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## Polymeric Precipitants for the Crystallization of Macromolecules

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Nine different water soluble polymers reported to strongly affect the properties and structure of water were evaluated for their use in crystallizing a series of 24 different proteins, viruses, and conventional small molecules. All of the polymers produced crystals of some of the molecules and viruses tested, and of the 24 molecules tested, 14 were crystallized. In a number of cases, crystals of the molecules and viruses were obtained under very different conditions than were ever previously used. Because the selection of polymers employed here represents only a sampling of those available to experimenters, we conclude that the potential range of such polymers useful in macromolecular and small molecule crystallization may be very broad.

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The ability to obtain crystals of sufficiently large size and quality for X-ray diffraction analysis continues to be a major obstacle to our understanding of macromolecular structure. Often, minor alterations in the composition or characteristics of the crystallization solution, or mother liquor, can make a significant difference in the results obtained. Inclusion of a small amount of detergent may effect crystallization (1); the system may be sensitive to small changes in pH or temperature (2,3); the nature of the precipitating agent may have a profound effect (3,4,5,6).

Precipitating agents for macromolecular crystallization generally fall into four different categories: salts, organic solvents such as ethanol, small molecular weight polyalcohols such as methyl pentane diol (MPD), hexane diol, or PEG 400, and large polymeric precipitating agents such as Polyethylene glycol (PEG). Only a very few classes of the latter category have been seriously evaluated for their usefulness in crystallizing proteins, nucleic acids, and viruses (5,7). They are even less studied for their utility with conventional small molecules.

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The large polymeric precipitating agents have been postulated to influence macromolecular solubility by altering the interactions between macromolecules and their surrounding solvent environment (8,9), and indeed, the ability of the agent to modify solvent structure and its physical properties appears to be a striking characteristic of this class of precipitants. For example, PEG dramatically reduces the viscosity of water at concentrations far below what can be explained by classical theories of fluid dynamics (10).

In the study described here, we collected a representative test set of polymeric agents that have been reported, like PEG, to alter water properties. Using an admittedly coarse screen of crystallization conditions, with little or no optimization, we attempted to determine if other polymeric precipitants might be useful for the crystallization of macromolecules as well as for a few selected conventional organic molecules as well.

### Materials and Methods

All polymers and buffer compounds were purchased from Fluka Biochemical Co. and solutions were filtered, where possible, and stored at 4° C to avoid microbial contamination, though no other anti microbial agents were added. In these experiments, nine unique polymers were evaluated for their utility in crystallizing proteins by constructing a rudimentary screen having only a single concentration of the polymer but combined with buffers at three pH values; 4.5, 6.5, and 8.5. All of the polymers used in these experiments yielded solutions having pH near neutrality and none apparently demonstrated significant buffering capacity, hence the measured value of the screening solutions was essentially that of the stock buffer solution used in formulating the screen.

The use of only a single concentration of the polymer is of course not the best that one might wish, but is justified to some extent by the observation that with this class of precipitants crystallization occurs over a broad range of concentrations centered on the optimum (11). The solutions comprising the crystallization screen are presented in Table 1.

Crystallization trials were performed at 22° C using Cryschem sitting drop vapor diffusion plates. The reservoirs contained 0.70 ml. of the precipitating solution. The drops were made by combining 3 ul of the protein solution with 3 ul of the reservoir with mixing. Plates were sealed with transparent, plastic tape (12). Crystallizations generally occurred in from one to ten days. Examination of the plates was carried out on a daily basis for the first week and every few days after that using a 30 x dissecting microscope. Photographs were made with an Olympus BH microscope and OM2 35 mm camera. The proteins, their sources, and the solutions in which they were dissolved are shown in Table 3.

### Results

Table 2 contains the 14 proteins, viruses, or biologically relevant conventional molecules of the 27 attempted that yielded crystals in the screen described above. It lists as well, those which failed to crystallize. Also found in Table 2 are the numbers of the reagents from Table

Table 1. Formulation of the polymeric precipitants. % v/v used in formulation. Buffers formulated as 1.0 M stocks and diluted with polymeric precipitants and no further pH adjustment.

