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Investigations on Protein Crystal Growth by the Gel Acupuncture Method

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Abstract

In this work we explore the possibilities of the gel-acupuncture technique, proposed previously for the growth of protein single crystals [García-Ruiz, Moreno, Viedma & Coll (1993). *Mater. Res. Bull.* **28**, 541–546]. The main advantage of the technique is that the crystals are obtained inside an X-ray capillary and, unlike classical microdiffusion techniques, it involves a very simple and accurate technical arrangement that permits the continuous monitoring of the crystals in their growth environment. In particular, we describe the growth of single crystals of lysozyme, concanavalin A and ribonuclease A. Different starting conditions have been used to grow single crystals of these proteins into different types of capillaries at several protein and precipitating-agent concentrations. It is demonstrated that the technique works for a wide range of precipitating agents commonly used in protein crystal growth, such as large polymers (PEG 4000 and PEG 6000), organic solvents (from methanol to butanol) and salts [NaCl, $(NH_4)_2SO_4$]. The range of inner diameter of the capillaries for which the technique works correctly has been also studied. The growth process and possible crystal movement was followed by video microscopy. Lysozyme crystals up to 3.1 mm were obtained but the average maximum linear crystal sizes were 2.0 mm for lysozyme, 0.4 mm for concanavalin A and 1.2 mm for ribonuclease, respectively. The waiting times to reach such a size, measured from the set-up of the experiments, were 72 h, 10 d and 5 d, respectively. Gels of tetramethoxysilane, tetraethoxysilane, sodium silicate, agar, high-strength agar and gel-gro have been tested in relation to their mechanical properties and their chemical interaction with the reactants. Finally, we discuss briefly the advantages of the gel-acupuncture technique and plausible applications other than crystal growth.

Introduction

The main experimental constraint on success in the crystallization of proteins and nucleic acids is the small amount of sample usually available (for a general picture of the problem see McPherson, 1982; Ducruix & Giegé, 1991). Therefore, all the crystallization techniques currently used have been developed to reduce (*a*) the required volume of protein solutions; (*b*) the risk of undesired perturbations of the experimental set-up; and (*c*) the manipulation of the crystals grown in order to minimize the loss of their physical properties when transferring them to the X-ray camera.

In current crystal-growth techniques, such as hanging (or sitting) drop as well as microdialysis, it is possible to reduce the amount of sample per experiment to less than 5 μl (Luft & DeTitta, 1992). The crystals grown by these techniques have to be transferred to X-ray capillaries, which requires some degree of dexterity to avoid breaking, dissolution or loss of structural order of the crystals, and the experimental set-up is not always free from undesired problems such as drop flattening or spreading and loss of sample during inversion of the cover slides due to mechanical instability in the experimental arrangement. Nevertheless, as discussed below, the main criticism of vapour-diffusion and button-microdialysis techniques lies in the way in which supersaturation is obtained.

We have previously presented a simple technique for obtaining protein single crystals in capillary volumes in the shape of a fibre (García-Ruiz, Moreno, Viedma & Coll, 1993). Crystallization by equilibration using capillaries is a technique previously used for protein crystal growth. Weber & Goodkin (1970) described the growth of some proteins in heavy-walled capillary tubes with 1.0 mm inner diameter and 6.5 mm outer diameter, using standard cellophane as a dialysis membrane. The method was also used by Pronk *et al.* (1985) but in this case thinner capillaries were used. Zeppezauer (1971) described the use of microdialysis cells consisting of capillaries closed by gel diaphragms made of polyacrylamide, which are later dipped into a solution of the precipitating agent. Our technique avoids the problems created by the shrinking of the gel and difficulties encountered in the manipulation of the apparatus. Another method of equilibration by diffusion in capillary glasses has been described by Salemne (1972) by putting the protein and precipitating agent solutions directly in contact. The same

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set-up was also used by Yonath, Müssig & Witlmann (1982) for the growth of ribosomal subunits. Unlike previous crystal growth techniques using capillaries, the main advantage of the new technique lies in its simple, versatile and accurate technical arrangement that permits continuous testing of the crystallization process including the performance of X-ray studies in the growth environment.

