THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 250, No. 4, Issue of February 25, pp. 1484-1489, 1975
Printed in U.S.A.

Affinity Partitioning

A METHOD FOR PURIFICATION OF PROTEINS USING SPECIFIC POLYMER-LIGANDS IN AQUEOUS POLYMER TWO-PHASE SYSTEMS*

(Received for publication, May 24, 1974)

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SUMMARY

We describe a method, called affinity partitioning, for the purification of proteins containing specific ligand binding receptor sites. This method adds specificity to the procedures for protein purification with aqueous polymer two-phase systems by introduction of a polymer derivative, coupled to an appropriate ligand. The addition of a polymer-ligand that partitions predominantly into one phase shifts the protein that binds this substance to the same phase. By performing countercurrent distribution in the presence of a polymer-ligand, the protein that binds the polymer-ligand can be separated from a heterogenous mixture.

One example of affinity partitioning used dextran as the polymer-ligand. Dextran was chosen since it is a constituent of the most commonly used system for partitioning proteins. In a dextran-poly(ethylene oxide) system, concanavalin A bound dextran and partitioned predominantly into the dextran-rich phase. The addition of the specific competitor, D-mannose, displaced the partition coefficient toward unity, while the application of L-fucose, a noncompetitor, had little effect.

Application of affinity partitioning to the purification of another protein required the synthesis of a specific polymer-ligand. To study this we synthesized dinitrophenyl-poly(ethylene oxide), which binds specifically to S-23 myeloma protein. Addition of dinitrophenyl-poly(ethylene oxide) to the dextran-poly(ethylene oxide) phase system shifted the S-23 myeloma protein into the poly(ethylene oxide)-rich phase. ε-*N*-dinitrophenyl-L-lysine, by competing with binding of dinitrophenyl-poly(ethylene oxide), antagonized the latter's effect on the partition coefficient of S-23 myeloma protein. By adding various amounts of dinitrophenyl-poly(ethylene oxide), we correlated the partition coefficient with concentration of polymer-ligand. A model of the action of polymer-ligand derivatives on the partition coefficient, derived from thermodynamic considerations, was found to be consistent with the experimental data relating the concentration of polymer-ligand and partition coefficient.

Affinity partitioning should prove to be a useful complement to affinity chromatography in the purification of mixtures of proteins. Since cells and subcellular particles may be purified with aqueous polymer two-phase systems, affinity partitioning might be applied to their fractionation by using polymer-ligands specific for unique surface receptors.

When solutions of appropriate concentrations of poly(ethylene oxide) and dextran are mixed, two aqueous phases are formed (1). The top phase is rich in poly(EO)[1]; the bottom is rich in dextran. Albertsson and others have successfully employed such aqueous polymer two-phase systems with countercurrent distribution to fractionate cells, membrane fragments, nucleic acids and proteins (1–7).

Partitioning of substances in these systems has been shown to be influenced by the addition of charged or hydrophobic derivatives of dextran or poly(EO) as well as salt composition and pH (1, 8). Walter *et al.* (9, 10) demonstrated that addition of the polycationic polymer DEAE-dextran to such systems markedly influenced the partition coefficients of mammalian erythrocytes. We recently demonstrated that effects of DEAE-dextran on partition coefficient are due to the binding of the polycation to the polyanionic red blood cell surface (11). We presented evidence that DEAE-dextran apparently changes the partition coefficient by altering the surface charge of the erythrocyte. Johansson *et al.* (3, 12) showed that charged derivatives of poly(EO) markedly influenced the partition coefficient of proteins in dextran-poly(EO) systems.

Albertsson demonstrated that the addition of a surfactant, containing a short chain of poly(EO), markedly changed the partition coefficient of hydrophobic proteins (13). He noted that the addition of Triton X-100 (*p-tert*-octylphenyl-poly(EO)) to dextran-poly(EO) systems resulted in the partitioning of

* This work was supported by Grant MH 18282 from the United States Public Health Service and a grant from the Alfred P. Sloan Foundation.

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[1] The abbreviations used are: poly(EO), poly(ethylene oxide) or poly(ethylene glycol); Dnp-poly(EO), 2,4-dinitrophenyl derivative of poly(EO) (see "Materials and Methods" for complete characterization); Con A, concanavalin A; ε-*N*-Dnp-lysine, ε-*N*-2,4-dinitrophenyl-L-lysine.

