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COUNTER-CURRENT DISTRIBUTIONS OF RENIN SUBSTRATE AND SERUM PROTEINS IN POLYETHYLENE GLYCOL–SALT SYSTEMS

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SUMMARY

Aqueous two-phase partitioning systems for proteins were prepared from polyethylene glycols and inorganic salt solutions. The effects of the molecular-weight range of the glycol, relative proportion of water, pH, temperature and nature of the inorganic cations and anions on the total solubilities and relative phase distributions of albumin, γ-globulin, total serum proteins and renin substrate were determined. These results led to the formulation of Na_2SO_4, $(NH_4)_2SO_4$ and Li_2SO_4 systems that were practical for counter-current distributions of renin substrate and unfractionated serum. Preliminary to the distribution of renin substrate, purification by batchwise partitioning was used. Substrate purities as high as 24300 Goldblatt units/g of protein were obtained. Serum was resolved into several groups of proteins, including γ-globulins with widely differing K values.

INTRODUCTION

Very few protein purification procedures have utilized counter-current distribution, although this technique has been widely applied to the purification of polypeptides. The failure to use counter-current distribution has primarily been due to a lack of satisfactory solvent systems, except in special instances. The ease with which protein structure, unlike that of most natural products, can be altered by organic solvents greatly restricts the choice of systems. Generally two-phase systems which do not harm proteins do not have adequate capacities or give practical K values for protein. CRAIG[1] and TAFEL AND SIGNER[2] have reviewed the theory and technique of counter-current distribution with application to the purification of several alcohol-stable proteins. Insulin and serum albumin were distributed in butanol–chloracetic acid systems and RNAase, lysozyme and serum albumin in ethanol–ammonium sulfate systems. Obviously, these solvents cannot be used with proteins that are sensitive to alcohol or chloracetic acids.

Abbreviation: PEG, polyethylene glycol.

In a recent monograph, ALBERTSSON[3] has described the development of 13 different aqueous two-phase systems for the distribution of cell particles and macromolecules up to the size of viruses. He found dextran–PEG systems to be particularly useful for partitioning proteins and presented counter-current distributions of phycoerythrin, ceruloplasmin and phycocyanin. ALBERTSSON also presented phase diagrams for systems not containing dextran, such as PEG–potassium phosphate, ammonium sulfate or magnesium sulfate, but did not use these systems for protein counter-current distributions. He noted that PEG systems showed no denaturing action on proteins and indicated that they may possibly prevent surface denaturation.

This report will describe the application of PEG–inorganic salt systems to the purification of renin substrate and to the distribution of human serum.

MATERIALS AND METHODS

The PEG used in the following solvent systems were various types of Carbowax*, prepared as 50% (w/v) aqueous stock solutions. The grades used and their average molecular weights were as follows: PEG 1000, mol. wt. 950–1050; PEG 1500, mol. wt. 500–600**; PEG 1540, mol. wt. 1300–1600 and PEG 6000, mol. wt. 6000–7500.

TABLE I

PEG–SALT SYSTEMS FOR COUNTER-CURRENT DISTRIBUTION OF PROTEINS

System	*Protein*	*Salts*	*50% (w/v) Glycol*	*pH*	*Buffer*	*Water*
A	Substrate	850 g Na_2SO_4	3000 ml PEG 1540	6.5 8.5	120 ml M Na_2HPO_4 10.9 g Tris	5400 ml 6000 ml
B	Substrate	1150 g $Li_2SO_4 \cdot H_2O$	3275 ml PEG 1540	4.8 8.0	65–75 ml M Na succinate 17.4 g Tris	3930 ml 3930 ml
C	Serum	2400 ml 4 M $(NH_4)_2SO_4$	3360 ml PEG 1000	8.0	—	3000 ml
D	Substrate	1260 g Na citrate $(NaC_6H_5O_7 \cdot H_2O)$	2220 ml PEG 1540	6.5 7.5	120 ml M NaH_2PO_4 —	4380 ml 4380 ml
E	Serum	2800 ml 2 M Na_2SO_4 2450 ml 0.9% NaCl	2800 ml PEG 1000	8.5	112 ml M Na_2HPO_4	210 ml

Protein was measured spectrophotometrically at 280 mμ if both ammonia and PEG were present, colorimetrically by the Pauly diazo histidine reaction if ammonia was absent, or by the Folin–Lowry phenol reaction if both interfering substances were absent. The colorimetric methods were specific adaptations for the Auto-analyzer***. In all cases, protein standards and blanks were prepared to contain the same concentrations of PEG and salts as the unknowns.

