METACHROMATIC GRANULES OF MICROORGANISMS

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Received for publication April 23, 1959

The exact nature and function of the metachromatic (volutin) granules seen in many microorganisms is still a matter of conjecture although polymetaphosphates (polymers of high-energy anhydride-linked phosphates) are a known constituent. The object of this paper is to re-examine volulin as a cytological entity, and to present a working hypothesis on the mechanism of formation of this substance.

The metachromatic granules (volutin) of microorganisms are usually considered to be a reserve source of food; their formation during periods of phosphate uptake has been noted (Lewis, 1941; Nagel, 1948). The pioneer work of Wiame (1946; 1947a, b, c; 1949) on Saccharomyces cerevisiae developed chemical criteria for the recognition of volulin, establishing this substance as a polyphosphate, the stainable chromotropic form (Michaelis, 1947) probably existing in the cell as a polymer of a hexametaphosphate unit. Hexametaphosphate has since been shown to be a mixture of polyphosphates of varying chain lengths (Kornberg et al., 1956). Whether intracellular metaphosphate is free or bound in an organic complex has been a moot question (Ingleman, 1950). Schmidt (1951) pointed out that (a) there was no evidence for binding with proteins or a close association with nucleates and (b) differences in extractability of metaphosphate with trichloroacetic acid could simply reflect diffusion properties of the cell. However, both qualitative and quantitative differences between the acid-soluble and the acid-insoluble (presumably bound) metaphosphate fractions of growing yeast cells have been demonstrated (Juni et al., 1947, 1948; Katchman and Fett, 1955).

There is a variety of opinion on the constitution and location of the volulin granules:

1. Volutin (in yeast) is metaphosphate, occurs in the vacuole, and coats the chromosomes prior to their division (Lindegren and Lindegren, 1946).

2. Metaphosphate (volutin) is transiently associated chiefly with cytoplasmic granules and to a lesser extent with the central vacuole. In young yeast cells, lipid-soluble dyes and metaphosphate accumulated at the same site (Hartman and Liu, 1954).

3. Volutin granules may be ribonucleic acid (RNA) charged with metaphosphate (Minck and Minck, 1949).

4. Volutin granules are chiefly metaphosphate and contain calcium phosphate in combination with RNA; whether they contain lipid or phospholipid is uncertain (König and Winkler, 1948; Winkler, 1953).

5. Volutin is formed during nutrient imbalance and consists mainly of substances such as RNA and protein which are merely coated or permeated with metaphosphate (Smith et al., 1954).

6. Volutin is a nutritionally controlled accessory structure consisting of inorganic phosphate but no RNA (Grula et al., 1954).

7. The metachromatic granules of yeast and bacteria may be mitochondria (containing phospholipid) with accumulated metaphosphate (Mudd, 1953; 1954). Bradfield (1956) believes there is no evidence to support the view that the metachromatic (volutin) granules are mitochondria or are associated with mitochondria.

A variety of functions have likewise been hypothesized for volulin:

1. Volutin is a vacuolar storage compound like starch, glycogen, and fat (Lewis, 1941; Henrici, 1947).

2. It is doubtful that metaphosphates exist only as a reservoir of inorganic phosphate (Winder and Denneny, 1954).

3. Volutin granules may function as donors of energy and phosphates (Wiame, 1949; Winkler, 1953; Winder and Denneny, 1954; Mudd et al., 1958).

4. Polymetaphosphates may regulate enzyme activity, growth, and cell division through their

This study was supported in part by a grant (E-1700) from the National Institutes of Health, U. S. Public Health Service.
ability to complex with cations (Katchman and Fetty, 1955; Nickerson and Schultz, 1957; Trevelyan and Harrison, 1956).

We have observed that volutin-rich cells of Aerobacter aerogenes, possessing an internal source of phosphorus, will (competitively) reverse sodium arsenate (0.05 M) inhibition of glucose fermentation in paraffin-sealed agar tube cultures. Cells without volutin failed to grow in 0.01 M concentration.

In microorganisms which normally accumulate volutin (yeast, Corynebacterium sp., Spirillum sp., Mycobacterium sp.), and in some which do not (such as Aerobacter aerogenes), it is possible to obtain massive intracellular concentrations of volutin by manipulating the nutrient environment. A simple method is to follow a period of inorganic phosphate starvation with inorganic phosphate feeding in the presence of suitable carbon, nitrogen, and sulfur sources, magnesium and potassium ions. Smith et al. (1954) feel that volutin may be an "intermediary metabolite which is formed in the course of phosphate assimilation... and normally used for growth so rapidly that it does not accumulate." Thus, increasing acidity, which limits cell growth, would also tend to increase volutin accumulation.

