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THE PROTEIN MATERIX AND THE EMBEDDED MICROORGANISMS IN THE RENAL CALCULI

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ABSTRACT

To study the process of formation of renal calculi, 85 patients with calculi were divided into 6 groups according to the chemical composition of surgically removed stones. Isolation and identification of the embedded microorganisms in the cores of stones were carried out. It was found that *Pseudomonas* was the bacteria that infected the interstices and cores of the smost stones. *E. coli* (non - urease producer) and *Staphylococcus epidemidis* came next .

Proteus mirabilis was found to be the bacteria embedded in the cores of the struvite stones in group 6 (Ca Ox - Ca P-MAP-U stones) , as urea - splitting organisms . *Klebsiella* was not isolated from any stones in spite of the fact that it is urease producer. Urine protein, immunoglobulin fractions and the amount of proteins in the stone matrix were determined . It was noticed that the high level of proteins and immunoglobulins in urine samples may reflect an inflammation or infection in many cases of the studied groups compared to the normal subjects. This was particularly noticed in the groups having struvite stones with the presence of high protein content in stone matrix, associated with high bacterial infection . In all types of stones that contain proteins it was found that the embedded bacteria were protease producers . These findings may imply that the embedded bacteria, its excreted products and the protein contents in the stone matrix may be acting as a precipitant and as connective substances for the crystals to grow and aggregate .

INTRODUCTION

In stone formation the salts crystallize within an organic matrix, the nature of which is still not well know **Mc Kee et al** [1] reported that the organic matrix of renal calculi has been considered to influence the crystal growth that occurs in the pathological mineral deposits. Most urinary stones contain matrix as organic substances which constitute from 2-3% of the stone components **Grouer et al** [2] believed that two thirds of the matrix of all urinary stones consist of proteins. **Fraij** [3] found that albumin, transferin and Tamm Horsfall mucoprotein were accounted for greater than 40% of the matrix proteins. The investigator suggested that these proteins may play a role in stone formation and growth. While **Dussol et al** [4] detected that albumin, α_1- acid glocoprotein, α_1-microglobulin, immunoglobulin A (IgA) , IgG , IgM, a poliprotein A_1 , transferrin and α_1-antitrypsine were abundant proteins present in 5 types of renal calculi . Also , they found that not all proteins of normal urine are present in the protein of the matrix .

Although, macroglobulin was unable to pass into the urine and can not be participated in the formation of the matrix of stone. Fibrinogen and immunoglobulin G have been found in both matrices of uric acid, MAP stones and in urine samples [5] . Four to six different antigenic protein components could be detected in both matrices of calculi and urine by **Zahran et al** [6], with the suggestion that proteinuria may play a role in the formation of stones in patients with urolithiasis. **Spirnak and Resnick** [7] showed that serum and urinary proteins provide a framework for deposition of crystals. Total protein in urine increases in glomerulonephritis, lesions and chronic pyelonephritis [8]. Glomerular damage allows the escape of serum proteins of intermediate molecular size into the urine.

Antibodies of IgG , IgA and IgM are produced in response to most bacteria and viruses, they aggregate and coat the small soluble foreign proteins , such as bacterial toxins [9].

The ultrastructural ecology of bacteria associated with struvite calculi indicated that the interstices and external surface of such struvite aggregates contain a large number of bacterial cells [10] . These organisms grow as microcolonies and thick biofilms within extensive fibrous organic matrices. The bacterial cells and their secreted products appear to provide initial foci for crystal development .

In the present study , isolation and identification of the microorganisms from the cores of urinary stones, the presence of protein in the organic matrix of the stones and in the urine samples, were carried out to predict the role of bacterial cells and urinary proteins in the calculus formation.

MATERIAL AND METHODS

The study involved eighty five patients suffering from urolithiasis (with 87 stones) were admitted to Kasr El-Aini University Hospital , as well as 17 healthy persons served as a control group. The patients were divided according to the different chemical composition of stones into 6 groups .

Group 1 (12 cases) with calcium oxalate (Ca Ox) stones.

Group 2 (26 cases) with calcium oxalate + calcium phosphate (Ca Ox - CaP) stones.

Group 3 (15 cases) with calcium oxalate+ uric acid (Ca Ox - U) stones.

Group 4 (16 cases) with calcium oxalate+ calcium

phosphate + uric acid (Ca Ox - Ca P- U) stones.

Group 5 (9 cases) with calcium oxalate + calcium phosphate + magnesium ammonium phosphate (Ca Ox - CaP - MAP) stones.

Group 6 (7 cases) with calcium oxalate + calcium phosphate + magnesium ammonium phosphate + uric acid (Ca Ox - CaP - MAP-U) stones.

In 54 cases the stones were localized in kidney , in 28 cases the stones were present in ureters , while in 5 cases the stones were found in bladder .

Before the initiation of any treatment, urine samples were collected. Total protein levels in urine samples were determined and its fractions were evaluated by Agarose simple electrophoresis [11] . The reagents were obtained from Sigma Diagnostic Company USA.

Determination of immunoglobulins G, M, A was carried out by the Single Radial Immunodiffusion Technique [12]. The reagents were obtained from Boehring, Germany.

The surgically removed renal calculus was washed several times with normal saline solution. It was immediately scrubbed with a sterile bruch to remove surface debris . A saw cut was made through the center of the calculus and the following tests were then carried out .

