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Development of a new technique for the extraction of crystals from synovial fluids

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The presence of crystals in synovial fluid is associated with inflammation and destructive changes in the joint. The crystals can be isolated from the fluid and identified using a number of techniques. However, the processes are often lengthy and generally involve difficult procedures. This study describes the development of a simple and reliable method using hypochlorite solution to isolate crystals from joint fluids without causing any apparent changes to the crystals. Scanning electron microscopy and microanalysis are then used for accurate identification of the crystal deposits.

1. Introduction

A variety of crystals and particles can be found in synovial fluids. Their presence is often associated with inflammatory responses and progressive destructive changes within articulating joints [1]. Severe arthritis may develop without much inflammation due to the direct damaging effect of crystal deposits both within and on the surface of the cartilage. A knowledge of the size, morphology and quantity of crystals present is important with respect to an understanding of this response.

The analysis of synovial fluids and subsequent identification of crystals can be carried out using a number of techniques. The most common and routinely employed method is examination using polarized light microscopy (PLM). This technique was first used for the identification of monosodium urate monohydrate (MSUM) and calcium pyrophosphate dihydrate (CPPD) in the early 1960s [2, 3]. More recently, basic calcium phosphates such as hydroxyapatite (HA) have also been detected using staining of synovial fluids and PLM [4]. PLM is thus regarded as providing a highly sensitive and specific method for detection and identification of crystals [5]. However the technique is limited in that it is difficult to resolve particles of less than 1–2 μm in size. A recent study carried out to assess the reliability of synovial fluid analysis using PLM determined the threshold concentration of crystals for reliable identification and the sensitivity and specificity of observers to different crystals. Large numbers of false positive and false negative results were recorded at low crystal concentrations [5].

The scanning electron microscope (SEM) provides magnifications and resolutions much higher than those possible with the light microscope. Use of the SEM allows a three-dimensional morphological study of crystals to be carried out as well as elemental analysis using energy dispersive techniques (EDA). One advantage of SEM is that the threshold mass of crystals required for detection is much less than for either X-ray diffraction or light microscopic analysis where the quantity of crystals present in a joint fluid may be too low to allow for either of these two techniques to be applied [5, 6]. EDA is particularly useful for the analysis of individual particles in that the elements present in a particle can be analysed and their elemental ratio calculated. EDA cannot differentiate between different salts with the same elemental ratio, but used in conjunction with morphological appearances this technique can be employed to clearly identify different crystals.

Before a detailed analysis of synovial fluid crystals can be carried out, the organic phase needs to be removed. A number of methods have been used; these include enzymatic methods [7–9], the use of hydrazine [6], cytocentrifugation and microincineration [10]. The main disadvantages of the techniques employed are the risk of crystal dissolution during aqueous phase digestion and the length of time involved (over 24 h for the hydrazine method). The aim of this study was therefore to develop a simple and rapid technique for the extraction of crystals from synovial fluids prior to analysis using SEM and EDA. Initial work was based on a method developed for the removal of organic material from tissues and for the disaggregation of bone into crystals [11].

2. Materials and methods

Synthetic crystals provided by the Rheumatology Unit, Bristol Royal Infirmary and the Dental School, Bristol were used to check for the introduction of crystal changes and artefacts during the development stage of the experimental techniques. Synovial fluids were collected from patients attending outpatient clinics at the Royal National Hospital for Rheumatic Diseases, Bath. The patients showed a large age distribution and a variety of diagnoses.

Following aspiration the synovial fluid was placed in plain sterile tubes. These were spun at 5000 rpm for 20 min; the supernatant was discarded. 10 ml of 2.6%

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sodium hypochlorite solution were added to the remaining pellet. The tubes were placed in a beaker of ice-cold water and sonicated in an ultrasonic bath for 15 min. The solution was transferred to 1.5 ml Ependorf tubes. These tubes were spun down for two minutes at 13 000 rpm and then allowed to settle for a further 1 min. Approximately 1 ml of the supernatant was removed from each tube. HA saturated distilled water was filtered through a 0.22 μm millipore filter and 1 ml of this added to each Ependorf tube. The solution was then mixed and spun down as before. This washing procedure was repeated a total of three times using crystal saturated water and followed by three similar washes in 99% ethanol. The extracted crystals, suspended in ethanol, were dropped onto aluminium SEM dishes and allowed to dry in air. These dishes were then coated with gold/palladium for morphological studies or carbon coated for microanalysis in the SEM. Carbon-coated samples were examined using a JEOL JSM 35C SEM with a link AN 10000 energy dispersive analysis system; an accelerating voltage of 15 kv was used. Ca and P ratios were calculated from the spectra obtained. Gold/palladium coated samples were examined in a JEOL JSM T330 SEM with an accelerating voltage of between 10 and 15 kv.

