ELECTRON-MICROSCOPICAL

and

LIGHT-MICROSCOPICAL STUDIES

in

BACTERIAL CYTOLOGY

PART I.

by

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Part I.

A study of the factors influencing the production of volutin in *Aerobacter aerogenes*.

Contents.

The occurrence of strongly basophilic staining granules in bacteria was first recorded by Ernst (1888) in the case of Corynebacterium xerosis. In the following year Babes investigated the staining reaction of this and other bacteria and found the phenomenon not only in C.xerosis but also in C.diph-:theriae, V.cholerae and Myco.leprae. In honour of these two workers the inclusions became known as "Babes-Ernst" granules. Babes (1889) stated that on staining with Loeffler's methylene blue, the granules were coloured not blue like the bacilliary protoplasm, but either dark red or dark violet. This staining reaction was described as "metachromatic" and the granules were named "metachromatic" granules by Babes (1895). Meyer (1904) named the substance of the gran-:ules volutin, after the finding of such inclusions in Spirillum volutans by his pupil Grimme (1902). Subsequent investigations have shown that similar inclusions are found in algae, fungi, yeasts and protozoa, though, according to Lewis (1941) not in plants higher than the Thallophyta.

Table I indicates organisms in which volutin has been stated to occur. This list is not exhaust-:ive and may contain some instances which are doubt-:ful since the demonstration of volutin could not be

TABLE I.

Aerobacter aerogenes.	Duguid, J. P. (1948).
Azotobacter beijerinickii.	Lewis, I. M. (1937).
Azotobacter chroococcum.	Lewis, I. M. (1937).
Bacillus alvei.	Neide, E. (1904).
Bacillus anthracis.	Ebel, J.-P. (1949).
Bacillus asterosporous.	Neide, E. (1904).
Bacillus ellenbachensis.	Neide, E. (1904).
Bacillus fusiformis.	Neide, E. (1904).
Bacillus lacticola.	Neide, E. (1904).
Bacillus lactis.	Neide, E. (1904).
Bacillus robur.	Neide, E. (1904).
Bacillus sphaericus.	Neide, E. (1904).
Bacillus globiforme.	Mitchell, R. B. & Clark, F. E. (1941).
Bacillus.	Wilson and Miles, (1946a).
Clostridium.	Wilson and Miles, (1946b).
Cristospira.	Wilson and Miles, (1946c).
Coryne. diphtheriae.	Babes, V. (1889).
Coryne. equi.	Karlson et al, (1940).
Coryne. fimi.	Mitchell, R. B. & Clark, F. E. (1941).
Coryne. hoagii.	Mitchell, R. B. & Clark, F. E. (1941).
Coryne. murium.	Wilson and Miles, (1946d).
Coryne. ovis.	Wilson and Miles, (1946e).
Coryne. renale.	Wilson and Miles, (1946f).
Coryne. simplex.	Mitchell, R. B. & Clark, F. E. (1941).
Coryne. tumensens.	Mitchell, R. B. & Clark, F. E. (1941).

TABLE I (contd.)

Coryne. ulcerans.	Mitchell, R. B. & Clark, F. E. (1941).
Coryne. typhi.	Wilson and Miles, (1946g).
Coryne. xerosis.	Ernst, P. (1888).
Lacto. acidophilus.	Wilson and Miles, (1946h).
Lacto. bifidus. I.	Wilson and Miles, (1946i).
Lacto. bifidus. II.	Wilson and Miles, (1946j).
Lacto. bulgaricus. B.	White & Avery, (1910).
Myco. leprae.	Babes, V. (1889).
Neisseria catarrhalis.	Elser, W. J. & Huntoon, F. M. (1909).
Neisseria gonorrhoea.	Marx & Woithe, (1900).
Neisseria meningitidis.	Elser, W. J. & Huntoon, F. M. (1909).
Pseudomonas sp?	Neide, E. (1904).
Spirochaeta.	Wilson and Miles, (1946k).
Pasteurella.	Ebel, J.-P. (1949).
Spirillum giganticum.	Ellis, D. (1902-03).
Spirillum serpens.	Lewis, I. M. (1940).
Spirillum itersonii.	Lewis, I. M. (1940).
Spirillum tenue.	Lewis, I. M. (1940).
Spirillum undula.	Lewis, I. M. (1940).
Spirillum virginianum.	Lewis, I. M. (1940).
Spirillum volutans.	Grimme, A. (1902).
Aspergillus.	Mann, T. (1944).
Yeasts.	Lindegren, C. C. (1948).
Protozoa.	Lewis, I. M. (1941).

established with certainty prior to the introduction of the more specific staining techniques.

METHODS FOR THE DEMONSTRATION OF VOLUTIN.

(a) Methods depending upon metachromatic staining.

Babes (1889) used Loeffler's methylene blue to demonstrate the presence of volutin. This imparted a metachromatic dark red or violet colour to the granules and a pale blue tint to the remainder of the bacillus. Ernst (1888) used warm methylene blue to stain his smears and recommended the use of a counter-stain, Bismark brown, to be applied after the methylene blue stained smear had been washed with water. This gave a dark blue stain to the granules and a brownish cytoplasm; apparently the volutin differed from the cytoplasm in not allowing the replacement of the methylene blue with the counter-stain.

The demonstration of volutin came to have an important practical application as a means of recog-:nising the diphtheria bacillus in throat cultures, and for this, a variety of improved staining tech-:niques were developed. Neisser introduced his stain in 1897; he incorporated methyl violet in the methylene blue solution and applied chrysoidin as a

counter-stain; this gave a brownish-yellow bacillus with almost black volutin granules.

(b) Methods depending on the combined action of a metachromatic stain and iodine.

Albert (1921) stained the smears with a solution of toluidine blue and methyl green and, after washing, treated them with iodine; this imparted a black colour to the volutin granules and a green colour to the remainder of the bacillus. The methyl green was replaced by malachite green in a modification of Albert's method by Laybourn (1924). Christensen (1949) developed another stain in which he substi-:tuted safranin for malachite green and applied it after the iodine.

(c) Methods depending on the impregnation of the volutin granules with lead of cobalt salts.

Wachstein and Pisano (1950) and Bravo Oliva and Piedrola Gil (1951) applied lead impregnation in the demonstration of volutin. The method consisted of treating a heat fixed smear with a soluble lead salt, washing thoroughly with tap water and then applying ammonium sulphide. After washing with tap water, basic fuchsin was applied as the counter-stain, thus the granules appeared black against a red protoplasm. It was supposed that the phosphate of the volutin

combined with the lead giving lead phosphate which was converted to black lead sulphide by the action of the ammonium sulphide.

Macary (1951) used a similar method involving the impregnation of the granules with cobalt. He found that when volutin containing cells were treated with a soluble cobalt salt, the cobalt was fixed by the volutin presumably as cobalt phosphate (pink). Subsequent treatment with ammonium sulphide converted the cobalt phosphate to the sulphide which is black and so the volutin granules stood out clearly.

FURTHER STAINING AND MICROSCOPICAL PROPERTIES OF VOLUTIN.

Meyer (1904) with the help of his pupil Grimme (1902) studied the properties of the volutin found in Sp.volutans and B.alvei:

(a) When the volutin-containing bacteria were stained with methylene blue, the volutin granules assumed a dark red or a dark violet colour, which they retained even after treatment with 1 per cent sulphuric acid. Thus the granules were slightly acid fast.

(b) After staining the volutin-containing bacteria

with carbol fuchsin, the smears were treated with 1 per cent sulphuric acid. The cytoplasm and the nucleus faded leaving the volutin as dark red gran-:ules. When, however, 5 per cent sulphuric acid was employed the granules also slowly disappeared leaving a vacuole.

(c) Volutin was found to dissolve out of the cells on boiling for about 5 minutes, on heating for 5-10 minutes at 80°C or on standing for 2-3 days in the cold.

(d) If volutin containing cells were treated with formalin for 30 minutes previous to boiling, the volutin did not dissolve. Alcohol had the ability to fix volutin in 2 hours and osmic acid also fixed it completely.

(e) Volutin was soluble in 1 minute in 5 per cent hydrochloric acid, 5 per cent sulphuric acid or 25 per cent nitric acid, and in 24 hours at 28°C in $\frac{1}{2}$ per cent sulphuric acid.

(f) Volutin was soluble in 5 per cent sodium carbonate in 5 minutes.

(g) Volutin was not soluble in alcohol, chloroform or ether.

(h) Concentrated ammonia did not stain volutin.

(i) The staining of the volutin of B.alvei was improved by the addition of acetic acid to the stain.

Lewis (1941) stated that the volutin granules were more refractile than the cytoplasm and less refractile than spores. Macary (1951) recorded the fact that volutin could be observed by the phase contrast microscope, whereas no granules could be seen in the non-volutin containing control cells.

King and Beams (1942) studied the effect of subjecting Sp.volutans to ultracentrifugation. They found that the volutin granules were forced into the troughs indicating that these granules were heavier than the cytoplasm.

INFLUENCE OF CULTURAL CONDITIONS ON THE PRODUCTION OF VOLUTIN.

Volutin is produced by certain bacteria under normal conditions of culture, although for the best yield the bacterium might have to be grown on a special medium, thus C.diphtheriae shows the granules most abundantly when grown on Loeffler's inspissated serum medium.

Studies on the influence of the composition of the medium on volutin production by certain bacteria and yeasts appear to show that the presence of phos-:phate and an easily utilisable carbohydrate is necessary.

In their review of the meningococci, Elser and Huntoon (1909) stated that the number of volutin granules demonstrated by Neisser's stain varied with the strain but was fairly constant within one strain. They noted that the granules were produced in the presence of glucose or maltose but not in the absence of a sugar.

Zikes (1922) studied the production of volutin in several species of yeasts grown on synthetic media with different contents of phosphate, carbohydrate and nitrogen source. He found that the presence of phosphate was essential for volutin production and that, within limits, the amount of volutin was pro-:portional to the phosphate content of the medium. The amount of volutin produced was also found to depend on the ease of utilisation of the carbohydrate, e.g. glucose which was easiest metabolised gave the largest yield of volutin. With most yeasts volutin production was best with peptone as the nitrogen source, less with ammonium sulphate and least with

asparagine.

Lindegren (1947) showed that in the case of the yeasts Saccharomyces, Schizomyces and Torulopsis volutin production depended upon the phase of growth. Volutin appeared from a few minutes to several hours after they had been placed in a fresh medium containing adequate nutrient with carbohydrate, nitrogen source, phosphate and other substances. Each cell showed enormous volutin-encrusted chromosomes in the nuclear vacuole, but as division proceeded the amount of volutin diminished. In rapidly growing cultures on poor media the volutin might disappear from the chromosomes of nearly every cell, but if growing rapidly in a very good, well aerated medium, volutin might remain visible on the chromosomes throughout the period of multiplication and disappear after the cessation of growth.

Jeener and Brachet (1944) found that by growing yeast cells on a medium without phosphate, the cells lost their normal basophilic substance to a great extent or even completely. If, however, these phosphate-starved cells were transferred to a fresh medium containing phosphate, the basophilic substance was synthesised in excess of its normal level. Wiame (1945) repeated the experiment and obtained

the same result. In 1946b he, Wiame, stated that normal yeast cells were orthochromatic to toluidine blue but that phosphate-starved cells after transfer to a medium containing phosphate and a source of energy became metachromatic to toluidine blue; presumably this was due to the formation of volutin.

Duguid (1948) carried out a series of experiments on B.aerogenes grown on synthetic media. He was able to produce "nuclear" cells (i.e. cells with deeply staining nuclear bodies and poorly staining cytoplasm) by culture on a medium deficient in either the phosphate or the nitrogen source. On subculture of the phosphate-starved "nuclear" cells on to nutrient agar (containing phosphate), rapid and abundant volutin production was observed. The "nuclear" cells from a nitrogen-deficient medium did not produce volutin on transfer to nutrient agar, nor did the normal staining sugar-starved cells. These findings for a bacterium parallel Wiame's findings for yeast that abundant volutin production occurs when phosphate is supplied to phosphate deficient cells.

In the same paper, Duguid studied the effect of growing B.aerogenes on a sucrose-containing medium without adequate buffer. When growth was

not limited by phosphate or nitrogen deficiency, and when the phosphate concentration was below 0.1 per cent, the pH fell during growth to less than 4.5, the cells tending to be longer than usual and containing numerous volutin granules. Apparently acidity provides another condition inducing excess volutin production.

THE CHEMICAL NATURE OF VOLUTIN.

(a) Evidence that volutin is nucleic acid or nucleoprotein.

The early investigators such as Meyer (1904) expressed the view that volutin was a nucleic acid compound. Meyer based his assumptions on the comparison of the reactions of the volutin in Sp. volutans and B. alvei with the nucleic acid compound extracted from volutin-containing yeast buds. He found that both volutin and nucleic acid were stained with methylene blue and that they resisted destaining with 1 per cent sulphuric acid; both compounds were soluble in boiling water, sulphuric acid, hydrochloric acid, Javelle water, and chloralhydrate; Fehling's solution had no effect on either; alcohol fixed volutin in 2 hours and precipitated the nucleic acid from solution; formalin fixed volutin in 30 minutes i.e. volutin

was rendered insoluble in hot water and fixed nucleic acid overnight. From the above, Meyer concluded that volutin was a nucleic acid compound. Further he decided that volutin was not a nucleo-:protein as it did not give the biuret reaction. Therefore Meyer concluded that volutin was a nucleic acid compound other than nucleoprotein.