1-Chemical Analysis of Calculus :

The dust was analyzed for crystalline composition as described before [13] .

2-Microbiological Investigations :

Crushed fragments from the core of stone was cultured on different media as: Nutrient agar with addition of 1% dimethyl sulfoxide solution which was found to enhance the bacterial growth from the stones, Baird Parker medium, Mannitol salt agar, MacConkey agar , Shigella - Salmonella (S.S.) agar , Endo agar, Eosin Methylene - Blue Lactose Sucrose (EMB) agar and Xylose Lysine Deoxycholate (XLD) agar. The selected colonies were kept on slants of T.S.I agar and Nutrient agar (+1% dimethyl sulfoxide). Procedures of identifications were carried out according to procedures previously described [14-16] .

3-Determination of stone matrix and its protein content :

The halves of the stone were mixed together in visking cellophane casing and agitated in a vaccum-driven apparatus containing 5% (wt/ vol.) ethylenediamine- tetraacetic acid disodium salt (EDTA), adjusted to PH 7.8 with sodium hydroxide .

Decrystallization was grossly complete in 72 to 96 hrs. The cellophane casings were mechanically dialyzed against distilled water and the non-daily dialyzable matrix was recovered by lypholization. This dry powder was weighted then resuspended in distilled water.

Determination of total protein in the matrix of stone was done by using the Folin Phenol Method [17] .

RESULTS AND DISCUSSION

Results shown in Figure (1a) revealed that the urinary total protein levels are significantly increased in all the studied groups with urolithiasis as compared to the normal values. In patients with struvite stones (groups 5 and 6) the total protein level was higher than that in the other groups. This may be due to the bacterial infection, inflammation and damage of kidney tissues, resulting in impaired glomerular permeability and proteinuria .

Albumin (having intermediate molecular size) was the main protein lost in urine of all groups of stone-formers (Fig. 1 b), and in particular patients in groups 5 and 6 . **Latner** [18] stated that, albuminuria is known to be associated with the destruction or inflammation of kidney tissues.

Compared to our healthy subjects, the levels of α_1-β- Gama - globulin in urine samples are significantly increased (Fig. 1c , 1e , 1f). Certainly, a positive relationship was found between the incidence of infection of stones and the level of gamma globulin . It was increased several folds in patients with bacterial infection and repeated stones, specially in MAP containing stones (groups 5 and 6) .

Alpha$_2$-globulin showed high increments in most groups of stone - formers (Fig. 1d) . These findings may tend to support the observations that glomerular function remains largely unimpaired in these groups or may be considered to be primarily associated with renal tubular dysfunction. This fraction was absent in urine samples of group 3 , as in the normal healthy subjects. Group 3 with Ca Ox-U stones had the lowest incidence of urinary tract infection.

Urinary 1gG, IgM (high molecular weight protein) and IgA levels were relatively high in great number of cases with urolithiasis .

Figure (2 a) showed that IgG levels were very high in all groups specially groups 2 and 5. It was produced in response to the existed bacterial infection. Since immunoglobulin A (Fig. 2b) protects the mucosa from bacterial infection [9] its significant increase in all groups specially in group 5 indicates the bacerial infection . This was supported by the occurrance of certain infectious microorganisms in the urine samples of all patients of this group .

Zagazig J. Pharm. Sci., December 1998
Vol. 7 , No. 2, pp. 15 - 26

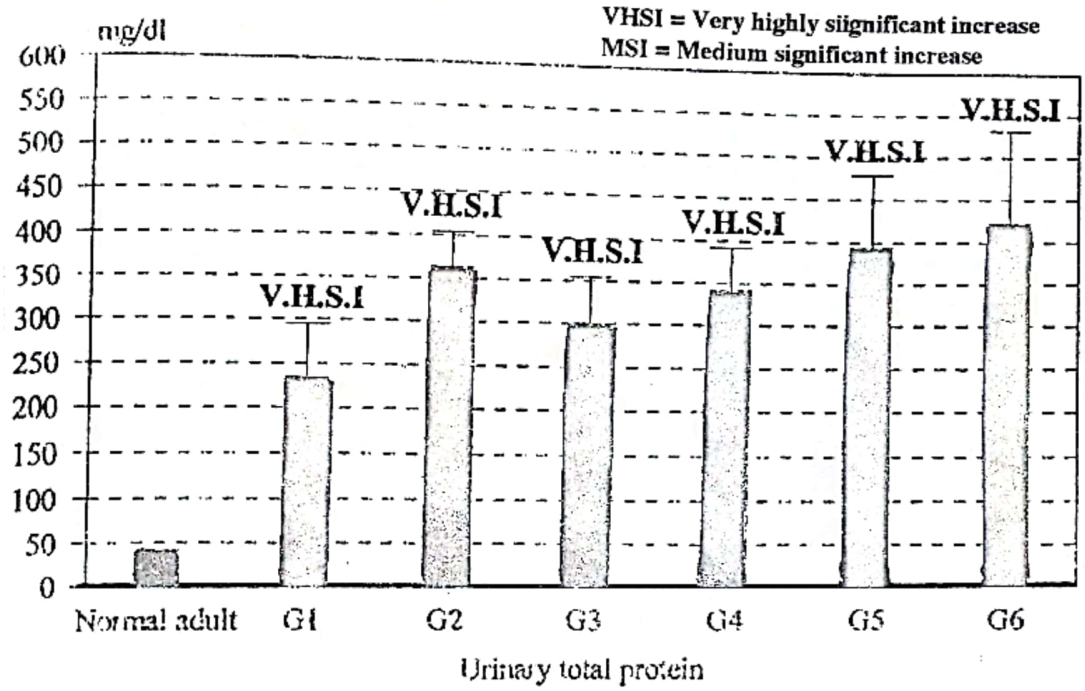

Fig. (1a) : The mean values ± S.E. of urinary total protein concentration (mg/dl) in the different studied groups.

