

The Interaction of Monosodium Urate with Connective Tissue Components

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ABSTRACT Monosodium urate deposits almost exclusively in the connective tissues of patients with gout. Acetone dried homogenates of bovine nasal cartilage, but not of other tissues, markedly enhances the solubility of urate in buffers having molarities and hydrogen ion concentrations similar to that of most body fluids. The components of cartilage responsible for this effect are the proteinpolysaccharides, compounds of protein and chondroitin sulfate, called PPL. A progressive increase in PPL concentration results in a corresponding increase in urate solubility. If, on the other hand, unbound chondroitin sulfate or PPL digested by trypsin is used, then no significant augmentation of urate solubility occurs indicating that the integrity of the molecule is essential. One subfraction of PPL, PPL_s, causes an even more exaggerated response while another, PPL_l, causes a lesser one. These proteinpolysaccharide macromolecules also inhibit the crystallization of urate from a supersaturated medium. The mechanism of the solubilizing phenomenon is not known. It is suggested that some type of physical or chemical binding is responsible. When, as a result of normal or accelerated connective tissue turnover, PPL is enzymatically destroyed, urate crystals then precipitate from the saturated tissue fluids.

INTRODUCTION

Monosodium urate deposits almost exclusively in the connective tissues of patients with gout (1). The crystals may be found in cartilage, synovia, tendon sheaths, the subcutaneous layers of the skin, and even the interstitial areas of the kidneys. They are conspicuously absent, however, from the muscular tissue, brain, liver, spleen, and lungs. Although it is recognized that urate microcrystals are capable of inducing an acute inflam-

matory reaction, no satisfactory explanation exists for the mechanism of their deposition or for their preference to deposit in some tissues and not in others.

Probably the earliest significant discussion of urate precipitation in gout was by Sir William Roberts in 1892 (2). After the 3 day incubation of pig tarsal bones in a saturated solution of sodium biurate, he noted the encrustation of tophaceous-like material on the cartilagenous articulating surfaces. He proposed that precipitation occurred because of relatively high sodium concentration in connective tissue as compared to the parenchymal organs. For the next half century, almost no work of this nature was performed. In 1961, Greiling utilizing titration studies tried to show that chondroitin sulfate acting as a weak acid served as a cation exchanger by replacing the sodium ion of monosodium urate with hydrogen and thus causing the conversion to uric acid (3). He cited the lesser solubility of the latter as a cause for its precipitation in polysaccharide-rich connective tissues. However, although uric acid is, indeed, less soluble in distilled water, it is more soluble in the tissue fluids at pH 7.43 (4). Furthermore, it is recognized that urate and not uric acid crystals are found in these tissues and that chondroitin sulfate is not present in a form containing associated hydrogen ion, that is, as a free acid.

Laurent postulated on the basis of solubility studies that the excluded volume effect of macromolecules such as chondroitin sulfate accounted for the decreased solubility of urates in the connective tissue in gout provided that "urate is produced within or in the neighbourhood of the tissue" (5). Solutions of progressively higher concentrations of chondroitin sulfate were agitated with excess urate for 48 hr. He found an inverse relationship between the dissolved urate and the polysaccharide in solution, that is, the higher the chondroitin sulfate concentration, the lower the urate

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solubility. The conclusions drawn from that study are open to question because it is more likely that the uricase action of certain bacterial contaminants and not an excluded volume effect accounts for this phenomenon.¹ In any case, the excluded volume effect of PPL to a small molecule like urate is negligible unless the urate is bound to a larger molecule. The purpose of this report is to show an apparent affinity of urates for connective tissue and one of its components, PPL.

PPL is a principle proteinpolysaccharide of connective tissue. One form is readily isolated from bovine nasal cartilage while others occur in varying concentrations in human and articular cartilage, human costal cartilage, and other tissues (6, 7). It is obtained from cartilage by homogenization and ultracentrifugation and may be separated into a series of fractions, PPL₂, 3, 4, 5, and 6 (8). They each have distinctly different properties and compositions and are numbered in increasing order of sedimentability at 100,000 *g*. All consist mainly of protein, chondroitin sulfate, and small amounts of keratan sulfate. These different forms may exist as such in native cartilage or may be artifacts of the preparative process. The PPL molecule is large, highly diffuse, and in solution the polysaccharide chains tend to be widely separated. Recent work has indicated that it is present in minute amounts in serum (9).