Escherichia coli phospholipase A1, a hydrophobic protein, into the poly(EO)-rich phase. Albertsson explained this result by hypothesizing that the hydrophobic region of the detergent interacts with the hydrophobic region of the protein while the poly(EO) region interacts with the poly(EO)-rich phase causing the detergent enzyme complex to partition into the poly(EO)-rich phase.

None of the polymer additives thus far studied causes a change in partitioning by binding to a unique receptor site. We reasoned that the addition of polymer derivatives with unique affinity for specific binding sites might be valuable for the purification of proteins and that such a method would prove a useful complement to affinity chromatography (14). In the present report, we demonstrate that the partition coefficients of proteins containing receptor sites for dextran or a derivative of poly(EO) are markedly influenced by such binding; and that this "affinity partitioning" effect can be used for the purification of specific proteins.

MATERIALS AND METHODS

Polymer Solutions—Dextran T-500 (Lot 17) was obtained from Pharmacia and poly(ethylene oxide) 6000 from Matheson Coleman and Bell. Stock solutions were prepared as described by Albertsson (1). The concentrations of dextran solutions were calibrated by polarimetry, specific rotation $[\alpha]_D^{25} = +199°$. The final composition of the systems is described under "Results" and in the legends. In most cases, a 2X phase system of poly(EO) and dextran was prepared, and then diluted with the appropriate buffers and polymer derivatives. For countercurrent distribution, the complete phase systems were prepared and separated into poly(EO)-rich and dextran-rich phases, then added to the apparatus.

Proteins—Concanavalin A and normal rabbit γ-globulin (Fraction II) were obtained from Miles Laboratories, Inc. William Geckler, Salk Institute, generously provided a sample of S-23 myeloma protein, which binds di- and trinitrophenyl compounds. This protein purified by affinity chromatography was derived from serum of mice injected with myeloma cells.

Polymer Derivatives—The ethylenediamine derivative of poly(EO) was synthesized to allow coupling of dinitrofluorobenzene to this polymer. Synthesis of the ethylenediamine derivative is based on the procedure of Johansson (3, 12) for the synthesis of trimethylamino-poly(EO). One gram of bromo-poly(EO), the generous gift of Dr. Göte Johansson, University of Umeå, Umeå, Sweden, was dissolved in 10 ml of absolute ethanol at 70° and 2 ml of ethylenediamine (Matheson Coleman and Bell) were added. After continuing reaction for 48 hours at 70°, excess ethylenediamine was removed by bubbling with dry nitrogen for 3 hours at 70°. The polymer was then precipitated by the addition of 5 ml of diethyl ether for 24 hours at 4°. The flocculent precipitate was collected on Whatman No. 50 filter paper, washed with 200 ml of diethyl ether at room temperature, and the precipitate was placed under vacuum at 70° for 4 hours.

Dinitrophenyl-poly(EO) was synthesized from ethylenediamino-poly(EO) by reacting it with dinitrofluorobenzene (Sigma). A solution containing 0.8 g of ethylenediamine derivative of poly(EO) and 0.75 ml of dinitrofluorobenzene in 25 ml of absolute ethanol was heated at 70° for 3 hours. The insoluble residue was removed by decanting and 12.5 ml of diethyl ether were added to precipitate the polymer. The mixture was maintained at 4° for 24 hours and the precipitate was collected on Whatman No. 50 filter paper, washed with 350 ml of diethyl ether, and then with 150 ml of absolute ethanol, and then again with 50 ml of diethyl ether all at 4°. The precipitate was dried under vacuum for 4 hours at 70°. After the molten polymer cooled, it was further purified by dissolving in 0.15 M NaCl and gel filtrating on Sephadex G-25. The void volume peak, constituting 90% of the dinitrophenyl absorption at 360 nm, was then mixed with phase solutions. The amount of dinitrophenyl incorporated into poly(EO) was measured by the absorption of dinitrophenyl at 360 nm. We assumed that the molar extinction coefficient of dinitrophenyl conjugated to the primary amine of the ethylenediamine derivative of poly(EO) is identical with that of ϵ-*N*-Dnp-lysine. We measured the optical density of Dnp-poly(EO) at pH 7.4 and assumed a molar extinction coefficient of 17,530 (15). ϵ-*N*-Dnp-lysine was obtained from Sigma. There was 0.27 mmol of dinitrophenyl per g of poly(EO), indicating that there were 2.0 dinitrophenyl groups per 7,400 molecular weight. In all experiments where the concentration of Dnp-poly(EO) is given, this really indicates the concentration of dinitrophenyl moieties, all of which are bound to polymer. For the concentration of polymer molecules which contain dinitrophenyl, the indicated concentrations may be divided by 2.0.

