting peptide and some of the side chain esters used for this work.

this material, backbone of desired size can be obtained. The precise details of fractionation varied somewhat with the lot of the starting material, but the general procedure employed was similar for all samples. A typical example is as follows: To 12 liters of a 2.5 per cent solution of the sodium salt of 34,000 number average molecular weight peptide in 0.6 molar sodium chloride solution, pH 6.5, 5.0 liters of 95 per cent ethyl alcohol was added with stirring. On standing overnight at 0°C., a heavy oil, containing high molecular weight peptide, separated out and settled to the bottom of the container. The supernatant was siphoned off and 300 ml. of ethyl alcohol added to it. On standing overnight in the cold room a second cut was obtained by another heavy oil. The supernatant was separated by decantation. This second oil was diluted with water to make a 4 per cent solution of peptide and sufficient hydrochloric acid was added to make the final acid concentration half normal. The peptide precipitated out on standing overnight in the cold room and was collected by filtration, washed with water, alcohol and ether, and dried *in vacuo*. It is sometimes advantageous to remove coloring matter by treatment with charcoal prior to acidification. The supernatant of the second oil contains low molecular weight peptide which may be recovered by addition of more alcohol. In this fractionation the following yields were obtained: 143 gm. of 72,000 molecular weight, 50 gm. of 48,000 molecular weight, and 50 gm. of 30,000 molecular weight.

The middle cuts were used as backbones and these fractions were more precisely characterized with respect to size by an alcohol fractionation similar to the above in which, however, the alcohol concentrations were so adjusted that the first and third fractions each represented 5 to 10 per cent of the total. The molecular weights of these small fractions essentially constitute the upper and lower limits of the backbone molecular weight.

Preparations of Side Chains.—The side chains were prepared for conjugation as the methyl esters. Purified peptide of 12,000 to 15,000 molecular weight was suspended in 7 N dry methanolic HCl, using 2 liters of solvent per 100 gm. of peptide, and the mixture was allowed to stand at room temperature for 10 hours with occasional shaking. After a few hours the peptide went completely into solution. As much as possible of the methanol and HCl was removed by vacuum distillation for 10 hours at room temperature. The remaining thick viscous solution of polyester was converted to a dry white powder by a few ether precipitations from methanol. This powder was extracted with pyridine and the pyridine-soluble portion was used for conjugation.

Preparation and Purification of Polymer.—The conjugation and saponification of conjugate were carried out as previously described (2). After saponification unreacted side chain was separated from conjugate by alcohol fractionation. To the crude neutralized solution of saponified conjugate at pH 7.0 was added sufficient sodium chloride and 95 per cent ethanol to make the final concentrations 0.6 M and 40 per cent respectively. On standing overnight at 0°C. the sodium salt of conjugate separated out as a heavy oil. After decantation of the supernatant, which contained the unreacted side chain, the conjugate bearing oil was diluted and similarly reprecipitated twice more. The final oil contained very little unreacted side chain as evidenced by the concentration of free amino groups which were determined by the use of Sanger's reagent on a suitable aliquot. On lyophilizing the oil to remove alcohol, the sodium salt was left as a water-soluble white solid. This was dissolved in saline and final concentrations were adjusted to 0.85 per cent with respect to sodium chloride and 2.0 to 2.5 per cent with respect to conjugate. The pH was adjusted to 7.2. The solution was sterilized by Seitz filtration and tested for sterility and pyrogenicity before use.

The patients tested were all admitted to the hospital for conditions unrelated to the circulatory or renal systems, and all had normal renal and cardiac function. All had been afebrile throughout their hospital stay prior to the test, except for one patient with Hodgkin's disease whose afternoon temperature usually rose to 99–100°F. During the day of the test the patients were kept on a low calorie, fat-free diet. Red cell counts and hematocrit deter-

minations were performed by standard methods. White cell counts and differential counts were done with the previously described slight modification of the customary method (3). Clotting time was determined by a modified Lee White method (5). The bleeding time was determined by the Duke method (6).

