

# A Free Interface Diffusion Technique for the Crystallization of Proteins for X-Ray Crystallography

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Received February 18, 1972; accepted May 8, 1972

A free interface diffusion technique for the crystallization of proteins for X-ray crystallography is described. The procedures and apparatus are simple and allow experiments to be carried out routinely with small quantities of protein. Detailed procedures for the crystallization of cytochrome  $c_2$  (*Rhodospirillum rubrum*), cytochrome  $c'$  (*Rhodopseudomonas palustris*), cytochrome  $c'$  (*R. rubrum*), and glyceraldehyde 3-phosphate dehydrogenase (*Palinurus*) are described to exemplify the technique.

Recent advances in protein structure determination have created great interest in improved techniques for obtaining large single crystals (average linear dimension  $\approx 0.2$  mm) suitable for X-ray diffraction work. The most desirable features of such a technique are that it be sufficiently simple and flexible to easily allow exploration of a wide range of crystallization parameters such as solvent pH, dielectric constant, ionic strength, or the effect of various additives, and that it allow experiments to be carried out with small amounts of starting material. Reports of a number of techniques fulfilling these criteria have appeared in the literature (1-4).

The method described in this communication utilizes free diffusion between a concentrated protein solution and a precipitant solution to attain the conditions of protein supersaturation requisite for the nucleation and subsequent growth of large single crystals. Protein and precipitant solutions are layered directly over each other in small crystallization cells or tubes, and allowed to diffuse to equilibrium. At the onset of interdiffusion of the protein and precipitant layers, the protein in the immediate vicinity of the interface is exposed to a transient supersaturating concentration of precipitant. This transient protein supersaturation induces the formation of nuclei which serve

as crystal growth loci when equilibrium is reached. At equilibrium the overall precipitant concentration in the total volume is substantially less than that required to spontaneously precipitate the protein, facilitating the gradual growth of a few large crystals from the nuclei created at the initial interface. The free diffusion method offers manipulative advantages because of its simplicity.

## MATERIALS AND METHODS

*Reagents.* "Special Enzyme Grade" ammonium sulfate was obtained from Mann Research Laboratory, New York, NY. Solutions of ammonium sulfate were prepared by dissolving a specified weight of salt in 1 liter of solution at 25°C according to the nomogram of Dixon (5). All other chemicals were standard analytical reagent grade. Doubly glass-distilled water was used for the preparation of all solutions. Parafilm is a deformable polyethylene film manufactured by the American Can Co., Neenah, Wisconsin.

*Proteins.* Cytochrome  $c_2$  of *R. rubrum* was isolated by a modification of the method of Horio and Kamen (6). Cytochrome  $c'$  of *R. rubrum* and cytochrome  $c'$  of *R. palustris* (7) were the gift of Dr. Robert Bartsch. Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) was isolated from commercially obtained frozen South African spiny lobster (*Palinurus*) tails by the method of Allison and Kaplan (8).

Concentrated stock solutions of proteins were prepared by centrifuging suspensions of micro-

crystals grown in ~90% saturated ammonium sulfate, and dissolving the resulting pellet in a minimum volume of cold water or buffer. The concentration of these solutions was assayed spectrophotometrically and adjusted to the desired "stock" concentration with the addition of more buffer. The final concentration of the stock cytochrome solutions ( $MW = 13,000-16,000$ ) was 30 mg/ml. The concentration of *Palinurus* glyceraldehyde 3-phosphate dehydrogenase ( $MW = 144,000$ ) was 50 mg/ml. Stock protein solutions were minimally buffered so that crystallization conditions could be varied by adjustments of the pH and ion content of the precipitant solution. Protein solutions prepared from centrifuged salt precipitates contained residual ammonium sulfate (5-10% saturated), which did not normally interfere with the course of the experiments unless crystallization from organic solvents was attempted. In this case, the residual salt was removed by dialysis. The conditions for crystallization are reported later and did not require desalting of the stock solutions prior to crystallization. In the case of *Palinurus* glyceraldehyde 3-phosphate dehydrogenase, the presence of the residual ammonium sulfate appeared to enhance the stability of the protein during storage. Concentrated protein solutions were stored at 5°C in plastic centrifuge tubes. The solutions were centrifuged for 10 min at 10,000g prior to the removal of aliquots for crystallization in order to pellet any dust or debris which was present in the solution.

