CRYSTAL GROWTH OF MONOSODIUM URATE MONOHYDRATE

BY

YOUGESH CHANDER DUTT

Diploma in Pharmacy, Panjab University, 1969
B. Pharm., Panjab University, 1973
M. Pharm.(Pharmaceutics), Panjab University, 1975

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Faculty of Pharmaceutical Sciences)

Division of Pharmaceutics

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

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Department of Pharmaceutical Sciences

The University of British Columbia
1956 Main Mall
Vancouver, Canada
V6T 1Y3

Date April 25, 1985

ABSTRACT

Hyperuricemia and local temperature changes in the joints of the extremities are known to be responsible, in part, for the development of gouty arthritis. No satisfactory explanation is yet available for (1) the selective deposition of monosodium urate monohydrate (MSUM) crystals in connective tissues (2) the increased incidence of gout in the later years of life and (3) the increased incidence of MSUM crystal deposition in connective tissues after trauma and in joints with preexisting disease. It is possible that the alterations in composition of the non-fibrillar matrix of cartilage and synovial fluid which are thought to occur with ageing, trauma or preexisting disease, may predispose these tissues to crystal deposition. The objectives of this study were to determine the effect of the cartilage and synovial fluid components, chondroitin sulfate, hyaluronic acid, proteoglycan monomer, proteoglycan aggregate, phospholipids and albumin on the growth of MSUM.

The degradation of MSUM solutions was studied under sterile and non-sterile conditions to determine the possible causes of degradation and to define the time span of crystal growth experiments. The rate of degradation of MSUM solutions increased with an increase in temperature. The concentration of MSUM in

solution fell sharply after autoclaving and solutions stored in containers with rubber closures showed greater degradation of MSUM than autoclaved solutions stored in all-glass containers. Rubber stoppers apparently absorbed MSUM from solution. The degradation of MSUM solutions was thought to be due to both bacterial consumption and chemical decomposition in non-sterile solutions but was due only to chemical decomposition in sterile solutions.

The aqueous solubility of MSUM was determined at different temperatures and in the presence of varying concentrations of sodium chloride. Sodium chloride suppressed MSUM solubility.

The aqueous solubility of MSUM was also determined in the presence of several connective tissue components at 37^{o}. Chondroitin sulfate (CS) decreased the saturation solubility of MSUM probably due to the sodium present in the CS samples. Proteoglycan aggregate, proteoglycan monomer, hyaluronic acid and albumin resulted in very slight increases in the solubility of MSUM.

The growth kinetics of MSUM was studied using the seeded growth technique. An equation of the general form:

$$R_g = K_o(obs)\ S\ (C-Cs)^n$$

was used to determine the overall growth rate constant, $K_o(obs)$ or rate constant, K' ($K' = K_o(obs)S$).

Linear plots of the integrated form of the second order growth equation gave the best fit between the points and gave reasonably constant values for K_o(obs) determined at a given initial supersaturation concentration and varying seed amounts.

An induction period or a period of slow growth was observed at both the initial supersaturation concentrations studied. The length of the induction period was inversely proportional to the added seed amount.

Differing concentrations of additives were included in the growth medium and K' determined.

Chondroitin sulfate (CS) significantly increased the growth rate constant for MSUM growth. However, the proportion of CS decreases in aged and osteoarthritic cartilage and thus a decreasing proportion of a growth accelerator is unlikely to be a factor in the deposition of MSUM in cartilage. CS has been found in the synovial fluid of arthritic joints and may act as an MSUM growth accelerator in this medium.

Hyaluronic acid (HA) and albumin caused significant inhibition of the growth of MSUM crystals. This effect may be due to the adsorption of these molecules onto the MSUM seed crystals resulting in the poisoning of the active growth sites on the crystal surface. Cartilage HA and synovial fluid albumin levels are increased in aged and/or diseased joints. Increased proportions of growth inhibitors do not offer likely explanations of crystal deposition in joint tissues.

At concentrations of 0.1-1.0 mg mL^{-1} proteoglycan monomer (PGM) and proteoglycan aggregate (PGA) slightly increased the MSUM growth rate constant but this increase was statistically insignificant.

The two phospholipids, phosphatidylcholine and phosphatidylserine increased the growth rate constant of MSUM. Phosphatidylserine, however, did not significantly increase the growth rate constant at the concentrations studied. It is possible that the raised levels of phospholipids in aged or diseased cartilage and synovial fluid could accelerate the growth of MSUM crystals resulting in MSUM deposition in these tissues.

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SYMBOLS AND ABBREVIATIONS

s'	Degree of supersaturation
C	Concentration of solute in the solvent at some given temperature
Cs	Equilibrium saturation concentration of solute in the solvent at a given temperature
Ci	Solute concentration at the crystal-solution interface
W	Total quantity of the work required to form a stable crystal nucleus
W_s	The work required to form the surface of the surface of the crystal
W_V	The work required to form the bulk of the crystal
δ	The surface energy of the spherical particle per unit area
a	Surface area of the particle
Δp	The pressure difference between the vapour phase and the interior of the liquid droplet
v	Volume of the particle
r	Radius of the droplet
pr	Vapour pressure over a liquid droplet of radius r

p^*	Vapour pressure over a flat liquid surface
M	Molecular weight of the substance
ρ	Density of the droplet
T	Absolute temperature
R	Gas constant
G	Overall excess free energy
G_s	Surface excess free energy
G_v	Volume excess free energy
G_{crit}	Overall excess free energy, G, at maximum where $r = r_c$
r_c	radius of a critical size nucleus
N	Rate of nucleation or the number of nuclei formed per unit time
A	Constant of nucleation rate
ΔG	Overall free energy change for the formation of a partical
ΔG_{crit}	Overall free energy change associated with homogeneous nucleation
$\Delta G'_{crit}$	Overall free energy change associated with heterogeneous nucleation
ϕ	A constant related to the interfacial energy
σ_{sl}	The interfacial energy between the surface of the seed and the liquid
σ_{cs}	The interfacial energy between the surfaces of the crystallizing phase and the seed surface
σ_{cl}	The interfacial energy between the

crystallizing phase and the liquid

a_i Area of the i th face of the crystal

g_i Surface free energy per unit area of the i th face

b_o Burgers vector

R_g Rate of crystal growth

A Temperature dependent constant of the growth rate

B Temperature dependent constant of the growth rate

S Surface area of the solid

D Coefficient of diffusion of solute

h Thickness of the liquid film

R_t Rate of arrival of solute at the surface by diffusion from the bulk solution

R_s Rate of integration into the solid surface

K_t Transport rate constant

K_s Surface integration rate constant

K_o(obs) Observed overall growth rate constant

K' Observed growth rate constant, ($K' = K_o S$)

K'(add) Observed growth rate constant for MSUM growth in the presence of additive

K'(control) Observed growth rate constant in the absence of additive

n Order of growth reaction

e_{S_t} Effective surface area at time t

K_T	Growth rate constant dependent on temperature
$f(o_t)$	Term dependent on the supersaturation
c_t	Total mass of solid at time t
S_t,S	Geometric total surface area at time t
C^-,C^+	Concentrations of anions and cations at the supersaturation concentration
Cs^-,Cs^+	Concentrations of anions and cations at the saturation concentration
P	Partial pressure of adsorbate
Po	Saturated pressure of adsorbate
Nu	Avogadro's number (6.023×10^{23})
Vc	Volume of calibration gas
Pa	Ambient pressure in atmosphere
A	Signal area or desorption count of calibration gas
Acs	Cross sectional area of adsorbate molecules in square meters
λ	Wavelength of X-rays
d	Spacing between the crystallographic planes of a crystal
n*	Is an integer in equation 27
θ	Angle of the incident X-rays
ΔH	Heat of solution
PGA	Proteoglycan aggregate
PGM	Proteoglycan monomer
PGs	Proteoglycans
HA	Hyaluronic acid

CS	Chondroitin sulfate
PC	Phosphatidylcholine
PS	Phosphatidylserine
MSUM	Monosodium urate monohydrate
CPPD	Calcium pyrophosphate dihydrate
HAP	Hydroxy apatite, Apatite, Calcium hydroxy apatite

ACKNOWLEDGEMENTS

I wish to thank Dr. H.M. Burt for her supervision and encouragement during the course of this study.

I am grateful to Dr. A.G. Mitchell for his valuable questions and suggestions and to Dr. J. McNeill for encouragement throughout this project.

Sincere thanks to:

Dr. Mark Adams, Department of Medicine, for his suggestions and for providing the proteoglycans.

Mr. R. Butters, Department of Metallurgy, for the X-ray diffraction analysis.

Ms. M. Mager, Department of Metallurgy, for the scanning electron microscopy.

Mr. R. Burton, Faculty of Pharmaceutical sciences, for computer analysis.

Several friends (who must remain anonymous) for their friendship.

Financial support from the University of British Columbia, Medical Research Council of Canada and Stanley Drug Products is gratefully acknowledged.

1 INTRODUCTION

The nucleation and growth of crystals from supersaturated solutions is an important process in biological systems, for example, in the deposition of calcium hydroxyapatite crystals in bones and teeth. Other compounds such as calcium oxalates, calcium pyrophosphates, uric acid, sodium urate etc. may be found as pathological deposits in the body. A crystal deposition disease may be defined as a pathological condition associated with the presence of crystals which then contribute to the tissue damage.

Gout is associated with the appearance of monosodium urate monohydrate (MSUM) crystals in the synovial fluid and/or in other connective tissues of the joint. Hyperuricemia is required for the development of gouty arthritis. Increased urate levels in blood may result from: metabolic disorders, impaired renal excretion and ingestion of drugs and alcohol. Since the mean plasma urate levels in healthy males are nearly the same as the saturation solubility of urate in plasma, local temperature changes may be a factor in the formation of tophi in peripheral sites and acute crystallization in joints.

Hyperuricemia and temperature variations alone, however, are insufficient to explain the observed patterns of MSUM crystallization, such as, (1) the selective deposition of MSUM crystals in connective tissues, (2) the increased incidence of gouty arthritis in the later years of life and (3) the involvement of connective tissues exposed to trauma in this disease.

There are a number of biochemical and metabolic changes which occur in the articular cartilage and synovial fluid of joints as a result of factors such as ageing, trauma and preexisting disease. Alterations in the composition of the cartilage matrix and synovial fluid caused by ageing and disease may predispose these tissues to crystal deposition.

Although there have been some studies of the factors affecting the solubility and nucleation rates of MSUM, the factors which may influence the crystal growth of MSUM are poorly understood.

The objectives of the present study were:

1. To investigate the crystal growth kinetics of MSUM.

2. To determine the effect of supersaturation and seed amount on the MSUM crystal growth kinetics.

3. To determine the effect of cartilage and synovial fluid components on the MSUM growth kinetics.

There are a number of methods for studying the kinetics of crystal growth, but the seeded growth technique has been shown to be highly reproducible and was employed in these studies. Supersaturated sodium urate solutions were seeded with well-characterised seed crystals of MSUM under conditions of constant temperature and agitation.

2. LITERATURE REVIEW

2.1 CRYSTAL DEPOSITION DISEASES

Gout as a disease has been known for several centuries. The ancient accounts of gout were summarized by Francis Adams in 1844 in his commentaries in the translation of "The seven books of Paulus Aegineta" (Hartung, 1957).

Hippocrates (400 BC) described this disease with remarkable accuracy (Pritchard, 1981). In his aphorisms on gout, Hippocrates referred specifically to the hereditary predisposition, the periodicity of acute episodes, the worsening of the disease in the spring and fall, the rarity before puberty, the appearance in women after the menopause, the immunity of eunuchs to gout and the benefit of cold applications (Hartung, 1957). Gout has been traced to the first century of the christian era. In a cemetery in Nubia, Egypt, Simith and Jones (1910) found a skeleton of an elderly male whose great toe and other joints showed large tophi of urate crystals (Hartung, 1957).

The first microscopic description of the crystals derived from a gouty tophus was given by Antoni von Leewenhoek in early 1700 (McCarty, 1970), but it was only in 1797, when Wollaston

demonstrated that the extrusions from gouty tophi were of "lithiated soda" (Hartung, 1957). "Lithic acid" is now known as uric acid.

Crystals, as the cause of the inflammatory response were advocated by Sir Alfred Baring Garrod in 1859 (Pritchard, 1981). He stated that acute gouty arthritis is an inflammatory reaction to crystals of sodium urate. Freudweiler (1901) and Hiss (1900) reported experiments on crystal-induced inflammation. Evidence of crystalline deposits in and around joints was noted in radiological examinations as early as 1907 by Painter. This was followed by reports of inflammation related to these deposits (Schmitt, 1921; Sandstorm, 1938). The nature of the precipitates in tophaceous gout was described by Brandenberger et al.(1947).

It was not until the early 1960's that active research was initiated in this field by Hollander and McCarty. The terms "crystal deposition diseases" and "crystal-induced inflammation" were coined after crystals of sodium urate in the synovial fluid were observed using polarized light microscopy (McCarty and Hollander, 1961). This method of synovial fluid examination led to the discovery of calcium pyrophosphate dihydrate (CPPD) crystals in joints (McCarty et al., 1962; Kohn et al., 1962). This arthropathy was called "pseudogout" because of its similarities to gout.

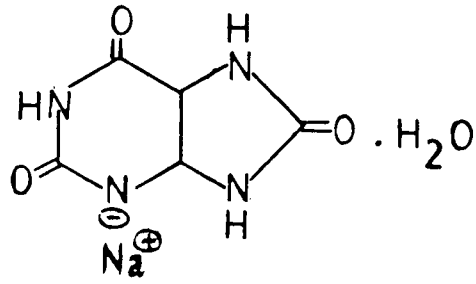
2.1.1 GOUT

This disease is caused by the deposition of monosodium urate

monohydrate (MSUM) crystals in cartilage or in the joint fluid. The chemical structure of MSUM is shown in Figure 1a. Crystals of MSUM have been extracted from gouty tophi and synovial fluid and examined by polarized light microscopy (McCarty and Hollander, 1961). These crystals belong to the triclinic crystal system (Howell et al., 1963) and have an acicular or needle shaped crystal habit. The crystals are between 2-20 μm in length and 0.5-2 μm in width (Dieppe and Calvert, 1983). The crystal structure of MSUM has been determined by Mandel and Mandel (1976) and Rinaudo and Boistelle (1982). They have shown that the MSUM crystal consists of urate anions stacked in parallel sheets interspersed with sodium ions which bond to four neighbouring urate anions via the oxygen atoms. The water molecules form hydrogen bonds with the purine rings (Figure 2).

Recent studies have shown that the crystals of MSUM possess a net negative charge (Dieppe et al., 1981a; Burt et al., 1983). The electrophoretic mobility of these crystals in physiological saline solution has been found to be 1 um s^{-1} V^{-1} cm (Dieppe and Calvert, 1983).

MSUM crystallizes only in connective tissues. The major sites are articular cartilage, periarticular soft tissues, bursae, on the ear and in tendon sheaths (Dieppe and Calvert, 1983). In acute gout, the synovial fluid contains numerous MSUM crystals often within the leucocytes (McCarty and Hollander, 1961; Weinberger et al., 1979; Agudelo et al., 1979; Gordon et al., 1982). In chronic gout a large number of MSUM



$\{C_5H_3O_3N_4Na \cdot H_2O\}$

(A)

$Ca_2P_4O_7 \cdot 2H_2O$

(B)

$Ca_{10}(PO_4)_6(OH)_2$

(C)

Figure 1. Chemical formulae of
(A) Monosodium urate monohydrate,
(B) Calcium pyrophosphate dihydrate, and
(C) Hydroxy apatite.

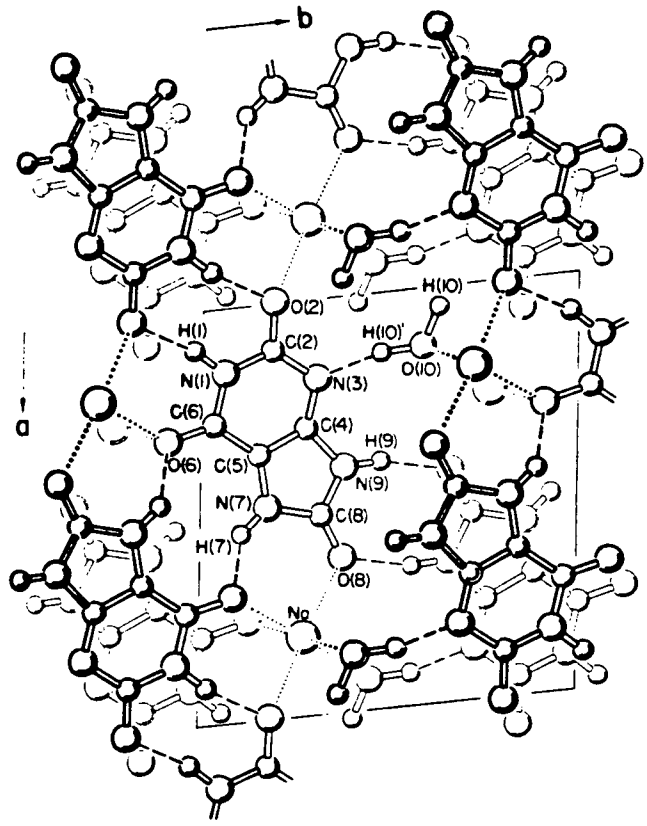


Figure 2. A view of the crystal structure of MSUM viewed down the needle axis

crystals are located in a tophus (Dieppe and Calvert, 1983).

2.1.2 PSEUDOGOUT

This disease is caused by the deposition of calcium pyrophosphate dihydrate (CPPD) crystals. The chemical formula for CPPD is shown in Figure 1b. There are two polymorphic forms of CPPD, one belonging to the triclinic crystal system and the other to the monoclinic crystal system. Both these forms are deposited in the joints (McCarty et al., 1962; McCarty et al., 1966; Jacobelli et al., 1973; Ellman et al., 1981a,b). CPPD crystals have dimensions of between 0.1-10 μm.

2.1.3 HYDROXY APATITE (HAP) DEPOSITION DISEASE

The chemical formula of HAP is shown in Figure 1c. Calcium hydroxy apatite (HAP) has been identified in degenerated articular cartilage by electron probe and scanning electron microscopy (Ali and Wisby, 1975), in tendons, cartilage and other connective tissues (McCarty and Gatter, 1966; McCarty et al., 1966; Pritzker and Luc, 1976) and synovial fluid (Dieppe et al., 1976). The common sites are the shoulder joint, hip joint and spine (Dieppe and Doherty, 1982; Andres and Trainer, 1980). The crystals of HAP are hexagonal and are usually between 0.1-2 um in size. The crystals generally form small spherical clusters composed of many HAP crystals (Dieppe and Calvert, 1983).

2.1.4 MISCELLANEOUS

Dicalcium phosphate dihydrate (Utsinger, 1977; Faure

et al., 1977), corticosteroids (Kahn et al., 1970) and cholesterol crystals (Zuckner et al., 1963) have also been identified in joint tissues. Uric acid and calcium oxalate crystals are responsible for renal stones.

2.2 CRYSTAL-INDUCED INFLAMMATION

The physical presence of crystals in the joint fluid produces an acute arthropathy, seen in gout, pseudogout and in some cases of inflammatory osteoarthritis.

Synthetic crystals of MSUM and CPPD, when injected into joints provoke an inflammatory response similar to the natural material (Boyle and Seegmiller, 1971). Crystals of HAP produce an inflammatory response when injected intradermally into human volunteers (Dieppe, 1977).

The size of the individual crystals is known to be an important factor in acute inflammation. For example, different sized urate crystals cause a varying degree of edema after being injected into a rat's paw (Dieppe and Calvert, 1983). These workers reported that very small crystals (<0.1 μm) and large crystals (>20 μm) are relatively ineffective in producing inflammation, whereas crystals of about 5 μm in length are most reactive. The nature of the crystal surface is thought to be the most important factor in determining the inflammatory effect of the crystals. The structural basis of the inflammatory potential has been investigated by Mandel (1976). He concluded that the key factor is the surface roughness on an atomic scale. Thus

'smooth' crystals like cysteine are inactive, whereas urate, with many charged groups protruding from the surface is very phlogistic.

The negative surface charge on the crystal faces is thought to be responsible for a part of the inflammatory effect of the crystals. Kellermeyer (1965) proposed that the negative surface charge of urate crystals could initiate the Hageman factor and induce inflammation. Dieppe et al. (1981a,b) reported that all the crystals capable of causing inflammation were negatively charged. They also reported that sodium urate, which has the highest inflammatory potential, also has the highest negative surface charge. Heating and grinding significantly reduces the surface charge and consequently the inflammatory potential (Dieppe et al., 1981a,b). Burt et al. (1983) studied the membranolytic effect of MSUM crystals and reported a decrease in both the zeta potential and membranolytic effect of MSUM crystals after heating and rehydration.

Various pathways involved in the mediation of an acute inflammatory attack are shown in Figure 3 (Dieppe and Calvert, 1983). Following the release of crystals into the synovial fluid, protein becomes bound to the crystal surface. In-vitro studies show that MSUM crystals adsorb a variety of proteins (Kozin and McCarty, 1976, 1977; Hasselbacher, 1978, 1982; Hasselbacher and Schumacher, 1978), particularly immunoglobulin, IgG, (Kozin and McCarty, 1976, 1977). Protein binding is likely to change the surface characteristics of the crystals and may

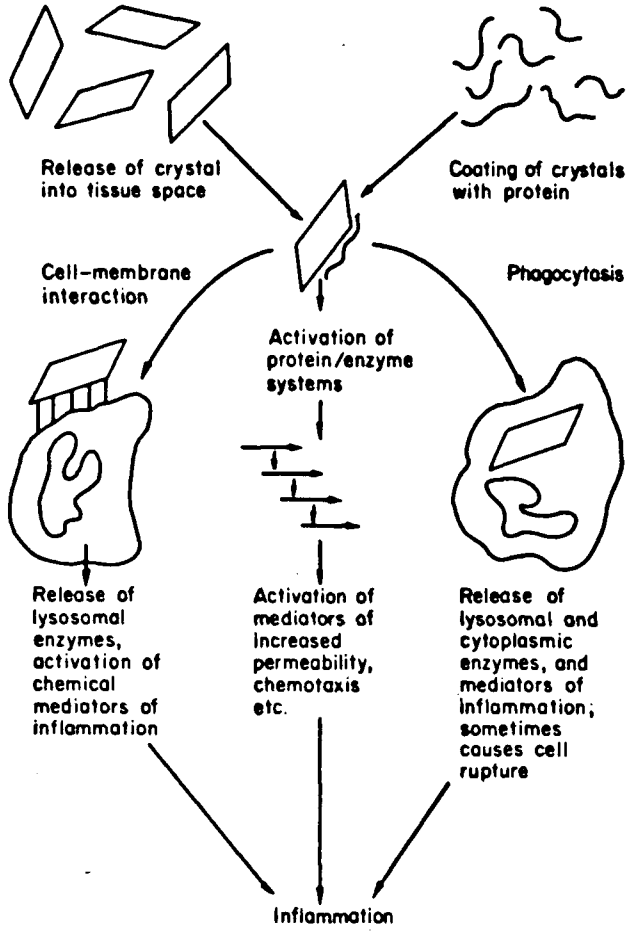


Figure 3. Possible steps involved in crystal-induced inflammation.

enhance the interaction of the crystals with cell membranes, phagocytosis and activation of cell free inflammatory mechanisms (Doherty et al., 1983; Giclas et al., 1979).

Several workers have reported the central role played by the phagocytic cells in crystal-induced inflammation. A mechanism was proposed by McCarty (1979). The evidence for the role of phagocytic cells includes, (a) depleting the phagocytic cells reduces the inflammatory response in experimental animals and (b) the predominance of polymorphonuclear cells with active phagocytosis in crystal-induced inflammation (Phelps and McCarty, 1966).

Several authors have documented the events that occur on incubation of crystals with phagocytic cells in-vitro. Rajan (1975) observed the reaction between polymorphs and sodium urate crystals using cine-microphotography. He demonstrated that polymorphs engulfed or attempted to engulf the urate crystals. Once inside the cell, the crystal was surrounded by a membrane (phagosome). Lysosomes then fused with the phagosome membrane. Lysosomal enzymes were transfered into the phagosomes but with no apparent effect. Polymorphs rapidly died after that resulting in both the release of crystals for further inflammatory reaction and lysosomal release. Release of lysosomal enzyme is thought to be the major cause of inflammation.

2.3 HYPERURICEMIA AND EPIDEMIOLOGY

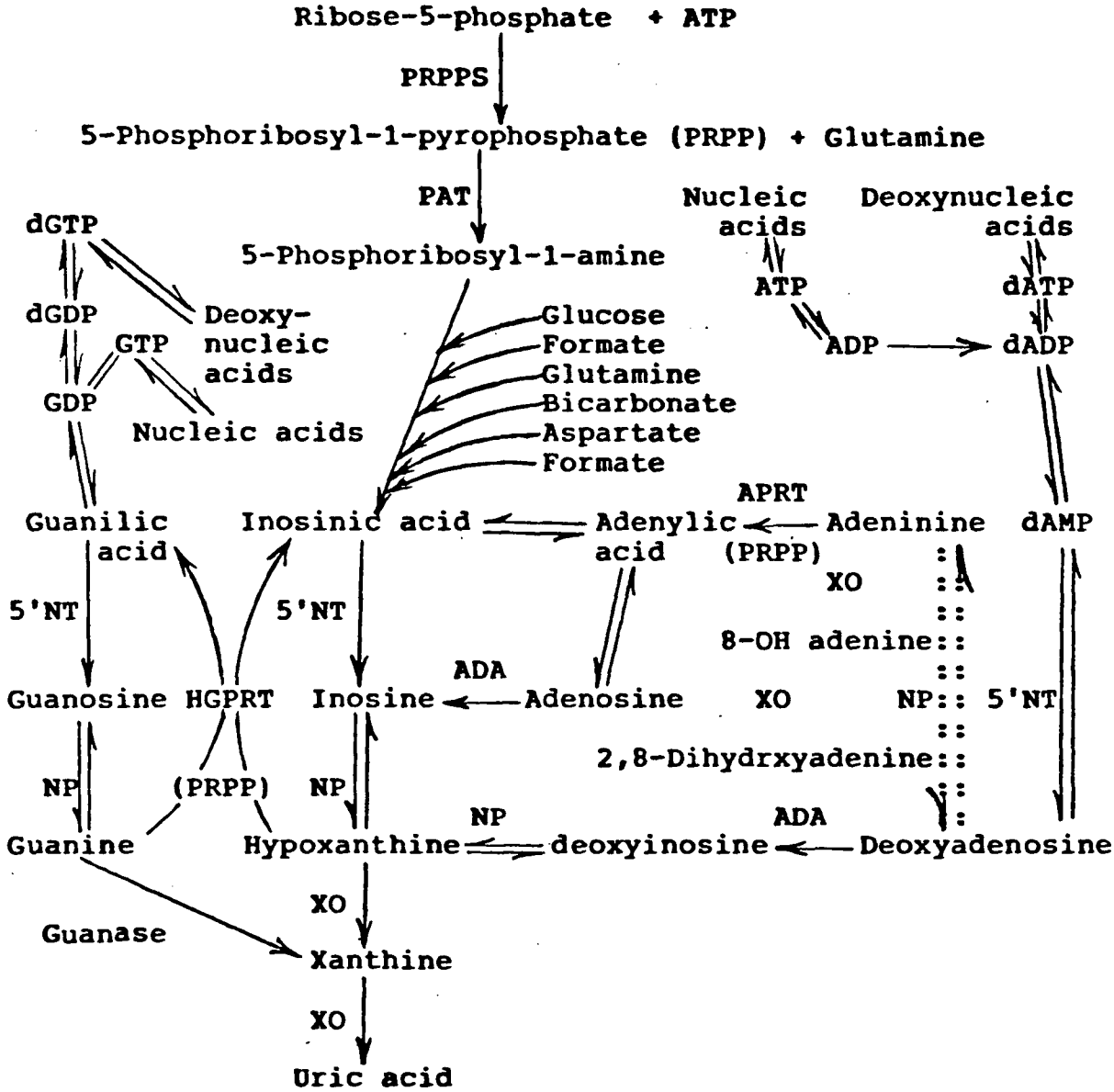
Uric acid is the final product of catabolism of the purines,

adenine and guanine. The purine metabolic pathways are known in detail (Boyle and Seegmiller, 1971; Wyngaarden and Kelley, 1978; Nuki, 1979). The steps involved in purine metabolism are shown in Figure 4. At physiological pH more than 90% of the uric acid is ionized and it is common to represent the plasma or serum uric acid concentration as plasma or serum urate concentration.

Numerous epidemiologic studies have appeared in the literature over the last twenty years. More recent reports include, Munan et al. (1976); Munan et al. (1977); Fessel and Barr (1977); Sturge et al. (1977); Yano et al. (1977); Simons and Jones (1978); Scott (1980); Akizuki (1982); Glynn et al. (1983) and Hall et al. (1983). These studies show that the serum urate levels vary with factors such as geographical area, race, social status, age and sex.

Mean serum urate levels are generally higher in normal adult males than normal adult females. A sex difference in the serum urate levels is usually not observed in childhood but appears during adolescence, when serum urate levels increase more in men than in women (Nishioka et al., 1974; Mikelson et al., 1965; Munan et al., 1977). Lower mean serum urate levels generally makes females less prone to gouty attacks than males until they reach the menopause age where, due to unknown factors, the mean serum urate level in females rises making them susceptible to gouty arthritis.

Normal serum urate levels in healthy males is between 5.5 mg



ADA = Adenosine deaminase.
APRT = Adenine phosphoribosyl transferase.
HGPRT = Hypoxanthine-guanine phosphoribosyl transferase.
NP = Nucleoside phosphorylase.
5'NT = 5'nucleotidase.
PAT = Phosphoribosyl amidotransferase.
PRPPS = Phosphoribosyl pyrophosphate.
XO = Xanthine oxidase.

Figure 4. Pathways of purine metabolism in man (Nuki, 1979).

dL^{-1} to 7.0 mg dL^{-1}. In hyperuricemia the urate levels are higher than normal. Hyperuricemia and gout are closely related (Hall et al., 1967). The presence of hyperuricemia during an attack of acute gout is an important diagnostic indicator. However, many individuals have hyperuricemia for years without ever having an attack of gouty arthritis.

2.4 CAUSES OF HYPERURICEMIA

2.4.1 INBORN ERRORS OF METABOLISM

Enzyme deficiencies are known in patients who are primary over producers of urate. However, only about 5 percent of all cases of gout are due to these deficiencies. A strong family history exists in all types of primary gout (Graham and Scott, 1970). The best known of these deficiencies is the total or partial deficiency of the enzyme, hypoxanthine guanine phosphoribosyl transferase (HGPRTase), a familial neurological disorder and associated with hyperuricemia. This disorder was first described by Lesch and Nyhan in 1964 and is inherited as an x-linked recessive trait. The enzyme defect was identified by Seegmiller et al. (1967). Mothers of the affected children are heterozygotes and have two populations of fibroblasts, one with a normal phosphoribosyl transferase (PRTase) enzyme and others without enzyme activity (Rosenbloom et al., 1967).

Another condition which may lead to gout by the age of 10 years is the Type-I glycogen storage disease. This disease is due to the deficiency of the enzyme glucose-6-phosphatase and

occurs in infancy. Patients with this disease are unable to produce free glucose resulting in hyperlactic acidemia and ketosis. This condition has an inhibitory effect on the renal tubular excretion of uric acid (Yu et al., 1957).

Deficiency of an enzyme, erythrocyte glutathione reductase (EGRase) has been reported in conjunction with hyperuricemia in negro males (Long, 1967). An autosomal mode of inheritance is indicated but the connection between the two abnormalities is not understood.

2.4.2 IMPAIRED EXCRETION

Only ten percent of the total urate passing through the kidneys is finally excreted. There is a general disagreement as to the relationship between gout and kidney function. In a review, Steele (1979) has implied that hyperuricemia alone has a deleterious effect on kidney function. Berger and Yu (1975) failed to detect any harmful effect of untreated hyperuricemia on renal efficiency. However, Klinenberg et al. (1975) claimed that hyperuricemia may induce renal impairment even in the absence of symptoms.

Moderate hyperuricemia frequently occurs in chronic renal disease (Gresham et al., 1971). However, chronic renal insufficiencies are found only in a small proportion of gouty subjects (Sarre, 1964; Richet et al., 1965). A number of renal diseases appear to be associated with gout, for example, chronic lead nephropathy (Emmerson, 1963; Morgan et al., 1966),

polycystic kidney disease (Newcombe, 1973; Martinez-Maldonado, 1974) and renal amiloidosis (Richet et al., 1965).

Although acute gouty arthritis has been reported infrequently and even questioned as a complication of chronic renal disease, it is believed that impaired renal excretion is the major cause of hyperuricemia (Currie, 1979; Sorensen, 1980; Gibson et al., 1980).

2.4.3 DRUGS AND ALCOHOL

A number of drugs may interfere with the renal tubular handling of urate. The hyperuricemia associated with diuretic therapy has been found to be due to salt and water loss (Steele and Oppenheimer, 1969).

Salicylates, phenylbutazone, sulphinpyrazone and probenecid reduce urate excretion at low doses, whereas, at higher doses these drugs increase the excretion of urate (Wyngaarden and Kelley, 1978).

The ingestion of ethanol may precipitate an attack of gout (Wyngaarden and Kelley, 1976). This effect of alcohol has been attributed to decreased renal clearance of uric acid secondary to the hyperlactic acidemia associated with ethanol metabolism (Lieber and Davidson, 1962; Lieber et al., 1962; Beck, 1981; Yu et al., 1957; Maclachlan and Rodnan, 1967). Alcohol intake has also been implicated in the increased production of uric acid (Grunst et al., 1977; Delbarre et al., 1967a,b; Faller and

Fox, 1982).

There is a higher prevalence of gout amongst individuals who consume ethanol regularly (Saker et al., 1967; Pell and D'Alonzo, 1968) and populations with a higher incidence of gout have a high proportion of people who consume ethanol in excess (Gibson and Grahame, 1974).

2.5 DEPOSITION OF CRYSTALS

2.5.1 ORIGIN OF CRYSTALS AND THEIR RELATIONSHIP TO JOINT DISEASE

The relationship between joint diseases and crystal deposition is not well understood. A number of complex systems are present in the body to activate crystal formation where it is necessary, for example in the bones and teeth and inhibit it where it is undesirable, for example in the excretory organs, saliva and the urinary tract, all of which contain compounds which inhibit crystal growth. There are several instances where abnormal functioning of a system may cause a solute excess leading to crystallization. Patients with hyperuricosuria may develop renal stones. In joint disease there may be either a local or a generalized solute excess. For example, in CPPD deposition disease there is a localized excess of inorganic pyrophosphate in the joint, and in gout, there is an increased level of serum urate. However, true solute concentrations at various sites cannot be established because of the complex nature of the biological system.

Some of the suggested mechanisms related to MSUM crystal formation are listed in Table.1. (Scott, 1983). Little is known about these factors except those related to supersaturation, such as temperature.

The relationships between crystal deposition and joint disease have been studied by Dieppe (1982) and Dieppe and Calvert (1983). They have proposed various pathways involved in crystal related joint diseases (Figure 5). They believe that joint damage may also result in nucleation and crystal growth because of the removal of the inhibitors or introduction of some activators. Dieppe et al. (1983) have proposed some possible relationships between crystal deposition and joint disease (Figure 6). These include, (1) joint disease causes crystallization, (2) crystals cause joint disease, (3) crystal deposits are a by-product of a process causing arthritis, (4) crystal deposition and joint disease are independent and a chance relationship exists, and (5) joint damage and crystal deposition interact together.

2.6 CARTILAGE

Joints contain a variety of connective tissues such as ligaments, tendons, synovial membrane and cartilage. With the exception of cartilage, little is known about the composition of these tissues.

Almost, all vertebrate cartilages consists of a combination of connective (or skeletal) tissue cells and extracellular

Table 1. Suggested mechanisms related to MSUM *in-vivo* crystal formation (Scott, 1983).