Plasma volumes were determined by the Evans blue due method (7) or by plasma protein dilution or both. When the former method was used the determination was based on samples taken at 10, 20, and 30 minute intervals after injection of the dye. In many control trials plasma volumes obtained before, and 1 and 6 hours after a 1 liter infusion of saline fell within a 150 ml. spread. Plasma proteins were determined by Kjeldahl nitrogen analyses of the dissolved (in 2 N NaOH) washed, trichloracetic acid precipitate of heparinized plasma samples. Concentration of the polymer in plasma was determined by the method previously described (8).

The molecular weight of the conjugate was determined by osmometry using the half-sum method of Fuoss and Mead in a modified Fuoss-Mead osmometer (9, 10). Osmotic pressures of varying concentrations of conjugate in 1.0 M sodium chloride solution, pH 5.5, were measured at 25.00 ± 0.01°C. A wet gel cellophane membrane, No. 450, was used.[2] Cathetometer readings for both positive and negative applied pressures were taken at 10 minute intervals over a period of 2½ hours each. The maximum applied pressures did not exceed 3 cm from equilibrium pressure. In all cases the last three of the series of readings were essentially identical.

Osmotic pressures of polymer and of serum albumin–polymer mixtures were determined in a simple osmometer consisting of a Visking tubing tied at one end and plugged at the other by a two holed rubber stopper bearing a capillary tube and a filling outlet. The solutes were dissolved in isotonic saline at pH 7.2. After loading, equilibrium was obtained by rotating the osmometer in a 100-fold volume of saline for 12 hours at room temperature. Readings were then taken for a period of 12 to 24 hours with the osmometer in a constant temperature air bath. A measured capillary correction and a density correction were applied, and the values obtained were corrected to 25°C.

RESULTS AND DISCUSSION

Of the many peptides and polymers tested, the group shown in Table I in many ways provides the most promising basis for further development. It is evident from the data that two of the polymers, Nos. 55 and 59, gave satisfactory sustained plasma expansion. The 6 hour volume retention of these two were 75 and 90 per cent of their respective 1 hour retention volumes. For an expander with a volume retention half-life of 15 hours, the calculated value is 79 per cent retention in 5 hours if the rate of disappearance follows a first order reaction. The use of the 1 hour retention volume as a base line rather than the infusion volume is due to the fact that the infusion volumes in this exploratory series were necessarily somewhat arbitrary. It is therefore felt that the 1 hour volume expansion provides a more valid base line for evaluation of subsequent volume retention than does the actual volume injected.

While it is recognized that the data in no sense adequately characterize these conjugates with respect to molecular dimensions, they do provide on a comparative basis reasonably good clues as to the size of components which

[2] This was obtained from J. Stabin of Brooklyn.

are apt to yield a satisfactory product under the conditions of conjugation used in this work. Thus it is seen conjugates 55 and 59 both have backbones of number average molecular weights higher than 40,000 and side chains of greater than 3,000. It is evident that shortening the side chain, conjugate 58, or the backbone, conjugates 51 and 52, and possibly 56, seems to lead to a loss of ability to effect satisfactory retention of plasma volume expansion.

These findings are more or less substantiated by the data on plasma levels and urinary excretion of the polymers (Table II). The rate of clearance of polymer from the blood stream as evidenced by the percentage decrease in the total plasma polymer between 1 and 6 hours post infusion was slower for polymers 59 and 55, and more rapid for polymers 51, 52, 57, and 58.

TABLE I

Plasma Volume Expansion by Various Sized Polymers

Polymer	Dose	Molecular weights of backbones					Molecular weight of side chains	Plasma volumes–dye method			
		Upper limit		Lower limit		Entire		Preinfusion	Post infusion		6 hr. expansion / 1 hr. expansion × 100
								0 hr.	1 hr.	6 hr.	
	gm.	*mol. wt.*	*per cent*	*mol. wt.*	*per cent*	*mol. wt.*		*ml*	*ml.*	*ml.*	
51	27.2	65,000	8.5	13,000	11	34,000	3,000	2680	3580	3100	47
52	23.2	32,000	15	12,000	10	24,000	3,000	2780	3840	3220	42
55	25.5	64,000	5	38,000	25	46,000	3,200	3190	3980	3790	76
56	22.2	49,000	6	31,000	6	38,600	3,000	4170	4850	(lost)	
57	19.4	49,000	6	31,000	6	38,600	2,100	3390	4180	3800	52
58	23.6	67,000	6	18,000	6	42,900	2,500	3050	3730	3480	63
59	23.5	67,000	6	18,000	6	42,900	3,400	2880	3670	3600	90