Renin substrate activity was assayed as angiotensin produced after incubating with renin as described previously[4]. PEG, Na_2SO_4 or Li_2SO_4 did not interfere in the assay. When ammonia was present in the solvents, it was removed by dialysis before the renin incubation.

* Carbowax is the trademark of Union Carbide Chemicals Co., New York 17, N.Y., for its PEG products.

** PEG 1500 is commercially prepared from equal parts of PEG 300 and PEG 1540.

*** Trademark of the Technicon Instruments Corp., Chauncey, N.Y., for its equipment for automatic chemical analysis.

Counter-current distribution was carried out with a 200-tube automatic Craig-Post apparatus using 10 ml per phase.

The compositions of solvent systems which were used to distribute proteins are given in Table I. Systems A through C have been used extensively.

RESULTS AND DISCUSSION

Purification of hog renin substrate before counter-current distribution

Renin substrate is present in one or more of the globulin fractions of plasma. The action of the kidney enzyme renin on this protein produces a decapeptide, angiotensin I[4], which can be assayed for its pressor effect in the anesthetized rat[3]. Partial purifications of renin substrate from the horse[5] and the hog[6] have been described. In order to apply counter-current distribution to the further purification of hog substrate, additional intermediate purification steps were developed. Batchwise adsorption and elution from DEAE-cellulose and subsequent large-scale partitioning in a PEG–Na_2SO_4 system were carried out as follows.

Crude substrate, with a specific activity of 150–250 units/g, was prepared from hog plasma. In general, the procedure described previously for horse plasma[3] was used with the exception that angiotensinase was destroyed at pH 2.5 instead of pH 3.8. Following dialysis of the crude substrate in an artificial kidney, an electrodialysis step reduced the conductivity of the protein solution at 25° to less than

