VOLUTIN PRODUCTION IN AEROBACTER AEROGENES DUE TO NUTRIENT IMBALANCE

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Metachromatically staining granules of volutin, first observed in *Corynebacterium xerosis* and *Corynebacterium diphtheriae*, have since been demonstrated in a wide variety of bacteria, yeasts, fungi, and algae. In the species normally forming volutin, the amount produced is influenced greatly by the conditions of culture. Moreover, there are some species which form the granules only in exceptional circumstances. Thus, *Aerobacter aerogenes* forms no volutin when grown on ordinary nutrient media, but much when grown under acid conditions (pH 4.0 to 5.0) on a poorly buffered medium containing sugar and a small amount of phosphate (Duguid *et al.*, 1954). Volutin production is induced also in *A. aerogenes* by certain conditions of nutrient imbalance not involving development of acidity; this paper describes an investigation of such conditions.

There is evidence that volutin accumulates when cell growth is limited. Pesch (1924) found that volutin production in *C. diphtheriae* on glucose blood agar was increased when the amount of growth was decreased by reduction of the blood content. Winkler (1953) noted that the granules of corynebacteria and mycobacteria were small in the early stages of growth and enlarged greatly in the later stages. Stich (1953) observed that *Acetabularia mediterranea* formed large cytoplasmic granules of volutin ("metaphosphate") when its growth was terminated naturally or artificially. Similarly, in the present study, *A. aerogenes* was found to form volutin when its growth was halted by exhaustion of the nitrogen or sulfur source.

The restoration of phosphate supply following phosphate starvation constitutes another form of nutrient imbalance inducing production of volutin. Jeener and Brachet (1943) found that yeast cells grown on a phosphate deficient medium lost most of their normal basophilia; when transferred to a fresh medium containing phosphate, such cells synthesized much basophilic substance within a short time. Wiame (1946*a*) showed that the basophilic substance formed in the subcultures of phosphate starved yeast, unlike that in normal yeast, gave the metachromatic reaction with toluidine blue which is characteristic of volutin. Duguid (1948) found that volutin formation was induced similarly in *A. aerogenes*, granules appearing abundantly in the first hour after transfer of the phosphate starved bacilli onto nutrient agar. The present paper reports a further study of this mode of volutin formation.

Wiame (1946*a*, *b*; 1947*a*, *b*) obtained convincing evidence that the volutin ("metachromatic substance") of yeasts is a polymerized inorganic metaphosphate. Duguid *et al.* (1954) obtained similar findings for the volutin of *A. aerogenes* grown in acid conditions. Synthesis of volutin requires adequate provision of certain nutrient factors, including favorable sources of energy and phosphorus (Elser and Huntoon, 1909; Zikes, 1922; Duguid *et al.*, 1954). The requirement of potassium ions for uptake of phosphate prior to metaphosphate formation by bakers' yeast was demonstrated by Schmidt *et al.* (1949).

MATERIALS AND METHODS

The observations were made with a capsulate, nonmotile strain (A3) of *A. aerogenes* which had the following properties: methyl-red negative, producing acetylmethylcarbinol, utilizing citrate, not producing indole, not liquefying gelatin, and fermenting glucose, lactose, sucrose, mannitol, inositol, and glycerol with production of acid and gas.

Surface cultures were grown on 50 ml amounts of agar medium in 9 cm petri dishes. A "complete" medium was used to give growths limited by exhaustion of the carbon and energy source (glucose); the cells so grown are termed "carbon-deficient." This medium contained per 100 ml distilled water: glucose, 0.2 g; phosphate (mixture of 3 parts by weight Na_2HPO_4 and 1 part NaH_2PO_4; pH = 7.3), 1.0 g; NH_4Cl, 0.1 g; NaCl, 0.2 g; Na_2SO_4, 0.01 g; KCl, 0.01 g; $MgCl_2$,

0.001 g; $CaCl_2$, 0.001 g; $FeCl_3$, 0.0001 g; washed agar fiber, 2.0 g. For growths limited by exhaustion of other nutrient factors, similar media were used with a reduced content of phosphate (sole phosphorus source), NH_4Cl (sole nitrogen source), Na_2SO_4 (sole sulfur source), or KCl (sole potassium source). For extremely deficient cultures no addition of the nutrient compound was made, the deficient nutrient being provided by traces of contamination substances in the agar, which were estimated according to the amount of growth supported. The phosphate content of 1.0 g per 100 ml effectively buffered the pH of the culture, preventing fall below 6.5. When the phosphate content was smaller, use was made of a bicarbonate buffer (pH 7.4); 0.3 per cent (w/v) $NaHCO_3$ was included in the medium and 20 per cent (v/v) CO_2 added to the air of a sealed 8 L tank in which the culture plate was incubated with lid ajar. The agar plate was inoculated uniformly over its surface with two loopfuls of a light suspension in saline of a 24 hr culture on nutrient agar. The plates were incubated at 35 C with the lids raised 2 mm to ensure free aeration.

Estimation of amount of volutin. A loopful of culture was mixed into a drop of 5 per cent formalin to give a dense suspension; this was smeared on a slide and dried. If growth was slight, a piece of agar bearing the culture was smeared directly on the slide. The smears were fixed by flaming and stained by Albert's method as modified by Laybourn (1924). The amount of volutin was assessed according to the number and size of purple-black granules present and recorded on an arbitrarily standardized scale: − for none, ± for small granules in a few cells, + for small granules in most cells, ++ and +++ for medium sized and large granules in most cells.

Estimation of pH value of culture. A small block of agar was cut from the culture plate and placed for two hours in a little distilled water. The pH of the water then was estimated with indicator dyes.

The *amount of growth* was measured in terms of the total nitrogen content of the cells. These were removed from the agar, suspended in saline, and washed twice by centrifugation and resuspension in fresh saline. Their nitrogen content was measured by a micro-Kjeldahl digestion and colorimetric estimation of the ammonia produced, using the Spekker absorptiometer. The *phosphorus content* of the washed cells was measured by digestion with concentrated H_2SO_4 and HNO_3, and colorimetric estimation of the resulting inorganic orthophosphate, by the method of Fiske and Subbarow (1925).