Winkler and König (1948) studied the effect of temperature on the volutin granules of C.diphtheriae. In a vacuum oven the granules were unaltered up to 500°C but at 600°C and over the granules were altered and on cooling showed up as empty vacuoles. (The granules were similarly altered in the electron microscope). As the granules were soluble in acid, 36 hour cultures of C.diphtheriae grown on Claudberg II medium were treated with 20 per cent sulphuric acid for an hour. After spinning, the acid extract was analysed and it was found to contain a larger amount of calcium and phosphate than a corresponding extract from a culture of Esch.coli. In order to study the effect of these two radicals on the prod-:uction of volutin, the authors added them to sheep's blood agar on which C.diphtheriae normally gives very few granules, and a large number of granules were produced. It was also found that

the addition of calcium and phosphate to sheep's blood agar stimulated the production of volutin in Esch.coli which is normally volutin-free. Winkler and Kőnig therefore concluded that volutin was a calcium salt of a nucleic acid. However, it must be noted that photochemical alteration of volutin at 600°C in a vacuum oven is not a recognis-:ed property of nucleic acids, and the observation thus does not prove that volutin is a nucleic acid. Furthermore the acid treatment of the cells which by the light microscope merely appears to remove the volutin granules, may remove other substances in addition. The conclusions of Winkler and Kőnig are therefore not to be accepted unreservedly.

(b) Evidence that volutin is metaphosphate.

The other view that volutin is or contains polymerised metaphosphate, arose from the study of the metachromatic reaction of volutin and that of some phosphorus compounds.

By staining a volutin-containing organism with methylene blue or toluidine blue the volutin has been found to be metachromatic in nature, i.e. it assumed a pinkish purple colour as opposed to the normal pale blue. Michaelis and Granick (1945) stated that all metachromatic effects of chemical

substances were either diminished or abolished by raising the temperature and that they returned on cooling. Wiame (1946b) found that the metachromatic appearance of yeast volutin underwent the same changes on heating so yeast volutin appears to be truly metachromatic in nature.

As the phosphate content of the medium has been found to be so important for the production of volutin, Wiame (1947b) examined various phosphates for their reaction with toluidine blue. He noted that if a 0.1 per cent solution of hexametaphosphate was mixed with an excess of a 0.5 per cent solution of toluidine blue a precipitate was given (colour not stated) but that trimetaphosphate, pyrophosphate and orthophosphate did not give any reaction under similar conditions. If a dilute solution of hexametaphosphate (10^{-2} - 10^{-4}M) was mixed with a dilute solution of the dye (10^{-4}M) no precipitate was produced but a purple colour resulted and this was studied spectro-:scopically. It was found that the adsorption maximum for toluidine blue fell from 630 mμ in water to 530 mμ in hexametaphosphate. Metachromasy was defined as $\frac{\epsilon\ 530\ m\mu}{\epsilon\ 630\ m\mu}$ where ϵ is the adsorption and 530 mμ the wavelength, and this was maximum when the concentration of the hexametaphosphate was 8 times

that of the toluidine blue. The other phosphate compounds mentioned above did not give this meta-:chromatic reaction, and it was found that nucleic acid and potassium sulphate inhibited metachromasy. The metachromatic reaction was also known to be given by the ester sulphates, therefore Wiame (1946c) investigated the effect of sulphate ions on the pro-:duction of volutin in yeasts. He found sulphate ions to have no effect on the amount of volutin pro-:duced, so he concluded that the metachromatic re-:action of volutin did not depend upon the presence of a sulphate. The metachromatic reaction is given by volutin, hexametaphosphate and estersulphate; phosphate is crucial for the production of volutin but sulphate is not, therefore it would appear that yeast volutin contains hexametaphosphate or a polymer of hexametaphosphate. The volutin of bacteria might well be metaphosphate in nature also.

(c) Evidence that volutin granules contain metaphosphate and nucleic acid compounds.

The question arises as to whether volutin is merely an inert chemical compound of a relatively simple nature such as metaphosphate or if it might be an animate material which only reacts metachro-:matically when the metaphosphate is deposited upon

it. Bringmann (1950-51) who studied the volutin granules of C.diphtheriae by the light and electron microscopes, would seem to support this latter theory when he stated that volutin was composed of ribonucleic acid and deoxyribonucleic acid together with an inorganic portion. When the volutin-containing cells were stained with pyronin, the granules stained red, therefore the granules were concluded to contain ribonucleic acid. The granules gave a negative Feulgen reaction but a positive reaction with methyl green (this depends upon the reaction of the methyl green with the polymerised deoxyribonucleic acid), so it was concluded that they contained deoxyribonucleic acid in addition to ribonucleic acid. The failure of the Feulgen reaction was attributed to the effect of the hydro-:chloric acid treatment of the cells prior to the staining with basic fuchsin, as,if the granules were first treated with lanthanum acetate they then gave a positive Feulgen reaction and this was taken as further proof of the presence of the deoxyribonucleic acid. The cells on treatment with Merck trypsin appeared to lose both their granules and their nucleic acid content; if however they were first treated with 40 per cent lead acetate, the

granules remained intact, although the nucleic acid was removed. It was thought that the unfixed granules were dispersed throughout the cells on enzyme treatment, but that the fixed granules were not; for this reason the remaining substance was thought to be inorganic in nature. The appearance of the compound in the electron microscope was found to correspond most nearly to that of metaphosphate when compared with metaphosphate, carbohydrate and protein. It was concluded that the volutin granules consisted of a complex of ribonucleic acid, deoxyri-:bonucleic acid and an inorganic compound which probably was metaphosphate.

THE OCCURRENCE OF METAPHOSPHATE IN OTHER MICROORGANISMS.

Macfarlane (1936) studied phosphorylation in living yeast and found that 30 per cent of the phosphate was present as a complex containing iron, nucleic acid and metaphosphoric acid. Mann (1944) identified metaphosphate in Aspergillus. Houlahan and Mitchell (1948) found a pyrophosphate or meta-:phosphate in Neurospora similar to or identical with that found in yeasts by Wiame.

Schmidt et al. (1949) found that the amount of

metaphosphate produced by yeasts depended upon the concentration of potassium ions in the medium. If the culture were deficient in magnesium, sodium ions were absorbed in preference to potassium ions but in both cases the negative charge of the phosphate ions absorbed was balanced by the positive charge of the potassium or sodium ions. The authors did not know whether the potassium ions were concerned with the physico-chemical absorption of the phosphate ions or if they were concerned with the transformation of the orthophosphate to metaphosphate inside the cell.

The metaphosphate content of the coliform ba-:cilli and the staphylococci was measured by Ebel (1949) and found to account for 5 per cent of the total phosphate. He also studied the distribution of the metaphosphate in the diphtheria bacillus in relation to the amount of volutin produced, and found that, when the organism was grown on a medium con-:taining serum, both the volutin content and the metaphosphate content were increased as compared with those grown on a serum-free medium. Unfortunately this publication did not give details of the methods or tables of results, so that the work cannot be repeated or its value assessed.

Schmidt et al. (1947) found that fresh yeast

cultivated in the presence of ammonium sulphate and phosphate rapidly assimilated phosphate at rates exceeding by far those of growth on a normal medium, and that the acid-soluble and insoluble phosphorus fractions increased in similar proportions. The considerable amounts of metaphosphate which appeared during the first two hours disappeared rapidly thereafter; this is in contrast with the behaviour of metaphosphate in yeast treated according to the method of Jeener and Brachet.

ESTIMATION OF PHOSPHATE COMPOUNDS IN YEASTS.

In 1945 Wiame applied the method of Jeener and Brachet for the production of volutin in yeasts i.e. by transfer of phosphate-starved cells to a phosphate containing medium. Using the method of Johnson and Harkiss (sodium hydroxide extraction) for the extraction of deoxyribonucleic acid he obtained a substance which was found to contain relatively more phosphorus and pentose than deoxyribonucleic acid. It resembled nucleic acid in its precipitation and properties but contained 15.6 per cent phosphorus as opposed to 9.45 per cent in nucleic acid. Wiame then compared the rate of hydrolysis of this nuclear fraction with that of nucleic acid from

normal cells and found it to be ten times greater. This rate of hydrolysis resembled that of the two labile phosphate groups in A.T.P. but as it differed from A.T.P. in its other properties, the polyphosphate was not A.T.P. (Wiame (1946a).) However it might be an energy store and Wiame (1946c) suggested it was metaphosphate.

Schmidt et al. (1946) also found that they were able to extract a substance which contained a higher percentage of phosphate than normal nucleic acid, from phosphate-enriched yeast. This fraction corresponded to the acid-insoluble, non-lipid fraction of the yeast. The compound was completely precip-:itated from acqueous solution by barium acetate at pH 4. Hydrolysis at 100°C with normal hydrochloric acid for seven minutes liberated 85 - 90 per cent of its phosphate and the original compound coagulated white of egg - a property of inorganic metaphosphate.

Wiame (1947a) treated phosphate-rich yeast with either trichloracetic acid or sodium hydroxide and isolated the metaphosphate by barium precipitation. He found the phosphorus content of this metaphosphate to be 19.6 per cent; that of chemical metaphosphate is 19.8 per cent.

Juni et al. (1947;1948) produced volutin rich

cells by the method of Jeener and Brachet, using radio-active phosphorus in the second medium. When such volutin-rich cells were treated with tri-:chloroacetic acid at 4°C, they gave rise to an acid soluble and an acid insoluble fraction, both of which were found to contain metaphosphate. These two metaphosphates differed in that the acid soluble one contained only normal phosphorus while the insoluble one contained radio active phosphorus, so the metaphosphates were classified as inactive and active respectively i.e. radio active phosphorus indicated activity of the phosphate. Wiame (1949) confirmed this activity of the insoluble metaphos-:phate. The insoluble metaphosphate was also considered to be active as it was preferentially used for the production of nucleic acid when cells which had previously accumulated metaphosphate were allowed to stand in a phosphate free medium.

INHIBITION OF VOLUTIN FORMATION BY PHYSICAL AND CHEMICAL AGENTS.

Wiame and Lefèbvre (1946) compared the action of the pH and temperature upon glycolysis and the synthesis of metaphosphate in yeasts. They found that glycolysis was not inhibited by a pH of 8.4 or greater but that the synthesis of volutin was

inhibited at pH 8.4. Lower pH values and temper-:atures did not differentiate the two processes.

Wiame (1947a) showed that azide was able to inhibit the production of metaphosphate in concentra-:tions insufficient to stop glycolysis. Spiegelman et al. confirmed this in 1948. Wiame (1949) found iodoacetate and fluoride also inhibited meta-:phosphate synthesis. The fundamental action of the azide may well be the inhibition of the form-:ation of the energy rich phosphate bonds thus stopping the production of metaphosphate.

BIOLOGICAL SIGNIFICANCE OF VOLUTIN.

Since the first demonstration of the inclusion bodies known as volutin in bacteria, there has been a great deal of speculation as to their biological significance. In 1888, Ernst put forward the idea that the volutin granules of C.xerosis were spores, as such organisms could remain viable in culture up to 80 days and could resist drying on silk threads for 5 days. Babes (1889) however found that C.xerosis was no more resistant than the non-volutin containing, non-sporing bacteria so Ernst's theory was found to be invalid. Then in 1889 Ernst stated that the granules were sporogenic

as he had seen them fusing to form spores. Marx and Woithe (1900) next tried to correlate the pre-:sence of these basophilic granules with the virulence of the bacterial strain, but this has been amply disproved by other workers e.g. Krompecher (1901).

In 1938 Groh advanced the theory that the gran-:ules of C.diphtheriae were living entities which, after the disruption of the parent cell, gave rise to rod forms.

As, until recently no nucleus could be demon-:strated in bacteria, some of the earlier workers thought that the volutin granules might represent the nucleus. This was regarded with some suspicion as the granules were not a constant feature of all bacteria. It was however finally disproved with the constant demonstration of the nuclei in all the bac-:terial species by the method of Robinow (1946).

Grimme (1902) recognised that volutin could be a reserve material as volutin was present in cells previous to sporulation and disappeared with the formation of the spores. Guilliermond (1910) observed the same building up of the volutin supply previous to sporulation in yeasts and fungi. He also, thought that the volutin was a reserve food material. While studying the occurrence of volutin

in the meningococci, Elser and Huntoon (1909) noticed that the volutin granules appeared in 6 hours, reached a maximum at 18 hours, all but disappeared in 48 hours and were definitely absent in one week and if an 18 hour culture were exposed to room temperature, very few granules remained after 6 hours. This together with the fact that they disappeared rapidly in the incubator after growth was complete would suggest that they represented a reserve food material which was used up when one factor favourable for growth was withdrawn - either the loss of a nutrient or a drop in temperature. Some of Wiame's work would also point to the same conclusion as in 1949, he recorded that the synthesis of nucleic acid was accompanied by the disappearance of the volutin from the yeast cell.

Knaysi (1948) studied the formation of volutin and lipid inclusions of Hansenula anomala in synthetic media. Both volutin and lipid were formed; the vol-:utin content diminishing with age. If the cells were grown on 2 per cent glucose, the growth was rapid but only lipid was formed. When, however, such cells were washed and incubated anaerobically in water, the fat disappeared and small granules of volutin were formed. This transformation of lipid

to volutin suggests that they are by-products of a related process.