There seems to be a general correlation between backbone size and rate of excretion. Conjugate 52 is by far the most rapidly excreted in the urine and has the smallest backbone. Conjugate 55, having a lower limit of 38,000 is excreted less rapidly than Nos. 56, 57, 58, and 59 having lower limits of 18,000 and 31,000. The slow rate of excretion of No. 51 is unexplained. In several cases plasma and urine levels were followed to complete disappearance of the peptide, which occurs in both fluids approximately 1 week after infusion.

It appears therefore that under the conditions of conjugation used, the minimal favorable sizes for side chains and backbones are number average molecular weight of 3,000 and 40,000–50,000 respectively, with a lower limit of 30,000 for the backbone.

As mentioned previously, this combination has several favorable qualities, particularly with respect to side chains. One aspect relates to the fact that side chains of this size are easily prepared for conjugation by the HCl methanol

esterification as described in methods, whereas larger side chains can only be prepared by the use of diazomethane for the esterification (2), which is not only more difficult but is also somewhat hazardous, especially in large scale preparations. Another advantage lies in the easy and sharp separation of

TABLE II

Plasma Levels and Urinary Excretion of Polymers

Polymer	Dose	Hr. post infusion	Plasma level	Total plasma polymer*	Per cent decrease from 1–6 hrs.	Cumulative urinary excretion
	gm.		*mg./ml.*	*gm.*		*per cent total infused*
51	27.2	1	6.4	23		3.1
		6	5.3	16.5	28	8.8
		20				11.6
52	23.2	1	5.2	20		6.6
		6	3.8	12.2	39	28.4
		20				41
55	25.5	1	4.9	19.5		3.9
		6	3.8	14.4	26	8.6
		20				16.5
56	22.2	1	4.0	19.4		9.0
		6	3.3			15.7
		20				26.6
57	19.4	1	4.0	16.7		10.3
		6	3.0	11.4	31	18.5
		20				29
58	23.6	1	4.9	18.2		10.6
		6	3.3	11.6	36	17.4
		20				30
59	23.5	1	5.9	21.6		10.6
		6	4.6	16.6	23	21
		20				30

* Total plasma polymer = mg./ml. × plasma volume obtained by Evans blue method.

conjugate from side chains of this size by alcohol fractionation as described in methods. In contrast, the side chains of number average molecular weight of 12,000 and larger are more difficult to separate from conjugate by this type of fractionation.

In all the cases tested it is apparent that rather low levels of plasma polymers are associated with considerable plasma expansion volumes. The data relating

TABLE III

Fluid Retained in Plasma Polymer

Polymer	Time	Plasma protein	Plasma polymer	Fluid retained/gm. polymer
	hrs.	*gm./liter*	*mg./ml.*	*ml./gm.*
51	0	69.0	0	
	1	48.5	6.4	47
	6	53.8	5.3	42
	20	57	3.8	46
52	0	70	0	
	1	51	5.2	52
	6	53	3.8	64
	20	66	2.3	25
55	0	69.5	0	
	1	52	4.9	51
	6	51	3.8	70
	20	60	3.00	46
56	0	70.5	0	
	1	54	4.0	58
	6	59.8	3.3	49
	20	62	2.0	61
57	0	69	0	
	1	54	4.0	54
	6	57.8	3.0	54
	20	63.8	1.99	38
58	0	68	0	
	1	48	4.9	60
	6	51.4	3.3	74
	20	58	2.92	50
59	0	68.5	0	
	1	50	5.9	46
	6	52.5	4.6	51
	20	61	3.0	37
Average				51

to the volume of fluid retained in the blood stream per gram of polymer are shown in Table III, with the results expressed as oncotic efficiency, $\frac{dv}{dw_2}$. In Table IV similar data and results are given for mixed solutions of polymer and serum albumin in isotonic saline at pH 7.4, in which the range of concentrations of polymer is the same as were found *in vivo*.