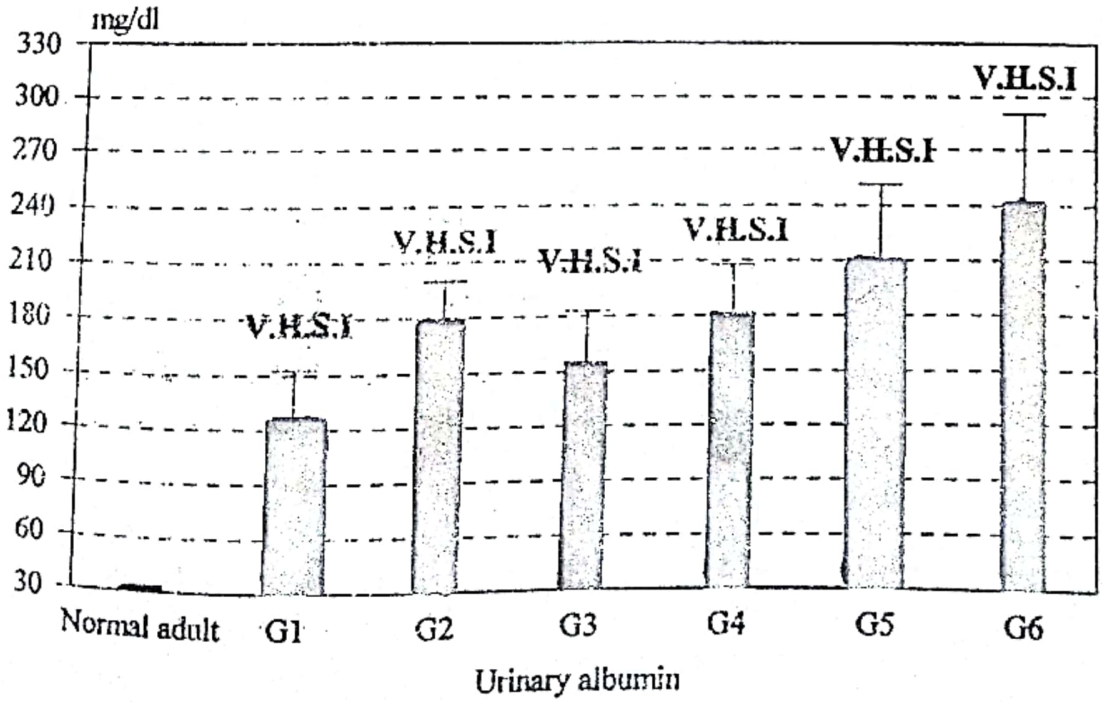

Fig. (1b): The mean values ± S.E. of urinary albumin concentration (mg/dl) in the different studied groups.

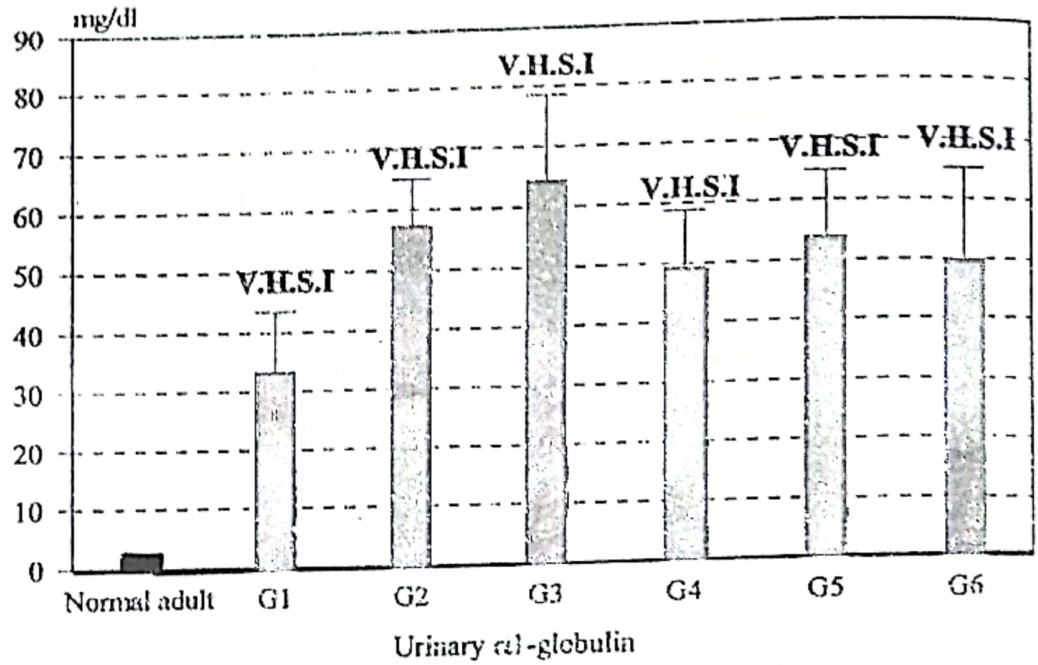

Fig. (1c) : The mean values ± S.E. of urinary α_1-G concentration (mg/dl) in the different studied groups.