RESULTS

Volutin formation in growths limited by different deficiencies. Table 1(a) shows the observations made during 96 hours' growth on five media deficient in their carbon, phosphorus, nitrogen, sulfur, and potassium sources, respectively. Growth was almost complete after the first 24 hours' incubation since the nitrogen contents were little greater after 48 and 96 hours. As compared with the carbon-source limited culture on the "complete" medium (i), the growths on media (ii) to (v) were only about a tenth as great due to their respective nutrient deficiencies. In each kind of culture the majority of cells was still viable after 48 hours' growth; viable counts made by the pour-plate method comprised 90 to 100 per cent of total counts made by use of a Thoma-type counting chamber and phase-contrast microscope. For all media the pH value after growth was between 6.5 and 7.3 so that any production of volutin could not be due to acid conditions.

Volutin was not produced at any stage by the carbon, phosphorus, or potassium limited cultures, but a moderate amount was produced by the nitrogen limited and sulfur limited cultures. Most cells in the latter formed a single small granule as growth was ending at 18 to 24 hours, and these granules persisted for one or two days (figure 1). Volutin production was not correlated with polysaccharide production; the phosphorus, nitrogen, and sulfur limited cultures were highly mucoid, while the carbon and potassium limited cultures were nonmucoid (c.f., Duguid and Wilkinson, 1953).

Volutin formation in young subcultures from growths limited by different deficiencies. The 48 hour cultures limited by the different nutrient deficiencies (table 1(a), i–v) were subcultivated on a fresh medium with the same composition as the "complete" medium (i). Thick streaks of the culture were inoculated on the surface of this second medium. Table 1(b) shows the amounts of volutin found after different periods of incubation at 35 C. No volutin was produced in the subcultures made from the carbon limited and potassium limited cultures. Also, there was no further production of volutin in the subcultures

TABLE 1

(a) *Amount of volutin formed during growth at 35 C on media deficient in different nutrient factors*

NUTRIENT CONTENT OF MEDIUM: G PER 100 ML AGAR BASE†					
Medium no.	(i)	(ii)	(iii)	(iv)	(v)
Glucose	0.2	0.2	0.2	0.2	0.2
Phosphate	1.0	0.0003	1.0	1.0	1.0
Ammonium chloride	0.1	0.1	0.003	0.1	0.1
Sodium sulfate	0.01	0.01	0.01	0.0001*	0.01
Potassium chloride	0.01	0.01	0.01	0.01	0.0001*
Growth limiting deficiency	carbon, energy	phosphorus	nitrogen	sulfur	potassium
GROWTH TIME					
hours					
0	−	−	−	−	−
6	−	−	−	−	−
12	−	−	−	−	−
18	−	−	⊥	⊥	−
24	−	−	+	+	−
48	−	−	+	⊥	−
96	−	−	⊥	−	−
Amount of growth at 48 hr, mg N	3.9	0.41	0.38	0.46	0.30

(b) *Amount of volutin present in subcultures on fresh medium (i) of cells from the above 48 hr growths*

SUBCULTURE TIME					
min					
0	−	−	+	⊥	−
5	−	+	+	⊥	−
15	−	++	+	⊥	−
60	−	+++	+	⊥	−
120	−	+++	+	−	−
240	−	++	−	−	−
480	−	⊥	−	−	−

* No Na_2SO_4 was added to (iv) or KCl to (v); the table shows the amount of these salts calculated equivalent to the contamination sulfur and potassium compounds in the medium.

† Other salts in medium as listed in text.

Amount of volutin: − nil, ⊥ trace, +, ++, and +++ increasing amounts.

made from the nitrogen limited and sulfur limited cultures; the volutin already present in these remained apparent during the first one to two hours of subculture and then disappeared in the progress of growth. In contrast, when the volutin-free cells of the phosphorus limited culture were subcultivated on the second medium, they rapidly produced a very large amount of volutin; granules appeared within a few minutes after transfer and increased in size and number so that in one to two hours almost every cell contained one or more large granules (figures 3 and 5). This volutin disappeared again in the course of further cell growth between 2 and 8 hours. When the subcultures were made on agar containing peptone and meat extract, instead of on the synthetic medium, the results were similar except that the volutin disappeared much more rapidly.

The conditions of volutin formation in subcultures from phosphate starved growths. Volutin was formed in subculture whenever the phosphate

