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Search Designs for Protein Crystallization Based on Orthogonal Arrays

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Abstract

In protein crystallography, the initial experimental problem is the identification of physical and chemical conditions that will support nucleation and crystal growth. Ideally, experiments to search for such conditions would be based on a full-factorial structure, with variation in the temperature and solution composition. However, consideration of even a moderate number of possibilities for the composition of the system will result in factorial experiments which may be prohibitively large. In this paper it is proposed that search experiments for protein crystallization might be based on orthogonal arrays. These are subsets of full-factorial experiments which possess a great deal of symmetry, such that a uniform distribution of points throughout the experimental region is preserved. Such experiments have reasonable size, explore the proposed experimental region in a systematic fashion, and form a logical basis for a sequential approach to the search for crystallization conditions. Examples of such initial search experiments are given, and their application to some recent protein crystallization problems in this laboratory is described briefly. The relationship of this approach to other protein crystallization search procedures is also discussed.

Introduction

Crystals form in supersaturated solutions in which the solute concentration exceeds its equilibrium solubility. Hence, all the physical techniques for crystallizing proteins involve bringing a protein solution into the supersaturated state by alteration of some property of the system. Typically, this is accomplished by the gradual introduction of substances which serve to reduce protein solubility (protein precipitants) *via* some diffusive process. Salts, simple organic compounds and long-chain synthetic polymers have all been used for this purpose.

From a supersaturated solution, equilibrium can be restored by phase separation. A solid phase can result either from the formation of disordered protein aggregates, leading to an amorphous solid or flocculate, or from the formation of crystalline aggregates, leading (once a critical size is reached) to nucleation and crystal growth (Feher & Kam, 1985). Protein crystals will form only given an appropriate degree of supersaturation, and only in an appropriate physical and chemical environment (defined by the temperature and the composition of the system). The initial experimental problem is to establish the environmental conditions favouring the formation of protein crystals rather than an amorphous solid or flocculate. This is essentially a search problem. Subsequent to this is the problem of producing the large single crystals required for X-ray diffraction studies. For most laboratories this experimental program is carried out using one of the available micro-methods for protein crystallization (see Ducruix & Giegé, 1992), and examining the results with an optical microscope.

Given the small amounts of protein often available, the need for efficient and economical search experiments is evident. In recent reviews of protein crystallization (Giegé & Mikol, 1989; Gilliland & Davies, 1984; Littlechild, 1991; McPherson, 1990; Ollis & White, 1990; Weber, 1991; Weigand, 1990) the need for better experiments to search for crystallization conditions has been emphasized, and a number of recent papers have described both informal experimental approaches to this problem (McPherson, 1992; Jancarik & Kim, 1991; Stura, Nemerow & Wilson, 1992) and more formal search procedures (Weber, 1990; Carter, 1992). The purpose of this paper is to suggest a systematic search method based on the use of orthogonal arrays, which follows from this latter work. The theoretical and practical background to the approach is given, and the application of these ideas discussed with reference to several recent protein crystallization problems in this laboratory.

The experimental problem

Before proceeding further, it is necessary to define clearly some general terms associated with the design of experiments. The experimental factors are the variables which influence (or are believed to influence) the attribute of interest in the experiment. Factors can be continuous (that is having a numerical value *e.g.* temperature, pH) or discrete (that is having a non-numerical value from a finite set of values *e.g.* precipitant type). In any experiment the effects of a factor will be evaluated at a number of levels (in the case of continuous factors), or over a number of classes or categories (in the case of discrete factors). However, the term 'level' is often applied to both continuous and discrete factors. A treatment combination, or simply a treatment, is one of the possible combinations of the levels of

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the experimental factors. A factorial treatment structure simply consists of all possible level combinations of all the factors included in the investigation.

The general procedure currently used to identify and optimize protein crystal growth conditions was assessed from an appraisal of recent crystallization communications and papers describing search experiments for protein crystallization. It can be described as follows:

(I) An initial search experiment is executed, aimed at determining a physical and chemical environment which will support nucleation and crystal growth. The nature of this search experiment is critical, since without the initial identification of conditions supporting crystal growth, no subsequent optimization can be undertaken. For difficult crystallization problems an extended sequential program of experimentation may be necessary, until appropriate solution conditions are identified. In addition to the approaches referenced above, it seems that in many laboratories, search experiments have been loosely based on a factorial structure (such experiments are often described as being based on multivariate arrays, matrices or grids, a terminology reflecting a development independent of the statistical literature).

(II) For each experimental factor the level(s) resulting in the best response are identified and retained.

(III) Further factorial experiments are conducted, centred around these remaining levels, typically with levels of the continuous factors spaced more closely than in the previous experiment.

(IV) Steps (II) and (III) are repeated until a satisfactory result is obtained.

Iterative optimization methods like this, which are not based on any formal model of the response, have not been extensively studied in the statistical literature. Underlying much of the statistical research work in experimental design has been the assumption that the experiment can be adequately described by a statistical model (Hunter & Steinburg, 1984; Lehmann, 1990). Recently, Wu, Mao & Ma (1990) considered some physical problems for which this assumption might not hold, and proposed a quite general class of model-free optimization methods, which they termed SEL (sequential elimination of levels). The procedure given above is closely related to these methods.

As noted above, the success of such a procedure is completely dependent on finding appropriate starting points for subsequent optimization [*i.e.* on the success of the initial search experiment(s)]. It is with this initial problem that this paper is concerned. However, we note that even given seemingly appropriate starting points, it may not be possible to grow crystals of a suitable quality for X-ray diffraction by simple diffusive techniques coupled with manipulation of the environmental variables. The potential importance of seeding procedures (Stura & Wilson, 1992) or of techniques for protein modification [*e.g.* proteolytic cleavage, or enzymatic deglycosylation (Baker, Day, Norris & Baker, 1994)] should not be overlooked.

Several criteria can be proposed which an initial search experiment needs to realize. Firstly, the experiment should be economical (having a practicable size). In many protein crystallization studies there will be a severe constraint on the amount of material available. With regard to the size of the experiment, the potential importance of sequential experimentation should be recognized. It should be evident that very large initial search experiments, involving many trials conducted in parallel, will typically be inefficient. This inefficiency arises from the failure to exploit information which could have been obtained if a more modest sequential strategy had been adopted. Hence sequential experimental procedures can have an important impact on the required experimental size (Ghosh, 1991). Since the time in which results from crystallization trials can be expected is relatively short (typically days or weeks), the attractiveness of sequential experimentation is increased.

