4. Title: Solubilities and the Mechanism of the Bladder Stone Formation.

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Introduction

The mechanism of calculogenis in stones of all kinds is not clear at the present time. Studies on morphology and compositions of stones in the urinary tract provide two possible hypotheses:

A. Vermeulen and Lansdale (1,2) and Prien (3) who were able to grow artificial stones from pure aqueous solutions believed that stones must be formed by the crystallization of some substances which supersaturate in the urine. The majority of these are calcium oxalate, calcium phosphates, uric acid and urates. It is also believed that the organic matrix existing in the stone is only an incidental inclusion.

B. The other investigators (4) proposed that inorganic substances did not just agglomerate to form stones but they combined with the organic matrix prior to the occurrences of precipitation. This proposal is based on 1) organic matrix is always found in every stone and (2) a large number of patients with hyperoxaluria failed to develop calculi. From a microradiograph and a photomicrograph of a calcium oxalate-apatite calculus (5) it is obvious that large, well formed surface crystals of weddelite show no trace of the influence of colloids on their growth; thus crystallization clearly dominated the development of calcium oxalate calculus, but colloids have played a secondary role in helping to bind the small outer crystals to the central mass.

According to the literature, it has been proposed that the solubility and the crystallization of the urinary inorganic compounds are influenced by two mechanisms:

1. A deficiency in some substances in the urine, (citrate, urea, potassium, sodium, sulfates, phosphates, chlorides, magnesium and colloids) which are essential for holding calcium and other minerals in the solution.

2. In the urine of active stone formers, there are some unusual substance(s) which enhance(s) the precipitation of calcium salts or other minerals in the urine.

Thomas (6,7) demonstrated that urine from selected patients with renal stones could induce mineralization of incubated hypertrophic cartilage but no mineralization could be demonstrated in urine from normal subjects despite high concentration of Ca^{++} and $PO_4^{\equiv 3}$. He suggested that there probably are one or more relatively low molecular weight inhibitors in these patients. Furthermore, it has also been demonstrated that adding pyrophosphate (2.5 to 40 mg/litre of urine) could prevent mineralization of hypertrophic cartilage in vitro (8). According to Hodkinson (9), this calcification inhibitor was dialyzable and ultrafiltratable stable; to heat at 100°C for 1 hour and stable to acidity at pH 1 for 1 hour or to both heat and acidity together. This substance was thought to be acidic peptides with a negative charge containing some 25 ninhydrin positive materials such as serine glycine, alanine, threonine and cysteic, aspartic and glutamic acids.

Fleisch (10) confirmed that, when urine was added to the solution containing calcium and phosphate ions it could inhibit the precipitation of calcium phosphate. He also observed that the important factor in the urine was pyrophosphate which was believed to be a potent inhibitor of the precipitation of calcium phosphate and calcium oxalate; its urinary concentration varies between $1-7\times10^{-5}$M. In addition he also found that injections of pyrophosphate and polyphosphates could prevent the calcification of the aorta due to vitamin D (11) and of skin in rats (12). He proposed the inhibition of calcification by pyrophosphate could be explained as a blockage of crystal growth centers by adsorption of the compound on to the apatite crystal at the site of calcification (13). Howard (14) supported the results of Hodkinson that phosphate which could inhibit calcification is in the form of an acidic phosphorylated peptide, and in order that inorganic phosphate could be an effective calcification inhibitor it must be ingested in the body first.

Citrate has also been suspected to play some roles in the pathogenesis of urolithiasis. Johnson (15). reported that administration of citric acid could prevent ammonium magnesium phosphate calculus formation. Miller (16) demonstrated that citric acid is particularly effective in increasing the solubility of calcium oxalate and Howard (14) showed that the addition of 1 g of sodium citrate per litre could change an evil urine into a good one which could prevent the calcification. However, Packett gave a contrary result that citrate did not prevent calculi formation (17). Vermuelen (1) also reported that citrate could change the appearance of calcium oxalate crystal. This compound was found to give a beneficial result in the treatment of renal stone and it was reported that after the kidney was constantly perfused with the citrate solution for a period of several weeks some success had been observed in patients with stones of calcium phosphate group (18)

In addition to phosphates and citrates some reports suggested that the increase in solubility of calcium oxalate is due to a high concentration of urinary urea (19). This compound also was able to inhibit the formation of both dentrical and artificial calculus (20).

Some people have believed in the protection of inorganic precipitation by the urinary colloid while in contrast several people have believed some colloidal substances could facilitate the precipitation. According to Boyce (21) the calculus formation was caused by a condensation of mucopolysaccharides and mucoprotein into coagulated molecules or by albuminous matrix material in which inorganic substances diffused and deposited. He proposed this idea on the basis that the central core of every calculus was composed of one or more dense, circular whorls of parallel fibers and accompanying amorphous material. They also found the increase in urinary excretion of mucoprotein in patients with renal stones (22, 23, 24). Berger (25) also reported that decalcified rat femurs, studied in vitro, could accumulated Ca^{++} where acid mucopolysaccharides were present. Studies of Baker and Howard (26, 27) suggested a possibility that calcigerous urinary calculi might originate in the presence of a chondroitin sulfate or other ion binding mucopolysaccharides. Later a substance designated "Substance A" was found by immunological means to be present in stone matrix and in the urine of active stone formers, but not in normals or calculous disease subjects not actively forming stones (28).

In Thailand, bladder stone has been a problem for a long time as reported by several investigators (29—35). In order to disclose the etiology of this disease, both survey studies and biological studies have been carried out. According to the composition study (34), the majority of bladder stones in Thailand were composed of calcium oxalate, uric acid and urates. In addition, from the work of Nordin (36), the only crystalline substances observed in samples of fresh urine were calcium oxalate and one form of calcium phosphate. Other compounds previously described in urine, notably ammonium urate, calcium hydrogen phosphate (steller calcium phosphate, brushite) and magnesium ammonium phosphate, were only seen when the slides or incubated samples were examined several hours after voiding; so it was suggested that many or most of the crystalline materials hitherto described in urine develop after voiding when the urine is left to cool and the pH is allowed to rise. From his work it was found that crystals found in the stone have the same size as those found in the urine so it is possible that stones are formed by the aggregation of crystals more than by crystal growth.

In accordance with various works mentioned above, the solubility and the crystlization of calcium oxalate and uric acid should be performed first. This study will enable us to elucidate the mechanism of not only the bladder stone formation but also of stones found in other parts of the body.

Purpose

The role of this investigation is to study the effect of various compounds suspected to be involved in the solubility of calcium oxalate and uric acid. Compounds used in this study were sodium orthophosphate, sodium pyrophosphate, ADP, ATP, sodium citrate, sodium sulfate, sodium chloride and urea.