**Crystallization in microcells.** An apparatus useful in searching for proper crystallization conditions in cases where only a small amount of protein (<5 mg) is available is shown in Fig. 1. The apparatus consists of a disposable Pasteur pipette (VWR Scientific, 9 in.  $\times$  7 mm o.d., No. 14672-040) which has been cut off so that the large diameter section is about 2 cm long. The cell is loaded by drawing a premeasured volume of the least dense solution into the capillary bore of the pipette by means of a screw-type pipetting aid (Clay-Adams A2473) so that the lower meniscus of the solution volume remains even with the end of the capillary. A premeasured volume of the more dense solution is then drawn into the capillary bore so that both solutions form a continuous liquid volume having a sharp interface. With the tube maintained in a vertical orientation, the protein/precipitant volume is drawn higher into the capillary bore. The end is sealed with hard wax. The pipetting aid is carefully removed and the top of the tube is sealed with Parafilm. The useful capacity of these gradient tubes ranges from 10 to 100  $\mu$ l. The quantity of protein suitable to perform an individual test in a microcell ranges

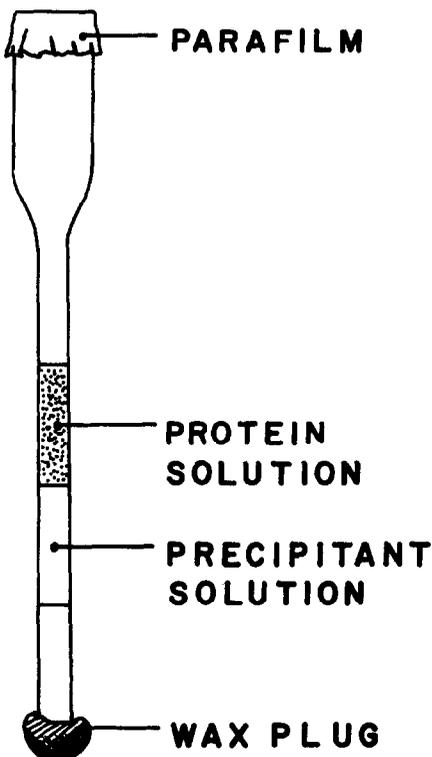


FIG. 1. A free diffusion microcell manufactured from a cut-off Pasteur disposable pipette. The cell is stored in a vertical position to allow even diffusion between protein and precipitant layers.

from 0.06 to 0.6 mg. Since the capillary bore of the pipette tapers near the top, the time required to reach equilibrium between the protein and precipitant layers may be altered by adjusting the height of the total solution volume in the capillary bore (Fig. 2). Diffusion to equilibrium in these capillaries, as observed with colored cytochrome solutions ( $MW$  approx 13,000) layered over 50-80% saturated ammonium sulfate, takes place within about an hour, depending upon conditions. The method is adaptable to smaller scale or longer diffusion times by drawing the capillary bore to a smaller diameter.

**Larger scale crystallization.** In cases where more than a few milligrams of protein are available for experimentation, gradients may be set up in miniature Pyrex test tubes (VWR Scientific, No. 60820-068, 6  $\times$  50 mm). The commercially available tubes may be cut down to a length of 10-25 mm to facilitate introduction and removal of solutions (Fig. 3). Protein and precipitant solutions are carefully layered in with a micro-pipette. The tube is sealed with Parafilm. Diffusion to equilibrium usually takes place within 1 hr of