Secondly, the experiment should define clearly the experimental region, and explore it comprehensively. Evidently, the requirements to limit the experimental size and to perform a comprehensive search of the experimental region are exclusive of one another, and a suitable compromise will always need to be found. Search experiments having a factorial structure define clearly the experimental region, with the probability of locating regions which will support protein crystallization (if they exist) related to the spacing of the levels of the continuous factor(s). This is the reasoning behind the successive automated grid searches described by Weber (1990). However, as the number of factors increases, the size of factorial experiments can quickly become very large (the size will be an integer multiple of the number of levels of each factor).

Finally, the experiment should be flexible. Proteins may vary markedly in their stability and ability to maintain biological activity as a function of temperature, pH and solvent composition. As a consequence of this dissimilar physical behaviour, it is difficult to design initial search experiments which will have a widespread applicability. However, there are practical advantages associated with the use of standard initial search designs. Principal among these are increased speed of experimentation, a decreased probability of systematic experimental error, and a ready association with automated crystallization systems. Evidently, a standard initial search design should be sufficiently flexible to accommodate a likely diversity of physical behaviour. One way to achieve this flexibility is to ensure that the overall design can be partitioned into a number of smaller designs (*i.e.* is modular). For example, a search experiment might be constructed so that it was comprised of a set of smaller experiments, each covering a limited pH range. This would enable a quite general

search experiment to be adapted to a specific protein crystallization problem.

Full-factorial experiments (multivariate arrays or matrices) are attractive because they clearly define and explore the experimental region. However, the consideration of even a modest number of possibilities for the composition of the system may result in factorial experiments that are very large. It should also be noted that there may be redundancy within the factorial structure, in the sense that in a given case, crystal nucleation and growth may not be critically dependent on some of the factors considered in the initial search experiment.

Therefore, it is logical to consider whether a subset of the possible factorial level combinations might be selected which is in some way representative of the full-factorial set (that is, the available experimental points are spread throughout the candidate region as regularly or evenly as possible). This subset could then form the basis of an initial search experiment. It may be possible to stop the search after executing this subset. Even if this is not the case then the information gained (principally concerning the protein solubility) serves to illuminate the problem, and can be used in designing better subsequent experiments, and in the consideration of directions for further search. Consequently, such experiments have the potential to significantly reduce the experimental cost.

For a situation in which the experimental factors are all continuous, the problem of spacing the available experimental points 'uniformly' throughout the experimental region was considered by Kennard & Stone (1969). They implemented an algorithm to achieve this by sequentially choosing that point furthermost from the current design points. The problem of systematic search in high-dimensional spaces was considered more formally by Aird & Rice (1977) and Sobol (1979), who presented algorithms to generate designs in rectangular domains, based on some measure of dispersion in the set of selected points. However, for a situation in which some of the experimental factors are discrete (qualitative) a more general approach is required.

Rao (1947) identified a special class of subsets with a great deal of symmetry. These subsets (or fractions) are known as orthogonal arrays. The formal definition of an orthogonal array is as follows. An orthogonal array of strength d, with k_i columns with entries from a set of s_i symbols ($i = 1, \ldots, r$), is an $N \times m$ matrix ($m = k_1 + \ldots + k_r$) in which all possible combinations of the symbols in any d columns appear with equal frequency (Rao, 1947, 1973). Such an array can be denoted OA(N, m, $s_1^{k_1} x s_2^{k_2} x \ldots x s_r^{k_r}$, d).*

N, m, s_i and d are said to be the parameters of the array. N refers to the size of the array, m to the number of constraints or factors, and d to the strength of the array. A simple example should serve to make this definition clear.

Consider a factorial experiment involving three factors, each at two levels. This is a $2 \times 2 \times 2$ factorial experiment, having a total of eight possible level combinations. This is said to be a symmetrical factorial experiment since each factor has the same number of levels. Following convention, we denote the levels of these factors with the integers 0 and 1. Then the factorial structure can be represented by the following 8×3 matrix,

$$\begin{matrix} 1 & 1 & 1 \\ 0 & 1 & 1 \\ 1 & 0 & 1 \\ 0 & 0 & 1 \\ 1 & 1 & 0 \\ 0 & 1 & 0 \\ 1 & 0 & 0 \\ 0 & 0 & 0 \end{matrix}.$$

The columns of this matrix correspond to the experimental factors, the entries in the columns correspond to the levels of the factors and the rows correspond to the level combinations (experimental runs).

Now consider the following 4×3 matrix,

$$\begin{matrix} 0 & 0 & 0 \\ 0 & 1 & 1 \\ 1 & 0 & 1 \\ 1 & 1 & 0 \end{matrix}.$$

The rows of this matrix are a subset of those in the preceding 8×3 matrix. This 4×3 matrix is an OA(4, 3, 2^3, 2). Note that consistent with the definition of an orthogonal array, in each possible pair of columns the possible level combinations [(0,0), (0,1), (1,0), (1,1)] occur the same number of times (once). Orthogonal arrays are matrices whose columns possess certain balancing properties. A more complex example is given in *Appendix* I, together with some properties of orthogonal arrays useful in their manipulation which follow from the definition.

The development of methods of construction of orthogonal arrays is an active field of research. Known orthogonal arrays and their methods of construction were reviewed and catalogued by Dey (1985). Note that because of the strict conditions relating the parameters of an orthogonal array, such an array cannot exist for all possible values of these parameters.

The principal use of orthogonal arrays (and the original justification for their construction) has been in the planning of comparative experiments. Their properties of balance result in orthogonal (uncorrelated) estimates of effects for the linear models commonly used in the analysis of such experiments. In this context they are often referred to as orthogonal fractional factorial designs.

* Some authors prefer to use the term orthogonal array exclusively for the special case $s_1 = s_2 = \ldots = s_r = s$, and refer to the more general case as an orthogonal array with variable symbols. No such distinction is made here. Note also that an orthogonal array may be defined in a transpose fashion to that given here (*i.e.* with rows and columns interchanged).

An additional property of orthogonal arrays is that the points in an orthogonal array are usually spread regularly throughout the factor space. This suggests that appropriate orthogonal arrays might be used as the basis of initial search experiments in protein crystallization. The idea of using orthogonal arrays as the basis of a search procedure has been suggested in a more general context by Wu, Mao & Ma (1990).