Method

The solubility method developed by Higuchi was used in this study. A detailed discussion of this method has been given by Kostenbander and Chulkaratana (37, 38). The solution is maintained

saturated with respect to one component (calcium oxalate or uric acid 5×10^{-2} M/L) in a series of containers into which increment amounts of solubilizing agent (range 0.1×10^{-2} and 0.1×10^{-3}) were added. The system was shaken (75 times/min) in the 37°C constant temperature bath approximately 48 hours unless the compounds decomposed, then the system was shaken for only 10 minutes. The solution was filtered through the filter paper (Carl Schlercher & Schuell Co. Army No. 4360500 for calcium oxalate and through the Millipore RA for uric acid). The solution was pipetted immediately as soon as it passed through the filtering system in order to avoid any variation in the temperature which greatly effect the solubilities of the substances. The filtrates were analysed by the Atomic Absorption Spectrophotometer (Perkin Elmer 290) for calcium and by U.V. Spectrophotometer (Beckman DU—2) at 292 mu for uric acid. If complexes formed are not highly soluble, the plot of the soluble substance (calcium or uric acid) vs. the amount of the complexing agent (solubilizing agent) added will increase proportionally until it reaches the saturated solubility of the complex itself then the curve will level off as shown by B-C in Figure I. At C all solid has gone into the complex forms and the complexing agent starts to combine with free calcium oxalate or uric acid in the solution hence causes the curve of the solubility plots to decrease until it reaches the solubility of the complex at D. If the complex formed is very insoluble, the AB range may not be detected or, if a very soluble complex is formed, the curve A-B will never reach a plateau but the stoichiometric formula of the complex can be investigated.

It can be seen that for all soluble complex formation which is first order with respect to the complexing agent added, the linear relationship will be derived when the concentration of soluble substrate (calcium oxalate or uric acid) is plotted vs. the concentration of the compound added (as shown in Figure 2). In this case $K_{1:1}$ (equilibrium constant of the complex formation) will be equal to slope/1-slope.

When a second order complex, with respect to the complexing agent, is formed, $K_{1:2}$ = Amount of complex formed/(free complexing agent)^2 ($K_{1:2}$ = equilibrium constant of 1:2 complex). In this case a plot of the concentration of the complex formed vs. (the concentration of free complexing agent)^2 will yield a straight line, with the slope $K_{1:2}$ (as shown in Figure 3) but the solibility curve will be a concave one as shown in Figure 4.

Results and Discussion

Effects of Various Compounds on Calcium Oxalate: According to the results it is obvious that ATP, ADP, sodium pyrophosphate and sodium citrate formed soluble complexes with either calicum ions or calcium oxalate because they increased the solubility of calcium from calcium oxalate significantly (Figure 5). Among the four compounds mentioned, ATP was the most effective one even though it has the biggest molecule, the steric effect did not appear to significantly obstruct the binding abilities. It is possible that one molecule of ATP might combine with more than one molecule of calcium or calcium oxalate since the solubility plots of soluble calcium against ATP added was greater than one (1.15 as shown in Figure 5). ADP had less binding ability than ATP, as it can be seen from the same figure the solubility plots of ADP did not yield a straight line but leveled off when the concentration of the ADP added was higher. This is due to the fact that the binding force between ADP and either calcium ion (s) or calcium oxalate molecule (s) may not be strong enough to overcome the polymerization of ADP-molecule themselves which tends to occur at a higher concentration of ADP. However, the solubility plots of ADP yielded the slope of 0.81 which suggested that possibly only one molecule of calcium ion or calcium oxalate complexed with one molecule of ADP. According to the value of the slopes from the solubility plots of various compounds mentioned the number of phosphate groups appeared to be more significant than the steric effect from the bulky adenosine molecule. This may be because the phosphate groups in both ADP and ATP are at the end of the molecules so they are opened to be attacked by the calcium. If the site of complex formation differs from the phosphate groups, the results should be reversed and the slope derived from the graph of ATP should be less than that from ADP.

For inorganic pyrophosphate its binding strength with the calcium appeared to be lowest among the pyrophosphates (ADP, ATP and sodium pyrophosphate). Since the molecule of inorganic pyrophosphate was not too bulky the complexes formed appeared to be a mixture of the first and the second order complexes