Iodination of Proteins—S-23 myeloma protein and normal rabbit γ-globulin were iodinated by the method of McConahey and Dixon (16) using 10 μg of chloramine-T per mg of protein and various amounts of radioactive iodide. Approximately half of the added ^{125}I or ^{131}I was incorporated, resulting in specific activities in the range of 0.75 to 1.5 mCi per mg for ^{125}I-labeled S-23 myeloma protein and 0.15 to 0.5 mCi per mg for ^{131}I-labeled γ globulin. The iodinated proteins were dialyzed against 0.15 M sodium phosphate, pH 7.4. The dialyzed proteins were then repurified by gel filtration in Sepharose 4B. We collected the peak fraction corresponding to a molecular weight of about 180,000 for S-23 myeloma protein and the peak fraction corresponding to a molecular weight of about 150,000 for γ-globulin. For experimental data presented in in Table II, S-23 was repurified on Sephadex G-200 and the fraction collected for use was the void fraction which contained 90% of the ^{125}I-labeled S-23 myeloma protein, including higher molecular weight species.

Partition Coefficient Measurements—Phase systems were prepared by mixing stock solutions and appropriate buffers and additives. Upon addition of the protein to be separated, the system was shaken in a Vortex mixer every 10 min for 1 hour. The system was then placed in a syringe fitted with a valve. After 3 hours an aliquot was aspirated from the top phase, and an aliquot of the bottom phase was collected by dripping through the valve.

To estimate protein in the experiments with Con A we used a modified ninhydrin assay (17, 18). In this modification, we replaced *n*-propyl alcohol with water since *n*-propyl alcohol precipitated the polymers. The standard Folin-phenol protein assay could not be used because the polymers interfered. Aliquots of iodinated protein were counted in a Nuclear-Chicago γ-counter.

The partition coefficient of Dnp-poly(EO) was measured in phase systems of two salt compositions: (*a*) 36 mmol of NaCl, 25 mmol of sodium phosphate (pH 6.8) per kg of phase system; (*b*) 66.25 mmol of NaCl, 8 mmol of sodium phosphate (pH 6.8) per kg of phase system. Aliquots of the top and bottom phase of a system containing 5% (w/w) dextran T-500, 4% (w/w) poly(EO) 6000, and 0.19 mmol of Dnp-poly(EO) per kg of phase system were diluted in 0.1 N NaOH and the optical density at 360 nm was measured. Background absorption was subtracted and the optical density in top phase divided by the optical density in bottom phase was taken as the partition coefficient.

Countercurrent Distribution—We constructed a thin layer countercurrent distribution apparatus similar to that described by Albertsson (19). Each of the 60 chambers was filled with 0.6 ml each of top and bottom phases. Proteins to be distributed were mixed with phase solutions in chamber 1. The automated apparatus was set to provide a cycle of 20 s of gyratory shaking (1 inch diameter, 300 rpm) and 4 min settling time. After 60 transfers, the phases were broken by the addition of 0.6 ml of glass-distilled water, mixed by gyratory shaking, and aliquots removed for assay. Where double label experiments were performed with ^{131}I- and ^{125}I-labeled proteins, appropriate corrections were made for spill from the ^{131}I into the ^{125}I channel.

RESULTS

Partitioning of Concanavalin A—Since dextran is known to bind Con A, we first determined whether this specific interaction influenced the partition coefficient of this protein. In the system which we used here, Con A had a partition coefficient of 0.04 (Table I). This suggested that the great affinity of Con A for the dextran-rich phase is due to its binding to dextran. We confirmed this hypothesis by finding that the addition of D-mannose, a specific competitor of the binding of Con A (20) to dextran, substantially increased the partition coefficient of Con A whereas L-fucose had little effect (Table I).