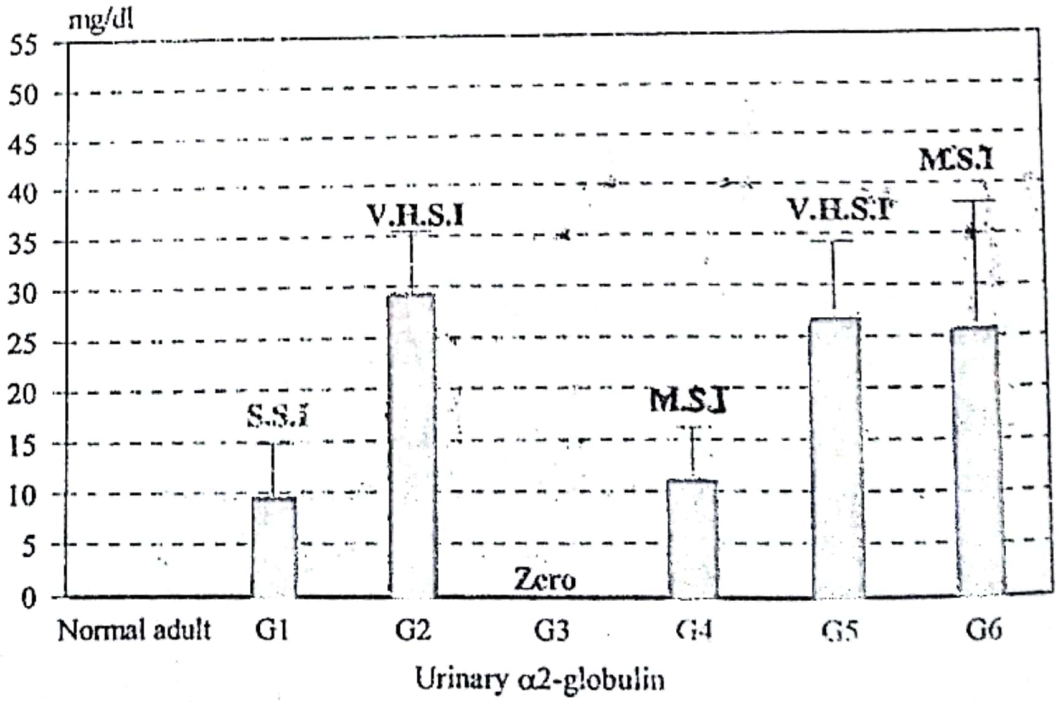

Fig. (1d) : The mean values ± S.E. of urinary α_2-G concentration (mg/dl) in the different studied groups.

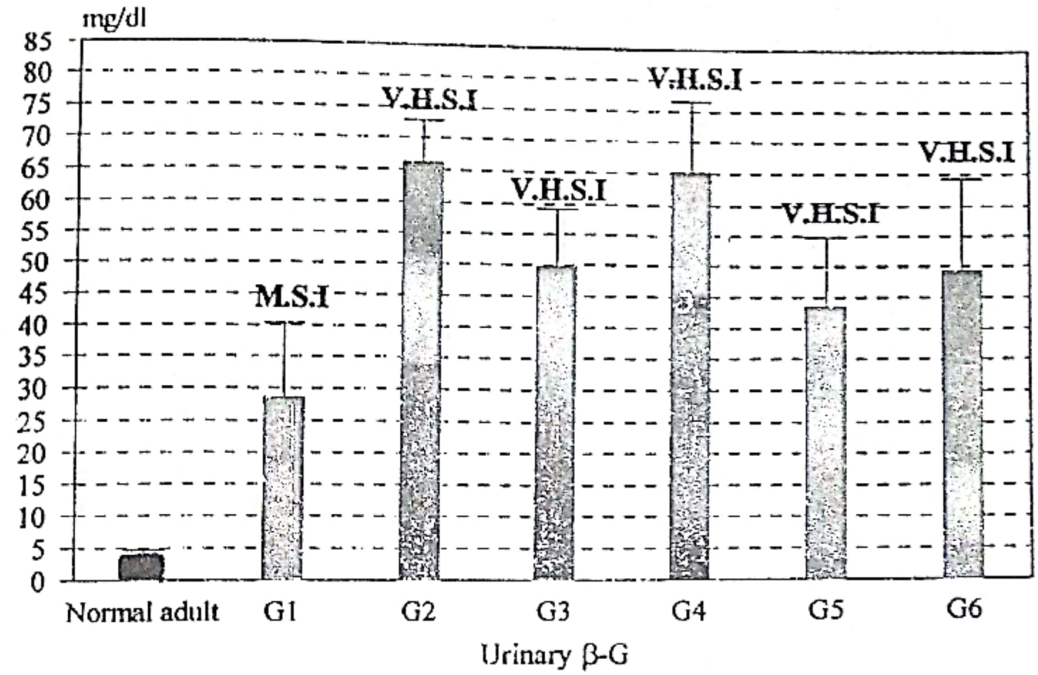

Fig. (1e) : The mean values ± S.E. of urinary β-G concentration (mg/dl) in the different studied groups.