FIG. 1

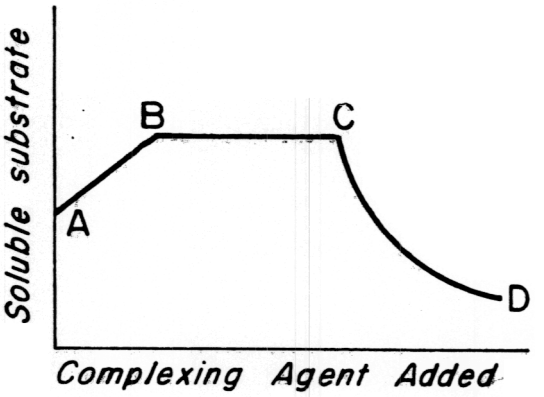


FIG. 2

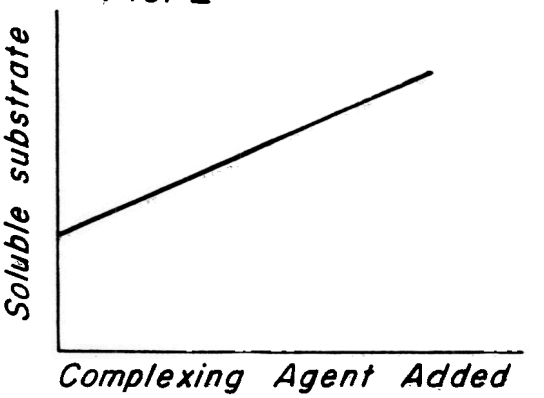


FIG. 3

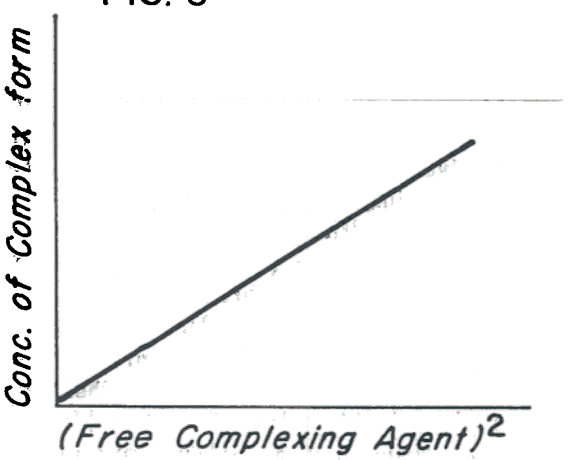


FIG. 4

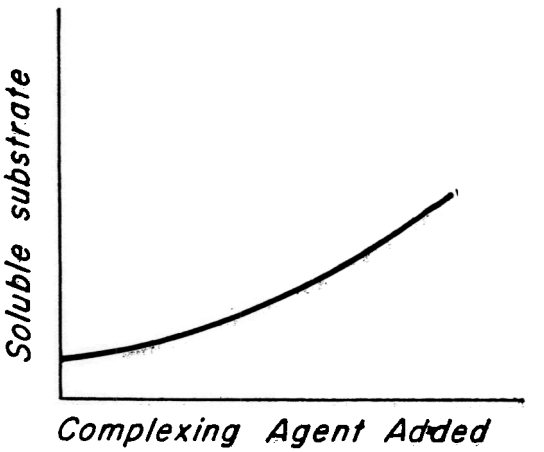
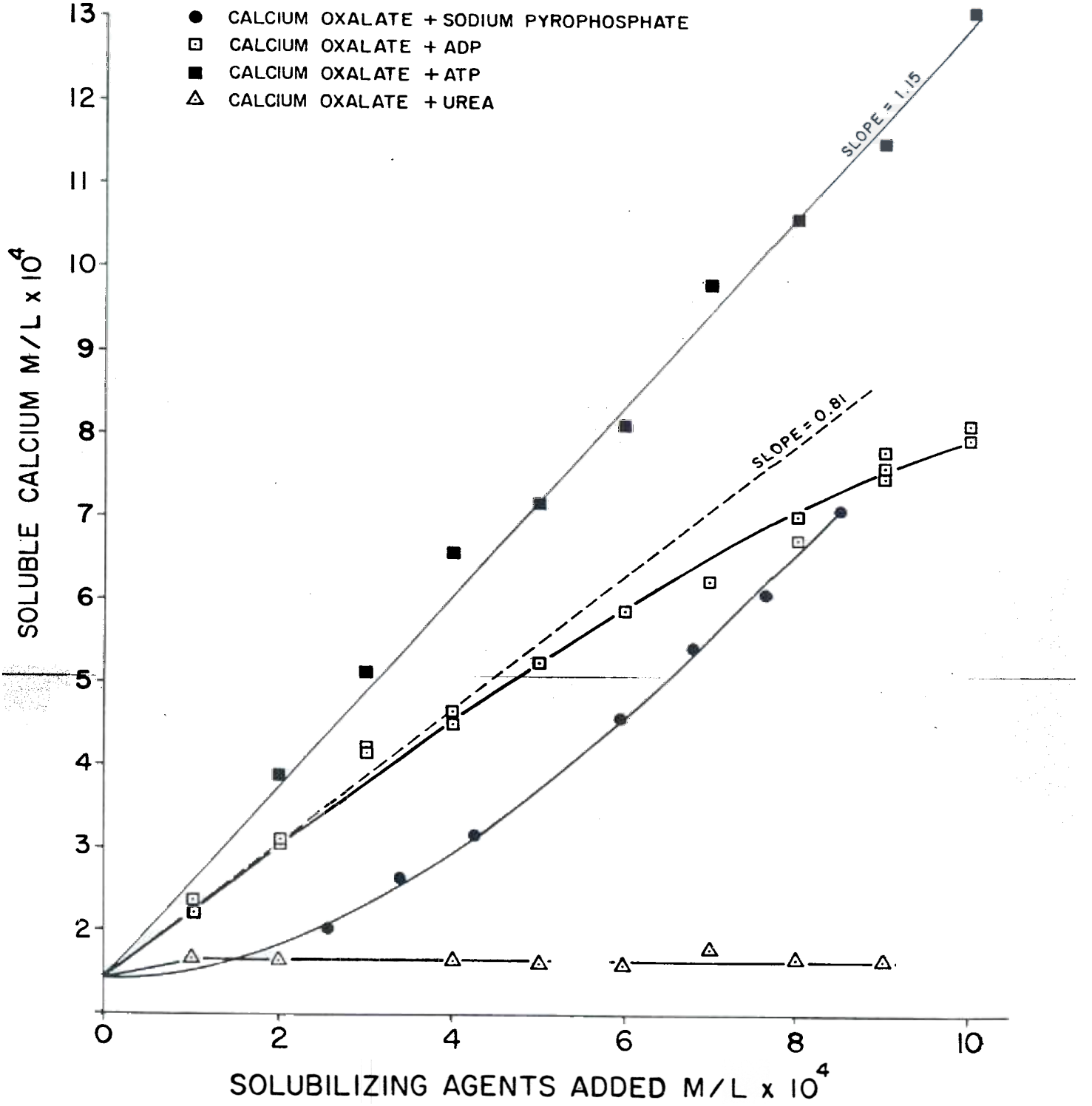


FIG. 5. EFFECTS OF VARIOUS COMPOUNDS ON THE SOLUBILITY OF CALCIUM (10 MINUTES)



with respect to the pyrophosphate because the solubility plot was a concave one (Figure 5). However, when the concentration of sodium pyrophosphate was higher, the polymerization of pyrophosphate themselves itself started to interfere and the curve changed to a convex one (Figure 6).

Sodium citrate also behaved similarly to ADP and the initial slope of the solubility plots was 0.45 (Figure 7) which showed that the solubilizing ability of citrate on calcium oxalate was weaker than those of the pyrophosphates. This result seems to disagree with Miller's report (16) in which citric acid was said to be more effective in increasing the solubility and in preventing the precipitation of calcium oxalate, than the phosphates. In this study only sodium orthophosphate failed to solubilize calcium oxalate while other phosphates did. The effect of sodium sulfate was rather negligible since the slope of the solubility plots was only 0.035 (Figure 6).

Urea and sodium chloride did not increase the solubility of calcium oxalate at all (Figure 7). This is in contrast with the work of Medes (19) who mentioned that a high concentration of urea in urine help to solubilize calcium oxalate in urine. Sodium orthophosphate somewhat decreased the solubility of calcium in this study. This is probably because of the alkalinity from the sodium orthophosphate itself. When sodium pyrophosphate was shaken with calcium oxalate for 48 hours the results were similar to those of sodium orthophosphate: the solubility of calcium from calcium oxalate was decreased (Figure 7). However, in Figure 5 where the systems were shaken for only ten minutes, sodium pyrophosphate increased the solubility of calcium from calcium oxalate significantly; thus it is rather reasonable to suggest that when sodium pyrophosphate is left in the solution it will decompose to sodium orthophosphate which may cause the precipitation of calcium.

