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# Laboratory handling of crystals

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## Introduction

The three main phases of matter are solid, liquid, and gas. Most solids are crystalline. Crystals are characterised by their ordered arrangement of molecules; each unit takes up the most stable position possible in relation to its neighbours, resulting in repetition of the three-dimensional structure throughout the substance—that is, internal symmetry. Internal symmetry results in predictable effects on transmitted electromagnetic rays, which aids identification of crystals and may give the particles colour and sparkle. The close packing of the molecules confers hardness and stability. Crystals are often thought of as solid, inert, ageless particles, impervious to physical force.

Crystals found in joint tissue form 'in vitro' via a phase change—from liquid to solid. Their formation and dissolution can often be affected by minor changes in physiological conditions because the solute concentrations are in the metastable region—that is, above saturation point, but below that at which crystal formation is inevitable. Biological crystals are usually small (less than 20 $\mu$m long), and therefore have a high surface area to weight ratio. This means that dissolution can occur rapidly. Their large surfaces are often charged or exhibit atomic roughness and 'dangling bonds''—that is, component atoms stick out from the surface. These surface properties may result in attachment of various other substances found in joint tissue or in the laboratory. Crystals related to joint disease are therefore far from being inert and stable. They can form and dissolve rapidly (dissolution is generally easier and faster than crystallisation) and attract other things to their surfaces. They are also susceptible to many physical forces applied during laboratory procedures.

Precautions have to be taken in the handling of specimens from joint and of those made in the laboratory if artefacts and false results are to be avoided.

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## Crystals in specimens of joint tissue or fluid

Examination of synovial fluids and tissue samples for crystals is now a routine diagnostic procedure. The report—crystals 'present' or 'absent'—is usually regarded as definitive. However, both false positive and false negative results may occur for a variety of reasons (Table 1).

### SYNOVIAL FLUID

Aspiration of synovial fluid involves passing a needle through skin, subcutaneous tissue, joint capsule, and synovium; it may strike the cartilage. Crystals could be dislodged from any of these sites during the procedure and seen in the fluid when they were not there before aspiration.

Table 1 *Some causes of false positive and false negative results when synovial fluids are examined for the presence of crystals*

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|---|
| False positive: |
| (1) Joint puncture introduces crystals into fluid from previously stable tissue deposit |
| (2) Crystals form in the fluid after aspiration due to changes in temperature or pH |
| (3) Non-crystalline material or contaminants mistaken for joint crystals |
| False negative: |
| (1) Sampling error, or crystals not seen due to poor technique |
| (2) Dissolution of crystals after aspiration |
| (3) Crystals too small to be seen by light microscopy |

Once the fluid is out of the joint it changes; this may result in formation or dissolution of crystals. The two most important factors are probably the pH and temperature of the fluid.

*Temperature* changes are particularly important in urate crystallisation. Reardon and Scott have reported that raising synovial fluid temperatures from 32°C to 37°C reduces crystal concentration—a finding that may have relevance to the gout attack in vivo, as well as to crystal identification in vitro.[1] Other authors have reported an increased yield of 'positive results' if synovial fluids are cooled.[2] This is hardly surprising in view of the rapid decrease in solubility as temperature falls. Recent reports of urate crystals in asymptomatic joints[3] should be viewed critically, and results may not be valid if fluids have been stored in the cold before examination.

Changes in pH are produced by loss of $CO_2$ after fluid aspiration. This may be reduced by covering samples with oil to avoid contact with the air. The increase in pH could favour crystallisation of various calcium phosphates, and we have observed in vitro formation of brushite crystals in fluids left to stand on the bench for 48 hours. Crystals which form after fluid aspiration may take on a star-shaped configuration (Fig. 1). Changes in pH also affect urate solubility; correction of gouty synovial fluid pH to 7·4 has been reported to result in increased crystal formation.[4]

Alterations in the crystal load after fluid aspiration may help explain the lack of correlation between crystal numbers and inflammation, and the occasional false positive or false negative result. Care must be taken in handling fluids, and early examination

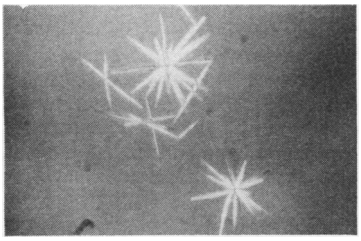

Fig. 1. *Crystals formed in synovial fluid left for 48 hours on laboratory bench. Initial examination gave negative results. Note star-shaped clusters of crystals which formed in vitro. They contained calcium and phosphate and were probably brushite. Polarised light microscopy × 100 (original magnification).*

with minimisation of pH and temperature changes is recommended.