The method for arriving at the amount of fluid retained in the circulating plasma per gram of polymer present, hereafter referred to as the oncotic efficiency of the polymer, is as follows. The volume fraction of the plasma retained by the plasma protein at any time, t, is made equal to $\frac{p_t}{p_o}$, in which p_t the plasma protein concentration at time t, and p_o = the plasma protein concentration prior to the infusion. The oncotic efficiency, dw_2, may then be expressed as follows:—

$$\frac{dv}{dw_2} = \frac{1 - \frac{p_t}{p_0}}{y} = \frac{p_0 - p_t}{p_0\, y} \qquad \text{in which}$$

y = the grams of polymer per milliliter of plasma.

TABLE IV

Oncotic Efficiency of Polymer in Mixed Solution with Serum Albumin

Concentration of albumin	Concentration of polymer	Osmotic pressure at 25°C.	$*b_{12}$	$\frac{dv}{dw_2}$	$\frac{dv}{dw_1}$
gm./liter	*gm./liter*	*mm./Hg*		*ml./gm.*	*ml./gm.*
51.3	2.9	25.1	0.0107	50.1	16.8
48.6	3.8	25.8	0.0125	50.2	16.7
46.0	4.7	25.3	0.0111	50.3	16.6
42.0	5.6	24.0	0.0097	51.7	16.9
40.5	6.5	25.7	0.0118	50.5	16.5
0	18.1	27.9		57	

* Average value of b_{12} = 0.011.

These equations are valid if we can make the following three assumptions. The first is that the osmotic pressure of the plasma is constant *in vivo*. The second is that the osmotic pressure of the plasma protein, especially that of the serum albumin which accounts for the major part of this pressure, is independent of its concentration over the pertinent range in an isosmotic mixed solution with the polymer. The third is that the composition of the plasma protein is not sufficiently changed by the infusion to alter significantly its oncotic efficiency.

The first assumption is easily accepted as true. The second has been shown by Scatchard (11) to be essentially true for mixed solutions of serum albumin and straight chain glutamyl polypeptide of number average molecular weight of 15,000. Using Scatchard's method, the development and application of which is discussed in the Appendix, we have demonstrated (Table IV) that the second assumption is also valid for mixed isosmotic solutions of serum albumin and polymer of number average molecular weight of 48,000. The third assumption may not be strictly valid. A comparison of the plasma protein dilution

values at 6 hours post infusion (Table III) with the expansion obtained by the Evans blue method at this time indicates compensatory migration of the protein to the extravascular spaces. If, as seems to be the case with other expanders, the albumin preferentially migrates, the composition and oncotic efficiency of the remaining plasma protein will have changed. It is felt, however, that the possible error thus introduced is inconsequential, and this seems to be borne out by the good agreement between the oncotic efficiency values for the polymer *in vivo* and *in vitro* (Tables III and IV).

The average value of the oncotic efficiency of the polymer determined *in vivo* was 51 ml./gm., and that obtained in vitro, 52 ml./gm. at 25.0°C. (= 54 ml./gm. at 37°C.), both being approximately three times that of serum albumin. It is of further interest that the oncotic effect of the polymer is additive to

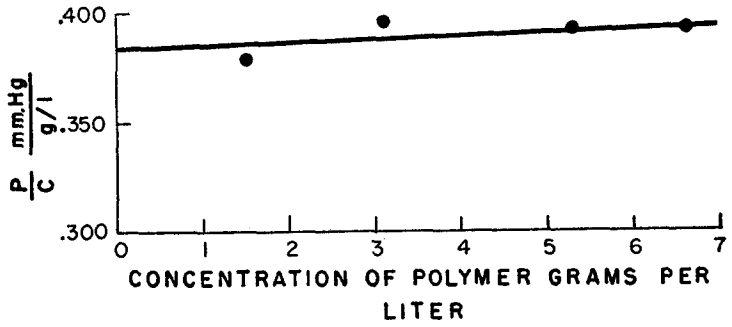

FIG. 1. Ratio of osmotic pressure to concentration versus concentration of polymer in 1.0 M sodium chloride at 25°C.

that of plasma protein and of albumin, with no indication of association between the polymer and proteins.

The possibility that the high oncotic potency of the polymer might be due to low molecular weight rather than to Donnan effects is excluded by the molecular weight of 48,000 calculated from the osmotic pressure concentration curve of Fig. 1.