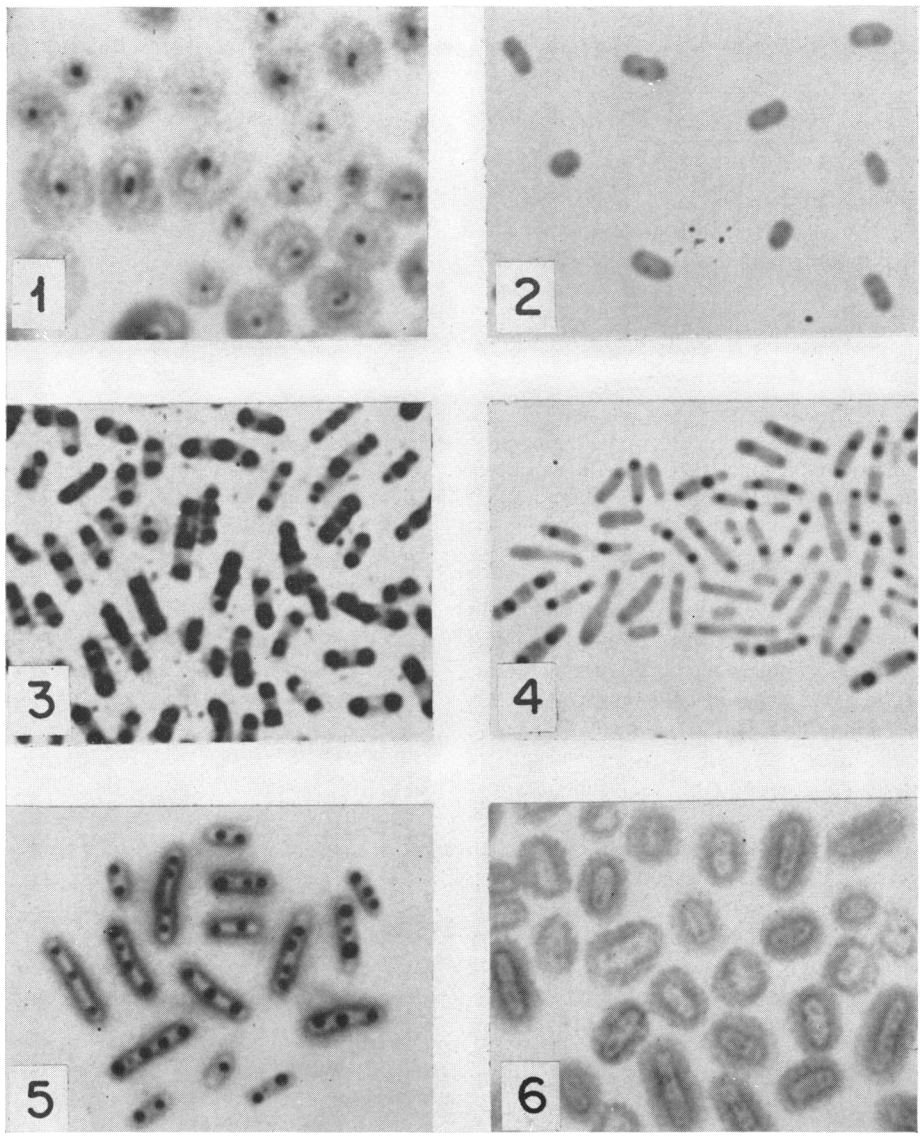

PLATE I

Aerobacter aerogenes, strain A3, by light microscope. × 3,000.

Figure 1. Grown for 48 hr on nitrogen deficient medium (table 1a, iii). Albert stain. One or two dark volutin granules in each cell. Capsules lightly stained.

Figure 2. Similar culture to no. 1, in unstained wet film by dark phase contrast. Single dark granule laterally in each cell.

Figure 3. Subculture for two hr on "complete" medium of cells from a phosphate starved growth (table 1b, ii). Albert stain. Several large dark volutin granules in each cell.

Figure 4. Similar subculture to no. 3, in unstained wet film by dark phase-contrast. Dark granules in some cells only.

Figures 5 and 6. Subcultures for two hr of phosphate starved cells in a "complete" medium (no. 5, showing volutin granules) and in a medium lacking magnesium (no. 6, showing no granules). See table 3, experiment 6.

TABLE 2

Amount of volutin formed by cells from growths on media with different phosphate contents when subcultivated on a phosphate-rich second medium

Phosphate in first medium, g per 100 ml		0.00001*	0.0001	0.0003	0.001	0.003	0.01	1.0
Amount of growth on first medium, mg N		0.01	0.15	0.42	0.96	2.6	4.3	4.5
Amount of volutin in subculture on second medium after different periods at 35 C	0 min	−	−	−	−	−	−	−
	3 min	+	+	+	⊥	−	−	−
	15 min	++	++	++	+	⊥	−	−
	1 hr	+++	+++	+++	+++	+	−	−
	2 hr	+++	+++	+++	+++	+	−	−
	4 hr	++	++	++	+	⊥	−	−
	8 hr	⊥	⊥	⊥	−	−	−	−
	24 hr	−	−	−	−	−	−	−

First medium: 0.2 g glucose, various amounts of phosphate and 0.1 g NH_4Cl per 100 ml agar base (bicarbonate buffer and other contents as in text). No phosphate was added to medium marked (*): the calculated amount of contamination phosphate is shown. Cultures grown for 48 hr at 35 C.

Second medium: 0.2 g glucose, 1.0 g phosphate, and 0.1 g NH_4Cl per 100 ml agar base (other contents as listed in text).

supply of the parent culture was small enough to limit the amount of growth to less than that supported by a similar medium with an excess of phosphate. An experiment demonstrating this is recorded in table 2. Cultures grown for 48 hours on media with different phosphate contents (0.00001 to 1.0 g per 100 ml) were subcultivated on a second medium with much phosphate (1.0 g per 100 ml). The final pH values were in all cases between 6.7 and 7.1. The parent growths on the media with 0.01 and 1.0 g phosphate per 100 ml were of the maximal amount supportable by the glucose supply and so were not phosphate starved; their subcultures did not produce volutin. The parent growths made with supplies of phosphate smaller than 0.01 g per 100 ml were decreased about proportionately in amount, showing that they were limited by phosphate starvation; the subcultures from these growths produced volutin abundantly.

Dependence of volutin formation on the presence of certain nutrient factors in the subculture medium was demonstrated in the experiments recorded in table 3. The subcultures were grown on square pieces of cellophane immersed in a liquid second medium. The inoculum of phosphate starved cells was grown for 48 hours on four cellophane squares (3 by 3 cm) covering the surface of 50 ml agar first medium containing 0.0003 g phosphate per 100 ml. On removal from the exhausted first medium, the cellophane squares and adhering culture were washed free of residual nutrients by two 7 minute immersions in large volumes of saline (0.85 g NaCl per 100 ml distilled water); most of the culture remained adherent to the cellophane. A single square bearing the growth from about 12.5 ml first medium then was immersed in 12.5 ml second medium in a 6 cm petri dish. After incubation for two hours at 35 C, the subculture was examined

TABLE 3

Amount of volutin formed by cells from a phosphate starved growth when subcultivated for 2 hours at 35 C on second media of varied nutrient content

AMOUNT OF VARIED NUTRIENT	SINGLE NUTRIENT VARIED IN SECOND MEDIUM					
	Expt 1 glucose	Expt 2 phosphate	Expt 3 NH_4Cl	Expt 4 Na_2SO_4	Expt 5 KCl	Expt 6 $MgCl_2$
g per 100 ml						
0.1	+++	+++	+++	+++	+++	+++
0.01	⊥	+++	+++	+++	+++	+++
0.001	−	⊥	++	+++	+	+++
0.0001	−	−	+	+++	⊥	⊥
0	−	−	+	+++	−	−

First medium: 0.2 g glucose, 0.0003 g phosphate, and 0.1 g NH_4Cl per 100 ml agar base (bicarbonate buffer and other contents as in text). Culture grown for 48 hours at 35 C.