3. Results

Synthetic crystals played an important part in the development of the hypochlorite preparation technique in that they were used at each stage to test the procedures. In this way the optimum times required for maximum cleaning without changes occurring to the crystals themselves could be established prior to treatment of the valuable patient material.

During the early stages of the development of the preparation techniques for analysis of synovial fluids a number of artefacts were observed that are of interest to report. Initially all the organic material was not completely removed from the samples. Consequently the crystal deposits were left covered in a 'blanket', as seen in Figs 1 and 2, making imaging and analysis difficult. As the technique was developed, artefacts in the form of both sodium chloride and silicon containing particles, were frequently found to be present. The NaCl appeared as cubic crystals 2–10 μm in size, often having round corners; a typical example is shown in Fig. 3. The more regular shaped NaCl crystals were generally cracked on the surface as shown in Fig. 4. This made it easy to distinguish them from the much smoother CPPD crystals. Silicon-containing particles were also seen but less frequently. These varied in size and shape but most commonly appeared as large rods; a typical example is shown in Fig. 5. As the preparation procedures were modified and improved both these artefacts were eradicated.

Having optimized the cleaning process it proved possible to isolate and identify a number of different crystals from the synovial fluid of patients with a range of arthropathies. The most commonly occurring crystals that could be positively identified were those of CPPD and HA. Typical samples are shown in Figs 6–9. Both rhomboid and rod forms of CPPD were identified, the rods being typically 0.5–10 μm in length and the rhomboids 0.5–5 μm, although the rods were found in greater numbers in all the fluids examined. As can be seen in Figs 6 and 7, the rods were of varying length and diameter. An unusual feature of some of the rods is illustrated in Fig. 8 where the surface of the CPPD rods appeares to be striated. A further, less common observation was the layered appearance of some of the rods as seen in Fig. 9. The HA appeared primarily as amorphous clumps ~ 4 μm in diameter

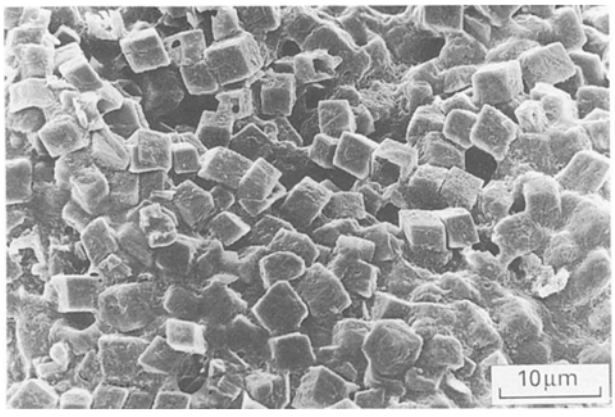

Figure 1 SEM of NaCl crystals covered with a 'blanket' of organic material.

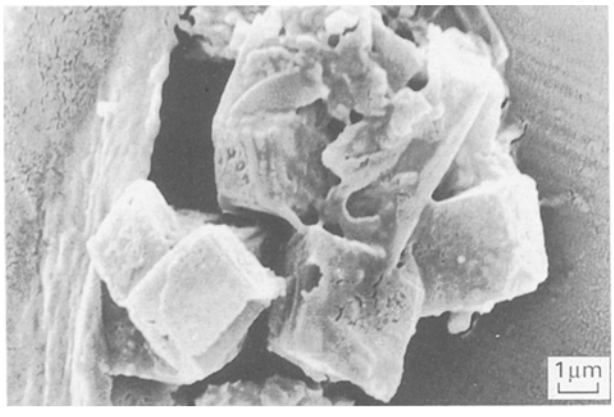

Figure 2 NaCl crystals and organic material seen during SEM examination of a poorly prepared synovial fluid sample.