This calculated molecular weight of 48,000 is, however, surprisingly low when it is remembered that the polymer backbone molecular weight was 46,000 and that side chains of molecular weight of 3,000 were presumably attached to approximately 25 per cent of the carboxyl group in the conjugation. One possible explanation is that this result may be due to error in the molecular weight determination caused by the high concentration of charges associated with the polymer. In this case 48,000 would be a minimum value. A second possibility is that the value of 48,000 is correct, and that breakdown of the backbone occurred in the polyazide stage. The azides are notoriously unstable and a Curtius rearrangement of an azide group would be apt subsequently to result

in a break in the chain. There are about 300 azide groups in a backbone of molecular weight of 40,000 and the rearrangement of 6 or 7 of these, if evenly distributed, would lead to final products of molecular weight of 50,000 if there were 25 per cent coverage with side chains of 3,000 molecular weight. Further work will be needed to determine the correct explanation of this value. Whichever explanation is the correct one, it is evident that the Donnan activity of these polymers is a real factor in relation to their usefulness as expanders.

The clinical response in these seven trials was essentially uneventful. There was no significant increase in temperature or respiration, and a slight transient rise in pulse rate and blood pressure during the early period of expansion. Other than an expected increase in sedimentation rate and decrease in hematocrit value and red cell count following the infusion, there was no effect on the red cells. There was no significant change in platelet counts, clotting, or bleeding times. In two cases there was a rise in white count to 14,000 at 6 and 24 hours, and in two cases there were vague mild complaints of abdominal distress and some anorexia starting the day following the infusion and continuing intermittently for 2 or 3 days. Since these were not new symptoms in these patients, their significance is difficult to evaluate. The possibility of allergic manifestation is of course present but will require larger numbers of tests for final evaluation. In some 35 previous infusions with peptide and polymer prepared in our laboratory no similar complaints or other evidence of sensitivity have been encountered, and in a small series of observations no evidence of positive precipitation test or complement fixation tests between the peptide or conjugate and either preinfusion or 2 to 3 week postinfusion sera have been found. More extensive clinical and immunological studies are in progress. Should any positive allergenic properties of the conjugate manifest themselves, the recent synthesis of alpha (12, 13) and gamma (14) glutamyl peptides offer the possibility of preparation of synthetic conjugates composed of homogeneous d- or l-glutamic acid, either of which might be immunologically inert in humans should the bacterial peptide, which is a mixture of D-, L-glutamic acid prove otherwise. The bacterial peptide is economically attractive, however, thanks to the excellent fermentation yields made available by the studies of Houseright and Thorne and their associates (15) who have developed conditions of deep culture on modified Santon's medium which lead to yields as high as 10 to 16 gm. of peptide per liter of deep culture.

SUMMARY

A series of polymers of polyglutamic acid have been tested as plasma volume expanders. The results indicate that those polymers prepared from backbones of 40,000 molecular weight or over and side chains of 3,000 molecular weight or over, will have plasma volume retention half-life of 15 hours or longer.

Measurements of the oncotic efficiency of these polymers *in vivo* indicated a

blood stream fluid retention of approximately 51 ml./gm. of polymer. Similar measurements of polymer–serum albumin solutions *in vitro* showed a retention of 52 ml./gm. of polymer.

The clinical response to infusion of solutions of these polymers indicated no untoward pharmacological properties. Although the number of trials presented is too small for ultimate conclusion regarding either the physiological or clinical effects of these polymers, they do provide a strong indication of the desirability of further clinical testing of this polymer and a sound basis on which to plan the larger scale preparation of polymer. Both measures are currently under way.

APPENDIX

The oncotic efficiency, $\frac{dv}{dw_{2(p,w_1)}}$ of a non-diffusible substance in solution has been defined by Scatchard (11) "as the number of cc.'s added to an infinite volume of the solution for each gram of the substance added when the osmotic pressure, the quantities of all other non-diffusible substances inside the membrane and the concentration outside the membrane of each diffusible substance is kept constant." He further states that in a mixed solution of two non-diffusible substances, "in the concentration range in which the osmotic pressure is a quadratic function of the total concentration when the ratio $\frac{c_1}{c_2}$ is kept constant, the osmotic pressure is expressed by the relation:—

"(1) $p = a_1c_1 + a_2c_2 + b_{11}c_1^2 + b_{22}c_2^2 + 2b_{12}c_1c_2$

in which

"p = mm. of Hg
"c_1 = gm. of first non-diffusible substances per kg. of water (albumin)
"c_2 = gm. of second non-diffusible per kg. of water (polymer), and
"a_1, a_2, b_{11}, b_{22}, and b_{12} are constants."