In this paper we report a study of two factors affecting the growth process in our technique: the types of gel and the size and shape of the capillaries. The study was also designed to test, with our method, the three groups of precipitating agent most frequently utilized in vapour-diffusion and micro-dialysis techniques. All these tests were carried out using three proteins with well known crystallization conditions.

Experimental

A brief description of the technique, schematically shown in Fig. 1, is as follows: a crystallization vessel with a diameter of 50 mm and 30 mm high is filled with a sol of silica (pH 6) up to a level of 15 mm in height. Then, capillary fibres are gently filled with a solution of protein, using capillary forces, and one of their ends is then sealed with wax or clay. After gelation of the sol, the gel layer was perforated with the open ends of the capillary fibres keeping them in a vertical position. The typical penetration length is 7 mm. After placing a number of capillary fibres with this technique, the gel is a overlayered with an aqueous solution of the precipitating agent. Finally, the growth vessel containing the capillary tubes is kept in a closed environment at constant temperature

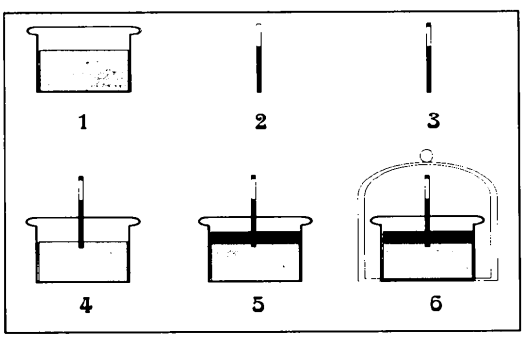


Fig. 1. The steps in a typical experimental set-up of the gel acupuncture technique: (1) Pour in a reservoir a volume V_0 of a sol prepared previously. (2)–(3) Fill the capillary fibres with the protein solution and seal the top of the capillaries with wax or clay. (4) After gelation of the sol, the open ends of the capillary fibres are punctuated in the gel layer, keeping them in a vertical position. The typical penetration length is 7–8 mm. (5) After placing the capillary fibres, pour a volume V_p of the precipitating agent. (6) In order to reduce evaporation, the growth vessel containing the capillary tubes is kept at constant temperature in a closed environment.

to reduce evaporation of the precipitating agent. The volumes of the gel and precipitant agent are variables that can be selected by the operator.

Three proteins were crystallized: hen egg-white lysozyme (Fluka), concanavalin A (Sigma) and ribonuclease A (Sigma). The proteins were used as purchased without further purification and we did not use buffers. The chemicals for gel preparation were sodium silicate (SS, Panreac), tetramethoxysilane (TMOS) and tetraethoxysilane (TEOS) (Fluka), agar, gel-gro (ICN), high-strength agar (Sigma), and acetic acid (Quimon). As precipitating agents we used sodium chloride and ammonium sulfate (Panreac), methanol, ethanol and *n*-butanol (Riedel-deHaën), *n*-propanol (Fluka), *t*-butanol and 1,3-propanediol (Sigma), and polyethylene glycol (PEG) 4000 and 6000. All the non-protein materials were reagent grade or better. De-ionized and bi-distilled water was used throughout this study and all the experiments were conducted under controlled temperature [295 (1) K].

Five types of gel were tried. Silica gel was prepared starting with a sodium silicate solution with specific gravity 1.06 g cm ^{3} and pH of 11.6. This solution was acidified to pH 6 or 6.5 by adding CH_3COOH (1*N*). This silica sol was then poured into a crystallization vessel until a layer of 15 mm in height was obtained. The gelling time was 20 min. Agar gels, agar gel of high strength and gel-gro were prepared at 1%(*w*/*v*) under continuous stirring at 363 K. Their pH values (measured at 323 K) were 5.0, 7.4 and 6.1, respectively. Gels of tetramethoxysilane at 5 and 10%(*v*/*v*) were used. They were prepared by mixing under continuous stirring water and TMOS at the appropriate proportions. For TMOS 5%(*v*/*v*), the stirring time was 60 min, the gelling time 24 h and the gel pH 4.5. For TMOS 10%(*v*/*v*), the stirring time was 120 min, the gelling time 10 h and the pH 4.3. We have also used TMOS gels made with propanol–water mixtures with a ratio 4:1(*v*/*v*), and stirring time of 60 min. In this case the working temperature was 313 K in order to reduce the gelling time to 48 h.