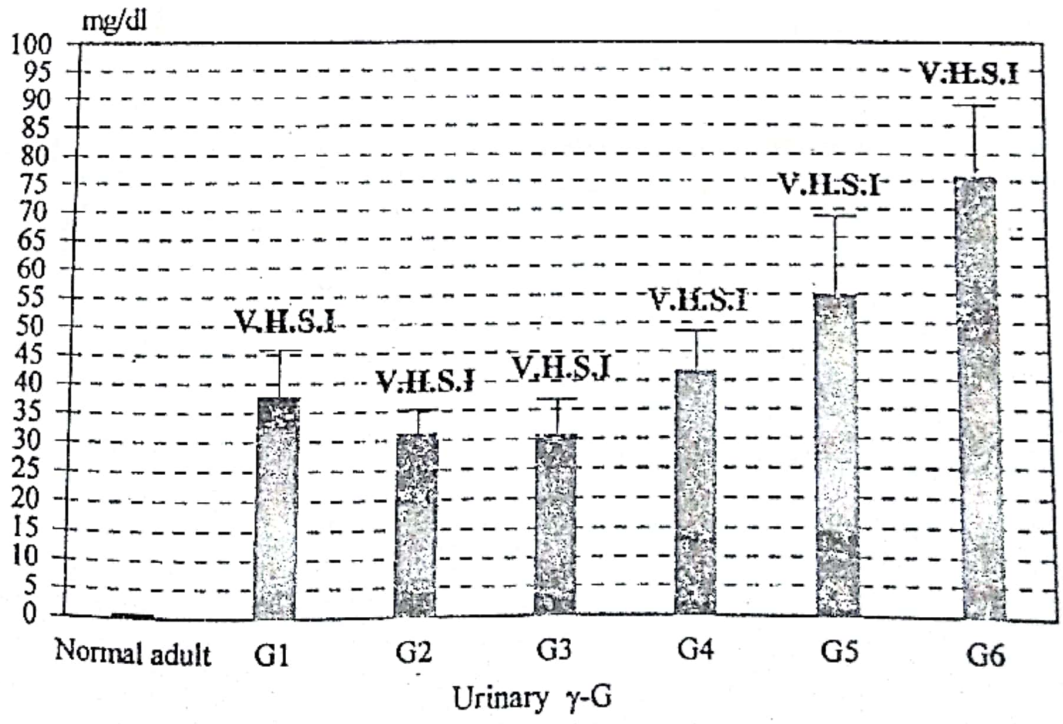

Fig. (1f) : The mean values ± S.E. of urinary γ -G concentration (mg/dl) in the different studied groups.

Zagazig J. Pharm. Sci., December 1998
Vol. 7 , No. 2, pp. 15 - 26

Figure (2c) showed increased values in the urinary IgM concentration in the different studied groups, specially in group 5. Its high molecular weight prevents its passage into extra- vascular species in normal healthy persons . IgM contains antibodie against Gram - ve bacteria .

Table (1) showed that the urinary stones contain matrix as organic substances which constitute 3 to 3.66% of the stone components. Protein represented 23.5 to 38% of this matrix . **Grouer and Resnick** (2) belived that two thirds of matrix of all urinary stones consist of proteins .

Different views had proposed for the possible role of such proteins in renal stone formation. It has been observed that a high content of protein matrix was found in the struvite calcui . Numerous bacterial cells, large amounts of organic matrix as well as crystals are thought to be important components in the infection stone formation.

Stone recurrence rate was high in some patients . This may be due to the presence of a high amount of different urinary proteins and immunoglobulins that form the matrix of the stones . The matrix was infected by bacteria. These bacterial cells and their secreted products appear to provide initial foci for crystals development.

Figures (3, 4 and 5) showed that *Pseudomonas* was found to be of a high frequency in 29.73% of the infected stones. Although it appeared negative in the urease test, it was isolated from the crushed fragments of the cores of 4 types of stones, specially from Ca Ox -Ca P MAP-U stones. *E. coli* (non - urease producer) and *Staphylococcus epidemidis* (18.92% , 16.22% , respectively) coming next . They were found in all types of stones except in Ca Ox-CaP - MAP-U stones.

Table (1) : The percentage of matrix, protein content in different types of stones in relation to percentage of infected cores.

The stones of different groups	% of matrix content	% of matrix protein content	% of infected cores
Group 1	3.40	23.50	33.33
Group 2	3.45	29.28	32.14
Group 3	3.62	31.40	46.67
Group 4	3.66	32.90	37.50
Group (5+6)	3.00	38.00	69.07

Sabinski and Leusmann (19) reported that the recent bacteriological studies using the scanning electron microscope have also shown microorganisms to be present in the different urinary stones , including Ca Ox stones. These microorganisms were assumed not to be able to split urea.

Proteus mirabilis was the organism isolated mostly from the cores of Ca Ox- CaP - MAP - U stones. **Arai et al.** (20) concluded that *P. mirabilis* play a significant role in the formation of struvite stones.

Pseudomonas , E. coli , Staphylococcus and *P. mirabilis* were proteases producers . Specially the struvite , the stones were found to contain proteins. Thus , they were suitable media for these organisms.