METHODS

Materials. The PPL was isolated from bovine nasal cartilage homogenates according to the procedure of Gerber, Franklin, and Schubert (8). The potassium salt of chondroitin sulfate was obtained employing the method of Einbinder and Schubert (10). Commercial chondroitin sulfate, trypsin, and bovine tissue homogenates were purchased from the Pentex Corporation.² Ultracentrifugation was used to fractionate PPL₃ and PPL₅ from whole PPL (11). Crystals of monosodium urate were prepared following a previously described standard technique with the exception that urate solutions were immediately cooled (12).

Analyses. Uric acid was determined by enzymatic differential spectrophotometry using a Hitachi-Perkin-Elmer spectrophotometer, Model No. 139 (Perkin-Elmer Corp., Norwalk, Conn.). All readings were taken at a wave length of 292 μ , the absorption peak (13). Urate crystals were identified by a Leitz polarizing microscope (E. Leitz, Inc., New York).

The solubility of monosodium urate in bovine tissue homogenates. Acetone dried homogenates of bovine tissues were dialyzed against buffer for 3 days at 4°C. 120 mg each of dried nasal cartilage, liver, brain, heart, and kidney was mixed in 4.0 ml, 0.021 M potassium phosphate buffer containing 0.130 M sodium chloride. This was used throughout the study. The final pH was 7.40 \pm 0.03. These suspensions were then stored overnight at 4°C. To each was added 10.0 mg of sodium urate, and the resultant mixture was placed in a sealed ampoule and agitated continuously at 4°C for 24

hr. The uric acid of the supernatant was determined after the excess urate was removed by centrifugation at 3500 rpm for 15 min. If periodic culturing of the media revealed any evidence of bacterial contamination, the material was discarded. After trypsin was added, the supernatants were incubated at 4°C for 16–20 hr. Enzymatic action was satisfactory at this temperature as at 37°C, but bacterial growth was eliminated.

The solubility of monosodium urate in polysaccharide solutions. Excess amounts of monosodium urate (equivalent to 2 mg/ml) were weighed into ampoules and sterilized by dry heat (100°C) overnight in order to eliminate growth of uricase-producing organisms. To the ampoules were added solutions containing varying concentrations of PPL, PPL₃, PPL₅, chondroitin sulfate, albumin, and methyl cellulose in sterilized potassium phosphate buffer. Sterilization of the polysaccharides was also achieved by dry heat. Their biochemical properties were not affected. The vessels were agitated continuously for periods extending to 4 days. The mixture was centrifuged for 20 min at 3500 rpm, and the uric acid of the supernatant was determined. PPL did not interfere with the uricase reaction. After centrifugation, although some of the solutions were opalescent, no crystals were identifiable by polarizing microscopy.

In order to determine the final solubility starting from a supersaturated level, solutions were prepared by dissolving monosodium urate (350 mg/100 ml) in 0.021 M potassium phosphate buffer in a boiling water bath. NaCl was added to give a final nonurate molarity of 0.161 and a pH 7.40 \pm 0.03. After the solutions cooled to room temperature, varying amounts of heat-sterilized PPL, PPL₃, PPL₅, chondroitin sulfate, albumin, and methyl cellulose were dissolved. Maximum concentrations used were 10.0, 10.0, 10.0, 100.0, 120.0, and 10.0 mg/ml respectively. The solutions were kept at 4°C and agitated in a Kahn shaker for 1 hr, 4 times daily. They were then centrifuged until maximal separation of crystals occurred (10 min). As before, the solution containing PPL remained opalescent after centrifugation, but urate crystals could not be identified microscopically in the supernatant. However, if allowed to warm to room temperature, the opalescence cleared though no further crystallization occurred.

Enzyme studies were performed in order to destroy the integrity of the PPL molecule. These were done without readjusting the pH of the supernatant in order to avoid the precipitation of urate crystals. Trypsin or hyaluronidase was added to the supernatant and incubated overnight at 4°C as in the first part of the study. Cetylpyridinium chloride (4 mg/ml) was mixed with the urate-saturated supernatant of PPL or buffer and allowed to stand overnight at 4°C. Centrifugation at 3500 rpm of the mixture was followed by determination of uric acid.

RESULTS

The solubility of monosodium urate in bovine tissue homogenates. The solubility of monosodium urate in saline solutions at 4°C was 5.7 mg/100 ml measured as uric acid. When a homogenate of bovine nasal cartilage was first dispersed in the buffer, the final solubility increased to 25.5 mg/100 ml. Without appreciable bacterial contamination, the results of urate solubility at 37°C were equivalent to those obtained at 4°C. On the other hand, when homogenates of other organs were similarly added, minimal augmentation of solubility

¹ Katz, W. A., and R. Levy. The uricase action of certain bacteria. To be published.