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1. 22% polyacrylic acid 5100 sodium salt, 0.1 M sodium citrate pH 5.6
2. 22% polyacrylic acid 5100 sodium salt, 0.1 M imidazole pH 6.5
3. 22% polyacrylic acid 5100 sodium salt, 0.1 M Tris pH 8.5
4. 22% polyvinylpyrrolidone K15, 0.1 M sodium citrate pH 5.6
5. 22% polyvinylpyrrolidone K15, 0.1 M imidazole pH 6.5
6. 22% polyvinylpyrrolidone K15, 0.1 M Tris pH 8.5
7. 22% polyacrylic acid 2100 sodium salt, 0.1M sodium citrate pH 5.6
8. 22% polyacrylic acid 2100 sodium salt, 0.1M imidazole pH 6.5
9. 22% polyacrylic acid 2100 sodium salt, 0.1M Tris pH 8.5
10. 4% polyvinyl alcohol 15,000, 0.1 M sodium citrate pH 5.6
11. 4% polyvinyl alcohol 15,000, 0.1 M imidazole pH 6.5
12. 4% polyvinyl alcohol 15,000, 0.1 M Tris pH 8.5
13. 2% carboxymethylcellulose sodium salt medium viscosity, 0.1 M sodium citrate pH 5.6
14. 2% carboxymethylcellulose sodium salt medium viscosity, 0.1 M imidazole pH 6.5
15. 2% carboxymethylcellulose sodium salt medium viscosity, 0.1 M Tris pH 8.5
16. 45% polyethylene glycol 2000 dimethyl ether, 0.1 M sodium citrate pH 5.6
17. 45% polyethylene glycol 2000 dimethyl ether, 0.1 M imidazole pH 6.5
18. 45% polyethylene glycol 2000 dimethyl ether, 0.1 M Tris pH 8.5
19. 45% polypropylene glycol P400, 0.1 M sodium citrate pH 5.6
20. 45% polypropylene glycol P400, 0.1 M imidazole pH 6.5
21. 45% polypropylene glycol P400, 0.1 M Tris pH 8.5
22. 1% carboxymethylcellulose sodium salt high viscosity, 0.1 M sodium citrate pH 5.6
23. 1% carboxymethylcellulose sodium salt high viscosity, 0.1 M imidazole pH 6.5
24. 1% carboxymethylcellulose sodium salt high viscosity, 0.1 M Tris pH 8.5
25. 9% carboxymethylcellulose sodium salt ultra low viscosity, 0.1 M sodium citrate pH 5.6
26. 9% carboxymethylcellulose sodium salt ultra low viscosity, 0.1 M imidazole pH 6.5
27. 9% carboxymethylcellulose sodium salt ultra low viscosity, 0.1 M Tris pH 8.5