Some general design considerations

In the construction of a protein crystallization search experiment based on a suitable orthogonal array, the preliminary step is the specification of the underlying factorial structure describing the experimental region. This involves consideration of both the factors to be included in the initial study, and the specification of appropriate levels for these factors.

The selection of a suitable physical method to bring the protein solution into the supersaturated state is assumed (see Ducruix & Giegé, 1992). The choice of physical procedure together with the volume and geometry of the experimental arrangement will influence the crystallization process, principally through the kinetics of equilibration processes (Mikol & Giegé, 1992). However, such considerations seem likely to be of secondary importance to the definition of the temperature and composition of the system.

Even in the simplest system there will typically be three components, the protein, a hydrogen-ion buffer to maintain the solution pH, and a protein precipitant. For membrane proteins a detergent to solubilize the protein is also required because of their characteristic hydrophobic surface features (Garavito & Picot, 1991; Kühlbrandt, 1988). Detergents may also be useful or necessary for the crystallization of water-soluble proteins (as originally proposed by McPherson *et al.*, 1986). In this paper the simpler three-component system is considered. The experimental problem is then to determine an appropriate precipitant and buffer, together with the ranges of temperature, pH and concentration of the solution components that will support nucleation and crystal growth. Experimentally, it is usually most convenient to work at a fixed protein concentration and govern the degree of supersaturation in the system by manipulating the concentration of the other solution components. Considering here the situation in which the buffer concentration is also fixed, the experimental factors to be considered are precipitant type, buffer type, precipitant concentration, pH and temperature. Experimentally, because it does not involve variation in the solution composition, temperature can be treated differently from other experimental factors.

With regard to the protein precipitant, three classes of compounds (inorganic salts, synthetic polymers and alcohols) have found widespread use in the growth of protein crystals. While in all cases the reduction in protein solubility is associated with the exclusion of the precipitant from the immediate domain of the protein, the principal sources of this effect differ for the differing classes of precipitant (Arakawa & Timasheff, 1988). Consequently, it is physically sensible to consider each class of precipitant within the framework of a separate search experiment. Because the number of potentially useful compounds within each class is very large, a representative selection will have to be made.

The control of pH during crystallization requires the presence of a suitable buffering system. However, for a given buffer, effective buffering capacity is limited to pH values close to its pK_a, so it is unlikely to be useful over the entire pH range of interest in an initial search experiment. The consequence of this for an experiment having a factorial structure is that the factor 'buffer type' will need to be nested within the factor 'pH' (*i.e.* the levels of the factor 'buffer type' will be dependent upon the level of the factor 'pH').

For the continuous variables (precipitant concentration and pH), the spacing of the levels is clearly very important. Recent work by Weber (1990) suggests that in some cases, protein crystallization may occur over reasonably large bounded ranges of these variables. Once the number of levels has been decided upon it would seem reasonable, in the absence of other information, to space the levels evenly with neither the highest or lowest level being set at the extreme of the feasible range.

The concentration of the precipitant will govern the degree of supersaturation in the system. A principal difficulty arises in relating the commonly used expressions of concentration (molarity, molality and volume percentage) to the relative effectiveness of compounds in reducing protein solubility, a problem related to the lack of adequate physical models of this phenomenon. For example, consider non-ionic polymers such as polyethylene glycol (PEG). At a fixed concentration of PEG, the solubility of proteins increases with decreasing average molecular weight of the polymer (see Atha & Ingham, 1981). Physical models have been proposed (based on simple volume-exclusion effects) which can account qualitatively for this behaviour (Mahadaven & Hall, 1990). However, from an experimental perspective, for polymers having differing molecular weight it is still not clear what relative concentrations are required to effect the same decrease in protein solubility for a given protein. This makes specification of reasonable level settings difficult.

Search designs based on orthogonal arrays

A description is now given of an initial search design for protein crystallization, based on an orthogonal array, which illustrates the general approach adopted here. In the example given, non-ionic polymers are used

as protein precipitants. The experiment is based on a factorial structure, with variation in polymer type, polymer concentration, pH and buffer type. By necessity the buffer type is nested within the pH. The factorial structure is given below.

Factor	Levels	
Polymer type	Polyethylene glycol *MW* 6000	
	Polyethylene glycol monomethyl ether *MW* 5000	
Polymer concentration [%(*w*/*v*)]	7	
	14	
	21	
	28	
pH (buffer type)*	4.9 (Acetate	Citrate)
	5.5 (Succinate	Malate)
	6.1 (MES	Cacodylate)
	6.7 (PIPES	Bis-tris propane)
	7.3 (HEPES	MOPS)
	7.9 (EPPS	Tris)
	8.5 (TAPS	Bis-tris propane)
	9.1 (AMPSO	Borate)

* Abbreviations employed: MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; Bis-tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; HEPES, *N*-(hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); MOPS, 3-(*N*-morpholino)-propanesulfonic acid; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid); Tris, [tris(hydroxymethyl)aminomethane]; TAPS, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; AMPSO, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid.

The two non-ionic polymers employed are polyethylene glycol (PEG) M_r = 6000 and polyethylene glycol monomethyl ether (PEG-mme) M_r = 5000. The use of PEG-mme was suggested by recent work at the University of York (Brzozowski, 1993). Because these polymers have a similar average molecular weight, the volume exclusion effect should be similar for each (Mahadavan & Hall, 1990), so neglecting differences in polymer binding to the protein (Arakawa & Timasheff, 1985), similar concentrations should be required to effect the same decrease in protein solubility. Polymer concentration is varied over the range 7–28%(*w*/*v*), employing four equally spaced levels.

The pH range of the experiment is 4.9–9.1, employing eight equally spaced levels. At each pH two buffers are employed. The buffers, being obtained as free acids or bases, were titrated to the appropriate pH with KOH or HCl, respectively. The buffer concentration is fixed at 0.2 *M*. [Note that the addition of aqueous PEG solutions at high concentration to buffered solutions can cause a change in the measured pH (Atha & Ingham, 1981).]

The experiment as it stands would involve 2 × 2 × 4 × 8 = 128 runs. Using columns *A*, *B*, *E* and *F* of the OA(64, 6, 2^4 × 4 × 8, 3) (see *Appendix* I), a subset (or sample) of 64 runs is selected, forming the basis of an initial protein crystallization search experiment. (Column *A* has been assigned to polymer type, column *B* to buffer type, column *E* to polymer concentration, and column *F* to pH.) The resulting experiment is given in *Appendix* II. Since there are only four factors involved it is possible to represent this experiment diagrammatically (see Fig. 1). It can be seen that the subset selected by employing this orthogonal array is evenly distributed.