² Pentex Biochemical, Kankakee, Ill.

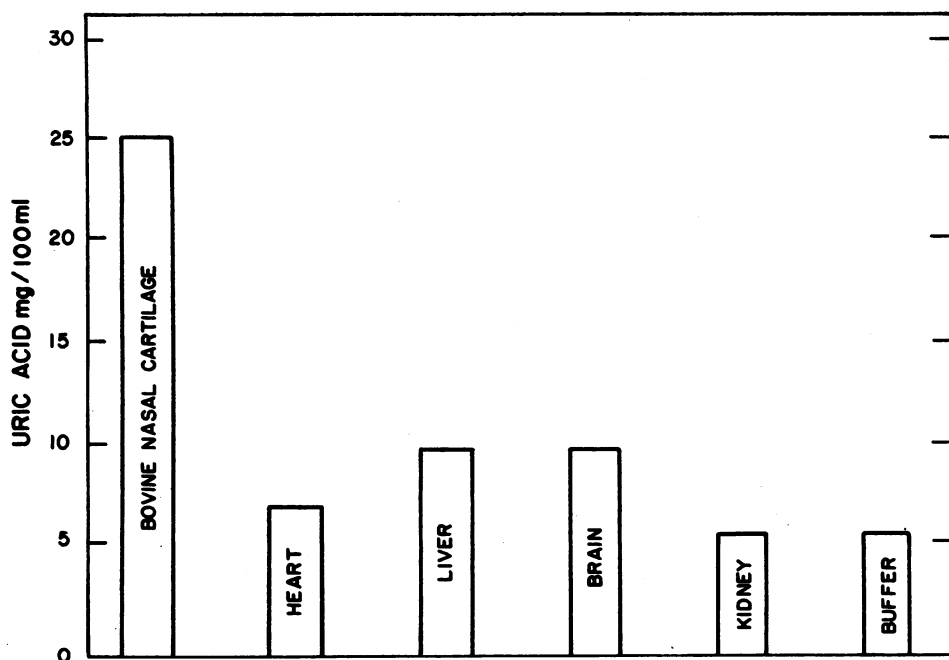


FIGURE 1 Effect of bovine tissue homogenates on the solubility of monosodium urate in pH 7.4, 0.021 M potassium phosphate buffer containing 0.13 M sodium chloride.

occurred with liver and brain but not with heart, muscle, and kidney (Fig. 1). After the incubation of a solution of urate-saturated bovine nasal cartilage with trypsin, visible precipitation of crystals occurred within a few hours. Simultaneously, the uric acid concentration of the supernatant after centrifugation was identical to that of the control buffer (5.7 mg/ml). Trypsin, when added to the solution containing homogenates of other bovine tissues, of course, did not change the uric acid concentration.

The solubility of monosodium urate in polysaccharide solutions. Urate solubility is greatly enhanced by the presence of PPL. As shown in Fig. 2, at higher concentrations of PPL, more urate enters the solution. At relatively lower concentrations, the relationship between the urate solubility and PPL concentration is linear. However, at greater than 8 mg/100 ml, the slope of the curve levels off. In comparison, PPLs had a slightly greater ability to increase urate solubility. In Table I, it is demonstrated that at concentrations of 8 mg/ml, PPLs held 3.5 times as much urate in solution as the control buffer while PPL held only 3 times as much. In contrast, PPLs caused relatively little change of urate solubility. Unbound chondroitin sulfate in concentrations as high as 80 mg/ml, 10 times that of PPL, resulted in only a slight increase in solubility; and similar changes occurred with methyl cellulose and albumin even when 120 mg/ml of the latter was used. PPL from bovine humeral articular cartilage, used in a limited

number of experiments, caused a solubilization of urate similar to that from nasal septum. Expanded studies were not performed because of difficulty in obtaining the material. Although agitation of powdered urate with solutions containing these substances was allowed up to 4 days, maximal solubility was achieved in 6–24 hr; therefore, most experiments were performed overnight as a matter of convenience. Equilibrium studies designed to demonstrate the final solubility starting from a supersaturated level revealed that maximal crystallization of urate occurred at 5–6 days (Fig. 3). After that time, the final solubility, when complete crystallization had occurred, was the same as that achieved in the standard

TABLE I
The Effect of Polysaccharides and Proteins on the Solubility of Monosodium Urate in pH 7.4, 0.021 M Potassium Phosphate Buffer Containing 0.130 M Sodium Chloride

Added substance	Concentration mg/ml	Uric acid mg/ml
PPL	8	16.8
PPL _s	8	7.1
PPL ₅	8	19.6
Chondroitin sulfate	80	7.7
Albumin	120	7.3
Methylcellulose	8	7.4
None	—	5.7

solubility studies. Albumin and glycerin (to test the effect of viscosity alone) inhibited the rate of crystallization of urate but did not otherwise alter urate solubility.