Experimental results

Types of gel and shape and size of the capillaries

Different types of capillaries were used to test how their geometry and size affect the experimental results. None of the capillaries were siliconized. We have used: (*a*) X-ray capillaries with a height of 60 mm and an inner diameter of 0.5 mm; (*b*) melting-point capillaries 65 mm tall with an inner diameter of 1.2 mm; (*c*) capillaries with a rectangular section of 4 × 0.3 mm and 50 mm tall. To test the mechanical properties of the gels and the mechanism of transport of the precipitating agent through them and

Table 1. *Heights attained by the precipitating agent (PA) in sealed (S) and unsealed (U) capillaries*

Gel	PA	Capillary	S/U	Height (mm)
TMOS (5%)	$(NH_4)_2SO_4$	mp	S	19
TMOS (5%)	$(NH_4)_2SO_4$	mp	U	28
TMOS (5%)	$(NH_4)_2SO_4$	Plate	S	20
SS pH 6	PEG 6000	mp	S	0
SS pH 6.5	PEG 6000	mp	U	25
SS pH 6.5	PEG 6000	Plate	S	16
Agar	NaCl	mp	S	1
Agar	PEG 6000	mp	S	1
Agar	$(NH_4)_2SO_4$	mp	S	13
SS pH 6*	NaCl	mp	S	0
SS pH 6*	NaCl	mp	U	33
SS pH 6*	NaCl	Plate	S	0
SS pH 6*	NaCl	X-ray	S	30
TMOS-pro	NaCl	mp	S	3
TMOS-pro	NaCl	mp	U	13
TMOS-pro	NaCl	Plate	S	1

* In these cases the column of precipitating solution was 10 mm tall.

through the capillaries, unfilled capillaries were punctuated into the gels and the precipitating agent was later poured on the gel layer to form a column 10 mm in height. The height attained by the precipitating solution inside the capillaries was then measured after 48 h and the results are shown in Table 1. Note that we differentiate between sealed and unsealed capillaries.

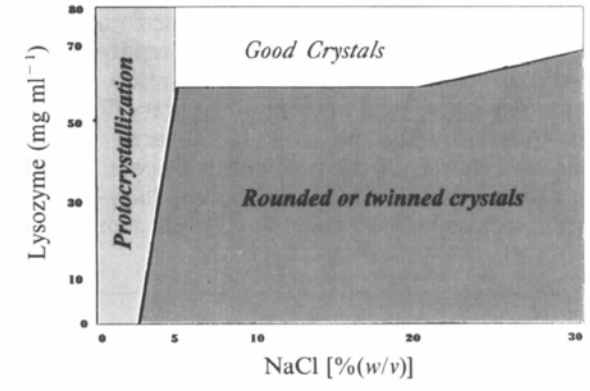
The best results (*i.e.* those reducing the rise of the solution through the capillaries) were obtained for sodium silicate and agar gels. We have observed that the density and volume of the cracks provoked by the punctuation seem to be relevant variables controlling the mechanism of transport of the precipitating agent. When the volume of free solution provoked by the cracks is large, capillary forces rising up the precipitating solution have to be considered. Otherwise, there is a perfect-contact gel capillary that constrains mass transport to diffusional phenomena. The properties of the silica gel made from sodium silicate are the most appropriate for supporting the capillaries mechanically. In addition they permit the use of a wide range of pH. Unlike sodium silicate gels, gel-gro gels were unable to maintain the capillaries raised while high-strength agar gels were unable to be used because the capillaries were rejected when we tried to perform the punctuation. The results of both gels are, therefore, not shown in Table 1. Agar gels were also found to be appropriate for this technique.

Type of precipitating agent, protein and precipitating-agent concentration

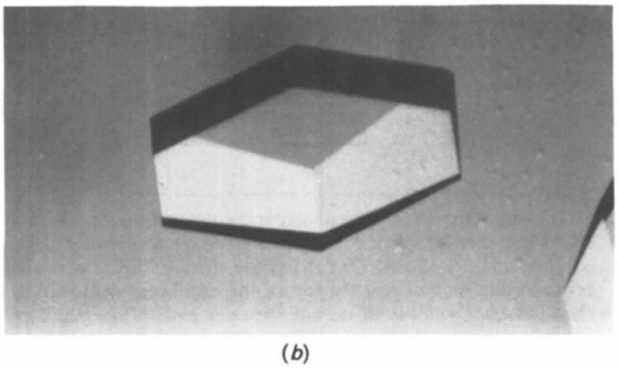
We have tested the use of different types of precipitating agents currently used in the literature. In particular, we have studied the efficiency of several salts [NaCl, KCl, CsCl and $(NH_4)_2SO_4$], large polymers (PEG 4000 and PEG 6000) and several organic solvents (methanol, ethanol, *n*-propanol, *n*-butanol, *t*-butanol and 1,3-propanediol).