In 1948, Lindegren studied the formation of volutin in normal yeast cells by means of a stain consisting of toluidine blue and formalin, the pH of which was adjusted to 2.5 in order to obviate the staining of the cytoplasmic proteins. On placing the resting cells in the new medium the first compo-:nent to show the metachromatic staining reaction was the chromosomes which became coated with volutin. These chromosomes joined on to the unstained nucleolus after it had enlarged; the nucleolus now becoming stainable and the chromosomes colourless indicating a transfer of the volutin from the chromosomes to the nucleolus. When phosphate-starved cells were con-:sidered, the cytoplasm was found to be colourless due to a loss of basophilic protein. After sub-cul-:ture of such cells on a medium containing both phos-:phate and a metabolisable substrate, the chromosomes were again the first to show the metachromatic re-action. Then the cytoplasm showed volutin which decreased rapidly till the cytoplasm gave the normal pink colouration. It has been shown in the cells of higher plants and animals that the nucleolus is the centre of the production of the ribosenucleoprotein

which after sythesis travels through the membrane into the cytoplasm. From the above work, Lindegren has produced the following hypothesis which has still to receive a full experimental backing. He thinks that the metaphosphate is synthesised on the chromo-:somes from the orthophosphate and transferred to the nucleolus where the synthesis of ribosenucleoprotein depends on the supply of metaphosphate. It has been shown that the apoenzyme (protein) components of cellular enzymes are responsible for their specificity so Lindegren offers the suggestion that the poly-:merised metaphosphate when it is transferred to the nucleolus from the chromosomes, may carry over certain specificity conferring groups, which influence the production of the ribonucleoprotein in the nucleolus. Previously Lindegren (1947) had suggested that due to the phosphate-rich energy bond content of metaphosphate it might act as an energy source and this was supported to a certain extent by the fact that non-volutin containing cells showed a lag on being sub-cultured in fresh media.

Summarising it would appear that the most likely explanation of the significance of the occurrence of volutin would be either as a reserve material or as a specific group carrier.

Purpose of the present investigation.

Since much evidence has been obtained on the occurrence of volutin in yeasts the purpose of this present work is to study the formation of volutin in bacteria.

In his paper Duguid (1948) noted the formation of volutin in Aero. aerogenes under two conditions, viz.,

(1) By growing Aero. aerogenes on an unbuffered sucrose medium whose pH fell as the sugar was metabolised.

(2) By the transfer of phosphate-starved cells of Aero. aerogenes from the phosphate-deficient medium to nutrient agar.

The object of this paper is to investigate further the factors influencing the production of volutin in Aero. aerogenes and allied organisms by morphological observations and chemical analyses and to try to correlate the amount of volutin production estimated microscopically with the amount of metaphosphate production estimated chemically.

(a) VOLUTIN PRODUCTION DUE TO ACIDITY.

METHODS.

Strain:- The present observations were all made with a single strain, A3, of Aero. aerogenes; this was a typical type I Bacterium aerogenes according to the classification of Wilson (1935), being methyl-red negative; Voges-Proskauer positive; citrate-utilisation positive; indol negative; Eijkman negative; it produced acid and gas from glucose, lactose, sucrose, mannitol, inositol and glycerol, but it did not ferment dulcitol; it did not liquefy gelatin; it was capsulate and gave a mucoid growth on MacConkeys' medium at 15°C and at 37°C, and it was non-motile.

Certain observations were repeated for a variety of other strains and species.

Medium:- By varying the amount of phosphate buffer in the following medium, well-buffered and poorly-buffered cultures were obtained:-

various amounts of Phosphate (1 part KH_2PO_4 to 3 parts Na_2HPO_4 giving a pH of 7.3)

0.1gm. Glucose.

0.5gm. Ammonium sulphate.

0.2gm. Sodium chloride.

0.1gm. Potassium sulphate.

0.01gm. Magnesium sulphate.

0.0001gm. Calcium chloride.

0.0001gm. Ferrous sulphate.

2gm. washed Agar

100ml. Distilled water.

To ensure that all the contamination phosphate was removed, the agar was washed by allowing it to stand in a large volume of distilled water for three one hour periods. The small amount of added phosphate was the only buffering agent present, so that the pH fell as the sugar was fermented. The phosphate, sugar, ammonium sulphate and salts were all A.R. reagents. The medium was sterilised by steaming for an hour. When examining volutin production by other bacterial strains and species 0.05 per cent peptone was included in the above medium to ensure the growth of the more fastidious strains.

Preparation of cultures:- The medium was poured into Petri dishes in 50ml. amounts and allowed to solidify. Each plate was inoculated uniformly over its surface with two loopfuls of a light saline suspension from an agar slope culture. To

allow free aeration three bent pins were placed over the edge of the Petri dish thus holding the lid raised about 1mm. The plates were incubated at 35°C for three days.

When a bicarbonate - carbon dioxide buffer was employed, as in the control experiments, 5 mls. of the medium containing the appropriate amount of sodium bicarbonate was poured into an 120ml. bottle and the agar allowed to solidify as a shallow layer. After inoculation with one loopful of the light bacterial suspension the bottle was closed by means of a screw cap (it was calculated that the bottle contained sufficient oxygen for complete metabolism of the sugar present) and the required amount of carbon dioxide was added by injection with a syringe through the rubber washer of the bottle cap. For a concentration of 0.1 per cent sodium bicarbonate in the medium 7 per cent of carbon dioxide was added and for less sodium bicarbonate proportionately less carbon dioxide.

A citric acid - sodium hydroxide mixture was also added to the medium in order to buffer it. Here the alkali released by the metabolism of the "citrate" balanced the acid formed by the fermentation

of the sugar. The media was poured in 50ml. amounts into Petri dishes and inoculated as described above for the unbuffered media. The "citrate" consisted of 40mls. 25 per cent anhydrous citric acid and 15.2mls. 10N sodium hydroxide which gave 1 per cent "citrate" when incorporated in 1 litre of medium. The pH of the "citrate" was 6.5.

For observations on the bacterial strains and species other than Aero. aerogenes A3, 10mls. of the medium was poured into 1oz. universal bottles and the agar allowed to solidify in a sloping position. These cultures were incubated open in a large glass tank to allow ample aeration; the tank itself being sealed.

Methods for observing the pH of the medium:-

A cube of the medium was removed from the plate by means of a sterile scalpel and placed in a well washed test-tube. The agar was covered with distilled water. After standing for two hours in order to allow the salts to diffuse out of the agar, the pH of the water was measured using colormetric indicators and a Lovibond comparator. Alternatively the surface of the agar was flooded with distilled

water which was allowed to stand for two hours before being decanted and its pH measured as before.

Methods for observing volutin production:- The smears were made by cutting out a cube of the agar with a sterile scalpel and placing it growth downwards on a slide - thus giving an impression. This smear was fixed by heat and stained by Laybourn's modification of Albert's method (Laybourn 1924). Volutin granules were stained black and the cytoplasm green.

The amount of volutin present was recorded by taking into account the number of cells showing granules, the number of granules per cell and the size of the granules. The amounts were designated according to a standard scale using the following symbols:- -no volutin, ± trace, +, + +, + + +, + + + +, increasing amounts of volutin. + + + + indicates very large granules in nearly every cell as in fig. iv.

Method for measuring the amount of growth:- The amount of growth from each culture was estimated by making an opacity measurement on an aqueous suspension of a culture prepared in parallel on the same medium. The growth produced on one 50ml.

plate of medium was washed off with distilled water; the volume made up to 100mls. and the opacity measured by means of the Hilger Spekker Photoelectric Absorptiometer. The amount of growth was recorded as the opacity which would have been produced if the growth from 10mls. of agar had been suspended in 10mls. of distilled water.

Method for the estimation of the total nitrogen content of the cells:-

The total nitrogen content of the culture was measured by the micro-Kjeldahl method incorporating Nessler's method for the estimation of the ammonia produced. The resulting yellow colour was measured by means of the Hilger Spekker Photoelectric Absorptiometer using the violet filter no.601.

Method for the estimation of the total phosphorus content of the cells:-

The total phosphorus content of the culture was measured by the estimation of the resulting inorganic orthophosphate by the method of Fiske and Subbarrow (1924) after the digestion of the cells with concentrated sulphuric and nitric acids. The blue colour produced was measured on the Hilger Spekker Photoelectric Absorptiometer using the red filter no.608.

Method for the fractionation of the phosphate compounds in the cultures:-

The cultures were fractionated after the method which Juni et al. (1948) applied to yeasts. Juni et al. were studying the distribution and the turnover of the phosphate compounds in yeasts on the transfer of phosphate-starved yeast to a medium containing phosphate. They used radio active phosphorus in the "Second Medium" and studied its distribution by the means of a Geiger Müller counter. In addition, they estimated the amount of phosphorus present by a chemical method. In the analysis of Aero. aerogenes the scheme of Juni et al. was followed throughout, but it was found that the large concentration of trichloroacetic acid in the acid soluble fraction tended to obscure the effect of heating the fraction with sulphuric acid when estimating the total phosphorus, as the trichloroacetic acid fumes were evolved in addition to those of the sulphuric acid. This was obviated by precipitating the phosphate present in this fraction as barium phosphate at a slightly alkaline pH in the presence of two volumes of alcohol. The barium precipitate was dissolved in hydrochloric acid and the barium precipitated as barium sulphate.

The barium sulphate precipitate was washed with dilute hydrochloric acid (o.1N) and the 2 centrifugates combined. This, after adjusting the pH to neutrality was examined for its total phosphorus content. In all cases the pH of the fraction was adjusted to neutrality and where barium was present either as barium phosphate or in solution it had to be removed prior to the phosphorus estimation due to the presence of the sulphuric acid in the Fiske and Subbarrow reagent. In all cases the total phosphorus content of the fraction was estimated as only one estimation could be carried out on the smaller fractions.

TABLE II.

The influence of different phosphate concentrations on the growth, pH and volutin production of Aero.aerogenes A3 in a poorly-buffered medium containing glucose at 35°C.

TIME. HOURS.	PERCENTAGE pH 7.3 PHOSPHATE ADDED TO THE MEDIUM.																	
	NONE		0.0001		0.0003		0.001		0.003		0.01		0.03		0.1		1.0	
	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.
0	6.2	—	6.2	—	6.2	—	6.4	—	6.4	—	6.3	—	6.6	—	4.0	—	4.2	—
6	5.9	+	6.0	—	6.0	—	6.3	—	5.8	—	6.3	—	6.6	—	6.8	—	4.2	—
16	4.4	—	4.6	—	4.5	+	4.6	+++	4.2	+++	4.6	+++	5.2	+++	6.2	—	4.1	—
24	4.3	—	4.1	—	4.2	+	4.4	++	4.2	+++	4.6	+++	4.6	+++	6.2	—	4.1	—
48	4.3	—	4.1	—	4.2	+	4.3	++	4.3	+++	4.3	+++	4.6	++	6.1	—	4.0	—
72	4.3	—	4.1	—	4.3	+	4.3	+	4.2	++	4.3	+++	4.6	++	6.1	—	4.0	—
OPACITY.	0.019		0.045		0.082		0.10		0.108		0.240		0.480		0.860		0.960	

VOL. = AMOUNT OF VOLUTIN PRODUCED.

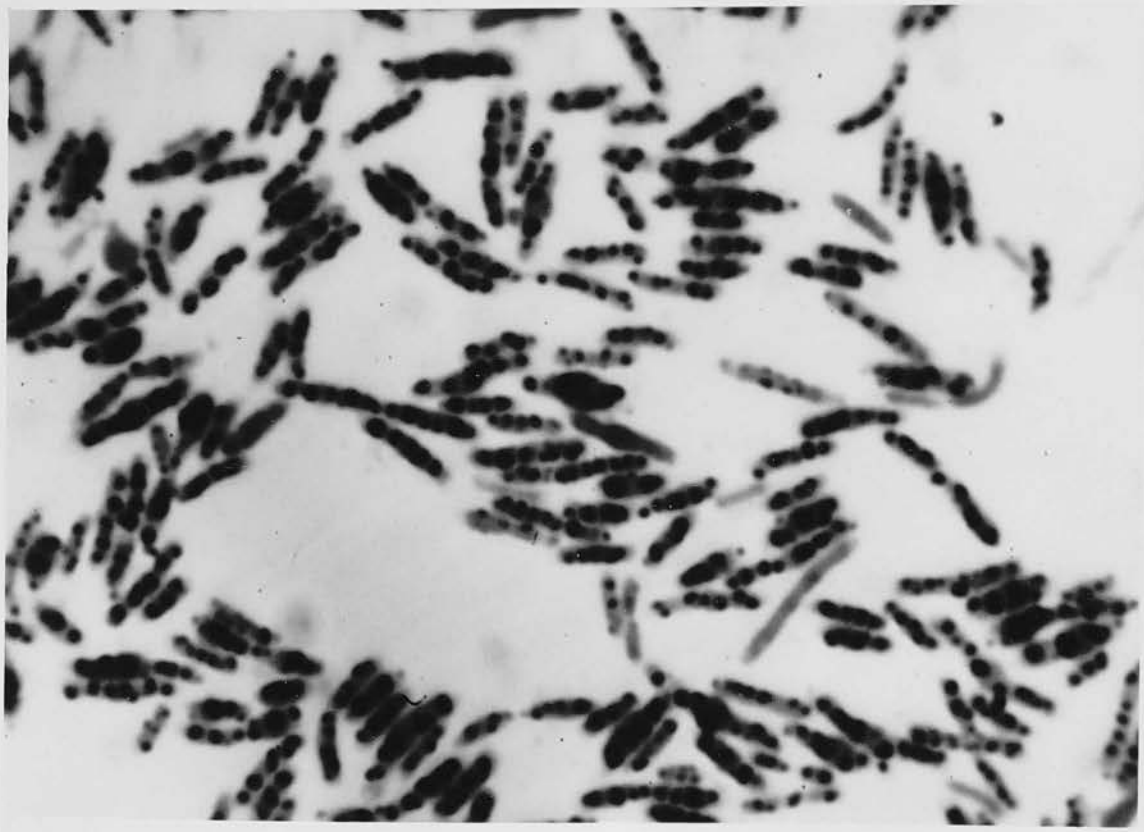


Fig. i. Aero. aerogenes A.3 showing volutin due to acidity developed in glucose media - Albert's stain.