Yeast was isolated only from samples of the core of Ca Ox-CaP -MAP - U stones. **Durlach et al.** (21) isolated *Candida albicans* from one case of urine with struvite stone which was found to be incorporated in the developing stone .

In spite of *Klebsiella* is the urease producer it has not been isolated from any of the cores of the different stones.

In conclusion, the urinary calculi often lead to inflammation or injury of the surrounding tissues associated with proteinuria. Urinary albumin was the protein that excreted mostly in urine samples of patients with urolithiasis. Also α_1-β and gamma - globulin had a significant increase in all studied groups as compared to normal values . α_2- globulin was absent in group 3 as in the healthy persons . This group had the lowest urinary infection than all the other groups.

The increased levels of α_2-and β- glycoprotein, specially in the group with struvite stones, was associated with the high bacterial infection and the incidence of acute inflammation. Gamma - globulin increased several folds in patients with bacterial infection and repeated stones.

Urinary immunoglobulins increased in all groups. The high values of immunoglobulins reflect the infection and inflammatory response .

The composition of stones was found to be associated with an organic matrix. The substances of this matrix are present in urine, as urinary proteins, which incorportated into the matrix material then acts as a framework for deposition of crystals.

Pseudomonas was the most isolated organisms from the cores of stones specially from Ca Ox-CaP-MAP-U stones. *P. mirabilis* was the common microorganism isolated from the cores of these stones. This was followed by *E. coli* and *Staphylococcus* which were found in all types of stones except in the Ca Ox-CaP -MAP - U stones.

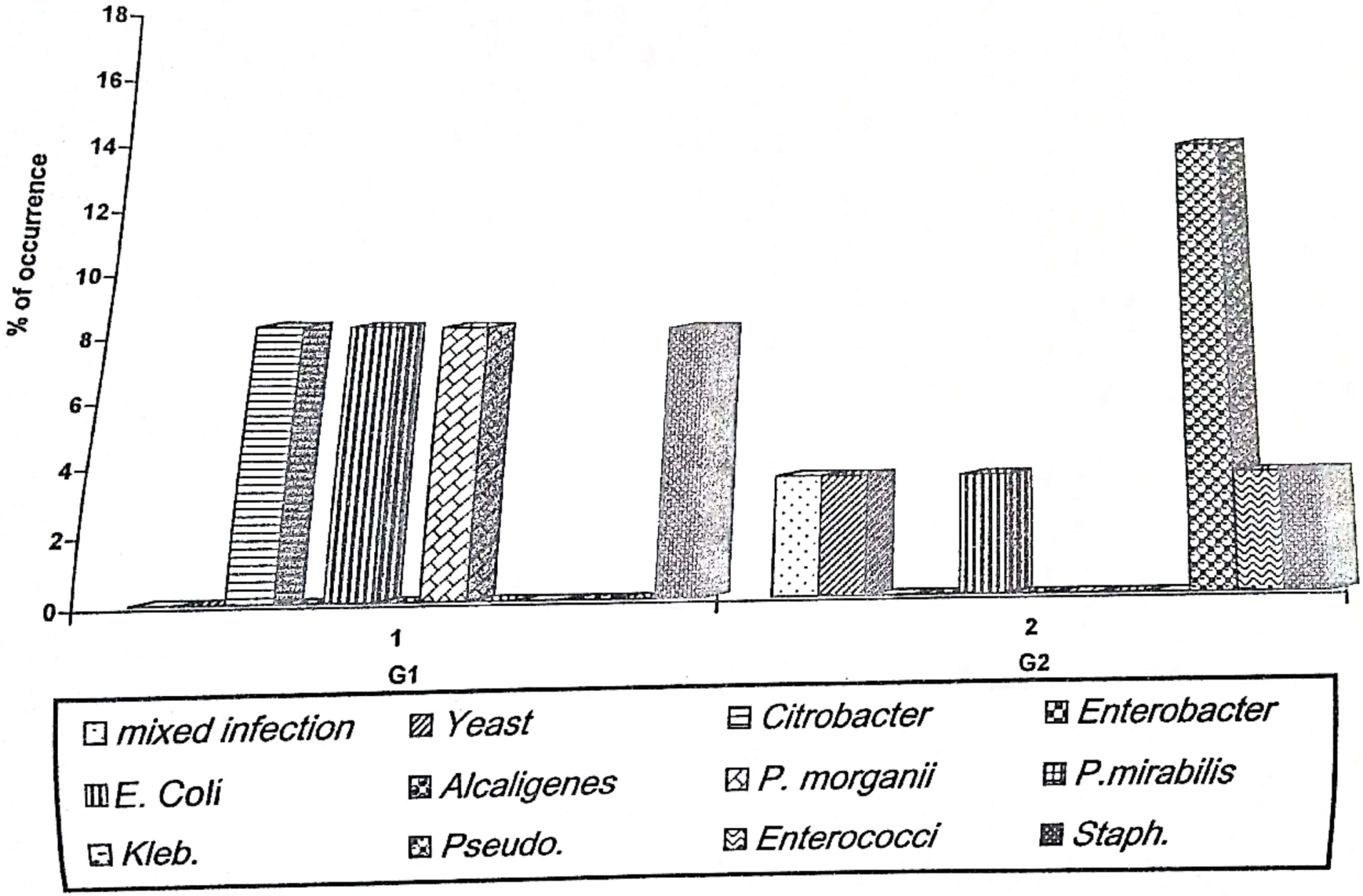

Fig. (3) :Percentage of occurrence of different microorganisms in stone (core) samples with Ca - Ox (G1) and Ca - Ox + Ca -P (G2).