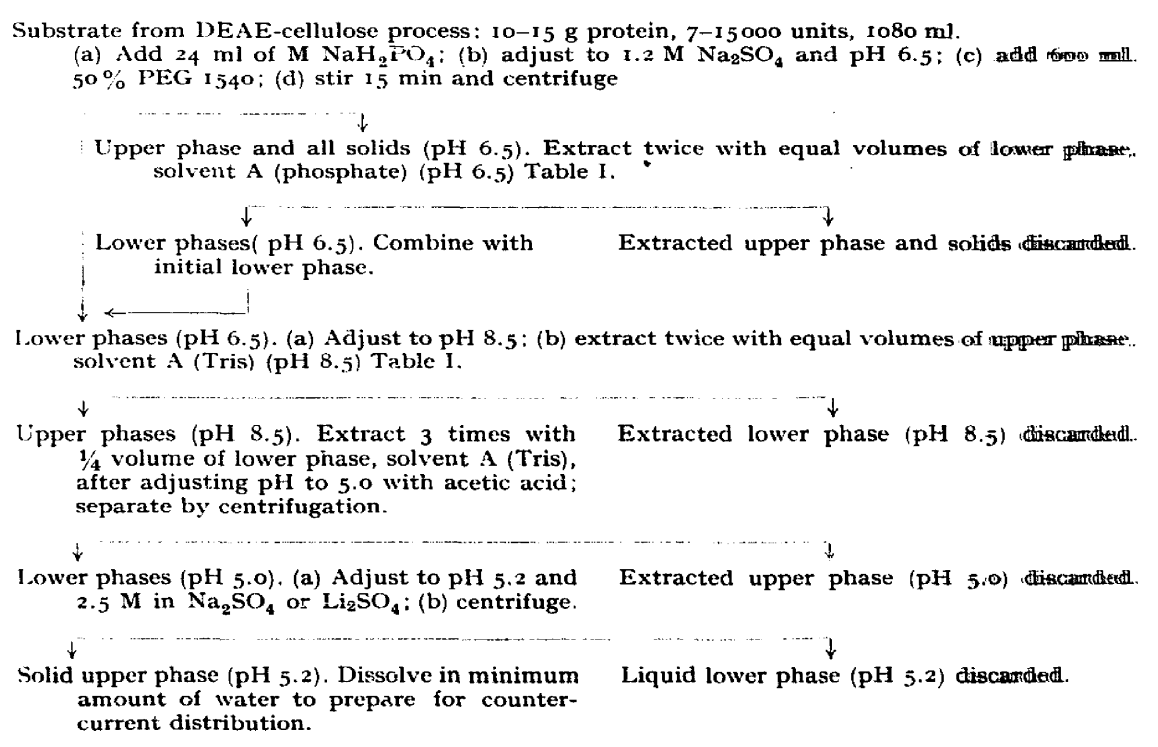

Fig. 1. Two-phase aqueous partitioning scheme for batch purification of renin substrate.

120 μmhos/cm². As much as 80% of the inactive protein could be removed by centrifuging the salt-free suspension at pH 5.2. The substrate protein (40–80 g) was next adsorbed from the supernatant solution at pH 8–9, using 5 mequiv DEAE-cellulose/g protein. A considerable amount of inactive protein was eluted at pH 5.0 (measured in the stirred suspension) without any loss of substrate protein. The active material was obtained by 3 extractions of the adsorbent with 0.03 M Na_2HPO_4 at pH 7.0–7.5. The substrate protein was collected from the combined extracts as a precipitate after adding $(NH_4)_2SO_4$ to 2.5 M and adjusting the pH to 5.2. Ammonia was removed by extracting the precipitate 3 times with 2.5 M Na_2SO_4 at 30°. A solution containing 10–15 g of this protein preparation (approx. 10000 units) was then processed by the scheme outlined in Fig. 1. The final product contained 40–50% of the starting substrate activity at a 4–5-fold increase in specific activity. The rest of the active material was distributed among several side fractions and was not purified further.

Counter-current distribution of renin substrate in a PEG 1540–Li_2SO_4 system

A 54-ml sample, obtained as the product of the scheme of Fig. 1 and containing 2810 mg protein and 3600 units was prepared for distribution by mixing with 47.5 ml of 50% PEG 1540, 11 ml of water, 19.7 g of $Li_2SO_4 \cdot H_2O$ and 1.0 ml of 1 M sodium succinate at pH 6.5. One solid phase and 2 liquid phases were obtained after centrifuging. The upper liquid layer, containing 189 mg of protein and 900 units in 60 ml, and the lower liquid layer, containing 1060 mg of protein and 2120 units in 85 ml,

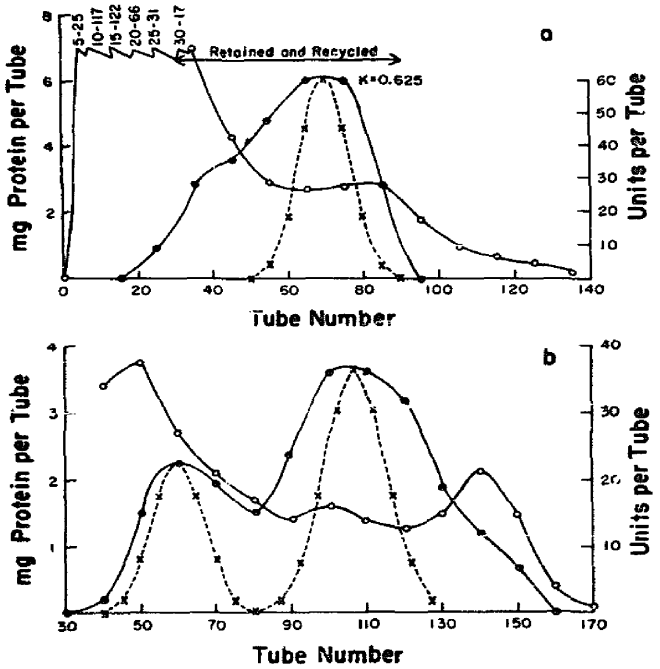

Fig. 2. Counter-current distribution of protein renin substrate in solvent system B (succinate) of Table I, at 10° and pH 6.5. The substrate had previously been purified by the method of Fig. 1. O—O, protein by Pauly diazo histidine; ●—●, substrate activity; ×---×, theoretical distribution of substrate activity. a, after 182 transfers the indicated band being retained for further distribution; b, after 326 total transfers (with recycling).