CELLS AND TISSUE SAMPLES

Crystal phagocytosis is regarded as a central factor in the inflammatory response to particles (P Platt and W C Dick, p. 4). Uptake of crystals is often reported on specimens examined by light microscopy for diagnostic purposes, and has been afforded some significance. In our experience it is almost impossible to differentiate between cells with crystals attached to their surface and those with crystals inside them. Examination by scanning electron microscopy (SEM) and thick section scanning transmission electron microscopy (STEM) suggests that attachment without internalisation is common.

Tissue samples (usually synovial biopsy specimens) need careful handling to preserve crystal deposits. Fixatives and stains should be buffered to avoid crystal dissolution due to pH changes. Tissue sectioning may result in crystals dropping out of the sample; this is particularly common in thin sectioning for electron microscopy. Many of the published data include photographs showing holes where crystals might once have been (Fig. 2). Thick sectioning and high voltage EM can reduce this difficulty.[5]

The increasing use of the electron microscope to study crystal deposition diseases has highlighted two other problems: (*a*) sampling errors are a major problem, as only a tiny piece of tissue can be examined, and crystal deposits are often patchy; (*b*) the variability in the morphology and types of crystal sometimes found in a single specimen presents difficulties in identification. Heterogeneity in crystal deposits makes confident statements about the species and size of crystal found in a sample seem naive.

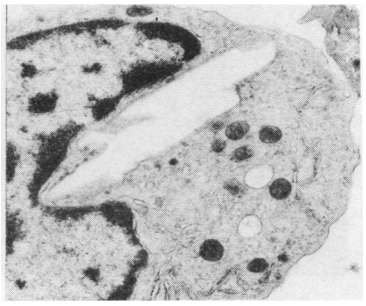

Fig. 2 *Transmission electron micrograph of phagocyte with a cleft in cell. This is typical of published pictures of crystal-cell interactions. A crystal may have occupied the cleft and fallen out during thin sectioning of the sample. It is impossible to say what principle (if any) was there. Thick sectioning techniques sometimes overcome this problem. Transmission electron microscopy × 1500 (original magnification).*

**Crystals used for experimental work**

It is difficult to extract large quantities of a pure crystalline preparation from biological samples. Processes required to remove organic material may ruin the crystals, and yields are generally small. Most experimental work therefore uses crystals manufactured in vitro.

In most laboratories (including ours) a variety of techniques have been applied to the preparations in order to obtain a batch of sterile particles of the right size and in a form that is easy to use. Many of these techniques are now known to alter the crystals, thereby invalidating many of the published data on the biological effects of crystals. Crystals age and are susceptible to heating and grinding; they also 'pick up' extraneous matter from the environment, changing their active surfaces. Furthermore, no two batches are ever quite the same.

HEAT

Van Armen suggested that crystal-induced inflammation might be mediated by attachment of bacterial pyrogens to the surface.[6] Most workers have therefore heated crystal preparations to remove pyrogen (usually to about 180°C for three hours). Most of the crystals in question are hydrated, and there is extensive evidence to show that the water of crystallisation is lost, and that the crystal lattice and surface charge are both altered extensively by heating.[7 8] It has recently been shown that bacterial lipopolysaccharide coating needs to be very extensive to alter crystal reactivity. If care is taken to avoid contamination, heating is probably unnecessary.[9]

GRINDING

Grinding crystals to reduce their size also alters their inflammatory potential. This is probably related to the change in surface charge which also occurs on grinding (Table 2). The mechanism for this is unclear.

AGING OF CRYSTAL PREPARATIONS

We have regularly observed a change with time in the biological activity of a batch of crystals. Preparations of monosodium urate monohydrate usually lose their phlogistic activity slowly over a period of months. Brushite appears to be particularly susceptible to aging, and some of its cellular effects increase strikingly with age (Fig. 3). Crystal lattices and surfaces may change slowly, phase transitions may occur—that is, change from one type of crystal to another—and the crystal surfaces may pick up contaminants in the laboratory. This aging phenomenon deserves more investigation, and means that great care must be used to obtain fresh 'age matched' samples for any comparative work.

BATCH VARIATION

Comparisons cannot be made if two different crystal preparations are used, because the activity of different batches of the same crystal is never identical. Thus preparations of urate crystals made in the same way on different days may look the same and have the same characteristics on analytical screening, but may affect

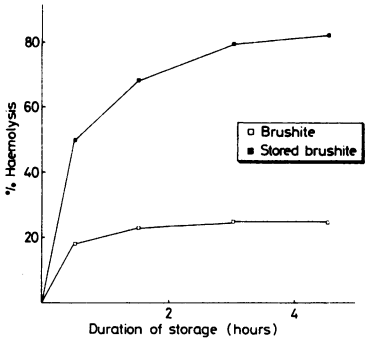


Fig. 3 *Effect of storing brushite crystals on their ability to lyse red cell membranes. Fresh brushite caused 20% haemolysis after four hours' incubation of crystals and red cells at 37°C. Same preparation caused 80% haemolysis after three months' storage in laboratory. This is a striking example of effect of aging on crystal reactivity. Most other particles slowly lose activity with age.*

biological systems differently. Minor changes in size, available surface area, and surface characteristics probably account for this.