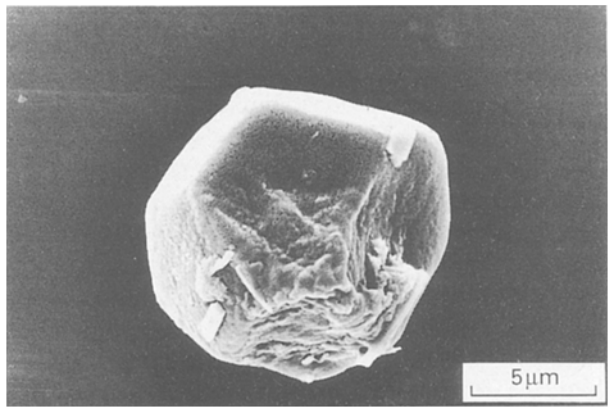

Figure 3 SEM of a typical NaCl crystal found in synovial fluid during the initial development of preparation procedures.

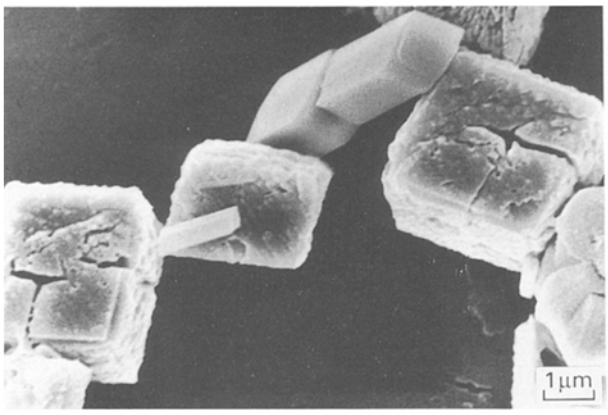

Figure 4 Smooth CPPD and cracked NaCl crystals found together during SEM examination.

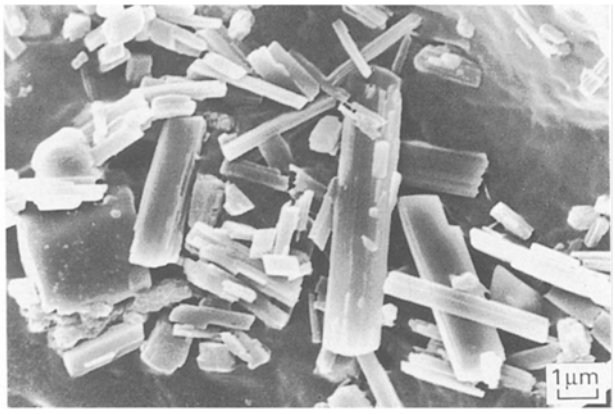

Figure 7 SEM of CPPD crystals from synovial fluid, showing the range of sizes observed.

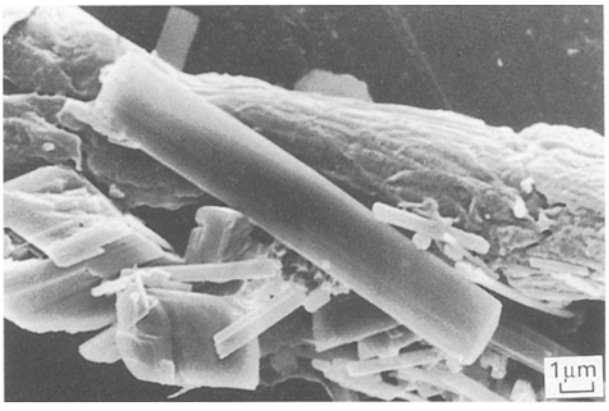

Figure 5 SEM micrograph of a large silicon-containing particle with its typical smooth and rounded appearance.

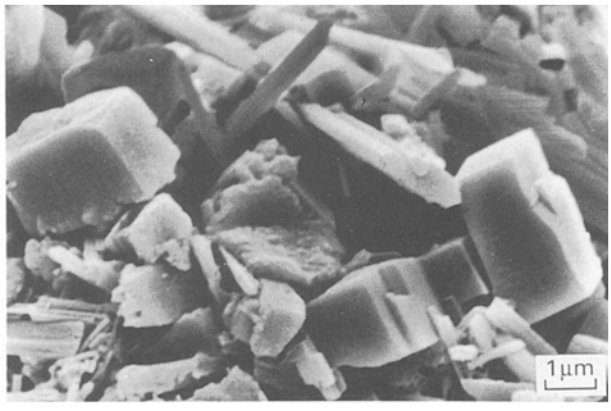

Figure 8 SEM showing rhomboidal CPPD crystals with striated surfaces.