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1. Supersaturation of serum or synovial fluid with MSUM.
2. Protein binding of urate.
3. Turnover of proteoglycans.
4. Temperature.
5. Trauma and exercise.
6. Altered hydrogen ion concentration.
7. Resorption of extravascular fluid.
8. Ageing and avascularity.

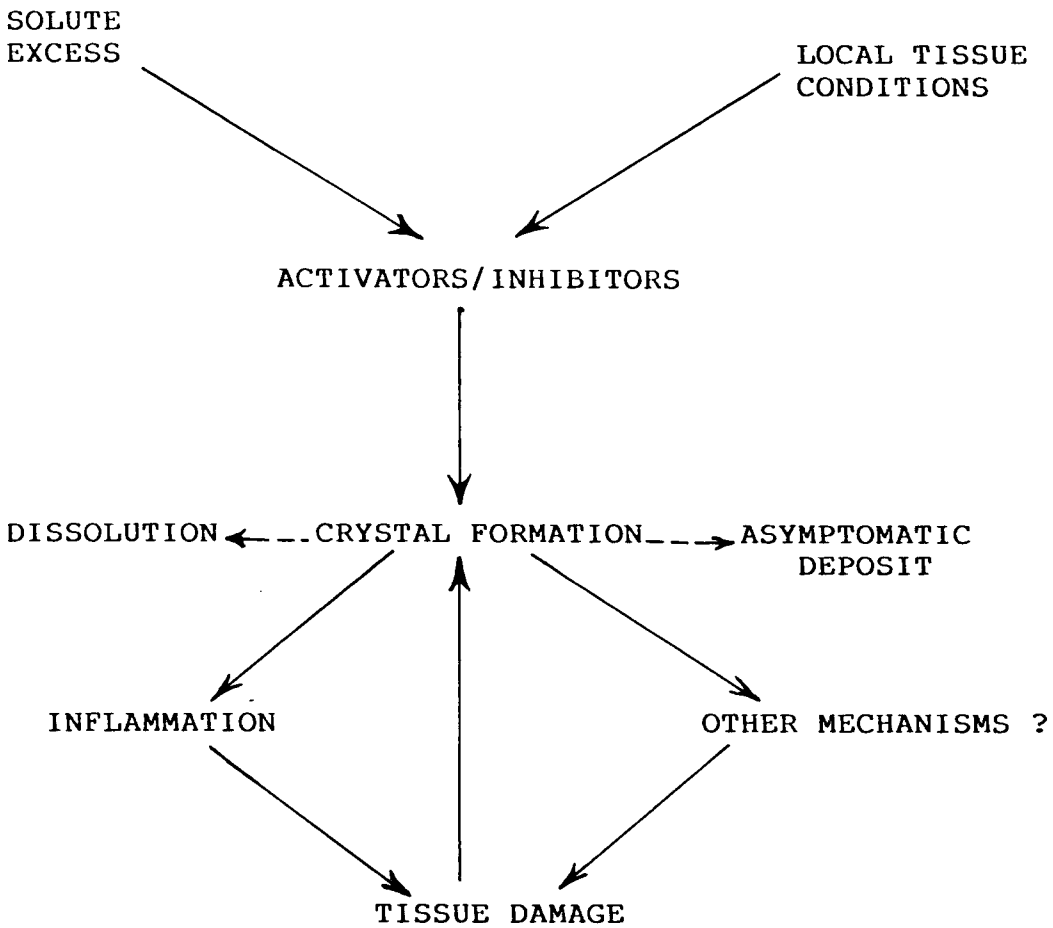


Figure 5. Possible pathways involved in crystal related joint diseases (Dieppe and Calvert, 1983).

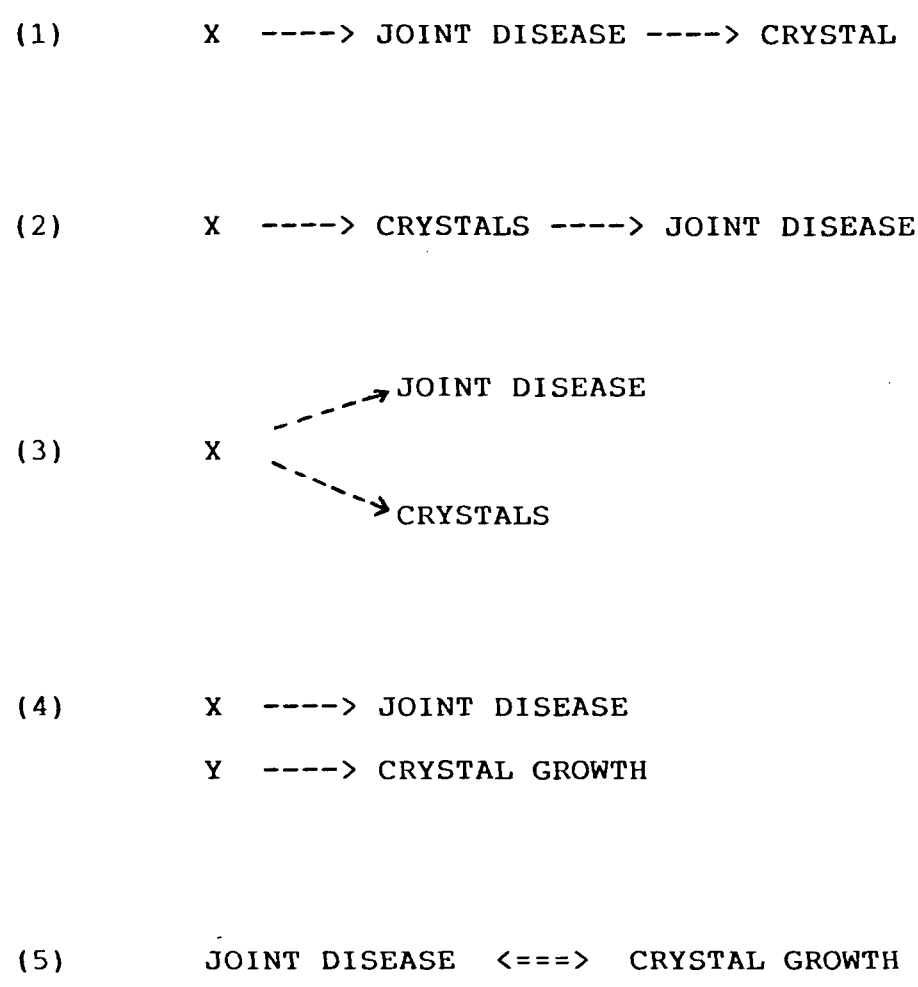


Figure 6. Possible relationships between crystal deposition and joint diseases (Dieppe et al., 1983).

substance (fibrous matrix and ground substance) (Moss and Moss-Salentijn, 1983).

Articular cartilage is an avascular tissue in which the cells (chondrocytes) are sparsely distributed in a stiff and abundant matrix (Edwards and Chrisman, 1979). In the joints, this tissue can withstand very high compressive loads.

2.6.1 COMPOSITION OF CARTILAGE

Articular cartilage consists mainly of three components, water, collagen and proteoglycan aggregates. The composition of articular cartilage varies markedly with depth and localization (Muir, 1979).

(A) WATER

The water content of articular cartilage is about 65-75% (Venn and Maroudas, 1977; Maroudas, 1979). The presence of water in conjunction with proteoglycans and collagen makes cartilage tough and elastic.

(B) COLLAGEN

Collagen is the major component of the extracellular matrix of all cartilages and comprises approximately 10% of the wet weight and 40-50% of the dry weight of the tissue (Anderson et al., 1964 ; Mayne and Vonder-Mark, 1983). Collagen appears as a fine mesh work of fibrils. The nomenclature for the collagen chains and their molecular organization was proposed by Bornstein and Sage (1980). Various types of collagens found in different

tissues have different chains and molecular organization.

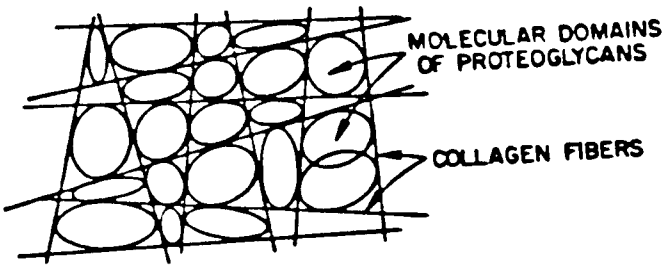
Proteoglycans can either interact with collagen directly (Smith et al., 1967) or possibly through a protein such as chondronectin (Hewitt et al., 1980) and a cartilage matrix protein (Paulsson and Heinegard, 1979, 1981).

(C) PROTEOGLYCAN AGGREGATE (PGA)

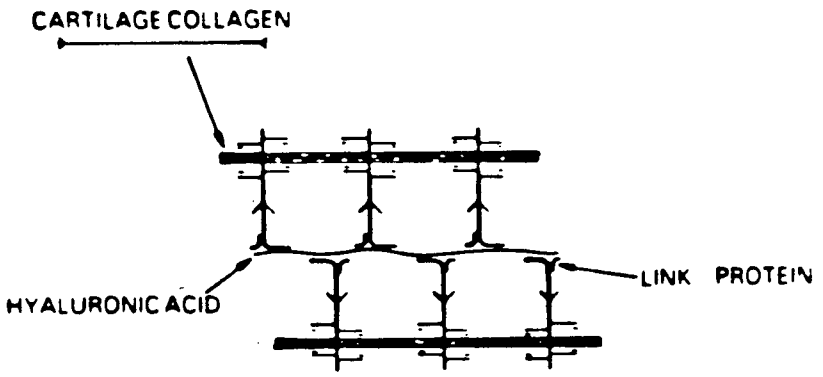
In normal articular cartilage, collagen forms a fibrous network, within which a random macromolecular mesh is present. This macromolecular mesh is an aggregate of some 30-40 proteoglycan subunits (monomers) each with a molecular weight of approximately one million and hyaluronic acid. Figure 7 shows a schematic representation of the domains of collagen and its regions of interaction with proteoglycan aggregate (Hascal and Heinegard, 1974; Hardingham and Muir, 1974). The ultrastructure of the proteoglycan aggregate was reported by Rosenberg et al. (1975). Figure 8 shows the most accepted model of the proteoglycan-hyaluronic acid aggregate (Hardingham et al., 1976). The biological function of proteoglycan aggregation is not known. However, the size of the aggregate immobilizes them in the collagen network and presumably has a role peculiar to the function of cartilage.

(I) PROTEOGLYCAN SUBUNIT (MONOMER)(PGM)

These are macromolecules which contain one or more glycosaminoglycan (GAG) chains, normally attached to protein.



(a)



(b)

Figure 7. Structure of articular cartilage:
(a) Domains of proteoglycan aggregate and collagen.
(b) Regions of interaction between proteoglycan aggregate and collagen.

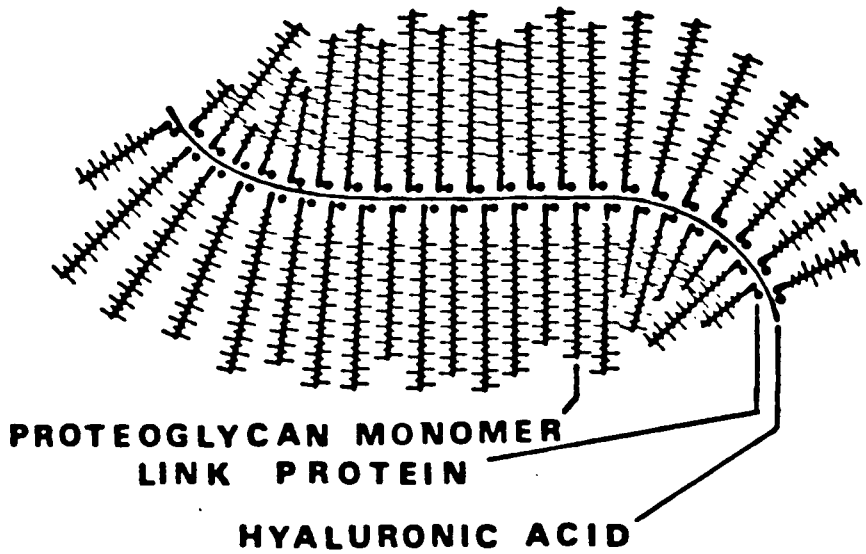
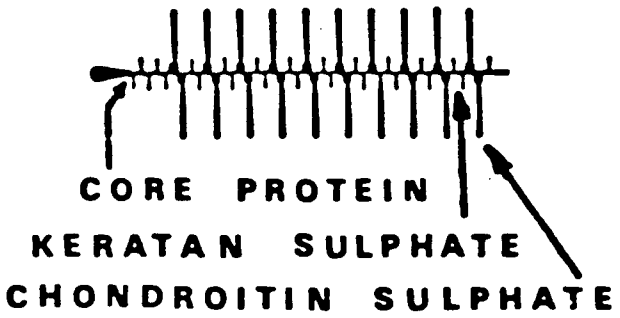


Figure 8. Structure of proteoglycan aggregate.

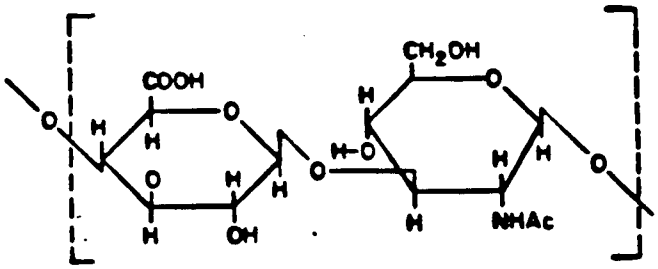
Different classes of proteoglycans (PGs) vary in the nature and size of the core protein, in the type, number, average size and degree of sulfation of the GAG chains, and the types of other oligosaccharides present on the core protein. Several reviews of the structure and functions of proteoglycans have been published (Hascall, 1981; Muir, 1980; Roden, 1980).

The cartilage PGs are composed of a protein core generally comprising about 10-20% of the weight of the molecule, to which a large number of GAG chains are attached laterally (Figure 9a). The structure of PG was first proposed by Mathews and Lozaityte (1958). In the cartilage PGs, chondroitin sulfate (CS) is the major GAG with smaller but variable amounts of keratan sulfate (KS).

Most of the PG-monomers can form an aggregate with hyaluronic acid (HA). However, there are minor PGs that cannot interact with HA to form an aggregate and these PGs can be extracted from cartilage by non dissociating salt solutions of physiological ionic concentration (Hardingham and Muir, 1974; Hardingham et al., 1976). These PGs are heterogeneous in molecular size and chemical composition (Brandt and Muir, 1971) and are of relatively low molecular weight (Tsiganos and Muir, 1969). They contain less protein and less KS than the majority of PGs (Hardingham and Muir, 1974; Brandt and Muir, 1969; Simunek and Muir, 1972a,b; Mayes, Mason and Griffin, 1973).



(a)



(b)

Figure 9. Structures of:
(a) Proteoglycan monomer,
(b) hyaluronic acid.

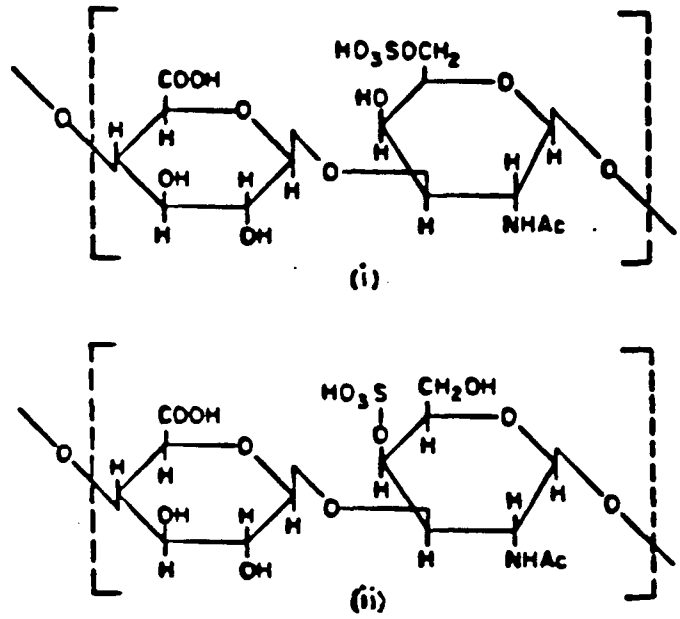
(Ia) CHONDROITIN SULFATE (CS)

Chondroitin sulfate consists of repeating units of glucuronic acid and N-acetyl galactosamine. An average chain of CS contains about 25-30 disaccharide units and about one sulfate group per disaccharide unit in one of the two isomeric positions, forming either chondroitin-6-sulfate or chondroitin-4-sulfate (Figure 10a). The distribution of the sulfate residues is not uniform along the chain, being fewer in the region near the linkage of carbohydrate to protein (Wasteson and Lindahl, 1971). Chondroitin-6-sulfate appears to predominate in human and bovine articular cartilage (Mourao et al., 1976; Murata and Bjelle, 1977), whereas in growth cartilage there are approximately equal proportions of the two isomers (Mourao et al., 1976).

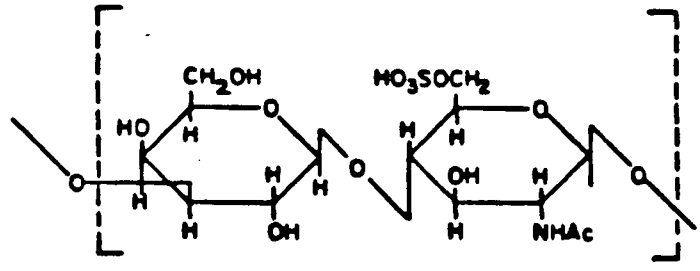
The biological significance of the position of the sulfate group is not clear. Both CS isomers show highly ordered helical conformations. The sulfate groups project further from the chain in chondroitin-6-sulfate than in chondroitin-4-sulfate (Isaac and Atkins, 1973; Atkins, 1977). It has been suggested that the presence of chondroitin-6-sulfate allows PGs to interact more strongly with the basic groups of collagen and other proteins.

(Ib) KERATAN SULFATE (KS)

Keratan sulfate is composed of disaccharide repeating units of galactose and N-acetylglucosamine (Figure 10b). Histochemical (Stockwell, 1970) and X-ray microprobe analysis (Maroudas, 1972) methods indicate that in the middle and deep



(a)



(b)

Figure 10. Structures of:
(a) Chondroitin (i:-6-; ii:-4-) sulfate,
(b) Keratan sulfate.

layers of articular cartilage the proportion of KS relative to CS varies.

KS is more variable than CS in both chain length and in degree of sulfation (Muir and Hardingham, 1975). Two populations of KS are thought to exist. One population has one sulfate group per disaccharide repeating unit, while the other population contains considerably more sulfate (Hjertquist and Lempberg, 1972). The skeletal KS has an average molecular weight of 5000-10,000 corresponding to about 13 disaccharide units (Robinson and Hopwood, 1973; Hascall and Riolo, 1972). Other sulfated cartilage GAGs include dermatan sulfate, heparin sulfate and heparin. The composition of various sulfated GAGs and their sources have been reviewed recently by Lash and Vasan (1983).

(II) HYALURONIC ACID (HA)

HA from cartilage has been isolated and fully characterized (Hascall and Heinegard, 1974; Hardingham and Muir, 1974; Hjertquist and Lempberg, 1972).

HA is a non-sulfated GAG composed of equimolar quantities of glucuronic acid and N-acetylglucosamine. Electron microscopic studies (Fessler and Fessler, 1966) show HA to consist of a single chain. Figure 9b shows the structure of HA.

In cartilage, HA plays an essential role in the aggregation of proteoglycans. It is present mainly as a component of PGAs and not in the free form.

(D) LIPIDS OF CARTILAGE

Lipid is found in the cells and in the matrix of human articular cartilage. Intracellular lipid is considered to be a normal constituent of cartilage because it is found in the absence of degenerative changes in the cells (Collins et al., 1965). Extracellular lipids have been identified in healthy articular cartilage from individuals in the second decade of life upwards (Ghadially et al., 1965; Stockwell, 1965; Zbinden, 1952; Schott, 1963; Marotti, 1963). Little is known about the nature of the lipids of articular cartilage. Staining characteristics and chemical analysis indicate that they are partly comprised of natural fats: triglycerides, cholesterol, cholesterol esters and phospholipids (Bonner et al., 1975; Pearse, 1968).

2.6.2 FACTORS AFFECTING THE COMPOSITION OF CARTILAGE

(A) EFFECT OF AGE

Adult articular cartilage shows increased degenerative changes with age (Collins and Meachim, 1961). Other changes include, a decrease in water content of cartilage with age (Ruttner et al., 1974) and a decrease in the collagen content with age on a dry weight basis (Werner et al., 1976).

The elasticity of cartilage is responsible for the resistance to compression and is related to the content and the structure of the proteoglycans in the cartilage matrix (Harris et al., 1972; Scott, 1973, 1975). The resistance to

compression of the cartilage decreases with age, probably due to the alterations in the proteoglycan structure of the articular cartilage (Schofield and Weightman, 1978; Armstrong et al., 1979).

Evidence indicates that the composition of the matrix of human articular cartilage changes with age (Elliot and Gardener, 1978, 1979; Venn, 1978; Inerot et al., 1978). The following is a summary of the effects of ageing on the composition of cartilage.

(i) Biochemical studies of changes in the nature and quantity of cartilage matrix glycosaminoglycans during ageing have shown different results. Little or no changes in total GAG content of articular cartilage were reported by Maroudas et al. (1973). Elliott and Gardner (1979) reported a decrease in the GAG content of the surface zone cartilage from 15% (dry weight) at about 4 years of age to about 7% by 70 years of age. This observation of a decrease in the total GAG content was also noted by Inerot et al. (1978), and Roughley and White (1980).

(ii) The hyaluronic acid content of the cartilage increases gradually with ageing. During the first decade of life, very little HA is detected. After ten years the HA content increases to 2% and reaches 6% by 60 years (Elliott and Gardner, 1979).

(iii) The proportion of proteoglycans that can be extracted with 4 molar guanidinium chloride decreases with

increasing age (Inerot et al., 1978; Roughley and White, 1980). However, Bayliss and Ali (1978a,b); Simunek and Muir (1972b) and Sweet et al. (1977) reported an increase in the extractable proteoglycan fraction with age.

(iv) The average molecular weight of chondroitin sulfate decreases from about 20,000 in young individuals to 16,000 in adults and the elderly (Hjertquist and Wasteson, 1972; Garg and Swan, 1981; Sweet et al., 1979). Ageing also results in a decrease in the proportion of chondroitin sulfate in the extracted proteoglycans (Strider et al., 1976; Bayliss, 1976; Venn, 1978). However, the relative proportion of chondroitin-6-sulfate to chondroitin-4-sulfate increases with age in adult human and bovine cartilage (Hjertquist and Wasteson, 1972; Hjertquist and Lempberg, 1972; Mankin and Lippiello, 1971; Lust and Pronosky, 1972; Lempberg et al., 1974; Murata and Bjelle, 1979; Rougley and White, 1980; Garg and Swan, 1981).

(v) The keratan sulfate content of the extractable proteoglycans increases wih age (Bayliss and Ali, 1978a,b; Inerot et al., 1978; Sweet et al., 1977; Elliott and Gardner, 1979). During ageing the superficial zone of adult cartilage seems to accumulate a higher percentage of KS (Elliott and Gardner, 1979). This increase has been thought to be due to a faster turnover of the GAG in the cartilage surface zone caused by wear during the mechanical functioning of the synovial joints (Elliott and Gardner, 1979).

(vi) Both intracellular and extracellular lipid

concentrations show a distinct increase with advancing age (Ghadially et al., 1965; Marotti, 1963; Bonner et al., 1975). The estimated increase per year is shown in Table 2 (Bonner et al., 1975; Hirsch and Peiffer, 1957; Ravetto, 1964).

(B) EFFECT OF INJURY

Studies using the articular cartilage of the rabbit showed that after a superficial injury, the first change occured in the matrix with a loss of proteoglycans along the margin of injury followed by enhanced GAG synthesis (Meachim, 1963).

After injury, the chondrocytes produce enzymes capable of degrading the cartilage matrix (Chrisman and Fessel, 1962). A cathepsin-like protease seems to be the enzyme responsible for cartilage degradation (Dingle and Dingle, 1980; Ziff et al., 1960).

(C) EFFECT OF JOINT DISEASE

Primary osteoarthritis is usually but not exclusively a disease of old age. Osteoarthritis secondary to disease or trauma often occurs in young and middle aged individuals. The changes in the joint cartilage are similar in both primary and secondary osteoarthritis (Inerot et al., 1978; Chrisman, 1969).

Kempson et al. (1971) showed that the elasticity of the cartilage decreases in osteoarthritic individuals. Since the elasticity of cartilage is largely related to the content and structure of proteoglycans, it is therefore likely that the

Table 2. The estimated increase in the lipid content of cartilage per year of age (Bonner et al., 1975; Hirsh and Peiffer, 1957; Ravetto, 1964).

Lipids	Estimated increase per year (% dry weight)	
	Superficial layer	Deep layer
Total lipid	0.046	0.03
Triglycerides	0.007	0.006
Cholesterol	0.008	0.003
Phospholipids	0.001	0.004

composition of the proteoglycans is changed in osteoarthritis. It has been suggested that degradation of the proteoglycans is an important step in the development of osteoarthritis (Mankin et al., 1971. Several studies have reported the following changes in the composition of cartilage in osteoarthritis.

(i) The degenerated osteoarthritic cartilage has a higher water content (McDevitt and Muir, 1976; Mankin and Zarins-Thrasher, 1975; Lipshitz et al., 1976).

(ii) Proteoglycan monomers from degenerated cartilage are reported to be smaller than those from normal cartilage of the same age (Inerot et al., 1978). The degradation of proteoglycans may be due to the increased enzyme activity of cathepsins (Ali and Evans, 1973) or neutral proteases (Martel-Pelletier et al., 1984).

(iii) The total GAG content is slightly lower (not significant) in degenerated than in normal cartilage of the same age (Inerot et al., 1978). Similar observations were also made by Bollet and Nance (1966) and Mankin and Lippello (1970).

(iv) In osteoarthritis the concentration of chondroitin-4-sulfate increases and the concentration of keratan sulfate decreases (Mankin and Lippiello, 1971).

2.7 SYNOVIAL FLUID

The synovial fluid is responsible for the nutrition and lubrication of the joint tissues. The synovial membrane

regulates both the volume and the macromolecular composition of the synovial fluid (Horst and Walitza, 1980).

2.7.1 COMPOSITION OF SYNOVIAL FLUID

The constituents of synovial fluid are derived from three possible sources (Swan, 1978).

(A) SOLUBLE CONSTITUENTS DERIVED FROM THE BLOOD

The electrolyte components, glucose and all but a few protein constituents are derived directly from the plasma. The relative proportions of the constituents depends primarily on their molecular weights. The relationship between molecular weight and concentration of serum protein in synovial fluid is given in Table 3 (Kushner and Somerville, 1971).

Albumin, the low molecular weight protein of plasma, constitutes about 75% of the total protein content of synovial fluid (Sandson and Hamerman, 1958).

(B) CONSTITUENTS SECRETED BY THE JOINT TISSUES

(I) HYALURONIC ACID (HA)

The HA of the synovial fluid is similar to the HA of cartilage. Only a small amount of HA is present in the synovial fluid and it is thought to be manufactured by the cell lining of the synovial membrane (Baxter et al., 1973). The function of HA in the synovial fluid is not clear but it is thought to maintain the viscosity of the synovial fluid.

Table 3. The relationship between molecular weight and concentration of serum proteins in synovial fluid.

Component	Molecular weight ($\times 10^3$)	Normal plasma concentration ($mg.mL^{-1}$)	Normal SF/Serum ratio
a_1-acid glycoprotein	44	0.75- 1.0	0.23 +0.09
Transferin	74	2.0 - 3.2	0.24 +0.08
Ceruloplasmin	160	0.27- 0.39	0.16 +0.04
a_2-macroglobulin	820	2.2 - 3.8	0.033+0.028
IgG	158	12.0 -18.0	0.13 +0.07
IgM	1000	---	0.045+0.024

(II) LUBRICATING GLYCOPROTEINS

The purified lubricating proteins present in the synovial fluid represent about 0.5% of the total protein in the synovial fluid (Swan and Radin, 1972).

(C) PRODUCTS DERIVED FROM THE CATABOLISM OF JOINT TISSUES

Disease states such as osteoarthritis and rheumatoid arthritis result in persistant PG loss from the articular cartilage. Synovial fluid removes the degradation products by lymphatic uptake or phagocytic uptake. Chondroitin sulfate is present in synovial fluid as a normal constituent (Silpananta et al., 1967).

2.7.2 LIPIDS OF SYNOVIAL FLUID

Normal synovial fluid contains some phospholipids, cholesterol and some neutral lipids. Among the phospholipids, phosphaphatidylcholine, sphingomyelin, lysolecithin, phosphatidylinositol and cephalins have been identified (Bole, 1962; Chung et al., 1962).

2.7.3 FACTORS AFFECTING THE COMPOSITION OF SYNOVIAL FLUID

(A) EFFECT OF JOINT DISEASE

The total synovial fluid protein content is usually increased in degenerative joint disease and rheumatoid arthritis (Currey and Vernon-Roberts, 1976). The total protein content increased

from 1.8-2.1 g dL^{-1} in normal joints to 2.9-3.9 g dL^{-1} in osteoarthritis and to 4.2-4.9 g dL^{-1} in rheumatoid arthritis (Markowitz, 1983). However, the percentage of albumin decreased from about 56-63% in normal joints to 42-52% in rheumatoid arthritis and the percentage of high molecular weight proteins (globulins) increased (Markowitz, 1983).

Chondroitin sulfate is present in the normal synovial fluid, and is probably released as a result of the catabolic process in articular cartilage (Silpananta et al., 1967). The levels of chondroitin sulfate in the synovial fluid are elevated in degenerative joint disease and rheumatoid arthritis (Seppala et al., 1972; Barker et al., 1966).

Diseased joints in osteoarthritis and rheumatoid arthritis show greatly increased lipid levels (Bole, 1962; Chung et al., 1962; Small et al., 1964; Newcombe and Cohen, 1965). The increase in triglyceride content is less than the increase in phospholipid and cholesterol content (Bole, 1962). The concentration of phospholipids in the synovial fluid also shows a distinct change in disease states. Chung et al.(1962) reported a decrease in the levels of phosphatidylcholine and an increase in cephalin and sphingomyelin levels in the synovial fluid in osteoarthritis or rheumatoid arthritis.

2.8 THEORY OF CRYSTAL GROWTH

The deposition of a solid crystalline phase from solution can only occur if some degree of supersaturation or supercooling

has first been achieved in the system. Any crystallization process can be considered to be comprised of three basic steps:

(A) Achievement of supersaturation or supercooling.

(B) Formation of crystal nuclei.

(C) Growth of the crystals.

2.8.1 SUPERSATURATION

A solution which is in equilibrium with the solid phase is said to be saturated with respect to that solid. However, a solution containing more dissolved solid than that represented by the saturation solubility is said to be supersaturated. Uncontaminated solutions in clean containers, cooled slowly, without disturbance, in a dust free atmosphere, can readily be made to show appreciable degrees of supersaturation. The state of supersaturation is an essential feature of crystallization. Below the saturation solubility, all solutions are stable.

Ostwald in 1897 introduced the terms 'labile' (unstable) and 'metastable' supersaturation. These terms refer to supersaturated solutions in which spontaneous deposition of the solid phase in the absence of solid nuclei, will and will not occur, respectively. A solubility-supersolubility diagram is shown in Figure 11. The lower continuous line is the normal solubility curve for the particular salt. Temperatures and concentrations at which spontaneous crystallization occurs are represented by the upper broken curve, referred to as the supersolubility curve. This curve is not as well defined as the solubility curve and

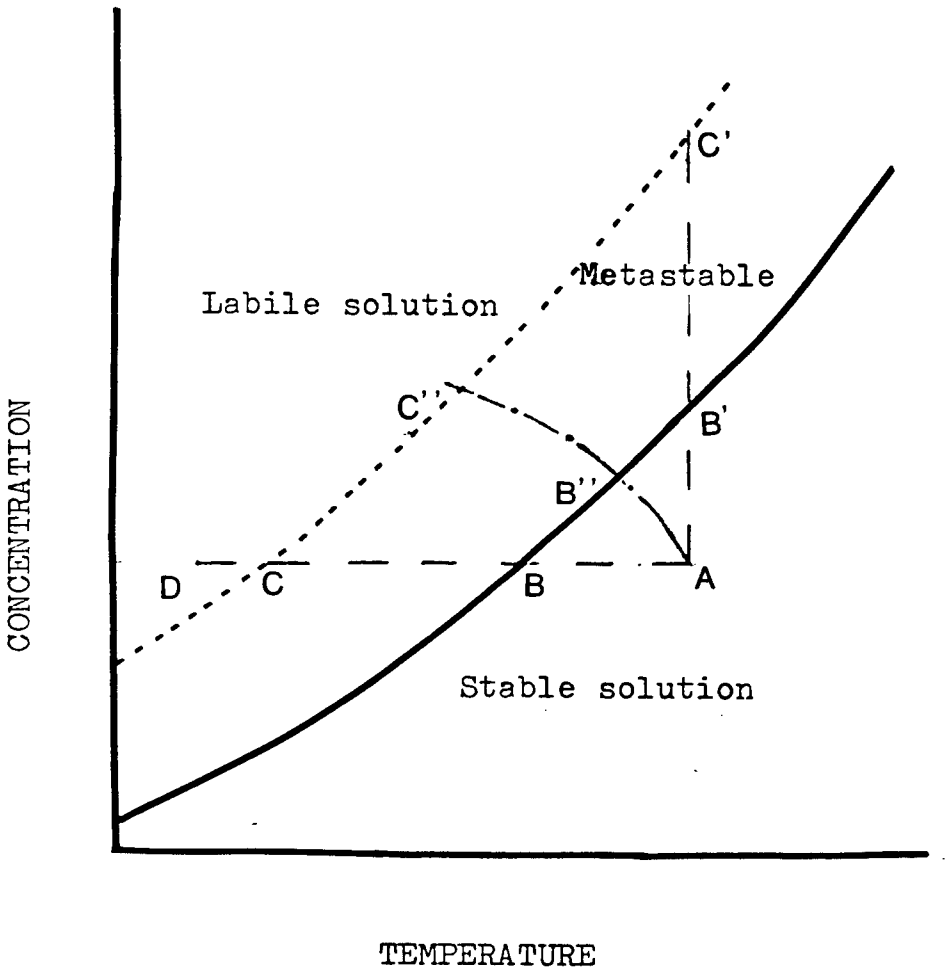


Figure 11. The solubility-supersolubility diagram.

its position on the diagram depends on the degree of agitation of the solution. Despite the fact that the supersolubility curve is ill-defined, there exists a region of metastability in the supersaturated region above the solubility curve. In the metastable region, spontaneous crystallization is improbable. However, if foreign particles (seed crystals) are introduced into such a solution, crystal growth occurs until the concentration of solute in solution reaches the saturation solubility. When the supersaturation is greater than the limits of the metastable region, a region of unstable solution is attained, where spontaneous crystallization is probable.

If a solution represented by point A in Figure 11 is cooled without loss of solvent (line ABC), spontaneous crystallization cannot occur until conditions represented by point C are reached. At this point, crystallization may be spontaneous or it may be induced by seeding, agitation or by mechanical shock. Further cooling to point D may be necessary before crystallization can be induced, particularly with highly soluble substances.

Supersaturation can also be achieved by removing the solvent from the solution by evaporation carried out at constant temperature (line A B'C') in Figure 11.