Strain grown on 0.003% phosphate, 0.1% glucose, 0.5% ammonium sulphate, salts agar for 16 hours at 35°C. The black granules represent the volutin in this field which is rated as + + + x 3,000

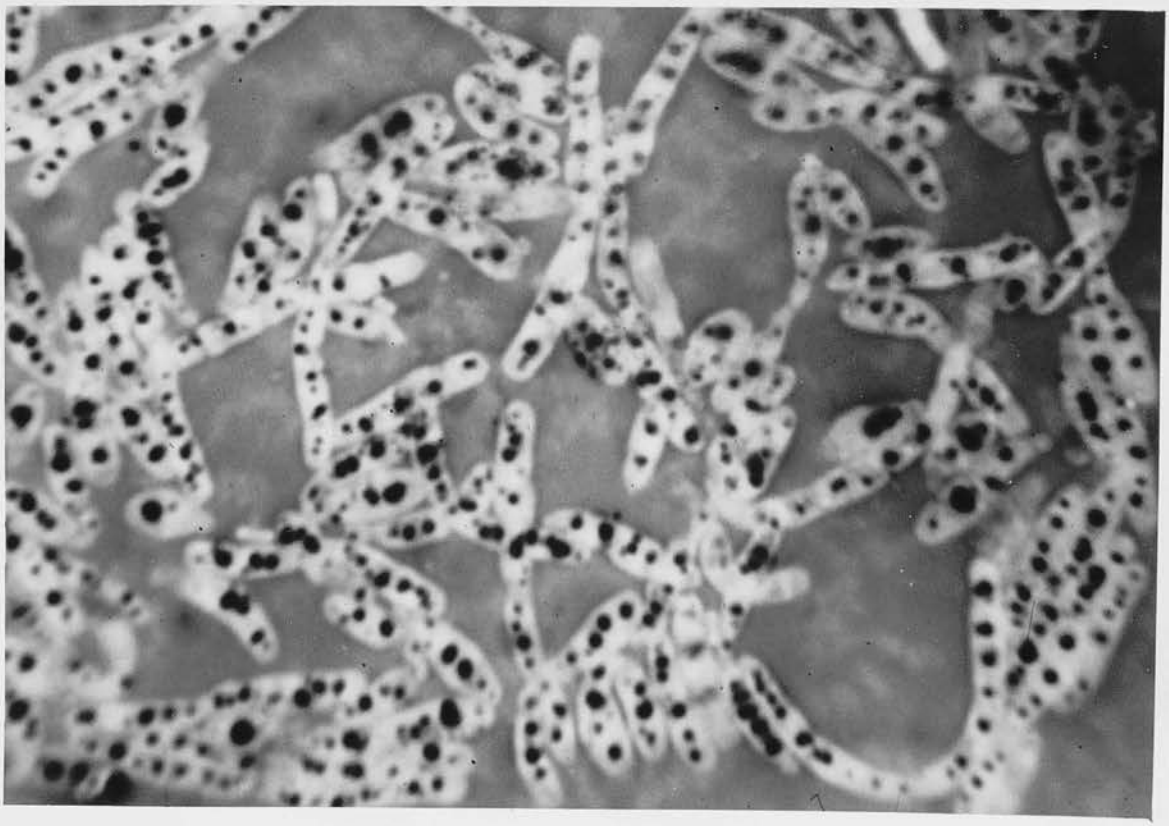


Fig. ii. Aero. aerogenes A3 showing volutin due to acidity developed in glucose media after 16 hours - Albert - Nigrosin stain.

Here the outline of the organism is sharply defined by the nigrosin. Strain grown on 0.02% phosphate, 0.1% glucose, 0.5% ammonium sulphate, salts agar for 16 hours at 35°C x 3,000

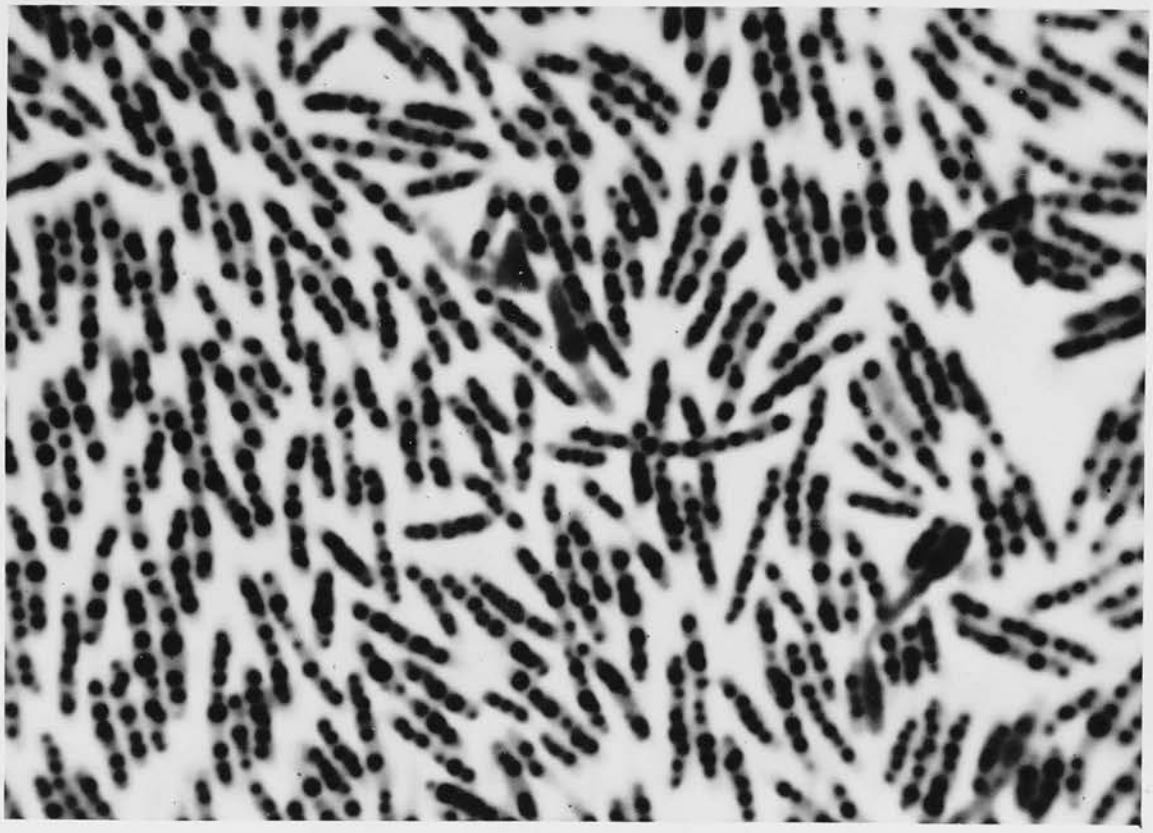


Fig. iii. Aero. aerogenes A3 showing volutin due to acidity developed in glucose media after 16 hours - Albert stain.

Strain grown on 0.02% phosphate, 0.1% glucose, 0.5% ammonium sulphate, salts agar for 16 hours at 35°C x 3,000

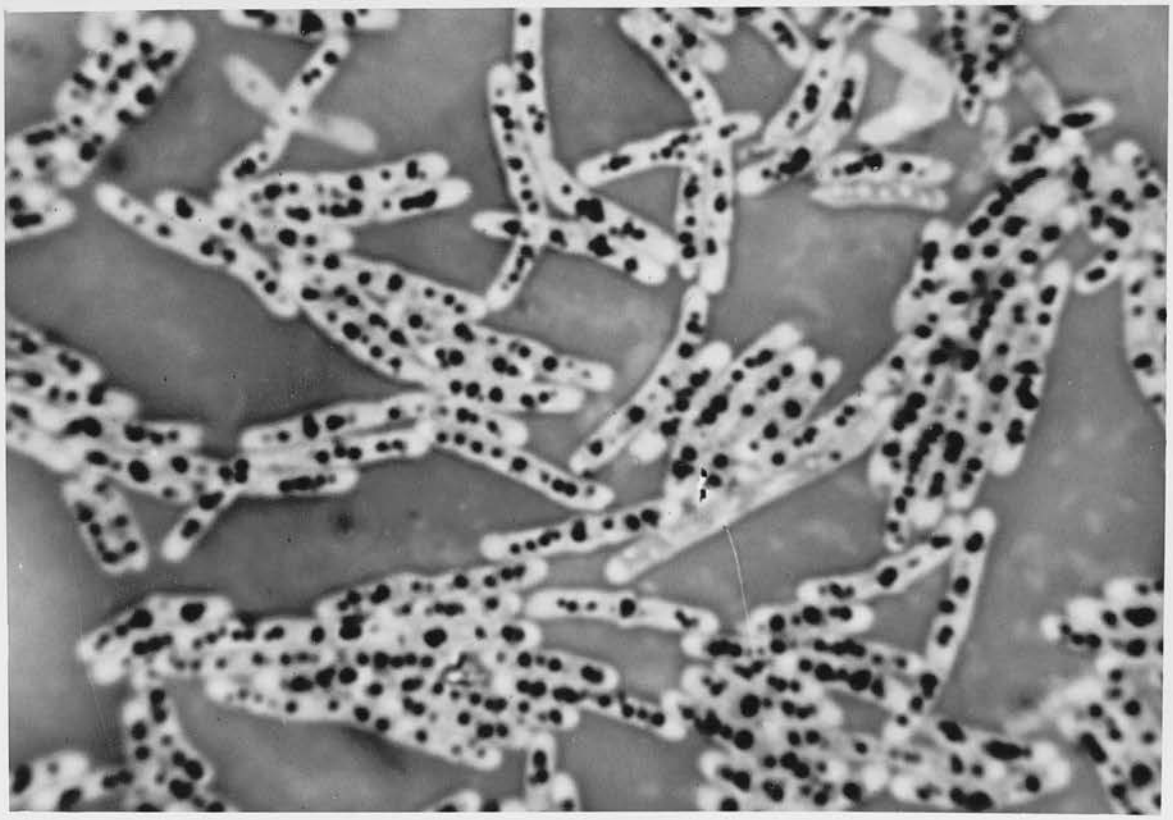


Fig. iv. Aero. aerogenes A3 showing volutin due to acidity developed in glucose media after 24 hours - Albert - Nigrosin stain.

Strain grown on 0.02% phosphate, 0.1% glucose, 0.5% ammonium sulphate, salts agar for 24 hours at 35°C x 3,000

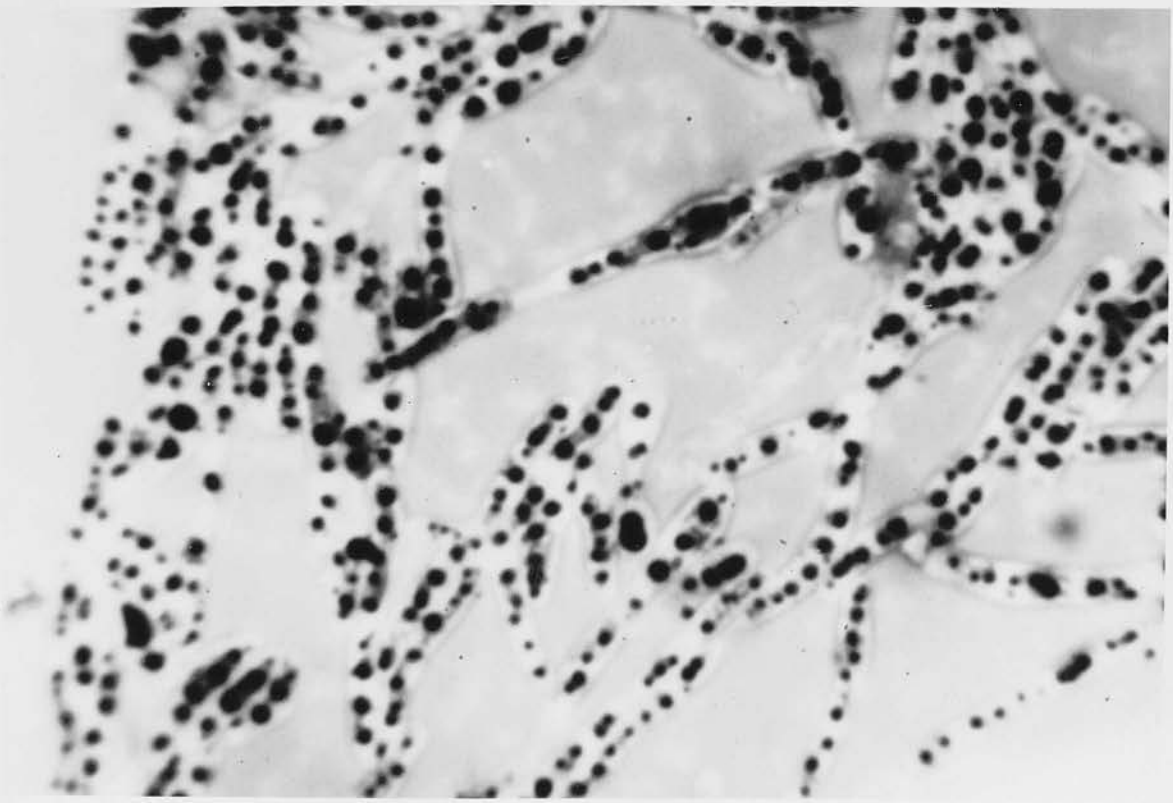


Fig. v. Aero. aerogenes A3 showing volutin due to acidity developed in glucose media after 48 hours - Albert - Nigrosin stain.