In this study those compounds which seemed to decompose (sodium pyrophosphate, ADP, ATP) were shaken only 10 minutes instead of shaking until equilibrium (48 hrs). This still serves the purpose of this study quite well since the urine is continuously excreted and is not kept in the urinary system as long as 48 hours. In addition when samples were shaken for ten minutes they seemed to attain the equilibrium already as it can be seen that the plots were not scattered at all and the solubilities of calcium from calcium oxalate derived from both shaking times were nearly the same (1.65 M/L at 37°C).

In case there are some interferences from the intermolecular forces between the molecules of complexing agents, (as in the case of ADP, sodium pyrophosphate and sodium citrate) which will be greater as the concentration of these compounds are higher, the solubilizing ability of these compounds on the substrate (calcium oxalate) will be gradually decreased. This is shown clearly by the curve of sodium citrate in Figures 7 and 6. In the latter one in which the concentration of sodium citrate is ten times higher than that in the former one, the curve deviated more and the initial slope was less (0.33 for Figure 6 and 0.45 for Figure 7).

Effects of various compounds on uric acid As shown in Figure 8 the solubility of uric acid in the presence of either sodium orthophosphate or sodium pyrophosphate was different when the samples were shaken for only ten minutes and for 48 hours. In the latter case none of the uric acid in the solution could be detected thus suggesting the decomposition of uric acid in the solutions, especially in the prescence of phosphates. Orthophosphate appeared to catalyse the degradation of uric acid better than the pyrophosphate, and the degradation in the presence of either compound was surprisingly fast at 37°C after 5 hours (as shown in Figure 9). However, the kinetic study of uric acid by various urinary constituents is beyond the scope of this study and it should be performed later.

The systems of uric acid with sodium citrate and with urea yielded different results from those of uric acid with phosphates; in the concentration range $0—1.0 \times 10^{-2}$ M/L, after shaking for 48 hours, soluble uric acid was still detectable and the solubility plots showed the formation of insoluble complexes which were more stable than the uric acid itself. According to the graphs (Figure 8) if the concentration of the citrate or urea was higher than 1.0×10^{-3} M/L the precipitation of insoluble uric acid complexes may occur even if the concentration of total solubie uric acid may rise to approximately to 5.1×10^{-3} M/L in the presence of sodium citrate and to 2.0×10^{-3} M/L in the presence of urea. However, these values should be reinvestigated especially when the system is shaken for only 10 minutes.

FIG. 6. EFFECTS OF VARIOUS COMPOUNDS (AT HIGHER CONCENTRATION) ON THE SOLUBILITY OF CALCIUM. (10 MINUTES)

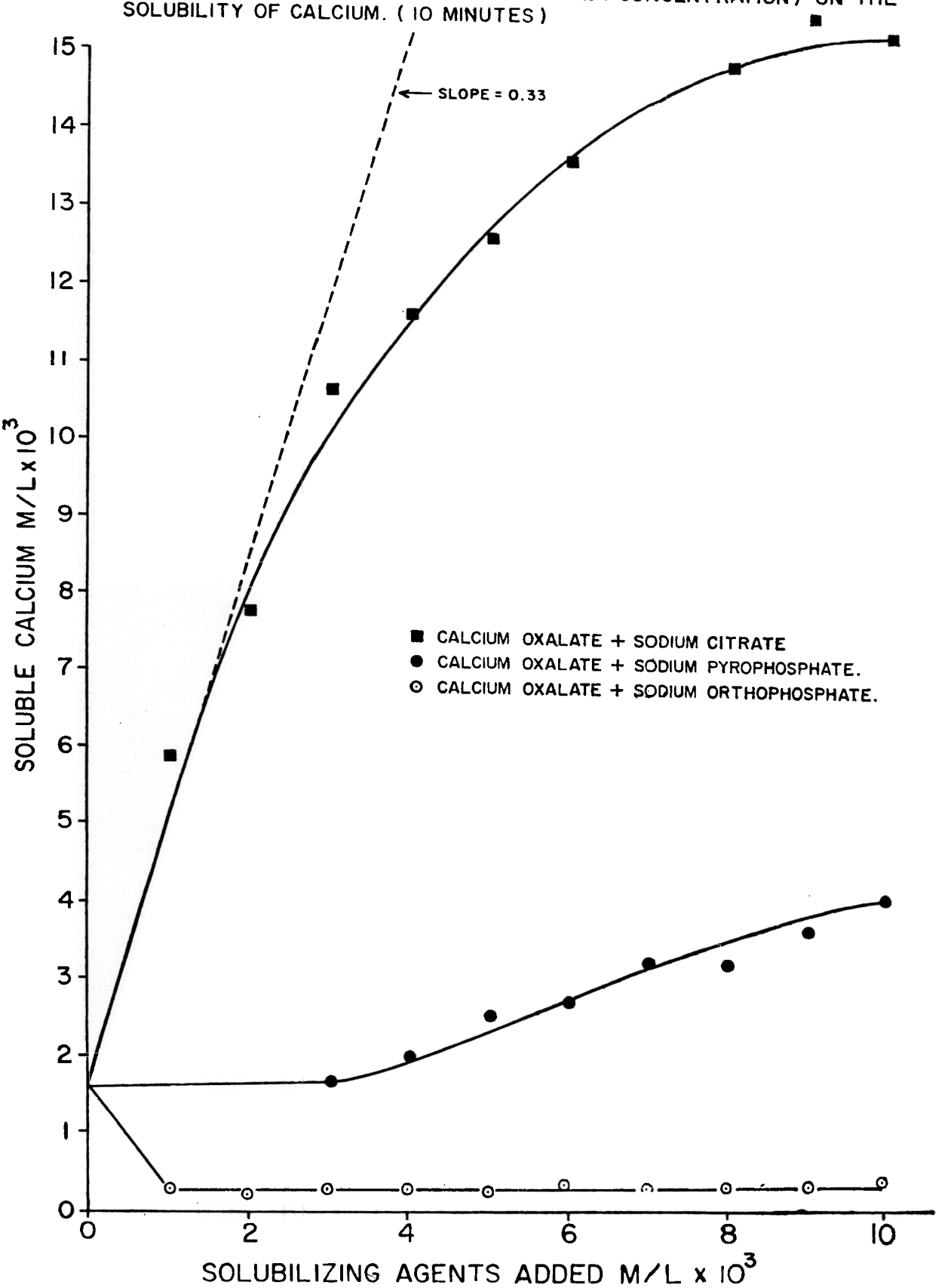


FIG. 7. EFFECTS OF VARIOUS COMPOUNDS ON THE SOLUBILITY OF CALCIUM (48 HOURS)