A coefficient or degree of supersaturation, s', can be defined by:

$$s' = \frac{C}{Cs} \qquad (1)$$

where C is the concentration of the substance in solution at some given temperature, and Cs is the equilibrium saturation concentration of solute in the solvent at the same temperature.

deCoppet in 1872 produced evidence to show that the length of time of stability of a supersaturated solution was inversely proportional to the degree of supersaturation.

2.8.2 NUCLEATION

Supersaturation alone is not sufficient for a system to begin to crystallize. Before crystals can grow there must exist in solution a number of minute solid bodies known as centers of crystallization, seeds, embryos or nuclei. Nucleation may occur spontaneously or it may be induced artificially, referred to as homogeneous and heterogeneous nucleation respectively.

(A) HOMOGENEOUS NUCLEATION

Exactly how a crystal nucleus is formed within a homogeneous fluid is not known with any degree of certainty. The condensation of a supersaturated vapour to the liquid phase is only possible after the appearance of microscopic droplets. However, as the vapour pressure at the surface of these minute droplets is very high, they evaporate rapidly even though the surrounding vapour is supersaturated. New nuclei form while old ones evaporate, until eventually stable droplets are formed either by coagulation or under conditions of very high supersaturation. The mechanism of homogeneous nucleation is

thought to be as follows:

Minute structures are formed, first from the collision of two ions or molecules, then from a third with the pair, and so on, to form clusters. For crystallization to occur, the energy barrier for nucleation must be exceeded.

The formation of a liquid droplet or solid particle within a homogeneous fluid requires the expenditure of a certain amount of energy in the creation of the liquid or solid surface. The total quantity of work, W, required to form a stable crystal nucleus is equal to the sum of the work required to form the surface, W_s (a positive quantity), and the work required to form the bulk of the particle, W_v (a negative quantity), so that

$$W = W_s - W_v \qquad (2)$$

For the formation of a spherical liquid droplet in a supersaturated vapour, for example, equation 2 can be written as

$$W = a\sigma - v\Delta p \qquad (3)$$

where σ is the surface energy of the spherical droplet per unit area, Δp the pressure difference between the vapour phase and the interior of the liquid droplet, and a and v are the surface area and volume, respectively, of the droplet. If r is the radius of the droplet, then

$$a = 4\pi r^2$$

$$v = \frac{4}{3}\pi r^3$$

and $$\Delta p = \frac{2\sigma}{r}$$

therefore equation 3 becomes

$$W = \frac{4}{3}\pi r^2 \sigma \qquad (4)$$

The increase in the vapour pressure of a liquid droplet as its size decreases can be estimated from the Gibbs-Thomson equation

$$\ln \frac{pr}{p^*} = \frac{2M\sigma}{RT\rho r} \qquad (5)$$

where, pr and p* are the vapour pressure over a liquid droplet of radius r and a flat liquid surface, respectively, M is the molecular weight, ρ, the density of the droplet, T, the absolute temperature and R, the gas constant.

The term, pr/p* is a measure of the supersaturation, s', of the system, so equation 5 becomes

$$\ln s' = \frac{2M\sigma}{RT\rho r} \qquad (6)$$

$$\text{or} \qquad r = \frac{2M\sigma}{3(RT\rho \ln s')} \tag{7}$$

Substituting for r in equation 4

$$W = \frac{16\pi\sigma^3 M^2}{3(RT\rho \ln s')^2} \tag{8}$$

Equation 8 gives a measure of the work of nucleation in terms of the degree of supersaturation of the system. When s'=1, (a saturated solution), ln s'= 0 and the amount of energy required for nucleation is infinite.

The free energy changes associated with the process of homogeneous nucleation are as follows. The overall excess free energy ΔG, between a small solid particle of solute and the solute in solution is equal to the sum of the surface excess free energy, ΔG_s, that is the excess free energy between the surface of the particle and the bulk of the particle, and the volume excess free energy ΔG_v, that is the excess free energy between a very large particle ($r = \infty$) and the solute in solution. ΔG_s is a positive quantity and is proportional to r^2. In a supersaturated solution ΔG_v is a negative quantity proportional to r^3. These relationships are shown in Figure 12. As r increases, the overall excess free energy, ΔG, reaches a maximum value when the nucleus achieves a critical size (r_c), that is,

$$\Delta G_{crit} = \frac{4\pi\sigma (r_c)^2}{3} \tag{9}$$

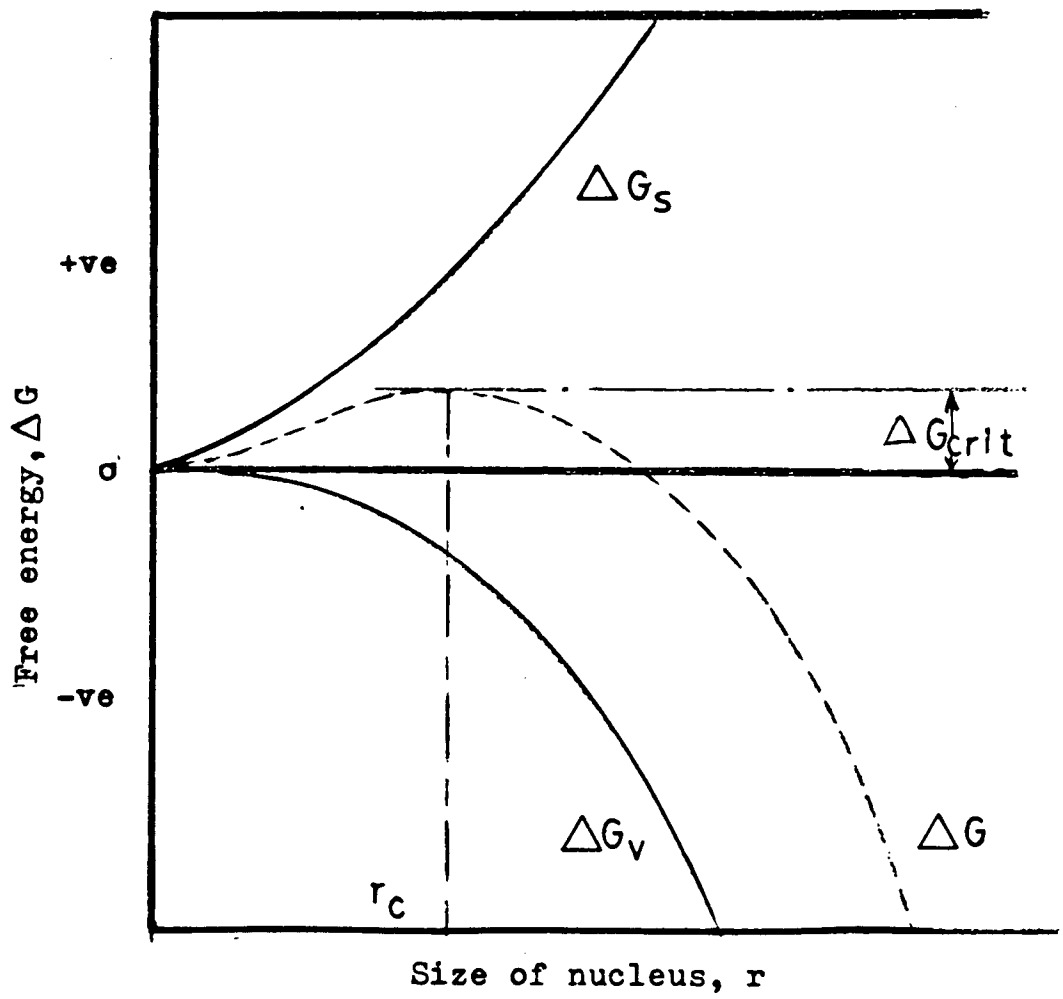


Figure 12. Free energy diagram for nucleation explaining the existence of a critical nucleus.

The critical size r_c, represents the minimum size of a stable nucleus. Particles smaller than r_c will dissolve.

The rate of nucleation, N (the number of nuclei formed per unit time per unit volume), is given by,

$$N = A \exp(-\Delta G/RT) \tag{10}$$

where A is a constant and ΔG is the overall excess free energy of the particle, that is, the work of nucleation W, from equations 8 and 10,

$$N = A \exp\left(\frac{16\pi\mathcal{E}^3 M^2}{3R^3T^3\rho^2(\ln s')^2}\right) \tag{11}$$

This equation shows the dependence of the rate of nucleation on the temperature, T, the degree of supersaturation, s', and the interfacial tension, $\mathcal{E}$.

A plot of the nucleation rate, N versus the degree of supersaturation, s', is shown in Figure 13. A rapid increase in the rate of nucleation is evident once a critical level of supersaturation is exceeded.

(B) HETEROGENEOUS AND SECONDARY NUCLEATION

Crystallization may be induced by inoculating or seeding a supersaturated solution with small particles of the material to be crystallized. Effective seed crystals do not have to be the

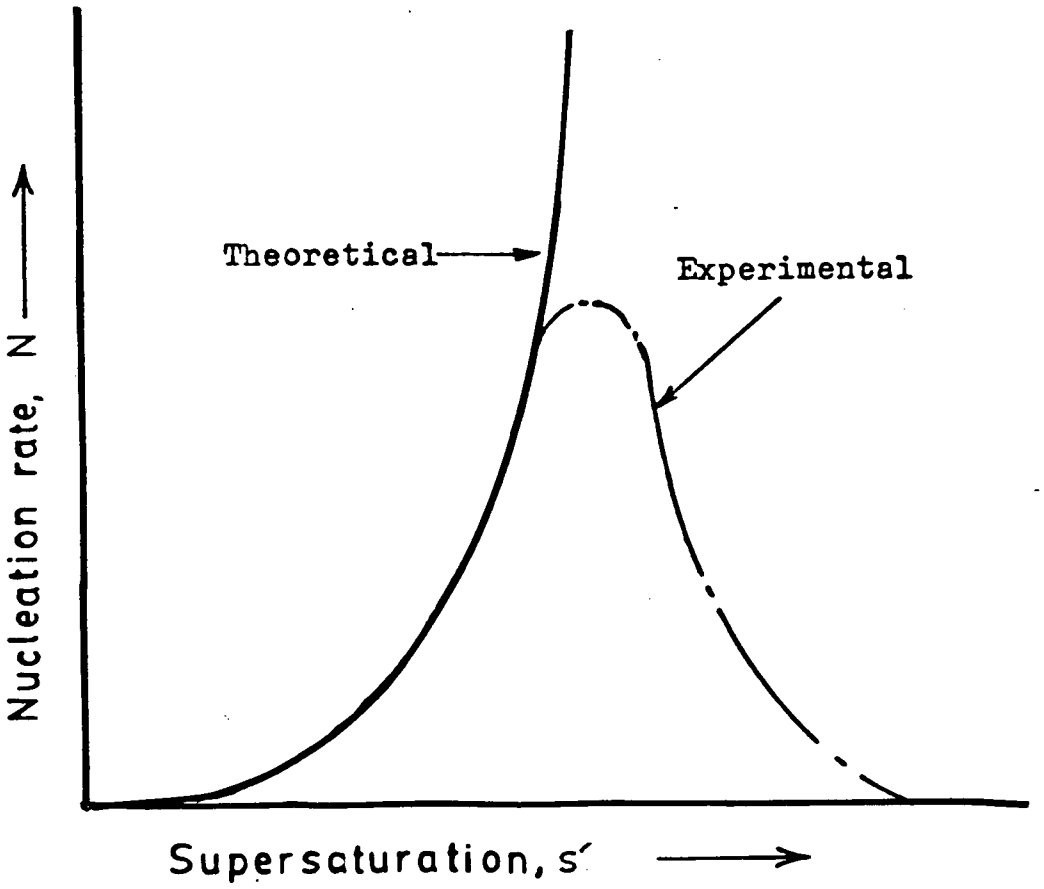


Figure 13. Effect of supersaturation on the nucleation rate.

material being crystallized. For example isomorphous compounds will frequently induce crystallization of the supersaturated solution. There is some evidence to show that nucleation by seeding is dependent on the surface charge of the nucleating substrate (Edwards and Evans, 1962).

The presence of a suitable seeding material induces nucleation at degrees of supersaturation lower than those required for homogeneous nucleation. The overall free energy change associated with the formation of a critical nucleus for heterogeneous nucleation, $\Delta G'_{crit}$, is less than the corresponding free energy change, ΔG_{crit}, associated with homogeneous nucleation is given by,

$$\Delta G'_{crit} = \Delta G_{crit} \phi \qquad (12)$$

where ϕ is less than unity, and is given by equation 13 (Volmer,1939).

$$\phi = \frac{(2 + \cos\theta)(1 - \cos\theta)^2}{4} \qquad (13)$$

where $\cos\theta = \dfrac{\gamma_{sl} - \gamma_{cs}}{\gamma_{cl}}$

γ_{sl} = the interfacial energy between the surface of the seed and the liquid,

γ_{cs} = the interfacial energy between the

surfaces of the crystallizing phase and the seed surface.

σ_{cl} = the interfacial energy between the crystallizing phase and the liquid, and

$\sigma_{sl} = (\sigma_{cs} + \sigma_{cl} \text{ Cos } \theta)$

2.8.3 CRYSTAL GROWTH

As soon as stable nuclei, that is particles larger than the critical size are formed in a supersaturated system, they begin to grow into crystals of a visible size. Three theories of crystal growth have been proposed.

(A) SURFACE ENERGY THEORIES

A droplet of a liquid is most stable when its surface free energy and thus, its surface area is a minimum. Gibbs (1928) suggested that the shape of a growing crystal would be such that the surface energy was a minimum. The total free energy of a crystal in equilibrium with its surroundings at constant temperature and pressure is a minimum for a given volume. If the volume free energy per unit volume is assumed to be constant throughout the crystal, then

$$\sum_{1}^{n} a_i g_i = \text{minimum} \qquad (14)$$

where a_i is the area of the i th face of the crystal bounded by n faces, and g_i is the surface free energy per unit area of the i th face. If a crystal is allowed to grow, it

should develop into an equilibrium shape to ensure minimum total surface free energy for a given volume of the crystal. Wulff (1901) showed that the equilibrium shape of a crystal is related to the free energies of the faces. He suggested that the crystal faces would grow at rates proportional to their respective surface energies. Laue (1943) modified Wulff's theory pointing out that all possible combinations of faces must be considered to determine which of the overall surface free energies represent a minimum. There is litle quantitative evidence to support the surface energy theories and they have not been generally accepted.

(B) ADSORPTION LAYER THEORIES

A theory of crystal growth based on the existence of an adsorbed layer of solute atoms or molecules was proposed by Volmer (1939). Contributions and modifications to Volmer's theory have been made by Brandes (1927); Stranski (1928) and Kossel (1934).

Atoms, ions or molecules will attach themselves onto the crystal surface where the attractive forces are greatest, that is they migrate towards positions where a maximum number of like elements are located (Figure 14a). This stepwise build up will continue until a whole plane surface is completed (Figure 14b). Before the crystal surface can continue to grow, another "center of crystallization" must be formed on the plane surface. It was suggested that a monolayer island nucleus (two dimensional nucleus) was created on the crystal surface (Figure 14c).

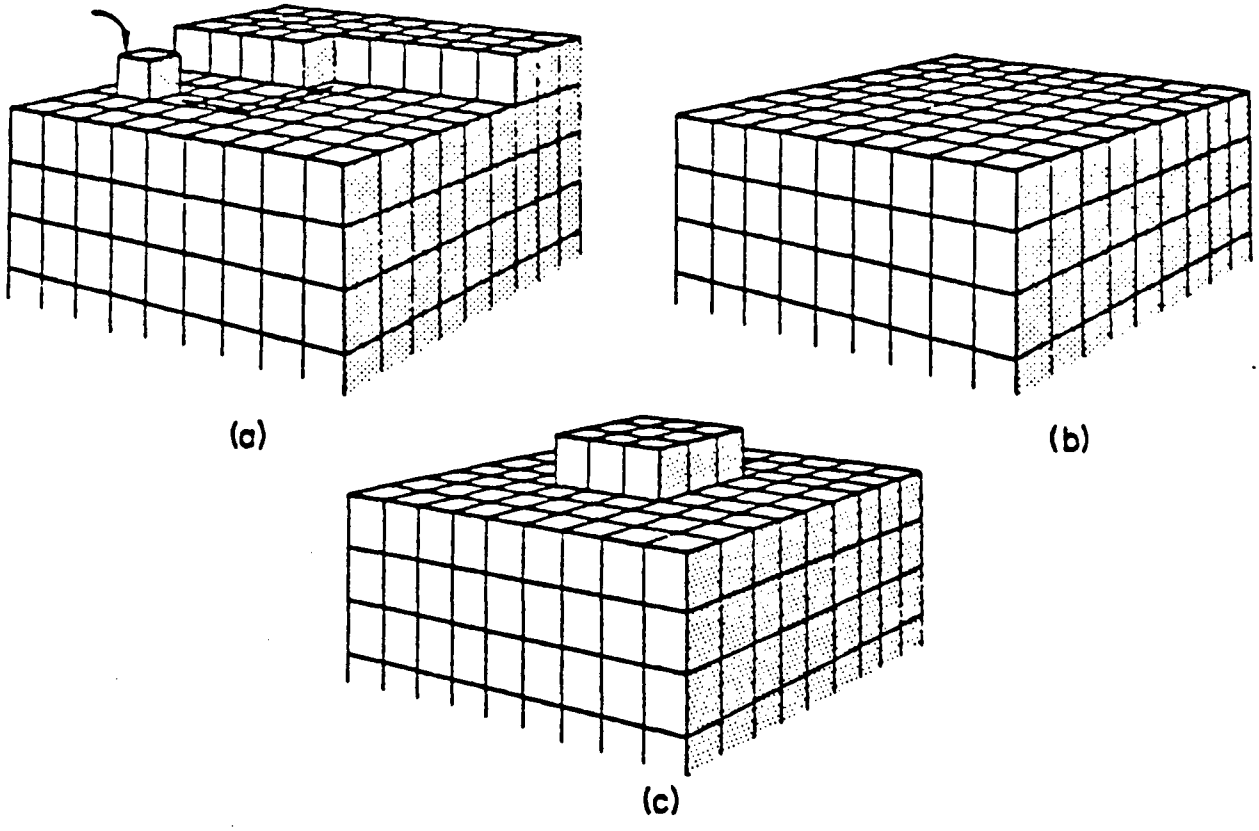


Figure 14. Crystal growth without dislocations:
(a) migration towards desired location;
(b) completed layer;
(c) surface nucleation.

However, a high degree of supersaturation is necessary for this type of two dimensional nucleation to occur (Mullin, 1961).

Kossel (1934) proposed a model of a growing crystal face (Figure 15). The flat surface is divided into two regions by a monatomic step and the step itself may be incomplete, showing one or more kinks. In addition there are loosely adsorbed growth units on the crystal surface and vacancies in the surface and steps. Growth units are more easily incorporated at a kink site. The face is completed by the movement of the kink along the step and a fresh step is created by surface nucleation. According to this theory, a crystal should grow fastest when its faces are entirely covered with kinks.

(I) DISLOCATIONS

Most crystals contain dislocations. Line defects or dislocations are one dimensional defects. A dislocation is a region where the atoms are not properly surrounded by neighbours. There are two types of dislocation, the edge dislocation and the screw dislocation. A dislocation can be defined with the aid of a Burgers circuit. This is any atom-to-atom path made in a crystal which forms a closed loop. If the circuit is made in an ideal dislocation-free crystal, the vector to complete the circuit is zero. If the Burgers circuit encloses a dislocation, the closure failure is the Burgers vector (b_o). In edge dislocations the Burgers vector is perpendicular to the dislocation and in screw dislocations it is parallel (Figure 16)

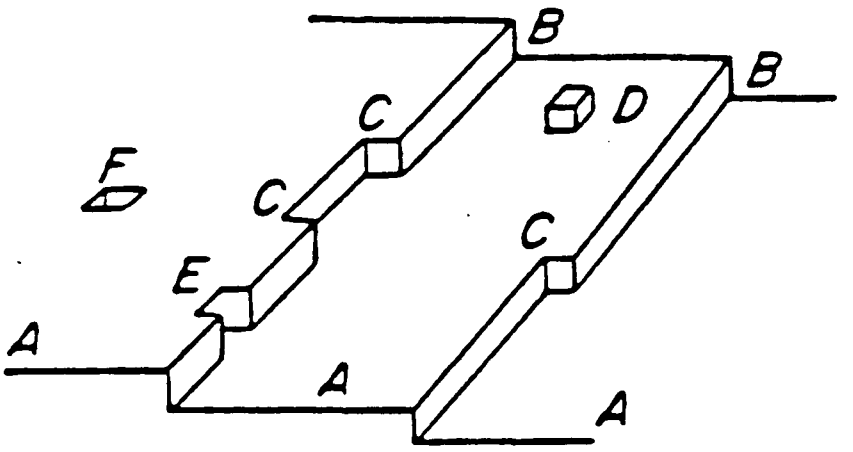
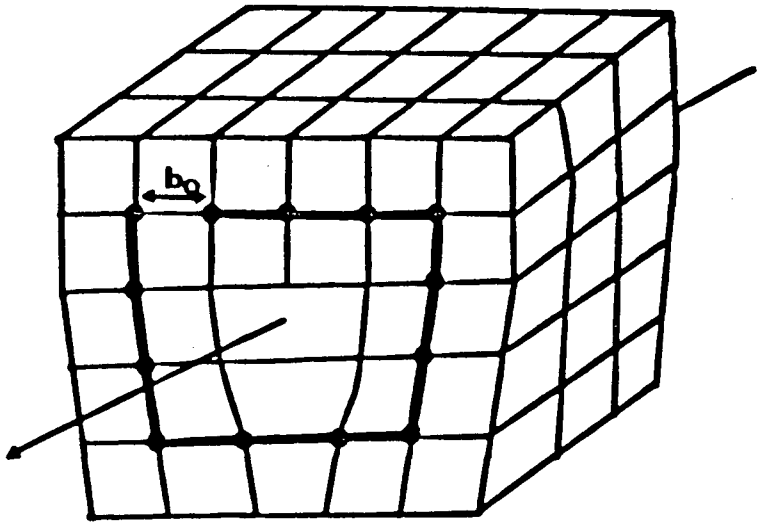


Figure 15. Kossel's model of a growing crystal surface: (A) flat surfaces; (B) steps; (C) kinks; (D) surface-adsorbed growth units; (E) edge vacancies; and (F) surface vacancies.



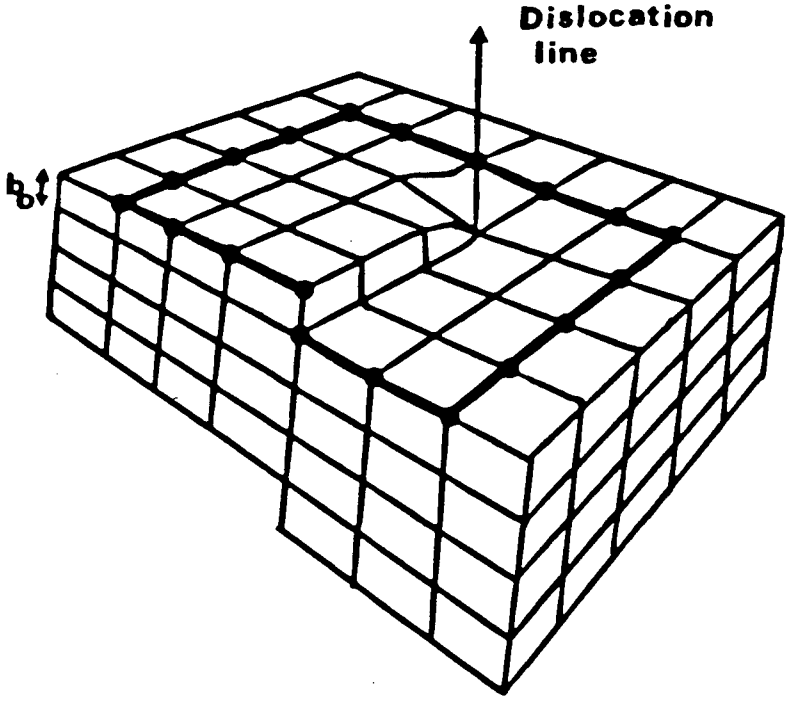


Figure 16. Dislocations in crystal:
(a) an edge dislocation, and
(b) screw dislocation.

Frank (1949) proposed several ways in which dislocations could arise during the growth of a crystal and these have been reviewed by Nabarro (1967) and Albon (1963).

The dislocations are formed during the growth process under the influence of surface and internal stresses. Screw dislocations are considered important for crystal growth, since they eliminate the need for surface nucleation for crystal growth to continue.

Screw dislocations give rise to a particular mode of growth, first postulated by Frank (1949). Once a screw dislocation has been formed, the crystal face can continue to grow. Figure 17 (a-c) shows the successive stages in the development of a growth spiral from a screw dislocation. Frequently, complex spirals develop when several dislocations grow together (Verma, 1953; Read, 1953). Burton, Cabrera and Frank (1951) developed a kinetic theory based on the screw dislocation mechanism of crystal growth and were able to calculate the growth rate at any supersaturation. The Burton, Cabrera and Frank (B.C.F) relationship is given by

$$R_g = A\sigma^2 \tanh (B/\sigma) \qquad (15)$$

where R_g is the growth rate, σ = s'-1 and s'= C/Cs. A and B are complex temperature dependent constants.

At low supersaturation concentrations, R_g is proportional to σ^2, but at high supersaturation concentrations R_g is

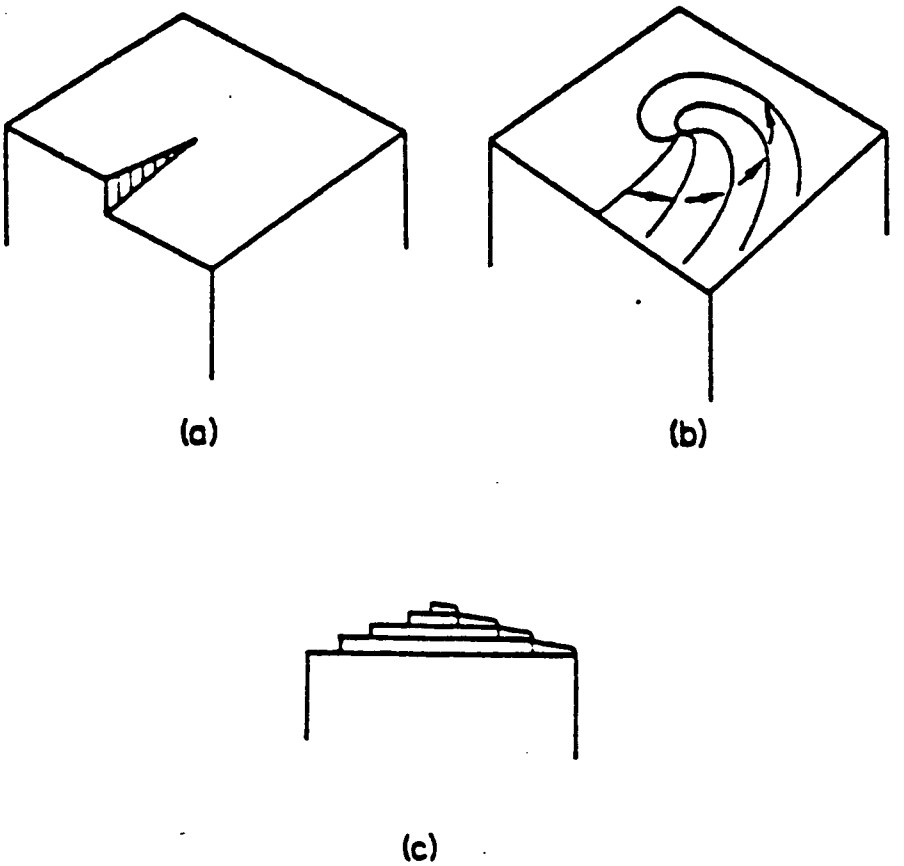


Figure 17. Development of a growth spiral starting from a screw dislocation.

proportional to σ, or the relationship changes from a parabolic (square) growth law to a linear growth law as the supersaturation increases.

(C) DIFFUSION THEORIES

Noyes and Whitney (1897) proposed that the deposition of solid on the face of a growing crystal was the reverse of the dissolution process and was essentially a diffusion process. According to them the rates of crystal growth or dissolution were governed by the difference between the concentrations at the solid surface and in the bulk of the solution, given in the form

$$R_g = K_t \, S \, (C-Cs) \qquad (16)$$

where R_g is the growth rate, S is the surface area of the solid, C is the solute concentration in the supersaturated solution, Cs is the saturation solubility and K_t is the transport rate constant.

Nernst (1904) modified the Noyes-Whitney equation with the assumption that there was a thin stagnant film of liquid adjacent to the growing face, through which solute molecules diffused. The modified relationship was:

$$R_g = \frac{D}{h} \, S \, (C - Cs) \qquad (17)$$

He concluded that $K_t = (D/h)$, where D = coefficient of diffusion of solute and h = thickness of the liquid film.

The thickness of the liquid film depends upon the relative solid-liquid velocity, that is on the degree of agitation. Marc (1908, 1909a,b, 1910) observed that K_t did not increase indefinately with increasing solution velocity but reached some limiting value. He suggested that crystallization is not necessarily the reverse of dissolution and that film diffusion alone is not sufficient to explain the mechanism of crystallization. Berthoud (1912) and Valeton (1923a,b; 1924) consequently modified the diffusion theory of crystal growth and suggested that crystal growth involved two steps. Firstly, a diffusion process, whereby solute molecules are transported from the bulk of the fluid phase to the solid surface, followed by a first order reaction where solute molecules are incorporated into the crystal lattice. These two steps, occuring under the influence of different concentration driving forces can be represented by the equations:

$$R_t = K_t S (C-Ci) \qquad (18)$$

and

$$R_s = K_s S (Ci-Cs) \qquad (19)$$

where K_t is the transport rate constant (coefficient of mass transfer by diffusion), K_s is the surface reaction rate constant, Ci is the solute concentration at the crystal-solution interface, R_t is the rate of arrival of solute at the surface by diffusion from the bulk solution and R_s is the rate of integration into the solid lattice (surface reaction).

Equations 18 and 19 are of little use in practice because

the concentration Ci is difficult to measure. It is possible to eliminate the term Ci since at steady rate, $R_t = R_s = R_g$. Then the growth rate can be written as:

$$R_g = K_o(obs)\ S\ (C-Cs) \qquad (20)$$

where $K_o(obs)$ = observed overall growth rate constant,

$$\text{and} \quad \frac{1}{K_o(obs)} = \frac{1}{K_t} + \frac{1}{K_s}$$

Equation 20 describes crystal growth when the two consecutive reactions, transport of solute by diffusion and a surface reaction step, are of comparable velocity.

For the crystallization of an ionising solute from an aqueous solution, the two consecutive step mechanism of crystal growth may not explain the growth because of the involvement of several processes simultaneously (Mullin, 1972). Some of these processes include:

- Bulk diffusion of solvated ions through the diffusion layer.
- Bulk diffusion of solvated ions through the adsorption layer.
- Surface diffusion of solvated or unsolvated ions.
- Partial or total desolvation of ions.
- Integration of ions into the lattice.
- Counter diffusion of released water through the adsorption layer.

- Counter diffusion of water through the boundary layer.

Any of these processes may become rate-controlling and render the crystal growth phenomenon very complex.

Marc (1908) investigated the rate of crystallization of a number of salts and found that in many cases the dependence of growth rate on supersaturation was greater than first order. Thus equation 20 may be written as:

$$R_g = K_o(obs)\ S\ (C-Cs)^n \qquad (21)$$

where n = the order of the overall growth process.

2.8.4 FACTORS AFFECTING CRYSTAL GROWTH RATES

(A) EFFECT OF SEED CRYSTAL SIZE AND SEED SURFACE AREA

The rate constant K_s for the surface integration process is expected to be independent of mass transfer in the solution, and therefore is independent of crystal size. However, K_t, behaves as a normal mass transfer coefficient and thus should show dependence on crystal size and solution velocity (Phillips, 1973).

In the seeded growth technique, reduction in the size of seed crystals would result in an increase of the growth rate. During crystal growth in the presence of seeds the surface area of the seeds increases progressively. Little is known about the effect of that increase on the crystal growth kinetics. In a

recent report, Barone et al. (1983) discussed the relationship between the increase in surface area and mass deposited on the growing crystal. The following is a brief summary of their discussion.

The rate of crystal growth for a seeded system can be interpreted in terms of empirical (Nancollas, 1978) or theoretical expressions (O'Hara and Reed, 1973; Christoffersen and Christoffersen, 1976; Christoffersen, 1980). The functional form of the rate equation (equation 20) for many ionic compounds growing in an aqueous solution has been detailed by Barone et al.(1983) and can be written as,

$$R_g = -\frac{dc_t}{dt} = K_{(T)}\ {}^{e}S_t\ f(o_t) \qquad (22)$$

where c_t is the total mass of solid at time t, $K_{(T)}$ a rate constant dependent on the temperature T, ${}^{e}S_t$ the total effective surface area at time t, and $f(o_t)$ a term dependent on the supersaturation, $o_t = (C - Cs)/ Cs$, where C and Cs are the concentrations of precipitating ions or molecules in solution at time t and at saturation respectively.

The effective surface area, ${}^{e}S_t$, is different from geometric total surface area (S_t) which depends only on the geometry of the crystal and is equal to the quantity measured, for example by BET gas adsorption. ${}^{e}S_t$ depends on the

dimensional type of the growth, and is the quantity which determines the rate of reaction. For 1-dimensional growth of cubic shaped crystals, growth occurs only along two parallel faces resulting in no increase in effective surface area. Similarly, for 3-dimensional growth $S_t = {}^eS_t$ and for two dimensional growth $S_t > {}^eS_t$

As the seed crystals grow, the effect of an increase in surface area on the rate of crystal growth is appreciable for some salts (Barone and Nancollas, 1978) and negligible for others (Liu and Nancollas, 1970). In cases where the rate of growth changes as the surface area increases the relative increase in surface area (S_t/S_o) or effective total surface area (${}^eS_t/{}^eS_o$) has a direct relationship which is dependent on the type of growth dimension of the crystal. For uniform three dimensional growth

$$S_t/S_o = {}^eS_t/{}^eS_o = (m_t/m_o)^{2/3} \qquad (23)$$

where m_o and m_t are the total seed masses at zero time and at time, t.

(B) EFFECT OF DEGREE OF SUPERSATURATION

From the growth equation, crystal growth rate is directly proportional to supersaturation. Schott (1980) studied and confirmed the direct relationship between supersaturation and growth rate. Theoretical calculations indicate that for

dislocation controlled growth, the growth rate is proportional to the square of the driving force while surface-nucleation controlled growth will result in an exponential dependence (Mullin, 1961).

The degree of supersaturation can also affect the habit of a crystal. A high degree of supersaturation can cause the preferential growth of a crystal in one particular direction, leading to the formation of needle shaped crystals. This habit allows for a fast rate of heat dissipation from the solid phase when a high degree of supersaturation is present (Mullin, 1961).

(C) EFFECT OF TEMPERATURE

The relationship between the growth rate constant, K_o(obs), and the absolute temperature is given by the Arrhenius equation (Arrhenius, 1880).

The effect of temperature on crystal growth rate has been extensively studied and the energy of activation for the growth reaction determined. Some of the studies have been referenced by Phillips and Epstein (1974). The growth rate of crystals increases with an increase in temperature and is generally controlled by the surface reaction mechanism at low temperatures, whereas at high temperatures the crystallization is generally controlled by the diffusion process (Mullin, 1961).

(D) EFFECT OF DEGREE OF AGITATION

The rate at which a crystal grows at a given temperature under constant conditions of supersaturation and surface area of seed crystals can be altered by agitating the solution. The rate of growth increases considerably in the initial stages as the relative velocity between crystal and liquid increases, but conditions are soon reached when further agitation has no effect. An increase in the agitation rate of the solution decreases the thickness of the film on the crystal surface for diffusion controled growth. Therefore at high rates of agitation the rate of crystal growth depends solely on the rate of the surface reaction process.

