The Isolation, Characterization, and Identification of the Crystalline Inclusions of the Large Free-Living Amebae*

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PLATES 115 TO 117

(Received for publication, June 25, 1959)

ABSTRACT

Amoeba proteus, Amoeba dubia, and Chaos chaos all contain similar plate-like and bipyramidal cytoplasmic crystals. Isolated crystals, purified by recrystallization from water, yield plate-like crystals which have been shown to be identical with synthesized carbonyl diurea (triuret) with regard to physicochemical properties, elemental analysis, x-ray diffraction patterns, infrared spectra, and optical properties. The birefringent plates found in the cytoplasm are carbonyl diurea. While the exact composition of the isotropic plates and of the bipyramids is not clear at present, the major constituent of these crystal types is also carbonyl diurea. It is suggested that carbonyl diurea is a nitrogen excretion product, not previously found in living organisms, and probably represents an end product of purine metabolism in amebae.

INTRODUCTION

Crystalline inclusions are found in the cytoplasm of most of the large free-living amebae. These crystals are particularly apparent in the carnivorous amebae, Amoeba proteus, Amoeba dubia, and Chaos chaos, while the lack of crystals is one of several characteristics that distinguish the herbivorous ameba, Pelomyxa palustris, from the species above (10). Many speculations have been made as to the chemical nature of the crystals and as to their function in the cytoplasm. Most authors have considered them to be either food reserves or excretory products (1, 2, 3, 5, 11, 12, 16, 18).

Mast and Doyle (12) suggested, on insufficient evidence, that the plate-like and bipyramidal crystals found in Amoeba proteus were leucine and a magnesium salt of glycine respectively. Bernheimer (4) studied the refractive index, melting point, and solubility properties of bipyramidal crystals from various ameba species. He demonstrated that the crystals of Chaos diffluens (A. proteus), Trichamoeba villosa, and Cochliopodium bilimbosum were similar, but differed as a group from those of Mayorella sp. and other protozoa which he studied. A recent study by Grunbaum, Max Møller, and Thomas (9) will be discussed more fully below.

Until recently (14, 15) the culture methods for amebae were not sufficiently productive to allow isolation and purification of the crystalline material. The need for relatively large quantities of material led the author, before undertaking the present work, to develop controlled mass culture methods that would be suitable for the sustained production of very large populations of amebae (7, 8).

Materials and Methods

In the present investigation, crystals from the three species, Amoeba proteus, Amoeba dubia, and Chaos chaos (Chaos carolinensis, Pelomyxa carolinensis) have been compared. The amebae were grown on Tetrahymena by improved mass culture methods, described in (7) and (8). The strains used, PROT 1, PROT 4, DUB 1, and CH 1, are described more fully there. Isolation of the crystalline inclusions was carried out in the following manner. The amebae were concentrated by centrifugation and small pellets were frozen with dry ice and acetone and lyophilized in a high vacuum system at room temperature. The dried material was...
dispersed by grinding in di-n-butyl phthalate with an agate mortar and pestle. When microscopic examination showed that most of the crystals were free from attached cytoplasm, the dispersed material was centrifuged in carbon tetrachloride. This solvent is intermediate in density between the heavier crystals and the lighter cytoplasm. The supernatant was then poured off and the heavy material washed 3 times with carbon tetrachloride. The cytoplasmic material floating in the supernatant was again dispersed and centrifuged to obtain remaining crystals. Crystals prepared by the above technique are contaminated with small amounts of cytoplasmic material (protein, nucleic acid, etc.). However, such amorphous material does not interfere with petrographic or x-ray diffraction analysis (see Figs. 5 and 8), and comparative studies of the crystals from the three species were made using such preparations.

The crystalline material was further purified for chemical studies. Since, as will be shown later, the material is the same in the three species, crystals from A. proteus were used for this purpose. This species grows rapidly and contains more crystals than A. dubia or C. chaos. The crude preparation described above was placed in a dialysis sack with a small amount of water and dialysed for a few hours at 60 to 70 °C. against a relatively large volume of distilled water. The dissolved crystalline material passed through the dialysis membrane and was recovered when the external volume of water was concentrated by boiling under reduced pressure. After macromolecules had been removed from the preparation in this way, the material was further purified by recrystallizing 3 times from minimal volumes of boiling water. “Millipore filters” (HA) were used as an analytical sample.