Strain grown on 0.02% phosphate, 0.1% glucose, 0.5% ammonium sulphate, salts agar for 48 hours at 35°C x 3,000

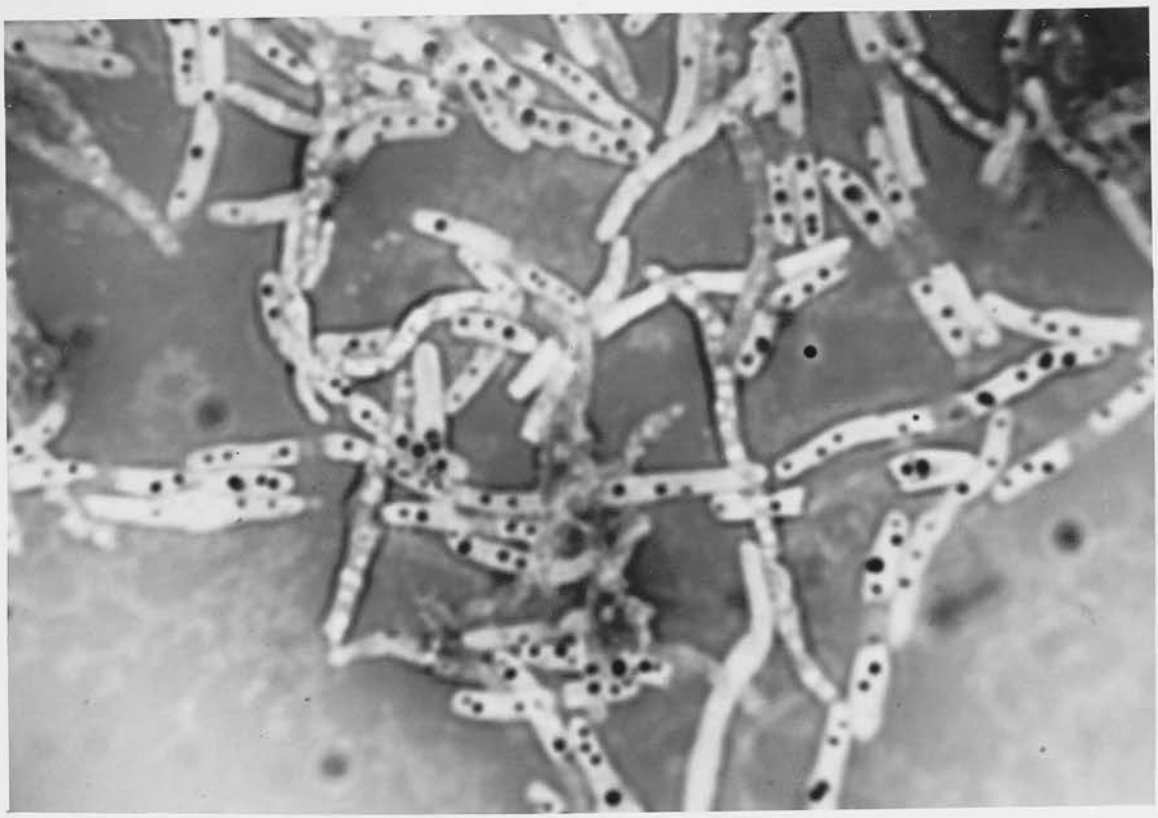


Fig. vi. Aero. aerogenes A3 showing volutin due to acidity developed in glucose media after 72 hours - Albert - Nigrosin stain.

Strain grown on 0.02% phosphate, 0.1% glucose, 0.5% ammonium sulphate, salts agar for 72 hours at 35°C x 3,000

RESULTS.

The influence of the phosphate concentration in the medium on the volutin production.

Experiments were made to determine the effect of various concentrations of phosphate on the growth, pH and volutin production of *Aero. aerogenes* cultures on sugar-containing media. The results for the glucose media where phosphate was the sole source of buffer are shown in table II. When the phosphate was present only in small amounts, the pH fell during growth due to the fermentation of the glucose. In media where the concentration of phosphate was 0.1 per cent or greater, the pH did not fall below 6.1, growth was maximal (opacity 0.86 and 0.76) and no volutin was produced. When the phosphate concentration lay between 0.001 and 0.03 per cent, the pH fell below 5.2 (finally 4.2 - 4.6), the growth was much reduced (opacity 0.1 to 0.48) and much volutin was produced (see fig. i.). The amount of volutin produced was maximal at 16 hours and it gradually diminished to 72 hours (see figs. ii. to vi.). When the concentration of phosphate was 0.0003 per cent or less the final pH was again low (4.1 - 4.3) and the growth minimal (opacity 0.019 to 0.082), but no significant

amount of volutin was produced. When such cells were stained with methyl violet those from media containing 0.0001 per cent or less phosphate gave the "nuclear" appearance considered indicative of phosphate-starvation. The cells from 0.0003 per cent phosphate medium did not have the "nuclear" appearance and were assumed to be on the borderline of phosphate-starvation. These last findings suggest that volutin synthesis requires a certain small amount of phosphate as well as a low pH.

A similar experiment was carried out using sucrose as the energy source and the results are recorded in table III. The results obtained were similar except that the pH did not fall so far, rather less volutin was produced on the most favourable medium (see fig.vii.) and the volutin production was maximal at 24 hours instead of at 16 hours as in the case of glucose.

The glucose experiment was repeated at room temperature (15°C - 20°C) instead of at 35°C. (See table IV.). The results show that except for the growth being considerably slower, the effect of growth on a poorly-buffered, glucose containing medium was the same, i.e. volutin was produced when

TABLE III.

The influence of different phosphate concentrations on the growth, pH and volutin production of Aero. aerogenes A3 in a poorly-buffered sucrose containing medium at 35°C.

TIME HOURS	PERCENTAGE pH 7.3 PHOSPHATE ADDED TO THE MEDIUM.																	
	NONE		0.0001		0.0003		0.001		0.003		0.01		0.03		0.1		1.0	
	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.
0	6.3	–	6.2	–	6.3	–	6.3	–	6.3	–	6.4	–	6.4	–	6.9	–	7.2	–
6	5.8	⊥	5.9	+	5.9	+	5.8	–	5.9	–	6.4	–	6.4	–	6.8	–	7.1	–
16	5.8	–	5.9	–	5.8	–	4.3	++	4.6	++	5.3	++	5.3	⊥	6.2	–	7.1	–
24	5.4	–	5.9	–	4.6	⊥	4.6	++	4.6	+++	4.6	+++	4.6	–	6.2	–	7.1	–
48	5.2	–	5.3	–		⊥	4.4	++	4.5	++	4.5	++	4.8	⊥	6.2	–	7.0	–
72	4.9	–	4.5	–	4.5	⊥	4.4	+	4.5	++	4.4	+	4.6	⊥	5.9	–	7.0	–
OPACITY.	0.044		0.062		0.178		0.240		0.280		0.220		0.660		0.980		1.060	

VOL. = AMOUNT OF VOLUTIN PRODUCED.

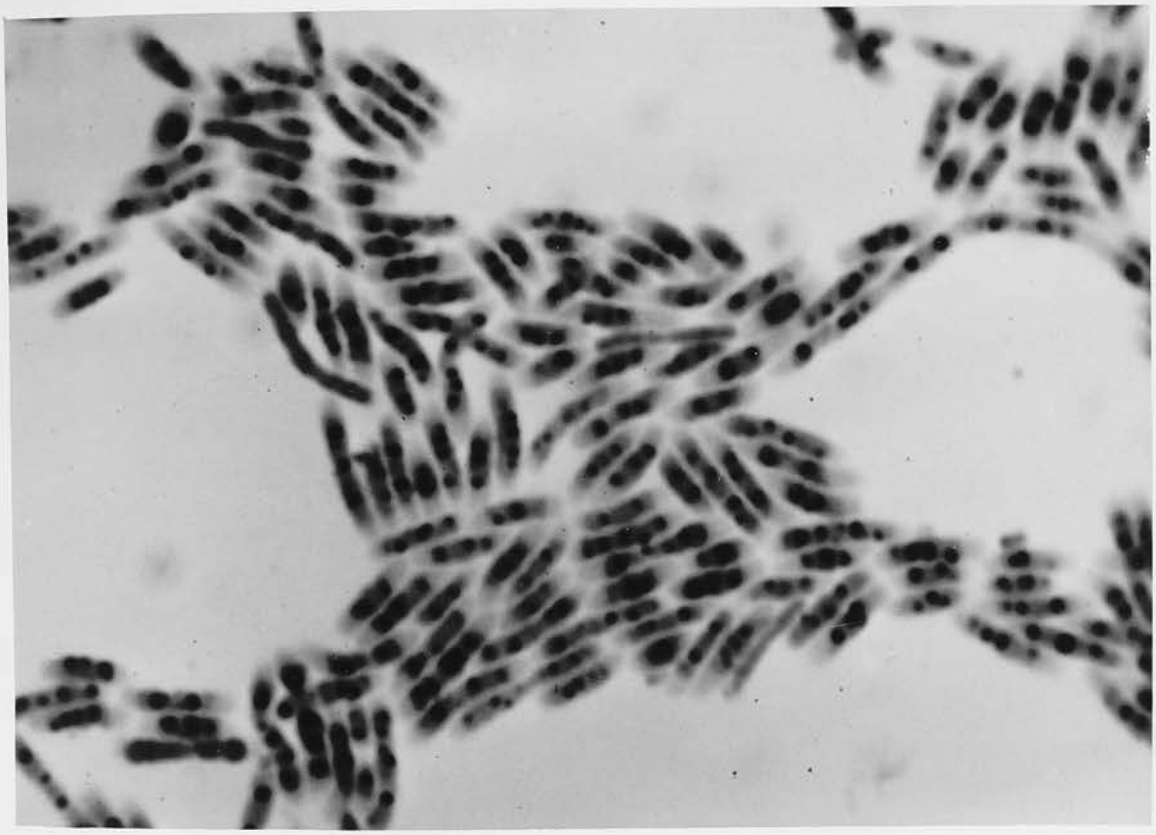


Fig. vii. Aero. aerogenes A3 showing volutin due to acidity developed in sucrose media - Albert's stain.

This field is rated as + +
Strain grown on 0.003% phosphate, 0.1% sucrose, 0.5% ammonium sulphate, salts agar for 16 hours at 35°C x 3,000

TABLE IV.

The influence of different phosphate concentrations on the growth, pH and volutin production of Aero.aerogenes A3 in poorly-buffered glucose containing media at room temperature (15° - 20°C).

Time Days.	Percentage pH 7.3 phosphate added to the medium.																	
	None		0.0001		0.0003		0.001		0.003		0.01		0.03		0.1		1.0	
	pH	Vol.	pH	Vol.	pH	Vol.	pH	Vol.	pH	Vol.	pH	Vol	pH	Vol.	pH	Vol.	pH	Vol.
0	6.1	–	6.1	–	6.3	–	6.4	–	6.4	–	6.5	–	6.7	–	6.9	–	7.2	–
2	4.6	–	4.5	–	4.4	⊥	4.3	+	4.3	++	4.3	++	4.3	+	6.2	–	7.2	–
3	4.4	–	4.7	–	4.4	–	4.4	++	4.3	+++	4.5	++	4.8	++	6.2	–	7.0	–
4	4.3	–	4.1	–	4.3	+	4.3	++	4.3	+++	4.2	++	4.3	⊥	6.5	⊥	7.1	–
6	4.3	–	4.2	–	4.2	+	4.2	++	4.2	+++	4.3	++	4.3	+	6.2	+	7.1	–
Opacity.	0.10		0.18		0.388		0.456		0.448		0.480		0.828					

Vol. = amount of volutin produced.

the phosphate concentration lay between 0.001 and 0.03 per cent and the pH fell to 4.3 or less.