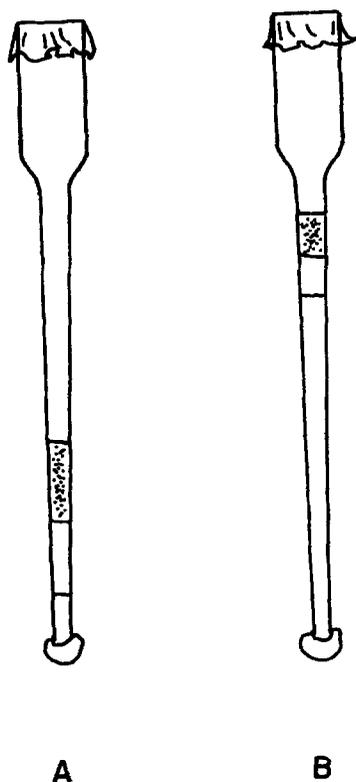


FIG. 2. The time required to reach equilibrium between protein and precipitant layers in a microcell may be decreased by drawing the protein/precipitant volume into a part of the capillary having a larger cross section. Cells A and B contain the same total solution volume. The area of the interface is greater and the diffusion path length shorter in Cell B than in Cell A. As a result, approach to equilibrium is more rapid in Cell B.

setting up the gradient. The total volume used in a single tube ranges from 50 to 200  $\mu$ l and contains 0.6–2.5 mg of protein.

Although crystals may be grown in either microcells or small tubes, it is advantageous to perform experiments in tubes as soon as approximately correct crystallization conditions are established. The large number of crystals required for X-ray work can then be grown by simultaneously preparing several tubes under conditions identical to those which experimentally gave good results.

*Crystallization conditions.* The conditions giving large well-formed crystals were empirically determined by systematic variation of the properties of the precipitant solution introduced into the gradient cells within the range of pH, ion content, and temperature required for the maintenance of

the conformational homogeneity of the protein. Ammonium sulfate was used as the precipitant since it rendered both the cytochromes and glyceraldehyde 3-phosphate dehydrogenase reversibly insoluble with negligible loss of activity, and could easily be buffered over the range of pH 5–8 in which these proteins were known to be conformationally stable.

A rough estimate of the ammonium sulfate concentration required to precipitate the protein was made by mixing 1  $\mu$ l of concentrated protein solution with 50  $\mu$ l of ammonium sulfate solution of various concentrations in small test tubes. The minimum ammonium sulfate concentration required to precipitate the protein determined the conditions for the initial crystallization experiments. These were carried out by layering equal volumes of protein (3–5%) and ammonium sulfate solutions into microcells or small test tubes as described above. The ammonium sulfate concentration was adjusted so that when the cell reached equilibrium, the total ammonium sulfate concentration in the cell equaled the minimum precipitating concentration found in the solubility tests.

The best results were obtained when a slight turbidity (which subsequently disappeared when the cell reached equilibrium) initially appeared at the protein/precipitant interface. In some cases it was observed that conditions which gave a slight turbidity at the initial interface did not result in crystal formation, while experiments using slightly higher precipitant concentrations gave a flocculent precipitate at the interface.

In this case, conditions were adjusted to reduce the initial precipitant concentration gradient at the interface, while simultaneously maintaining

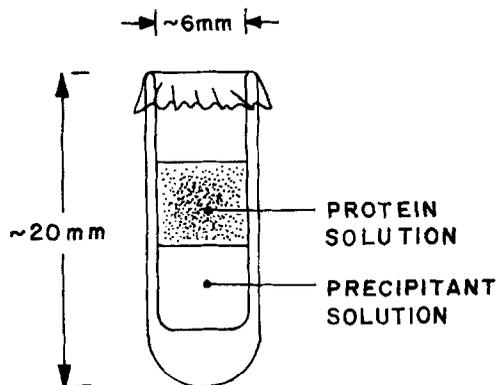


FIG. 3. Free diffusion crystallization in a small test tube. The rate at which the cell reaches equilibrium may be varied by altering the relative dimensions of the tube, in analogy to that described in Fig. 2.

sufficiently high overall precipitant concentration to promote crystal growth after the cell had reached equilibrium. This was done by increasing the volume of the precipitant layer relative to the protein solution volume. The precipitant solution concentration was accordingly reduced so that the final equilibrium precipitant concentration equaled that used in the equal volume experiment which gave excessive precipitation at the interface. This procedure both lessens the initial gradient in ionic strength imposed upon the protein at the interface and reduces the equilibrium protein concentration in the cell.