Since the crystals are small, immersion refractometry with a phase contrast microscope was used for determining refractive index rather than the Becké line technique. When a rotatable polaroid was introduced above the phase condensor, the refractive index could be determined for a single crystallographic direction in the birefringent crystals.

**RESULTS**

**Observations on Living Cells:**

The number of crystals per cell generally appeared to increase when amebae were transferred from “stock” infusion cultures to cultures fed on *Tetrahymena*. Two morphologically distinct types of crystals are found in the three species: bipyramidal crystals, often truncated, and thin square or rectangular plates. The long dimension of the largest crystals usually found and the approximate ratios of crystal types in the cytoplasm are listed below for the three species. (See also Figs. 1 to 3).

<table>
<thead>
<tr>
<th></th>
<th>Bipyr-</th>
<th>Plates</th>
<th>Approximate ratio Bipyr-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>amids</td>
<td></td>
<td>mids/Plates</td>
</tr>
<tr>
<td>1.</td>
<td>A. dubia</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>2.</td>
<td>A. proteus</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>3.</td>
<td>C. chaos</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

While some of the crystals are contained in vacuoles, careful observations on uncompressed cells with bright field, phase contrast, and television microscopes indicated that many crystals have no visible surrounding membrane. Care must be taken in such observation since compression can cause vacuoles to appear around crystals (cf. 1).

**Optical Crystallography:**

The bipyramidal crystals from all three species are isotropic, have similar shapes, and show refractive indices of 1.638 to 1.642. By contrast, most of the plates in *A. dubia* are highly birefringent. The refractive indices were >1.72 and <1.52. When the plates were tilted, the spread of refractive indices was even higher so that precise values could not be obtained. Many of these plates showed a characteristic twinning pattern, which proved to be a useful diagnostic feature. Some plates, however, were not twinned, and others were apparently isotropic (See Fig. 5). The plates found in *A. proteus* and *C. chaos* were small and apparently very thin. An extensive search, however, revealed some plates that were birefringent.

When *A. proteus* crystals (mostly isotropic bipyramids) were put in solution, they recrystallized as birefringent plates identical in optical properties with the plates found in *A. dubia*. The two compared closely in the spread of refractive indices, external morphology (when carefully recrystallized), and characteristic twinning pattern (See Figs. 5 and 6). Large crystals (0.2 to 1.0 mm.) of this material could be formed by slow cooling (Fig. 4). When examined petrographically, they proved to be uniaxial, negative, and probably of hexagonal symmetry. The most prominent cleavage plane forms an angle of 45° with the c axis. The flat surfaces of the birefringent plates are oriented parallel to this cleavage plane, which explains the higher spread of refractive indices of such plates when they are tilted.
X-ray Crystallography:

The x-ray diffraction patterns of concentrated crystal preparations were found to be the same for all three species (See Figs. 9 and 10 for patterns from A. dubia and A. proteus and Table I for d-spacings). The recrystallized material exhibited a pattern different from that of the naturally occurring crystals (compare Figs. 10 and 11). In the isolated crystal preparations washed in carbon tetrachloride, bipyramidal crystals were close to the ideal size and thickness for x-ray powder photographs (3 to 5 μ). The plates, however, were less than 0.4 μ thick even in A. dubia (computed from independent direct measurements of both birefringence and retardation) and would be expected to give comparatively weak bands. With the exception of a few weak lines in the A. dubia pattern, the diffraction patterns of the naturally occurring crystals seem to be due to the bipyramidal crystals. It can be seen from the number of bands present and their spacing that the crystal symmetry is not cubic and is probably of a higher order of symmetry, even though the bipyramidal crystals appear to be isotropic.

**Physicochemical Properties:**

Several properties of the recrystallized material obtained from A. proteus were investigated.

Visible and Ultraviolet Absorption Spectra.—By heating aqueous solutions it was possible to analyze concentrations of approximately 10 mg./100 ml. (10 times the concentration usually used for UV spectra). At this concentration and below, no absorption peaks were found. This result immediately eliminated several classes of compounds which might be found in living cells.