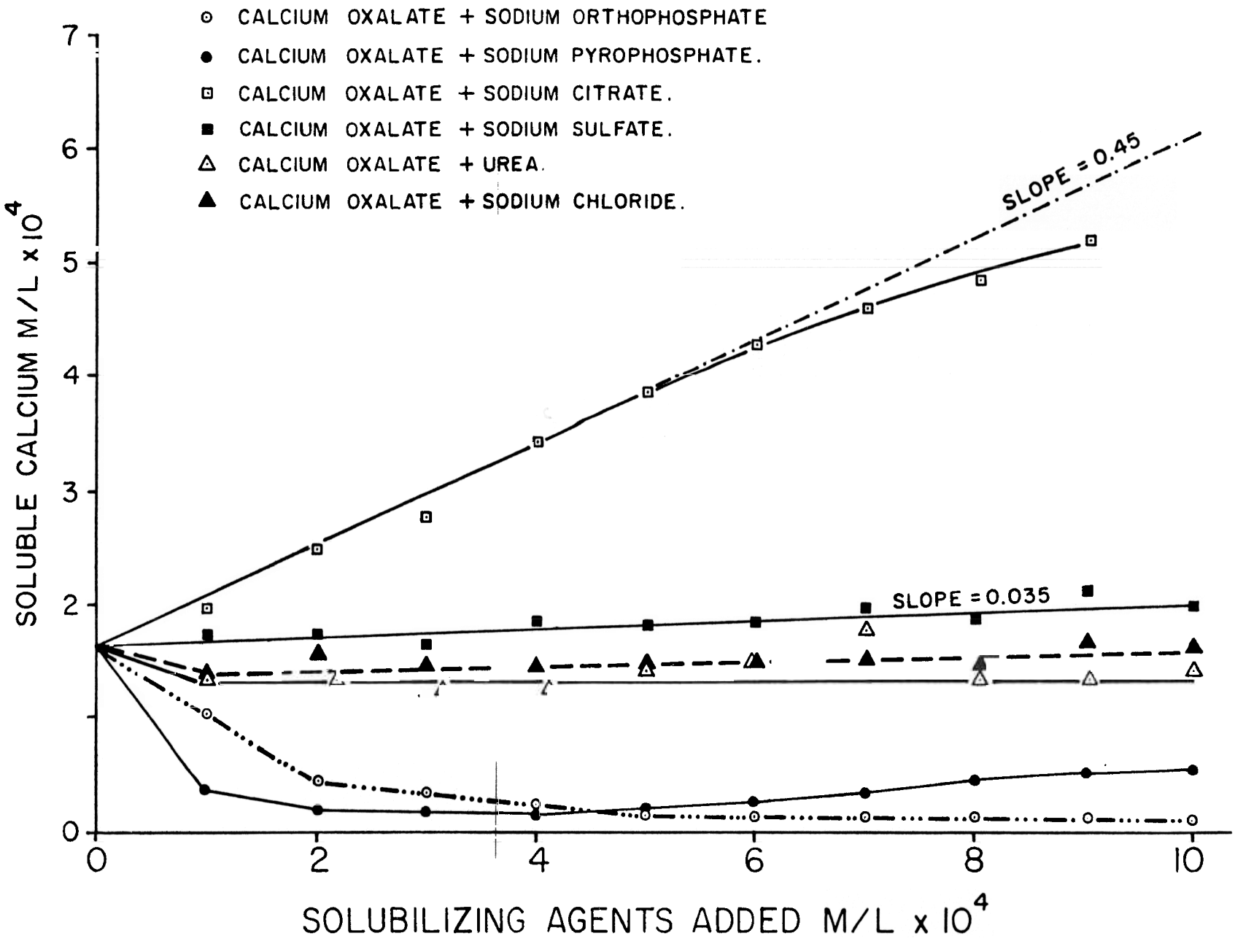


FIG. 8 EFFECTS OF VARIOUS COMPOUNDS (AT HIGHER CONCENTRATION) ON THE SOLUBILITY OF URIC ACID.