(E) EFFECT OF IMPURITIES

The presence of impurities can have a profound effect on the growth of a crystal. Some impurities suppress growth, some enhance growth and others may modify the crystal habit.

Impurities can change the properties of the solution or the equilibrium saturation concentration. They can change the characteristics of the adsorption layer at the crystal-solution interface. They may be adsorbed onto the crystal faces and exert a blocking effect and thus disrupt the flow of growth layers across the faces. They may be built into the crystals or may interact chemically with the crystals.

Impurities that have a common ion accelerate the growth

process by reducing the solubility according to the law of mass action. Impurities without a common ion can accelerate growth at low concentrations but reduce it at high concentrations. It has been suggested that these impurities act as diffusion retarders. Impurities of organic origin retard the growth by being adsorbed onto the crystal faces.

It has been suggested that the action of impurities is due to:

(1) The electric field of the ions (governed by ionic charge and radius),
(2) chemical interaction between the impurity and the crystal, or
(3) formation of complex aquo ions,

resulting in a decrease in the growth rate of crystals (Mullin, 1972).

2.8.5 DETERMINATION OF CRYSTAL GROWTH RATE

(A) FACE GROWTH RATES

The different faces of a non-isotropic crystal may grow at different rates under identical environmental conditions. A small crystal is mounted on a tungsten wire in a particular orientation. A solution of known supersaturation is circulated around the mounted crystal and the rate of advance of the crystal face is observed through a travelling microscope (Mullin, 1972).

(B) OVERALL GROWTH RATES

The measurement of crystal growth rates as a function of the mass deposited per unit time per unit area of crystal surface is convenient. This can be done by determining the mass deposited on a known mass of seed crystals under carefully controlled conditions. Two methods have been used to obtain reliable information on crystal growth kinetics.

In the fluidised bed method the seed crystals are kept suspended in a supersaturated solution by controlling the solution velocity in a vertical glass column. Solution concentrations can be determined at intervals or continuously by means of a recording density meter. The details of the apparatus and method are given by Mullin (1972).

A second method involves the use of a stirrer to suspend the seed crystals in a supersaturated solution. This method was first used by Davies and Jones (1949) to study the precipitation kinetics of silver chloride from aqueous solutions. Many reports have appeared in the literature over the last several years using this technique to study the kinetics of crystal growth of many electrolytes.

Davies and Jones (1949) proposed a model for crystal growth and suggested that the rates of adsorption for the cations and anions were directly proportional to the surface area of seed crystals. They proposed that in the event of simultaneous arrival of positive and negative ions at the growth site, the

rate of growth is given by:

$$R_g = K_o(obs)\ S\ (C^+ - Cs^+)(C^- - Cs^-) \quad (24)$$

where C^+, Cs^+, C^- and Cs^- are the concentrations of cations and anions at the supersaturation and the saturation concentrations of the electrolyte. For equal ion concentrations, this equation reduces to:

$$R_g = K_o(obs)\ S\ (C-Cs)^2 \quad (25)$$

The rate constant for the growth of ionic compounds has been generally found to be independent of the stirring rate and the fluid dynamics under experimental conditions (Liu and Nancollas, 1970). Thus the transport rate of solute by diffusion is not the rate controlling mechanism. During these experiments, the mass of solid deposited may be as much as two to three times the weight of seed crystals added initially. Therefore there is a considerable increase in the surface area during the experiments. However, the kinetic equation appears to adequately describe the growth data without introducing a term to account for the increase in surface area into the rate equation.

There is sufficient evidence to show that in many systems the effective growth area becomes constant at some stage in the

growth process even though the crystals increase in size (Nancollas and Purdie, 1961). The rate constant (K') is, however, directly proportional to the amount of seed crystals (Doremus, 1958).

Two models of crystal growth for electrolytes have been proposed (Doremus, 1958). According to the first model, adsorbed ions combine to form a neutral salt molecule which then diffuses to the growth step. The rate of formation of the surface molecules was proportinal to $(C-Cs)^3$ for 1:1 and 2:2 electrolytes and $(C-Cs)^4$ for 2:1 electrolytes. In the second model, the oppositely charged ions are incorporated into the crystal surface alternately, directly from the adsorbed layer at a kink in a growth step. The rate of crystal growth is then proportional to $(C-Cs)^2$ for 1:1 and 2:2 electrolytes and $(C-Cs)^3$ for 2:1 electrolytes. The rate constant in equation 25 includes all factors such as probability of incorporation of molecules into the crystal lattice and frequency of collision with growth sites.

2.9 MSUM SOLUTIONS

Uric acid is a dibasic acid (pKa 5.75 and 10.3) (Bergmann and Dikstein, 1955). Alkaline solutions of uric acid are readily obtained forming the salt of uric acid and alkali. MSUM is formed when solutions of sodium hydroxide and uric acid (pH 8.9, 50°) are mixed and left to stand. Monosodium urate dissociates partially in aqueous solution to sodium and urate ions and shows

typical reactions of uric acid in alkaline conditions.

2.9.1 DEGRADATION OF MSUM SOLUTIONS

Urates are decomposed by a number of bacteria, molds, some plants and a number of animal tissues. An enzyme uricase is responsible for this decomposition.

Organisms belonging to the Clostridium genus, for example, Cl.acidiurici convert uric acid anaerobically into ammonia, carbon dioxide and acetic acid (Barker, 1938). Uricase has been identified in the tissues of a number of animals (Truszkowski and Goldmanowna, 1933; Mikhlin and Ruizova, 1934; Fanelli et al., 1970). Extracts from breast and kidney tissues cause anaerobic uricolysis and form urea (Mikhlin and Ruizova, 1934). Soybean seeds contain uricase which converts uric acid into allantoin (Echevin and Brunnel, 1937).

In-vitro experiments have shown that the end products of uricase oxidation of uric acid depends on the conditions of the reaction. In phosphate buffer (pH 7.2-8.5) uricase oxidizes uric acid to allantoin, whereas in borate buffer at pH 7.2 urea and alloxanic acid are formed (Cananllakis and Cohen, 1955).

All alkalis cause decomposition of uric acid (Austin, 1903; May, 1911; Stevens and May, 1911). This decomposition was reported to be similar to the decomposition caused by uricolytic enzymes present in organ extracts. Water at 200^{o}, converts uric acid, in part to a mixture of pteridines and pyrimido(5,4g) pteridines.

Uric acid in alkaline solution oxidizes rapidly to allantoin, oxonic acid, allantoxadin or oxaluric acid depending on the conditions (Dalgliesh and Neuberger, 1954).

2.9.2 SOLUBILITY OF MSUM

The solubility of MSUM in water has been the subject of several studies. The solubility of MSUM was determined by the equilibrium solubility method (Loeb, 1972), hot stage microscopy (Wilcox and Khalaf, 1975) and by seeding a supersaturated solution of MSUM followed by repeated analysis of the solution until a constant result was obtained (Fiddis et al., 1983). The solubility of MSUM is a function of temperature in the presence of a physiological sodium ion concentration. Figure 18 shows the relationship between the temperature and the solubility determined by various groups of workers.

Amorphous MSUM has an apparent solubility of 2.03 g L^{-1} at 18^{o}. On storage of this solution the apparent solubility decreases and becomes that of the crystalline form, which is about 0.85 g L^{-1} at 18^{o} (Barkan, 1922).

Supersaturated solutions of MSUM prepared by heating solutions containing excess solid to 70^{o} followed by cooling were found to be true solutions (Kohler 1913). However, when MSUM was autoclaved in water at 121^{o} for 30 minutes the formation of a supersaturated colloidal dispersion resulted (Schade, 1922). The colloidal form of sodium urate in water is converted into a stable granular form after storage (Barkan,

1924). MSUM in a supersaturated solution may exist in a metastable condition, in which spontaneous precipitation does not take place. The upper limit of this metastable state for a pure solution of MSUM was found to be about 2.5 times the saturation concentration (Kohler, 1913). Sodium urate is ten times more soluble in pure water than it is in a 1% solution of sodium chloride, but in a 1% sodium chloride solution the upper limit of the metastable state for sodium urate is 5 times the saturation concentration for this solution.

The saturation solubility of MSUM in plasma and synovial fluid from normal and arthritic patients has been determined. There is a small difference in the solubility of MSUM in plasma and synovial fluid in healthy individuals. However, this difference is greater in patients with various arthritides (Dorner et al., 1981).

Bovine nasal cartilage has been shown to have an appreciable solubilizing effect on MSUM in buffer (Katz, 1978). Katz and Schubert (1970) studied the effect of proteoglycans, chondroitin sulfate and albumin on the solubility of MSUM and reported that the MSUM solubility was greatly enhanced by small amounts of proteoglycan, whereas chondroitin sulfate and albumin produced a slight increase in solubility even when present in large amounts. The effect of chondroitin sulfate on the solubility of MSUM was in disagreement with Laurent (1964) who reported a decrease in the solubility of MSUM in the presence of chondroitin sulfate.

Perricone and Brandt (1978, 1979) studied the enhancement of urate solubility by connective tissue components and found that proteoglycan aggregate facilitated the dissolution of about 2.5 times more sodium urate than non-aggregated proteoglycans. This effect of proteoglycan on urate solubility was abolished by digestion of the aggregate with an enzyme hyaluronic acid B 1-> 3 hydrolase. This indicated that to increase urate solubility the proteoglycan must exist as a large macromolecular aggregate (Perricone and Brandt, 1978). They also reported that the aggregate did not sustain sodium urate concentration in supersaturated solutions after 24 hours. Perricone and Brandt (1979) reported that the enhancement of sodium urate solubility by proteoglycan aggregate (prepared in the presence of potassium ions) was due to the cationic exchange of potassium with sodium ions. Potassium entered the solution and potassium urate being more soluble than sodium urate resulted in the observed increase in sodium urate solubility.

2.10 NUCLEATION AND CRYSTAL GROWTH OF MSUM

Khalaf and Wilox (1973) and Wilcox and Khalaf (1975) developed the hot stage microscope technique to observe the nucleation of MSUM and found that calcium and hydrogen ions increased the nucleation rate of MSUM as determined by the time for the appearance of observable nuclei. Cupric ions were found to have a mixed effect on the number of nuclei formed. Synovial fluid from gouty patients increased the number of nuclei formed. However, synovial fluid from rheumatoid patients was found to

inhibit nucleation. Tak et al. (1980) studied the nucleation of MSUM under physiological conditions of temperature, pH and ionic concentration and found that the nucleation of MSUM was greatly enhanced by synovial fluid from gouty patients, whereas synovial fluid from degenerative joint disease patients moderately enhanced nucleation and fluid from rheumatoid arthritis patients inhibited the nucleation. Tak et al. (1980) also reported that hyaluronic acid and purines had a minimal effect on urate nucleation and other connective tissue components had no effect. Nucleation of sodium urate has been shown to be more sensitive to urate ion concentration (Tak and Wilcox, 1980) and addition of physiologic quantities of potassium and magnesium ions slightly inhibited the nucleation of MSUM.

The growth of MSUM crystals from supersaturated solutions has been studied. Allen et al.(1965a,b) reported that the growth of the needle-shaped crystals was inhibited by surface active agents. The linear growth rate of MSUM crystals in the presence of 0.1% benzalkonium chloride was reduced (Allen et al., 1965a). Dyes such as bismarck brown and methylene blue were shown to inhibit the crystal growth of MSUM (Allen et al., 1965b).

The seeded growth technique was used by Erwin and Nancollas (1981) to determine the rate of growth of MSUM and the effect of additives on the growth of MSUM. They showed that the growth of MSUM followed the square law which indicated a surface reaction controlled growth process. Methylene blue , a cationic dye,

significantly reduced the rate of crystal growth of MSUM at 13 ppm, whereas, organic phosphonate and sodium heparin had a negligible effect.

Fiddis et al. (1983) studied the poisoning effect of neutral red dye and serum albumin on the crystal growth of MSUM. They determined the crystallization time at 37^{o} in the presence of the additives. Addition of 33 mmol L^{-1} neutral red to a 70 mmol L^{-1} sodium urate solution increased the crystallization time by 50 minutes. Albumin at a concentration of 10 g L^{-1} increased the crystallization time by a factor of 4. Heparin (0.01%) was shown to be an effective inhibitor of urate cystallization. Fiddis et al. (1983) reported the dependence of the linear growth rate of MSUM on the supersaturation. Expressed as a power law, the linear growth rate was found to vary with supersaturation defined as:

$$\left[\frac{((Na+)(HU-))^{1/2}}{((Na+)(HU-))^{1/2} - 1} \right]^{4.5}$$

which they proposed fitted the screw dislocation model of crystal growth.

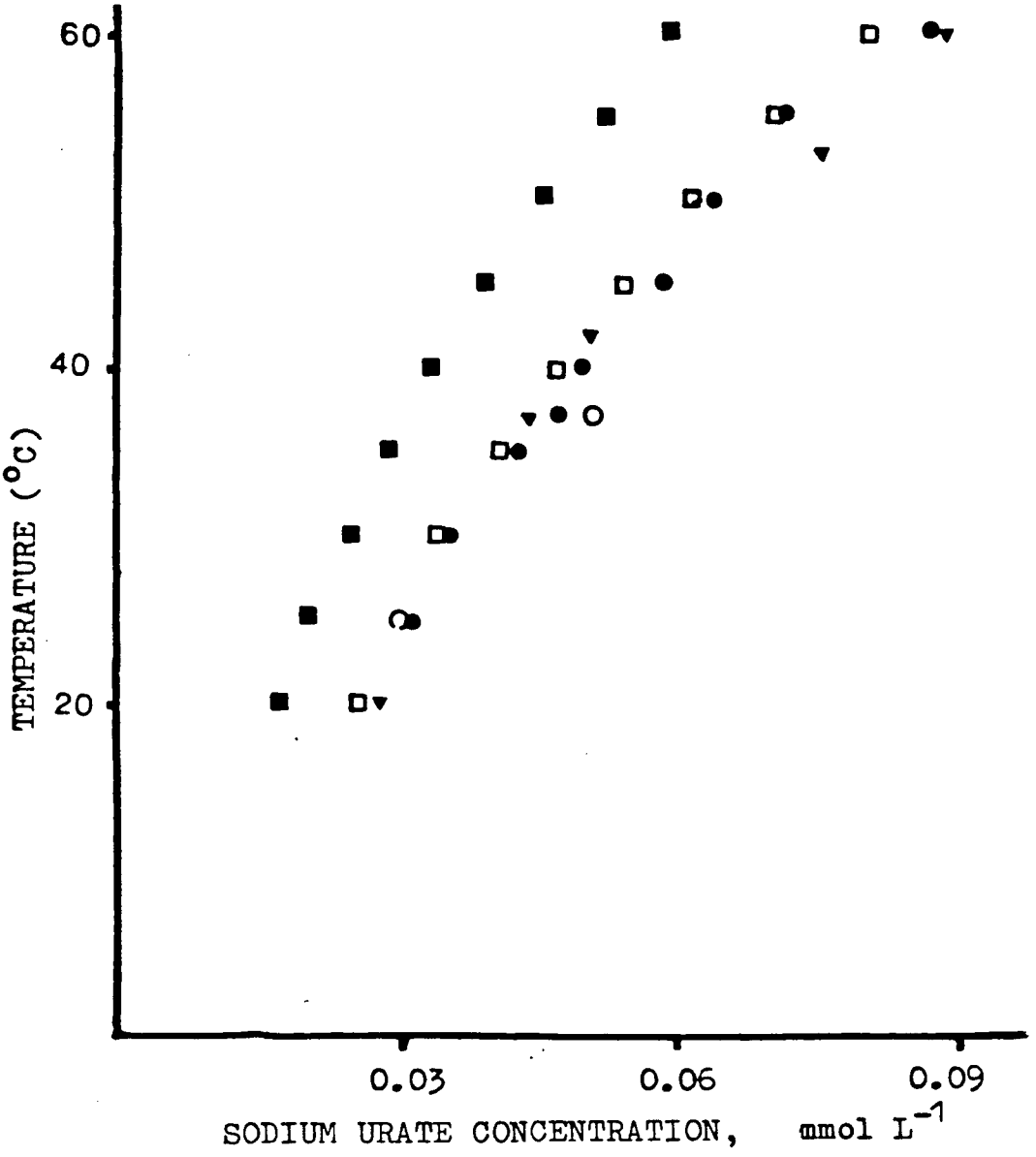


Figure 18. Solubility of sodium urate in normal saline.

(■) Wilcox et al., 1975 by hot stage; (□) Wilcox et al., 1975; (●) Allen et al., 1965; (○) Erwin and Nancollas, 1981; and (▼) Fiddis et al., 1983.

3 EXPERIMENTAL

3.1. INSTRUMENTS

Autoclave, AMSCO General purpose, American Sterilizer.

Atomic Absorption Flame Emission Spectrometer, Jerald Ash.

Cahn Electrobalance, Gram Ventron Corporation.

Constant Temperature Water Circulator, Haake FT.

Differential Scanning Calorimeter, DSC-1B, Perkin Elmer.

Electronic Stroboscope, Flashtac Electronic Corporation.

Infra red Spectrophotometer, IR 10, Beckman.

Oven, Isotemp, Fisher Scientific.

pH Meter, Accumet, Fisher Scientific.

Rotary Vacuum Evaporator, Rotavapor, Buchi Laboratories.

Scanning Electron Microscope, ETEC Autoscan.

Stirrer, Fisher Stedi Speed Stirrer, Fisher Scientific.

Surface Area Analyzer, Quantasorb , Quantachrome Corporation

U.V Spectrophotometer, Becman Model 24.

Ultrasonic Cleaner, Mettler Electronics.

Water Bath Shaker, Aquatherm, New Brunswick Scientific Co. Inc.

Water Bath Cooling Unit, Fridgedflow Bath Circulator, New Brunswick Scientific Co. Inc.

X-Ray Diffractometer, Wide Angle, Philips.

3.2. MATERIALS

Albumin, fraction-V, from bovine serum, Sigma Chemicals.

Aseptic filtration unit, Millipore Corporation.

Calcium chloride, B.D.H.

Chondroitin sulfate, sodium salt, from Whale or Shark cartilage (99%), Sigma Chemicals.

Disposable filtration units, (Millex-GS,0.22um, Millex-HA,0.8 um), Millipore Corporation.

Hyaluronic acid, salt (0.1% sodium, 9.7% potassium), from Human Umbilical Cord, Sigma Chemicals.

Membrane filters, Millipore Corporation.

Phosphatidylcholine (40%), from Soybean, Sigma Chemicals.

Phosphatidylserine (80%), from Bovine Brain, Sigma Chemicals.

Potassium chloride, Analar, B.D.H.

Proteoglycan aggregate, (A1 fraction)*.

Proteoglycan monomer, (A1D1 fraction)*.

(*) obtained from Dr. Mark Adams, Faculty of Medicine, University of B.C. Method of preparation given in Appendix.

Sodium chloride, ACS, Fisher Scientific.

Sodium hydroxide, ACS, Fisher Scientific.

Sterile evacuated glass tubes, red top Vaccutainer, Becton Dickinson.

Uric acid (99%), Sigma Chemicals.

3.3 METHODS

3.3.1 PREPARATION OF MSUM

Crystals of MSUM were grown according to the method described by Denko and Whitehouse (1976). A solution of uric acid (6.0 g L^{-1}) and 1 M sodium hydroxide at 55^{o} and pH 8.9 was left to stand overnight at room temperature. The crystals formed were separated by suction filtration and were rinsed several times with cold distilled water and dried at 60^{o} for 12 hours in a circulating hot air oven. These crystals were used for the preparation of solutions in various experiments and as seed crystals in the preliminary crystal growth experiments (batch A seed crystals).

Batch B seed crystals, used to study the effect of supersaturation and presence of additives on crystal growth, was prepared by a modification of the method described above. A solution of uric acid (6.0 g L^{-1}) and 1 M sodium hydroxide at 55^{o} and pH 8.9 was filtered through a 0.22 μm Millipore membrane filter while hot. The filtrate was left to stand overnight. The crystals were separated, rinsed several times with cold, filtered, distilled water and dried at 60^{o} for 12 hours in a circulating hot air oven.

Drying of MSUM samples resulted in the formation of a hard cake. Samples were therefore ground in a glass mortar and

pestle prior to their use in various experiments. The ground batch B seed crystals were passed through a set of sieves (# 50/80, US standard) of mesh size 180 μm to 300 μm in an attempt to obtain a greater degree of size uniformity of seed crystals.

3.3.2 CHARACTERIZATION OF MSUM CRYSTALS

(A) ULTRA-VIOLET SPECTROSCOPY

Approximately 10 mg of ground MSUM crystals were dissolved in 100 mL of distilled water. A suitable dilution of this solution was scanned from 320 nm to 230 nm on an ultra-violet spectrophotometer.

(B) INFRA-RED SPECTROSCOPY

Approximately 2 mg of an MSUM sample was mixed with approximately 200 mg of anhydrous potassium bromide. A portion of this mixture was compressed into a disc at 10 ton pressure using a die and punch in a hydraulic press. The compressed disc was scanned in the 625-3800 cm^{-1} range using an infrared spectrophotometer.

(C) X-RAY DIFFRACTION

Approximately 100 mg of a ground MSUM sample was tightly packed in a glass sample holder (thickness = 0.1cm) and exposed to CuKα radiation a wide angle X-ray diffractometer at a scanning rate of 2 degrees of 2θ per minute.

The location and intensity of the peaks at different values

of 2θ were studied.

(D) DIFFERENTIAL SCANNING CALORIMETERY

Ground samples of MSUM were analysed using a differential scanning calorimeter. Samples of 1-5 mg were weighed on a Cahn-Gram electrobalance and analysed at a scanning rate of 20° per minute in open aluminum sample pans. Vaporization of the water of hydration from the open pans was estimated quantitatively by weighing the pan after the appearance of the endothermic peak, and the percent water loss calculated.

(E) SCANNING ELECTRON MICROSCOPY

A small quantity of an MSUM seed crystal sample was dispersed on an SEM sample holder. The sample was coated with spectrographic graphite under vacuum. Scanning electron micrographs were taken at 4000 x or 8000 x magnification using a Scanning Electron Microscope.

(F) DETERMINATION OF SURFACE AREA OF MSUM SEED CRYSTALS

The surface areas of the two batches of MSUM seed crystals were determined using the Quantasorb surface area analyser. Three point BET surface area determinations were made using 0.072%, 0.104% and 0.184% mole fraction krypton in helium gas mixtures.

An accurately weighed sample (approximately 0.1g) was placed in a glass cell and degassed at the out gassing port for one hour

at 60^{o} under a slow stream of nitrogen gas. The cell was transfered to the adsorbing gas port and cooled with liquid nitrogen. Krypton from one of the three krypton-helium mixtures was allowed to adsorb onto the MSUM sample for 30 min. Krypton was desorbed by removing the liquid nitrogen flask and quickly bringing the cell to room temperature by dipping the cell in water at room temperature. The desorption count was recorded. The procedure was repeated three times for each of the three krypton-helium mixtures. The surface area was calculated from the mean desorption count (at each krypton concentration) calibrated with the desorption counts accumulated from measured volumes of nitrogen gas using the following equation

$$S = (1 - \frac{P}{Po})(\frac{A}{Ac})\, Vc(\frac{Nu\ Acs\ Pa}{RT}) \quad \text{metre square} \qquad (26)$$

where P = partial pressure of adsorbate,

Po =saturated pressure of adsorbate,

Nu = Avogadro's number = 6.023 x 10^{23},

R = gas constant = 82.1 cc atm./mole.degree,

Vc = volume of calibration gas,

Pa = ambient pressure in atmosphere,

A = signal area (desorption count of sample),

Ac = area of calibration (desorption count of calibration gas,

Acs= cross sectional area of adsorbate molecules in square meters (for krypton, 19.5 x10^{-5} meter

square per atm.),

T = temperature of calibration volume for ambient temperature in degree Kelvin.

3.3.3 ANALYSIS OF MONOSODIUM URATE MONOHYDRATE

Urate concentrations in solution were analyzed by measuring the absorbances at 292 ηm using an ultra-violet recording spectrophotometer.

A urate standard curve was prepared by dissolving an accurately weighed, 200 mg sample of MSUM in water in a volumetric flask and making up to 100 mL with water. After further dilution, the absorbances were immediately read at 292 ηm.

3.3.4 DEGRADATION OF MONOSODIUM URATE MONOHYDRATE SOLUTIONS

(A) NON-STERILE SOLUTIONS

A 0.2 g L^{-1} solution of MSUM in water was prepared and 125 mL of the solution added to each of five, 150 mL glass Erlenmeyer flasks fitted with ground glass stoppers. One flask was stored in the refrigerator (4^{o}), one flask was kept at room temperature (22^{o}), one flask was stored in a water bath equilibrated at 45^{o} and remaining flasks were stored in a circulating hot air oven at 35^{o} and 65^{o}. Aliquots of 3 mL of solution were withdrawn from each flask after 6, 24, 48, 72, and 96 hours, and after further dilution, the absorbance of the solutions was read at 292 ηm.

(B) STERILE SOLUTIONS

All solutions were prepared with freshly distilled, deionized water which had been sterilised by autoclaving at 121^{o} for 30 minutes.

A 0.2 g L^{-1} solution of MSUM in water was prepared and approximately 10 mL of solution filtered through a glass syringe Millipore filtration unit fitted with a 0.22 µm filter directly into each of six sterile, red top Vacutainer tubes (Becton Dickinson). The tubes were stored at 4^{o}. After 6 hours, a 3 mL sample was removed from one tube using a sterile disposable syringe and needle and the remaining solution in the tube discarded. Samples were also taken at time intervals of 24, 48, 72, and 96 hours. The samples were diluted and the absorbance of the solutions was read at 292 nm. This procedure was repeated for tubes stored at 22^{o}, 35^{o}, 45^{o} and 65^{o}.

An MSUM solution (0.2 g L^{-1}) was filtered through a large scale Millipore filtration unit fitted with a 0.22 um filter and 125 mL of the solution placed into each of five, sterile Erlenmeyer flasks with glass stoppers. The MSUM solution was also filtered directly into sterile, red top Vacutainer tubes as described above. The flasks and tubes containing MSUM solution were then autoclaved at 121^{o} for 35 minutes. The flasks and tubes were allowed to cool to room temperature and the solutions in one of each of the flasks and Vacutainer tubes were

immediately analyzed for the urate concentration. The remaining tubes and flasks were stored at 22°, 35°, 45°, and 65° and samples removed as described above using sterile, disposable syringes and needles, after 12, 24, 48, 72 and 96 hours. Samples were further diluted and the absorbance of the solutions was read at 292 nm.

3.3.5 DETERMINATION OF SATURATION SOLUBILITY OF MONOSODIUM URATE MONOHYDRATE

(A) EFFECT OF TEMPERATURE

Into each of three 125 mL Erlenmeyer flasks containing freshly washed and dried glass marbles, was placed 100 mL of distilled water and the flasks fitted with ground glass stoppers. The flasks were equilibrated at a given temperature in a water bath shaker fitted with heating and cooling units. An excess (200-500mg sample) of MSUM was added to each of the flasks and shaken vigorously. Aliquots of approximately 2 mL were withdrawn from each flask after various time intervals, immediately filtered through a 0.22 μm Millex-GS disposable filter unit and the filtrate assayed for the MSUM concentration after suitable dilution. Sampling was terminated when three consecutive assays gave identical results. Solubilities were determined at 4.4°, 9.0°, 15.1°, 24.7°, 29.9°, 35.0°, 37.0°, 40.0°, 41.8°, 51.0° and 55.0°.

(B) EFFECT OF ELECTROLYTES ON THE SATURATION SOLUBILITY OF MONOSODIUM URATE MONOHYDRATE IN WATER

To determine the effect of sodium chloride and other electrolytes present in plasma and synovial fluid on the solubility of MSUM, various concentrations of sodium chloride (0.2, 0.4, 0.6 and 1.0 % w/v, final concentration), and a mixture of sodium chloride (0.78% w/v), potassium chloride (0.03 % w/v) and calcium chloride (0.035% w/v) (all are final concentrations) were added to 125 mL Erlenmeyer flasks containing previously washed and dried glass marbles and 100 mL of distilled water which had been equilibrated at a given temperature as described previously. MSUM samples (200mg) were added to these flasks and shaken vigorously. Sample withdrawal and analyses were carried out as described in section 3.3.5.(A). Solubilities were determined at 4.4^{o}, 10.4^{o}, 17.6^{o}, 24.7^{o}, 29.9^{o}, 41.8^{o} and 51.1^{o}.

(C) EFFECT OF CHONDROITIN SULFATE, HYALURONIC ACID, ALBUMIN, PROTEOGLYCAN MONOMER AND PROTEOGLYCAN AGGREGATE ON THE SATURATION SOLUBILITY OF MSUM

The saturation solubility of MSUM in water at 37^{o} was determined in the presence of these additives at several concentrations. The additive was added to 125 mL Erlenmeyer flasks containing previously washed and dried glass marbles and 100 mL of distilled water. The flasks were equilibrated at

37°, MSUM (200 mg) added and the solubility determined as described in section 3.3.5(A). Either 0.45 μm or 0.8 μm Millex disposable filters were used to filter solutions containing HA and PGs.

The following additives were used:

chondroitin sulfate (20, 40, and 60 mg dL^{-1})
hyaluronic acid (10, 20 and 40 mg dL^{-1})
albumin (10, 20, 40, 60 and 100 mg dL^{-1})
proteoglycan monomer (10, 20, 40 and 100 mg dL^{-1})
proteoglycan aggregate (20, 40 and 100 mg dL^{-1})

3.3.6 CRYSTAL GROWTH OF MONOSODIUM URATE MONOHYDRATE

A diagram of the crystal growth apparatus is shown in Figure 19. The supersaturated MSUM solution (1000 mL or 50 mL) was placed in the 1000 mL or 50 mL capacity glass reaction vessel and equilibrated in a water bath at 37° using a constant temperature water circulator. The reaction vessel was fitted with a cover to minimize solvent evaporation. An all glass stirrer with two paddles was lowered into the solution through a central port to a constant depth (2.5 cm from the bottom for the 1 L vessel and 1 cm from the bottom for the 50 mL reaction vessel) and rotated at a constant speed (200 rpm) by means of a motor. Constant checks on the rotation speed were made throughout all the experiments using an electronic stroboscope. The temperature of the supersaturated solution was monitored

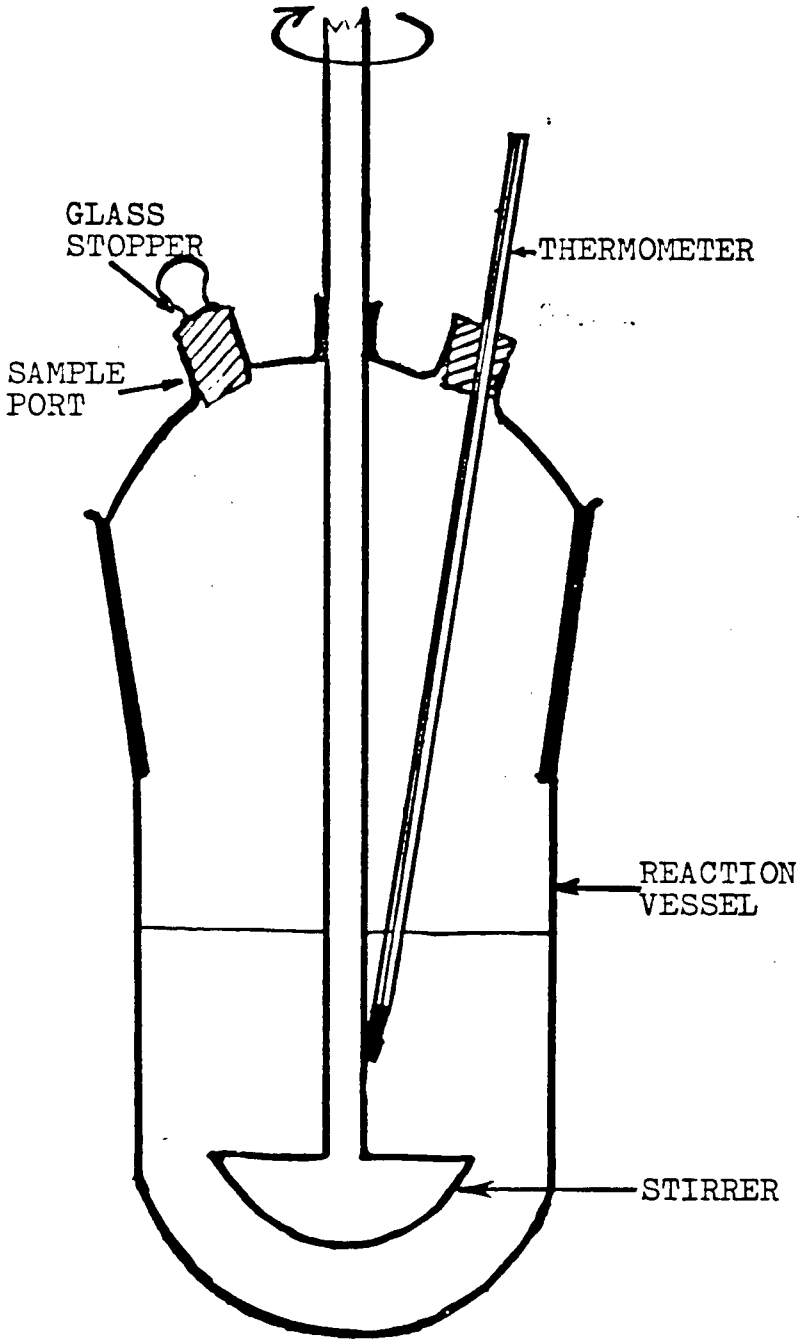


Figure 19. Crystal growth apparatus.

continuously using a thermometer reading to 0.1^{o} placed through the second port. The third port was fitted with a ground glass stopper and was used for sample withdrawal.

Solutions of differing degrees of supersaturation were prepared by dissolving accurately weighed samples of MSUM (4.0 g, 5.0 g, 6.0 g) in distilled water (950 mL) with the aid of heat (85 to 90^{o}) and constant stirring. The solution was cooled slowly to about 45^{o}, the pH was adjusted to 7.4 and the volume made up to 1 L with pH 7.4-adjusted distilled water. The solution was filtered through a 0.22 μm membrane filter and transfered to the reaction vessel.

(A) EFFECT OF SUPERSATURATION CONCENTRATION, SEED CRYSTAL BATCH AND SEED AMOUNT ON THE CRYSTAL GROWTH KINETICS OF MONOSODIUM URATE MONOHYDRATE

Initial crystal growth studies were carried out using 1 L of a supersaturated solution in a 1 L reaction vessel. To determine the effect of concentration of seed crystals, experiments were carried out by adding either 100 mg, 200 mg, 300 mg, 500 mg or 1 g of seed crystals to the supersaturated solution in the reaction vessel. Aliquots of 1 mL of growth medium were withdrawn at predetermined time intervals up to 360 minutes, filtered immediately through a 0.22 μm Millex-GS disposable filter unit, diluted and analyzed for MSUM content as described in section 3.3.5.(A).

These experiments were repeated using a smaller scale

apparatus. Supersaturated solutions (50 mL) containing 5 or 6 g L^{-1} MSUM were prepared as described above and placed in a 50 mL glass reaction vessel. To the supersaturated solutions were added either 5 mg, 10 mg, 20 mg, 30 mg, 40 mg, or 50 mg of seed crystals. Aliquots of 0.3 mL were withdrawn, filtered through a 0.22 um Millex-GS disposable filter unit, diluted and analysed for MSUM content (see section 3.3.5 (A)).

(B) EFFECT OF ADDITIVES ON THE CRYSTAL GROWTH OF MONOSODIUM URATE MONOHYDRATE

The small scale (50 mL) growth apparatus was used for all the experiments.