Second medium: 0.2 g glucose, 1.0 g phosphate, 0.1 g NH_4Cl, 0.2 g NaCl, 0.01 g Na_2SO_4, 0.01 g KCl, 0.001 g $MgCl_2$, 0.001 g $CaCl_2$, and 0.0001 g $FeCl_3$ per 100 ml distilled water. The amount of one of these nutrients was varied in each experiment (1 to 6).

in stained smears. The basic composition of the second medium was as follows: glucose, 0.2 g; phosphate, 1.0 g; NH_4Cl, 0.1 g; NaCl, 0.2 g; Na_2SO_4, 0.01 g; KCl, 0.01 g; $MgCl_2$, 0.001 g; $CaCl_2$, 0.001 g; $FeCl_3$, 0.0001 g; distilled water, 100 ml. In each experiment one of these nutrients was varied in amount and the others kept unchanged. The media with less than 1.0 g phosphate per 100 ml were buffered by inclusion of 0.03 per cent (w/v) $NaHCO_3$ in the medium and 2.5 per cent (v/v) CO_2 in the atmosphere of incubation. The pH values of the two hour subcultures were in all cases between 6.7 and 7.2. These experiments showed that volutin formation in subcultures of phosphate starved cells was prevented entirely by absence from the subculture medium of glucose, phosphate, potassium ions, or magnesium ions (figure 6); it was reduced much by the absence of the nitrogen source but undiminished by absence of the sulfur source. The findings of Schmidt *et al.* (1949) with bakers' yeast suggested that magnesium ions might be required merely for their action in enabling selective uptake of potassium ions from medium with an excess of sodium ions. However, we found that absence of magnesium still prevented volutin formation when potassium salts were substituted for all the sodium salts in the subculture medium. The amounts of glucose, phosphate, potassium, and magnesium required in the subculture medium for full volutin production by the subcultured cells were much more ($\times 3$ to 30) than the amounts required for the original growth of the same cells. Amounts just insufficient to support volutin formation were still adequate to support some further degree of cell growth. The volutin forming subcultures lost their volutin again between the second and eighth hours, except those deficient in nitrogen or sulfur wherein the volutin continued to increase.

Volutin production also occurred in subcultures on media in which the glucose was replaced by sucrose, pyruvate, or lactate as the carbon source, and on media buffered at different pH values from 4.5 to 8.5. Volutin formation in subcultures with glucose was inhibited by 0.0002 M dinitrophenol, a concentration at which glucose assimilation and polysaccharide synthesis were halted, but the rate of oxygen consumption undiminished. Similar results were obtained with 0.02 M sodium azide. These findings accord with observations in yeasts by Wiame (1947*a*) and Yoshida and Yamataka (1953).

Cytological observations. In Albert stained smears the volutin containing cells showed their granules stained purple-black, their protoplasm light green, and their capsules violet-brown (figures 1, 3, and 5). Volutin-free cells showed the green protoplasm and brown capsules but no black granules (figure 6). The granules ranged in diameter from the smallest resolvable about 0.2 μ to the largest about 0.9 μ; the bacilli were mostly from 0.5 to 0.8 μ in width. In the nitrogen deficient and sulfur deficient cultures, most cells contained a single small granule (0.2 to 0.4 μ) situated centrally or laterally near the cell equator, and some contained two granules (figure 1). In the two hour subcultures of phosphate starved growths, most cells contained two to four large granules (0.4 to 0.9 μ), two of which commonly occupied terminal or subterminal positions (figures 3 and 5). Corresponding granules, staining dark purple, were demonstrated clearly in wet films with the toluidine blue-formalin-acetic acid stain of Lindegren (1948). In dried smears stained with methylene blue or toluidine blue, the granules were darker than the rest of the cell; subsequently, they proved weakly acid-fast, resisting destaining by one per cent sulfuric acid for one minute. The granules did not stain as fat or as polysaccharide.

Volutin containing cultures examined in unstained films by the phase-contrast and electron microscopes exhibited dense granules corresponding generally in size and situation with the volutin granules seen in stained smears. No granules were thus demonstrable in any of the volutin-free cultures. In wet films by phase-contrast, circular granules were seen which were much denser than the rest of the cell although the granules in some of the cells were not thus apparent (figures 2 and 4). Those newly formed in 10 to 30 minute subcultures of phosphate starved cells were generally indistinguishable by phase-contrast although clearly stained by the Albert and Lindegren methods. Dried films, not shadowcast, were examined by the electron microscope (Metropolitan-Vickers, EM3) at 75 kv. Granules ranging from 0.03 to 0.6 μ in diameter were seen. They appeared much more opaque (electron-scattering) than the rest of the cell, were mostly circular or ovoid in shape, and generally possessed sharply defined, smoothly contoured margins (figures 7 to 13). Occasionally, the site, which would show a single large granule by the light microscope, was seen by the electron microscope