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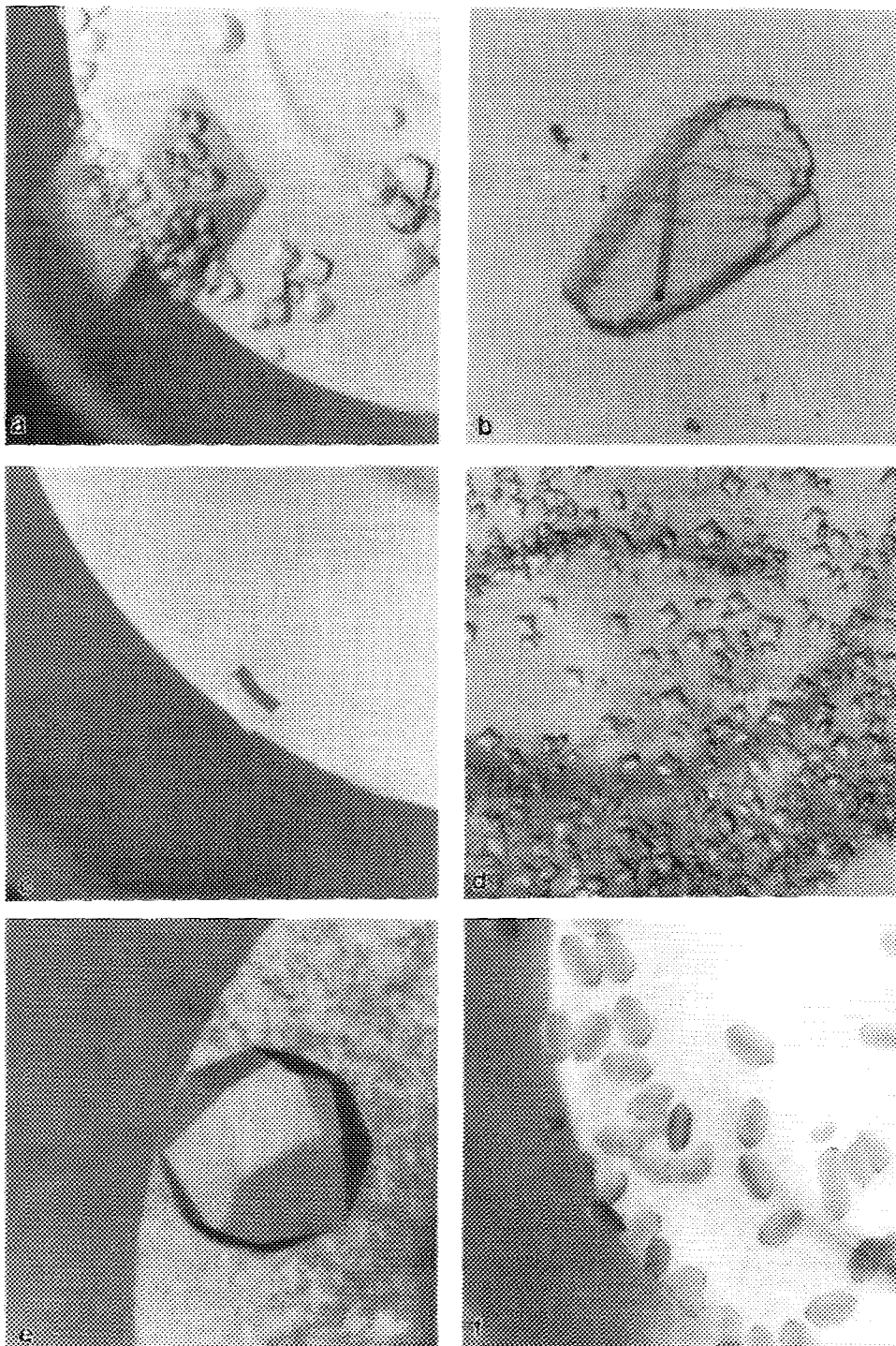
which produced those crystals. Perhaps most striking, is the effectiveness of the various polymers in the crystallization of the viruses (TYMV, STMV, PMV), and poring, the only membrane protein included in the screen. In figure 1 are seen photographs of some of the crystals of proteins and viruses obtained in these experiments, and fig. 2 photos of the crystals of two conventional biological molecules also grown. Obviously, many of the crystals obtained here are of poor quality and ill suited for immediate X-ray diffraction analysis. The purpose of a screen such as this however, is to simply identify suitable conditions for subsequent optimization. Judged in that light, and given the constrained starting set, this particular screen was remarkably successful.

Table 2. Samples, sources, the polymeric solutions which produced crystals, and sample concentration. All samples were prepared in water and where necessary a trace of ammonium hydroxide was added to produce dissolution.