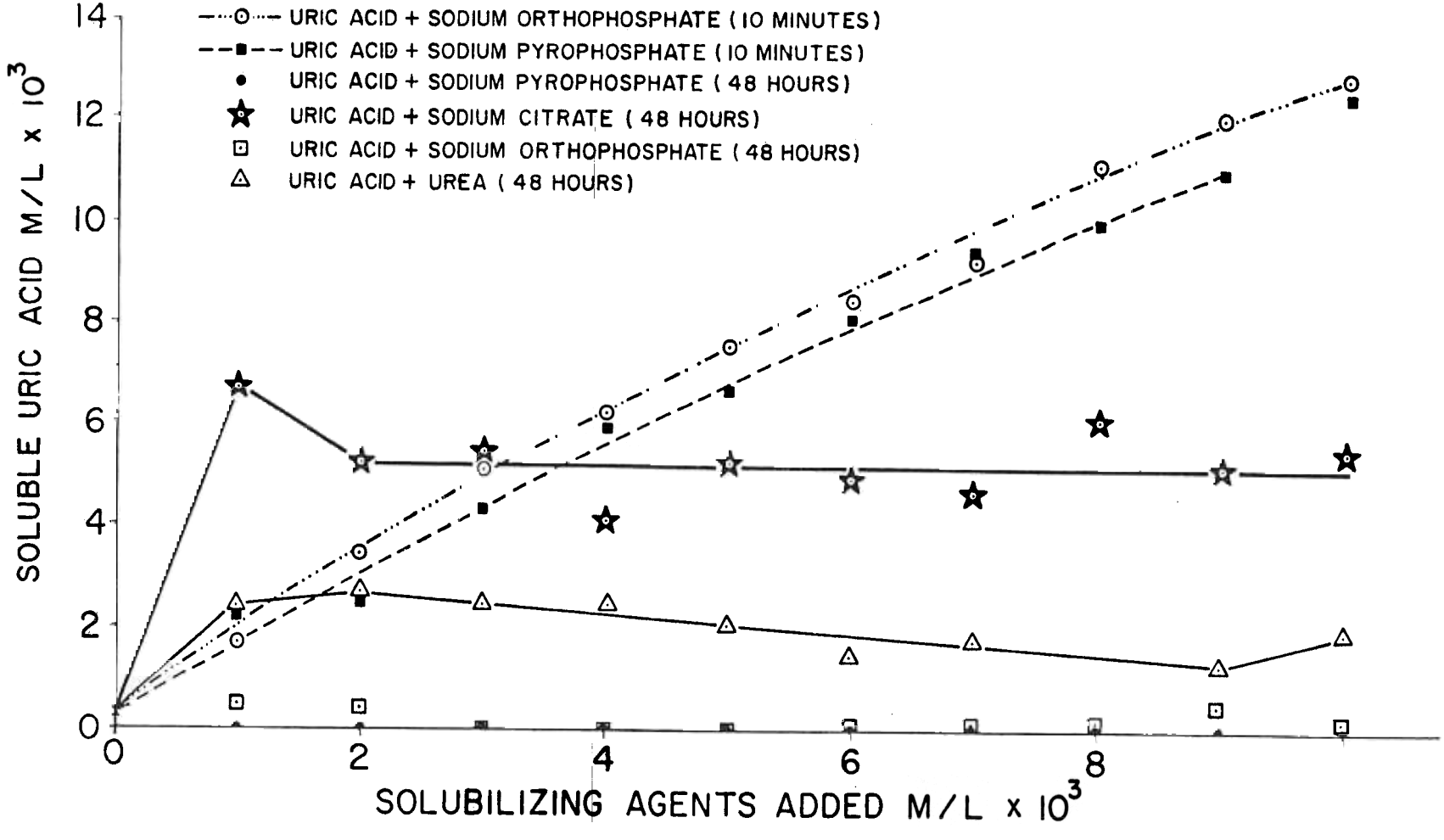
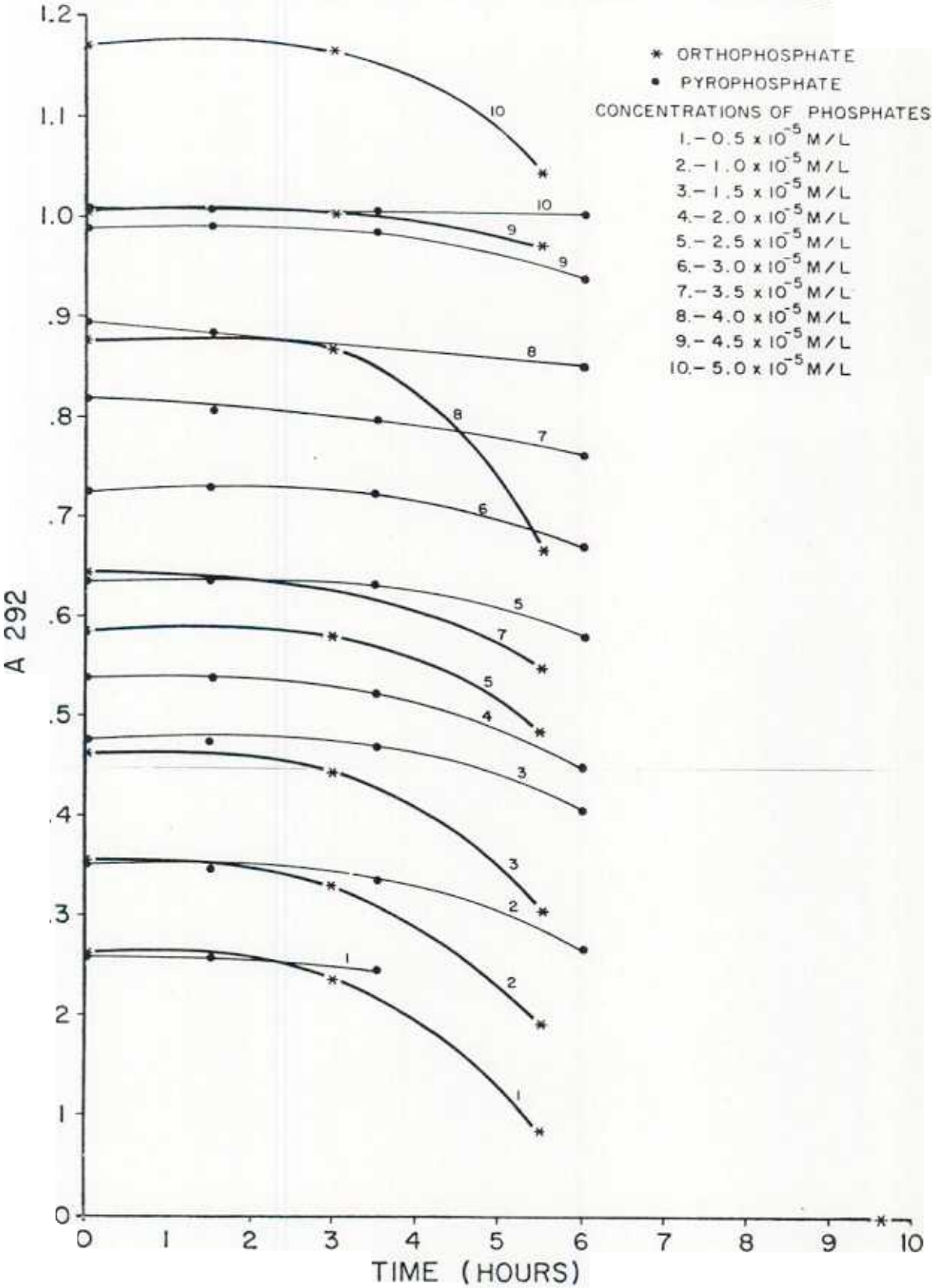