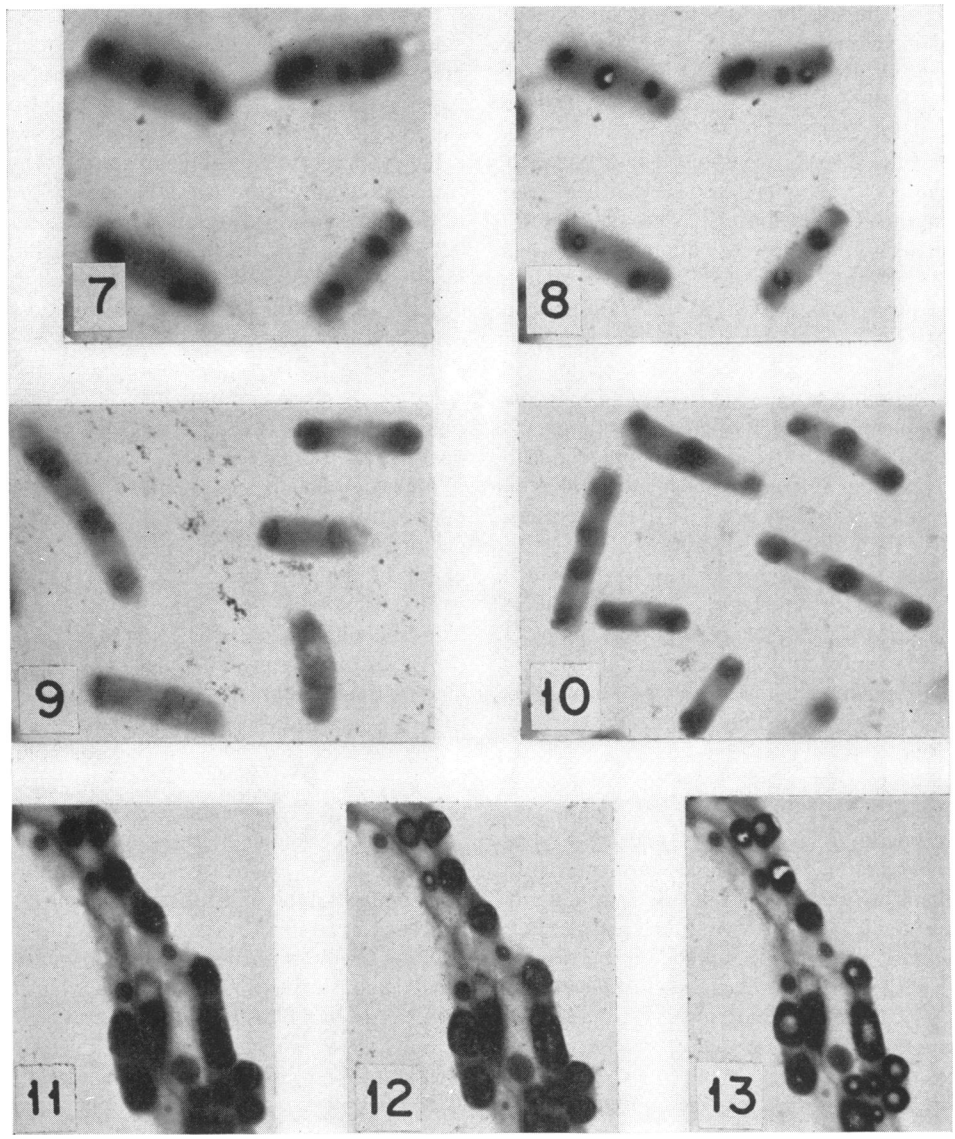

PLATE II

Aerobacter aerogenes with volutin granules by electron microscope. × 9,000

Figures 7 and 8. Subculture for 15 min on "complete" medium of cells from a phosphate starved growth of strain A3 (table 1b, ii). No. 7 before, and no. 8 after exposure to maximal beam. Small dark granules present; some in no. 8 are partially disintegrated.

Figures 9 and 10. Similar subcultures to no. 7, after incubation for 60 and 120 min, respectively. Not exposed to maximal beam. Large dark granules in all cells.

Figures 11, 12, and 13. Subcultures for 120 min in "complete" medium of cells from a phosphate starved growth of strain A3(0) (table 5c). No. 11, before exposure to maximal beam, shows uniformly dense granules. Nos. 12 and 13, of same field after short and long exposures to maximal beam, show partial disintegration of granules.

to contain a cluster of several small granules. Normally the granules were almost uniformly electron-scattering from center to periphery (figures 7, 10, and 11). If, however, the electron beam was increased suddenly to maximal intensity, many of the granules at once underwent a partial disintegration like that observed in *C. diphtheriae* by Winkler and Kčnig (1948); each developed several small or one large clear area, or the former changed to the latter (figures 8, 12, and 13). At the same time the rest of the cell substance became less dense so that the granules were seen in clearer contrast. The dense electron-scattering matter left in the granules was not dispersed on continued exposure to the maximal electron beam.

Albert stained smears were compared with parallel smears stained for nuclear bodies by the method of Robinow (1944). The volutin granules did not seem to correspond with the nuclear bodies; generally they were more numerous and differently situated, often lying asymmetrically to the side of the cell. Wet preparations stained with triphenyltetrazolium, neotetrazolium, and Janus green B were examined in order to determine whether the volutin granules corresponded with the mitochondria-like bodies observed in a variety of bacteria by Mudd *et al.* (1951*a*, *b*). These reagents in concentrations of 0.005 to 0.25 per cent were incorporated in the culture medium or were dispensed in a solution with glucose and phosphate in which the volutin containing cells were incubated for an hour. Irregular results were obtained. Usually only a minority of the cells showed stained granules, and these did not correspond in number, size, or situation with the volutin granules. The tetrazolium stained granules in some cases appeared to lie on the outside of the cell surface.

Volutin formation in other strains and species. A variety of strains was tested for volutin production under two conditions: (1) in nitrogen limited cultures grown for 48 hours on medium containing 0.003 g NH_4Cl per 100 ml, and (2) in young subcultures on "complete" medium of phosphate starved cells from 48 hour growths on medium containing 0.0003 g phosphate per 100 ml. Twelve further strains of *A. aerogenes* were examined. In the nitrogen limited cultures, 8 of these formed small amounts of volutin. In the subcultures of phosphate starved cells, all formed volutin; 11 mucoid strains formed large amounts, and a rough colony strain, a small amount. Volutin also was formed, generally in small amount, by some strains of *Serratia marcescens*, *Aerobacter cloacae*, and *Escherichia coli*. In the nitrogen limited cultures it was formed by 6 of 10 *A. cloacae* strains, 6 of 14 *E. coli* strains, and in the subcultures of phosphate starved growths by 8 and 7 of these strains, respectively.