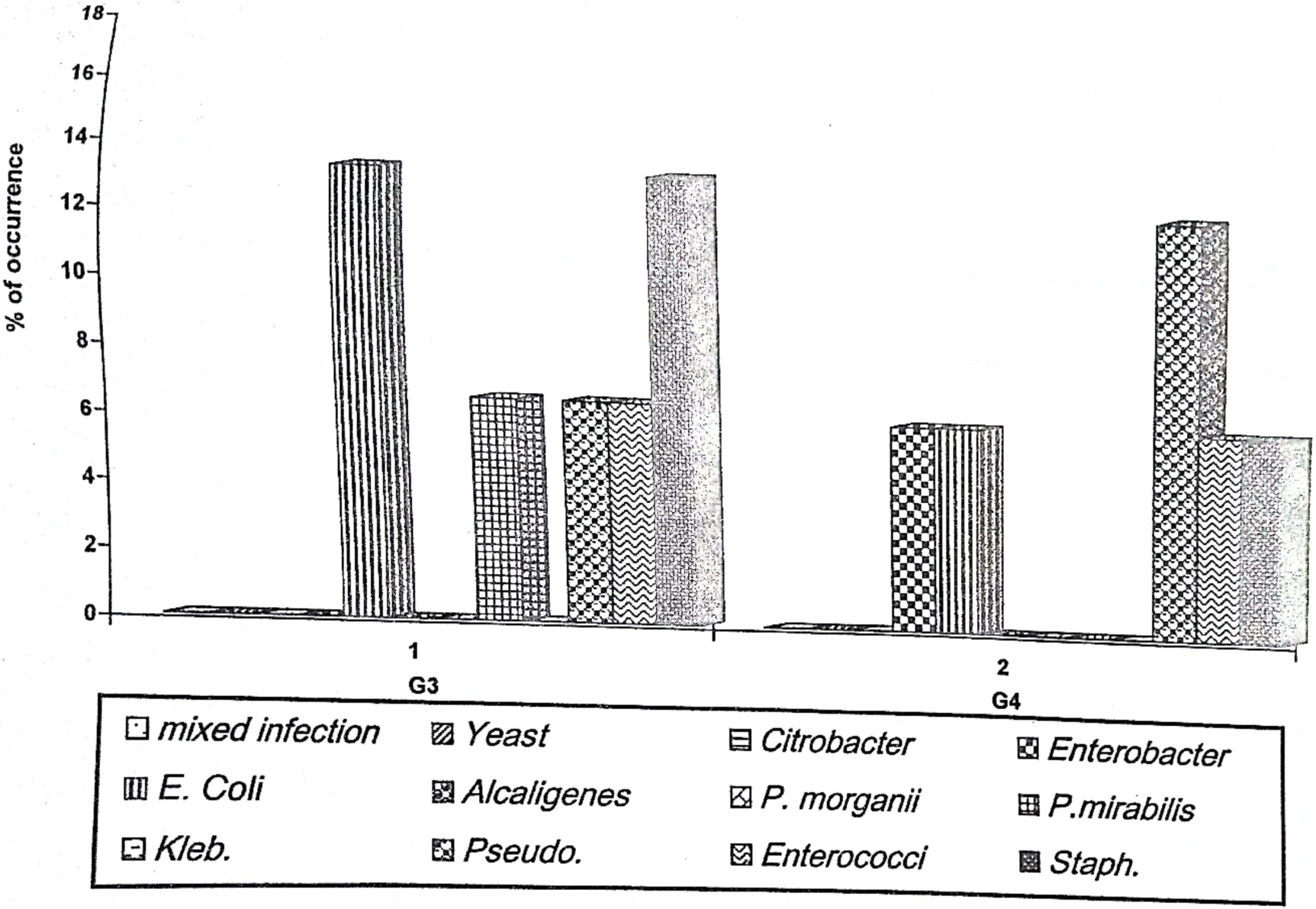

Fig. (4) : Percentage of occurrence of different microorganisms in stone (core) samples with Ca - Ox + Uric acid (G3) and Ca - Ox+ Ca P+ Uric acid (G4).