Optical Rotation.—At the above concentrations no optical rotation was detected in 1 cm. cells.

Solubility.—The recrystallized material and both types of crystals formed in amebae had similar solubility properties. They were soluble in hot water, dimethyl formamide, dimethyl sulfoxide, and glacial acetic acid; were slightly soluble in cold water, and were insoluble in carbon tetrachloride, alcohol, ether, acetone, oils, and di-n-butyl phthalate (cf. 4, 9, 12).

Infrared Spectroscopy.—The purified crystals gave a characteristic infrared spectrum (Fig. 13) which indicated the probable presence of an aliphatic carbonyl group and an O—H or an N—H bond and the absence of C—H bonds. Since spectra of unknowns are difficult to analyze in detail, this spectrum was used chiefly for comparative purposes.

The spectrum of material obtained by solution of native crystals, dialysis, and evaporation to dryness was the same as that of the fully purified material. No dialyzable organic breakdown product, other than that in the plates, was present. However, during the change from bipyramidal to plates, either a gaseous, or a non-absorbing, or a non-dialyzable hypoproduct could have been formed.

**Decomposition Point.**—Decomposition points were determined on a microscope hot-stage using a rate of temperature increase of 2° per minute. The recrystallized material (from A. proteus) decom-

### TABLE I

*d*-Spacings Calculated from X-ray Diffraction Patterns

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<tr>
<th>A. proteus</th>
<th>Relative intensity</th>
<th>A. dubia</th>
<th>A. proteus recryst.</th>
<th>Relative intensity</th>
<th>Carbonyl diurea</th>
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The Results of Elemental Analysis*

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<th>Sample 1 vacuum-dried 80°C. 36 hrs. (per cent)</th>
<th>Sample 2 vacuum-dried 20°C. 30 hrs. (per cent)</th>
<th>Sample 3 vacuum-dried 20°C. 20 hrs. (per cent)</th>
<th>Computed for C₆H₂₂N₄O₄ (per cent)</th>
<th>Sample 1 vacuum-dried 80°C. 24 hrs. (per cent)</th>
<th>Sample 2 vacuum-dried 80°C. 21 hrs. (per cent)</th>
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</table>

*p I would like to thank Joseph F. Alicino, P.O. Box 267, Metuchen, New Jersey, who did the microanalyses. The analytical methods of Pregl (modified) were used (cf. Niederl, J. B., and Niederl, V., Micromethods of Quantitative Organic Elementary Analysis, New York, J. Wiley and Sons, Inc., 1938). All tests were quantitative. Nitrogen was determined by the micro Dumas method and basic groups by perchloric acid in non-aqueous media.

posed slowly between 225° and 230°C. At the time of decomposition long birefringent rods, stable to 360°C., appeared on the slide. Above 200°C. stacks of plates would sometimes fly suddenly apart, apparently because of evolved gases trapped between them. The relatively large crystals from A. dubia, both flat plates and bipyramids, could be seen to decompose between 225° and 230°C. to form birefringent rods like those above (up to 100 μ long, 10 μ wide). By interruption of heating and subsequent inspection, crystals from A. proteus and Chaos chaos were found to be visibly unchanged after exposure to 225°C. and to be decomposed, with the formation of rods, after exposure to 230°C.

Elemental Analysis:

The data obtained by microanalysis is presented in Table II. The first sample of purified crystalline material (4 mg. vacuum-dried at 80°C.) showed no metals or halogens present and carbon, hydrogen, and nitrogen were present in proportions which fitted an empirical formula of C₆H₂₂N₄O₄. This combination of high nitrogen and high oxygen could not be fitted into a structural formula. Since the compound had appeared to give off gas while decomposing (see above), the second sample was vacuum-dried at room temperature. This sample gave a negative test for sulfur and phosphorous, and the nitrogen was significantly higher. Using the proportions of carbon and hydrogen previously found, the new proportion of nitrogen was compatible with the empirical formulas C₆H₂₂N₄O₄ and C₆H₂₂N₄O₆. A search of the chemical literature revealed one compound which matched the unknown in chemical properties and fit the formula C₆H₂₂N₄O₆. This compound, carbonyl diurea (triuret), NH₂–CO–NH–CO–NH–CO–NH₂, was synthesized using a modification of the procedure of Schittenhelm and Warnat (17).