Analysis of phosphorus compounds in volutin containing and volutin-free cultures limited by different deficiencies. Since the volutin of *A. aerogenes* grown in acid conditions had been identified as a metaphosphate, fractionations were now made of the phosphorus compounds of the cultures grown in conditions of nutrient imbalance. Strain A3(0) was used (Wilkinson *et al.*, 1954, *in press*), this nonmucoid, smooth colony variant of strain A3 formed volutin in the same way as its parent strain but was more convenient for the fractionation procedure due to its lack of extracellular polysaccharide. To supply sufficient quantities of cells, liquid cultures were grown for 24 hours at 37 C in 5 L flasks aerated after the sixth hour. The "complete" medium used for growing carbon deficient cells contained per 100 ml distilled water: glucose, 0.5 g; Na_2HPO_4, 0.9 g; KH_2PO_4, 0.3 g; NH_4Cl, 0.5 g; NaCl, 0.1 g; K_2SO_4, 0.1 g; $MgCl_2$, 0.03 g; $CaCl_2$, 0.001 g; $FeCl_3$, 0.0001 g; glutamic acid, 0.002 g. For growing cells deficient in other factors, the following modifications of this medium were used: (1) *nitrogen deficient*, the NH_4Cl content was reduced to 0.005 g per 100 ml; (2) *sulfur deficient*, no K_2SO_4 was added; (3) *potassium deficient*, sodium salts were substituted for the potassium salts and 0.0001 g KCl added per 100 ml; (4) *phosphorus deficient*, the only phosphate added was 0.0002 g KH_2PO_4 per 100 ml, and the medium was buffered by addition of 1.0 g citric acid and 0.58 g NaOH per 100 ml with adjustment of pH to 6.6 (the acid formed from the glucose was balanced by base formed in metabolism of the citrate). The pH value after growth was in all cases between 6.4 and 7.2. Part of the culture was taken for estimation of the total cell nitrogen, and the remainder was fixed by addition of formalin to give a concentration of 0.1 per cent formaldehyde. The distribution of phosphorus compounds was not altered by the treatment with formalin, and similar results were obtained without it, both for phosphorus deficient cultures

TABLE 4

Phosphorus content of fractions of cultures limited by different nutrient deficiencies

Strain A3(0) grown for 24 hours at 37 C in aerated liquid media.

FRACTION AND ITS PROBABLE NATURE	μG P IN FRACTION PER MG N IN UNFRACTIONATED CELLS				
	Growth limiting deficiency:				
	Carbon, energy	Phosphorus	Nitrogen	Sulfur	Potassium
Trichloracetic acid soluble fractions					
S2 Organic, including free nucleotide	13.3	11.7	60.2	57.2	23.5
S3 Inorganic orthophosphate	5.9	1.6	13.2	10.1	8.2
R3 Inorganic metaphosphate	0.3	0.3	1.2	0.8	0.2
Trichloracetic acid insoluble fractions					
S4 Phospholipoid	12.6	6.1	17.1	24.8	16.1
R6 Desoxyribonucleic acid	20.4	11.7	23.4	28.5	20.3
S7 Ribonucleic acid	75.2	35.6	150.5	214.7	99.0
R7 Pyrophosphate and phosphoprotein	0.9	0.3	3.4	5.5	0.8
R8 Unknown	5.8	2.0	4.8	9.7	2.3
S9 Unknown	6.4	2.2	10.9	13.4	6.6
R9 Inorganic metaphosphate	0.1	0.1	8.5	27.3	0.1
μg total P per mg total N*	156	80	340	420	184
Volutin staining	−	−	+	++	−

* 86 to 96 per cent of the total phosphorus was recovered in the fractions.

and for carbon deficient cultures. The cells were deposited in a Sharples centrifuge, thrice washed with saline using an angle centrifuge, and suspended in 20 ml distilled water. The ratio of the total phosphorus content to the total nitrogen content (P/N ratio) and the volutin staining were estimated on 0.5 ml of the suspension. Trichloracetic acid (TCA) was added to the remainder, giving a concentration of 5 per cent, and the cells were fractionated by the method of Juni *et al.* (1948) as used by Duguid *et al.* (1954).

Table 4 shows the fractionation results for the cultures limited by the different nutrient deficiencies. The distribution of phosphorus compounds was generally similar in the three kinds of volutin-free cultures: the carbon, phosphorus, and potassium deficient. As might be expected, the P/N ratio of the phosphorus deficient cells was exceptionally low, being only about half that of the carbon or potassium deficient cells. In all three cultures the content of trichloracetic acid insoluble metaphosphate (R9) was negligible, and there was no appreciable barium precipitate at pH 3.5; the stated value in the table of 0.1 μg P per mg N is a maximum. The volutin containing cells grown in the nitrogen deficient and sulfur deficient media had a P/N ratio much increased above that of the volutin-free carbon deficient cells. Presumably this was due to protein formation being limited by lack of nitrogen and sulfur containing amino acids. Corresponding to the increase in the P/N ratio, there was an increase in the amount of phosphorus in all fractions. However, the increase did not exceed 6-fold in any fraction except the trichloracetic acid insoluble metaphosphate fraction (R9); this was increased over 80-fold in the nitrogen limited culture and 250-fold in the sulfur limited.

Analysis of phosphorus compounds in volutin forming subcultures of phosphate starved cells. Fractionations were made on young subcultures in a "complete" medium of cells from a phosphate starved growth. Two methods were employed: (a) Phosphorus deficient cells were grown for 24 hours at 37 C in 5 L aerated liquid medium as described in the previous section. The culture was cooled below 5 C in an ice bath. After centrifugation and washing twice in cold saline, the cells were suspended in 200 ml of the "complete" medium and incubated with aeration at 37 C for various times as shown in table 5. The suspension then was cooled in a freezing mixture and centrifuged. The deposited cells were washed

TABLE 5

Phosphorus content of different fractions of cells from carbon limited and phosphorus limited growths after subculture in a sufficient medium

Strain A3(0) in aerated liquid media at 37 C. (a) and (c) formalin fixed before fractionation.