TABLE I

Partitioning of concanavalin A in dextran poly(ethylene oxide) aqueous polymer phase systems

Partitioning of concanavalin A in dextran T-500, 6.28% (w/w), poly(EO) 6000, 4.45% (w/w), 1.1 mol of NaCl per kg per phase system, and the indicated monosaccharide concentrations. Con A was added to phase system to a final concentration of 0.6 mg per g of phase system. The system was then shaken in a Vortex mixer repeatedly for 1 hour and then allowed to settle for 3 hours. Aliquots were assayed for protein by a modified ninhydrin assay.

Monosaccharide	Partition coefficient
None	0.04
L-Fucose (86 mM)	0.08
D-Mannose (29 mM)	0.23
D-Mannose (57 mM)	0.44
D-Mannose (86 mM)	0.46

Since the bottom phase is dextran-rich in this system, we considered the possibility that Con A was simply precipitated in the presence of dextran. To evaluate this, phase systems containing Con A were diluted to a composition below the binodial curve, where only a homogeneous phase is formed; and were centrifuged at 500 × *g* for 15 min. No precipitate was observed and an assay of the supernatant showed that the supernatant protein concentration was identical in the absence and presence of 85.7 mmol of mannose per kg of phase system. Apparently the high concentration of dextran precludes precipitation of Con A. The validity of the "affinity partitioning" effect is further strengthened by results described below where the shift in partitioning is from bottom phase to top.

Effect of Addition of Dnp-Poly(EO) on Partitioning of S-23 Myeloma Protein—The partition coefficient of Dnp-poly(EO) was determined in two systems as described under "Materials and Methods." In both systems studied it was 3.1, which is close to that published for the unsubstituted poly(EO), 3.5 (21). This is consistent with the findings of Johansson that the phase diagrams of charged derivatives of poly(EO) are close to that of unsubstituted poly(EO) in dextran-poly(EO) systems under conditions similar to those used here (12).

Addition of Dnp-poly(EO) markedly affected the partition coefficient of ^{125}I-labeled S-23 myeloma protein (Table II). The partition coefficient of ^{125}I-labeled S-23 myeloma protein was found to be 2.8 in the absence of Dnp-poly(EO) or ε-*N*-Dnp-lysine. In the presence of 0.18 mmol of Dnp-poly(EO) per kg of phase system, the myeloma protein was shifted markedly into the poly(EO)-rich phase. This change in partition coefficient could be reversed by the addition of ε-*N*-Dnp-lysine, indicating that it is the result of the specific interaction of Dnp-poly(EO) with S-23 myeloma protein. Experiments not shown indicate that the partition coefficient of normal γ-globulin was not changed by the addition of either Dnp-poly(EO) or ε-*N*-Dnp-lysine.

The potential utility of a polymer-ligand like Dnp-poly(EO) in purification of a protein with which it reacts was shown by countercurrent distribution of the protein in the presence and absence of polymer-ligand. Mixtures of ^{125}I-labeled S-23 myeloma protein and ^{131}I-labeled γ-globulin were fractionated in a countercurrent apparatus in a two-phase system in the presence of Dnp-poly(EO) or ε-*N*-Dnp-lysine. In the presence of Dnp-poly(EO) there was a striking shift in the partitioning of the S-23 myeloma protein but no effect on the partitioning of normal γ-globulin (Fig. 1).

TABLE II

Partitioning of ^{125}I-labeled S-23 myeloma protein

^{125}I-Labeled S-23 myeloma protein was preincubated in buffer for 1 hour with or without Dnp-poly(EO) and ε-*N*-Dnp-lysine, as indicated. A 2X polymer system was then added and the mixture was agitated and then allowed to settle for 3 hours. Aliquots were removed for counting. The final phase system contained 50 g of dextran T-500, 40 g of poly(EO) 6000, 37.5 mmol of [Cl^-], 37.5 mmol of phosphate, 9 mmol of [K^+], 94.5 mmol of [Na^+], and 10^{-7} mol of S-23 myeloma protein per kg of phase system.