After the PPL solution saturated with urate was incubated with trypsin for 2-3 hr, the appearance of crystals was noted. Crystallization was complete within 24 hr. A corresponding decrease in uric acid concentration of the supernatant was observed (Table II). The adjusted final urate concentration was 7.1 mg/100 ml, the same as found with chondroitin sulfate alone. A similar phenomenon was found when hyaluronidase was added; however, the final solubility did not quite fall to the level seen with chondroitin sulfate. Under the conditions of the experiment (pH 7.4), hyaluronidase action is not optimal and this could account for its smaller effect. The addition of cetyl pyridinium chloride to precipitate PPL resulted in urate crystallization and again a corresponding diminution in urate concentration after only a few minutes.

DISCUSSION

Urate crystals are not ubiquitous in the normal human body. By definition their presence indicates a pathological condition—gout. Hyperuricemia is almost always coexistent; however, despite common belief, it is as a sole factor not the cause of gout. The majority of patients with serum uric acid elevations never develop joint disease nor do they show any evidence of urate crystallization (14). Even when they occur, the deposits tend to be found only in the connective tissues. The nature of this apparent affinity has never been clearly demonstrated although numerous hypotheses have been proposed. Avascularity of connective tissues, notably cartilage, is often held as the predisposing factor in urate deposition but, indeed, urates may also deposit in the synovial membrane which is moderately vascular, especially when inflamed. It is assumed that in avascular areas glycolysis is the primary source of energy resulting in the production of large quantities of lactic acid. After the urate-induced inflammatory process is established, then even more lactic acid is produced. As the hydrogen