It will be seen from tables II and III that, at the phosphate concentrations which gave volutin production, the amount of growth, expressed as an opacity measurement, was cut to about half the maximum obtained with abundant phosphate. As stated above, when the phosphate concentration was as much as 0.1 or 1.0 per cent the pH did not fall below 6.1 and no volutin was produced. If, however, acid phosphate (KH_2PO_4) at pH 5.2 was substituted for the pH 7.3 phosphate mixture, the pH fell to 4.5 and volutin was produced (table V); this showed that a high phosphate concentration, apart from the effect on the pH, was not inhibitory to volutin formation. By comparison of the opacity measurements in table V. with those of the extreme right hand columns of tables II. and III. it will be seen that the substitution of acid phosphate cut the growth to about one fifth because of the lower pH.

The influence of various energy sources on volutin production.

Various other energy sources were incorporated in the medium in place of glucose and the results

TABLE V.

The influence of a high concentration of acid phosphate on the growth, pH and volutin production by Aero.aerogenes A3 on poorly-buffered sugar containing media at 35°C.

Time Hours	Glucose				Sucrose			
	Percentage pH 5.2 phosphate in medium							
	0.1		1.0		0.1		1.0	
	pH	Vol	pH	Vol	pH	Vol	pH	Vol
0	5.2	–	5.2	–	5.2	–	5.2	–
6	5.2	–	4.8	–	5.1	–	4.9	–
16	4.5	–	4.6	–	4.6	⊥	4.9	–
24	4.5	⊥	4.5	+	4.6	+	4.6	⊥
48	4.3	⊥	4.3	++	4.4	++	4.4	++
72	4.3	⊥	4.4	++	5.1	⊥	4.4	++
Opacity	0.078		0.16		0.148		0.280	

Vol. = amount of volutin produced.

are shown in table VI. In all cases the medium had a low initial pH due to the presence of 1.0 per cent acid phosphate (pH5.2) and the pH of the acids employed were adjusted to 5.2 also. It will be seen that only in the case of glucose and sucrose did the pH fall below 4.6 and an appreciable amount of volutin was produced. When "citrate" was the source of energy, the pH started at 5.2 and rose to 5.4 in 24 hours when volutin was produced but on further incubation the pH rose still higher and the volutin disappeared. With the hexose-di-phosphate, glycerophosphate, pyruvate and lactate, the amount of volutin produced was negligible. In all of these cases except the last where the pH rose, the pH remained constant throughout the experiment. When the calcium salt was employed as in hexose-di-phosphate and glycerophosphate the calcium would presumably exert a neutralising effect so that the pH would not tend to fall and this might explain the absence of the volutin. Where the pH tended to rise (lactate, "citrate" and pyruvate) the growths were much greater than those on glucose or sucrose. This possibly explained the absence of volutin as volutin is only formed in such cultures when growth

TABLE VI.

The influence of various energy sources on the growth, pH and volutin production of Aero.aerogenes A3 when it was grown on a medium containing 1% acid phosphate pH 5.2 at 35°C.

TIME HOURS.	ENERGY SOURCE													
	GLUCOSE		SUCROSE		CITRATE		HEXOSE – DI – PHOSPHATE		CALCIUM GLYCERO PHOSPHATE		SODIUM PYRUVATE		LACTIC ACID	
	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.
0	5·2	–	5·2	–	5·2	–	5·2	–	5·2	–	5·1	–	5·1	–
6	4·8	–	4·9	–	5·2	–	5·1	–	5·2	–	5·0	–	5·2	–
16	4·6	–	4·9	–	5·3	–	5·1	–	5·2	+	5·0	–	5·2	–
24	4·5	+	4·6	⊥	5·4	++	5·1	–	5·2	+	5·0	–	5·2	–
48	4·3	++	4·4	++	5·9	⊥	5·1	⊥	5·2	⊥	5·4	+	6·2	+
72	4·4	++	4·4	++	6·0	⊥	5·3	+	5·1	–	5·4	–	6·4	–
OPACITY.	0·16		0·28		1·07		0·056		0·072		0·612		1·06	

VOL. = AMOUNT OF VOLUTIN PRODUCED.

is prematurely halted due to a low pH.

The influence of anaerobsis on the production of volutin.

The glucose experiment was also carried out under anaerobic conditions in the presence of 0.01 per cent phosphate at pH 7.3 which gave the best volutin production under aerobic conditions. The anaerobic results are shown in table VII. Comparing these results with those obtained under aerobic conditions (see table II) it is seen that the growth, expressed as an opacity, was cut from 0.24 to 0.18. The pH did not fall so quickly under anaerobic conditions, reaching only 4.5 as opposed to 4.3 at 72 hours under aerobic conditions. The amount of volutin produced rose to a maximum at 48 hours but was not so large as that under aerobic conditions. The volutin-containing cells were noted to be exceptionally long whilst the volutin-free cells were normal short bacilli. Possibly a number of these short cells were dead.

The influence of added buffer on volutin production.

Cultures were made to show the effect of the addition of increasing amounts of buffer to the medium most favourable for volutin production (see tables VIII. and IX.). The addition of 0.05 per

TABLE VII.

The influence of culturing Aero.aerogenes A3 anaerobically at 35°C on the growth, volutin production and pH of the medium which contained 0.01% phosphate, 0.1% glucose, 0.5% ammonium sulphate, salts and agar.

TIME HOURS	pH	VOLUTIN
0	6.6	–
24	4.9	+
48	4.6	++
72	4.5	+
OPACITY	0.148	

TABLE VIII.

The influence on the growth, pH and volutin production of Aero.aerogenes A3 by the addition of various concentrations of bicarbonate and citrate buffers to a poorly-buffered glucose containing medium at 35°C. The medium contained 0.01 per cent phosphate mixture pH 7.3 together with nitrogen source and the usual salts.

TIME HOURS	PERCENTAGE BICARBONATE BUFFER ADDED TO THE MEDIUM												PERCENTAGE CITRATE BUFFER ADDED TO THE MEDIUM.											
	NONE		0·01		0·02		0·03		0·05		0·1		NONE		0·01		0·02		0·03		0·05		0·1	
	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.	pH	VOL.
0	6·6	–	6·9	–	7·0	–	7·4	–	7·4	–	7·4	–	6·4	–	6·8	–	6·8	–	6·8	–	7·1	–	7·0	–
6	6·4	–	6·8	–	6·8	–	6·8	–	6·8	–	6·9	–	6·4	–	6·7	–	6·8	–	6·8		7·1	–	7·2	–
16	5·4	+++	5·2	+	5·4	+	6·4	–	6·6	–	6·8	–	4·7	+++	4·6	++	5·3	+	5·4	⊥	6·0	–	6·2	–
24	5·2	++	4·2	++	4·2	+	6·0	–	6·6	–	6·8	–	4·5	+++	4·5	+++	5·2	⊥	5·8	⊥	6·6	–	7·4	–
48	4·4	++	4·2	++	4·3	++	6·1	–	6·3	–	6·8	–	4·5	+++	4·9	++	5·9	⊥	6·4	⊥	7·3	–	8·2	–
72	4·3	++	4·2	+++	4·4	+	5·9	–	6·2	–	6·8	–	4·5	+++	4·9	++	5·9	⊥	6·5	⊥	7·4	–	8·3	–
OPACITY													0·210		0·724		0·824		0·812		0·778		0·946	

VOL. = AMOUNT OF VOLUTIN PRODUCED.

TABLE IX.

The influence on the growth, pH and volutin formation of Aero.aerogenes A3 by the addition of various concentrations of bicarbonate and citrate buffer to a poorly-buffered sucrose containing medium at 35°C. The medium contained 0.01 per cent phosphate together with the nitrogen source and the usual salts.

TIME HOURS	PERCENTAGE BICARBONATE BUFFER ADDED TO THE MEDIUM.												PERCENTAGE CITRATE BUFFER ADDED TO THE MEDIUM.											
	NONE		0.01		0.02		0.03		0.05		0.1		NONE		0.01		0.02		0.03		0.05		0.1	
	pH	VOL	pH	VOL	pH	VOL	pH	VOL	pH	VOL	pH	VOL	pH	VOL	pH	VOL	pH	VOL	pH	VOL	pH	VOL	pH	VOL
0	6.6	–	6.6	–	6.9	–	7.1	–	7.3	–	7.4	–	6.3	–	6.4	–	6.5	–	6.4	–	6.6	–	6.6	–
6	6.1	–	6.3	–	6.6	–	6.8	–	6.9	–	6.0	–	6.3	–	6.6	–	6.4	–	6.6	–	6.4	–	6.2	–
16	5.4	++	5.4	+	5.4	⊥	6.2	–	6.4	–	6.8	–	5.2	++	5.4	++	5.4	⊥	5.5	⊥	5.2	–	<6.0	⊥
24	5.2	++	5.3	++	5.3	⊥	6.0	–	6.4	–	6.8	–	5.4	++	5.3	++	5.3	⊥	5.9	⊥	5.9	–	6.2	–
48	4.7	+	4.1	++	4.3	⊥	5.6	⊥	6.0	–	6.8	–	4.8	+	5.2	+	5.2	–	6.1	⊥	6.6	–	7.6	–
72	4.3	⊥	4.3	+	4.6	⊥	5.6	–	6.2	–	6.8	–	4.6	–	5.2	+	5.2	–	6.1	–	6.9	–	7.8	–
OPACITY.													0.286		0.666		1.058		1.144		1.232		1.086	

VOL. = AMOUNT OF VOLUTIN PRODUCED.

cent sodium bicarbonate in the presence of 3.5 per cent carbon dioxide was sufficient to hold the pH of the medium at or above 6.0 and no volutin was produced (see figs. viii. and ix.), while 0.03 per cent was barely sufficient. In the case of the citric acid - sodium citrate buffer, 0.05 per cent inhibited volutin production although the pH fell to 5.2 in the early stages before the alkali released by the utilisation of the "citrate" had been liberated (see fig. ix.). The bicarbonate - carbon dioxide buffer was considered to be the more satisfactory buffer as it was not metabolised and the pH did not rise as it did when the "citrate" was used.

The viability of the volutin-containing cells.

The viability of the volutin-containing cells was studied by making parallel total and viable counts. The results of the experiments carried out with 0.01 and 0.02 per cent phosphate in the medium are recorded in tables X. and XI., together with the opacity measurements, pH estimations, and volutin production. The percentage of viable cells fluctuated during the course of growth and was not constant in repeated experiments. In the case of