(I) CHONDROITIN SULFATE AND ALBUMIN

Accurately weighed quantities of 10 mg, 20 mg, or 30 mg of chondroitin sulfate or 10 mg, 50 mg, 100 mg, or 200 mg, of albumin were dissolved in 5 mL of distilled water and filtered through a 0.22 μm Millex-GS filter unit directly into the reaction vessel. To the reaction vessel were added 45 mL of a supersaturated solution such that a final MSUM concentration of 5.0 g L^{-1} was obtained. The solution was equilibrated at 37^{o} for 30 minutes, stirring constantly at a stirrer speed of 200 rpm. Seed crystals (40mg) were then added and 0.3 mL aliquots of growth medium withdrawn at predetermined times up to 360 minutes. Samples were filtered immediately and assayed for MSUM content as described in section 3.3.5 (A).

(II) HYALURONIC ACID AND PROTEOGLYCAN MONOMER

A 5 mg, 10 mg or 20 mg sample of hyaluronic acid or 5mg or 10 mg of proteoglycan monomer (A1 D1 fraction from bovine cartilage) was added to the reaction vessel containing 5 mL filtered (0.22 μm Millex-GS filter) distilled water and dispersed in the water. To the reaction vessel were added 45 mL of a supersaturated solution to obtain a final MSUM concentration of 5.0 g L^{-1} or 6.0 g L^{-1}. After equilibrating at 37° for 30 minutes and a stirrer speed of 200 rpm, 30 mg, 40 mg, or 50 mg of seed crystals were added. Aliquots of 0.3 mL of growth medium were removed at predetermined time intervals, filtered, diluted and assayed for MSUM content (see section 3.3.5 (A)).

(III) PROTEOGLYCAN AGGREGATE

A sample of proteoglycan aggregate (10 mg to 50 mg) was dispersed in 5 mL of filtered (0.22 μm filter) distilled water in the growth cell. To the growth cell were added 45 mL of a previously filtered (0.22 μm filter) supersaturated solution of MSUM to obtain a final concentration of 5.0 g L^{-1}. The stirrer speed was 200 rpm and the mixture was equilibrated at 37° for 30 minutes. An accurate quantity (40 mg) of seed crystals were added and aliquots of 0.3 mL of growth medium were withdrawn at predetermined time intervals. The samples were immediately filtered through 0.8 um Millex-HA filters, diluted and assayed for MSUM content as described previously (section 3.3.5 (A)).

(D) PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLSERINE

A 10 mg, 20 mg or 30 mg sample of phosphatidyl choline or 10 mg or 20 mg of phosphatidyl serine was dissolved in 10 mL of a chloroform - methanol mixture (2:1) in a 50 mL round bottomed flask. The solvent mixture was evaporated under vacuum at 60^{o} using a rotary vacuum evaporator. The thin film of phospholipid formed on the inside wall of the flask was dispersed in 5 mL filtered (0.22 μm filter) distilled water using an ultrasonicator. The phospholipid suspension was transferred into the reaction vessel and a supersaturated MSUM solution (45 mL) was added to obtain a final concentration of 5.0 g L^{-1}. After equilibrating at 37^{o} for 30 minutes and stirrer speed of 200 rpm, 40 mg or 50 mg of seed crystals were added. Aliquots of 0.3 mL of growth medium were filtered, diluted and assayed for MSUM content as described in section 3.3.5 (A).

3.3.7 DETERMINATION OF SODIUM AND/OR POTASSIUM CONTENT OF HYALURONIC ACID, CHONDROITIN SULFATE AND PROTEOGLYCAN MONOMER SAMPLES

An accurately weighed 100 mg quantity of sodium chloride or potassium chloride was dissolved in distilled water in a volumetric flask and the volume was made up to 100 mL. Sodium or potassium standard solutions were made by further diluting these solutions to obtain a concentration range of 0.2 mg dL^{-1} to 2.0 mg dL^{-1} of sodium chloride or potassium chloride. The percent emission of these solutions was measured on a flame photometer at

the maximum wavelength of 585 nm for sodium and 765 nm for potassium. A calibration curve of percent emission versus concentration of sodium ion or potassium ion was plotted.

A 10 mg sample of chondroitin sulfate, hyaluronic acid, proteoglycan monomer or proteoglycan aggregate was dissolved in distilled water in a volumetric flask and the volume made to 100 mL. The percent emission for chondroitin sulfate and proteoglycan was measured at 585 nm and for hyaluronic acid, percent emission was measured at 765 nm and 585 nm. The concentrations of sodium ion and potassium ion in the samples were determined from the standard curve.

3.3.8 EFFECT OF SODIUM AND POTASSIUM IONS ON GROWTH OF MSUM

A 0.7 mL volume of 0.1 M sodium hydroxide (equivalent to 1.6 mg sodium ion) representing 5.6% w/w sodium in a 30 mg sample of the sodium salt of chondroitin sulfate, was added to 45 mL of a supersaturated solution of MSUM (pH 7.4). The volume of the solution was adjusted to 50 mL by the addition of distilled water (pH 7.4) so that the final MSUM concentration of the supersaturated solution was 5.0 g L^{-1}. The solution was filtered through a 0.22 um Millex-GS disposable filter unit into the growth cell and equilibrated at 37^{o} for 30 minutes. Seed crystals (40 mg) were added and the growth experiment carried out as described in section 3.3.6.

This experiment was repeated using 0.34 mL of a 0.1 M potassium hydroxide solution (equivalent to 1.3 mg potassium ion)

representing 6.5% w/w of potassium in a 20 mg sample of hyaluronic acid. The concentration of the supersaturated solution was 5.0 g L^{-1} and 50 mg of seed crystals were used.

3.3.9 CHARACTERIZATION OF MSUM CRYSTALS AFTER CRYSTAL GROWTH

Impurities and additives in the growth medium can result in a change in crystal morphology or crystal structure. To determine whether the additives had caused any change in the crystal habit or structure, scanning electron microscopy and powder X-ray diffraction studies were carried out on the MSUM crystals obtained after various crystal growth experiments. Where ever possible, the supersaturated solution containing the crystals was filtered through a 0.22 μm membrane filter and the crystals on the filter were dried in a circulating hot air oven at 60^{o}. The dried crystals were ground in a glass pestle and mortar and subjected to SEM and powder X-ray diffraction analyses as described in section 3.3.2(E) and 3.3.2(C).

4 RESULTS AND DISCUSSION

4.1 CHARACTERIZATION OF MONOSODIUM URATE MONOHYDRATE

Ultra-violet and infra-red absorption spectroscopy methods are used to determine and confirm the functional groups present in known compounds. The ultra-violet spectrum for an aqueous solution of MSUM (Figure 20) showed the λmax at 235 and at 292 ηm. These two values coincide with the λmax values of ionised uric acid (West, 1970) indicating the formation of the sodium salt of uric acid.

The infra-red spectrum of MSUM (Figure 21) showed peaks at 3580 cm^{-1} (-OH); 2850-3150 cm^{-1} (=CH); 1750 cm^{-1} (=C=O); 1750-1650 cm^{-1} (=C=C=, =C=N-,-COO^-); 1610 cm^{-1}; 1525 cm^{-1}; 1425 cm^{-1}; 1390 cm^{-1}; 1250 cm^{-1}; 1000 cm^{-1} and 900 cm^{-1}. The IR absorption spectra of MSUM was similar to that reported in the literature (Dieppe and Calvert, 1983).

The relationship between the wavelength of the X-rays, λ, and the spacing between the crystallographic planes, d, of a crystal is given by Bragg's law:

$$n \lambda = 2d \sin\theta \qquad (27)$$

where n is an integer and θ is the angle of the incident X-rays.

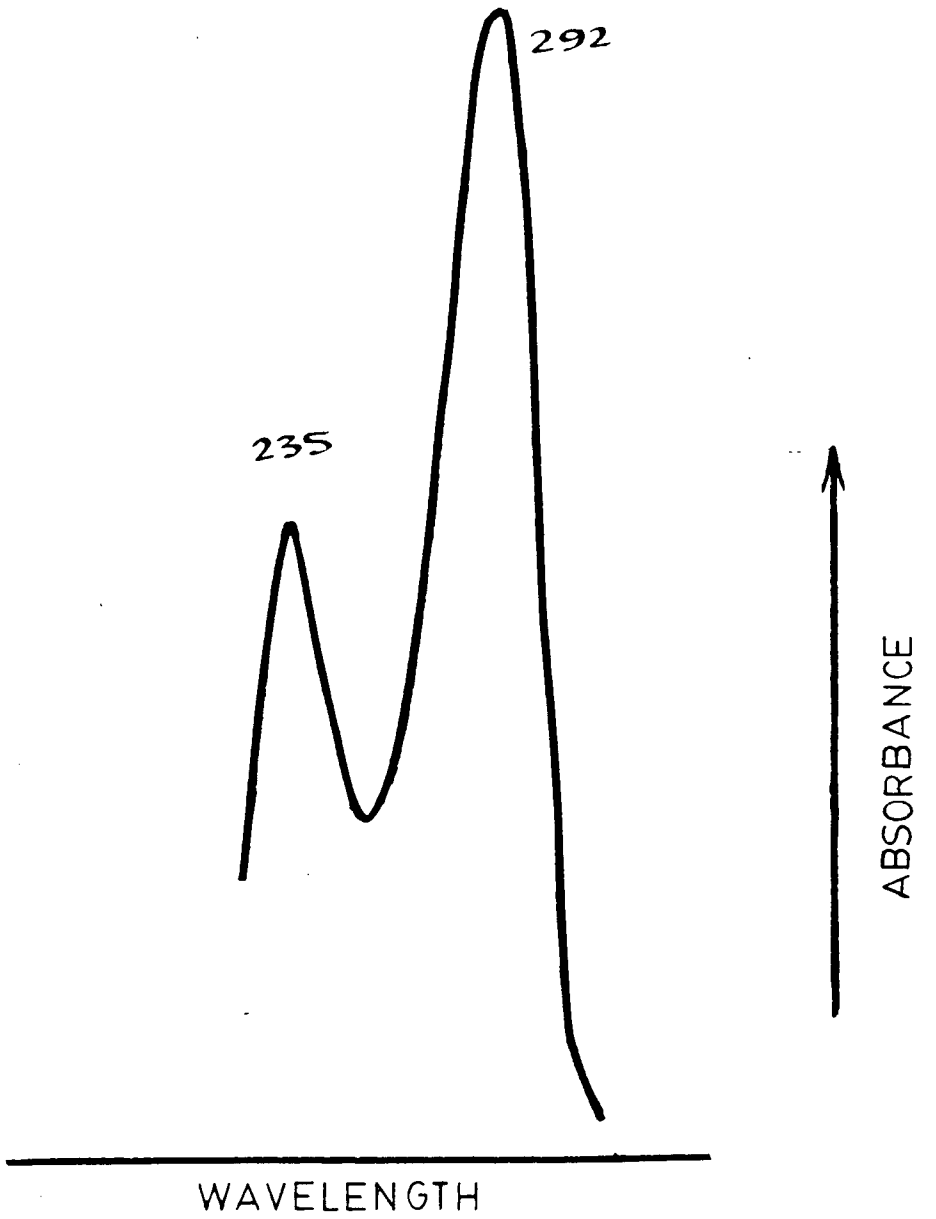


Figure 20. Ultra-violet spectrum of monosodium urate monohydrate solution.

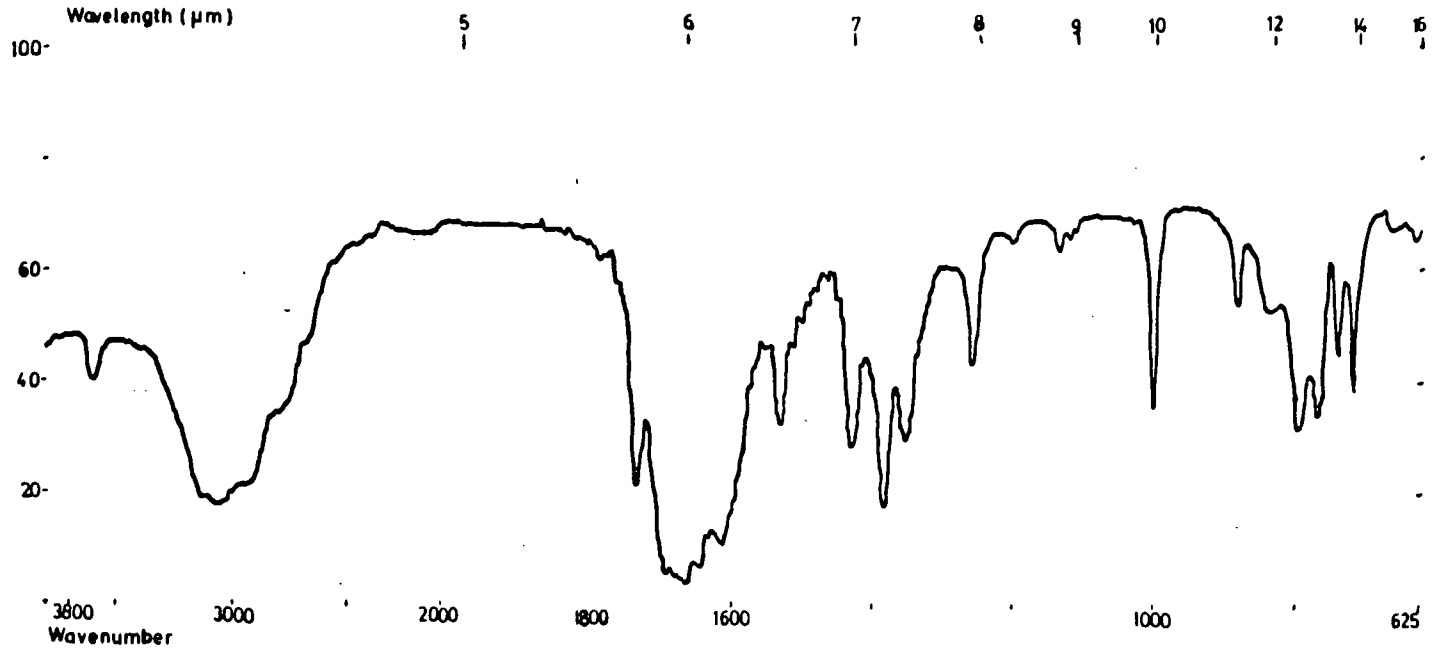


Figure 21. Infra-red spectrum of monosodium urate monohydrate.

Since sin θ must be between 0 and 1 there are usually no more than one or two diffraction orders if λ is comparable to d.

The powder X-ray diffraction pattern of MSUM is shown in Figure 22. The calculated d-values are shown in Table 4. The d-values were characteristic of monosodium urate monohydrate (Selected Powder Diffraction Data for Minerals, 1974; Rinaudo and Boistelle, 1982).

A scanning electron micrograph of the monosodium urate monohydrate crystals is shown in Figure 23. The crystals had a long, well-formed needle-shaped or acicular crystal habit.

A typical DSC scan of an MSUM sample is shown in Figure 24. A broad endothermic peak between 180^{o} to 240^{o} accounted for the loss of 8.78 % w/w of water of hydration. The percent water loss corresponded to the loss of one mole of water, confirming that the crystals were the monohydrate. The theoretical value of percent water loss for MSUM is 8.65% w/w.

4.2 ASSAY OF MONOSODIUM URATE MONOHYDRATE IN SOLUTION

The standard curve for an MSUM solution in water is shown in Figure 25. The plot was linear over a concentration range of 0.002 g L^{-1} to 0.034 g L^{-1}, the correlation coefficient, r^2, was 0.999 and the slope (absorptivity, absorbance/ concentration/cell width) was 56.8 L $g^{-1}cm^{-1}$.

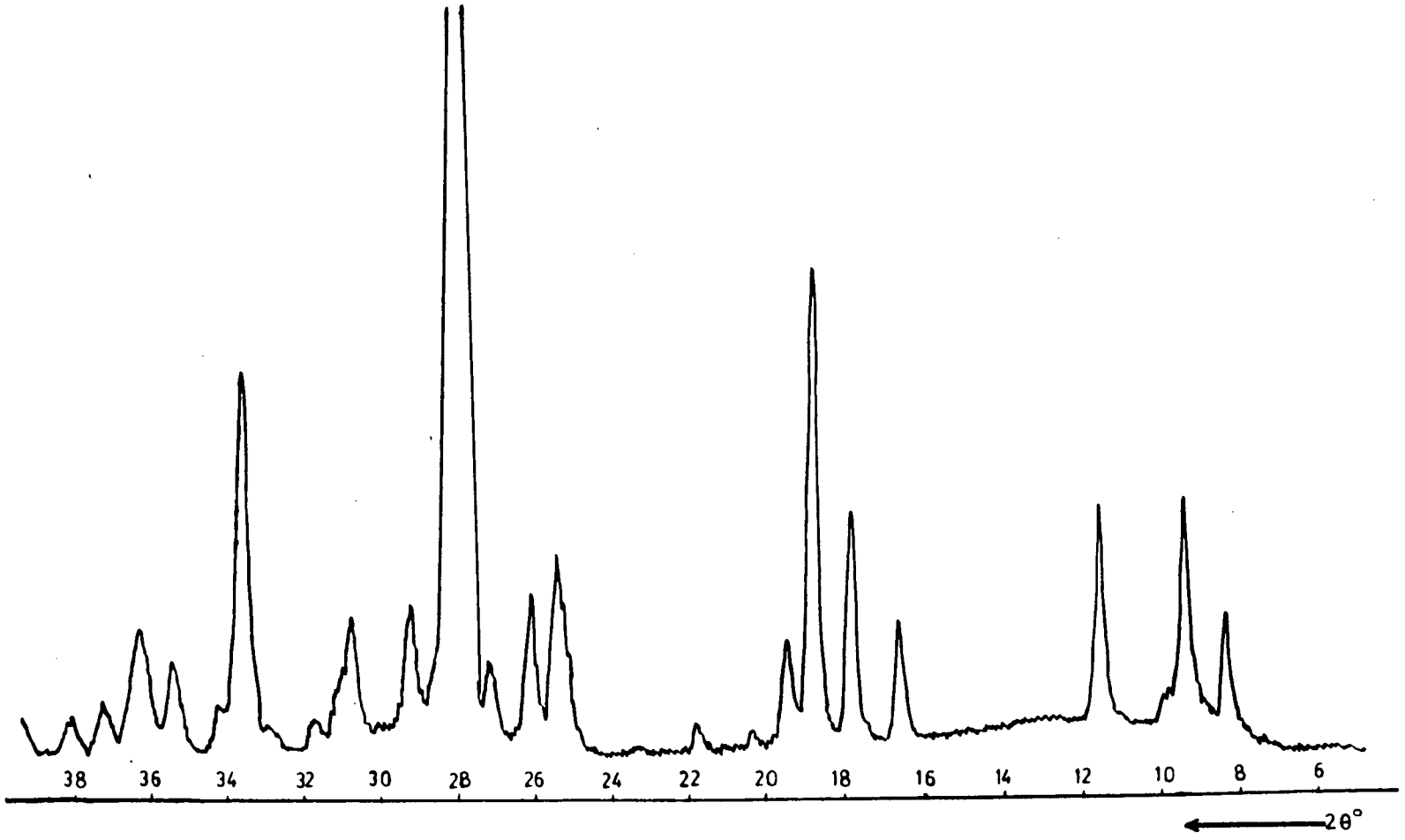


Figure 22. X-ray diffraction pattern of monosodium urate monohydrate.

TABLE 4. Powder X-ray diffraction pattern of monosodium urate monohydrate.

Interplanar distance (d-spacing, Å)	Relative intensity[a]
10.52	W
9.4	MS
7.55	MS
5.27	W
4.91	M
4.68	S
4.56	W
3.52	W
3.46	W
3.38	W
3.18	VS
3.02	W
2.97	W
2.65	M
2.61	W
2.53	W
2.47	W
2.42	W
2.36	W

a: VS = very strong
S = strong
MS = medium strong
M = medium
W = weak

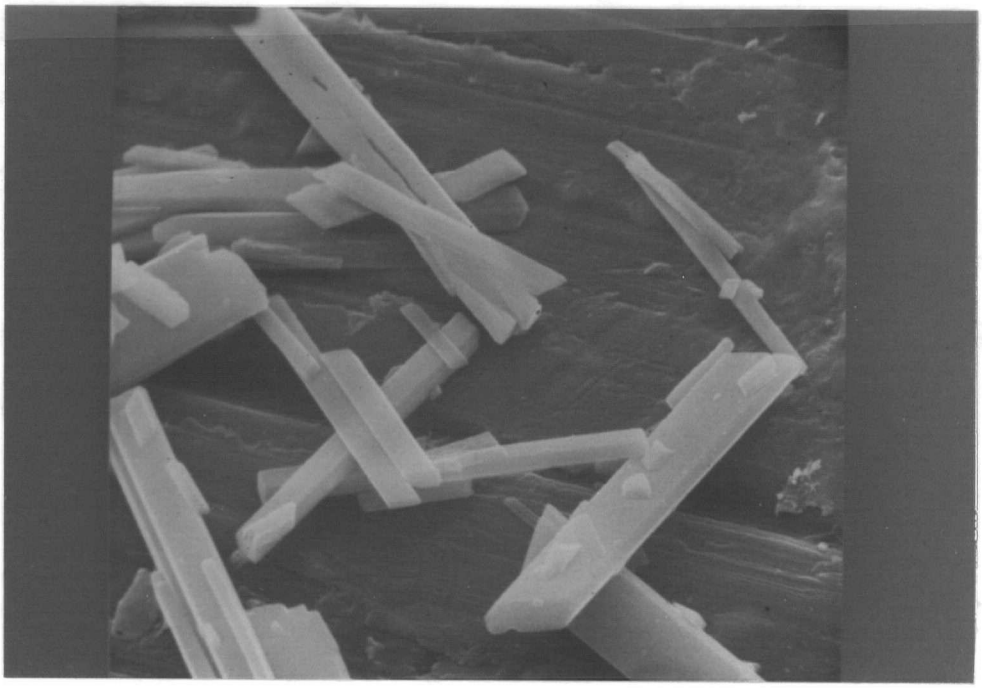


Figure 23. Scanning electron micrograph of monosodium urate monohydrate.

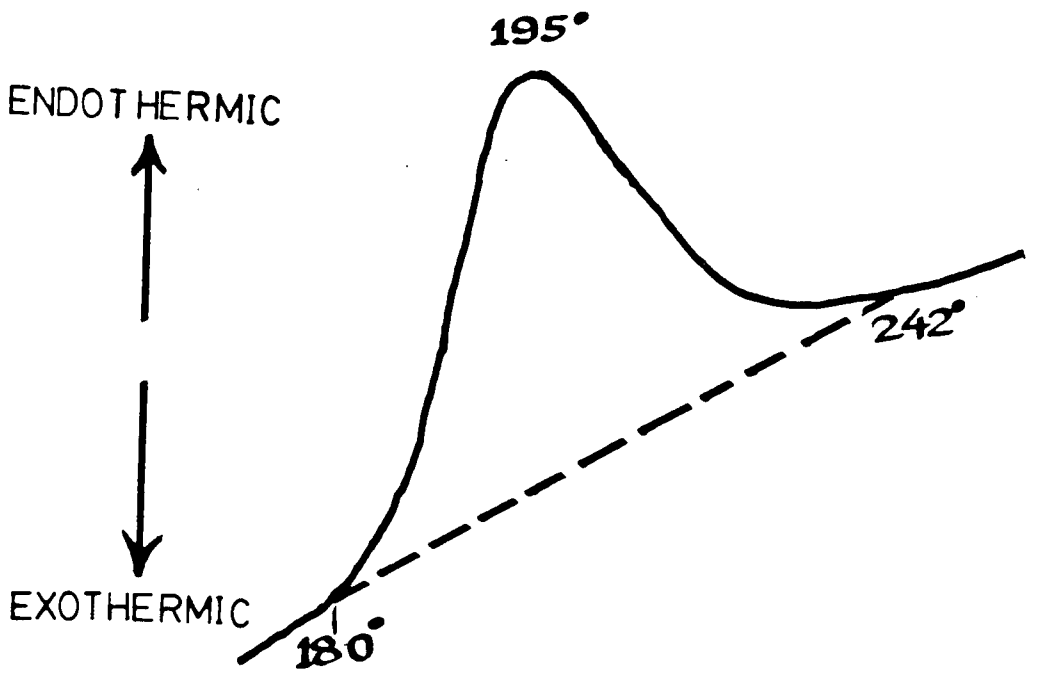


Figure 24. DSC scan of monosodium urate monohydrate.

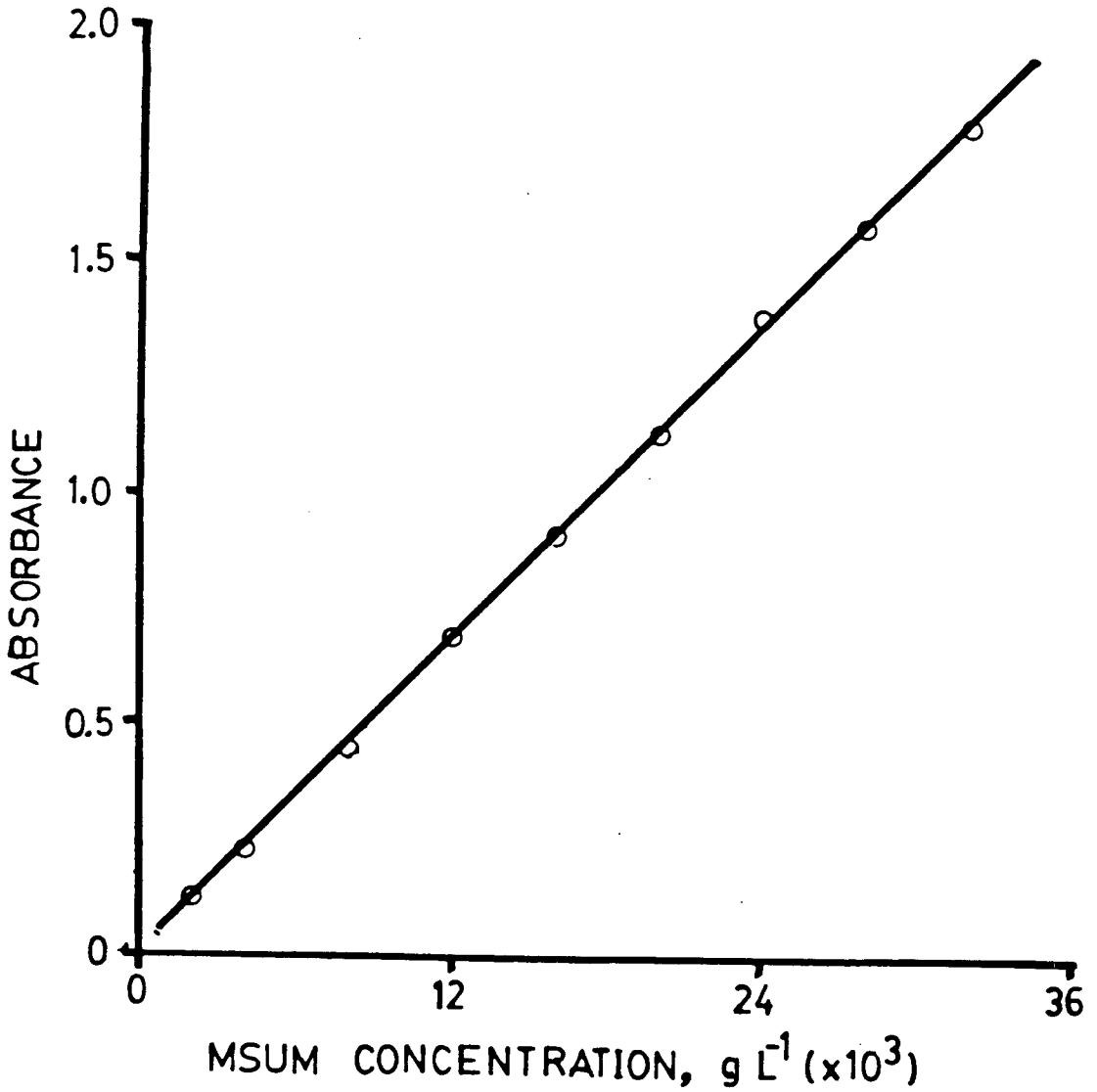


Figure 25. A standard curve for MSUM solution.
(n = 5; r = 0.999)

4.3 DEGRADATION OF MONOSODIUM URATE MONOHYDRATE IN SOLUTION

The results of degradation studies are given in Figures 26-29 and are plotted as concentration of MSUM remaining in solution versus time of incubation.

Figure 26 shows the degradation of non-sterile MSUM solutions. MSUM solutions stored at 4^{o} were relatively stable, showing little change in MSUM concentration with time. At 22^{o}, 35^{o}, 45^{o} and 65^{o}, there was a gradual decrease in the concentration of MSUM as the time of incubation was increased. As the temperature of incubation was increased, degradation of MSUM occured more rapidly, with the exception of solutions stored at 45^{o}, which were more stable than solutions stored at 35^{o}. Similar results were given by solutions sterilized by aseptic filtration through Millipore filters into rubber stoppered Vacutainers (Figure 27). However, whereas non-sterile solutions showed a smooth decline in MSUM concentration with time, the sterile solutions stored in Vacutainers showed some fluctuations in urate concentration, particularly for solutions stored at 22^{o}, 35^{o} and 65^{o}. These experiments were repeated several times, and the solutions sterilized by filtration and stored in Vacutainers always showed variability in urate concentration at each time interval.

During the course of these studies, it was found that the presence of rubber or plastic containers or closures appeared to cause a loss of MSUM from solution and that this effect was more

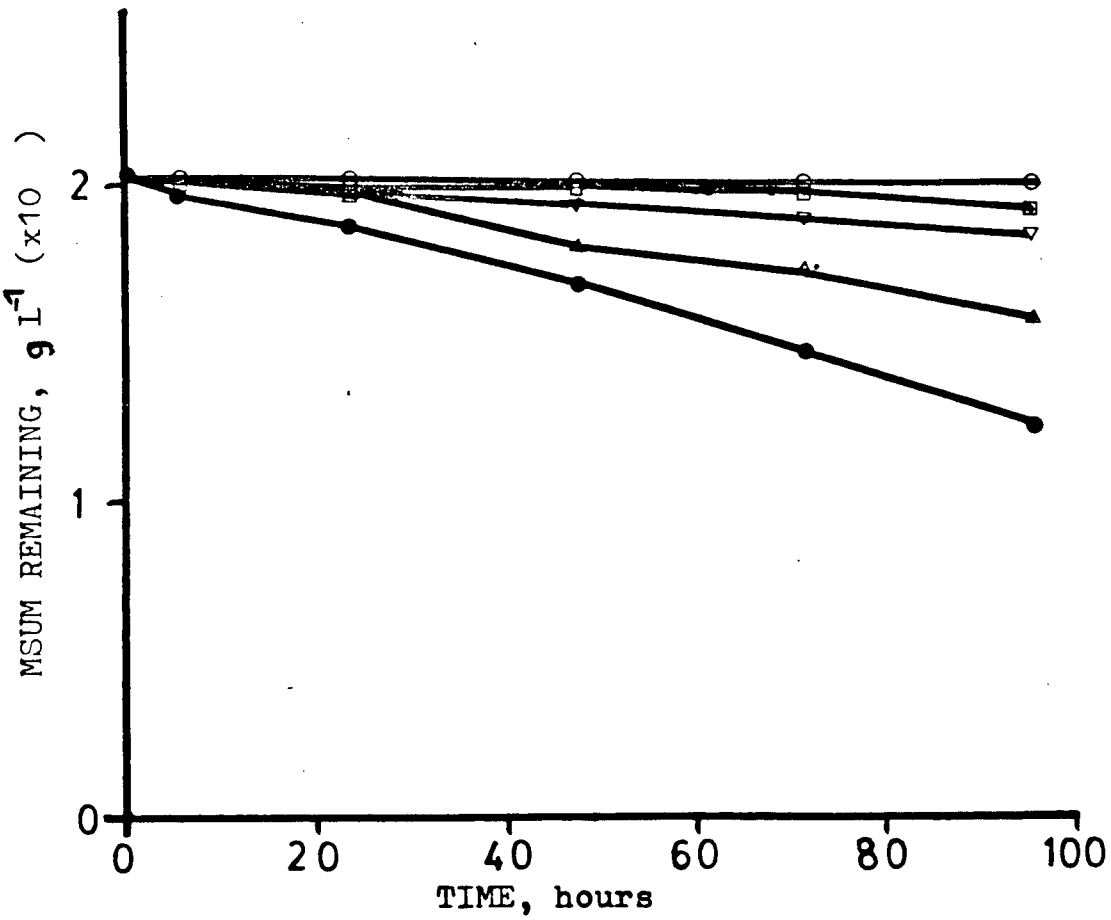


Figure 26. Degradation of non-sterile MSUM solutions. 4° C (○);;22° C (□); 35° C (△); 45° C (▽); 65° C (●)

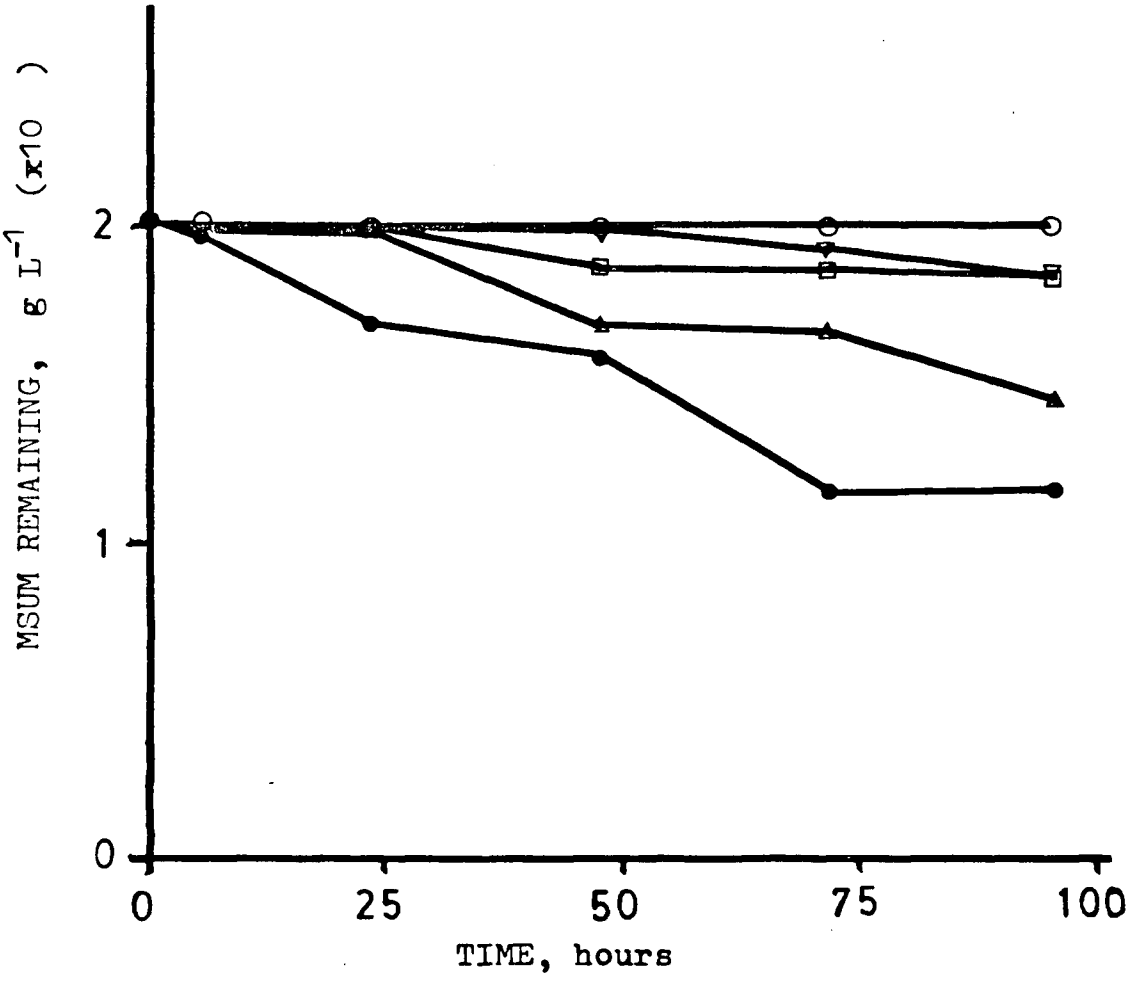


Figure 27. Degradation of sterile (0.22 um filter) MSUM solutions in Vacutainers. 4°C (○); 22°C (□); 35°C (△); 45°C (▽); 65°C (●).