Metaphosphate is a component of normal yeast and may be formed anaerobically (Wiane, 1949). Inhibitors of glycolysis, such as fluoride or arsenate ions, will prevent metaphosphate formation. Volutin does not appear in Corynebacterium diphtheriae grown under anaerobic conditions but is present under aerobic conditions (Winkler, 1953). The production of metaphosphate from inorganic phosphate is very rapid and is most efficiently linked to oxidative phosphorylation since it has been shown with cell-free systems that an enzyme catalyzing adenosine triphosphate (ATP) → phosphate "primer" = adenosine diphosphate (ADP) + metaphosphate exists in yeast and Escherichia coli (Hoffman-Ostenhof et al., 1954; Kornberg et al., 1956; Kornberg, 1957). Therefore, inhibitors of oxidative phosphorylation (such as dinitrophenol, sodium azide) will also inhibit volutin formation. The identity of the phosphate primer is unknown. A working hypothesis is presented to show that during nutrient imbalance or under anaerobic conditions, 2-phosphoenolpyruvate may function as the precursor and pyrophosphate as the primer in polyphosphate formation.

**MATERIALS AND METHODS**

A stock strain of A. aerogenes (U.N.C. II) when grown on corn meal agar (BBL) produced high concentrations of volutin in 1 to 3 days at 37 C. It would not produce volutin when grown on simple peptone or peptone-phosphate media in the absence of carbohydrate. Volutin was also formed on defined media as follows: the organism was first cultured on a phosphate deficient medium (NH4Cl, 5 g; glucose, 10 g; MgCl2-6H2O, 0.03 g; FeSO4-7H2O, 0.3 g; KCl, 0.5 g; NaCl, 0.5 g; riboflavin, trace; agar, 15 g; water, 1 L; autoclave; final pH 4.5) for 8 to 24 hr at 37 C, then transferred to the same basal medium with 10 g of K2HPO4-3H2O per L added (final pH 7.2). Heavy concentrations of volutin were found in from 2 to 8 hr at 37 C. For comparative purposes, Corynebacterium xerose, 24-hr cultures from Loeffler slants, were examined.

Smears were made on no. 0 cover slips, air dried, flamed, and then subjected to various staining procedures in Columbia dishes. These procedures included the Gram stain, Kinyoun acid-fast stain (with counterstain omitted), periodic acid-Schiff stain, volutin stain (Laybourn, 1924), a stain for free lipid (0.25 per cent Sudan black B in ethylene glycol for 10 min at 60 C), and a stain for "masked" or bound lipid. Bound lipids were demonstrated by placing the air-dried smears in a fresh mixture (1:1) of 5 per cent citric acid and 0.25 per cent Sudan black B in ethylene glycol for 10 min at 60 C then quickly passing them through graded alcohols (70 to 50 to 30 to 10 per cent) to water (Davis et al., 1953). RNA was located by staining with acidified 0.001 per cent toluidine blue (39 per cent dye content) and subsequently noting abolition of staining in control smears pretreated with ribonuclease. Stained smears were run to water and mounted in polyvinyl pyrrolidone (Burstone, 1957) for immediate inspection, Gram-stained smears were blotted, flame, and mounted in Mersol (Arthur H. Thomas Company, Philadelphia).

Extractive and (enzyme) hydrolytic procedures were run as follows: (a) extraction of smears with water: water at 80 C for 10 min, or 60 C for 1 hr, (b) lipid extraction: pyridine or ether-alcohol (1:1) at 60 C for 1 hr, (c) tryptic digestion: 1 per cent trypsin (Difeo, 1:250) in phosphate or Veronal buffer at pH 8 at 37 C for 1 hr, and (d) ribonuclease (Nutritional Biochemicals, Cleveland): 0.2 per cent aqueous at 37 C for 1 hr.
Figures 1–8
Photographs were taken through a 90× (1.40 N.A.) apochromatic objective lens, matching condenser, and a 15× compensating eyepiece on 35 mm Kodak microfilm. This primary magnification of 1350× was increased 3× in printing to give final magnification of 4050 diameters.

RESULTS AND DISCUSSION

Figures 1, 2, and 3 demonstrate the volutin granules stained by Laybourn's method which are found in *A. aerogenes* and *C. xerose*. Figures 1A and 1B show cells comparable to those in figure 1 stained with Sudan black B and citric acid-Sudan, respectively. Table 1 summarizes the tinctorial, extractive, and enzymatic procedures which serve to help characterize these granules.

Most investigators agree that the volutin granules of microorganisms are composed, at least in part, of polyphosphate. The chief area of disagreement lies in the nature of the other components. Therefore, the literature does not define the structure of the volutin granule or how the polyphosphates are related to it.