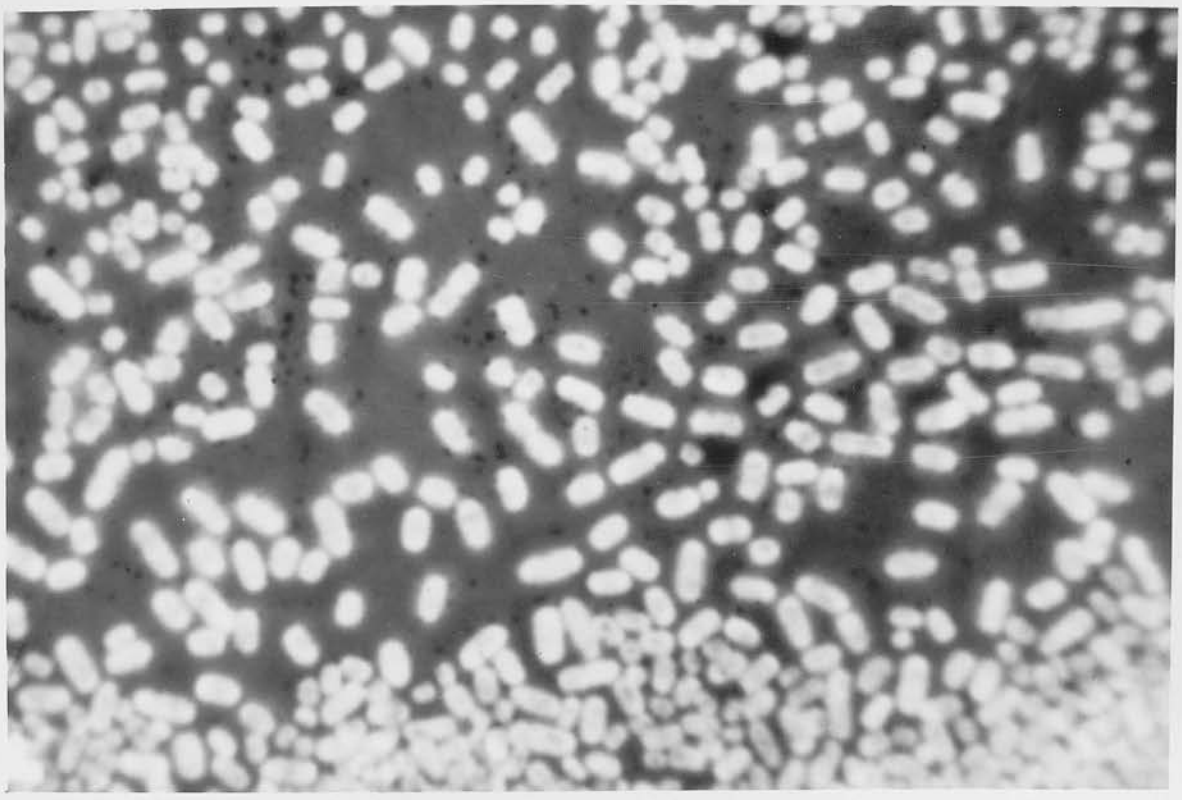
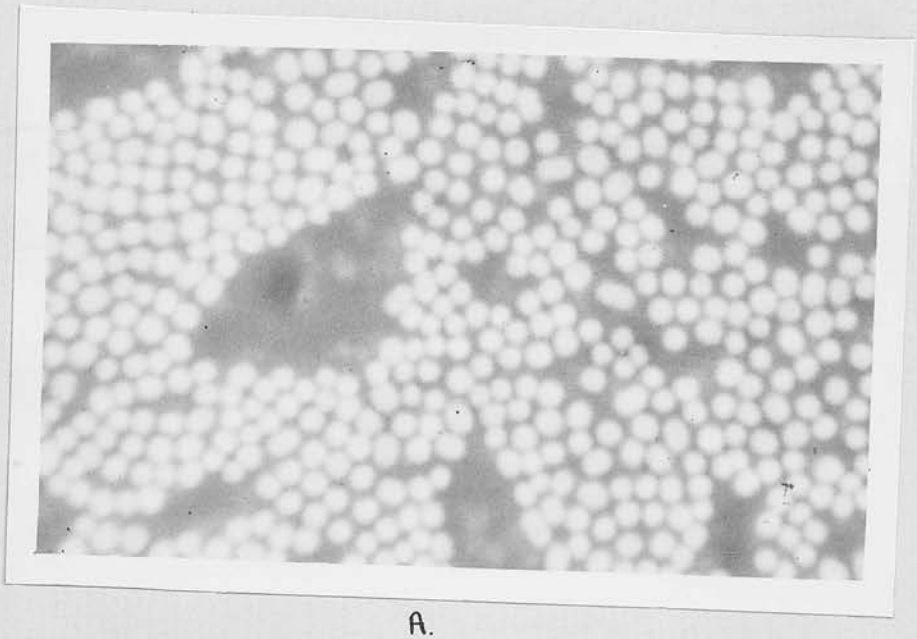
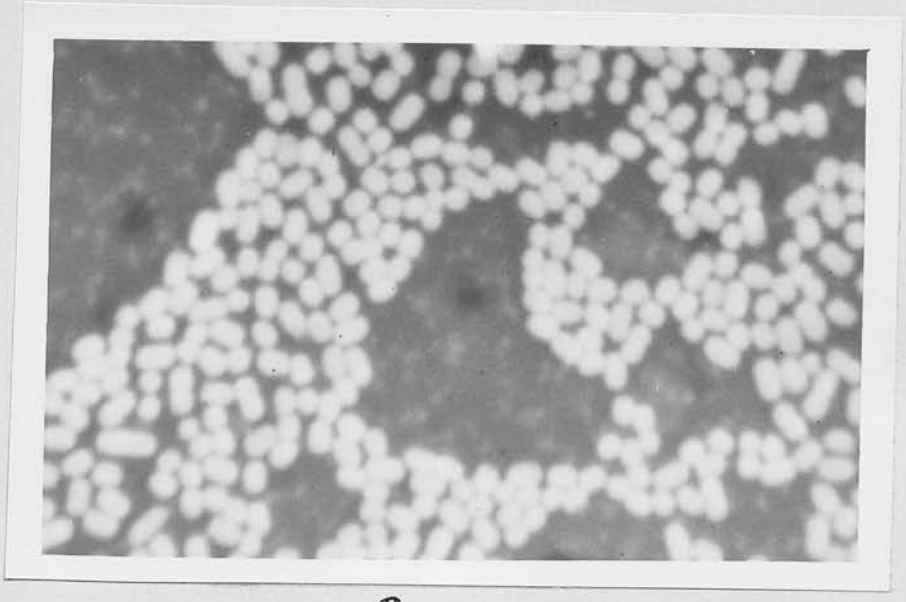


Fig. viii. Aero. aerogenes A3 showing absence of volutin in a glucose medium buffered against development of an acid reaction - Albert - Nigrosin stain.

Strain grown on 0.02% phosphate, 0.1% glucose, 0.5% ammonium sulphate, salts agar buffered by the addition of 0.3% bicarbonate to the medium and 20% carbon dioxide to the atmosphere for 24 hours at 35°C x 3,000



A.



B.

Fig. ix. A & B. Aero. aerogenes A3 showing absence of volutin in glucose medium buffered against development of an acid reaction - Albert - Nigrosin stain.

Strain grown on 0.02% phosphate, 0.1% glucose, 0.5% ammonium sulphate, salts agar with the addition of (a) 0.3% bicarbonate or (b) 0.05% citrate as buffer for 24 hours at 35°C x 3,000

TABLE X.

To show the viability of Aero. aerogenes A3 cells grown on a poorly-buffered medium containing glucose and 0.01% phosphate at 35°C.

TIME HOURS	VIABLE COUNT	TOTAL COUNT	pH	VOLUTIN PRODUCTION	OPACITY.
6	4.945×10^6	—	6.4	—	—
16	8.12×10^8	14×10^8	5.0	+++	0.238
24	3.05×10^8	13×10^8	4.6	+++	0.216
48	4.56×10^8	20×10^8	4.6	++⊥	0.296
72	6.01×10^8	22×10^8	4.6	++	0.276

TIME HOURS	VIABLE COUNT	TOTAL COUNT	pH	VOLUTIN PRODUCTION	OPACITY
6	0.03×10^6	—	6.6	—	—
16	11.4×10^8	22×10^8	4.5	+++	0.344
24	2.54×10^8	23×10^8	4.3	++++	0.348
48	9.8×10^8	25×10^8	4.6	+++⊥	0.402
72	3.6×10^8	26×10^8	4.8	++⊥	0.344

TABLE XI.

To show the viability of Aero.aerogenes A3 grown on a poorly-buffered glucose containing medium in the presence of 0.02% phosphate at 35°C.

TIME HOURS	VIABLE COUNT	TOTAL COUNT	pH	VOLUTIN PRODUCTION	OPACITY
6			6·8	—	
16	$19{\cdot}1 \times 10^8$	21×10^8	5·2	++⊥	0·164
24	$18{\cdot}8 \times 10^8$	19×10^8	4·6	++⊥	0·226
48	$19{\cdot}3 \times 10^8$	34×10^8	4·5	+++	0·266
72	$8{\cdot}8 \times 10^8$	34×10^8	4·5	++⊥	0·254

TIME HOURS	VIABLE COUNT	TOTAL COUNT	pH	VOLUTIN PRODUCTION	OPACITY
6	$5{\cdot}25 \times 10^6$	—	6·8	—	—
16	$3{\cdot}4 \times 10^8$	20×10^8	5·1	++⊥	0·192
24	$1{\cdot}43 \times 10^8$	18×10^8	4·5	++⊥	0·252
48	$14{\cdot}1 \times 10^8$	35×10^8	4·4	++	0·408
72	$1{\cdot}46 \times 10^8$	25×10^8	4·6	—	0·458

cultures on 0.01 and 0.02 per cent phosphate the viability of the cells at 16 hours ranged from 17 to 90 per cent; and at 72 hours between 5 and 25 per cent. Since volutin was present in almost all cells even in the cultures showing high viability (e.g. 90 per cent) it must be concluded that the exhibition of volutin granules occurs commonly in living cells and is not indicative of cell death.

The result of the transfer of the volutin-containing cells to media of different pH.

As many of the volutin-containing cells were not dead, an experiment was made to determine the fate of the volutin on transfer to fresh media. The results are shown in table XII. The crop from one 50ml. plate was spun down in buffer saline (0.4 per cent sodium chloride and 0.1 per cent phosphate pH 7.3) and the residue transferred to a 50ml. plate containing 0.4 per cent sodium chloride, 1.0 per cent phosphate and 2 per cent agar. This agar was buffered at pH 4, with phosphate, citric acid mixture and at 5, 6 and 7 with phosphate. At pHs 4 and 5 the volutin was still present after 24 hours and there was no evidence of cell division.

TABLE XII.

The effect of transferring centrifuged 24 hr. volutin-containing organisms from 0.01% phosphate, glucose medium to agar containing 0.4% sodium chloride and 1% phosphate at 35°C.

Hours on "Second Medium"	pH 4	pH 5	pH 6	pH 7	pH 8
	Volutin	Volutin	Volutin	Volutin	Volutin
0	+++	+++	+++	+++	+++
1	+++	+++	+++	+++	+++
2	+++	+++	++	++	++
4	+++	+++	+ *	+ *	+ *
8	++	+++	⊥	++	+
24	+++	++	—	⊥	⊥

* CELL DIVISION HAD OCCURRED IN THESE CULTURES.

At pH 6 and 7 the volutin disappeared after 4 hours on the fresh medium and the cells, which were initially long had undergone cell division and become coccoid. This disappearance of the volutin granules on the fresh media appeared to depend on the occurrence of renewed cell growth. The fact that the volutin remained apparent for the first few hours on the fresh medium was proof that exhibition of volutin was not a reversible staining artefact solely dependent on the concurrent low pH, but represented a true deposition of inclusion granules during growth at a low pH.

Volutin production in other bacterial strains and species.

Various other bacterial strains and species were examined for their ability to produce volutin under the acid conditions arising from growth in a poorly buffered glucose containing medium. Initially, the experiments were carried out using a synthetic medium containing phosphate, glucose, ammonium sulphate and salts, but finally 0.05 per cent peptone was added to allow the growth of the more fastidious organisms. The experiments were controlled by cultures on an identical medium which

was buffered with the bicarbonate-carbon dioxide mixture. The results for the organisms found to produce volutin are recorded in tables XIII. and XIV. It will be seen that most Aero. aerogenes strains, K. pneumoniae and Ser. marcescens produced a large amount of volutin while the rough Aero. aerogenes D(301) Aero. cloacae (typical and non-gelatin liquefying strains) and Ser. marcescens gave poor volutin production (see figs. x. to xiii.). Organisms which gave no volutin production on the standard medium and on a wide range of poorly buffered sugar containing media supplemented with various growth factors included Proteus X19, Ps. aeruginosa, S. typhi, S. paratyphi A, S. paratyphi B, Sh. shiga, Sh. sonne, Sh. flexneri Y, Esch. coli, strains A55, A93, A100, A101, A102, B. subtilis, B. cereus, B. anthracoides, B. mycoides, Staph. aureus, Staph. albus, Staph. citreus, Strept. faecalis, V. cholerae var bombaii, Sarc. lutea, and Myco. phlei.

TABLE XIII.

The influence of growth on poorly-buffered and well-buffered glucose containing media at 35°C for 24 hours on the pH and volutin production by other strains of Aero. aerogenes. The poorly-buffered media contained 0.003% phosphate, 0.1% glucose, 0.5% ammonium sulphate, 0.05% peptone, salts agar and this with the addition of 0.3% bicarbonate and 20% carbon dioxide constituted the well-buffered one.

ORGANISM	POORLY-BUFFERED MEDIA		WELL-BUFFERED MEDIA		ORGANISM.	POORLY-BUFFERED MEDIA		WELL-BUFFERED MEDIA	
	pH	VOL	pH	VOL.		pH	VOL	pH	VOL
AERO. AEROGENES A1	4·2	+++	6·4	—	AERO. AEROGENES A25	4·4	++	4·2	—
AERO. AEROGENES A3	4·2	+++	6·6	—	AERO. AEROGENES A26	4·3	+++	4·0	—
AERO. AEROGENES ABR	4·4	+++	6·4	—	AERO. AEROGENES A27	4·3	++	4·2	—
AERO. AEROGENES A4	4·3	+++	4·3	—	AERO. AEROGENES A29	4·4	++	4·1	—
AERO. AEROGENES A8	4·2	+++	4·2	—	AERO. AEROGENES D301	4·8	+	6·4	—
AERO. AEROGENES A9	4·4	+++	4·2	—	AERO. AEROGENES D301M	4·4	+	4·1	—
AERO. AEROGENES A14	4·3	+++	4·3	—	AERO. AEROGENES N418	4·2	++	4·1	—
AERO. AEROGENES A17	4·3	++	4·3	—	AERO. AEROGENES NS936	4·3	+	6·8	—
AERO. AEROGENES A20.	4·3	++	4·3	—	AERO. AEROGENES NS939	4·3	++	6·8	—
AERO. AEROGENES A 22.	4·3	+++	4·2	—	AERO. AEROGENES N8167	4·4	+	4·2	—
AERO. AEROGENES A 23	4·9	++	4·3	—	AERO. AEROGENES N8172	4·3	++	6·4	—

VOL. = AMOUNT OF VOLUTIN PRODUCED.

TABLE XIV.

The influence of growth on a poorly-buffered and a well-buffered glucose containing media at 35°C for 24 hours on the pH and volutin production by Aero.cloacae, Ser.marcescens and K.pneumoniae strains. The poorly-buffered media contained 0.003% phosphate, 0.1% glucose, 0.5% ammonium sulphate, 0.05% peptone, salts agar and this with the addition of 0.3% bicarbonate and 20% carbon dioxide constituted the well-buffered one.