A similar factorial structure can also be used for search designs based on other classes of protein precipitants, such as normal salts that do not possess an appreciable buffering capacity [*e.g.* $(NH_4)_2SO_4$, $(NH_4)NO_3$, LiCl *etc.*], or alcohols and poly-hydroxy compounds such as 2-methyl-2,4-pentanediol. The difficulty in assessing the relative effectiveness of such compounds in reducing protein solubility makes the setting of appropriate levels for the precipitant concentration difficult. In the case of salts, the relative effectiveness in altering protein solubility is reflected in the lyotropic or Hofmeister series (von Hippel & Schleich, 1969). When ion binding to the protein is negligible, the predominant protein–salt interaction is exclusion of the salt from the immediate domain of the protein, leading to greatly reduced protein solubility at high salt concentrations (salting-out behaviour) (Arakawa, Bhat & Timasheff, 1990). The likely physical basis of this exclusion is the increase in surface tension of water caused by the addition of salts. Accordingly, Melander & Horvath (1977) proposed that the differing relative effects of salts on protein solubility could be directly related to their effect on the surface tension of water (*i.e.* that surface tension might be used as the physical basis for a quantitative lyotropic scale). We have adopted this suggestion and have used surface tension as the basis for assigning relative salt concentrations in our experiments. However, complication of this simple physical picture will arise when there is significant salt binding to the protein as, for example, with the divalent cation salts such as $MgCl_2$, $BaCl_2$ and $CaCl_2$, which are known to be largely ineffective at salting out proteins despite having large molal surface-tension increments (see Arakawa, Bhat & Timasheff, 1990).

We have normally executed such experiments using vapour-diffusion techniques, in which a small volume of the buffer/precipitant solution (typically 1–10 µl) is mixed with an equal volume of protein solution (having a typical concentration of 5–50 mg ml^{-1}). This drop is then equilibrated in a sealed system against a much larger volume of the buffer/precipitant solution. Where possible the protein itself is suspended in water, unless this is impossible or undesirable because of constraints due to the stability, solubility or activity of the protein. Where control of the pH is required, low concentrations (10–20 m*M*) of an appropriate buffer are employed.

No mention has yet been made of the temperature at which these experiments are conducted. The OA(64, 6, 2^4 × 4 × 8, 3) has six columns, of which only four have been assigned to the variables governing the

composition of the system. One of the remaining two-level columns (columns *C* and *D*) could be assigned to temperature if this was desired. Alternatively, the experiment could be executed at a single temperature, and then again at another temperature if the results from the first experiment were not satisfactory. This matter is left to the discretion of the experimenter. It is, however, important to recognize that for some of the buffers included in this experiment, the pK_a values are temperature dependent, which may result in quite large pH changes with temperature.

If execution at a single temperature is considered, the experiment can be considered modular in construction. At each level of pH, the eight experimental points constitute an OA(8, 3, $2^2 \times 4$, 2). Further, for each pair of consecutive levels of polymer concentration, the four experimental points constitute an OA(4, 3, 2^3, 2). This should be apparent from inspection of Fig. 1. That is, the experiment can be considered to be comprised of a collection of smaller experiments, each covering more limited ranges of pH and precipitant concentration. Consequently, the overall experiment has a degree of flexibility. If there is a physical reason for avoiding a particular pH range then the appropriate runs can simply be omitted from the search experiment, without compromising the factorial basis of the experiment.