In addition to experiments in which the precipitant concentration, pH, or ion content was varied, several crystallization experiments were carried out at elevated temperatures. As Jacoby (9) has pointed out, proteins generally become less soluble in concentrated salt solutions as the temperature is increased. Consequently, it was possible to crystallize cytochrome  $c_2$  (*R. rubrum*) at 37°C from less concentrated ammonium sulfate solution than that required for crystallization at room temperature. This proved advantageous for the formation of heavy atom isomorphous derivatives, since the high ammonium sulfate concentration required for crystallization at room temperature inhibited derivative formation (see Ref. 10).

Zepezauer (3) has described a variety of alternative organic and inorganic precipitating agents covering a wide range of pH and dielectric constant. A summary of protein crystallization techniques illustrating the usefulness of several of these precipitants is given in the recent review by Eisenberg (11).

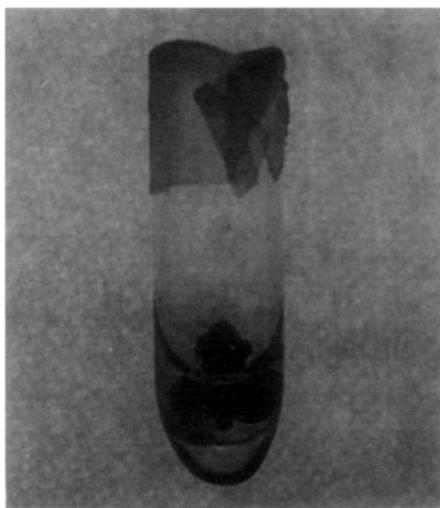


FIG. 4. Cytochrome  $c_2$  (*R. rubrum*) crystals grown at room temperature in a small test tube.

## RESULTS

Cytochrome  $c_2$  (*R. rubrum*,  $MW$  12,800) was crystallized from ammonium sulfate at pH 5.8 as flat rhombic plates. At 23°C, the crystals were grown by layering 50  $\mu$ l of 30 mg/ml protein solution in water, containing approximately 5% residual saturated ammonium sulfate over 100  $\mu$ l 85% saturated unbuffered ammonium sulfate (Fig. 4). At 37°C, crystals were grown by layering 50  $\mu$ l of protein solution over 50  $\mu$ l of 65% saturated unbuffered ammonium sulfate pH 5.8 (Fig. 5a). Both crystal types had similar habits and were isomorphous. Crystals generally required 2 weeks to 1 month to grow and commonly reached  $0.8 \times 0.6 \times 0.25$  mm in size. Crystals grown at room temperature under high salt conditions formed only one useful heavy-atom isomorphous derivative in approximately 200 attempts. The crystals grown at 37°C yielded five potentially useful derivatives in the first few derivatization attempts and made possible the completion of the crystal structure analysis of this protein.

Cytochrome  $c'$  (*Rps. palustris*,  $MW$  approx 15,000) crystallized in two distinct habits under differing conditions. This protein was crystallized at 37°C since its solubility characteristics were similar to those of cytochrome  $c_2$ . Crystals prepared by layering 50  $\mu$ l of 30 mg/ml protein solution over 100  $\mu$ l of unbuffered 65% saturated ammonium sulfate pH 5.8 and stored at 37°C grew as long, flat rectangular prisms having dimensions  $1.0 \times 0.5 \times 0.3$  mm (Fig. 5b). Crystals prepared by layering 50  $\mu$ l of 30 mg/ml protein solution over 50  $\mu$ l of 65% saturated ammonium sulfate 0.1 M in  $Mg(NO_3)_2$  pH 5.2 at 37°C, grew as beveled rectangular prisms (Fig. 5c) having dimensions  $0.6 \times 0.4 \times 0.3$  mm. Both crystal forms required about a month to grow to usable size.