SUB-CULTURE TIME	μG P IN FRACTION PER MG N IN UNFRACTIONATED CELLS										μG TOTAL P PER MG TOTAL N	VOLUTIN STAINING
	S2	S3	R3	S4	R6	S7	R7	R8	S9	R9		
(a) Carbon limited growth subcultured by transfer of washed cells to fresh medium												
min												
0	13.3	5.9	0.3	12.6	20.4	75.2	0.9	5.8	6.4	0.1	156	−
30	12.2	7.6	0.7	15.3	20.2	100.1	1.2	5.8	10.4	0.2	201	−
60	14.9	6.4	0.4	17.3	16.7	108.6	1.0	6.4	14.4	0.3	208	−
240	16.0	5.0	0.5	11.9	20.0	79.1	1.0	4.8	11.7	0.1	173	−
(b) Phosphorus limited growth subcultured by transfer of washed cells to fresh medium												
0	11.7	1.9	0.2	4.7	8.5	36.0	0.3	2.0	2.8	0.1	81	−
15	17.5	4.4	0.6	9.1	10.9	57.5	0.7	2.6	2.6	1.3	130	+
30	22.7	3.6	1.7	11.2	14.1	86.4	0.9	3.2	4.9	15.2	180	++
60	25.5	9.4	0.8	13.1	10.8	75.3	0.7	3.6	4.1	6.5	170	+
180	28.4	8.0	1.4	15.4	19.4	77.2	0.6	4.5	5.6	0.6	200	⊥
(c) Phosphorus limited growth subcultured by addition of glucose and phosphate to old culture												
0	11.7	1.6	0.3	6.1	11.7	35.6	0.3	2.0	2.2	0.1	80	−
10	14.7	4.1	0.9	9.3	12.5	61.4	0.6	2.4	2.5	0.9	122	+
20	18.6	10.2	1.0	12.8	17.4	111.2	1.6	5.4	5.6	3.4	203	+
30	31.2	6.3	1.5	12.6	20.7	180.9	3.3	5.8	9.6	9.1	308	++
60	48.1	21.6	1.0	15.0	25.4	298.0	4.2	7.7	7.0	24.4	481	+++
120	37.6	16.5	1.2	17.0	24.8	271.2	2.3	8.3	13.3	17.5	461	+++
180	39.5	6.2	0.8	22.8	27.4	203.3	2.3	10.5	12.0	8.9	387	++
240	25.4	8.8	0.6	16.4	22.9	141.2	1.8	7.3	12.5	2.1	260	+
360	15.9	4.1	0.5	12.7	22.2	92.5	1.2	4.4	9.9	0.5	173	⊥

twice with cold saline and finally suspended in 20 ml cold distilled water for fractionation and analysis as described in the previous section. For a control, carbon deficient cells were grown and treated similarly, except that they were subcultured in one L of "complete" medium and were fixed with formalin after incubation. The results are shown in table 5. The carbon deficient cells did not form volutin on subculture and showed only a small increase in the P/N ratio. The latter was due mainly to the increase of ribonucleic acid (S7) which is normal in the early stages of growth. The trichloracetic acid insoluble metaphosphate (R9) increased very slightly but never reached a value as high as in cells with volutin. In contrast, the phosphorus deficient cells formed volutin in increasing amount during the first 30 minutes of subculture and showed an increase of the P/N ratio up to the value of the control cells. The formation of volutin was paralleled by a very large increase (over 150-fold) in the trichloracetic acid insoluble metaphosphate (R9). The 30 minute subculture was of special interest since it contained much volutin yet had a P/N ratio and ribonucleic acid content (S7) slightly less than in the corresponding volutin-free control cells. Thus, the production of volutin was not related to a general increase of the phosphorus in all fractions, but specifically to the large increase in the R9 fraction.

(b) In the aforesaid method of subculture by washing and transfer of cells to a second medium, it was difficult to avoid some contamination with phosphate in the initial centrifugation and thus premature synthesis of volutin. Moreover, the volutin production was to some extent impaired, probably due to metallic contamination from the

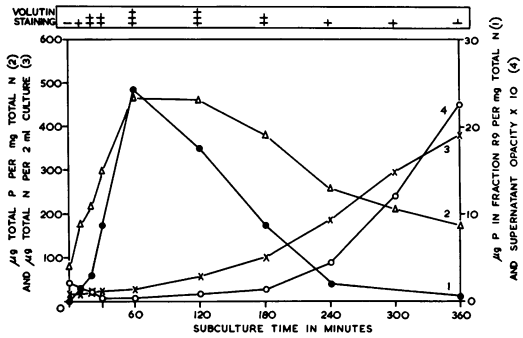

Figure 14. Total phosphorus and nitrogen contents, R9 metaphosphate content, supernatant opacity and volutin staining in a subculture from a phosphate starved growth of strain A3(0).

Sharples centrifuge. Therefore a second method of subculture was tested. A phosphorus limited culture was grown for 24 hours at 37 C in 5 L aerated liquid medium. After growth, 10 g glucose, 9 g Na_2HPO_4, and 3 g KH_2PO_4 were added to make the medium equivalent to a fresh "complete" medium. The "subculture" thus started was incubated at 37 C with aeration for various times up to 6 hours. The cells were fixed then by addition of formalin, centrifuged, washed, and examined as described previously. The results are shown in table 5(c) and figure 14. Volutin production was abundant and unimpaired, the maximal amount being formed in the first 60 minutes and most disappearing again between 120 and 360 minutes. Changes closely paralleled with these were shown by the P/N ratio and by the R9 fraction (increasing, respectively, 6-fold and 250-fold in the first hour). A sufficient amount of fraction R9 could not be obtained for its complete characterization. However, reasonable evidence for its identity with some form of polymerized metaphosphate was provided by the observations that it was precipitable by barium at pH 3.5, that it was 96 per cent hydrolyzable by N HCl at 100 C in 7 minutes, and that it gave a metachromatic reaction when mixed in the correct proportions with toluidine blue. Fraction R3, which in yeasts is believed to be trichloracetic acid soluble metaphosphate, did not occur in substantial amounts in the *A. aerogenes* cultures, either volutin containing or volutin-free. During the period of volutin formation, the bacilli became spontaneously agglutinable. The number remaining inagglutinable was measured by an opacity reading made with the Spekker absorptiometer on the supernatant of the subculture after centrifugation for 5 minutes at 500 rpm to remove the agglutinated cells. This reading fell to a low value after the first 30 minutes of subculture, indicating almost complete agglutination, and rose again as agglutinability was lost during subsequent growth (figure 14). Corresponding subcultures prevented from forming volutin by azide or dinitrophenol also became agglutinable during this period.