were loaded into the counter-current distribution apparatus. Using system B (succinate of Table I at pH 6.5 and 10°, 182 transfers were run in 94 h (Fig. 2a). The time required for the phases to separate decreased from 60 min to 30 min during the run. The active material moved ahead of the major part of the protein. In tube 70, the specific activity was 22700 units/g compared to 2420 units/g for the initial load. The contents of tubes 30–90 were retained, and the rest of the train was emptied and refilled with fresh solvent. After 144 more transfers (59.5 h with a 23-min phase separation time), the pattern appeared as in Fig. 2b. The material in tube 107, with $K = 0.49$, had a purity of 24300 units/g and had been purified 11-fold. The additional transfers had clearly resolved a second active band with its peak specific activity of 8350 units/g in tube 60. The calculated distribution curves for each active protein, based on the activity maxima, are shown by broken lines. Although the fit of the theoretical and experimental activity curves is poor in systems using Li_2SO_4, closely agreeing curves result when Na_2SO_4 is employed.

Purified substrate was recovered from the pooled upper phases of selected tubes by stirring with an equal volume of cold 4 M $(NH_4)_2SO_4$ at pH 5.2. After centrifugation, a thin interfacial pad of active protein was obtained; neither liquid phase contained significant amounts of protein, so both were discarded. The pad volume was estimated and 10 volumes of cold 50% PEG 1540 (pH 4.1) were added. After stirring, the mixture was centrifuged (13800 × *g*) in celluloid tubes until a clear supernatant resulted. The pad was then stirred in 5 volumes of 25% PEG 1540. The mixture was centrifuged to obtain a clear supernatant free of protein. This step was repeated, and then followed by suspending the solid phase in 2.5 volumes of 10% PEG 1540. At this stage, a small amount of low activity protein was dissolved in the supernatant. The protein residue had become a taffy-like solid that was next dissolved in the minimum volume of water and freed of small amounts of sediment by centrifuging. The critical stage of the scheme involved dissolving out impurities with a minimum amount of 10% PEG 1540 without dissolving appreciable active protein. There was no apparent denaturation of protein during the distribution; no solid appeared at the solvent interface, and the final salt-free protein product was completely soluble in water.

The sequence of operations involving DEAE-cellulose, batchwise partitioning in a PEG 1540–Na_2SO_4 system and finally counter-current distribution in a PEG 1540–Li_2SO_4 system has produced the highest purity renin substrate so far obtained, 24300 units/g. The distribution of active protein indicated the presence of at least two types of substrate.

Counter-current distribution of human serum proteins in a PEG 1000–$(NH_4)_2SO_4$ system

The widely investigated complex mixture of proteins in serum is very useful in studying the power of any technique to resolve proteins. Recovered protein fractions can be further studied by other techniques, such as paper or column electrophoresis, to help establish the identity of protein components. Accordingly, the ability of several PEG–salt systems to resolve whole serum was tested. The best of these systems was prepared from PEG 1000 and $(NH_4)_2SO_4$ (system C, Table I).

45 ml of pooled human serum containing 2853 mg protein was mixed with 84 ml of 50% PEG 1000, 60 ml of 4 M $(NH_4)_2SO_4$, and 30 ml of water. The pH was adjusted to 8.0 with 0.6 ml of 2.5 N NaOH; the mixture was chilled to 10° and centrifuged.