1.0 gm. of uric acid was added to 20 ml. of 5 per cent aqueous ammonia and refluxed 5 minutes, after which the flame was removed, 3 ml. of 10 per cent hydrogen peroxide added, and the reflux continued. This addition of peroxide was repeated four times at 10 minute intervals. When the suspension became clear (1.2 to 1.5 hours), it was filtered while hot and the crystals allowed to precipitate at room temperature. Yield: 0.12 gm. white crystals (decomposition point: 227°C. oil bath; 225 to 230°C., hot stage) (density: 1.745 ± 0.005 at 23.5°C.).

Comparison of Purified Crystals with Carbonyl Diurea:

The elemental analysis of synthetic carbonyl diurea and a third preparation of purified ameba crystals is shown in Table II along with computed
values for the empirical formula, \( C_3H_8N_4O_2 \). The agreement is clear. As expected, the nitrogen for a sample of synthetic carbonyl diurea which had been vacuum-dried at 80°C. was significantly low; some of the first sample of ameba crystals had apparently lost ammonia to form cyanuric acid (cf. 19), judging from the low nitrogen and hydrogen and the shape and melting point of the crystals formed during decomposition.

Infrared spectra for the synthesized material and that from amebae were identical (Figs. 13 and 14).

The x-ray diffraction patterns of synthetic carbonyl diurea and of recrystallized ameba crystals are presented in Figs. 11 and 12. The \( d \)-spacings computed from these strips are presented in Table I. They are the same.

The optical properties of crystalline carbonyl diurea are the same as those described for purified ameba crystals (Figs. 5 to 7).

These comparisons leave no doubt that the purified material from the cytoplasm of the large, free-living amebae is carbonyl diurea.

**DISCUSSION**

Two morphological types of crystals are found in the amebae studied: plates, often birefringent, and bipyramids, which appear to be isotropic. When a mixture of these two crystal types is dissolved in water and subsequently recrystallized, birefringent plates of carbonyl diurea are recovered. The naturally occurring birefringent plates, so obvious in *A. dubia*, are clearly carbonyl diurea. The identification of the isotropic plates and the bipyraridal crystals is more difficult.

In the original presentation of this work (Ph.D. thesis, 7), we concluded that the available evidence indicated a strong possibility that the plates and bipyramids were alternate crystalline arrangements of carbonyl diurea. Recently, Grunbaum, Max Møller, and Thomas (9) have published a study of the crystals of *A. proteus* and *Chaos chaos*, in which they reached somewhat different conclusions. (I would like to thank these authors and Dr. H. Holter, in whose laboratory their work was done, for generously allowing me to read the galley proof of their paper).

Outlined below is the evidence which originally led to the view that the isotropic crystals might be a different crystalline form of carbonyl diurea.

1. The decomposition points of all the crystal types found in amebae were the same as that of carbonyl diurea.
2. The solubility properties were also the same.
3. The isotropic crystals could not be a salt of carbonyl diurea, since this compound has no basic or acidic groups.
4. After solution of the native crystals and recrystallization, the only microscopically visible crystals were carbonyl diurea.
5. The infrared spectrum of dialyzed, dried crystals was identical with that of carbonyl diurea. No dialyzable breakdown product with a spectrum different from that of carbonyl diurea was formed on solution in water. This did not exclude a reaction producing a large molecule or the production of a gaseous or non-absorbing breakdown product.
6. Although carbonyl diurea formed only one crystal type when recrystallized from water, \( 1 \text{N HCl} \), \( 5 \text{per cent NH}_4\text{OH} \), dimethyl sulfoxide, dimethyl formamide, and glacial acetic acid, the conditions of crystallization in amebae are unknown and would be difficult if not impossible to duplicate.
7. Carbonyl diurea can be formed by oxidation of uric acid, probably through allantoin and allantoic acid (cf. 17). The bipyramids contain none of these compounds and such an oxidation is unlikely to proceed in water alone.
8. The birefringent plates in *A. dubia* were identified as carbonyl diurea. Some of the plates are isotropic rather than birefringent, while others are partially birefringent and partially isotropic. Since the isotropic portions of the latter plates show in the same relief in non-polarized light as the birefringent portions, the lack of birefringence is probably not due to extreme thinness. At the junctions of the interpenetration twins of carbonyl diurea, apparently isotropic areas blend into fully birefringent areas. The lack of birefringence of the isotropic plates or parts of plates could be due to:
   (a) some arrangement of molecules or crystal layers in such a manner that retardations cancel (as is probably the case in interpenetration twins);
   (b) a different arrangement of carbonyl diurea molecules, perhaps due to the presence of some other molecules or ions in the crystal or;
   (c) if the conclusions of Grunbaum et al. (discussed below) are correct, the isotropic crystals (both plates and bipyramids) are made up of a single compound which decomposes in water to form carbonyl diurea.