Dnp-poly(EO)	ε-*N*-Dnp-lysine	Partition coefficient
mM	mM	
0.0	0.0	2.8
0.0	0.50	2.8
0.18	0.50	3.1
0.18	0.25	5.4
0.18	0.12	6.1
0.18	0.0	7.0

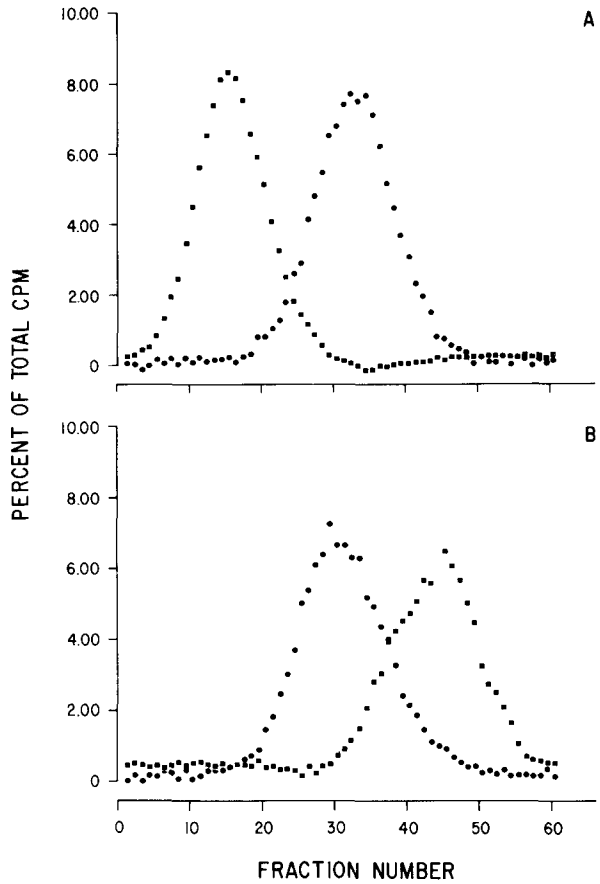

FIG. 1. Countercurrent distribution of ^{125}I-labeled S-23 myeloma protein (■) and ^{131}I-labeled γ-globulin (●): *A*, in the presence of 5.4 × 10^{-4} mol of ε-*N*-Dnp-lysine per kg of phase system; *B*, in the presence of 2.6 × 10^{-4} mol of Dnp-poly(EO) per kg of phase system. The phase system contained 55 g of dextran T-500, 40 g of poly(EO) 6000, 80.9 mmol of NaCl, and 8.7 mmol of sodium phosphate (pH 6.8) per kg of phase system; 10^{-9} mol of each protein were added to the first chamber of the apparatus.

Theoretical Analysis of Behavior of Interacting Components in Two-Phase System—To facilitate plotting of data on the effects of different concentrations of Dnp-poly(EO) on partitioning of S-23 myeloma protein, it was first necessary to analyze this interaction from fundamental thermodynamic considerations. Be-

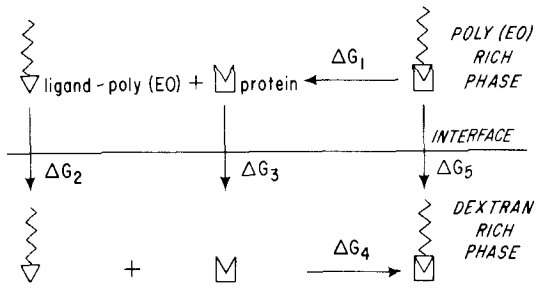

FIG. 2. Diagram depicting distribution of monovalent protein and an interacting monovalent polymer-ligand in an aqueous polymer two-phase system. For details see text.

havior of interacting components in a dilute system at equilibrium can be envisioned as in Fig. 2, if one assumes no adsorption of materials at the interface between the two phases, a monovalent polymer-ligand, and one biospecific binding site per protein.