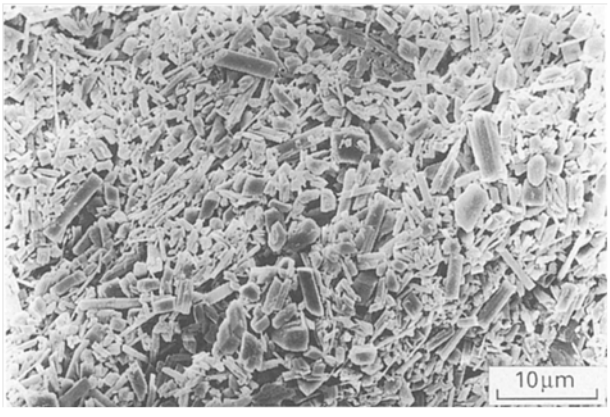

Figure 6 Typical clump of CPPD crystals found on SEM examination of hypochlorite-treated synovial fluid.

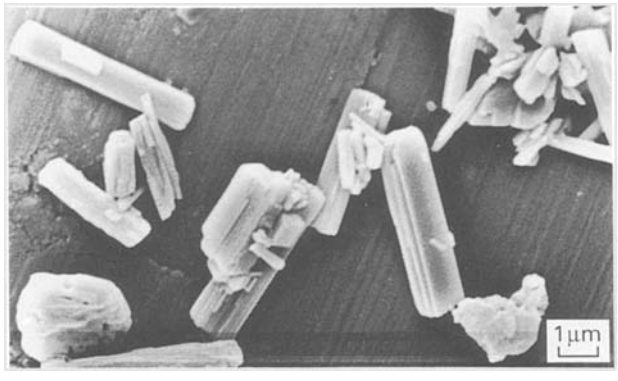

Figure 9 Rod-shaped CPPD crystals showing an unusual layered appearance.

or sometimes larger as seen in Fig. 10. Small individual particles approximately 0.1 μm across could be identified within the clump. It was not unusual to find mixtures of CPPD rods and amorphous clumps of HA as illustrated in Fig. 11. In some cases the presence of the amorphous clumps disguised the CPPD rods as seen in Fig. 12. The standard of Ca/P ratios for CPPD and HA are given in Table I and shown in Fig. 13 alongside typical ratios measured for the actual mineral deposits analysed in the SEM. EDA of the CPPD crystals gave a range of values for Ca/P of 1.0–1.3; corresponding values for HA were 1.5–1.95.

4. Discussion

Scanning electron microscopy has often been used to examine biological tissues. The high magnifications, resolutions and three-dimensional images increase detection levels and allow more detailed morphological studies to be carried out than is possible with polarized light microscopy. EDA techniques used in conjunction with SEM allow elemental analysis in addition to morphological detail to be obtained thus enabling positive identification of crystals. In the past the lengthy procedures often involved in the preparation have limited the use of analytical electron

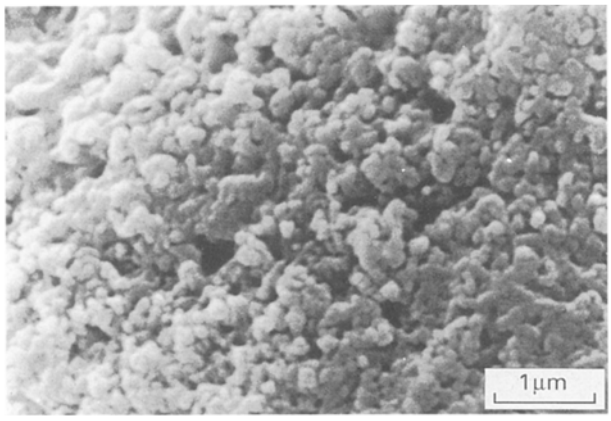

Figure 10 SEM showing a large amorphous clump of HA made up of a mass of smaller particles.

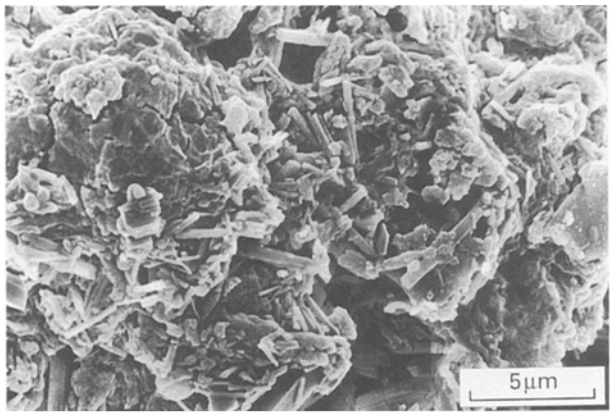

Figure 11 SEM of a mixture of rod-shaped CPPD crystals and amorphous HA.