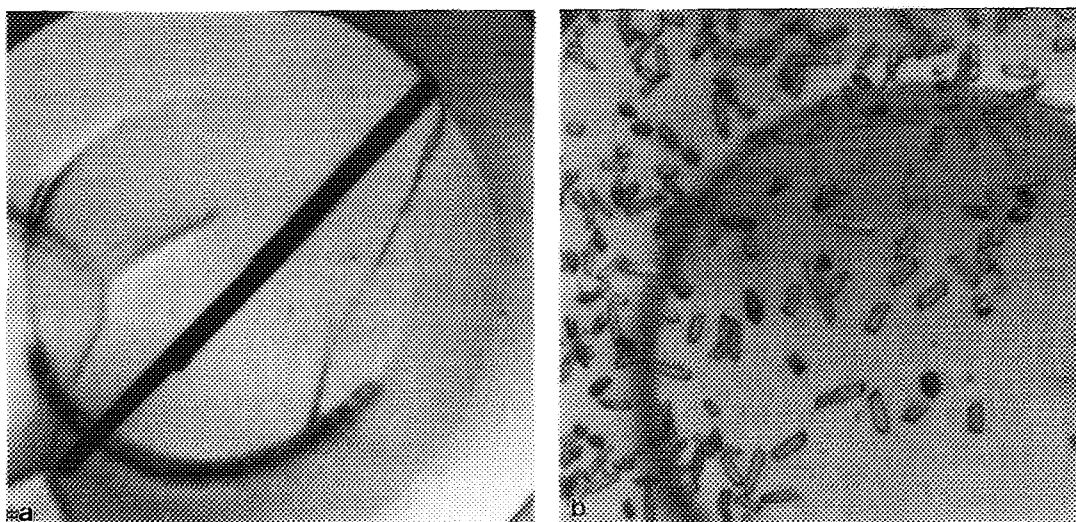
Molecule	Source of Molecule	Crystallized In Reagent from Table 1	Concentration of Molecule
<b>Successfully Crystallized Samples</b>			
<b>Protein</b>			
Lysozyme (hen egg)	Sigma	1,7,8,16,18	20 mg/ml
Insulin (porcine)	Sigma	1,2,3,8,9,11,20,22	20 mg/ml
Papain (papaya)	Sigma	18	20 mg/ml
Catalase (bovine liver)	Sigma	5,6,8,9,11,14, 25, 26, 27	25 mg/ml
Ribonuclease A (bovine pancreas)	Sigma	20	20 mg/ml
Beta-amylase (sweet potato)	Worthington	19,20,21	15 mg/ml
Canavalin (jack bean)	ref. 22	5,6,11,12,14,17, 23	30 mg/ml
TYMV (chinese cabbage)	ref. 23	5,7,8,9,10,12,13,15,22	16 mg/ml
STMV (tobacco)	ref. 24	5,10,11,13,14,16,17,18,19,20,21	7 mg/ml
PMV	ref. 25	1,4,5,6,10,12,14,15,20,21,22,27	10 mg/ml
$\beta$ sub unit LH (bovine)	gift, Prof John Pierce-UCLA	19,20,21	15 mg/ml
Myoglobin (equine)	Sigma	17	20 mg/ml
Pyruvate kinase (Chicken)	Sigma	22	20 mg/ml
Edestin (hemp seed)	Sigma	17,23,26	20 mg/ml
DNase I (bovine pancreas)	Boehringer	21	25 mg/ml
Concanavalin B	ref. 26	6,10,11,13,14,16,17,18,22,23,24	15 mg/ml
Porin	ref. 27	1,4,5,10,12,14,15,20,21	5 mg/ml
<b>Small Molecule</b>			
Vitamin B-12	Sigma	1	Saturated
Glycoursodeoxycholic acid	Sigma	1, 2, 3,5,11,13,16,17,19,22,23	25 mg/ml
<b>Failures</b>			
Pepsin (bovine)	Sigma	- (requires low pH)	25 mg/ml
Thaumatococcus (T. daniellii)	Sigma	- (prefers tartrate)	25 mg/ml
Phe t-RNA (yeast)	Sigma	- (requires Mg, polyamine)	10 mg/ml
Subtilisin Type VII (Bacillus)	Sigma	- (never crystallized)	25 mg/ml
Xanthine Oxidase (bovine milk)	gift, Dr. R. Hille-Ohio St.Univ.	- (never crystallized)	25 mg/ml
CAT FVX13 Ab (mouse)	ImmunoPharmaceutics	- (never crystallized)	7 mg/ml
ELAIO 102 Ab (mouse)	ImmunoPharmaceutics	- ( never crystallized)	7 mg/ml
Ribonuclease B (bovine pancreas)	Sigma	-	25 mg/ml
$\alpha$ amylase (B. subtilus)	Sigma	-	35 mg/ml
Seed Globulin (lima bean)		-	40 mg/ml
Carbonic anhydrase (bovine)	Sigma	-	25 mg/ml

## Conclusions and Discussion

A series of polymeric molecules chosen on the basis of their ability to alter the activity and properties of water were investigated for their utility in crystallizing a series of biological molecules, including conventional small molecules, proteins and viruses. It is clear that those

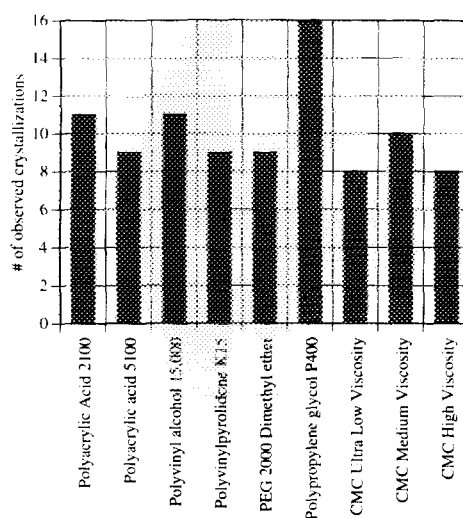


**Figure 1.** Crystals of a) canavalin grown in 22% polyvinylpyrrolidone K15, 0.1 M imidazole, pH 6.5; b) TYMV grown in 4% polyvinyl alcohol 15,000 0.1 M sodium citrate, pH 5.6; c) deoxyribonuclease type I grown in 45% polypropylene glycol P400, 0.1 M Tris, pH 8.5; d) leutenizing hormone ( $\beta$  sub unit) grown in polypropylene glycol P400, 0.1 M imidazole, pH 6.5; e) lysozyme grown in 45% polyethylene glycol 2000 dimethyl ether, 0.1 M Tris, pH 8.5; and f) catalase grown in 9% CMC ultra low viscosity, 0.1 M Tris, pH 8.5.