The evidence listed above, while it does not permit a definite identification, does show that the isotropic plates and the bipyramids are very similar.
to the birefringent plates of carbonyl diurea in most properties.

Grunbaum, Max Møller, and Thomas (9) confined most of their work to the native crystals of *A. proteus* and *Chaos chaos*. They concluded that there is only one crystal type in these two species and that the small plates found were actually bipyramids truncated to an extreme degree. However, this is clearly not the case in *A. dubia* and the fact that careful searching reveals some weakly birefringent plates in the former species leads us to conclude that all three species do form some plates of carbonyl diurea.

By microanalysis of native crystals from *A. proteus*, separated from most of the cytoplasm by density gradient centrifugation, Grunbaum *et al.* obtained values of (preparation D) C 26.25, H 4.50, N 26.66, ash 11.56, and (preparation E) C 27.70, H 4.61, N 24.75, ash 10.00 (values expressed as per cent of fresh weight, about 3 per cent moisture content). They disregarded the ash as being due to impurities and calculated an empirical formula of $C_4H_4N_4O_4$ (C 31.75, H 4.80, N 29.62).

A reinterpretation of this data is possible in the light of two further elemental analyses: carbonyl diurea (computed), C 24.66, H 4.14, N 38.35; and a relatively crystal-free, light fraction of lyophilized cytoplasm (carbon tetrachloride-washed, acetone-extracted 24 hours, vacuum-dried), C 44.6, H 7.2, N 11.8, ash 5.7. It will be seen that carbonyl diurea has the lowest C and H and the highest N, that the samples of native crystals are intermediate, and that the cytoplasmic sample has the highest C and H and the lowest N. From the method of isolation of the native crystals and the high ash content it is clear that some contaminants are present. Cytoplasmic contaminants would shift the C and H values up and the N value down. For example, preparation E, above, had a higher C and H and lower N than preparation D and so probably had a greater proportion of cytoplasm present. The foregoing evidence indicates a rather strong possibility that the native crystals contain less C and H and more N than Grunbaum *et al.* thought. Thus the empirical formula may well more closely approximate that of carbonyl diurea than they concluded.

Grunbaum, Max Møller, and Thomas found that the infrared spectrum of the native crystal preparation had 4 more absorption peaks than the spectrum of the "breakdown product" formed in water (carbonyl diurea). We have confirmed this with carbon tetrachloride-washed crystal preparations. This is evidence of a chemical change on solution in water, provided no strongly absorbing contaminant is present in the native crystal preparation. Further evidence will be necessary for a final decision. Since all the absorption bands in carbonyl diurea are present in the spectrum of the native crystal preparation, we can point out that all the groups of the carbonyl diurea molecule are present and absorb in the same way as in pure carbonyl diurea.

The isotropic plates and bipyramids may be: (a) a chemical compound that decomposes to form carbonyl diurea, (b) a crystalline complex of carbonyl diurea with some other organic or inorganic material, or (c) carbonyl diurea, contaminated with I-R absorbing material, arranged in a different crystalline lattice or in alternate crystal layers. A definite decision between these possibilities is not possible at present. However, it is clear that carbonyl diurea is a major constituent of the native crystals and if it is bound to or complexed with some other material, it is little altered by the binding and is not firmly held.