The upper phase contained 838 mg in 108 ml and the lower phase contained 1204 mg in 104 ml. The insoluble interfacial layer was dissolved in water and found to contain 811 mg, or 28.4% of the total added protein. 50 ml of the upper phase (388 mg) and 50 ml of the lower phase (579 mg) were loaded into tubes 0–4 of the train. Aliquots of the 2 phases and the redissolved interfacial solid were saved for paper electrophoresis.

The distribution was run for 194 transfers (162 h) at 10° in system C, Table I (pH 8.0). From 30 to 45 min were required for the phases to separate after equilibration. The distribution is shown in Fig. 3. Portions of the run were pooled as indicated, and dialyzed in bags against cold flowing tap water until Nessler tests

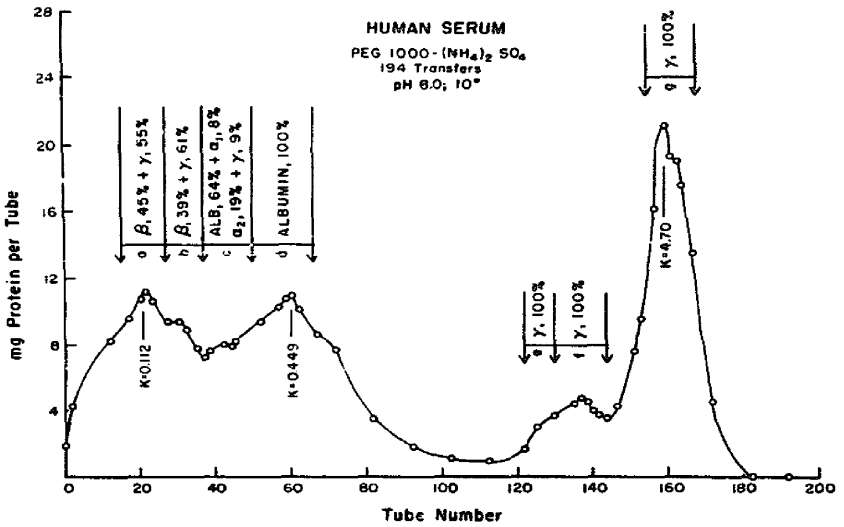

Fig. 3. Counter-current distribution of human serum proteins after 194 transfers in solvent system C of Table I, at 10° and pH 8.0. Protein by absorbance at 280 mμ. Pooled fractions were recovered as described in the text and components were identified by paper electrophoresis.

for ammonia were negative (21 h). All volumes doubled due to the osmotic effect of the glycol. The fractions were concentrated by dialysis against a large volume of 50–75% PEG 1540 with resultant precipitation of the protein from the salt-free, glycol-rich system. The bag contents were removed and centrifuged (13800 × *g*). The supernatant glycol solution was found to contain no protein and was discarded. The protein residue was then suspended in 10 volumes of 50% PEG 6000 and recentrifuged. The protein-free supernatant was discarded, and the residue was extracted with a small volume of 0.05 M Na_2HPO_4–0.1 M NaCl at pH 7.5. Paper electrophoresis in the Spinco system was used to establish the type of mobility and the percentage composition of each fraction. Patterns were obtained from aliquots analyzed separately as well as mixed with serum of known composition. Several γ-globulins with widely differing distribution characteristics were found. The high solubility and favorable *K* values for γ-globulins make this system useful for studying mixtures of these proteins.

A second distribution was run in order to confirm the resolution of serum into the fractions shown in Fig. 3, with essentially the same distribution pattern of proteins. When attempts were made to carry out this distribution at 25°, only two bands were

found: γ-globulin with high K values, and a mixture of the other serum proteins in the starting tubes.

The separation of serum proteins into electrophoretically distinct groups after approx. 200 transfers indicates the potential scope of counter-current distribution using these systems.