The cardinal point of the present investigation shows lipids to be a major component of the volutin granule since masked lipids and volutin occur at equivalent sites. Ribonuclease does not remove this lipid. The granule itself is periodic acid-Schiff negative but sometimes positive areas may overlay or lie close to it (figures 4, 5, and 6). Exclusion of RNA or carbohydrate as a binding agent would indicate that the lipid component is loosely bound to (or overlaid with) protein. This lipid is revealed by mild acid or trypsin hydrolysis of the granule; it is extracted by common fat solvents. In yeast, Smedley-Maclean and Hoffert (1923) showed that inorganic phosphate added to aerated sugar solutions would increase fat content markedly.

In agreement with Bringmann (1951; 1952) volutin granules were found to be trypsin digestable. This would indicate that the masking protein contains basic amino acids of the type found in histones.

In *Aerobacter*, volutin granules may be terminal, at one or both poles of the bacillus (figure 3), coinciding with the chief areas of tetrazolium salt reduction (figures 7 and 8), alkaline phosphatase activity (Schaechter et al., 1954), and polysaccharide accumulation (figure 4). Or they may be central or subterminal (figure 1) corresponding to the nuclear loci in predivision or dividing cells. At any rate, the sites of metaphosphate accumulation and tetrazolium salt reduction are not always in one-to-one correspondence. The location and typical appearance of the volutin granules will depend on growth conditions and age of culture.

In agreement with Minek and Minek (1949), the results of the present investigation show RNA to be a component of volutin. Ribonuclease treatment (or acid hydrolysis) abolishes polyphosphate staining as indicated by Laybourn's method but not the staining of bound lipid at the same site. Polyphosphate staining is also abolished by hot water treatment, but RNA staining is not abolished. This is demonstrable with dilute toluidine blue before and after ribonuclease treatment of hot water extracted cells. This indicates that the polyphosphates are RNA linked and the RNA is protein linked since lipid extraction diminishes neither the polyphosphate nor RNA stainability. The nature of the RNA-polyphosphate bond is unknown, but one may speculate that a divalent cation such as magnesium, essential for volutin production, is involved.Trevelyan and Harrison (1956) suggest that metaphosphate formation results in the

All photographs 4050X.

*Figure 1.* Cells (*Aerobacter aerogenes*) grown on corn meal agar 48 hr at 37C; Laybourn stain.

*Figure 1A.* Cells grown as above; Sudan black B stain. Cells are unstained, outlined by adsorbed dye.

*Figure 1B.* Cells grown as above; citric acid-Sudan black B stain. Volutin granules are stained.

*Figure 2.* *Corynebacterium xerose* after 24 hr on Loeffler slant; Laybourn stain.

*Figure 3.* Laybourn stain on *Aerobacter aerogenes* grown 24 hr in inorganic phosphate rich medium after inorganic phosphate starvation.

*Figure 4.* *Aerobacter aerogenes* grown on corn meal agar 48 hr at 37C; periodic acid-Schiff stain.

*Figure 5.* *Corynebacterium xerose* after 24 hr on Loeffler slant; periodic acid-Schiff stain.

*Figure 6.* Periodic acid-Schiff stain on *Aerobacter aerogenes* grown 24 hr in inorganic phosphate rich medium after inorganic phosphate starvation. Cell walls are uniformly stained. Contrast with figure 4.

*Figure 7.* *Aerobacter aerogenes* grown 48 hr on corn meal agar at 37 C; wet mount after 1 hr in 0.005 per cent triphenyltetrazolium chloride showing site of reduction to red formazan.

*Figure 8.* *Aerobacter aerogenes* grown 24 hr in inorganic phosphate rich medium after inorganic phosphate starvation; wet mount as above.
TABLE 1
Staining reactions of volutin granules in Aerobacter aerogenes and Corynebacterium xerose

<table>
<thead>
<tr>
<th>Stain</th>
<th>Volutin Loci in:†</th>
<th>Conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laybourn</td>
<td>+</td>
<td>Polyphosphates present</td>
</tr>
<tr>
<td>Gram</td>
<td>±</td>
<td>—</td>
</tr>
<tr>
<td>Kinyoun</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Sudan black B.</td>
<td>−</td>
<td>—</td>
</tr>
<tr>
<td>Citric-Sudan</td>
<td>+</td>
<td>Masked lipid present</td>
</tr>
<tr>
<td>Periodic acid-Schiff</td>
<td>−</td>
<td>Carbohydrate absent</td>
</tr>
<tr>
<td>Trypsin + Sudan</td>
<td>+</td>
<td>Lipoprotein present; basic amino acids present</td>
</tr>
<tr>
<td>Trypsin + Laybourn</td>
<td>−</td>
<td>Polyphosphates linked to protein (direct or indirect?)</td>
</tr>
<tr>
<td>Hot water + Laybourn</td>
<td>−</td>
<td>Polyphosphate loosely bound (link to protein indirect)</td>
</tr>
<tr>
<td>Ribonuclease + Laybourn</td>
<td>−</td>
<td>RNA† present; polyphosphate linked to RNA</td>
</tr>
<tr>
<td>Lipid extraction + Laybourn</td>
<td>+</td>
<td>Polyphosphates are not lipid based</td>
</tr>
<tr>
<td>Lipid extraction + citric-Sudan</td>
<td>±</td>
<td>Lipid is loosely bound</td>
</tr>
<tr>
<td>Hot water + toluidine blue</td>
<td>+</td>
<td>Acid component other than polyphosphate present</td>
</tr>
<tr>
<td>Hot water + ribonuclease + toluidine blue</td>
<td>−</td>
<td>Polyphosphate is RNA-based</td>
</tr>
</tbody>
</table>