The significance of these constants is best understood in relation to their determination. The osmotic pressure, p, of a single non-diffusible substance such as serum albumin, or the polymer, in isotonic salt solution can be defined by the relation

(2) $p = ac + bc^2$ or $\frac{p}{c} = a + bc$, in which c equals the concentration of the non-diffusible solute in grams per kilogram of water. If $\frac{p}{c}$ is plotted as ordinate against c, a curve is obtained whose intercept with the ordinate, where c is at 0, gives the value of the constant 'a'. The constant 'a' relates to the molecular weight of the solute as follows:—

$a\overline{W} = \frac{RT}{V_m^0}$, where R is the usual gas constant, T is the absolute temperature V_m^0, is the volume of the solution containing 1 kg. of water when c is 0, and $\overline{W}$ is the number average molecular weight of the solute in question (16-18).

The constant b is determined as the slope of the curve and represents the sum of three main components. These are (*a*) the effect of ionized groups and their associated charges, which effects account for the Donnan phenomena; (*b*) the interaction effects between the non-diffusible component and ionized salts present; and (*c*) the effects of interaction among the protein molecules themselves or the effects of the protein on its own activity coefficient.

The salt-protein interaction component is usually quite small. These three are combined in the one constant, b, which is a quadratic term and is determined in a salt-protein solution.

The values of the 'a' and 'b' constants for serum albumin (a_1 and b_{11}) are given by Scatchard (11) as 0.267 and 0.00256 respectively. The value of 0.384 for the polymer 'a' constant, a_2 is obtained from $\frac{p}{c}$ *vs.* c curve shown in Fig. 1. The data for this curve were obtained with a 1.0 M NaCl solution of polymer. In this concentration of salt the Donnan effects of the polyelectrolyte are suppressed so that the limiting value of $\frac{p}{c}$ should reflect only the molecular weight of the polymer. The value of the b polymer constant, b_{22}, was obtained from measurements of the colloid osmotic pressure of the polymer in isotonic saline at pH 7.4 which corresponds more closely to physiological conditions. The data are given in Table V. The positive deviations of osmotic pressure at the higher and lower concentrations of polymer are similar to those noted by Scatchard in measurements with the lower molecular

TABLE V

Osmotic Pressure of Polymer in Isotonic Saline

Concentration	Osmotic pressure
gm./liter	*mm.Hg.*
2.50	1.90
5.02	3.55
8.14	5.81
9.93	8.26
11.86	11.2
13.95	14.7
16.10	20.4
18.14	27.9

weight straight chain polyglutamic acid (11). Using these data and the independently determined value of a_2, Equation 2 can be solved for b_{22}, giving a value of 0.046.

In mixed solutions of two non-diffusible components, the term ac in Equation 2 becomes $\Sigma_N a_N c_N$ and bc^2 becomes $\Sigma_{MN} b_{MN} c_M c_N$, and Equation 2 expands into Equation 1. The first four constants of Equation 1 are given above. The b_{12} term can now be determined in Equation 1 by measurements of p for the mixed solution of the polymer and albumin. Values of b_{12} are given in Table IV. The average value was used for calculations of oncotic efficiency, $\frac{dv}{dw_2}$.

An equation expressing oncotic efficiency of the polymer, $\frac{dv}{dw_2}$ in the above terms can now be derived. By substituting $\frac{w_1}{v}$ and $\frac{w_2}{v}$ for c_1 and c_2 in Equation 1, and differentiating with w_1 and p as constants, the expression

$$\frac{dv}{dw_2} = 1000 \frac{a_2 + 2b_{12}c_1 + 2b_{22}c_2}{a_1c_1 + a_2c_2 + 2b_{11}c_1^2 + 2b_{22}c_2^2 + 4b_{12}c_1c_2} \tag{3}$$

is obtained.

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