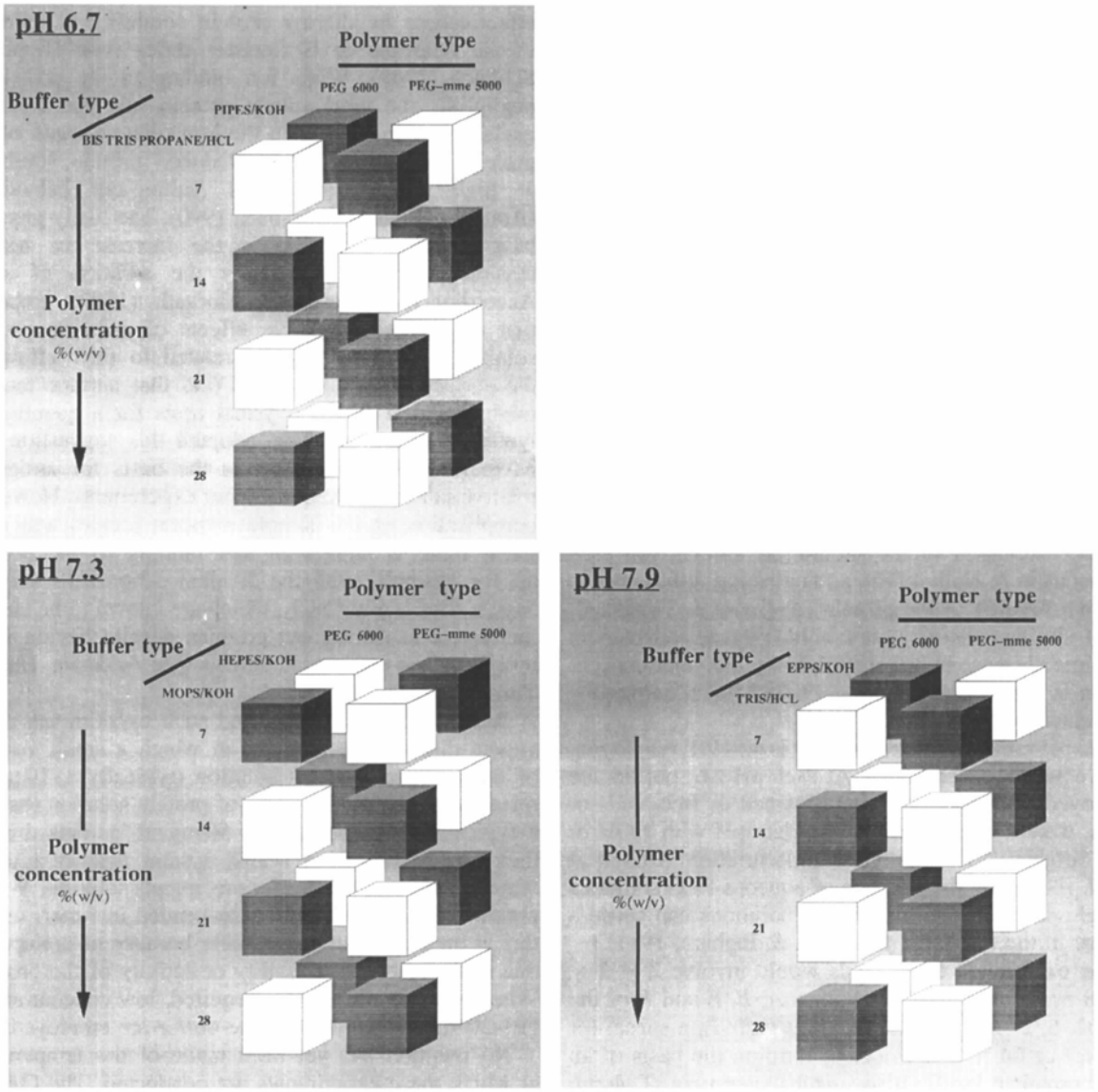

Fig. 1. A representation of part of an initial search experiment for protein crystallization, based on an orthogonal array, using non-ionic polymers as protein precipitants. Only the darkened locations are executed during the initial search.

A criticism that might be levelled against such designs is that they may be considered too large to be practicable. Where the quantity of protein available is very limited, we suggest two possible methods for the sequential execution of search designs based on orthogonal arrays, which could serve to reduce the overall experimental size

The first is simply to reduce the size of the experimental region initially explored. For example, the pH range explored in the initial search experiment might be reduced. Subsequent to this initial experiment, the search could be extended over a wider pH range if necessary, with some levels of precipitant concentration having been eliminated from further consideration.

A second procedure would involve reducing the strength of the orthogonal array used in the initial search experiment. In the example just given, use was made of the OA(64, 6, $2^4 \times 4 \times 8$, 3) to select a subset of 64 runs from a total of 128 (or 256 if execution of the experiment at two temperatures is considered). That is, use was made of an orthogonal array of strength 3. However, it would also be possible to use an orthogonal array of strength 2 to select a subset of 32 runs. Here, there would be only four experimental points at each level of pH, instead of eight (refer to Fig. 1). Reduction of the strength of the orthogonal array used in the initial search procedure substantially reduces the initial experimental size, but also reduces the completeness of the coverage of the experimental region, and increases the probability that solution conditions supporting crystal growth will not be located in the initial search.

Consequently, while either of these approaches (reducing the size of the experimental region to be explored, or reducing the strength of the orthogonal array used in the initial search experiment) may decrease the total required experimental size, they may concomitantly extend the length of time required to gain satisfactory results.

A final, and important, consideration is the direction for further searches should the initial experiment fail to identify suitable solution conditions. This is a difficult question to address, principally because negative results give so little information on the most promising neighbourhood for further searches. A well planned initial search experiment will at least serve to clearly define the protein solubility (*i.e.* to restrict the likely range of precipitant concentration to be investigated in further experiments). If we consider the experiments described in this paper, and focus on a particular class of protein precipitant, then extending the search might involve executing the remaining treatment combinations required to complete the factorial arrangement (or at least those that are appropriate based on the knowledge of protein solubility), extending the pH range of the search, or conducting similar experiments using related compounds (but again taking advantage of the information on protein solubility gained in the first experiment). However, the experiments described here employ orthogonal arrays of relatively high strength, with fairly close spacing of the levels of the continuous factors, and consequently cover the experimental region in a reasonably comprehensive fashion. If the results from such experiments are all negative, then it might be more worthwhile considering experiments based on a different class of precipitant, rather than pursuing the search further with the same or similar precipitants. As suggested, it might be preferred to use an orthogonal array of lower strength (and smaller size) as the basis of the initial search experiment. In this case, when results are negative, consideration should be given to performing a subsequent experiment to increase the overall strength of the array (*i.e.* to increase the coverage of the experimental region).

A principal advantage of the search experiments based on orthogonal arrays described in this paper over more empirical approaches, is that they provide (at least in a qualitative sense) a way of assessing the degree of coverage of the experimental region (through the concept of the strength of the array). They also provide a way of organizing a sequential experimental program for proteins which prove difficult to crystallize.

To illustrate the practical application of some of these ideas, the recent crystallizations of the enzymes glucose-fructose oxidoreductase (GFOR) (Zachariou & Scopes, 1986) and pyruvate decarboxylase (PDC) (Neale, Scopes, Wettenhall & Hoogenraad, 1987) from *Zymomonas mobilis* are described.

In the case of PDC two search experiments were conducted concurrently (in this case a reasonable quantity of purified protein was available to us). One was based on the use of polyethylene glycols as protein precipitants, as described in *Appendix* II. The second was based on the use of the salts $(NH_4)_2SO_4$ and LiCl. For this experiment, the same factorial structure was employed, with $(NH_4)_2SO_4$ and LiCl substituted for PEG 6000 and PEG-mme 5000. Relative concentrations for the two salts were set at (0.87, 1.65, 2.33 and 2.94 *M*) and (1.13, 2.24, 3.32 and 4.38 *M*), respectively, which should effect approximately the same change in the surface tension of water ($2, 4, 6, 8 \times 10^{-3}$ N m^{-1}).*

The protein was suspended in 10 m*M* MES/KOH buffer at pH 6 containing 1 m*M* $MgCl_2$ and 1 m*M* thiamin diphosphate (both co-factors of the enzyme), and concentrated to 10 mg ml^{-1}. Search experiments were executed using the hanging-drop vapour-diffusion technique, with 2 µl drops comprised of equal volumes of the protein and the buffer/precipitant solution, and were conducted at a single temperature (297 K). From these two search experiments (128 runs in total) crystals grew in only one drop. Small, clearly defined prismatic

* This calculation is based on fitting appropriate functions to the surface tension/molality and density/molality data available in the literature (Timmermans, 1960; Lobo & Quaesma, 1981).

crystals (approximately 0.05 mm in all dimensions) grew from 1.65 *M* ammonium sulfate in the presence of TAPS/KOH buffer at pH 8.5. The crystals appeared after 3-4 weeks. Efforts are currently underway to increase the crystal size using factorial experiments based on the variation of pH, buffer type and the concentration of all solution components.