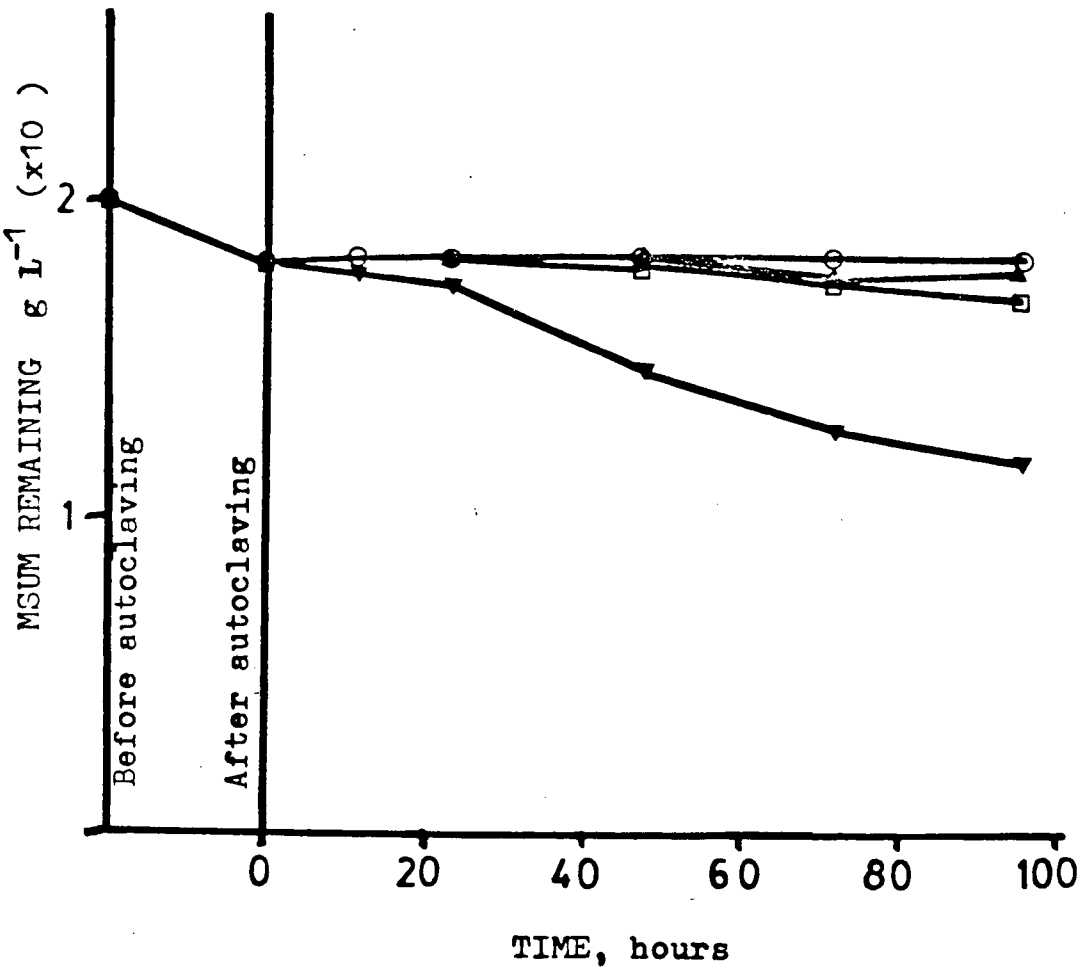


Figure 28. Degradation of sterile (by autoclaving) MSUM solutions in all glass containers. 22°C (○); 35°C (□); 45°C (▲); 65°C (▼)

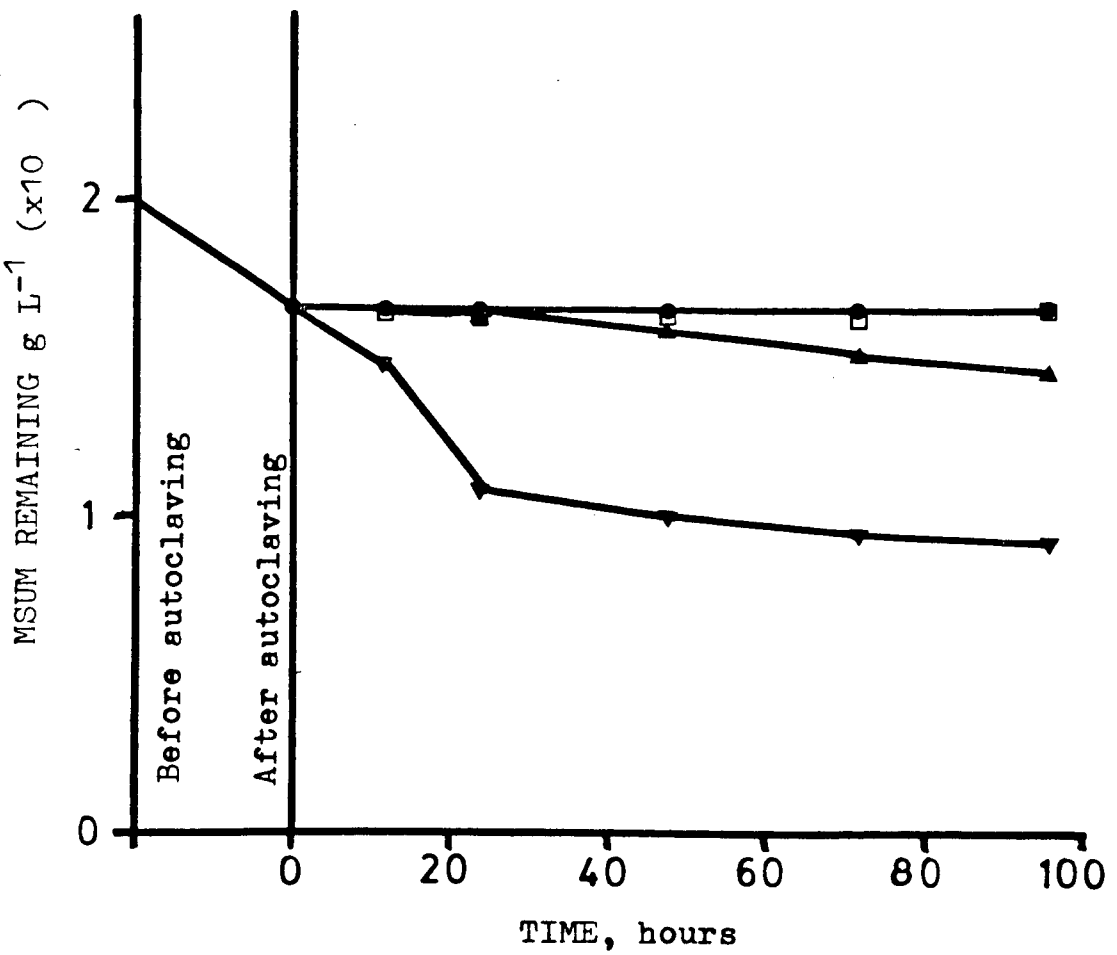


Figure 29. Degradation of sterile (by autoclaving) MSUM solutions in Vacutainers. 22°C (●); 35°C (□); 45°C (▲); 65°C (▼)

pronounced at higher temperatures. To confirm this observation, the degradation of sterile solutions of MSUM (sterilized by autoclaving) was studied in all-glass containers and Vacutainers with rubber closures. The results are shown in Figures 29 and 30. After autoclaving at 121^{o} for 35 minutes, the concentration of MSUM in solutions contained in all-glass vessels decreased from 0.198 g L^{-1} to 0.183 g L^{-1} (Figure 28) and from 0.198 g L^{-1} to 0.165 g L^{-1} for solutions in the Vacutainers (Figure 29). Solutions stored in glass flasks at 22^{o} and 45^{o} were relatively stable with little change in the concentration of MSUM in solution from 0 to 96 hours. At 35^{o} and 65^{o} the MSUM concentration gradually decreased to 0.168 g L^{-1} and 0.117 g L^{-1}, respectively, at 96 hours. Solutions stored in Vacutainers with rubber stoppers at 22^{o} and 35^{o} were relatively stable. At 45^{o} and 65^{o}, MSUM concentrations decreased to 0.147 g L^{-1} and 0.092 g L^{-1}, respectively, at 96 hours.

Heating the solutions in an autoclave gave a decrease in MSUM concentration. This decrease was greater for solutions stored in Vacutainers, indicating that some urate may have been lost by absorption into the rubber stoppers.

The data in Figures 26-29 indicate that non-sterile solutions and sterile solutions of MSUM undergo decomposition with time. In non-sterile solutions, MSUM concentration may have decreased in two ways, by bacterial consumption and chemical

degradation. For both non-sterile and sterile systems, solutions stored at 45^{o} were more stable than those stored at 35^{o}. The reason for this behavior is not understood. No studies were done to determine the degradation products of MSUM.

The degradation studies indicate that crystal growth studies may be conducted under non-sterile conditions for about 8 hours without any appreciable decrease in MSUM concentration due to decomposition.

4.4 SATURATION SOLUBILITY OF MSUM

The solubility of MSUM in-vitro is influenced by factors such as temperature, the presence of ions and the presence of impurities. The presence of amorphous material in commercially prepared MSUM has been reported to show an increased solubility (about 50% more) of MSUM (Kippen et al., 1974).

The MSUM prepared in our laboratory was used in all experiments to determine the effect of factors such as temperature and addition of sodium chloride, hyaluronic acid, chondroitin sulfate, proteoglycans and albumin on the aqueous solubility of MSUM.

4.4.1 EFFECT OF TEMPERATURE

The saturation solubility (Cs) of MSUM as a function of temperature is given in Table 5 and Figure 30. A van't Hoff plot (shown in Figure 31) of logarithm of saturation solubility versus reciprocal of absolute temperature should yield a straight line,

Table 5. Saturation solubility (Cs) of monosodium urate monohydrate at different temperatures.

Temperature (^{o}C)	4.4	9.0	15.1	24.7	29.9	35.0	37.0	40.0	41.8	51.0	55.0
Saturation solubility ($g\ L^{-1}$)	0.476	0.560	0.605	0.998	1.050	1.300	1.346	1.617	1.688	1.900	2.493

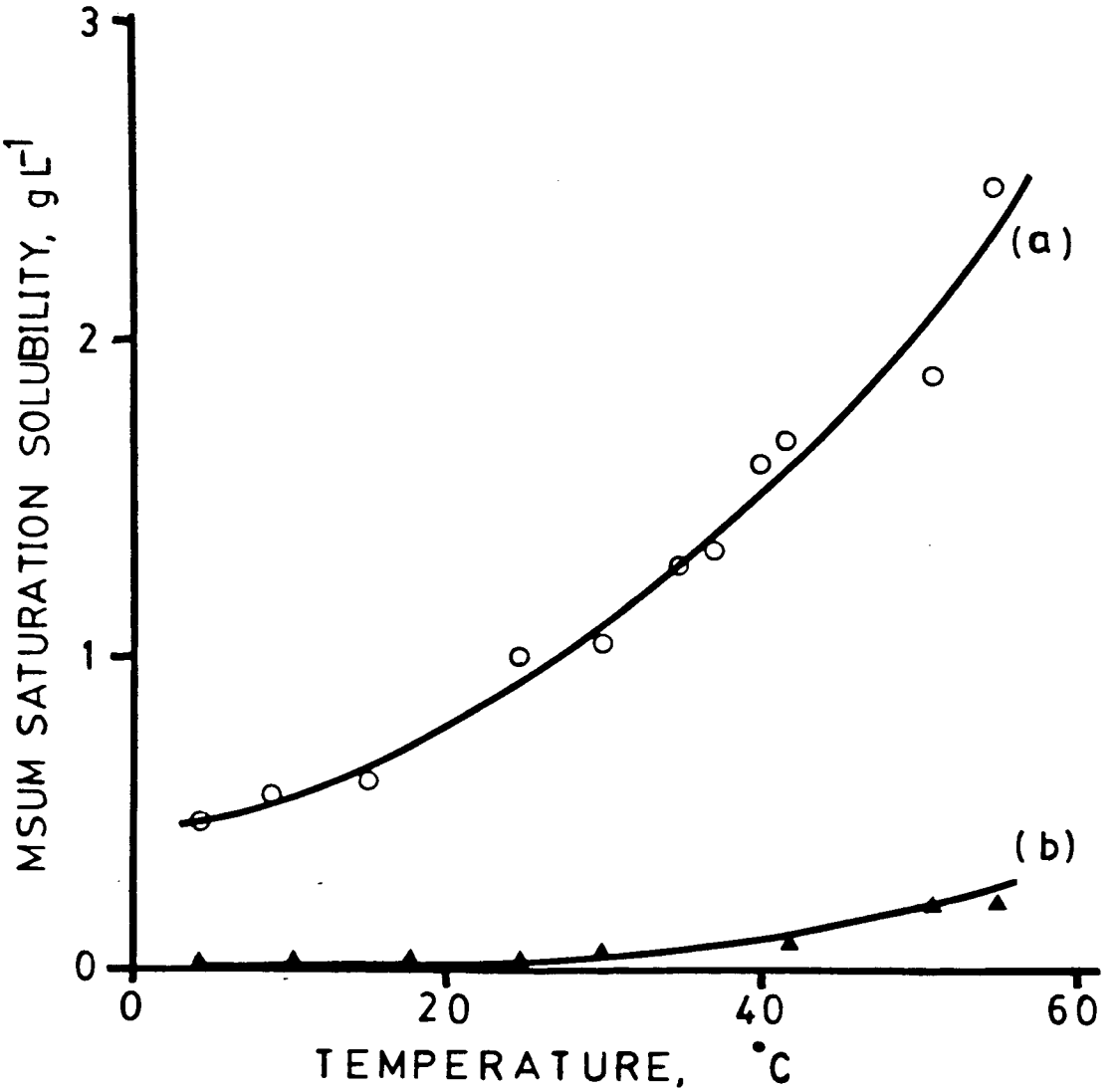


Figure 30. Effect of temperature on the saturation solubility of: (a) MSUM; (b) MSUM in the presence of physiological ion concentration.

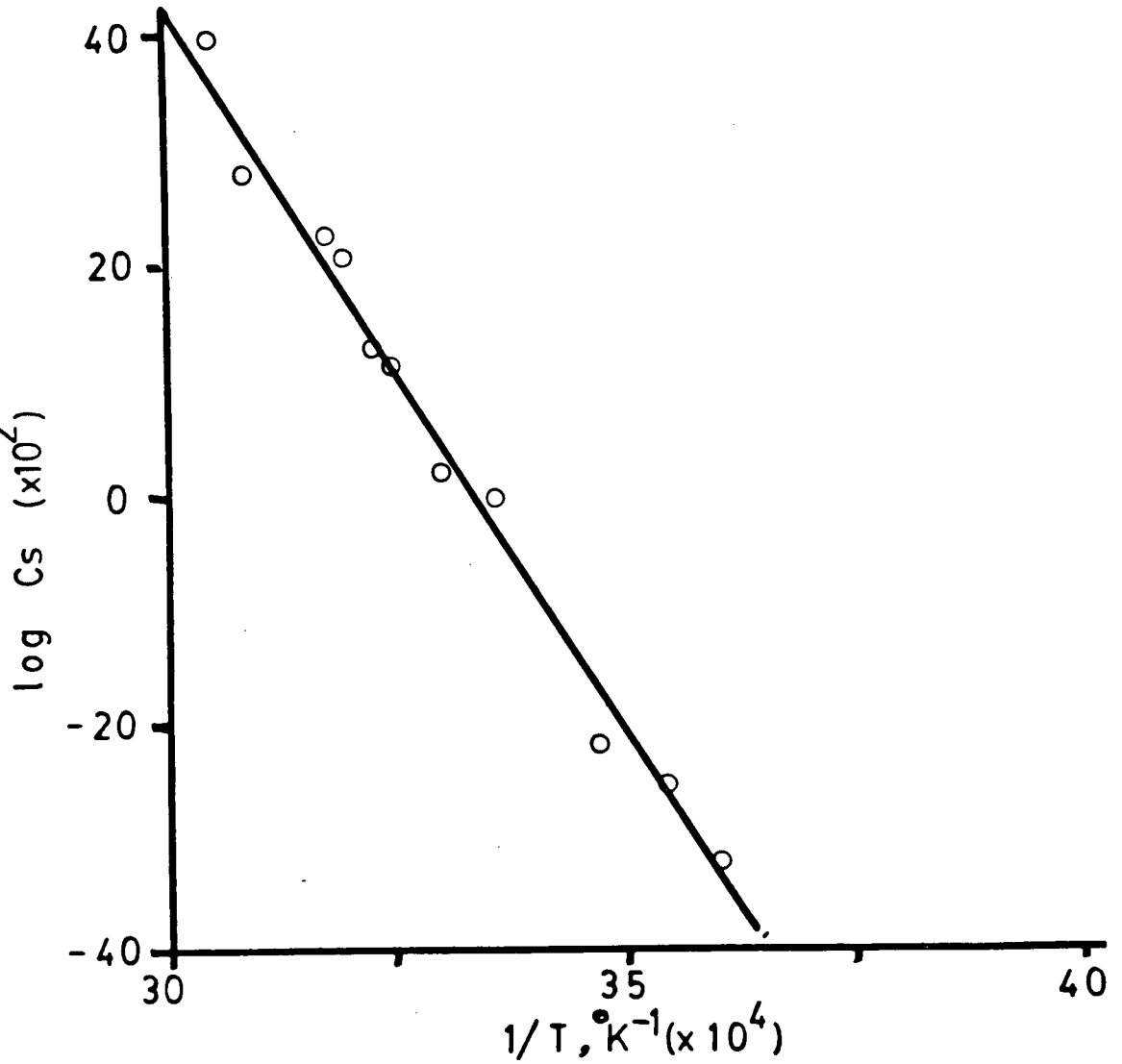


Figure 31. A van't Hoff plot for MSUM solubility in water.

the slope of which is - $\Delta H/2.303\ R$, where ΔH is the heat of solution. However, a requirement of this relationship is that the temperature range is not too large, so that ΔH remains a constant. Assuming that over the 50^{o} temperature range, the ΔH for MSUM in water is constant, the slope of the van't Hoff plot was found to be - 1267.7 ^{o}K, giving a ΔH of + 5.8 K cal mol^{-1} or + 24.3 KJ mol^{-1}. Allen et al. (1965b) found the slope of the van't Hoff plot for the solubility of MSUM in water to be - 1132 ^{o}K. The saturation solubility at 37^{o}, determined by interpolation of the van't Hoff plot was 1.379 g L^{-1}.

It is well documented that temperature has a significant effect on MSUM solubility (Loeb, 1972; Allen et al., 1965a,b; Wilcox et al., 1972; Fiddis et al., 1983). There are some variations in the reported values of the aqueous solubility of MSUM at different temperatures. This may be due to the different methods used in the determination of the saturation solubility. Our solubility data is within the range of values reported by other workers.

4.4.2 EFFECT OF SODIUM CHLORIDE

The saturation solubility of MSUM in the presence of differing concentrations of sodium chloride and the physiologic concentrations of chloride, sodium, calcium and potassium present in plasma or synovial fluid was studied as a function of temperature. The results are given in Table 6. The relationship

TABLE 6. Relationship between temperature and various concentrations of sodium chloride or physiologic ion concentration on the saturation solubility of monosodium urate monohydrate.

Temperature (°C)	Saturation solubility of MSUM[a] ($g\ L^{-1}$) in the presence of 0.2% sodium chloride	0.4% sodium chloride	0.6% sodium chloride	1.0% sodium chloride	0.88% NaCl 0.032%CaCl 0.03% KCl
4.4	0.047	0.023	0.018	0.012	0.015
10.4	0.664	0.035	0.031	0.013	0.021
17.8	0.106	0.053	0.032	0.022	0.030
24.7	0.143	0.064	0.042	0.027	0.035
29.9	0.240	0.116	0.072	0.042	0.063
41.8	0.471	0.229	0.155	0.096	0.139
51.1	0.599	0.336	0.241	0.142	0.216

a: Mean of 3 determinations.

between MSUM saturation solubility in water and temperature in the presence and absence of electrolytes is shown in Figure 30.

The saturation solubility of MSUM decreased significantly even in the presence of low concentrations of sodium chloride (Kippen et al., 1974). The concentrations of the different ionic species in plasma and synovial fluid are very similar and these ions also caused a large decrease in the solubility of MSUM at each temperature. Khalaf and Wilcox (1973) showed that calcium ions reduced the MSUM solubility in water, whereas potassium and cupric ions increased the solubility.

4.4.3 EFFECT OF CHONDROITIN SULFATE, HYALURONIC ACID, PROTEOGLYCANS AND ALBUMIN

The saturation solubility of MSUM in water was determined in the presence of these additives at 37^{o}. These values were subsequently used in the determination of rate constants for MSUM crystal growth in the presence of additives. The results are shown in Table 7.

The presence of 60 mg dL^{-1} CS decreased the saturation solubility of MSUM from 1.379 g L^{-1} (no additive) to 1.329 g L^{-1}. Laurent (1964) also showed a similar effect. However, Katz and Schubert (1970) reported a small increase in MSUM solubility in the presence of CS.

Chondroitin sulfate obtained commercially is the sodium salt of a mixture of chondroitin-6-sulfate and chondroitin-4-sulfate

TABLE 7. Saturation solubility of MSUM in the presence of additives at 37°.

Additive	Saturation solubility of MSUM[a] ($g\ L^{-1}$)				
	Additive concentrations ($mg\ dL^{-1}$)				
	10	20	40	60	100
Chondroitin sulfate	-----	1.358	1.347	1.329	-----
Hyaluronic acid	1.392	1.387	1.401	-----	-----
Albumin	1.382	1.391	1.397	-----	1.395
Proteoglycan monomer	1.402	1.397	1.399	-----	1.401
Proteoglycan aggregate	-----	1.408	1.397	-----	1.403

a: mean of three determinations.

containing about 5.6% w/w of sodium. The decrease in the saturation solubility of MSUM in water in the presence of chondroitin sulfate may be due to the presence of sodium in chondroitin sulfate sample (common ion effect).

Hyaluronic acid, albumin, proteoglycan monomer and proteoglycan aggregate caused very slight increases in the solubility of MSUM.

Katz and Schubert (1970) reported a slight increase in the solubility of MSUM in the presence of proteoglycans. They observed enhancement of MSUM solubility in the presence of small amounts of proteoglycans. Chondroitin sulfate and albumin were found to cause only a slight increase in MSUM solubility even in the presence of large concentrations of these additives. Perricone and Brandt (1978) reported an increase in urate solubility in the presence of PGA. However, the increase in MSUM solubility was not sustained beyond 4 hours.

Perricone and Brandt (1979) reported a greatly increased solubility of MSUM in the presence of proteoglycan aggregate (prepared in the presence of potassium ions). They concluded that the observed increase in solubility was due to the cationic exchange of potassium ions of the proteoglycan aggregate with sodium ions of MSUM.

4.4 CRYSTAL GROWTH OF MSUM

4.4.1 DETERMINATION OF SUPERSATURATION CONCENTRATION AND SEED AMOUNT FOR CRYSTAL GROWTH

Preliminary experiments conducted in the 1L apparatus showed no crystal growth at a supersaturation concentration of 4 g L^{-1}. The results of some of the experiments carried out at supersaturation concentrations of 5 g L^{-1} and 6 g L^{-1} are shown in Figures 32 and 33. No growth was observed when the supersaturation concentration was 5 g L^{-1} and the seed amount was 100 mg. In the presence of 200 mg seeds and a supersaturation concentration of 5 g L^{-1} there was slow linear growth followed by rapid non-linear growth showing a parabolic concentration-time curve. Similarly when the supersaturation concentation of MSUM was 6 g L^{-1}, the addition of 200 mg seeds showed slow growth for about 2 hours followed by a rapid non-linear growth phase. Addition of 500 mg and 1000 mg seeds to the supersaturated solutions, resulted in typical non-linear concentration versus time curves throughout the time course of the experiment.

Similar results were obtained using the 50 mL apparatus. Supersaturation concentrations necessary for crystal growth were 5 g L^{-1} and greater. Greater than 30 mg seed crystals were required to give the non-linear growth curves.

In the seeded growth studies of MSUM by Erwin and Nancollas (1981) no period of slow growth or induction period was observed.

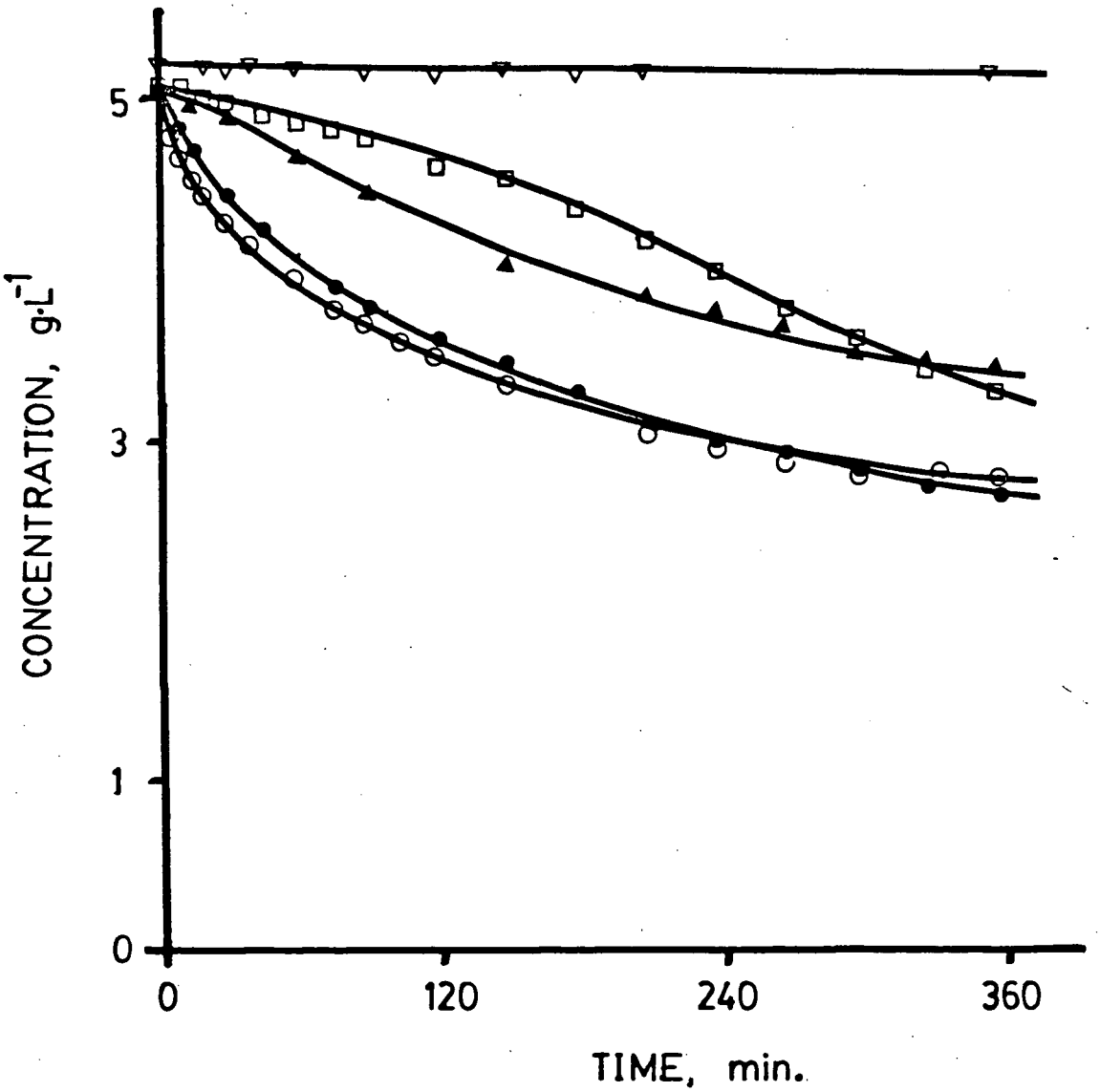


Figure 32. Seeded growth curves for MSUM at 37°, in 1 L capacity apparatus and an initial supersaturation concentration of 5 g L^{-1}. Added seed amount: 100 mg (▽); 200 mg (□); 300 mg (▲); 500 mg (●); 1000 mg (○).

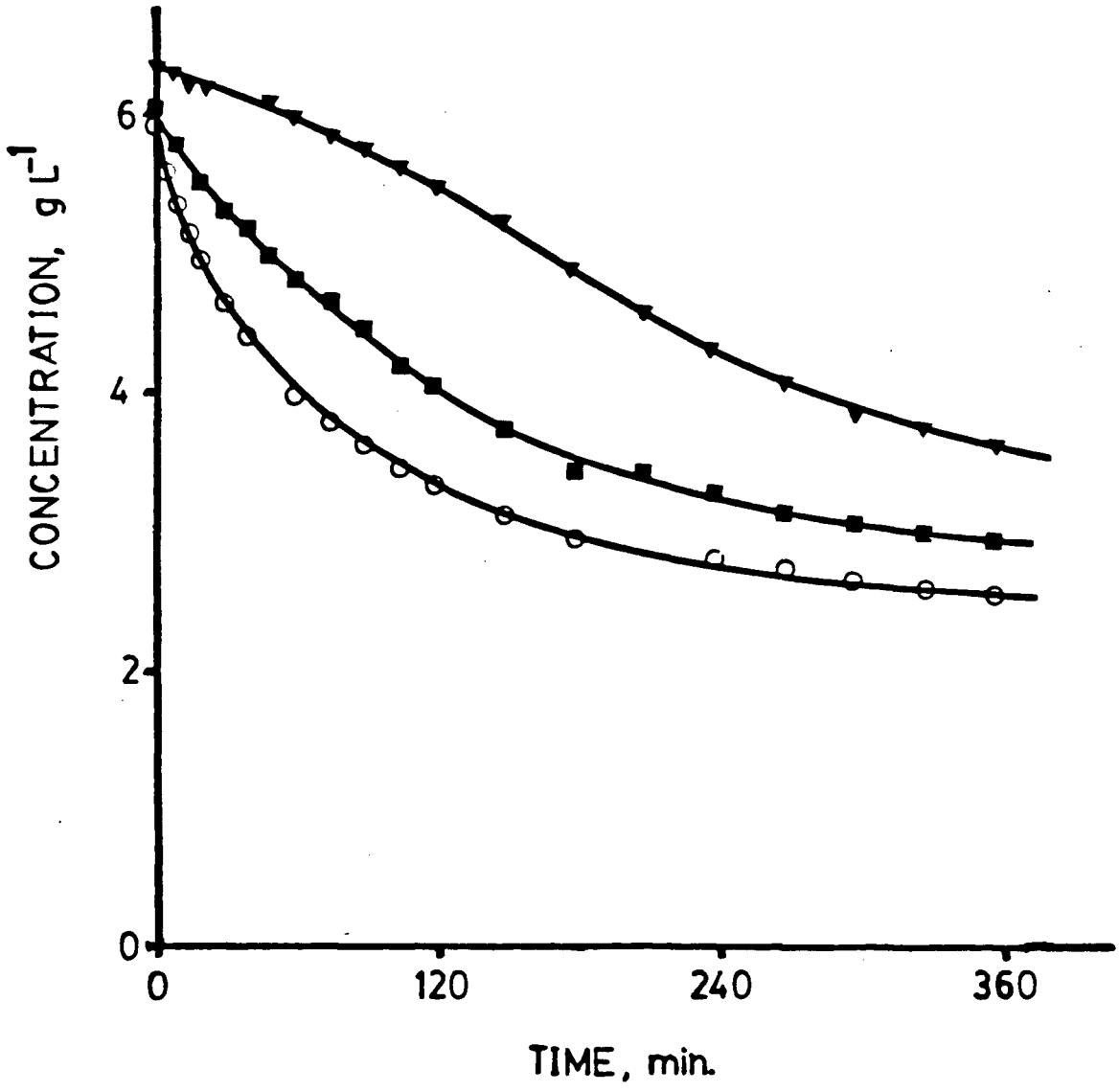


Figure 33. Seeded growth curves for MSUM at 37°, in 1 L capacity apparatus and an initial supersaturation concentration of 6 g L^{-1}. Added seed amount: 200 mg (▼); 500 mg (■); 1000 mg (O).

At an initial supersaturation concentration of approximately 2 g L^{-1} urate and seed concentration of 0.18 g L^{-1} and 0.54 g L^{-1} they observed the typical second order, non-linear relationship between urate concentration and time. Our results, however show that a very high initial supersaturation concentration of 5 g L^{-1} and large amount of seed crystals (greater than 200 mg) are prerequisites for non-linear growth curves.

Other studies have demonstrated induction effects in seeded growth systems. van Hook (1940) observed induction periods in the crystallization of silver chromate when supersaturated solutions were inoculated with seed crystals. He showed that either increasing the initial supersaturation concentration for a given seed amount or increasing the seed amount at a definite initial supersaturation concentration decreased the length of the induction period. He attributed his observations to a nucleation process. However, Howard and Nancollas (1957) subsequently showed that the induction periods in the growth of silver chromate were probably due to surface contamination by a hydrolysis product. Davies et al. (1955) studied the seeded growth of silver chloride and found that delayed crystallization occured when surface-contaminated seed crystals were added to supersaturated solutions. The induction period was inversely proportional to the quantity of seed added.

Even when impurities were absent, induction periods have still been observed. In the seeded growth of magnesium oxalate, Nancollas and Purdie (1961) showed that the rate constant for

growth depended on the number of crystallization sites present in the inoculating seed crystals. They found that the duration of the induction period was inversely proportional to the supersaturation concentration and bulk nucleation was observed during the time lag. It was thought that the high concentration gradients tended to build up solute by a diffusion process faster than it could be accommodated on the available growth sites. At lower supersaturation concentrations, or when the amount of inoculating seed was increased, the induction period disappeared, enabling second order growth to begin immediately.

Our observation that an increase in the amount of inoculating seed material at a given degree of supersaturation, reduced and finally abolished the induction period may be due to the achievement of a critical number of active growth sites on the seed crystals. As the amount of seed crystals added to the supersaturated solution is increased, achievement of the critical number of active growth sites would then lead to immediate second order growth of the seed crystals with the typical non-linear curves.

4.4.2 SELECTION OF THE METHOD TO DETERMINE THE RATE CONSTANT OF CRYSTAL GROWTH

The rate constants for crystal growth were determined using several different methods. The data from experiments without additives were used for this purpose.

An equation of the empirical form:

$$R_g = K'(C-Cs)^n \qquad (28)$$

where

R_g = the rate of growth (decrease in concentration per unit time),

K'(the rate constant of the reaction) = K_o(obs) S

K_o(obs)= observed overall rate constant of crystal growth,

S = surface area of the seed crystals,

C = supersaturation concentration of MSUM solution,

Cs= saturation solubility of MSUM at 37^o,

and n = order of reaction,

was used to calculate the growth rate constant as follows.

Logarithms of equation 28 were taken to give:

$$\log R_g = \log K' + n \log (C - Cs) \qquad (29)$$

Linear least square fits were performed on log R_g versus log (C - Cs) on the UBC computer. The rate constant of the reaction, K', was obtained from the intercept of the fit. The slope of the fit gave n, the order of the reaction. The values of the rate constants, K', and the order of the reaction, n, along with the correlation coefficients of the fits are given in Tables 8 to 11

In most of the experiments, the linear least-square fits gave poor correlation coefficients resulting in rate constant values which were in poor agreement for a given seed amount. The values of n, the order of the reaction, varied between 0.4 to

Table 8. MSUM growth rate constants, K', obtained from the linear regression of log R_g versus log (C-Cs).