DISCUSSION

The formation of volutin granules in *A. aerogenes* under conditions of nutrient imbalance was physiological in that it was not accompanied by cell death or impaired power of growth on subculture. Our observations suggest that volutin is a phosphorus compound requiring for its synthesis a source of energy and a supply of phosphate, potassium ions, and magnesium ions. Thus, while volutin was formed in aging cultures limited by exhaustion of their nitrogen or sulfur source, it was not formed in those limited by exhaustion of their carbon, phosphorus, or potassium source. Furthermore, volutin was produced in subcultures of phosphate starved cells only if sufficient glucose, phosphate, potassium ions, and magnesium ions were present in the medium. Production was inhibited by azide and dinitrophenol which probably act, as in the inhibition of glucose assimilation, by interfering with the synthesis of energy-rich phosphate bonds. Volutin appears to be an intermediary metabolite which is formed in the course of phosphorus assimilation, possibly as an energy-storage product, and is normally utilized for cell growth so rapidly that it does not accumulate. Accumulation of volutin in demonstrable amounts apparently depends on some factor hindering its utilization, while not preventing its synthesis. Our findings suggest that its utilization thus is hindered, first, when the enzymic constitution of the cells is unbalanced by previous phosphate starvation, and secondly, when growth is halted by exhaustion of the nitrogen or sulfur source, or by increasing acidity.

Chemical analysis of the volutin containing and volutin-free cells showed that formation of microscopically demonstrable amounts of volutin was accompanied usually by a large increase in the total phosphorus content of the cells relative to the amount of their protoplasm as measured by the nitrogen content. This increase involved

many different kinds of phosphorus compounds, including the large ribonucleic acid fraction. However, most of these were present in substantial quantities in the volutin-free cells and so were unlikely to be responsible for the volutin staining reaction. Our results suggest that the characteristic component of volutin granules is the metachromatically staining, trichloracetic acid insoluble metaphosphate found in the R9 fraction. Only minute amounts of this were present in the volutin-free cells, while substantial amounts were present in the volutin containing cells. The extent of the increase accompanying volutin formation was much larger for the R9 fraction (e.g., 200-fold) than for any other fraction (never over 10-fold). This identification agrees with that of Duguid *et al.* (1954) for the volutin of *A. aerogenes* grown in acid conditions and that of Wiame (1946*a*,*b*; 1947 *a*,*b*) for the volutin of *Saccharomyces cerevisiae*. It is clear, however, that metaphosphate is not the only component of the granules. In the cultures richest in volutin, the granules appeared to occupy over 20 per cent of the cell volume, while the metaphosphate comprised less than one per cent of the cells' dry weight. Probably the granules consist largely of other substances such as ribonucleic acid and protein and are merely coated or permeated with the metaphosphate which confers their peculiar staining character.

In staining phase-contrast microscopic and electron microscopic characters, the granules formed by *A. aerogenes* in conditions of nutrient imbalance resembled exactly those formed by this organism in conditions of acidity and also the classical granules of *C. diphtheriae*. Previous electron microscopic studies have shown that many bacteria contain highly electron-scattering granules with smooth, sharply defined margins: for instance, mycobacteria (Lembke and Ruska, 1940; Knaysi *et al.*, 1950; Mudd *et al.*, 1951*b*), *C. diphtheriae* (Morton and Anderson, 1941; König and Winkler, 1948; Bringmann, 1950–1951), *Staphylococcus flavocyaneus* and *Neisseria meningitidis* (Knaysi and Mudd, 1943), and *Bacillus mycoides* (Knaysi and Baker, 1947). König and Winkler proved the identity of the electron-scattering granules with the metachromatic volutin granules by examining the same Neisser stained film first by the light microscope and then by the electron microscope. Knaysi and his colleagues and Bringmann found evidence that the electron-scattering granules contained desoxyribonucleic acid and so corresponded to nuclear bodies. This view was controverted by Mudd (1953) and Winkler (1953). The comparative studies of Robinow and Cosslett (1948) and Hillier *et al.* (1949) showed that the nuclear staining sites of bacteria are normally less electron-scattering than the cytoplasm. However, the possibility is not yet excluded that in some circumstances the nuclei might become loaded with electron-scattering material. Mudd *et al.* (1951*c*,*b*) suggested that the electron-scattering granules of bacteria correspond to cytoplasmic granules which stain intravitally with tetrazolium salts and Janus green B. They consider that the granules represent organized centers of oxidative-reductive activity like the mitochondria of animal cells and are capable in certain conditions of accumulating volutin.

The volutin granules seen in our stained preparations of *A. aerogenes* corresponded in size, number, and situation with the dense granules seen by electron microscopy, but not with the nuclear bodies stained by Robinow's method nor with the mitochondria-like bodies stained by tetrazolium salts and Janus green B. The failure to show a correlation with mitochondria may have been due to unreliability of the mitochondrial staining methods. Weibull (1953) found that the colored reduced form of triphenyltetrazolium migrates from the sites of reduction and accumulates as secondary granules which are cytological artifacts. It is also possible that volutin may accumulate in more than one kind of cell structure. Its occurrence was observed in the cell vacuole, in cytoplasmic granules, and at other sites in the yeast cell by Lindegren (1948) and Hartman and Liu (1954). Lindegren found that when phosphate starved yeast was transferred to a phosphate containing medium, volutin appeared on the "chromosomes" within 3 minutes and passed to the cytoplasm in 20 minutes, the cells then dying. We were unable to demonstrate a comparable shift of volutin from one site to another in *A. aerogenes*.

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SUMMARY

Volutin was produced by *Aerobacter aerogenes* when grown under certain conditions of nutrient imbalance in cultures buffered near neutrality. Production occurred in the later stages of growth in cultures limited by exhaustion of the nitrogen or sulfur source, and also in early subcultures of cells previously starved of phosphate. In the latter case, volutin synthesis required the presence of an energy source such as glucose, and of phosphate, potassium, and magnesium ions; it was inhibited by dinitrophenol and azide in concentrations not inhibiting glucose oxidation. The volutin granules were examined by various staining methods and by the phase-contrast and electron microscopes. They did not appear to correspond either with the nuclear bodies or with the granules stained by tetrazolium salts. The volutin containing cells were mostly viable.

The phosphorus compounds of the cells were fractionated and estimated. Volutin formation generally was accompanied by an increase in the total phosphorus content of the cells relative to the nitrogen content. The increase affected most fractions, but the only one showing complete parallelism with the volutin staining was the trichloracetic acid insoluble inorganic metaphosphate; this was present in negligible amounts in the volutin-free cells and increased up to 250-fold in the volutin containing cells.

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