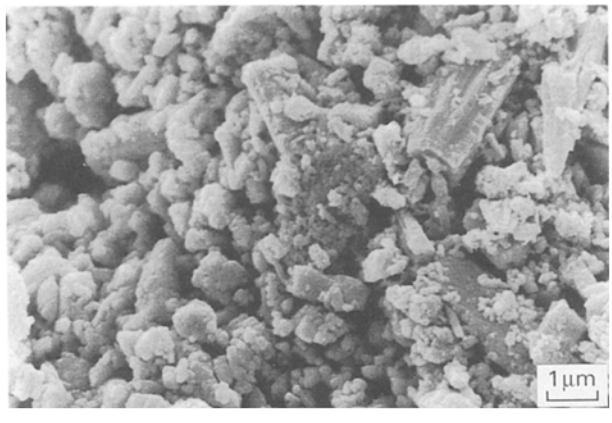

Figure 12 Amorphous HA clumps almost hiding the presence of the CPPD crystals.

microscopy in clinical diagnosis. The development of the sodium hypochorite technique as described above provides a quick and simple method of isolating fluid crystals using materials that are commonly available in most laboratories.

Development of the initial testing of the hypochlorite method was carried out using synthetic crystals. The size, shape, surface morphology and EDA ratios of the crystals were noted before and after each treatment with hypochlorite to check that there were no

TABLE I Theoretical and EDA measured Ca/P ratios for CPPD and HA crystals

Crystal	Theoretical ratio	Measured ratio
CPPD	1.00	1.0–1.3
HA	1.66	1.5–1.95

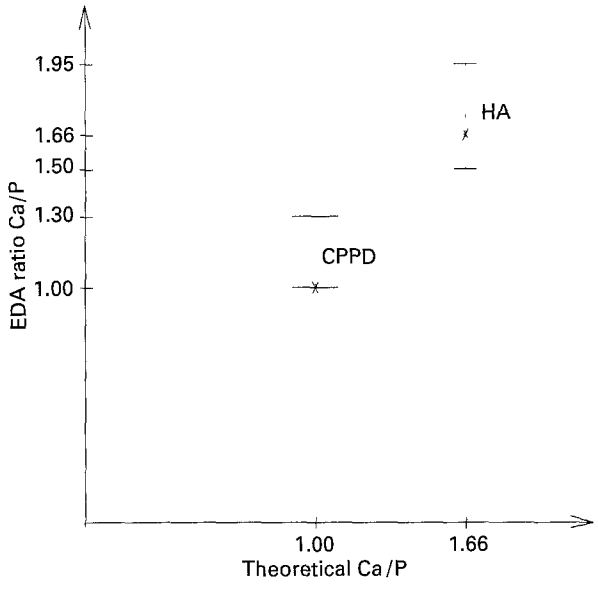

Figure 13 Standard Ca/P ratios for CPPD and HA relative to the ranges of ratios obtained from actual mineral deposits.

changes. Several variations to the technique were tried to obtain the most effective treatment and cleaning procedures possible without inducing any changes in the crystals themselves. During the early stages of development the introduction of two artefacts was discovered. These were the salt crystals and the silicon shown in Figs 3–5. The collection of synovial fluids in plastic tubes and the subsequent use of the plastic laboratory ware eliminated the silicon [12, 13]. More careful cleaning of the crystals after the hypochlorite treatment reduced the incidence of these artefacts significantly. The extraction technique had to be further refined when applied to joint fluids owing to the presence of the organic material. However, it was adapted without too much difficulty to enable the successful extraction of crystals from synovial fluids.

Morphological studies of crystals were carried out using SEM and were particularly successful in the study of CPPD crystals. A large range of sizes and a predominance of rod-shaped crystals were observed in all fluids. These findings were in general agreement with other previous studies [14, 15, 7, 8]. The surface characteristics of the CPPD crystals were interesting in that several different morphologies were observed within the same fluid, Figs 6–8. The CPPD crystals appeared not only as smooth rods but more often as rods with sections or corners missing or showing surface striations. In addition, both rhomboid and rod forms of CPPD crystals clearly showed a layered structure, Fig. 9. The significance of this different morphological form of CPPD is not known; similar layered structures have previously been reported for CPPD crystals in ligamentum flavum [16]. However,

studies of synovial fluid crystals have not generally shown this kind of layering in CPPD. In this study HA crystals were identified as amorphous clumps occasionally made up of smaller spherical particles. No crystalline needle-shaped crystals of HA were observed. Mixed crystal deposition was often observed with a mixture of both CPPD and HA found in one-third of the fluids examined. This again is in agreement with previous reports [15, 17–20].