Since the Gibbs' free energy is a state function, ΔG_5, the change in free energy required to transfer 1 mol of the polymer-ligand protein complex from the top to the bottom phase, can be calculated from the values of the free energy changes for dissociating the complex in the top phase, transferring the dissociated species across the interface, and then reassociating the complex in the dextran-rich phase. Therefore:

$$\Delta G_5 = \Delta G_1 + \Delta G_2 + \Delta G_3 + \Delta G_4 \qquad (1)$$

The dissociation constants K_1 and K_4 in the top and bottom phases, respectively, are related to ΔG_1 and ΔG_4 by

$$\Delta G_1 = -RT \ln K_1 \qquad (2)$$

$$\Delta G_4 = RT \ln K_4 \qquad (3)$$

K_2, K_3, and $K^{\S}$, the partition coefficients of free polymer-ligand, free protein, and protein-polymer complex, respectively, are related (Albertsson (1)) to ΔG_2, ΔG_3, and ΔG_5 by

$$\Delta G_2 = RT \ln K_2 \qquad (4)$$

$$\Delta G_3 = RT \ln K_3 \qquad (5)$$

$$\Delta G_5 = RT \ln K^{\S} \qquad (6)$$

Substituting Equations 2 through 6 into 1 we obtain

$$K^{\S} = (K_4K_2/K_1)\ K_3 \qquad (7)$$

If

$$(K_4K_2/K_1) \neq 1 \quad \text{then} \quad K^{\S} \neq K_3 \qquad (8)$$

and the binding of the polymer derivative will result in a change in the partition coefficient of the protein.

For a protein containing N binding sites, if we assume that the intrinsic dissociation constant is independent of the fraction of sites occupied, we derive an analogous expression

$$K_N^{\S} = (K_4K_2/K_1)^N\ K_3 \qquad (9)$$

where $K_N^{\S}$ is the partition coefficient of the protein-polymer complex containing N polymer derivatives per complex.

[A″] is now defined as the concentration of the free protein, with the superscript indicating the bottom phase, and [A′] is the concentration of free protein in the top phase. [P″], [P′] are the analogous expressions for the concentration of the free polymer-ligand and [AP″], [AP′] for the concentration of the species consisting of one polymer-ligand and the protein, and [AP_2″], [AP_2′] the expressions for the species containing two polymer-ligands per protein. By definition of the partition coefficient,

$$[A'] = K_3\ [A''] \qquad (10)$$

In the following, in addition to assumptions stated above, the protein is assumed to consist of two binding sites and the polymer-ligand is assumed to contain one ligand per molecule. This approximates the case we studied experimentally in that the fraction of S-23 myeloma protein we used had two binding sites per molecule. Although the Dnp-poly(EO) had two ligands per molecule we assume, to simplify these calculations, that only one S-23 myeloma binding site may be occupied by each Dnp-poly(EO) molecule. Given these assumptions the dissociation of AP″ can be written as:

$$AP'' \overset{K_4/2}{\rightleftharpoons} A'' + P'' \qquad (11)$$

The intrinsic dissociation constant K_4 is related to [A″], [AP″] by the usual expression for antibody-hapten binding,

$$[AP''] = 2\ [A'']\ [P'']\ /\ K_4 \qquad (12)$$

From Equation 7, and the definition of partition coefficients,

$$[AP'] = (K_2K_4/K_1)\ K_3\ [AP''] \qquad (13)$$

For the binding of the second polymer-ligand,

$$AP_2'' \overset{2K_4}{\rightleftharpoons} AP'' + P'' \qquad (14)$$

and

$$[AP_2''] = [A'']\ [P'']^2\ /\ (K_4)^2 \qquad (15)$$

then from Equation 9,

$$[AP_2'] = (K_2K_4/K_1)^2\ K_3\ [AP_2''] \qquad (16)$$

If we assume that the over-all concentration of protein is 1 unit, that the volume of each phase is 1 unit, making a total volume of 2 units, and that C, the over-all concentration of the polymer-ligand, is much greater than the protein concentration, then

$$[P''] = 2\ C/(1. + K_2) \qquad (17)$$

The total amount of protein is given by the sum of the concentration of the six species of protein and complexes in the top and bottom phases, each of which are expressed in terms of [A″] in Equations 10, 12, 13, 15, and 16. Therefore, [A″] can be expressed in terms of K_1, K_2, K_3, K_4, and [P″] giving

$$[A''] = \frac{2}{1 + K_3 + \frac{2[P'']}{K_4}\left(1 + K_1^{\S}\right) + \left(\frac{[P'']}{K_4}\right)^2\left(1 + K_2^{\S}\right)} \qquad (18)$$