For the case of lysozyme the effect of different ionic salts has been observed to fulfil the lyotropic Hofmeister's series, *i.e.* for the same anion (Cl^-) and the same salt concentration, better results were obtained with the salt of lower cation radius. Excellent crystals more than 2 mm in size and of good optical quality were obtained using a starting protein concentration from 50 to 200 mg ml^{-1} and starting concentration of NaCl from 10 to 20%(*w*/*v*). Lysozyme crystals display tetragonal shape and when the crystals were grown without external shape constraints (note that our crystals grow sometimes up to a size larger than the typical capillary diameter) the pyramidal {101} faces were more developed than the prismatic {110} faces. Fig. 2(*a*) shows a schematic plot of the crystallization conditions for lysozyme crystals.

Large polymers such as PEG 4000 and 6000 were demonstrated to diffuse up to the capillaries and work correctly as a precipitating agent for the case of concanavalin A. As noted by Mikol & Geigé (1989) rounded crystals as well as faceted crystals were obtained (Figs. 3*b*,*c*). Well faceted crystals were



(*a*)

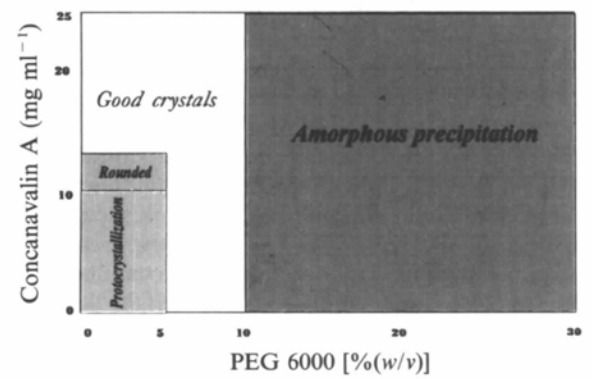


(*b*)

Fig. 2. (*a*) Crystallization conditions for lysozyme single crystals into melting-point capillaries using silica gel at pH 6 and a temperature of 296 K. (*b*) A crystal of lysozyme grown in a melting-point capillary.

obtained with PEG 6000 at 5%(w/v) and a protein concentration of 10 mg ml^{-1}. The quality and size of the crystals (up to a longest linear size of 0.4 mm) were found to be better than those obtained when using $(NH_4)_2SO_4$ as precipitating agent. Fig. 3(*a*) shows a schematic plot of the crystallization conditions for concanavalin A.

To test the use of organic solvents in the gel-acupuncture technique we used the information reported in the literature on the crystallization of ribonuclease A. Thus, methanol, ethanol, *n*-propanol, *n*-butanol, *t*-butanol and 1,3-propanediol were utilized as precipitating agents. The best results were obtained for a protein concentration in the range 10–20 mg ml^{-1} and using *n*-propanol as precipitant. These experiments were carried out with TMOS gels made at 10%(v/v) and using propanol–water mixtures with a ratio of 4:1(v/v) at pH 7. Our larger crystals of ribonuclease A do not improve the previous results obtained by Wlodawer, Bott & Sjölin (1982) by liquid–liquid diffusion, using *tert*-butyl alcohol at 40% and a protein concentration of 50 mg ml^{-1} at pH 5.3. Nevertheless, it has to be noted that the crystals were obtained by these authors at a different pH value and after a waiting time of 60 d, a value not attained in any of our experiments.