FIG.9 DECOMPOSITION OF URIC ACID IN THE PRESENCE OF PHOSPHATES



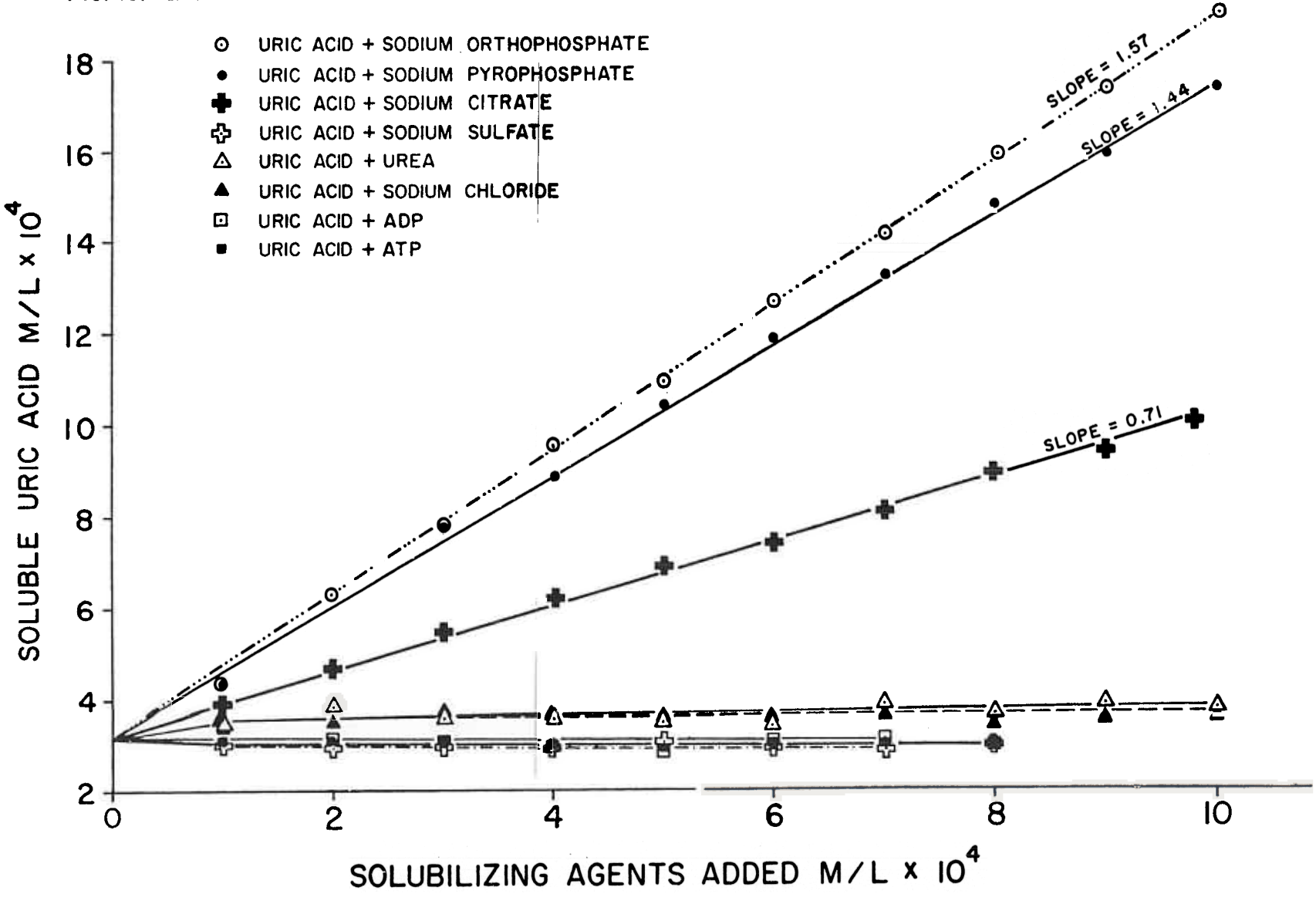
According to Figure 10 where all samples were shaken for only 10 minutes sodium orthophosphate and sodium pyrophosphate could significantly increase the solubility of uric acid. Even at higher concentation (Figure 8) the interference from the intermolecular forces between phosphate molecules was still rather negligible. In the concentration range $0-1.0 \times 10^{-3}$ M/L the solubilizing ability of inorganic phosphates on uric acid was greater than that of the citrate. It is possible that one molecule of phosphate can combine with more than one molecule of uric acid since the solubility plots yielded slopes greater than one: 1.57 for sodium orthphosphate and 1.44 for sodium pyrophosphate while that from the solubility plots of uric acid in the presence of sodium citrate was only 0.71. The data also revealed that even uric acid molecules could combine strongly with inorganic phosphate. The increase in solubility of uric acid by organic phosphates (ADP and ATP) was not observed at all (Figure 10). This may be explained either by the formation of very insoluble uric acid organic phosphate complexes or by the steric effect from both the bulky adenosine group and the uric acid molecule. When the molecules could not approach each other no interaction would occur; hence no complexes were formed and the slope of the solubility plots would be equal to zero. This is in contrast to the results from the system of calcium oxalate which was previously discussed.

The solubilization of uric acid especially by inorganic phosphates, appeared to be influenced by both the soluble complex formation and the increase in the alkalinity of the solution. However, when these two effects were compared, the former one was much more significant; as shown by Figure 11 in which the concentration of soluble uric acid was plotted against the hydroxyl ion concentration. The slopes were 66.7, 9.30 and 6.0 for pyrophosphate, orthophosphate and hydroxyl ion respectively. This showed that the pyrophosphate could form a more soluble complex with uric acid than could the orthophosphate. These results are in agreement with the results derived from the system of calcium oxalate in which the pyrophosphate has a stronger ability to form soluble complex than the orthophosphate. When uric acid was used as the substrate, the solubility results were apparently reversed: sodium orthophosphate appeared to be a stronger solubilizing agent because of its alkalinity.

The effects of phosphates on the solubility of calcium oxalate in this study seemingly support Cartier's idea (39) that calcium was distributed in various parts of the body in the form of pyrophosphate and this form was deposited at some tissues as a first step in calcification; this possibly by the interaction between phosphorus in the pyrophosphate and the amino group in tissue proteins, and that the pyrophosphate was discharged, possibly by the action of the pyrophosphates, to orthophosphate which was insoluble as was confirmed by this investigation. However, since ATP and pyrophosphate can solubilize calcium they may inhibit calcinosis in vitro while those orthophosphates may aggravate the calcinosis as reported by Gabbaini (40).

According to previous reports inorganic phosphates were beneficial in the prevention of stone recurrences (41, 42, 43) but the real mechanism of orthophosphate in this role has not been disclosed. Phosphates may reduce stone recurrence by promoting the calcium phosphorus balance thus in turn decrease the calcium excretion (as reported in 41, 43, 44). On the other hand phosphates may effect some metabolic cycles; this role is supported by the work at the SEATO Clinical Research Center (45) from which the results revealed that the orthophosphate supplementation reduced the urinary oxalate excretion in children inhabitating bladder—stone endemic area. The other point of view concerning the mechanism of phosphate on the stone formation is its ability to protect the urinary crystal formation. This possibility has also been supported by the investigators at the SEATO Clinical Research Center who found that the crystaluria in those village children also disappeared after the orthophosphates supplementation. In addition previous literature confirmed that the administration of orthophosphates will increase the urinary excretion of orthophosphate, pyrophosphate (22, 42, 45, 46) and citrate (42) so it may be reasonable to propose that even the disappearance of crystaluria may be due to the decrease in excretion of some substances such as calcium, oxalate and uric acid, which are believed to cause the crystal formation. It is quite possible that the solubilizing effect of phosphates and citrate play another role in the prevention of the crystallization of calcium oxalate and uric acid. This in turn will decrease the possibility of the stone formation and also will be valuable in the treatment by solubilizing stones in

FIG. 10. EFFECTS OF VARIOUS COMPOUNDS ON THE SOLUBILITY OF URIC ACID. (10 MINUTES)