Cytochrome  $c'$  (*R. rubrum*,  $MW$  approx 16,000) also crystallized in two distinct habits under different conditions. Crystals prepared by layering 50  $\mu$ l of 30 mg/ml protein solution over 50  $\mu$ l of 65% saturated unbuffered ammonium sulfate pH 5.8 at 37°C grew as long rods having a diamond-shaped cross section (Fig. 5d) having dimensions  $2.0 \times 0.2 \times 0.2$  mm. Crystals pre-

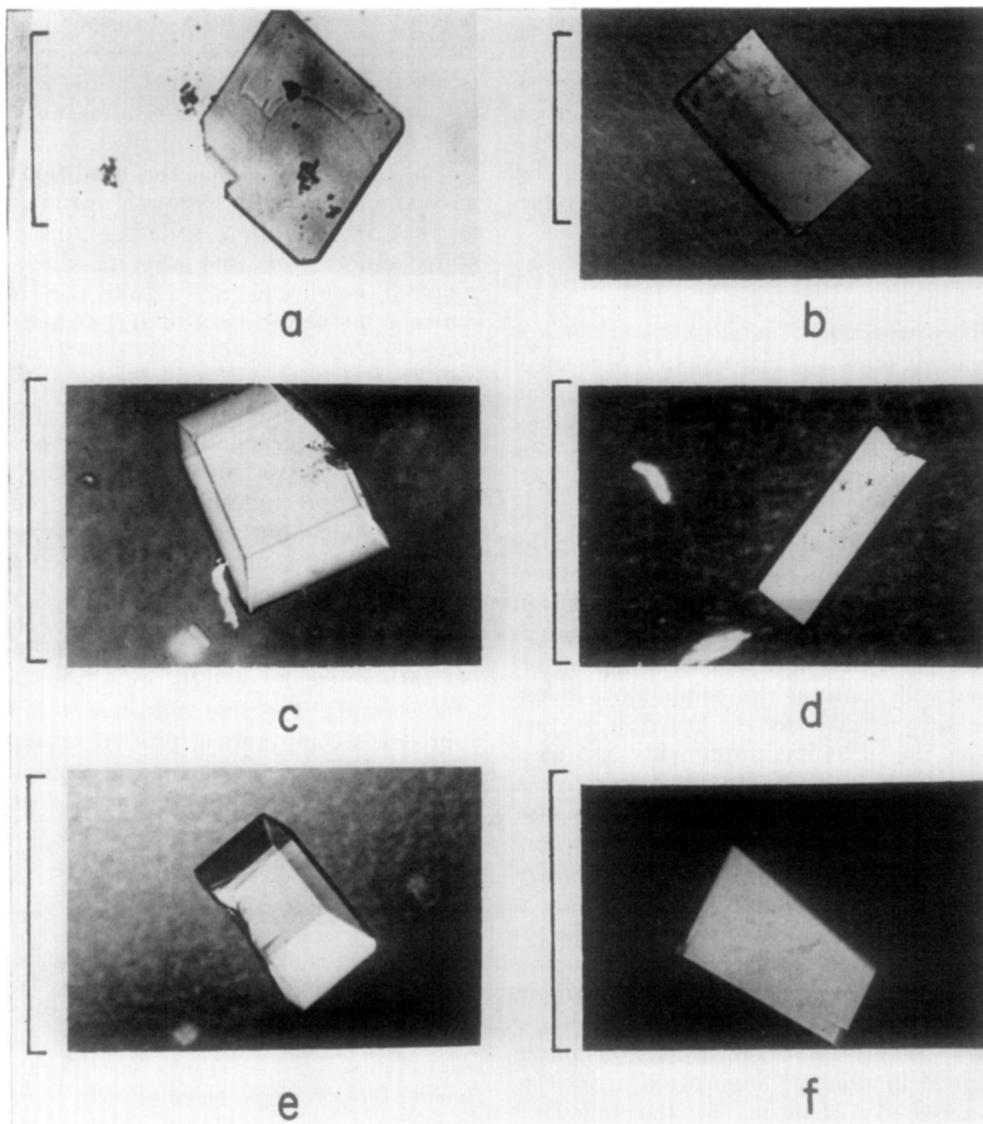


FIG. 5. Protein crystals. The scale is given by the 0.4-mm bar at the left of each picture. (a) Cytochrome  $c_2$  (*R. rubrum*); (b) Cytochrome  $c'$  (*Rps. palustris*); (c) Cytochrome  $c'$  (*Rps. palustris*),  $Mg(NO_3)_2$  form; (d) Cytochrome  $c'$  (*R. rubrum*); (e) Cytochrome  $c'$  (*R. rubrum*),  $Mg(NO_3)_2$  form; (f) Glyceraldehyde 3-phosphate dehydrogenase (*Palinurus*). Details of crystallizing conditions are given in text.

pared by layering 50  $\mu$ l of 30 mg/ml protein solution over 50  $\mu$ l of 65% saturated ammonium sulfate 0.1 M in  $Mg(NO_3)_2$  pH 5.2 at 37°C grew as beveled rectangular prisms (Fig. 5e) about  $0.4 \times 0.3 \times 0.2$  mm in size.