Vastly different crystallization behaviour was observed for GFOR. This protein was concentrated to 20 mg ml^{-1} in water, and search experiments were executed using the hanging-drop vapour-diffusion technique, as described above. A search experiment was conducted using polyethylene glycols as protein precipitants, which was similar (but not identical) to that described in this paper. It covered the pH range 5-8, with four equi-spaced levels, and four buffers at each pH. Again, an orthogonal array of strength 3 was used as the basis of the search experiment. It became apparent immediately that the solubility of GFOR in PEG solutions was not high; consequently, only the runs at the two lowest polymer concentrations were executed. Crystals grew across the entire pH range virtually independent of the buffer type, but were best defined at pH 5-6. The crystals appeared as masses of extremely thin stacked plates, growing in some cases within minutes at the higher [14%(*w*/*v*)] polymer concentration. Subsequent factorial experiments centred on the best conditions identified in this search experiment and involved variation in pH, and the concentration of the buffer and polymer. By careful control of the degree of supersaturation in the system it was possible to grow much larger, single plates (up to 1.5 mm in length and 0.2-0.3 mm in thickness), which diffract to beyond 2 Å resolution. The best crystals have been grown from succinic acid/KOH buffer pH 5.5 at 0.2-0.5 *M*, with a polymer concentration of between 4 and 6%(*w*/*v*) PEG 8000. It is also possible to grow small crystals of GFOR using salts as protein precipitants, but we have not pursued this further. The crystallographic results will be reported elsewhere.

In the case of GFOR, since it crystallized so readily, the nature of the search procedure was not critical to the successful resolution of the problem. However, the use of a balanced experiment allowed us to draw useful informal inferences about the crystallization process, for example that its behaviour appears essentially independent of the buffer type or the pH over the range 5-8.

If required these comparisons could be made more formally, by ranking the results in a semi-quantitative fashion and performing an analysis of variance. It is very difficult to extract this kind of comparative information from an empirical experiment with no factorial structure, such as that of Jancarik & Kim (1991). In the case of PDC, which proved much more difficult to crystallize, the nature of the search procedure was clearly more critical, and a less systematic or complete search procedure (or even employing an orthogonal array of lower strength) may well have failed.

This point was illustrated again during our recent attempts to crystallize the human $\alpha_2\varepsilon_2$ embryonic hemoglobin. Since the ε-globin chain has substantial sequence homology with the β-globin chain (Baralle, Shoulders & Proudfoot, 1980) several initial crystallization experiments were conducted based around solution conditions which support crystal growth of the human $\alpha_2\beta_2$ adult hemoglobin (Silva, Rogers & Arnone, 1992; Perutz, 1968). These factorial experiments involved variation in both pH and precipitant concentration, but resulted only in amorphous precipitation of the embryonic hemoglobin. Subsequently, search experiments based on orthogonal arrays were employed to try and identify suitable crystallization conditions. An initial search experiment used polyethylene glycols as protein precipitants, as given in *Appendix* II, but covering a reduced pH range (6.1-8.5) (40 runs from the proposed 64). The embryonic hemoglobin was concentrated to 40 mg ml^{-1} in water, and the experiment conducted as described above Crystals of the embryonic hemoglobin form in solutions buffered at pH 8.5, in the presence of high concentrations of polymers [>21%(*w*/*v*)]. At a lower pH, amorphous precipitation of the protein resulted. These crystals are still too small to study by X-ray diffraction. Subsequent experiments using salts as protein precipitants have not identified any further crystallization conditions. By way of comparison, a search experiment based on the solution conditions specified by Jancarik & Kim (1991) did not result in the identification of crystallization conditions.

Finally, attempting to design search experiments for protein crystallization quickly highlights one disadvantage of describing the experimental region with a factorial structure. That is that certain sub-regions of the proposed experimental region may be physically unrealizable, as a result of phase separation or precipitation of the solution components. If the experimental region is still to be described by a factorial structure, the ranges of some of the variables must be reduced. However, this approach can be unsatisfactory, because interesting parts of the original region may be excluded from the experiment. Further consideration needs to be given to the design of search experiments where the experimental region is irregular.

Discussion

The justification for the use of orthogonal arrays as search experiments in protein crystallization was that the points in such an array were likely to be evenly or uniformly distributed throughout the experimental region. Consequently, it is hoped that the response among these points will be indicative of the response over the entire experimental region, and hence regions supporting nucleation and crystal growth can be identified with

a smaller number of runs than would otherwise be possible.

The problem of designing systematic search experiments for protein crystallization has been addressed previously by Carter & Carter (1979), and discussed further in subsequent papers (Carter, Baldwin & Frick, 1988; Carter, 1992). In these papers an alternative procedure for constructing a subset of a factorial experimental design is proposed. In this procedure, the points comprising the subset are essentially chosen by simple random selection without replacement. Two restrictions govern this selection, firstly, that each factor is represented in the subset 'a nearly identical' number of times at each level, and secondly, that each possible pairwise combination of levels occurs at least once in the subset (Carter, 1992). Experiments resulting from this procedure have been termed incomplete factorial designs. It was proposed to analyse the results from such experiments using multiple linear regression.

This procedure has an interesting precedent in the statistical literature. In the late 1950's it was proposed (Satterthwaite, 1959; Budne, 1959; Anscombe, 1959) that useful experiments with a small number of runs could be derived from full-factorial experiments by selecting a suitably sized subset at random from the treatment combinations comprising the full-factorial experiment. Such experiments were known as random-balance or random-allocation (Dempster, 1960) experiments. One variant of this idea was that the random sampling be conditional on each factor being represented in the sample a prearranged number of times at each level. It is clearly possible to extend this technique to balancing with regard to the combinations of factors. Such an approach would then appear essentially equivalent to the incomplete factorial method of Carter & Carter (1979). Dempster (1960) suggested the name random allocation with partial balance for a procedure such as this.

In connection with model-based inference, there has been extensive discussion of the efficiency of such designs and of the potential difficulties associated with their interpretation (Tukey, Youden, Kempthorne, Box & Hunter, 1959; Herzberg & Cox, 1969; Kleijnen, 1975). In terms of design properties, necessary and sufficient conditions for orthogonality of linear model effect estimates were given by Addelman (1963), which may be useful in assessing the 'goodness' of designs generated by a random allocation or incomplete factorial procedure.

Here interest is centred on the (related) properties of arrays generated by random allocation as search designs in protein crystallization. For this purpose we require in some sense the even or uniform distribution of points throughout the experimental region. It is important to recognize that while random-allocation designs appeal because of their inherent simplicity, random selection alone will not necessarily ensure uniform distribution of the experimental points throughout the experimental region. A random sample is not a representative sample 'in the sense that the sample is like the population or is a typical cross section of the population' (Folks, 1984). Hence the importance of random selection conditional on certain balancing properties in the final sample (stratification of the sampling procedure), if uniform coverage of the experimental region is required.