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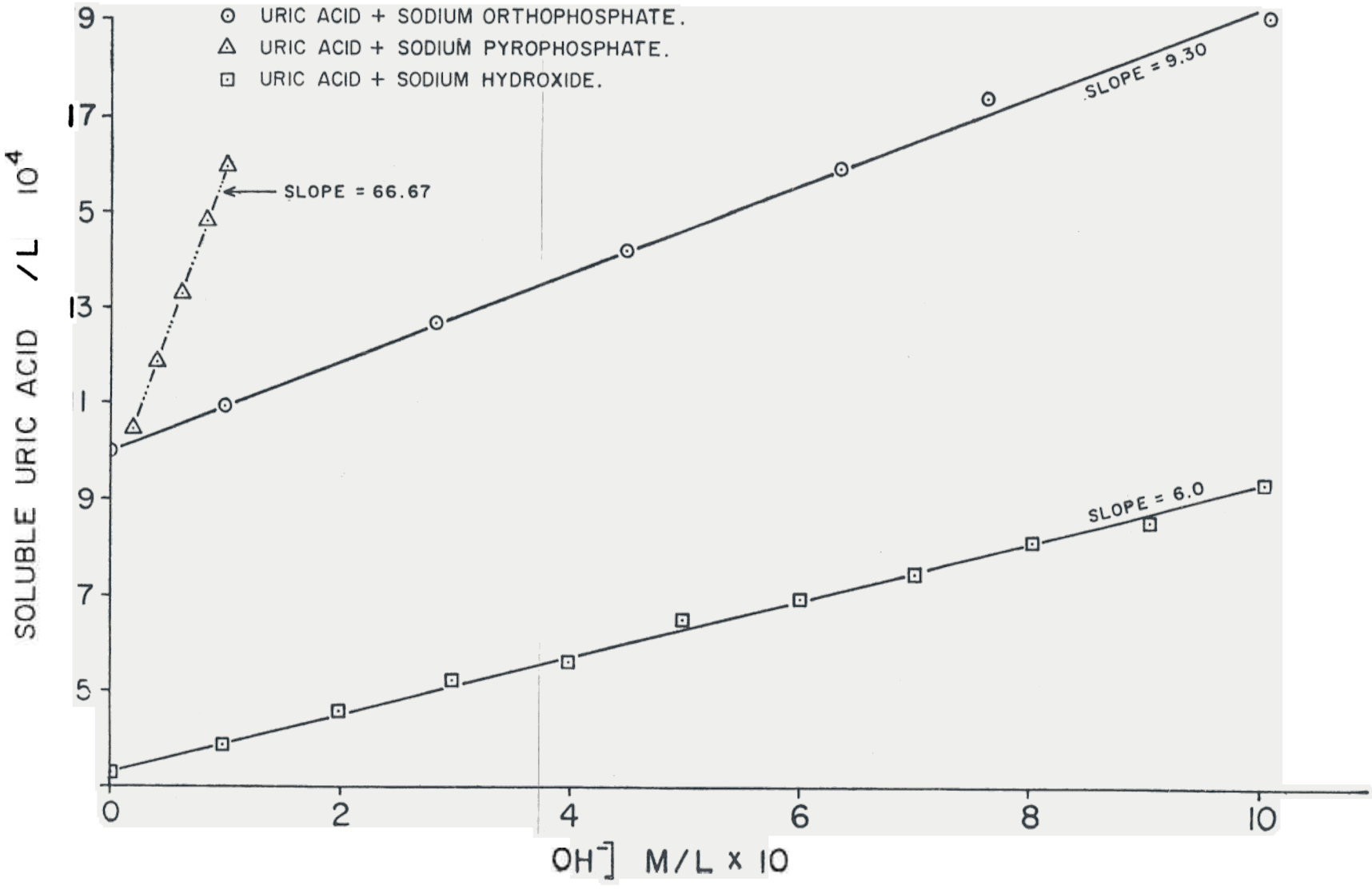

patients if the stones are mainly composed of calcium oxalate or uric acid. However, the effect of those compounds which did not appear to increase the solubility of calcium oxalate and uric acid in this study (for examples, urea and sodium sulfate) should not be ignored and should be further investigated because they may form very insoluble complexes which initiate stone formation.

Summary

Solubilizing properties on calcium oxalate and uric acid of various compounds suspected to be involved in the mechanism of stone formation were investigated in this study. These compounds are sodium orthophosphate, sodium pyrophosphate, ADP, ATP, sodium citrate, sodium sulfate, sodium chloride, and urea. The range of their concentration used were $0.0-1 \times 10^{-2}$ M/L and $0.0-1 \times 10^{-3}$ M/L. The concentration range $0.0-1 \times 10^{-3}$ M/L was used particularly for comparing the solubilizing effects of these compounds. This was to avoid the interference due to the polymerization or the intermolecular forces of these substances at higher concentrations. In order to avoid the decomposition of some compounds, the systems were shaken at 37°C for only ten minutes instead of until the equilibrium being attained (48 hours).

The solubility of calcium oxalate in water at 37°C found in this study was 1.65×10^{-4} M/L. Its solubility was increased significantly in the presence of the polyphosphates and citrate. Their solubilizing abilities on calcium oxalate were in the following order: ATP, ADP, sodium pyrophosphate, and sodium citrate; the initial slopes of their solubility plots were 1.15 for ATP, 0.81 for ADP and 0.45 for sodium citrate respectively. The increase in solubility by sodium sulfate was negligible since the slope of the solubility plots was only 0.035. Sodium chloride and urea did not increase the solubility of calcium oxalate at all while sodium orthophosphate apparently decreased the solubility of calcium oxalate.

The solubility of uric acid in water at 37°C found in this study was 3.26×10^{-4} M/L. When uric acid was left in the solution it decomposed, especially in the presence of inorganic phosphates, and it decomposed faster in the presence of sodium orthophosphate than sodium pyrophosphate. Only inorganic phosphates, sodium orthophosphate and sodium pyrophosphate, could increase the solubility of uric acid in water significantly. Organic phosphates, gave contrasting results to what were derived fram the system of calcium oxalate. ATP and ADP did not increase the solubility of uric acid at all. In the presence of sodium citrate and urea the solubility of uric acid could increase to approximately 5.1×10^{-3} M/L and 2.0×10^{-3} respectively. However, if the concentration of sodium citrate or urea was higher than 1×10^{-2} M/L the solubility of uric acid would not be increased higher than mentioned but it will precipitate in the form of insoluble complexes with citrate or urea. Sodium sulfate and sodium cloride did not effect the solubility of uric acid at all.

The results of this study suggest that the administration of orthophosphate should be greatly beneficial in the treatment and protection of the recurrence of stones of the calcium oxalate and uric acid type. Orthophosphate did not have direct benefit on calcium oxalate but in accordance with previous work it increased the urinary pyrophosphate which could effectively solubilize both calcium oxalate and uric acid. However, the results of this study reveal that there should be more than one urinary phosphate compound which act as inhibitors for crystal formation and for calcification.

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