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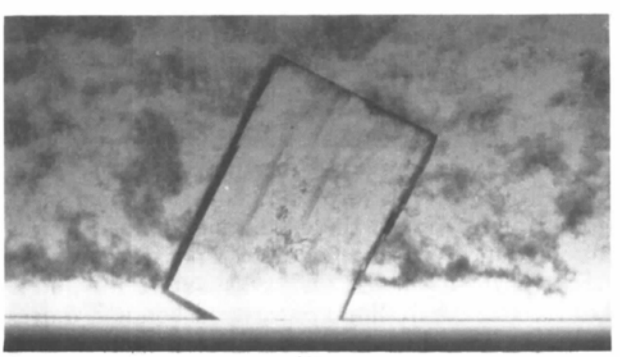

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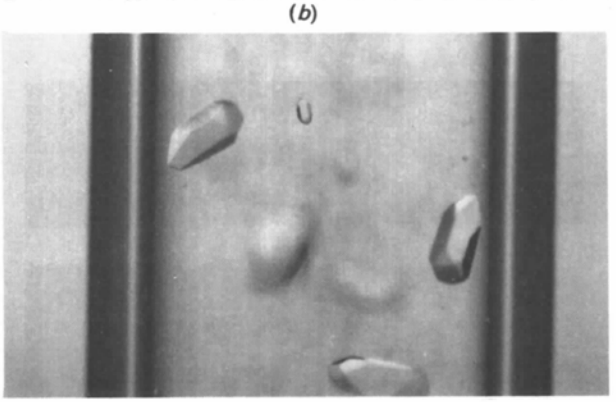

(*c*)

Fig. 3. (*a*) Crystallization conditions for concanavalin A single crystals in X-ray capillaries using 10%(v/v) TMOS gel at 296 K. (*b*), (*c*) Two different crystals of concanavalin A.

In summary, we have checked that all the precipitating agents currently used in the vapour-diffusion technique for protein crystal growth can be also used in the gel-acupuncture technique. It means that the knowledge already acquired in previous techniques can be used in further development of the technique described here. For comparative purposes, note that if the volume of the capillary (~50 μl) is neglected in relation to the volume of the gel V_g and that of the precipitating agent $V_p = V_g = 25$ ml, the final concentration of the precipitating agent in the capillary after equilibration can be considered to be half of the starting concentration.

Growth rates, crystal quality and maximum crystal size

As reported above, the size of the crystals varies with the starting precipitating agent and protein concentrations, and with the size and shape of the capillary column of protein solution. The nucleation density is generally very high in the lower part of the capillaries and decreases in the upper part (*i.e.* it is a function of the diffusion path) and the growth rate of the crystal is faster for crystals located in the lower part of the capillary. For the case of lysozyme we have measured the growth rate by using time-lapse recording with a television camera attached to a transmission optical microscope and video recorder. In Fig. 4 we have plotted the data from two crystals located at different heights in the capillary. Note that for the crystal growing in the upper part of the capillary the growth rate was 2.9×10^{-3} mm h^{-1}, while for that located in the lower part it was 2.5×10^{-2} mm h^{-1}. As expected and also shown in the figure, the waiting time for nucleation was higher for the later case. For this protein, the maximum crystal size measured in this work was 3.1 mm. The maximum average linear crystal sizes were 2.0 mm for lysozyme, 0.4 mm for concanavalin A and 1.2 mm for ribonuclease A. The waiting times to reach such a size measured from the set-up of the initial conditions were 72 h, 10 and 5 d, respectively.

For all the experimental runs in which single crystals were obtained, the optical quality of the crystals (as seen from their extinction behaviour under crossed polarizers) was observed to be similar to that obtained by other classical techniques (see Figs. 2–3). In some cases the crystals were attached to the capillary walls and their shape became rounded as they adapted to the capillary geometry, which could be expected to constrain the final size of the crystal. However, the crystals grow to fill the capillary and form a crystalline rod. Even in these cases the single-crystal quality is preserved as shown by the optical behaviour under crossed Nicols (Fig. 5). It is worth noting that, as previously reported (García-Ruiz, Moreno, Viedma & Coll, 1993), the *in situ* X-ray diffraction patterns of two lysozyme crystals studied with copper radiation show the existence of sharp diffraction spots up to 1.8 Å. A systematic study of crystal quality using synchrotron radiation is currently being designed.