Stratified sampling procedures have been studied in connection with computer experiments. Latin hypercube sampling was introduced by McKay, Beckman & Conover (1979). This is essentially random sampling subject to univariate stratification. Here the constraint is that for each factor, each level occurs with a fixed frequency in the final sample. A Latin hypercube sample is essentially an orthogonal array of strength 1. Owen (1992) showed that Latin hypercube sampling could be generalized using orthogonal arrays of any strength d, resulting in sampling plans that stratify on all d-variate margins simultaneously. In this paper, we have described search experiments for protein crystallization, based on the use of orthogonal arrays. The procedure of Carter & Carter (1979) will result in subsets which approximate the conditions of balance which orthogonal arrays of low strength fulfil exactly. Relaxing the mathematically restrictive requirement for orthogonality can result in a considerable reduction in experimental size, and designs produced in this fashion have proved useful for protein crystallization (Abergel *et al.*, 1991; Betts, Frick, Wolfendon & Carter, 1989; Lewit-Bentley, Doublié, Fourme & Bodo, 1989). However, by relaxing these conditions, the experimental region is necessarily covered less completely. Search experiments based on orthogonal arrays provide a more balanced coverage of the experimental region, but are more expensive in terms of the required number of runs. The work described in this paper is essentially a compromise between search experiments based on large full-factorial arrays (exemplified by the approach of Weber, 1990), and search experiments based on the small partially balanced arrays generated by the procedure of Carter & Carter (1979). It was motivated by our need for a systematic search procedure for proteins which might not crystallize readily [*i.e.* for proteins which crystallize over small bounded ranges of continuous variables such as pH and precipitant concentration, or proteins whose crystallization shows a marked dependence on the chemical nature of the solution components (*e.g.* buffer type)].

One advantage of random-allocation designs is that for any given factorial experiment, a random-allocation subset can always be quickly constructed. Notwithstanding this, useful orthogonal designs are increasingly accessible in the literature and elsewhere. For example, an easily constructed series of orthogonal fractional factorial designs (orthogonal arrays) for asymmetrical factorial experiments was given by Lewis (1982), while

Dey (1985) comprehensively catalogues construction methods for orthogonal arrays. A collection of C programs for the construction and manipulation of symmetrical orthogonal arrays has been deposited at statlib (lib.stat.cmu.edu).

This paper has concerned the construction of protein crystallization search designs for water soluble proteins, based on orthogonal arrays. In the example given, the size of the underlying factorial experiment was fairly modest (128 runs if conducted at a single temperature). In the case of membrane protein crystallization, a detergent is required. This additional solution component introduces a further two factors into the experimental structure (detergent type and detergent concentration). Even if each of these factors had only two levels, this would result in a factorial experiment with 512 runs, clearly much too large to be practical in most situations. Orthogonal arrays might be particularly useful in such an experimental situation. In fact an experiment of the nature just described could be accommodated in the OA(64, 6, $2^4 \times 4 \times 8$, 3) that has been employed here.

In conclusion, the use of experiments having an explicit factorial structure as search designs in protein crystallization seems well established. The attraction of such experiments is that they provide a wide 'inductive basis for our conclusions' (Fisher, 1935). The difficulty with such experiments is that they rapidly become extremely large. Here, what is in essence a sequential method of execution has been proposed, based on the use of orthogonal arrays. These arrays provide a unified framework for considering the problem of searching for the physical and chemical conditions which will support protein crystal growth. The concept of the strength of such arrays provides some measure of the degree of coverage of the experimental region. Such an approach allows for a systematic exploration of the experimental region while keeping the experimental size within reasonable limits, and may be particularly important as the complexity of the experimental problem increases, as it does, for example, with membrane protein crystallization.

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APPENDIX I

OA(64, 6, $2^4 \times 4 \times 8$, 3) (Dey, 1985; Dey & Agrawal, 1985)

	Factor					
	A	*B*	*C*	*D*	*E*	*F*
1	0	1	0	1	0	0
2	0	0	0	0	1	0
3	0	1	1	0	2	0
4	0	0	1	1	3	0
5	1	0	1	0	0	0
6	1	1	1	1	1	0
7	1	0	0	1	2	0
8	1	1	0	0	3	0
9	0	0	1	0	0	1
10	0	1	1	1	1	1
11	0	0	0	1	2	1
12	0	1	0	0	3	1
13	1	1	0	1	0	1
14	1	0	0	0	1	1
15	1	1	1	0	2	1
16	1	0	1	1	3	1
17	0	1	1	0	0	2
18	0	0	1	1	1	2
19	0	1	0	1	2	2
20	0	0	0	0	3	2
21	1	0	0	1	0	2
22	1	1	0	0	1	2
23	1	0	1	0	2	2
24	1	1	1	1	3	2
25	0	0	0	1	0	3
26	0	1	0	0	1	3
27	0	0	1	0	2	3
28	0	1	1	1	3	3
29	1	1	1	0	0	3
30	1	0	1	1	1	3
31	1	1	0	1	2	3
32	1	0	0	0	3	3
33	0	1	0	1	0	4
34	0	0	0	0	1	4
35	0	1	1	0	2	4
36	0	0	1	1	3	4
37	1	0	1	0	0	4
38	1	1	1	1	1	4
39	1	0	0	1	2	4
40	1	1	0	0	3	4
41	0	0	1	0	0	5
42	0	1	1	1	1	5
43	0	0	0	1	2	5
44	0	1	0	0	3	5
45	1	1	0	1	0	5
46	1	0	0	0	1	5
47	1	1	1	0	2	5
48	1	0	1	1	3	5
49	0	1	1	0	0	6
50	0	0	1	1	1	6
51	0	1	0	1	2	6
52	0	0	0	0	3	6
53	1	0	0	1	0	6
54	1	1	0	0	1	6
55	1	0	1	0	2	6
56	1	1	1	1	3	6
57	0	0	0	1	0	7
58	0	1	0	0	1	7
59	0	0	1	0	2	7
60	0	1	1	1	3	7
61	1	1	1	0	0	7
62	1	0	1	1	1	7
63	1	1	0	1	2	7
64	1	0	0	0	3	7

The following properties follow from the definition of an orthogonal array (Hedayat, 1990).

(I) Any array obtained from an orthogonal array by permuting columns, rows, or symbols in one or more columns will again be an orthogonal array with the same parameters.