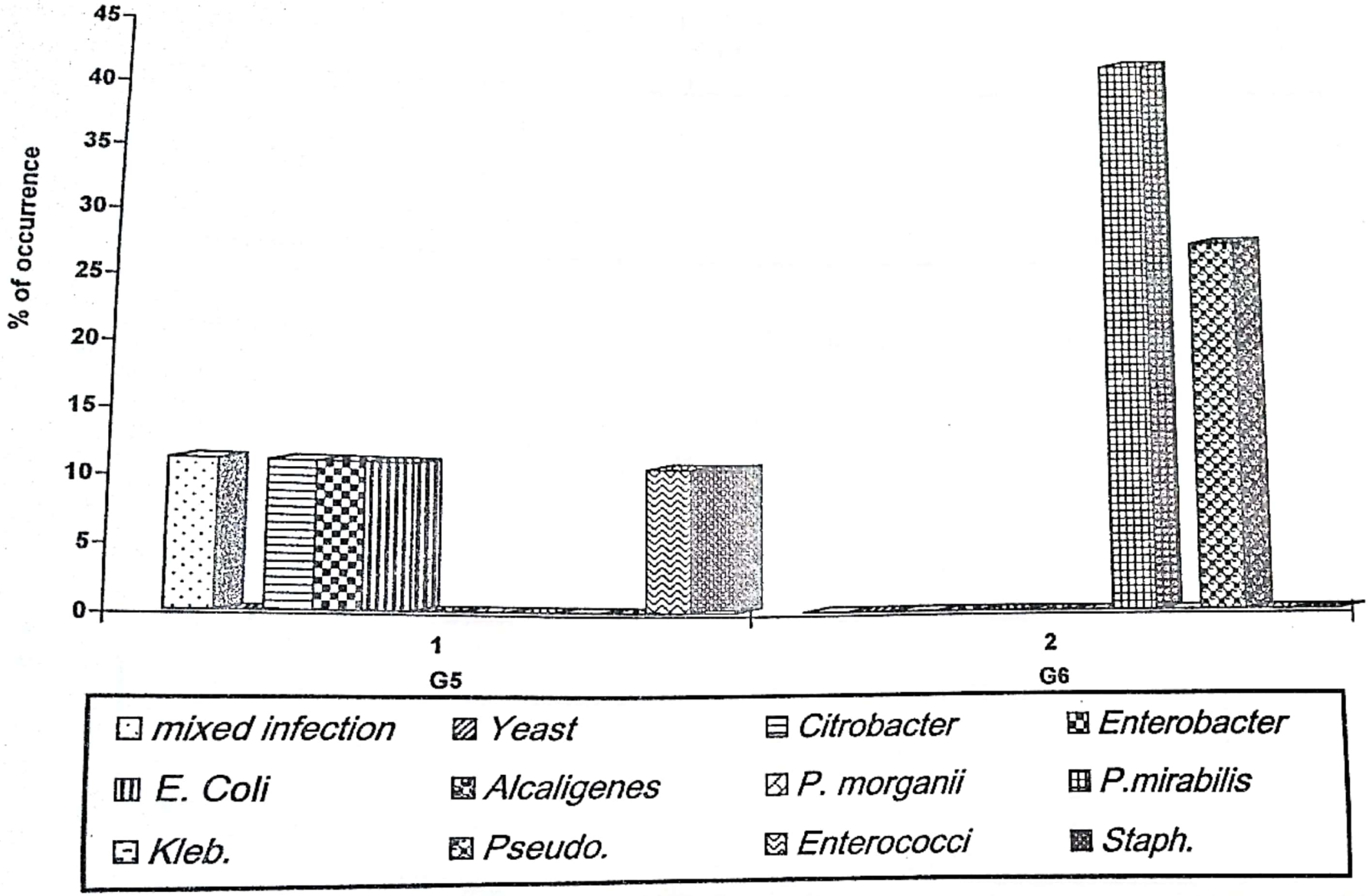

Fig. (5) : Percentage of occurrence of different microorganisms in stone (core) samples with Ca - Ox + Ca - P+MAP (G5) and Ca-Ox+ Ca- P+ MAP + Uric acid (G6).

Thus bacteria , their secreted products and protein matrix perhaps are acting as precipitant and connective substances for the salts. In this case these factors are the most essential cause of stone formation.

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المحتوي البروتيني والكائنات الدقيقة الكامنه في الحصوات البولية

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* الهئية العامه للمعاهد والمستشفيات التعليمية بالقاهرة –
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لدراسة أسباب تكون الحصوات البولية . فإن ٨٥ مريضاً بالحصوات قد قسموا إلى ٦ مجاميع تبعاً للتركيب الكيميائى للحصوات المنزرعة جراحياً.

فقد تم عزل وتصنيف الكائنات الدقيقة الكامنه داخل الحصوات فكانت السيدوموناس هى النوع السائد رغم أنه سالب لاختبار اليوريز . يأتى بعده عصويات القولون والستافيلوكوكاس ابيديرمدس . وقد وجد أن بروتيوس ميرابيلس كانت البكتريا الموجودة بكثرة داخل حصوات الإستروفيت فى المجموعة السادسة (حصوات أكسالات الكالسيوم + فوسفات الكالسيوم والماغنسيوم والأمونيوم + حامض اليوريك) وهى كائنات محلله لليوريا.

ومع أن الكليبسيلا من النوع المنتج لإنزيم اليوريز إلا أنه لم يتم عزلها من داخل أى نوع من الحصوات . تم تقدير كل من بروتينات وجلوبيولينات المناعة فى البول وكمية البروتين فى الحصوة ، فلوحظ ارتفاع فى معدلهم الذى قد يعكس وجود التهاب وعدوى فى حالات كثيرة من المرضى مقارنه بالمجموعة الضابطة ، خاصة فى مجاميع حصوات الإستروفيت التى تحتوى على كميات كبيرة من المحتوى البروتينى مصاحباً بارتفاع فى العدوى البكتثرية .

وقد لوحظ أن كل الحصوات التى تحتوى على بروتينات كانت البكتريا الكامنه داخلها منتجة للبروتيز . لذلك فإن وجود البكتريا ونواتجها الافرازية وكذلك المحتوى البروتينى داخل الحصوة يعملون جميعاً كمواد مرسبة ومجمعه للبلورات مما يساعد على نمو الحصاوى.