All of the cytochromes discussed above are stable and exist predominantly (>95%) in their oxidized form due to air oxidation. Preliminary attempts to grow large crystals of

the reduced forms of these cytochromes indicate that reduced cytochrome crystals do not form under the same conditions producing oxidized crystals, although in one case (*R. rubrum c2*) it is known that oxidized and reduced crystals are isomorphic.

Glyceraldehyde 3-phosphate dehydrogenase (*Palinurus*) was obtained as a 50 mg/ml solution by dissolving centrifuged

microcrystals in 0.05 M sodium phosphate pH 6.5 containing 0.05 M 2-mercaptoethanol and 0.005 M EDTA. Crystals were grown at 23°C by layering 50  $\mu$ l of stock solution over 100  $\mu$ l of identically buffered ammonium sulfate. The protein crystallized as flat rhombic plates (Fig. 5f). The largest crystals grown were 0.6  $\times$  0.5  $\times$  0.2 mm.

#### DISCUSSION

The production of large single crystals is dependent upon the introduction of a small number of nucleating sites into a solution which is saturated with protein. When growing crystals by batch methods, this condition is effected by slowly adding a precipitant to a concentrated protein solution until a slight turbidity appears, signaling the formation of microcrystalline or amorphously precipitated material. The solution is generally well-supersaturated with protein at this point and crystallization or precipitation will continue to completion unless more solvent is added to the dish to just remove the turbidity, presumably leaving a few nuclei to serve as growth points for a few large crystals. The same effect may also be obtained by putting a dish showing turbidity into the cold, which usually renders the protein more soluble, or by seeding a saturated protein solution with a few microscopic seeds obtained from crushing a previously grown crystal of the same protein (11).

The free interface diffusion technique described in this communication arrives at the required conditions in the following way: When the concentrated protein and precipitant layers begin to diffuse together at the interface, a transient condition of protein supersaturation sufficient to induce spontaneous nucleation of the protein is attained. As diffusion continues and the band of interpenetration of protein and precipitant widens, the relative concentrations decrease until finally at equilibrium the total solution volume is saturated with protein but unable of itself to induce spontaneous nucleation. A few of the nuclei formed at the initial interface may survive, however, and serve as growth sites for crystals. These nuclei usually fall to the

bottom or collide with the walls of the tube, although occasionally crystals are observed either to form in a ring on the tube walls at the location of the original interface or as an avalanche down one side of the tube.

The manner in which the conditions for nucleation and crystal growth are attained in the free diffusion technique discussed above differs from the way in which the required conditions are effected in other diffusion methods described in the literature. Both the Zeppezauer (1, 3, see also 2) micro-diffusion dialysis technique and the vapor diffusion technique described by Davies and Segal (4) gradually approach protein saturation by continuously increasing precipitant or precipitant/protein concentration. Crystallization is probably induced by the presence of some foreign particle acting as a heterogeneous nucleus for crystal growth, since sufficient protein supersaturation to induce spontaneous homogeneous nucleation usually results in a shower of microcrystals. The free diffusion technique approaches conditions of protein saturation from a transient condition of supersaturation sufficient to induce spontaneous nucleation. At equilibrium, a few of these nuclei remain to serve as growth points for crystals.

#### ACKNOWLEDGMENTS

The author is indebted to Prof. Joseph Kraut for his encouragement and for the use of the facilities required for this work, and to Prof. W. S. Allison for his assistance in the preparation of the *Palinurus* enzyme. Thanks especially to Dr. Robert Bartsch who unhesitatingly supplied quantities of highly purified bacterial cytochromes. This work was supported by Research Grants from the National Institutes of Health (GM 10928, GM 16717) and the National Science Foundation (GB 15684, GB 23054).

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