(crystal growth in 1L capacity apparatus; C = 5 g L^{-1})

Experiment #	Seed batch	Seed amount (mg)	Growth rate constant, K' x 10^5 (L $g^{-1}min^{-1}$)	Correlation coefficient	Reaction order (n)
4	A	100	-----	-----	---
5	A	200	592.9	0.128	1.0
6	A	300	65.5	0.574	1.7
7	A	500	63.7	0.893	2.8
14	B	500	40.0	0.414	1.9
15	B	500	34.7	0.465	2.7
16	B	1000	45.9	0.877	3.4
8	A	1000	36.9	0.934	3.4

Table 9. MSUM growth rate constants, K', obtained from the linear regression of log R_g versus log (C-Cs).

(crystal growth in 1L capacity apparatus; C = 6 g L^{-1})

Experiment #	Seed batch	Seed amount (mg)	Growth rate constant, K' x 10^5 (L $g^{-1}min^{-1}$)	Correlation coefficient	Reaction order (n)
9	A	200	355.6	0.364	0.6
3	A	500	15.1	0.215	0.4
10	B	500	80.4	0.903	2.3
13	B	500	97.5	0.966	2.1
11	B	1000	97.3	0.964	2.7
12	B	1000	89.5	0.958	2.8

Table 10. MSUM growth rate constants, K', obtained from the linear regression of log R_g versus log (C-Cs).

(Crystal growth in 50 mL capacity appratus; C = 5 g L^{-1}; seed crystals from batch B)

Experiment #	Seed amount (mg)	Growth rate constant K'x 10^5 (L $g^{-1}min^{-1}$)	Correlation Coefficient	Reaction order (n)
41	5	---	---	---
40	10	---	---	---
39	10	7.2	0.47	7.2
38	20	3.6	0.51	3.5
37	20	0.5	0.69	5.5
36	30	6.4	0.58	3.5
35	30	1.5	0.79	4.9
34	40	6.6	0.76	3.9
33	40	4.7	0.88	4.1
32	50	10.2	0.88	4.1
31	50	2.4	0.77	5.0
30	50	40.5	0.97	2.4

Table 11. MSUM growth rate constants, K', obtained from the linear regressions of log R_g versus log (C-Cs).

(Crystal growth in 50 mL capacity apparatus; C = 6 g L^{-1}; seed crystals from batch B)

Experiment #	Seed amount (mg)	Growth rate constant, K' x 10^5 ($L\ g^{-1} min^{-1}$)	Correlation coefficient	Reaction order (n)
29	5	385.3	0.03	--
22	10	289.2	o.45	0.8
21	10	202.3	0.21	0.9
20	10	372.4	0.05	1.0
26	20	80.0	0.85	1.7
24	20	61.5	0.74	2.0
23	20	49.9	0.71	2.0
28	30	89.3	0.94	2.0
27	30	60.1	0.91	2.3
19	50	47.0	0.89	3.0
17	50	62.2	0.96	2.8

7.2. However, when the correlation coefficients were good (>0.95), n was between 2 to 3.

Due to the poor correlation of the data points this method was not used to determine the effect of additives on the growth rate constant for MSUM.

Marc (1908) observed that the rate of crystal growth of highly and moderately soluble substances was usually proportional to $(C - Cs)^2$. A similar observation was made by Davies and Nancollas (1955). It has been suggested that this relationship is due to spiral step controlled growth close to equilibrium (Nielsen, 1964). The rate of crystal growth, R, then is given by

$$R_g = K' (C - Cs)^2 \qquad (30)$$

A number of 1:1 and 2:2 electrolytes have been shown to follow the same growth law. These include, silver chloride (Davies and Nancollas, 1955), magnesium oxalate (Nancollas and Purdie, 1961), calcium sulfate dihydrate (Liu and Nancollas, 1970). A number of other electrolytes which follow the same growth law have been referenced by Nancollas (1979). More recently, Erwin and Nancollas (1981) studied the dissolution and crystal growth of sodium urate and reported the applicability of the same equation over a range of supersaturation, s',(s'= C/Cs), of 0.9 to 3.0. Further evidence for the applicability of the parabolic rate law was shown by the independence of the rate of

crystallization on the stirring dynamics, indicating a surface controlled mechanism for the crystallization of sodium urate.

The integrated form of equation 30 has been used over the years to determine the reaction rate constant for various compounds. Integration of equation 30 gives the following equation:

$$\frac{1}{(C-Cs)} - \frac{1}{(Ci-Cs)} = K' t \qquad (31)$$

where C is the concentration at various time intervals and Ci is the initial supersaturation concentration.

Equation 31 can be rearranged to equation 32:

$$C = \frac{1}{K't + \frac{1}{(Ci-Cs)}} + Cs \qquad (32)$$

This equation shows the non-linear relationship between C and t. A computer program (non-lin, Metzler et al., 1974) was used to estimate the reaction rate constant, K'. The values of K' and the correlation coefficients obtained by this non-linear fit are given in Tables 12 to 15. Good correlation coefficients (>0.98) were obtained by this method.

If the order of the growth reaction, n, is 2 then a plot of

Table 12. MSUM growth rate constants, K', obtained from the non-linear computer program.

(crystal growth in 1L capacity apparatus; C = 5 g L^{-1})

Experiment #	Seed batch	Seed amount (mg)	Growth Rate constant, K'x10^5 ($L\ g^{-1}min^{-1}$)	Correlation coefficient
4	A	100	---	---
5	A	200	30.0	0.981
6	A	300	83.9	0.997
7	A	500	222.1	0.998
14	B	500	62.7	0.997
15	B	500	92.7	0.994
16	B	1000	306.1	0.999
8	A	1000	533.5	0.997

Table 13. MSUM growth rate constants obtained from the non-linear computer program.

(crystal growth in 1L capacity apparatus; C = 6 g L^{-1})

Experiment #	Seed batch	Seed amount (mg)	Growth rate constant, K'x 10^5 ($L\ g^{-1}min^{-1}$)	Correlation coefficient
9	A	200	34.3	0.986
3	A	500	49.4	0.998
10	B	500	138.6	0.998
13	B	500	134.4	0.998
11	B	1000	388.2	1.000
12	B	1000	402.0	0.999

Table 14. MSUM growth rate constants obtained from the non-linear computer program.

(crystal growth in 50 mL capacity apparatus; $C = 5\ g\ L^{-1}$; seed crystals from batch B)

Experiment #	Seed amount (mg)	Growth rate constant, $K' \times 10^5$ ($L\ g^{-1} min^{-1}$)	Correalation coefficient
41	5	109.1	0.939
39	10	218.1	0.987
40	10	150.9	0.984
38	20	61.1	0.995
37	20	305.8	0.984
36	30	126.0	0.996
35	30	118.6	0.997
34	40	152.9	0.998
33	40	156.4	0.998
32	50	405.1	0.996
31	50	391.2	0.997
30	50	402.4	0.997

Table 15. MSUM growth rate constants obtained from the non-linear computer program.

(crystal growth in 50 mL capacity apparatus; $C = 6\ g\ L^{-1}$; seeds from batch B)

Experiment #	Seed amount (mg)	Growth rate constant, $K' \times 10^5$ ($Lg^{-1}min^{-1}$)	Correlation coefficient
29	5	21.3	0.977
22	10	23.9	0.983
21	10	32.7	0.998
20	10	22.9	0.993
26	20	32.8	0.998
24	20	35.4	0.998
23	20	34.4	0.998
28	30	80.0	0.999
27	30	112.8	0.999
19	50	431.4	0.996
17	50	343.6	0.998

$$\frac{1}{(C-Cs)} - \frac{1}{(Ci-Cs)} \quad \text{versus } t$$

should give a straight line with a slope of K'. Typical plots for some of the experiments are shown in Figures 34 and 35. Linear regression analyses on the values of (1/(C-Cs)) - (1/(Ci-Cs)) and t were performed on a pocket calculator. Excellent correlation coefficients were obtained. The values of K' and the respective correlation coefficients for different experiments are given in Tables 16-19.

Both the methods, non-lin program and linear regression of (1/(C-Cs)) - (1/(Ci-Cs)) versus t (Tables 12-15 and 16-19 respectively) gave good correlation coefficients in most of the experiments (>0.99).

Since the added seed amount varied between experiments, then the surface area of seeds available for growth was also different. Therefore the growth rate constant, K', was corrected for the surface area of added seeds in each experiment. The observed overall crystal growth rate constant, K_o(obs), was determined from K'/S, where S is the surface area of added seed crystals. The surface areas of seed crystals from batches A and B were corrected for the weight of seeds taken in each experiment and these values were used as values of S to determine the observed overall growth rate constant, K_o(obs).

It is evident that for a set of experiments where the same supersaturation concentration and batch of seed crystals were

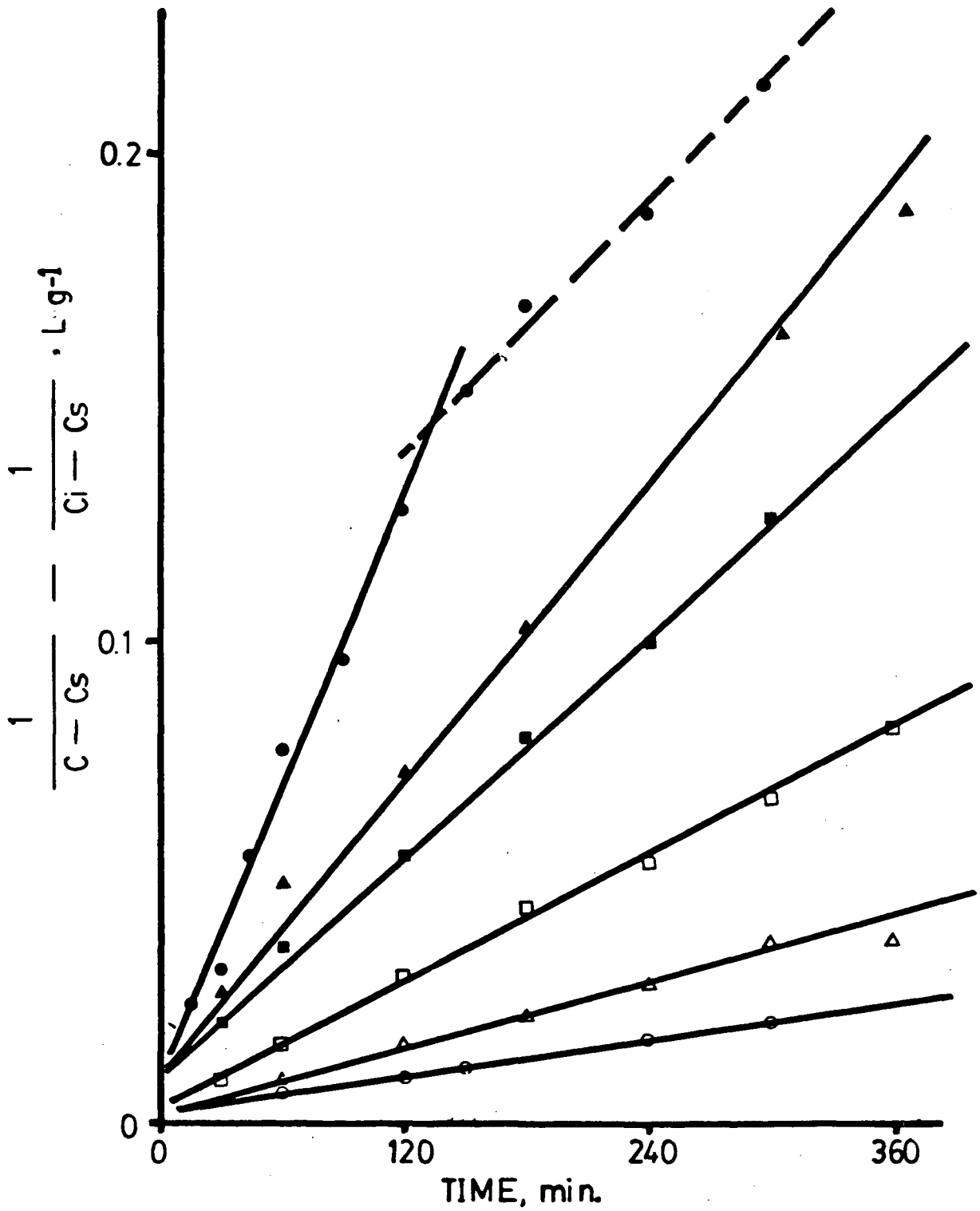


Figure 34. Second-order kinetic plots of the integrated form of growth equation for the seeded growth of MSUM at 37° in the 50 mL capacity apparatus and an initial supersaturation concentration of 5 g L. Seed amount: 5 mg, exp 41 (○); 10 mg, exp 39 (△); 20 mg, exp 38 (□); 30 mg, exp 36 (■); 40 mg, exp 34 (▲); 50 mg, exp 32 (●).

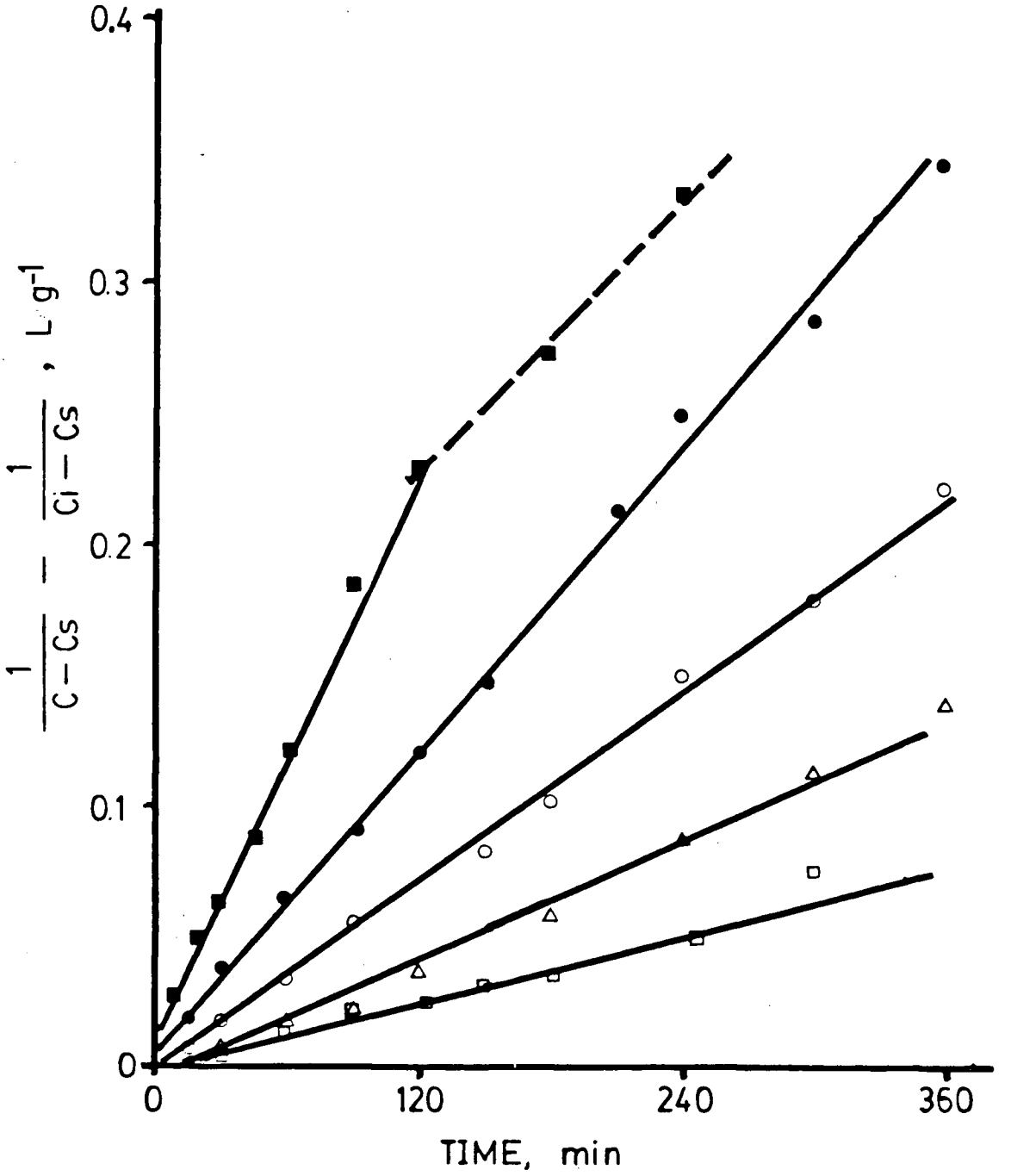


Figure 35. Second-order kinetic plots of the integrated form of growth equation for the seeded growth of MSUM at 37° in the 50 mL capacity apparatus and an initial supersaturation concentration of 6 g L^{-1} Seed amount: 5 mg, exp 29 (□); 10 mg, exp 20 (△); 20 mg, exp 26 (○); 40 mg, exp 27 (●); 50 mg, exp 30 (■).

Table 16. MSUM growth rate constants, K', obtained from plots of the integrated form of the second order growth equation.

(crystal growth in 1L capacity apparatus; C = 5 g L^{-1})

Experiment #	seed batch	Seed amount (mg)	Growth rate constant, $K' \times 10^5$ ($L\ g^{-1} min^{-1}$)	Correlation coefficient
4	A	100	----	---
5	A	200	69.9	0.998
6	A	300	62.3	0.997
7	A	500	135.2	0.996
14	B	500	69.9	0.996
15	B	500	72.0	0.990
16	B	1000	153.2	0.986
8	A	1000	121.9	0.991

Table 17. MSUM growth rate constants, K', obtained from plots of the integrated form of the second order growth equation.

(crystal growth in 1L capacity apparatus; C = 6 g L^{-1})

Experiment #	Seed batch	Seed amount (mg)	Growth rate constant, K'x 10^5 ($L\ g^{-1}min^{-1}$)	Correlation coefficient
9	A	200	44.8 (upto 180 min)	0.998
3	A	500	68.6	0.991
10	B	500	121.4	0.990
13	B	500	127.6	0.999
11	B	1000	170.7	0.986
12	B	1000	182.1	0.993

Table 18. MSUM growth rate constants, K', obtained from plots of the integrated form of the second order growth equation.

(crystal growth in 50 mL capacity apparatus; $C = 5\ g\ L^{-1}$ seed crystals from batch B)

Experiment #	Seed amount (mg)	Growth rate constant, $K' \times 10^5$ ($L\ g^{-1} min^{-1}$)	Correlation coefficient
41	5	7.7	0.946
39	10	10.3	0.986
40	10	12.1	0.977
38	20	22.6	0.995
37	20	18.6	0.988
36	30	38.8	0.990
35	30	34.6	0.986
34	40	48.4	0.988
33	40	42.6	0.993
32	50	62.7	0.975
31	50	51.4	0.978
30	50	72.3	0.995

Table 19. MSUM growth rate constants, K', obtained from plots of the integrated form of the second order growth equation.

(crystal growth in 50 mL capacity apparatus; $C = 6\ g\ L^{-1}$; seed crystals from batch B)

Experiment #	Seed amount (mg)	Growth rate constant, $K' \times 10^5$ ($L\ g^{-1} min^{-1}$)	Correlation coefficient
29	5	32.2	0.957
22	10	41.2	0.974
21	10	39.5	0.949
20	10	39.8	0.996
26	20	63.1	0.997
24	20	72.0	0.994
23	20	61.1	0.995
28	30	108.2	0.997
27	30	97.0	0.998
19	50	121.3	0.978
17	50	167.1	0.996

used, K_o(obs) values should be similar.

The values of K_o(obs) shown in Tables 20 to 23 were calculated from the values of K' generated by the two different methods.

The observed overall crystal growth rate constants, K_o(obs) estimated from the non-lin computer program show a wide variation in values for a given set experiments. However, K_o(obs) determined from the linear, reciprocal plot gave similar values within a given set of experiments. Therefore crystal growth rate constants, K_o(obs) were determined by the linear regression of (1/(C-Cs)) - (1/(Ci-Cs)) versus t for all further experiments on the effect of additives on the growth rate of MSUM.

4.4.3 EFFECT OF SEED CRYSTALS ON THE CRYSTAL GROWTH

Two batches of seed crystals A and B were used in the crystal growth experiments. The mean surface area of Batch A seed crystals was 1.0447 $m^2\ g^{-1}$ (n = 3; range 0.908 $m^2\ g^{-1}$ to 1.179 $m^2\ g^{-1}$) and the mean surface area of Batch B seed crystals was 1.4521 $m^2\ g^{-1}$ (n = 3; range: 1.417 $m^2\ g^{-1}$ to 1.548 $m^2\ g^{-1}$). The results of the effect on the rate constants for growth of these two batches of seed crystals are shown in Table 20. The seeds from the two different batches showed different growth rate constants.

The methods used in the preparation of the two batches

Table 20. Overall growth rate constants, K_o(obs) calculated from rate constants, K^o.

(crystal growth in 1L capacity apparatus; C = 5 g L^{-1})

Experiment #	Seed batch	Seed amount (mg)	Overall growth rate constant, K_o(obs) x 10^3 (L $g^{-1}min^{-1}m^{-2}$)	
			(a)	(b)
4	A	100	---	---
5	A	200	1.4	3.3
6	A	300	2.7	2.0
7	A	500	4.2	2.6
14	B	500	0.9	1.0
15	B	500	1.3	1.0
16	B	1000	2.1	1.1
8	A	1000	5.1	1.2

(a) Calculated from rate constant obtained from non-lin program.

(b) Calculated from rate constant obtained from linear regression of (1/(C-Cs) - 1/(Ci-Cs)) versus t.

Table 21. Overall growth rate constants, K_o(obs), calculated from rate constants, K'.

(crystal growth in 1L capacity apparatus; C = 6 gL^{-1})

Experiment #	Seed batch	Seed amount (mg)	Overall growth rate constant K_o(obs) x 10^3 ($L\ g^{-1}min^{-1}m^{-2}$) (a)	(b)
9	A	200	1.6 (upto 180 min)	-----
3	A	500	1.0	1.3
10	B	500	1.9	1.7
13	B	500	1.8	1.8
11	B	1000	2.7	1.2
12	B	1000	2.8	1.2

(a) calculated from rate constants obtained from non-lin program

(b) calculated from rate constants obtained from linear regression of (1/(C-Cs)-1/(Ci-Cs)) versus t

Table 22. Overall growth rate constants, K_o(obs), calculated from rate constants, K'.

(crystal growth in 50 mL capacity apparatus; C = 5 gL^{-1}; seed crystals from batch B)

Experiment #	Seed amount (mg)	Overall growth rate constant K_o(obs) x 10^3 ($L\ g^{-1}min^{-1}m^{-2}$) (a)	(b)
41	5	150.3	10.7
39	10	150.2	7.1
40	10	103.9	8.3
38	20	21.0	7.8
37	20	105.3	6.4
36	30	28.9	8.9
35	30	27.2	7.9
34	40	26.3	8.3
33	40	26.9	7.3
32	50	55.9	8.6
31	50	53.8	7.1
30	50	69.2	9.9

(a) Calculated from rate constants obtained from non-lin program.

(b) Calculated from rate constants obtained from linear regression of (1/(C-Cs)-1/(Ci-Cs)) versus t.

TABLE 23. Overall growth rate constants, K_o, calculated from rate constants, K'.

(crystal growth in 50 mL capacity apparatus; $C = 6\ gL^{-1}$; seed crystals from batch B)

Experiment #	Seed amount (mg)	Overall growth rate constant, $K_o \times 10^3$ ($L\ g^{-1}min^{-1}m^{-2}$) (a)	(b)
29	5	29.3	44.3
22	10	16.4	28.4
21	10	22.5	27.2
20	10	15.8	27.4
26	20	11.3	21.7
24	20	12.2	24.8
23	20	11.8	21.0
28	30	18.4	24.8
27	30	25.9	22.3
19	50	59.4	16.7
17	50	47.3	23.0

(a) calculated from rate constants obtained from non-lin program.

(b) calculated from rate constants obtained from linear regression of $(1/(C-Cs)-1/(Ci-Cs))$ versus t.

varied in several aspects (see section 3.3.1). Therefore it is likely that these variations in preparation led to significant differences in the type and number of defects present in the two batches of MSUM crystals. It is well documented that the defect content of crystals and their crystal growth rates are closely related (Van Batchelder and Vaughan, 1967; Brooks et al., 1968; Patel and Rao, 1979). The differences in growth rate constants shown by seeds from Batches A and B may be due to the differences in defect content of the seed crystals. Similar observations were reported by Erwin and Nancollas (1981) who showed that seed crystals of MSUM from different batches had different growth rate constants.

4.4.4 EFFECT OF SUPERSATURATION ON THE CRYSTAL GROWTH RATE CONSTANT

A change in the supersaturation concentration from 5 g L^{-1} to 6 g L^{-1} in the 50 mL apparatus, in the presence of seed crystals from the same batch increased the crystal growth rate constant values by about 3 to 4 fold. The growth rate constant value, K_o(obs), increased from approximately 7 x 10^{-3} L $g^{-1}min^{-1}$ m^{-2} to approximately 24 x 10^{-3} L g^{-1} min^{-1} m^{-2} when the supersaturation concentration was changed from 5 g L^{-1} to 6 g L^{-1} (Tables 22 and 23).

4.4.5 EFFECT OF SODIUM AND POTASSIUM IONS ON THE GROWTH RATE CONSTANTS

Chondroitin sulfate, hyaluronic acid, proteoglycan monomer

and proteoglycan aggregate contain a concentration of ions which are introduced during their preparation. The sodium and potassium contents of these cartilage components were determined by flame photometry and are shown in Table 24.

The effect on the growth rate constants of the concentrations of sodium or potassium present in the maximum quantities of additives used in crystal growth experiments was determined. Sodium or potassium ions equivalent to the highest concentration present in any of the additive studies were added to a supersaturated solution (5.0 g L^{-1}). Rate constants, K', were determined and are shown in Table 25.

In the presence of 1.6 mg (in 50 mL) of sodium the rate constant ranged from 42.5 x 10^{-5} L g^{-1} min^{-1} to 48.5 x 10^{-5} with a mean of 44.1 x 10^{-5} L g^{-1} min^{-1}. This value is not significantly different from that obtained in the absence of sodium ions. Similarly in the presence of 1.3 mg (in 50 mL) of potassium, a mean rate constant of 44.3 x 10^{-5} L g^{-1} min^{-1} was obtained.

It has been shown previously (section 4.4.2) that the addition of sodium ions suppresses the saturation solubility of MSUM, by the common ion effect. However, it is apparent that the small additional contribution made by the sodium or potassium ions present in the additives to the overall high concentration of sodium ions in the supersaturated solutions, was insufficient to cause any significant change in the rate constant for growth.

Table 24. Sodium and potassium content of additives.

ADDITIVE	SODIUM (% w/w)	POTASSIUM (% w/w)
CHONDROITIN SULFATE	5.6	---
HYALURONIC ACID	0.11	6.3
PROTEOGLYCAN MONOMER	5.8	---
PROTEOGLYCAN AGGREGATE	5.2	---

Table 25. Effect of sodium and potassium ions on MSUM crystal growth rate constant.

(crystal growth in 50 mL apparatus; C = 5 gL^{-1}

Experiment #	Seed amount (mg)	Sodium/ potassium added (mg)	Growth rate constant, K', x10^5 ($L\ g^{-1}min^{-1}$)
33	40	---	42.6
34	"	---	48.4
101	"	1.6(Na^+)	42.5
102	"	"	48.5
103	"	"	43.3
104	"	1.3(K^+)	44.9
105	"	"	45.7

4.4.6 EFFECT OF ADDITIVES ON CRYSTAL GROWTH RATE CONSTANT

The following method was used for the analysis of the data generated by crystal growth experiments in the presence of the additives, hyaluronic acid, chondroitin sulfate, proteoglycan monomer, proteoglycan aggregate, phosphatidylcholine and phosphatidylserine. The observed rate constant, K'(add), for the growth of MSUM in the presence of additives, was calculated from equation 31 as discussed previously (section 4.4.2), where Cs was taken to be the saturation solubility of MSUM in the presence of the same concentration of additive (see Table 7).

The ratio of observed rate constant in the presence of additive, K'(add) to K'(control) was calculated, where K'(control) is the control growth rate constant for MSUM in the absence of any additives, determined under identical conditions of supersaturation concentration, seed amount and sodium or potassium ion concentration. If the ratio K'(add)/ K'(control) was greater than or less than unity, the additive under study either increased or decreased the growth rate constant and hence altered the growth rate.

Statistical analyses were performed using a one way analysis of variance followed by a Neuman-Keul's test. The level of significance was $p<0.05$.

(A) EFFECT OF HYALURONIC ACID ON THE GROWTH RATE CONSTANT

The effect of HA on the growth rate constant of MSUM is shown in Table 26. In the presence of HA (5 mg to 20 mg) in the

Table 26. Effect of hyaluronic acid on the growth kinetics of MSUM in 50 mL apparatus.

Experiment #	Hyaluronic acid (mg)	K'(add)/K'(control)[a]
(a) Initial supersaturation concentration = 5 g L^{-1}.		
82	5	1.148
83	5	0.987
81	10	1.060
84	10	0.876
80	20	0.867
85	20	1.010
(b) Initial supersaturation concentration = 6 g L^{-1}.		
87	5	0.783
88	5	0.778
86	10	0.742
89	10	0.762

a: Ratio of the observed rate constant in the presence of HA to the observed rate constant in the absence of HA.

50 mL apparatus with an initial supersaturation concentration of 5 g L^{-1} and seed amount of 50 mg the ratios between the K'(add) and K'(control) showed only small variations from unity. However, when the initial supersaturation concentration was 6 g L^{-1} and the seed amount was 30 mg the K'(add)/ K'(control) decreased to 0.78 (±0.003) for 5 mg of HA and to 0.752 (± 0.010) for 10 mg of HA.

The analysis of variance and Neuman-Keul's test showed no significant difference in growth rate constants in the presence of HA from those of the controls (absence of HA) at the level of significance, $p < 0.05$ when the supersaturation concentration was 5 g L^{-1}. However, the same statistical analysis showed a significant decrease in the crystal growth rate constant in the presence of 5 and 10 mg of HA when the supersaturation concentration was 6 g L^{-1}.

Hence, although HA decreased the growth rate constant for MSUM growth at both 5 g L^{-1} and 6 g L^{-1} supersaturation concentrations, the decrease from control was significant only at the higher degree of supersaturation.

(B) EFFECT OF CHONDROITIN SULFATE ON THE GROWTH RATE CONSTANT

The ratios of K'(add) (rate constant in the presence of chondroitin sulfate) to K'(control) for different amounts of additive are given in Table 27. The ratio increased from 1.455 (± 0.004) for 10 mg chondroitin sulfate to 1.66 (± 0.040) for 30

Table 27. Effect of chondroitin sulfate on the growth kinetics of MSUM in 50 mL apparatus. Initial supersaturation concentration, 5 g L^{-1}.

Experiment #	Chondroitin sulfate (mg)	K'(add)/K'(control)[a]
91	10	1.734
92	10	1.496
98	10	1.414
93	20	1.094
96	20	1.520
97	20	1.624
94	30	1.624
95	30	1.697

a: Ratio of the observed rate constant in the presence of CS to the observed rate constant in the absence of CS.

mg chondroitin sulfate.

The analysis of variance and Newman-Keul's test showed a significant increase in the rate constant for MSUM crystal growth in the presence of chondroitin sulfate over that of controls at a level of significance, p, of <0.05.

(C) EFFECT OF PROTEOGLYCAN MONOMER ON THE CRYSTAL GROWTH RATE CONSTANT

The ratios of K'(add) (rate constant in the presence of proteoglycan monomer) to K'(control) at 5 mg and 10 mg added amounts of proteoglycan monomer are given in Table 28.

Since, the ratio is slightly greater than unity a small increase in the growth rate constant in the presence of 5 mg and 10 mg of PGM is evident. However, the analysis of variance and the Newman-Keul's test showed no significant difference between the control experiments and the growth experiments in the presence of PGM at the 5% level of significance.

(D) EFFECT OF PROTEOGLYCAN AGGREGATE ON THE CRYSTAL GROWTH RATE CONSTANT

The ratios of K'(add) (rate constant in the presence of proteoglycan aggregate) to K'(control) at different added amounts of PGA are given in Table 29.

In the presence of 10 mg, 20mg and 50 mg of PGA the ratios were 0.966 ($\pm$0.050), 0.987 ($\pm$ 0.140) and 1.163 ($\pm$ 0.025)

Table 28. Effect of proteoglycan monomer on the growth kinetics of MSUM in 50 mL apparatus. Initial supersaturation concentration , 5 g L^{-1}.

Experiment #	Proteoglycan monomer (mg)	K'(add)/K'(control)[a]
121	5	0.965
122	5	1.028
123	5	1.047
124	10	1.071
125	10	1.027

a: Ratio of the observed rate constant in the presence of PGMto the observed rate constant in the absence of PGM.

Table 29. Effect of proteoglycan aggregate on the growth kinetics of MSUM in 50 mL apparatus. Initial supersaturation concentration, 5 g L^{-1}.

Experiment #	Proteoglycan aggregate (mg)	K'(add)/K'(control)[a]
201	10	0.910
202	10	1.023
203	20	0.850
204	20	1.124
205	50	1.139
206	50	1.187

a: Ratio of the observed rate constant in the presence of PGA to the observed rate constant in the absence of PGA.

respectively. The one way analysis of variance followed by the Neuman-Keul's test showed no significant difference from control at the 5% level of significance.

(E) EFFECT OF PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLSERINE ON THE CRYSTAL GROWTH RATE CONSTANT OF MSUM

The results of the effects of the phospholipids, ph-choline and phosphatidyl serine expressed as ratios of K'(add) (rate constant in the presence of either of the two phospholipids) to K'(control) on the MSUM crystal growth are shown in Tables 30 and 31.

Phosphatidylcholine caused an increase in the growth rate constant in most of the experiments. The ratios (K'(add)/ K'(control)) were 1.120 (± 0.070), 1.325 (± 0.220) and 1.070 (± 0.220) in the presence of 10 mg, 20 mg and 30 mg of phohphatidylcholine respectively (Table 30).

As is evident from the results, large variations in the rate constant values were observed in the presence of ph-choline. The phosphatidylcholine used in this study was only 80% phosphatidyl-choline (label claim). Therefore the samples added to the supersaturated solution contained substantial levels of impurities. These impurities may have caused the high degree of variability in these experiments. It is also possible that the observed increase in the growth rate constant in the presence of phosphatidylcholine was due to a combination of both the phosph-atidylcholine and other impurities present in the phosphatidyl-

Table 30. Effect of phosphatidylcholine on the growth kinetics of MSUM in 50 mL apparatus. Initial supersaturation concentration, 5 g L^{-1}.

Experiment #	Phosphatidyl-choline (mg)	K'(add)/K'(control)[a]
53	10	1.050
54	10	1.190
55	20	1.293
56	20	1.357
57	30	1.299
58	30	0.862

a: Ratio of the observed rate constant in the presence of PC to the observed rate constant in the absence of PC.

Table 31. Effect of phosphatidylserine on the growth kinetics of MSUM in 50 mL apparatus. Initial supersaturation concentration, 5 g L^{-1}.

Experiment #	Phosphatidyl-serine (mg)	K'(add)/K'(control)[a]
71	10	1.171
75	10	1.071
72	20	1.027
73	20	1.047
74	20	1.028

a: Ratio of the observed rate constant in the presence of PS to the observed rate constant in the absence of PS.

choline sample.

Phosphatidylserine showed a very small increase in the growth rate constant. This is evidenced by a small increase from unity, in the ratios, K'(add)/K'(control) (Table 31). In the presence of 10 mg and 20 mg of ph-serine the ratios were 1.120 (± 0.050) and 1.030 (± 0.010).