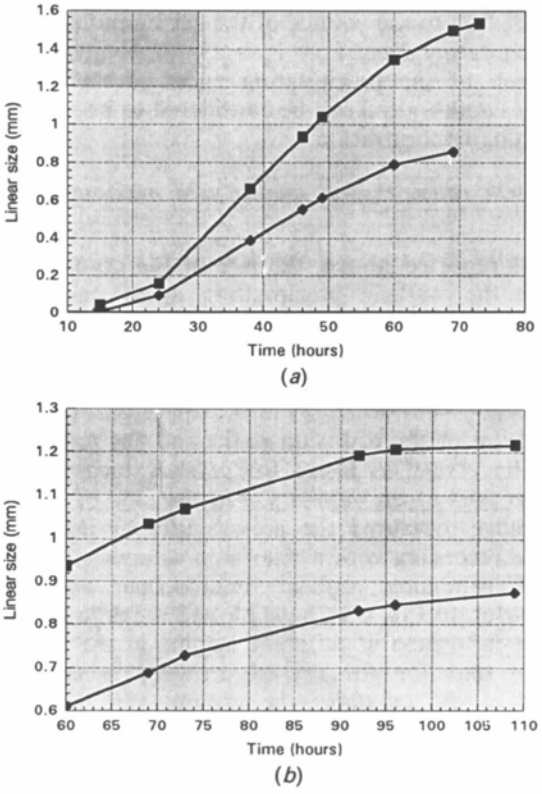

Fig. 4. (*a*) Growth rate of a lysozyme crystal located in the lower part of the capillary. The origin of the time axis is the set-up of the experiment. Experimental data: protein concentration 100 mg ml^{-1}; concentration of the precipitating agent (NaCl) 20%(*w*/*v*); silica gel at pH 6.0; capillary type: plane. The curves plotted belong to the largest and smaller linear sizes of the crystal shown in Fig. 2. (*b*) Growth rate of a lysozyme crystal located in the upper part of the capillary. Experimental data as for (*a*).

Discussion

The above results demonstrate that the three main groups of precipitating agent commonly used in the vapour-diffusion and microdialysis techniques work correctly with the gel-acupuncture technique. It is also clear that mass transport through the gel can be reduced to be diffusional provided that the mechanical properties of the gel preclude the formation of cracks during punctuation of the capillaries. Therefore, the technique permits the formation, in the lower part of the capillary, of a sharp interphase between the precipitating agent and the protein solution, providing the starting gradient for diffusional transport of the precipitating agent toward the upper part of the capillary. This point is important because, unlike the vapour-diffusion and microdialysis techniques, the gel-acupuncture method was designed to take advantage of (*a*) the possibility of decreasing convective flux by confining the mother solution into growth cells with small characteristic dimensions and (*b*) a geometry allowing a long diffusional path (the length of the capillary) and a very narrow area of the diffusion front (the cross-section of the capillary). In fact, the technique emulates the crystallization of new compounds under diffusional control in open systems approaching equilibrium (García-Ruiz, 1991). In this type of system not only supersaturation, defined as the ratio between the actual protein concentration and the equilibrium concentration,

$$[P]/[P_e] = \sigma,$$

but also the rate at which supersaturation increases $\Theta = \delta\sigma/\delta t$, are important crystallization variables. The variable Θ measures the change in the flux of growth units toward the crystal surface or towards any precritical cluster created by stochastic distribution of growth units in the metastable region and is the variable controlling how fast the solution moves in a solubility plot. Assuming a negative correlation of the protein solubility with the concentration of

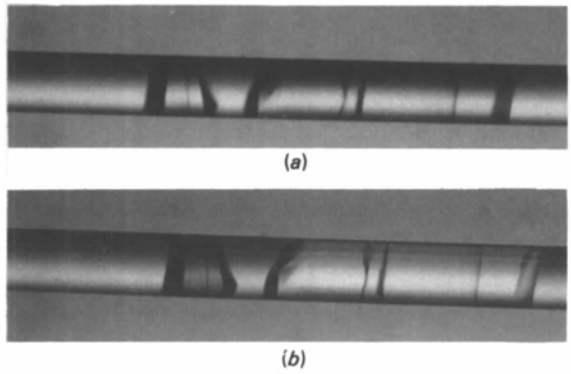

Fig. 5. Lysozyme single-crystal rod: (*a*) parallel and (*b*) crossed Nicols.