**Figure 2.** Crystals of a) Vitamin B-12 grown in 22% polyacrylic acid 5100, 0.1 M sodium citrate, pH 5.6, and b) glycooursodeoxycholic acid grown in 2% CMC, medium viscosity, 0.1 M sodium citrate, pH 5.6.

used in this study are indeed of value in that regard. 14 Of 24 molecules were crystallized and every polymer tested was useful in at least one, and generally several cases. Figure 3 presents the success distribution for all of the polymers tested. The successes of these polymers was



**Figure 3.** Number of observed crystallizations using the polymeric screen in Table 1 with the samples described in Table 2.

particularly noteworthy since only a very limited set of conditions was explored for each polymer and no optimization was carried out. It may be important that several of these polymers resulted in the crystallization of some proteins that had not previously been crystallized from PEG, MPD or hexanediol, the polymers and alcohols most commonly used (3,5,13,14). Because of the limited set of polymers examined here and the large number that exists, it seems fairly certain that there must be a vast number of other polymeric precipitants, now used for other purposes, that might be of utility in the crystallization process as well.

This class of reagents, we would note, may be useful as well not only for the crystallization of macromolecules but for their purification by conventional fractionation methods as well. Thus they may have additional utility in bioseparations and down stream industrial purification processes as well as for X-ray crystallography. It is relevant in this regard that the polymeric precipitants were useful over a very broad size range of target molecules, ranging from supramolecular assemblies such as TYMV and STMV to conventional molecules such as vitamin B12.

The behavior of all of the polymers was quite similar to PEG and shared many of the same advantages of that reagent. All yielded neutral solutions and had virtually no buffering capacity, thus accurate pH values could be established at otherwise low supplementary buffer concentrations. In experiments not presented here various detergents were combined with the polymeric precipitants and there was no incompatibility that we could observe. This was found to be true as well for the addition of a broad range of salts as well. Crystallizations were all carried out at room temperature and we have not yet determined if there is any particular temperature sensitivity. The concentration range over which crystals were observed varied from as low as 1% in the case of carboxymethylcellulose (insulin, canavalin, TYMV, PMV,  $\beta$ -amylase, STMV,  $\beta$ -LH) to 45% polypropylene glycol P420 and PEG 2000 dimethyl ether (papain, STMV). As with PEG's the times required for crystallization varied from several hours (insulin, catalase, canavalin, glycolic acid) to several weeks in some cases (DNaseI, lysozyme,  $\beta$ -LH). A significant property of several of these polymeric precipitating agents, for example carboxymethylcellulose, is that they are extremely viscous. Thus crystallization in their presence may resemble to some extent crystallization in a gel matrix (15,16,17) or in microgravity (18). We did not, however, note any clear correlation between the viscosity of a system and the time required for crystallization to occur.

Explanations can be offered for why 12 proteins failed to crystallize using these polymeric reagents. Some proteins that we know from previous experience can be crystallized



require some fairly special conditions or factors not utilized in this screen. Pepsin for example generally requires low pH of 2.5 (19), thaumatin requires tartrate ion (20), and yeast phe t-RNA usually demands  $Mg^{++}$  and a polyamine such as spermine (21). Cat FVX13 monoclonal antibody and ELAID 102 Ab are both intact immunoglobulins, a notoriously difficult class of proteins to crystallize. Other failures in the list such as Xanthine oxidase and Protease P5225 we have not crystallized by any other means.

Puzzling however, is the fact that ribonuclease B, carbonic anhydrase, lima bean globulin and bacterial  $\alpha$ -amylase were not crystallized using these polymers. All have been crystallized using conventional methods. Our conclusion, therefore, is that while this class of polymeric precipitating agents expands our range of available reagents for macromolecular crystallization, they do not yet represent a universal tool that can replace the need for many separate experiments using a broad spectrum of precipitants, pH's and additives.

Though much investigated for their applications in a wide range of formulations (cosmetics, surfactants, food products, chemical carriers, etc.) The properties of these polymeric molecules remain something of a mystery. In particular, their dramatic effects on the properties and structure of water, and their effects on other molecules that depend on this water environment for their solubility is poorly understood at best. Nonetheless, they provide a useful and, probably, expanding range of unique reagents for separation processes, and certainly for the crystallization of biological molecules and assemblies.

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