[A′], [AP′], [AP″], [AP_2′], [AP_2″] can be calculated from [A″] and [P″] by substituting into Equations 10 and 12 to 17. The measured partition coefficient of protein in the presence of polymer-ligand, K_T, is then given by

$$K_T = \frac{[A'] + [AP'] + [AP_2']}{[A''] + [AP''] + [AP_2'']} \qquad (19)$$

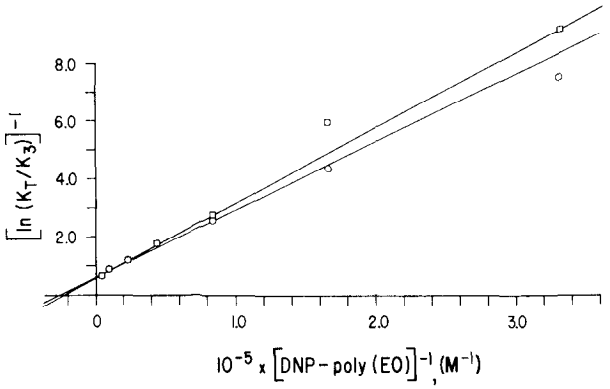

FIG. 3. Relationship between concentration of Dnp-poly(EO) and partitioning of ^{125}I-labeled S-23 myeloma protein. K_T is the measured partition coefficient in the presence of Dnp-poly(EO). K_3 is the measured partition coefficient in the absence of Dnp-poly(EO). Calculations by computer program show that for conditions used here a plot of $[\ln(K_T/K_3)]^{-1}$ *versus* [Dnp-poly(EO)]$^{-1}$ should closely approximate a straight line. The polymer concentrations were the same as in Table II. Two salt compositions were used: (□) 36 mmol of NaCl, 25 mmol of sodium phosphate (pH 6.8) per kg of phase system; (○) 66.25 mmol of NaCl, 8 mmol of sodium phosphate (pH 6.8). The ^{125}I-labeled S-23 myeloma protein used was the divalent form purified by gel filtration as described under "Materials and Methods." Its concentration was 10^{-7} mol per kg of phase system.

A computer program utilizing equations derived above to calculate K_T from values of the parameters K_1, K_2, K_3, K_4, and [Dnp-poly(EO)] showed that for values of parameters similar to those of data displayed in Fig. 3, the calculated relationship between K_T and [Dnp-poly(EO)] closely fit a straight line when plotted as $[\ln (K_T/K_3)]^{-1}$ *versus* [Dnp-poly(EO)]$^{-1}$ and was independent of K_3.

Given this analysis we calculated, by an iterative process, values for K_1 and K_4 that fit the linear regression from experimental data (Fig. 3). Studies were performed in systems with different salt compositions since this changed the partition coefficient of the protein. In the system containing 36 mmol of NaCl and 25 mmol of sodium phosphate (pH 6.8) per kg of phase system, the partition coefficient (K_3) of the free protein in the absence of Dnp-poly(EO) was 1.18. The data was fitted to a weighted linear regression with a y-intercept of 0.561 ± 0.08 and a slope of 2.831 ± 0.13 × 10^{-5} M. Based on computer calculations,[2] the linear regression fits the model when K_1 = 1.0 ± 0.15 × 10^{-4} M and K_4 = 8.2 ± 1.9 × 10^{-5} M. In the system containing 66.3 mmol of NaCl and 8 mmol of sodium phosphate (pH 6.8) per kg of phase system, K_3 was 0.75. When the data was fitted to a linear regression, the y-intercept was 0.617 ± 0.112 and the slope was 2.33 ± 0.14 × 10^{-5} M and K_1 = 8.07 ± 1.5 × 10^{-5} M, K_4 = 5.9 ± 2.5 × 10^{-5} M.