precipitating agent and by considering exclusively mass transport processes, it is clear that, for a given time value (t), σ decreases as we approach the top of the capillary, and that, for a given height (h) in the capillary, σ increases as a function of time. It means that for a single experiment, all possible values of σ are tested in a continuous way, the range of σ values tested being a function of the initial protein and precipitant concentration. It is also evident that the rate of equilibration Θ changes as a function of h and t, being slower as h and t increase. Now, if we consider that for a given couple of (h,t) values, conditions for precipitation occur, the concentration of protein will be locally depleted triggering a local concentration gradient that will have to be equilibrated by diffusional flux of protein molecules. In consequence, it is possible for the same experiment to move the system from a large σ and fast Θ leading to amorphous precipitation that provokes a strong depletion of the nutrient, toward σ values in the metastable region at slower Θ values. This interplay of mass-diffusion transport and precipitation phenomena permits the capillary to work as a growth cell with continuous variation of precipitation conditions. Thus, by varying the initial concentration of protein and precipitating agent, a finite but wide and continuous range of crystallization conditions (σ, Θ) can be screened.

The fact that diffusional transport dominates convection, or more exactly, that there is a concentration gradient along the capillary, can be deduced by three of our experimental results: (1) The nucleation density decreases along the capillary. In most of the cases, a polycrystalline precipitate of protein is formed in the lower part of the capillary, *i.e.* close to the end plugged into the gel. However, the diffusion of the precipitation agent continues throughout this protein plug which self-regulates the rate of equilibration. This is possible because of the open structure of the protein which lets the solution travel throughout the large pores of its structure. (2) The higher the height in the capillary the larger is the size of the crystals. (3) The growth rate of the crystals varies with their position in the capillary, being slower for crystals growing at a higher location. Nevertheless, these results should not be considered yet as a proof of pure diffusional control of mass transport in all the capillaries since the set of data shown in Fig. 4 does not fit clearly a linear relation between the advance of the face and the square root of time, and since homogeneous nucleation density and uniform crystal size distribution have been observed in some melting-point capillaries. Therefore, we plan to test capillaries in the range 0.5–1.2 mm diameter and the use of gelled solutions.

It is crucial to our technique that the growth of crystals takes place in the same location where they nucleated. This condition, which is usually fulfilled in gelled systems (Henisch, 1988), is a requirement to preserve the play of equilibration rates of the concentration profiles created in our diffusing-reacting capillary system. We have observed that, in many cases, the crystals adhere to the capillary walls and, therefore, there is no sedimentation. In other cases, the settling of the crystals has to be considered. For particle radii less than a few micrometers, the Reynolds numbers being less than unity, the classical Stokes law can be applied. Owing to the similarity in density between the protein crystals and their own solution [as small as $0.233\ g\ cm^{-3}$ for the case of lysozyme according to Privalov *et al.* (1989)] and because of the viscosity of concentrated protein solutions [several centipoises according to Azuma, Tsukamoto & Sunagawa (1989)], the terminal rate of settling of the nuclei can be estimated to be very low. In some cases we have observed that concanavalin A crystals 0.4 mm in size and fully embedded in the mother solution remain in the same position without significant sedimentation for a period of days. This behaviour has to be explained by the increment of the viscosity of the solution and because in our sealed thin-growth cells, the effect of capillary forces working against gravity has to be considered.

In the gel acupuncture method, the control of the growth process is a very simple procedure. The capillary containing the crystals to be tested is removed and after observation by optical microscopy or testing by X-ray diffraction, they can be re-punctuated into the gel layer and the crystal will continue to grow again. Also the arrangement shown in Fig. 1 permits the continuous monitoring of crystal growth using a binocular lens. Note that to avoid breaking the symmetry of the diffusion path, the repunctuation of the capillaries should not provoke the formation of cracks and, therefore, gels of higher viscosity are worth trying. Obviously, during periodic observation of the crystals, care has to be taken not to shake the capillaries.

Finally, although the gel acupuncture technique was designed to carry out crystallization, we are now exploring the use of the technique for other purposes. The same capillary used as a growth cell can be used later for soaking crystals with ligands prior to X-ray studies because, as demonstrated by Lebiola & Zhang (1992), equilibration of the ligand is correctly attained by diffusion through the mother liquid into a capillary volume. It also seems clear that this technique can be used successfully for the screening of crystallization conditions and for *in situ* diffusion of inhibitors, heavy atoms and substrates.

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