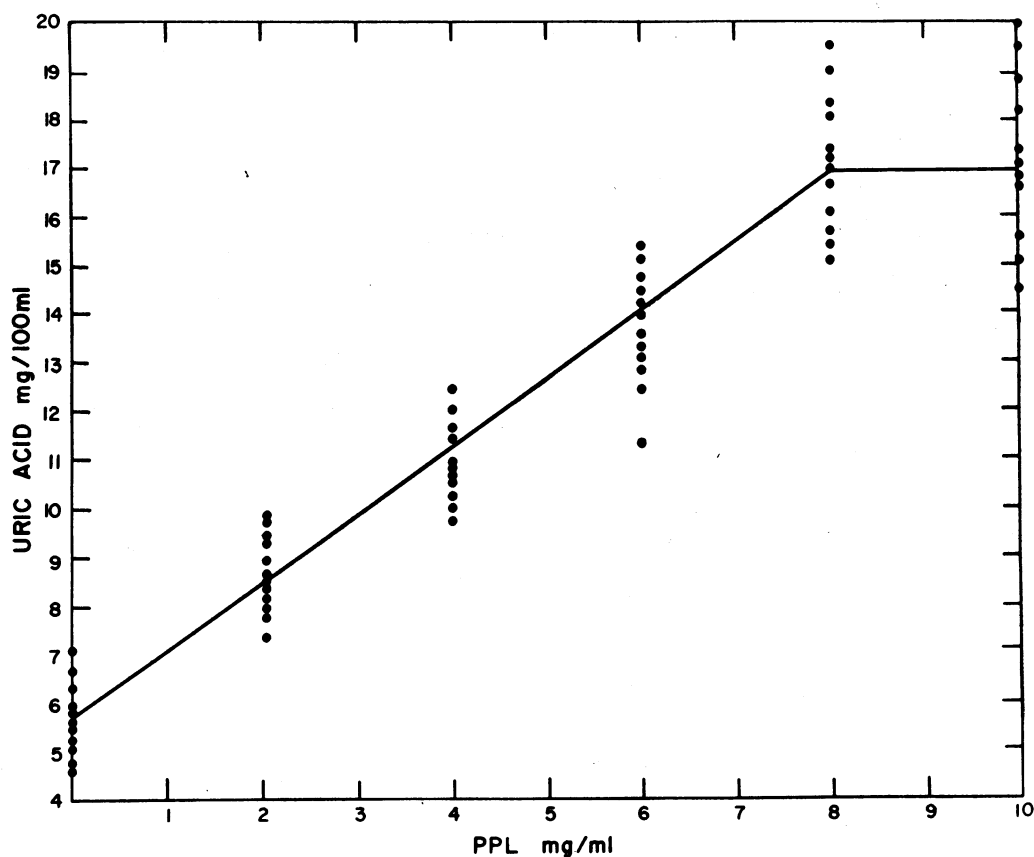


FIGURE 2 Effect of PPL on the solubility of monosodium urate in pH 7.4, 0.021 M potassium phosphate buffer containing 0.130 M sodium chloride.

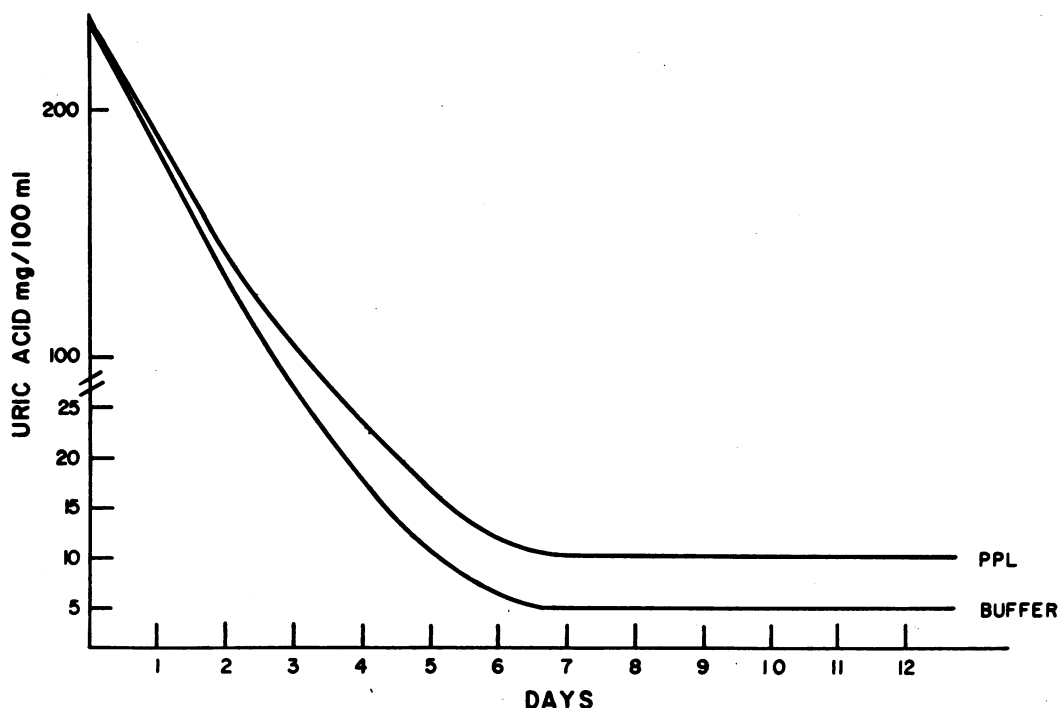


FIGURE 3 Changes in concentration of monosodium urate after crystallization from supersaturated solutions of PPL and pH 7.4, 0.021 M potassium phosphate buffer containing 0.130 M sodium chloride.

ion concentration rises, the resultant acid medium is thought to promote further urate crystallization (12). Although the theory is intriguing, provocative, and contributes to an understanding of the mechanism of urate deposition, there are the following certain inconsistencies: (a) the magnitude of pH change in synovial fluid is small, approximately 0.5 pH unit. Even if significantly greater ranges could be achieved at a subcellular level, then more likely uric acid rather than urate would form. (b) Joint diseases other than gout, such as rheumatoid arthritis, may cause acidification of synovial fluid (15). Yet, when hyperuricemia coexists in these patients, urates do not predictably deposit. (c) The theory in itself does not explain why some individuals with high serum uric acid levels develop gout and not others. (d) Although an acid environment might perpetuate urate crystallization, how does the first crystal form? Presumably, an acid medium is not present in the absence of leukocytic phagocytosis.