The analysis of variance and the Neuman-Keul's test on the results showed a significant increase ($p < 0.05$) in the growth rate constant in the presence of ph-choline over that of control. However, at the same level of significance there was no significant difference in the growth rate constant in the presence of ph-serine over that of the control.

(F) EFFECT OF ALBUMIN ON MSUM CRYSTAL GROWTH

The effect of albumin on the crystal growth of MSUM is shown in Figure 36. The growth rate constants for MSUM in the presence of albumin are not given because very poor correlations were obtained when the concentration-time data were substituted into equation 31.

At an initial supersaturation concentration of 5 g L^{-1}, the concentration-time plot showed a linear decrease in concentration with time in the presence of 10 and 50 mg of albumin. The growth rate in the presence of 10 mg and 50 mg of albumin was less than the growth rate of MSUM in the absence of albumin (control). Complete inhibition of crystal growth was seen in the presence of 200 mg of albumin.

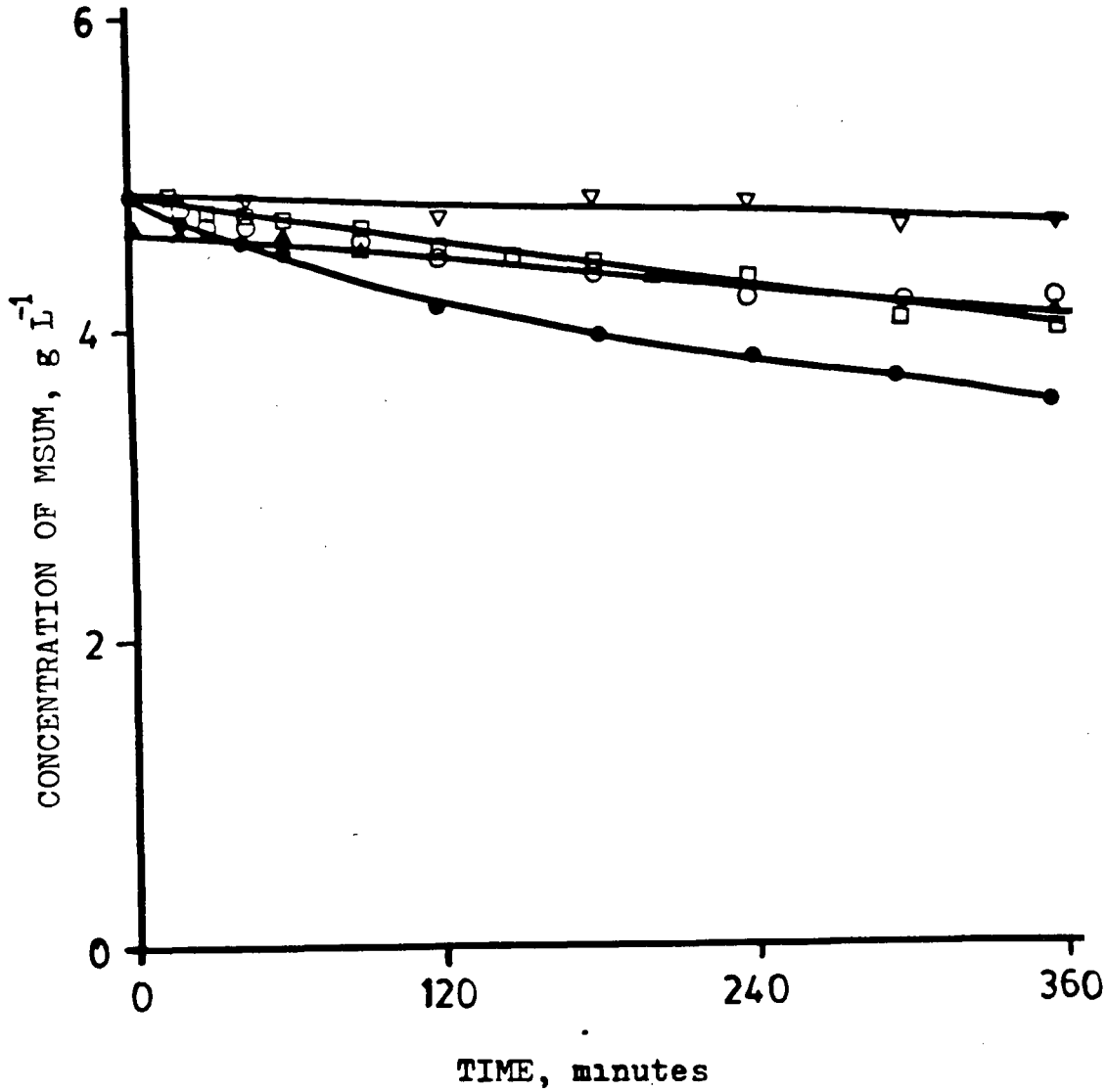


Figure 36. Seeded growth of MSUM in the 50 mL capacity apparatus and an initial supersaturation concentration of 5 g L^{-1}. Quantities of albumin added; 10 mg (○); 50 mg (□); 100 mg (▲); 200 mg (▽); control (●).

The presence of ions or molecules other than those of the material being crystallized can have number of effects on the growth kinetics or on the crystal morphology (see section 2.8.4). Atoms or molecules may enter the crystal lattice and can lead to the formation of defects in the crystals. Some compounds specifically adsorb onto a particular face of a crystal and inhibit the growth of the face, leading to a change in crystal morphology or habit. For example, surface active agents and trivalent ions such as Cr^{3+}, Fe^{3+} and Al^{3+} are commonly used to change crystal habits (Mullin, 1980).

Gron and Hay (1976) showed that the inhibitory effect of salivary secretions on the precipitation of dicalcium phosphate was associated with the macromolecular protein fraction of saliva. The stimulated saliva, which contains more protein was found to be about two times more inhibitory than unstimulated saliva. However, no inhibitory effect on the precipitation of dicalcium phosphate was observed when serum proteins (concentration range 0.6-5.5 mg mL^{-1}) were included in the growth medium.

Moreno et al. (1979) also observed a reduction in the rate of calcium hydroxyapatite precipitation in the presence of human salivary proteins. They suggested that the reduction in the growth rate induced by the macromolecules was produced by blocking of the crystal growth sites through adsorption of the macromolecules onto the surface of the calcium hydroxyapatite seed crystals.

Erwin and Nancollas (1981) studied the effect of heparin sodium on the growth rate of MSUM and reported no effect of this macromolecule at levels of 0.001-10 ppm.

In our studies both increased and decreased growth rates of MSUM were observed when the macromolecules, hyaluronic acid, chondroitin sulfate, proteoglycan monomer, proteoglycan aggregate and albumin were included in the growth medium. Hyaluronic acid and albumin inhibited MSUM growth, chondroitin sulfate and proteoglycan monomer accelerated MSUM growth and proteoglycan aggregate had no effect on MSUM growth kinetics.

Although chondroitin sulfate significantly increased the growth rate constant, this increase was not due to the sodium content of the CS sample as shown by the results in section 4.4.5 (Table 25).

MSUM has been shown to avidly bind with a number of proteins (Kozin and McCarty, 1976) and although MSUM crystals have a high negative surface potential, they bind significant amounts of negatively charged proteins such as the immunoglobulin IgG. Chondroitin sulfate is a highly negatively charged macromolecule, the negative charge arising from the sulfate (SO_4) groups on the molecule. It is possible that CS adsorbs onto the surface of the MSUM seed crystals and that following adsorption, CS acts as an accelerator of crystal growth. If the SO_4 groups on the CS molecule projected outwards away from the crystal surface these groups might attract sodium ions from the supersaturated solution

and these centers would then act as "catalysts" for the subsequent incorporation of urate ions into the MSUM crystal lattice. In this way, CS might function as a growth accelerator.

This proposed function of CS is similar to the role of proteoglycans in the calcification of bone. Before a tissue calcifies, it must be converted from a non-calcifiable to a calcifiable matrix (Urist, 1976). It is thought that the proteoglycans of cartilage bind calcium ions (Ca^{2+}) which then attracts phosphate ions (PO_4) and forms an inorganic nucleation center. It is also believed that the negatively charged, acidic phospholipids of matrix vesicles bind Ca^{2+} through a two-point electrostatic interaction which then allows the calcium ions to interact with phosphate ions in solution (Urist, 1976).

There is some evidence that preexisting disease such as osteoarthritis may lead to crystal deposition and also that the incidence of gouty arthritis increases with advancing age. Since the proportion of CS in both ageing and osteoarthritic cartilage decreases, it becomes difficult to explain how a decrease in the amounts of the growth accelerator, CS, could be a major cause of MSUM deposition in cartilage. However, it should be noted that in our growth experiments, a mixture of unknown composition of the two isomers of CS, chondroitin-4-sulfate and chondroitin-6-sulfate, was used. It is possible that the two isomers could have quite different effects on MSUM crystal growth which might correlate with the observed alterations in the ratios of chondroitin-6-sulfate to chondroitin-4-sulfate in aged and

osteoarthritic cartilage.

Crystals of MSUM can also precipitate directly in the synovial fluid. CS is found in the synovial fluid of patients with arthritis (Barker et al., 1966) but there is controversy as to whether CS is a normal constituent of synovial fluid (Barker et al., 1966; Silpananta et al., 1967). It is possible that the low levels of CS in pathological fluids may be sufficient to cause significant acceleration of MSUM crystal growth leading to the appearance of crystals in the synovial fluid.

The two phospholipids studied, phosphatidylcholine and phosphatidylserine, also increased the growth rate constant of MSUM. However, at the concentration levels of phosphatidylserine used in the growth experiments, the increase in the growth rate constant was not significant. Phosphatidylserine is an acidic phospholipid, bearing a net negative charge at physiologic pH, whereas phosphatidylcholine carries both a negatively charged secondary phosphate and a positively charged quaternary amine and is isoelectric in the pH range 3-10. Hence, these phospholipids may function as MSUM growth accelerators by the same mechanisms as described above for CS.

There is a significant increase in the intracellular and extracellular lipid content of articular cartilage with age and extracellular lipids are prominent in the surface layers of cartilage. These lipids are comprised of triglycerides, cholesterol or cholesterol esters, phospholipids and glycolipids. Normal synovial fluid contains small amounts of phospholipids

and cholesterol and synovial fluid from patients with rheumatoid arthritis and osteoarthritis show increased amounts of phospholipids, cholesterol and neutral lipids. The phospholipid composition of normal synovial fluid is similar to that of plasma with phosphatidylcholine being the major constituent. It is possible that the raised levels of phospholipids in aged or diseased cartilage and synovial fluid could accelerate the growth of MSUM crystals, resulting in MSUM deposition in these tissues.

Albumin and HA caused significant decreases in the MSUM growth rate constant, probably due to adsorption of these molecules onto the MSUM crystal surface with subsequent poisoning of the active growth sites on the crystal surface. Albumin levels have been found to be raised in the synovial fluids of gouty patients. Thus increased levels of the growth inhibitor albumin could not account for the appearance of MSUM crystals in gouty synovial fluids. Similarly, cartilage HA levels increase with advancing age and although HA has been shown to be a crystallization inhibitor, it is unlikely that HA is a significant factor in the crystallization of MSUM in articular cartilage.

At concentrations of proteoglycan monomer between 0.1-0.2 mg mL^{-1} and proteoglycan aggregate, 0.2-1.0 mg mL^{-1}, we found no significant effect of either PG monomer or PG aggregate on the growth kinetics of MSUM. Blumenthal et al. (1979) studied the effect of bovine nasal cartilage PG aggregate and PG monomer on the direct precipitation of hydroxyapatite from low concentration

calcium phosphate solutions. They found that with increasing concentration, the aggregate increased the time of onset (induction time) of hydroxyapatite formation compared to the PG-free control. The induction time was measured from mixing of reagents to onset of hydroxyapatite formation. The aggregate was more effective on a weight basis than the monomer in delaying the formation of apatite. Although the onset of apatite formation was affected by proteoglycans, the subsequent growth kinetics were not affected.

The latter observation is in agreement with our findings that proteoglycans did not significantly alter the growth kinetics of MSUM. The PG concentrations used by Blumenthal et al. (1979) were in the range of 0.1-1.5 mg mL^{-1}. Since the concentration of proteoglycans in cartilage was found by Maroudas (1975) to be in the range of 15-60 mg mL^{-1}, Blumenthal et al., (1979) predicted that such high PG concentrations would have a potent inhibitory effect on apatite deposition. It is possible that these high PG concentrations in cartilage, in addition to inhibiting the onset of apatite formation could also affect the growth kinetics of MSUM. Studies of the growth kinetics of MSUM employing high PG concentrations are needed to confirm this.

It is evident that the effects of the different cartilage and synovial fluid components on the seeded growth of MSUM from supersaturated solutions are very complex. Our results would suggest that alterations in the concentration of some components

as a result of ageing, preexisting disease or altered matrix metabolism could play significant roles in the growth of MSUM crystals in joints. However, crystal deposition may be a process involving the interplay of several factors. For instance, alterations in more than one cartilage or synovial fluid component may be necessary before MSUM growth proceeds. Factors such as local temperature and pH, concentrations of ions such as magnesium, calcium, sodium, pyrophosphate etc., water content of cartilage and other cartilage components not studied to date such as glycoproteins and keratan sulfate may also influence the deposition and growth of MSUM crystals.

4.4.7 CHARACTERIZATION OF MSUM AFTER GROWTH EXPERIMENTS

MSUM crystals after growth experiments both in the presence and absence of additives were characterized using scanning electron microscopy and X-ray diffraction.

Scanning electron micrographs of MSUM crystals after growth in the presence of additives are shown in Figures 37 to 41. The figures show that the needle shaped crystal habit of MSUM is retained on completion of the growth experiments.

The d-values for all MSUM samples after growth either in the presence or absence of additives were identical to d-values obtained for the original MSUM seed crystals, confirming that there was no alteration in the crystalline structure of MSUM after growth.

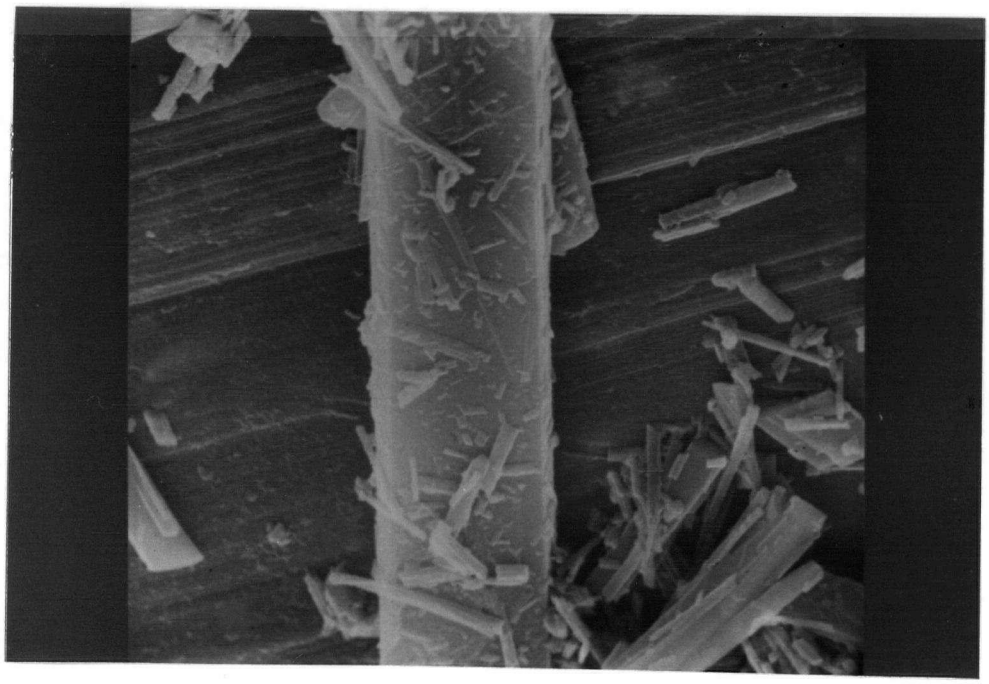


Figure 37. Scanning electron micrograph of MSUM crystals after growth. Initial supersaturation concentration 5 g L^{-1}.

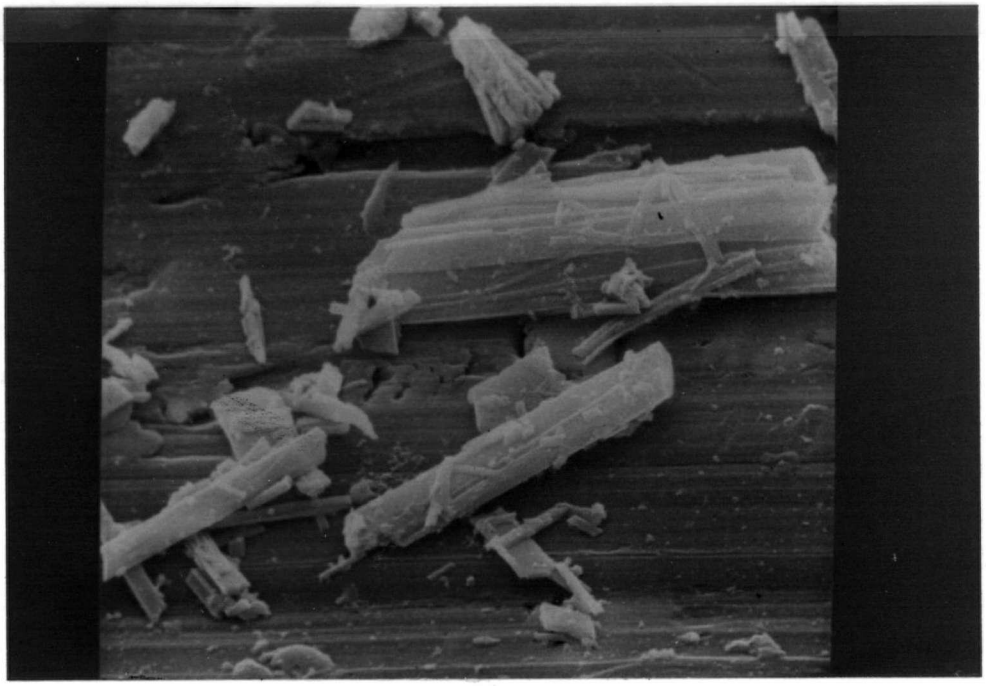


Figure 38. Scanning electron micrograph of MSUM crystals after growth. Initial supersaturation concentration, 6 g L^{-1}.

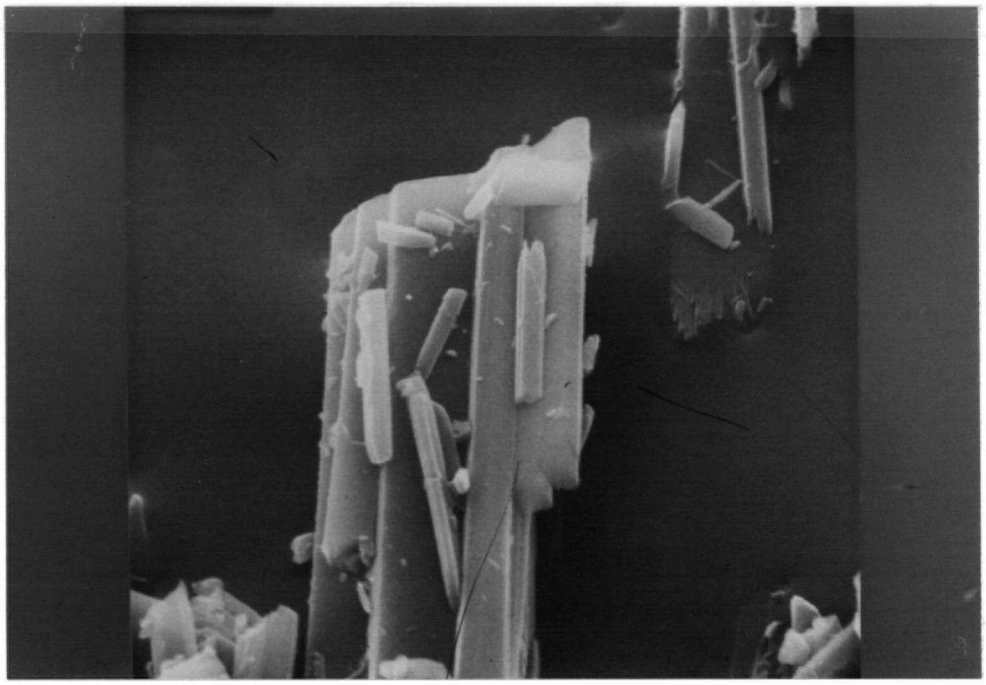


Figure 39. Scanning electron micrograph of MSUM crystals after growth in the presence of albumin (50mg). Initial supersaturation concentration, 5 g L^{-1}.

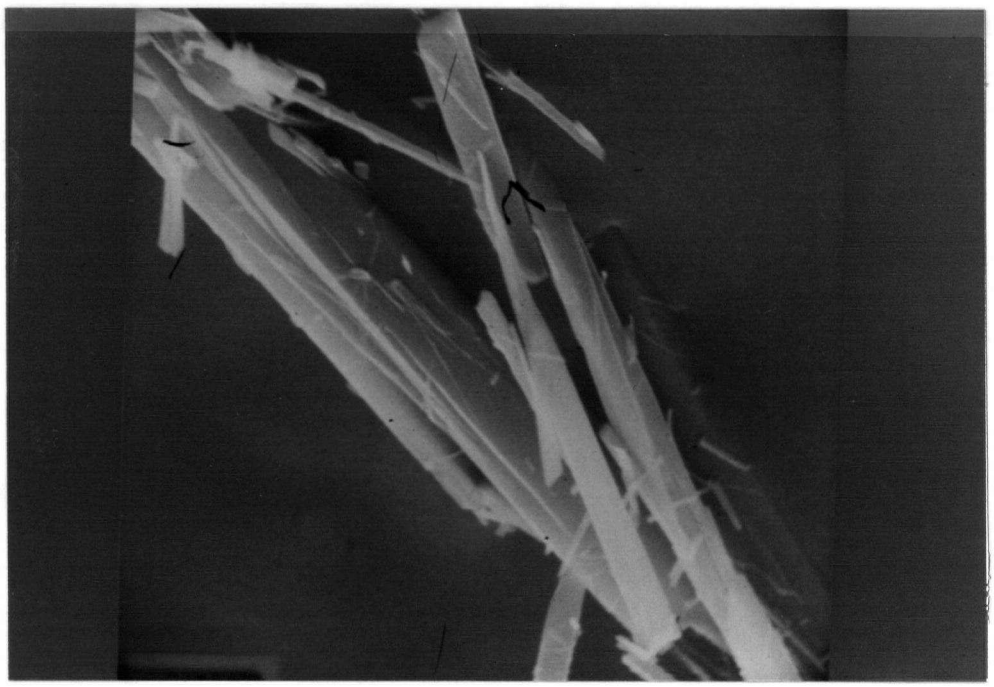


Figure 40. Scanning electron micrograph of MSUM crystals after growth in the presence of chondroitin sulfate (20 mg). Initial supersaturation concentration, 5 g L^{-1}.

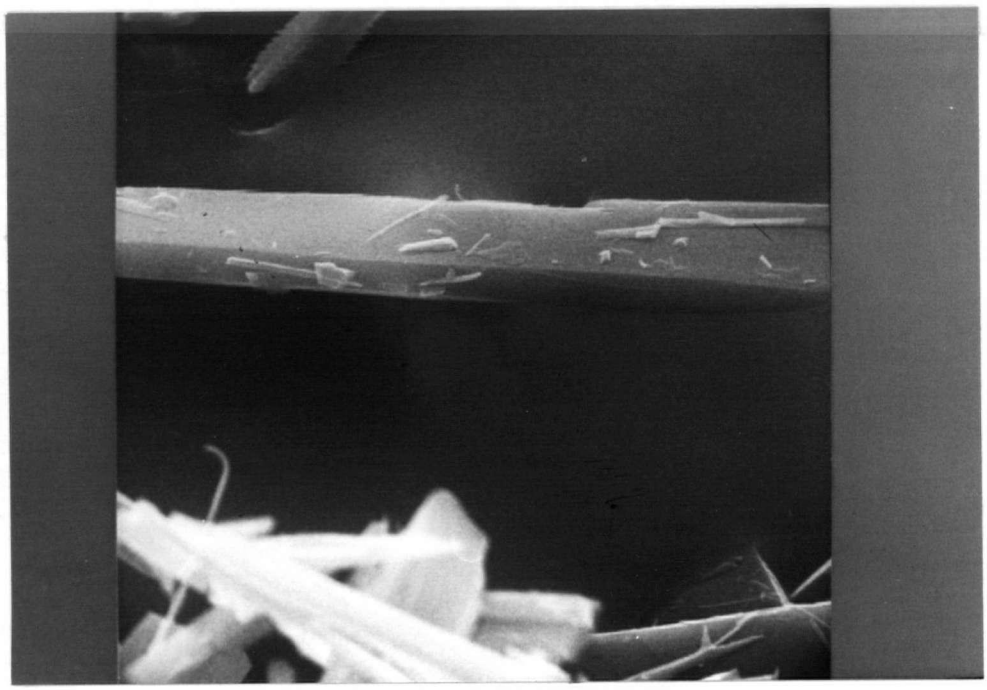


Figure 41. Scanning electron micrograph of MSUM crystals after growth in the presence of proteoglycan monomer (10 mg). Initial supersaturation concentration, 5 g L^{-1}.

5 SUMMARY AND CONCLUSIONS

(A) The degradation of MSUM in aqueous solutions was studied under non-sterile and sterile conditions. Sterile solutions were produced by (i) filtration through 0.22 um Millipore filters into rubber stoppered Vacutainers (ii) autoclaving in all-glass containers and (iii) autoclaving in Vacutainers. The solutions were incubated at different temperatures. The following results were obtained:

1. Non-sterile MSUM solutions stored in glass flasks with glass stoppers at 4^{o} were relatively stable, whereas at 22^{o}, 35^{o}, 45^{o} and 65^{o}, there was a gradual decrease in the concentration of MSUM. As the temperature of incubation was increased, degradation of MSUM occured more rapidly with the exception of solutions stored at 45^{o}, which were more stable than solutions stored at 35^{o}.

2. MSUM solutions sterilized by filtration through 0.22 um Millipore filters and stored in Vacutainers showed similar results to the non-sterile solutions. However, repeated determinations showed that these solutions demonstrated marked variability in urate concentrations at each time interval at

22^{o}, 35^{o} and 65^{o}.

3. MSUM solutions sterilized by autoclaving showed a decrease in urate concentration immediately after autoclaving. The decrease in MSUM concentration was greater for solutions stored in Vacutainers than for solutions stored in all-glass containers. This indicated that there was some urate loss in the presence of rubber stoppers, probably due to absorption into the rubber stoppers.

4. Autoclaved MSUM solutions in all-glass containers stored at 22^{o}, 35^{o} and 45^{o} were relatively stable upto 96 hours after an initial decrease in urate concentration during autoclaving, whereas solutions stored at 65^{o} showed a gradual decrease in urate concentration with time. In Vacutainers, the autoclaved MSUM solutions were relatively stable at 22^{o} and 45^{o} upto 96 hours after an initial loss in urate concentration during autoclaving, whereas solutions stored at 35^{o} and 65^{o} showed a gradual decrease in urate concentration from 0 to 96 hours.

5. Non-sterile and sterile MSUM solutions underwent decomposition with time. In non-sterile solutions, MSUM concentration may have decreased by both bacterial consumption and chemical degradation, whereas in sterile solutions the decrease in MSUM concentration was probably due only to chemical degradation.

It was evident from these experiments that the crystal

growth of MSUM could be studied upto 8 hours under non-sterile conditions at 37^{o} without appreciable degradation of MSUM.

(B) The saturation solubility of MSUM in water was studied at different temperatures and in the presence of differing concentrations of sodium chloride, chondroitin sulfate, proteoglycans, hyaluronic acid and albumin. The following is a summary of the results:

1. The heat of solution determined from a van't Hoff plot over a temperature range of 4.4^{o} to 55^{o} was 5.8 K cal $mole^{-1}$.

2. The saturation solubility of MSUM decreased significantly in the presence of sodium chloride.

3. Chondroitin sulfate decreased the solubility of MSUM, probably due to a common ion effect from the sodium present in the chondroitin sulfate.

4. Hyaluronic acid, proteoglycan monomer, proteoglycan aggregate and albumin caused very slight increases in the saturation solubility of MSUM at 37^{o}.

(C) The crystal growth kinetics of MSUM was studied by the seeded growth technique.

The effects of supersaturation concentration, seed amount and the additives, chondroitin sulfate, hyaluronic acid, proteoglycans (monomer and aggregate), albumin and phospholipids (phosphatidylcholine and phosphatidylserine) on the growth

kinetics of MSUM were studied. A brief summary of the results is given below:

1. Three methods were used to determine the growth rate constant, K' (K'= K_o(obs) S ; K_o(obs) = observed overall growth rate constant, S = surface area of the added seed crystals). An equation of the empirical form: $R = K' (C-Cs)^n$ was used for this purpose.

Least square fits were performed on log R versus log (C-Cs). The rate constant, K', was obtained from the intercept of the fit. In most of the experiments the linear least square fits gave poor correlation coefficients and resulted in rate constant values which were in poor agreement for a given seed amount. The values of n, the order of reaction, obtained from the slope of the curves varied between 0.4 to 7.2. However, when the correlation coefficient was good (>0.95), n was between 2 to 3.

A number of 1:1 and 2:2 electrolytes follow the second order (n=2) crystal growth equation. The integrated form of the growth equation with n = 2 was rearranged to obtain an equation which defined the non-linear relationship between the concentration and time (equation 32). A non-linear computer program (Metzler et al., 1974) was used to estimate the growth rate constant, K'. Good correlation coefficients were obtained by this method.

Since, the order of the growth process was assumed to be second, plots of the integrated form of the growth equation were constructed and the growth rate constants, K', were obtained from

the slopes of these straight line plots. Excellent correlation coefficients were obtained.

The growth rate constant, K_o(obs), was determined from K'/S. The growth rate constants, K_o(obs), estimated from the non-linear computer program showed wide variations in the values for a set of experiments at a given initial supersaturation concentration and seed amount. However, K_o(obs), estimated from the linear plots of the integrated form of the growth equation gave similar values within a given set of experiments.

The method employing the linear plot of the integrated form of the growth equation to determine K_o(obs) was used in all experiments involving the effect of additives on MSUM growth kinetics.

2. The growth of MSUM seed crystals required a high degree of supersaturation and a critical quantity of seed crystals. No crystal growth was observed at a supersaturation concentration of 4 g L^{-1}. In the 1 L capacity apparatus when the supersaturation concentration was 5 g L^{-1}, no growth was observed in the presence of 100 mg seed crystals. In the presence of 200 mg of seeds there was a slow linear growth for about 3 hours followed by rapid non-linear growth. Similarly when the supersaturation concentration was 6 g L^{-1}, a slow growth period for about 2 hours followed by rapid non-linear growth was observed when 200 mg seeds were present.

Similar results were obtained using the 50 mL capacity

apparatus. A period of slow growth (induction period) was observed in the presence of 5 mg to 20 mg seeds when the supersaturation concentration was 5 g L^{-1} and in the presence of 5 mg to 10 mg seeds when the supersaturation concentration was 6 g L^{-1}.

An increase in the added seed amount at a given degree of supersaturation decreased the length of the induction period until it was abolished at a certain "critical" added seed amount.

In the presence of seed crystals from the same batch, an increase in supersaturation concentration from 5 g L^{-1} to 6 g L^{-1} in the 50 mL capacity apparatus increased K_o(obs) by 3 to 4 fold.

3. The two different batches of seed crystals used in the crystal growth experiments showed different growth rate constants. The growth rate constants, K_o(obs), from batch A seeds were higher than the growth rate constants from batch B seed crystals. These differences in K_o(obs) values were thought to be due to the differences in the type and number of defects present in the two batches of seed crystals.

4. In the presence of sodium and potassium ions (1.6 mg in 50 mL and 1.3 mg in 50 mL respectively) there was no significant change in the growth rate constant values.

In the presence of 5 to 20 mg of hyaluronic acid the growth rate constant decreased at both 5 g L^{-1} and 6 g L^{-1}

supersaturation concentrations. However, the decrease from the control was significant only at 6 g L^{-1} supersaturation concentration. Chondroitin sulfate (10-30 mg) significantly increased the rate constant of MSUM growth. A small increase in the growth rate constant was observed in the presence of 5 mg to 10 mg of proteoglycan monomer. This increase in the growth rate constant was not significant at the 5% level of significance. Proteoglycan aggregate (5 mg to 20 mg) showed no significant change in MSUM growth rate constant. In the presence of the phospholipids, phosphatidylcholine and phosphatidylserine, an increase in the growth rate constant was observed. There was a significant increase in the growth rate caused by phosphatidylcholine, whereas for phosphatidylserine the increase was not significant.

Albumin (10 mg to 200 mg) caused inhibition of MSUM growth. At lower concentrations (10 mg and 50 mg), a decrease in the growth rate was observed from the concentration-time plot. Complete inhibition of MSUM crystal growth was seen in the presence of 100mg and 200 mg of albumin.

In these studies both increased and decreased growth rates of MSUM were observed when the macromolecules: hyaluronic acid, chondroitin sulfate, proteoglycans (monomer and aggregate) and albumin were included in the growth medium. These results indicated that the process of crystallization of MSUM from supersaturated solutions in the presence of these additives was complex. It is possible that alterations in the composition of

synovial fluid or cartilage as a result of ageing or preexisting disease or altered metabolism of the cartilage matrix could play a significant role in the growth of MSUM crystals.

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APPENDIX

EXTRACTION OF PROTEOGLYCANS FROM BOVINE NASAL CARTILAGE

The cesium chloride density gradient centrifugation method of Muir and Hardingham (1975) was used to isolate proteoglycans from the cartilage.

The bovine nasal cartilage was cut into small and thin slices. Ten volumes of 4 M guanidinium hydrochloride extraction buffer (see page 211) were added to each gram of the cartilage slices. The mixture was rolled for 24 hours in the cold room and the extract was separated from the residue by filtration through glass wool. Five volumes of the 4 M guanidinium hydrochloride extraction buffer was then added to the residue and the mixture was rolled for 3 hours at 4^{o}. The extract was separated from the residue by filtration through glass wool. The two extracts were pooled and were kept frozen till further treatment.

The extract was placed in a dialysing tube and dialysed against a large volume of 0.5 M guanidinium hydrochloride extraction buffer. The mixture was dialysed overnight. The extract was placed in a weighed measuring cylinder and the density of the mixture was adjusted to 1.69 g mL^{-1} by the addition of cesium chloride. The mixture was mixed gently to avoid foaming, placed in the ultracentrifuge tubes and ultracentrifuged for 48 hours at 100,000g at 20^{o}.

The centrifuge tubes were then cut into two portions:

(1) bottom 1/4 portion = A1

(2) top 3/4 portion = A2-4

To half of the A1 fraction was added equal volumes of 7.5 M guanidinium hydrochloride buffer and the density of this mixture was adjusted to 1.69 g mL^{-1}. The mixture was centrifuged at 100,000 g and 20^{o} for 48 hours. The tubes were cut into three portions:

(1) bottom 1/4 portion = A1D1

(2) middle 2/4 portion = A1D2-3

(3) top 1/4 portion = A1D4

The remaining half of the A1 fraction and the A1D1 fraction were dialysed against three changes of 0.2 M sodium chloride and the products lyophilized. The A1 fraction was proteoglycan aggregate and A1D1 fraction was proteoglycan monomer.

Extraction buffer:

4.0 M Guanidinium hydrochloride

0.05 M Sodium acetate trihydrate

0.01 M Tetrasodium EDTA

0.10 M Ethylamino caprolic acid.

0.005 M Benzamidine hydrochloride

0.001 M Phenylmethyl sulfonyl flouride

0.004 M n-ethyl maleimide