EDA results indicated that measurements for both the CPPD and the HA crystals fell within a range of ratios as shown in Fig. 13. Given that more than one type of crystal was often present this was not surprising. It is possible that the amorphous clumps comprised more than one form of calcium phosphate resulting in lowered EDA ratios. It is recognized that carbonate can substitute in the structure of HA to the same effect [12].

Frequently, in the first instance, PLM is used to check for the presence or otherwise of crystals in joint fluids. However, using the hypochlorite preparation technique crystals were often found in fluids where there had been a negative results using PLM. The threshold level for detection is therefore much lower for the new technique and this may therefore have clinical implications in terms of earlier diagnosis of changes occurring within the joint or the surrounding fluid.

A range of techniques is currently in use to isolate crystals from tissues and joint fluids. Many of the methods employed are complicated and highly time consuming (6, 7–10). The primary aim of this study was to develop a simple and reliable method for isolating synovial fluid crystals for microscopic study. The hypochlorite technique allows samples free from organic material and without any apparent change to the crystals to be prepared. Detailed SEM morphological studies combined with EDA allow clear identification of crystal deposits to be made. Hence, the hypochlorite method has been found to provide a simple, rapid and reliable method for isolating synovial fluid crystals prior to their analysis.

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References

1. P. DIEPPE and P CALVERT, in "Crystals and Joint Disease" (Chapman & Hall, London, 1983).
2. D. J. MCCARTY and J L HOLLANDER, *Ann. Int. Med.* **54** (1961) 452.
3. N N. KOHN, R E. HUGHES and D J. McCARTY, *ibid.* **56** (1962) 738.
4. H PAUL, A J REGINATO and H R SCHUMACHER, *Arthritis Rheum.* **26** (1983) 191.
5. C GORDON, A. SWAN and P. DIEPPE, *Ann. Rheum. Dis.* **48** (1989) 737.
6. T. CUNNINGHAM, D. UEBELHART, J M VERY, G H FALLET and T. L VISCHER, *ibid.* **48** (1989) 829.
7. G FAURE, P NETTER, B MALAMAN, J STEINMETZ J. DUHEILLE and A. GAUCHER, *SEM* **III** (1980) 163.
8. J C. GERSTER, A. GAUTIER, Y DROZ-GEORGET, F. FLACH, F ARDIZZONI, D; GROSS and G. GALLACCHI, *Schweiz. med. Wschr.* **116** (1986) 390.
9. P. B HALVERSON, H S CHEUNG, D J McCARTY, J GARANCIS and N MANDEL, *Arthritis Rheum.* **24** (1981) 474.
10. D V. DOYLE and P. R CROCKER, *Ann. Rheum. Dis.* **42** (1983) 108.
11. S WEINER and P. A PRICE, *Calcified Tissue International* **39** (1986) 365.
12. G. FAURE, P NETTER and M -C. BENE, *Ann. Rheum. Dis.* **44** (1985) 148.
13. T. BARDIN, H. R. SCHMACHER, J. LANSAMAN, S. ROTHFUSS and A. DRYLL, *ibid.* **43** (1984) 624.
14. M NAGAHASHI, *Nippon-Seikeigeka-Gakkai-Zasshi* **53** (1979) 793.
15. P DIEPPE, P. CROCKER, C. F. CORKE, D. V. DOLYE, E C HUSKISSON and D. A WILLOUGHBY, *Q. J. Med.* **48** (1979) 533.
16. M. OGATA, *J. Jpn. Orthop. Ass.* **59** (1985) 69.
17 P B. HALVERSON and D McCARTY, *Ann. Rheum. Dis.* **45** (1986) 603.
18. P DIEPPE, G CAMPION and M. DOHERTY, *Rheum. Dis. Clin. N. Amer.* **14** (1988) 415.
19. P. A. DIEPPE, D V. DOYLE, E. C HUSKISSON, D A. WILLOUGHBY and P R. CROCKER, *Brit. Med. J.* (1978) 150.
20. P. A. GIBILISCO, H. R SCHUMACHER, J. L HOLLANDER and K. SOPER, *Arthritis Rheum.* **28** (1985) 511.

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