Factors affecting the solubilities and distribution coefficients of proteins in PEG–salt systems

The proportion of water present, the pH, temperature, molecular weight of the PEG and the specific cations and anions all influence the behavior of proteins in these systems. Increasing the amount of water in the PEG–salt–water partition systems caused each phase to approach the same composition and to increase in capacity for protein. At the critical composition point, both phases became identical and the system homogeneous. ALBERTSSON[3] has shown that in the region of the critical point, other factors such as pH and temperature become of major importance in determining the composition of the phases and must, therefore, be rigidly controlled. This is particularly necessary during a counter-current distribution of extended duration. In order to be less restrictive of the pH and temperature and to decrease phase separation times to less than 60 min, the minimum water content compatible with adequate protein solubility and partitioning between phases was chosen. Exemplifying the large effect on K values of changes in water content, when the proportion of water in system E of Table I, was increased 4.3% (v/v), the K value for albumin increased from 0.08 to 0.22 and that for γ-globulin increased from 1.8 to 2.7. Total protein solubilities changed very little.

As the pH was increased within the range 4.5 to 8.5, protein K values frequently increased several-fold. The K for γ-globulin in system E of Table I, was 8.2 times greater (1.89) at pH 8 5 than at 5.5. However, the K for albumin (in the same system) was only 2.1 times greater at 8.5. In system C of Table I, renin substrate K values increased 3.5-fold, from 0.38 at pH 4.5 to 1.35 at pH 7.0. Total protein solubilities in all cases were essentially constant.

It was stated earlier that lowering the temperature of system C of Table I, from 25° to 5° altered the K values of some of the serum proteins sufficiently to permit their partial separation in 200 transfers. At 25°, no resolution of albumin, α_1-, α_2- or β-globulins occurred. An appreciable temperature variation in the K values of renin substrate was found using the same solvent system. The K values at 5° were 1.6–1.7-fold larger than at 25°. The capacity of the system for substrate at 5° was still 80% or more of that at 25°.

The degree of polymerization of the PEG markedly affects the solubility of protein in the organic phase. Although as much as 7.5 mg of renin substrate can be dissolved per ml of system C of Table I, when prepared with PEG 6000, none of the protein is soluble in the organic layer ($K = 0$). If PEG 6000 is replaced by PEG 1540 or PEG 1000, the K values increase to 0.96 and 1.35, respectively. In the case of albumin in system E of Table I, with PEG 1540, the K was 0.05; but with PEG 1000, it increased to 0.135. γ-Globulin in the same system showed a 5-fold increase in K using PEG 1500 instead of PEG 1540.

A systematic study of the effects of a number of ions on the solubilities and partitioning of albumin, γ-globulin and serum was made. Various combinations of

Na^+, NH_4^+ and Mg^{2+} with SO_4^{2-}, HPO_4^{2-} and $C_6H_5O_7^{3-}$ (citrate) were tested. Each system contained 5.6 ml of 50 % PEG 1000, 8 ml of 2 M salt (1.86 M sodium citrate) and 2 ml of 3 % (w/v) albumin, 3 % (w/v) γ-globulin or 7.3 % (w/v) serum proteins, adjusted to pH 8.0 at 25°.

Of the 3 sulfates, $(NH_4)_2SO_4$ provided the highest protein solubilities and K values. This was most apparent in the case of γ-globulin where solubility in the ammonium sulfate system was 100 % as compared with 1.1 % in Na_2SO_4 and 12.2 % in $MgSO_4$. The K's were 6.7, 0.1 and 0.0 for γ-globulin in the 3 salt systems. $MgSO_4$ gave no K value greater than 0.1 (with serum), and so other Mg^{2+}–anion combinations were not tried. The phosphates and citrates of both Na^+ and NH_4^+ gave results very similar to those obtained with the sulfates when testing albumin and serum. However, in the case of γ-globulin, the Na^+ salts were much poorer than the corresponding NH_4^+ salts in ability either to dissolve the protein or to give high K values. Among the NH_4^+ salts, the sulfate was outstanding in solubilizing γ-globulin (100 % *versus* 13 % for the phosphate, and 21 % for the citrate). The K values for γ-globulin were high in all NH_4^+ salt systems.

The data from these experiments showed that, in general, higher protein K values were obtained when the systems contained considerable water, low molecular weight PEG, $(NH_4)_2SO_4$ in preference to other salts, and when they were tested at high pH and low temperature. Greater protein solubilities were observed in ammonium sulfate systems when lower molecular weight PEG was used.

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