DISCUSSION

We have shown that affinity partitioning can be used for the purification of proteins containing biospecific receptors. In many cases a countercurrent distribution apparatus may be required to effect successful purification. However, in certain favorable cases, countercurrent distribution may not be necessary. For example, it may be calculated that in cases where the partition coefficient of protein bound to polymer-ligand is greater than 20 and the partition coefficients of contaminating proteins is less than 1, five sequential extractions, performed at an optimal ratio (22) of volume of top phase to bottom phase of $1/\sqrt{20 \times 1}$ would change the ratio of specific protein to impurities in the final top phase by a factor of 1,700:1. The yield of purified protein in the top phase in this case would be about 36%. High partition coefficients of the protein complex with polymer-ligand, like those assumed in this example, could be easily achieved with proteins containing multiple binding sites. For example, in the case of a protein with four available binding sites, if the value of K_2K_4/K_1 is greater than 4 and K_3 is greater than 1, then at saturating concentrations of polymer-ligand, the partition coefficient of ligand-binding protein would be greater than 4^4 or 256. Clearly, under these hypothetical conditions, a countercurrent distribution apparatus would not be required to provide a high degree of purity. High partition coefficients of the protein complex with polymer-ligand would also be favored if the polymer-ligand had a high molecular weight since partition coefficients of unsubstituted polymer in two-phase systems are a function of molecular weight (1). Ligand-poly(EO) of 15,000 to 20,000 molecular weight, which could be synthesized from available polymers, should have a partition coefficient of approximately 25, which is 1 order of magnitude greater than the poly(EO) of molecular weight 6,000 to 7,500 which we used here.

Even in cases far less favorable than those just considered, two advances in countercurrent distribution apparatus design should permit the application of affinity partitioning. The thin layer countercurrent distribution apparatus reduces the time required per transfer from 20 min, required with a Craig apparatus, to 5 min or less (19). The elution centrifuge, which has yielded resolution as high as 10,000 theoretical plates when applied in organic-aqueous phase systems, was recently applied to purification of polynucleotides in aqueous polymer two-phase systems (23, 24) and might be adapted for affinity partitioning.

Affinity partitioning would be most favorable in cases where the protein has a high affinity for polymer-ligand. However, purification might also be achieved in cases where the affinity is relatively low since large amounts of polymer-ligand may be added to the system. For example, in the system used here, if all of the poly(EO) is replaced with divalent ligand-poly(EO), the over-all concentration of ligand would be 10 millimolar. Since substantial shifts in partition coefficients occur at concentrations of polymer-ligand near the dissociation constant, it should be possible to purify proteins with binding sites that have dissociation constants in the 50 mM range. In proteins with more than two binding sites, purification should be possible even in cases where the dissociation constants are even greater.

Another potential application of affinity partitioning is in the purification of subcellular particles or cells. In this particular case, affinity partitioning might prove of much greater value than affinity chromatography with which it must always be compared. Affinity chromatography achieves purification by binding macromolecules to ligands attached to a solid matrix. We expect that the interaction of subcellular particles with such solid matrix should be less favorable than the interaction, in liquid phase, of polymer-ligand with particles containing specific receptors.

[2] A supplement documenting the computer calculations described is available as JBC Document Number 74M-735, in the form of one microfiche or 23 pages. Orders for supplementary material should specify the title, author(s), and reference to this paper and the JBC Document number, the form desired (microfiche or photocopy) and the number of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Maryland 20014 and must be accompanied by remittance to the order of the Journal in the amount of $2.50 for microfiche or $3.45 for photocopy.

Hence, affinity partitioning may allow the purification of cells or subcellular particles containing biospecific receptors.

A final application of affinity partitioning is as an analytical technique. Since the relationship between partition coefficients and concentration can be related to measurable parameters and fitted to other parameters, as shown above, it should be possible to use affinity partitioning to measure binding constants and the number of binding sites. This would be especially useful when only a small amount of a homogenous macromolecule is available. Under these circumstances the macromolecule could be labeled with a radioactive tracer of high specific activity and mixed with polymer-ligand in a two-phase system. From the partition coefficients at various polymer-ligand concentrations, binding constants and the number of binding sites could be calculated in circumstances where ordinary equilibrium dialysis is not practical.

Acknowledgments—We thank Mr. Gary Smith and Ms. Donna Reeve for technical assistance.

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J. Biol. Chem. 1975, 250:1484-1489.