(II) Consider an orthogonal array of size N, strength d, and having m columns. Any $N \times m'$ sub-matrix

formed by deleting some columns of this array is also an orthogonal array with strength $d' = \min(m', d)$

(III) Any orthogonal array of strength d is an orthogonal array of strength d', with $d' \leq d$.

(IV) Combining rows of OA(N_i, m, $s_1^{k_1} x s_2^{k_2} x \ldots x s_r^{k_r}$, d), $i = 1, 2$ leads to an OA(N, m, $s_1^{k_1} x s_2^{k_2} x \ldots x s_r^{k_r}$, d) where $N = N_1 + N_2$.

APPENDIX II

An example of a search experiment for protein crystallization, employing non-ionic polymers as protein precipitants

The experiment is based on the OA (64, 6, $2^4 \times 4 \times 8$, 3) (given in *Appendix* I). Note that by assigning one of the unallocated two-level columns of this array (columns *C* and *D*) to temperature it is possible to arrive at a plan to execute this experiment at two temperatures. Buffer concentration is fixed at 0.2 *M*.

Run	Precipitant type	Concentration [%(*w*/*v*)]	pH	Buffer type
1	Polyethylene glycol 6000	7	4.9	Citric acid/KOH
2	Polyethylene glycol 6000	14	4.9	Acetic acid/KOH
3	Polyethylene glycol 6000	21	4.9	Citric acid/KOH
4	Polyethylene glycol 6000	28	4.9	Acetic acid/KOH
5	Methoxypolyethylene glycol 5000	7	4.9	Acetic acid/KOH
6	Methoxypolyethylene glycol 5000	14	4.9	Citric acid/KOH
7	Methoxypolyethylene glycol 5000	21	4.9	Acetic acid/KOH
8	Methoxypolyethylene glycol 5000	28	4.9	Citric acid/KOH
9	Polyethylene glycol 6000	7	5.5	Succinic acid/KOH
10	Polyethylene glycol 6000	14	5.5	Malic acid/KOH
11	Polyethylene glycol 6000	21	5.5	Succinic acid/KOH
12	Polyethylene glycol 6000	28	5.5	Malic acid/KOH
13	Methoxypolyethylene glycol 5000	7	5.5	Malic acid/KOH
14	Methoxypolyethylene glycol 5000	14	5.5	Succinic acid/KOH
15	Methoxypolyethylene glycol 5000	21	5.5	Malic acid/KOH
16	Methoxypolyethylene glycol 5000	28	5.5	Succinic acid/KOH
17	Polyethylene glycol 6000	7	6.1	Cacodylic acid/KOH
18	Polyethylene glycol 6000	14	6.1	MES/KOH
19	Polyethylene glycol 6000	21	6.1	Cacodylic acid/KOH
20	Polyethylene glycol 6000	28	6.1	MES/KOH
21	Methoxypolyethylene glycol 5000	7	6.1	MES/KOH
22	Methoxypolyethylene glycol 5000	14	6.1	Cacodylic acid/KOH
23	Methoxypolyethylene glycol 5000	21	6.1	MES/KOH
24	Methoxypolyethylene glycol 5000	28	6.1	Cacodylic acid/KOH
25	Polyethylene glycol 6000	7	6.7	PIPES/KOH
26	Polyethylene glycol 6000	14	6.7	Bis-tris propane/HCl
27	Polyethylene glycol 6000	21	6.7	PIPES/KOH
28	Polyethylene glycol 6000	28	6.7	Bis-tris propane/HCl
29	Methoxypolyethylene glycol 5000	7	6.7	Bis-tris propane/HCl
30	Methoxypolyethylene glycol 5000	14	6.7	PIPES/KOH
31	Methoxypolyethylene glycol 5000	21	6.7	Bis-tris propane/HCl
32	Methoxypolyethylene glycol 5000	28	6.7	PIPES/KOH
33	Polyethylene glycol 6000	7	7.3	MOPS/KOH
34	Polyethylene glycol 6000	14	7.3	HEPES/KOH
35	Polyethylene glycol 6000	21	7.3	MOPS/KOH
36	Polyethylene glycol 6000	28	7.3	HEPES/KOH
37	Methoxypolyethylene glycol 5000	7	7.3	HEPES/KOH
38	Methoxypolyethylene glycol 5000	14	7.3	MOPS/KOH
39	Methoxypolyethylene glycol 5000	21	7.3	HEPES/KOH
40	Methoxypolyethylene glycol 5000	28	7.3	MOPS/KOH
41	Polyethylene glycol 6000	7	7.9	EPPS/KOH
42	Polyethylene glycol 6000	14	7.9	Tris/HCl
43	Polyethylene glycol 6000	21	7.9	EPPS/KOH
44	Polyethylene glycol 6000	28	7.9	Tris/HCl
45	Methoxypolyethylene glycol 5000	7	7.9	Tris/HCl
46	Methoxypolyethylene glycol 5000	14	7.9	EPPS/KOH
47	Methoxypolyethylene glycol 5000	21	7.9	Tris/HCl
48	Methoxypolyethylene glycol 5000	28	7.9	EPPS/KOH
49	Polyethylene glycol 6000	7	8.5	Bis-tris propane/HCl
50	Polyethylene glycol 6000	14	8.5	TAPS/KOH
51	Polyethylene glycol 6000	21	8.5	Bis-tris propane/HCl
52	Polyethylene glycol 6000	28	8.5	TAPS/KOH
53	Methoxypolyethylene glycol 5000	7	8.5	TAPS/KOH
54	Methoxypolyethylene glycol 5000	14	8.5	Bis-tris propane/HCl
55	Methoxypolyethylene glycol 5000	21	8.5	TAPS/KOH
56	Methoxypolyethylene glycol 5000	28	8.5	Bis-tris propane/HCl
57	Polyethylene glycol 6000	7	9.1	AMPSO/KOH
58	Polyethylene glycol 6000	14	9.1	Boric acid/KOH
59	Polyethylene glycol 6000	21	9.1	AMPSO/KOH
60	Polyethylene glycol 6000	28	9.1	Boric acid/KOH
61	Methoxypolyethylene glycol 5000	7	9.1	Boric acid/KOH
62	Methoxypolyethylene glycol 5000	14	9.1	AMPSO/KOH
63	Methoxypolyethylene glycol 5000	21	9.1	Boric acid/KOH
64	Methoxypolyethylene glycol 5000	28	9.1	AMPSO/KOH

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