* ± = Variable; progressive reduction of stain.
† RNA = ribonucleic acid.

binding of intracellular magnesium. The volutin granules may be considered to be similar to the protein-magnesium-ribonucleate complex held to be responsible for gram-positive staining. Indeed, the volutin granules in *A. aerogenes* are last to be decolorized by 95 per cent ethanol. If underdifferentiated, it is possible to stain the granules purple on a pink background. Ribonuclease treatment or lipid extraction results in loss of Gram stain retention by the discrete granule. Pearse (1953) believes reactive acid groups and a physical barrier such as a lipid or lipoprotein membrane is responsible for Gram staining.

In confirmation of Smith *et al.* (1954), the volutin granules of *A. aerogenes* were found to be acid-fast. *C. xerose* is not acid-fast, but many organisms in a smear from a 24-hr Loeffler slant culture exhibit volutin granules which, in parallel smears, resist acid alcohol in the acid-fast stain (Neisser, 1888). These granules are citric-Sudan positive and therefore contain bound lipid.

The simultaneous occurrence of lipid and high-energy phosphate in a discrete well-organized granule which apparently arises de novo indicates a common precursor substance. If inorganic phosphate uptake can be shown to operate exclusively through the Embden-Myerhof cycle, then the high energy bonds of metaphosphate must be derived by transfer (ATP) from 1,3-diphosphoglyceric acid or 2-phosphoenolpyruvate. It has been shown that a high inorganic phosphate level inhibits the hexose monophosphate shunt pathway of glucose metabolism but allows glycolysis to proceed via the Embden-Meyerhof scheme (Kravitz and Guarino, 1958). This is a situation obtained when inorganic phosphate-starved cells are transferred to an inorganic phosphate-rich medium. The phosphate is removed from this system by triose (3-phosphoglyceric acid) to form 1,3-diphosphoglyceric acid. This is the only route whereby inorganic phosphate is known to enter the cycle. The phosphate uptake is maintained by the phosphorylation of ADP by 1,3-diphosphoglyceric acid. The resultant ATP at once phosphorylates hexose to continue the cycle. After starvation,
the cellular ADP level may be so low as to hinder ATP formation from 2-phosphoenolpyruvate. This second ADP-ATP cycle must wait not only upon the availability of ADP, but also formation of phosphoenolpyruvate. This delay in operation of the second ADP-ATP cycle allows phosphoenolpyruvate to accumulate while the first cycle continues to operate. A coupled reaction now takes place during which polyphosphate (liberated during lipid synthesis) in the presence of ATP and the enzyme metaphosphate-kinase (Hoffman-Ostenhof et al., 1954), acts as the primer in generating polyphosphate and ADP. With the availability of ADP, balance is restored and both internal ADP-ATP cycles operate. Kornberg et al. (1956) have shown that polyphosphate can act as primer and be incorporated into polyphosphates. It is therefore hypothesized that under anaerobic conditions or during nutrient imbalance 2-phosphoenolpyruvate is the common precursor of the polyphosphate and lipid in the volutin granule.

In conclusion it may be said that the volutin (metachromatic) granules of microorganisms are complex structures consisting of lipoprotein, RNA, polyphosphates, and (probably) magnesium. A composite diagrammatic representation of a volutin granule is shown in figure 9.

SUMMARY

The constitution and postulated functions of the volutin (metachromatic) granules of microorganisms are reviewed. Protein-bound lipid is found in these granules as well as polyphosphates and ribonucleic acid. The coincidence of high-energy polyphosphate and lipids in the same granule indicates a common precursor. It is hypothesized that 2-phosphoenolpyruvate furnishes both high-energy phosphate for metaphosphate formation and, after decarboxylation, the “active acetate” for lipid synthesis in the granule. The phosphoenolpyruvate may accumulate during a time of nutrient imbalance when available adenosine diphosphate is phosphorylated by 1,3-diphosphoglyceric acid only and all available and newly formed adenosine triphosphate is channeled toward esterification of hexose. Addition of adenosine diphosphate would therefore restore balance and inhibit volutin formation.

REFERENCES


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