Some investigators believe that the common ion effect is responsible for urate deposition. Indeed, sodium urate is, of course, less soluble in solutions of high sodium concentration. Roberts demonstrated that cartilage contains sodium in concentrations significantly higher than in serum (2). He proposed, therefore, that sodium urate was more likely to deposit in connective

tissue because of its high sodium content. However, albeit that the sodium concentration is high, its activity is low. It is probably that avascularity, sodium content, and change in hydrogen ion concentration have small synergistic roles in the deposition phenomenon. Our studies represent an expanded search for the pathogenesis of urate deposition in gout.

These experiments establish the influence of bovine nasal cartilage on urate solubility. When suspended in physiologic buffer, it greatly enhances the amount of urate held in solution. In contrast, the presence of other bovine parenchymal organs has no significant effect. Free sodium ion does not alter solubility since all materials are thoroughly dialyzed against the same buffer. Because of their poor availability, connective tissues

TABLE II
The Effect of Enzymes and Cetyl Pyridinium Chloride on the Solubilization of Urate by PPL

Added substance	Uric acid mg/100 ml
Intact PPL, 8 mg/ml	16.8
Trypsin + PPL	7.1
Hyaluronidase + PPL	9.4
Cetylpyridinium chloride + PPL	6.1

other than bovine nasal cartilage were not used. What specific substance in connective tissue, then, is responsible for the enhanced solubility of urate? Of the major connective tissue components (cells, fibers, and matrix) we chose to investigate PPL, the prime constituent of the latter. It is readily available, soluble in physiologic buffers, and stable under standard laboratory conditions. The insolubility of the fibers, with the exception of soluble collagen, does not lend itself to solubility studies. It is even doubtful that significant quantities of soluble collagen exist at a tissue level in adults.

The studies demonstrate that PPL and at least one of its fractions have a marked solubilizing effect on monosodium urate. Although unbound chondroitin sulfate does enhance urate solubility slightly, a larger effect is dependent on the intact proteinpolysaccharide molecule. After the addition of trypsin or hyaluronidase, the solubilizing action is lost. Therefore, the integrity of the PPL molecule appears to be essential.

The nature of the solubilizing phenomenon is not known. It is conceivable that a chemical or physical bond, perhaps reversible, forms between the urate and PPL molecules. Studies on the binding of urate to protein substances in connective tissues have not been reported, but several investigators have been unable to establish an irreversible binding of urate to the proteins of serum (16-18). Alvsaker, on the other hand, has shown a reversible interaction between serum urate and albumin, low density beta-lipoprotein, beta macroglobulin, and an alpha globulin (19). The last named contains large amounts of uronic acid and may in part be a proteinpolysaccharide of the connective tissue variety (9, 20). Studies are currently underway to show that the urate binding in serum is caused by such a substance. It is unlikely that the relatively high viscosity of PPL in itself is responsible for its solubilizing action. Other polysaccharides and nonpolysaccharide solutions of high viscosity do tend to inhibit crystallization of urates but do not enhance urate solubility at all.

It is noted that the slope of the solubility curve is linear at low concentrations and levels off at higher concentrations (Fig. 2). Perhaps more and more PPL molecules become bound to urate until eventually all the binding sites are utilized.

The relationship of the ability of PPL to augment urate solubility in vitro to the actual in vivo mechanism of urate deposition in gout is unclear at this time. During the past century the mainstream of research in gout has been directed toward hyperuricemia, and only in the past decade in the reports of McCarty and Hollander and later Seegmiller was the importance of local factors reemphasized (21, 22, 12). These investigators noted the presence of microcrystalline monosodium urate in gouty synovial fluid. The crystals were capable of in-

ducing an acute inflammatory process in laboratory animals and human subjects alike. Little is known, however, about the mechanism by which urates deposit in order to initiate the now well-known cycle of crystal-induced inflammation. Our data indicate that there is biochemical support for the gross affinity that urates have for connective tissues in that PPL is formed almost exclusively in them. The relationship, however, is not straightforward. If, indeed, a true interaction exists between urates and connective tissue proteinpolysaccharides, then one would anticipate urate deposition to be inhibited rather than induced. However, the works of Dingle and others indicate that there is a constant turnover of the proteinpolysaccharide molecule (23, 24). We suspect, therefore, that ultimately crystallization of urate does occur because of alteration of this metabolic process in patients with gout. Experiments are now in progress to elucidate this phenomenon. We are in accord with A. B. Garrod who in 1876 stated, "Gouty inflammation is invariably attended with the deposition of urate of soda. . . . This fact I wish to impress forcibly upon my readers because in the constancy of such deposition lies the clue that has long been wanting; the occurrence of the deposit is at once pathognomonic and separates gout from every other disease which at first sight might appear allied to it" (25).

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