There have been conflicting reports as to whether the crystals are free in the cytoplasm or are surrounded by vacuole membranes (cf. 2, 9, 11, 12, 18). The presence or absence of vacuole membranes seems to depend on the strain of ameba used, on the culture conditions, and on the care with which the cells are handled during observations. Since carbonyl diurea is only slightly soluble in cold water, it seems probable that its production in the cytoplasm as an end product of nitrogen catabolism could result in the direct formation of crystals without a vacuolar concentration mechanism.

No previous references have been found to the natural occurrence of carbonyl diurea in living organisms. This compound has been synthesized by the action of strong oxidizing agents on uric acid and allantoin (cf. 13, 17, 19). Canellakis *et al.* have recently reported the formation of carbonyl diurea by the action of catalase and ethyl hydrogen peroxide on uric acid. They state that this system seems to represent a much stronger peroxidative system than three peroxidases also studied. Uric acid apparently is oxidized through allantoic and allantoinic acid to carbonyl diurea (17). In living organisms, the pathway of degradation of uric acid to allantoinic acid (the latter is excreted by teleost fishes) by the action of uricase and allantoinase has been well mapped out (cf. 20). One might speculate that an oxidative system (probably catalyzed by a specific enzyme), could act on
allantoic acid to produce carbonyl diurea in amebae.

This paper is part of a Ph.D. thesis submitted to the faculty of Princeton University. I would like to acknowledge the continued interest and encouragement of Professor R. D. Allen and the support of the Princeton Department of Biology.

I would like to thank the following people for their generosity in giving advice and technical assistance: Dr. C. E. Bricker, Dr. E. C. Taylor, and Mr. J. S. Driscoll, all of the Princeton Chemistry Department, Dr. I. Fankuchen of the Brooklyn Polytechnic Institute, and Mr. E. K. Oxburgh of the Princeton Geology Department.

REFERENCES
IDENTIFICATION OF AMEBA CRYSTALS

EXPLANATION OF PLATES

PLATE 115

Figs. 1. to 3. Photographs of the crystalline cytoplasmic inclusions of three species of amebae. Lyophilized amebae were mounted in immersion oil and photographed with a N.A. 1.25 oil immersion objective. Each unit of the scale (in Fig. 1) for Figs. 1 to 3 represents 10 microns. Fig. 1. *Amoeba proteus* (strain PROT 1). Fig. 2. *Amoeba dubia*. Fig. 3. *Chaos chaos* (strain CH 1).

Fig. 4. Relatively large crystals formed by very slow cooling of a hot, saturated solution of *A. proteus* crystals. Photographed by reflected light at low magnification (smallest scale units equal 10 microns).
(Griffin: Identification of ameba crystals)
Plate 116

Fig. 5. Isolated crystalline inclusions from *A. dubia*, photographed with a polarizing microscope, showing truncated bipyramids, isotropic plates, and birefringent plates, with and without the characteristic twinning pattern. Nicols crossed, with 1st order red compensator inserted.

Fig. 6. Recrystallized material from isolated *A. proiens* crystals to show the birefringence, right angle corners and twinning pattern of a typical plate. Nicols crossed, no compensator.

Fig. 7. Carbonyl diurea, crystallized from water, to show birefringent plates and twinning pattern. Compare with Figs. 5 and 6. Nicols crossed, no compensator.
Griffin: Identification of ameba crystals
PLATE 117

FIGS. 8 to 12. X-ray diffraction patterns. See Table I for d-spacings and other details.

FIG. 8. Long exposure of lyophilized *Chaos* *chaos* cytoplasm separated from most of the crystals to show that other components of the cytoplasm do not give sharp diffraction lines.


FIG. 10. Crystals isolated from *A. dubia*, washed in carbon tetrachloride. Compare the spacing and relative intensities of lines with those in Fig. 9.

FIG. 11. Crystalline material from *A. proteus*, dialyzed and recrystallized 3 times from water.

FIG. 12. Synthetic carbonyl diurea. Compare with Fig. 11.

FIG. 13. Infrared absorption spectrum of thrice recrystallized material from isolated crystalline inclusions of *A. proteus*. 0.299 mg. in 197 mg. of potassium bromide. (Run twice at slightly different gains.)

FIG. 14. Infrared absorption spectrum of synthetic carbonyl diurea. 1.38 mg. in 299 mg. potassium bromide.
Griffin: Identification of ameba crystals