Examination of most of the published reports on crystal-induced inflammation shows that samples have usually been heated or ground or both and precautions to avoid batch variation and aging have hardly ever been taken. Different results from different centres are therefore easy to explain. More importantly, much of the work and many of the theories may be invalid.

The two factors that may be most important in determining the activity of a crystal are probably the available surface area, and surface contamination.

**Experiments on surface area and surface contamination of crystals**

Weight-for-weight comparisons of crystals are meaningless because of differences in size and shape, and therefore of the available surface to interact with cells and proteins.[10]

Most recent work on crystal-induced inflammation has emphasised the role of absorbed proteins to crystal surfaces, especially IgG absorption resulting in Fc receptor activation on cells and complement activation.[11 12]

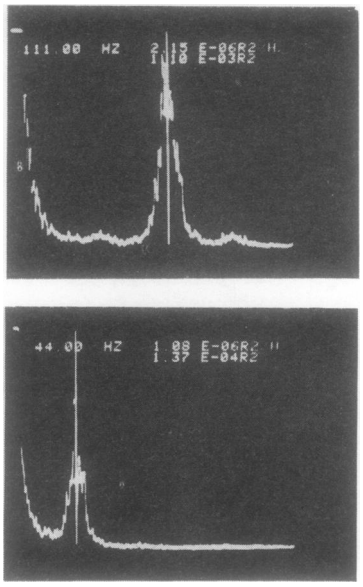


Fig. 4 *Electrophoresis of monosodium urate monohydrate crystals before and after incubation with Fab fragments of human IgG. Initial reading (top) shows that crystals are travelling rapidly to positive pole of an electrical field. After being coated with Fab fragments, mobility is much reduced (bottom). This reduction in mobility—that is, charge—may be reversed by washing protein off crystal. Fab-coated crystals have a reduced capacity to lyse red cell membranes (Measurements recorded on laser Doppler shift–axis represents the amount of shift and thus crystal mobility).*

We have examined the surface properties of crystals and the changes with protein coating by measuring electrophoretic mobility using a laser Doppler shift technique described elsewhere.[13] This may provide further understanding of contamination of the crystal surface and allow comparisons of the surface area and affinity of crystals for proteins.

Crystals are suspended in a balanced salt solution. A current is applied and the shift to negative or positive pole can be measured. Most biological crystals shift to the positive pole (they have a negative charge property). Previous incubation of crystals with a protein may result in attachment of the protein to the crystal, and a shift of the charge property towards that of the protein. The strength of the attachment may be assessed by washing the samples. We have recently shown that Fab fragments of IgG attach to urate crystals, inhibiting membranolysis[14] (Fig. 4). This provides a possible mechanism of self limitation of gouty inflammation (via cleavage of IgG to Fc and Fab fragments; the more positively charged Fab fragment attaches to the crystal, masking its active surface, and inhibiting its activity and ability to interact with Fc receptors). Histone is another protein avidly picked up by urate crystals (Fig. 5). The affinity of the particles for minute quantities of the protein illustrates the potential for laboratory contamination of crystals. The charge shift may also provide data on the available surface area.

It may be concluded from the data presented that crystals can attract a variety of proteins, that small quantities of surface contaminant can change their surface charge and biological activity, and that electrophoretic mobility can be used to investigate the available surface area of a particle. (Further experiments are under way, and full details of the methods used and data obtained are in preparation for publication elsewhere).

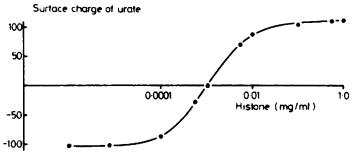


Fig. 5 *Electrophoretic mobility of urate crystals incubated in medium containing varying concentrations of histone. Note low concentration of protein required to shift charge property, and saturation of crystals negatively charged sites at a concentration of about 0·01 mg/ml. Minute amounts of protein can be shown to attach to, and change, crystal surfaces in this way. Shape of curve is a guide to affinity and active surface area of crystal.*

## Conclusions

(1) False positive and false negative results may occur when synovial fluids and tissue samples are examined for the presence of crystals. These errors may be minimised by careful handling of the samples.

(2) Many of the techniques used in the handling of crystals in the laboratory alter the surface of the particle and affect its biological activity. This factor has not been accounted for in most experiments on crystal-induced inflammation, and it invalidates much of the published work.

(3) Quantitative assessment of the affinity of crystals for proteins may be used to assess the surface area of biological significance.

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