Organism.	Poorly-Buffered Media		Well-Buffered Media		Organism	Poorly-Buffered Media		Well-Buffered Media	
	pH	Vol	pH	Vol		pH	Vol	pH	Vol.
Aero.cloacae N5920.	4.6	++	6.4	—	Aero.cloacae 63Y(2)	4.8	—	4.1	—
Aero.cloacae 035(1)	4.1	⊥	4.1	—	Aero.cloacae 636(2)	4.4	⊥	4.1	—
Aero.cloacae 919(1)	4.4	—	6.9	—	Aero.cloacae 889(2)	4.8	—	4.0	—
Aero.cloacae 989(1)	4.5	⊥	6.8	—	Aero.cloacae 439(1)	4.4	+	4.2	—
Aero.cloacae 602(1)	4.5	⊥	4.0	—	Aero.cloacae N8143	4.5	⊥	4.1	—
Aero.cloacae 438(1a)	4.5	⊥	4.0	—	K.pneumoniae N5054	4.8	+++	6.9	—
Aero.cloacae 493(1)	4.5	—	4.1	—	K.pneumoniae N5055	4.3	+	6.6	—
Aero.cloacae 259(1)	4.6	⊥	4.1	—	K.pneumoniae N5056	4.3	⊥	6.8	—
Aero.cloacae 034(3)	4.4	—	4.1	—	Ser. marcescens 2	4.6	++	6.8	—
Aero.cloacae 923(1)	4.5	⊥	4.1	—	Ser. marcescens N134Y	4.9	⊥	6.8	⊥
Aero.cloacae 439(2)	4.5	+	4.2	—	Ser. marcescens N8015.	4.4	⊥	6.8	—

Vol. = amount of volutin produced.

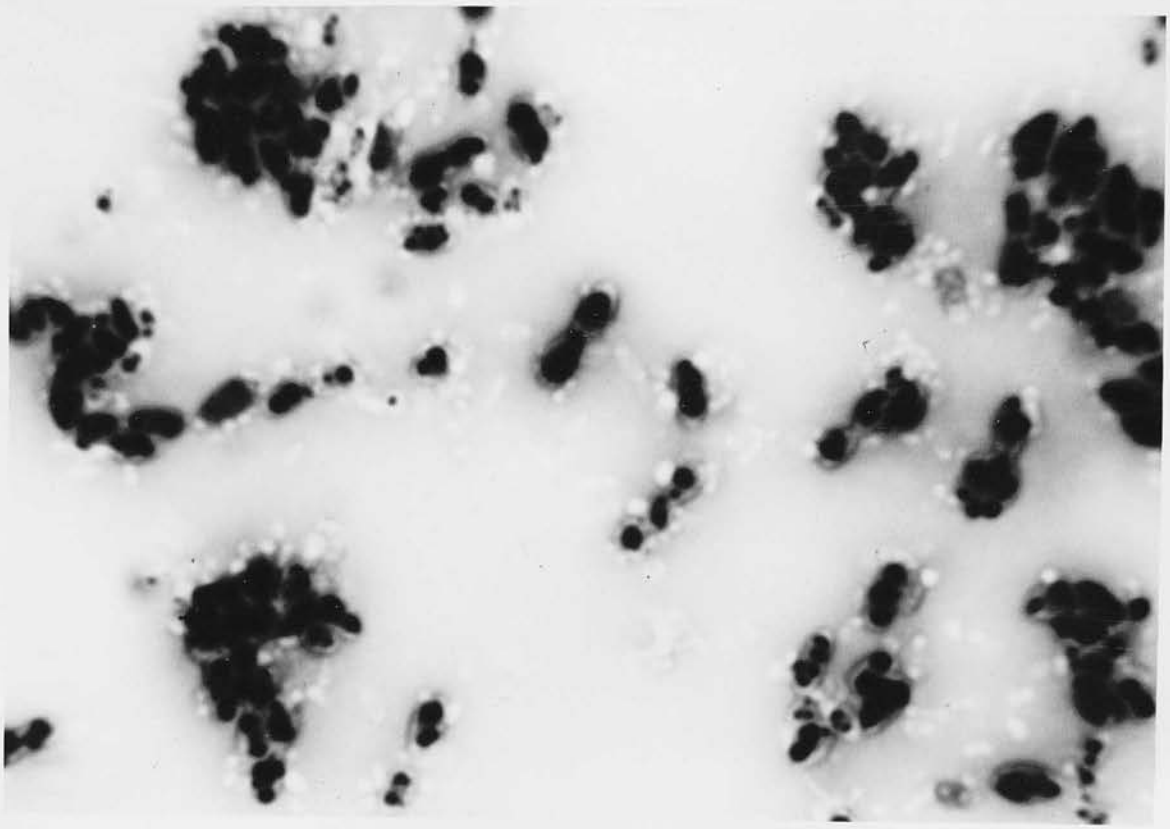
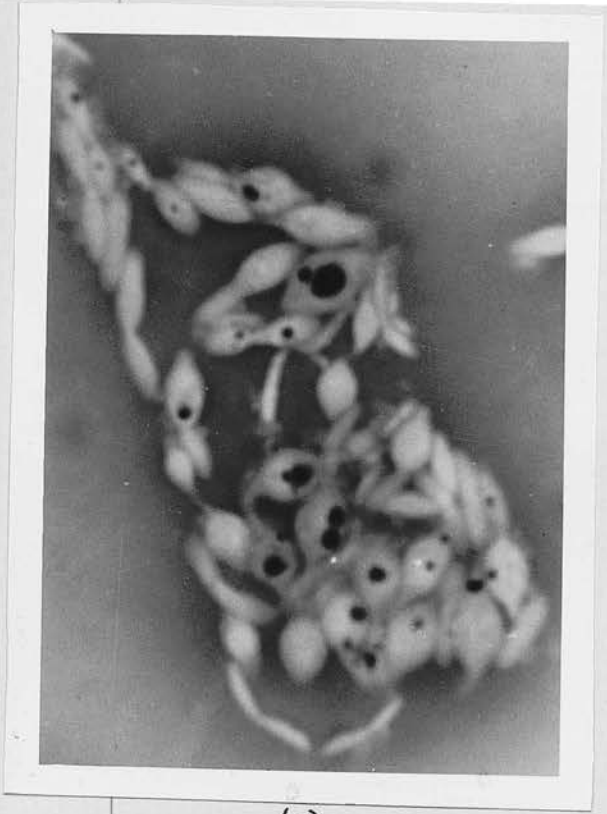


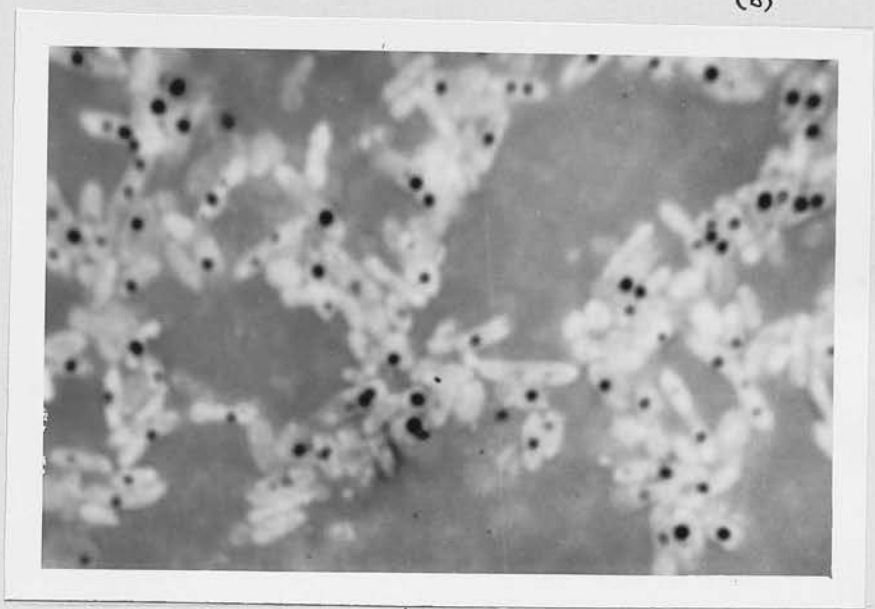
Fig. x. Aero. aerogenes A1 showing volutin due to acidity developed in glucose media - Albert - Nigrosin Stain.

Strain grown on 0.003% phosphate, 0.1% glucose, 0.5% ammonium sulphate, salts agar for 24 hours at 35°C x 3,000



(a)

(b)



(c)

Fig. xi. (a)(b)(c) Aero. aerogenes N.5936(a) N.5939(b) and Ser. marcescens(c) showing volutin due to acidity developed in glucose media - Albert - Nigrosin stain.

Strains (a) and (b) grown on 0.003% phosphate, 0.1% glucose, 0.5% ammonium sulphate, salts agar for 24 hours at 35°C. Strain (c) grown on the same medium with the addition of 0.05% peptone.

x 3,000

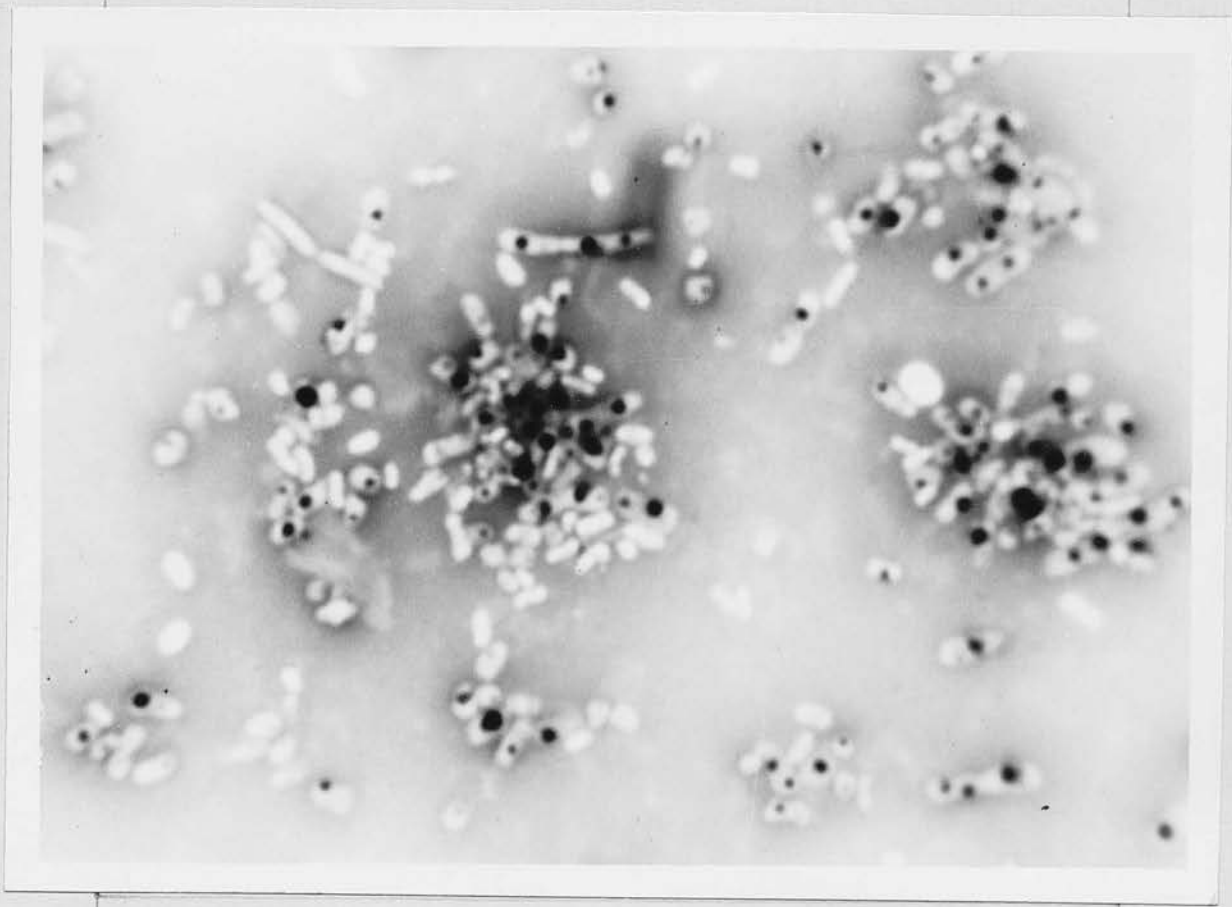


Fig. xii. Aero. aerogenes D.301 showing volutin due to acidity developed in glucose media - Albert - Nigrosin stain.

Strain grown on 0.003% phosphate, 0.1% glucose, 0.5% ammonium sulphate salts agar for 24 hours at 35°C x 3,000

Fig. xiii. Klebsiella pneumoniae N.5054 showing volutin due to acidity developed in glucose media - Albert - Nigrosin stain.

Strain grown on 0.003% phosphate, 0.1% glucose, 0.5% ammonium sulphate, salts agar for 24 hours at 35°C x 3,000

CYTOLOGICAL OBSERVATIONS ON THE VOLUTIN PRODUCED ON POORLY-BUFFERED, SUGAR CONTAINING MEDIA.

1(a) Optical microscope - Albert's method. (Modified by Laybourn 1924).

The granules stained black while the cytoplasm stained green. (Figures i. and iii.). The cells were seen to be 2 - 6 μ in length; each cell containing up to four large granules when the volutin production was good. Cells from the medium containing the lower concentrations of phosphate did not show stainable cytoplasm but had a green nuclear body surrounded by an unstained area in its turn surrounded by purplish - brown capsular material.

1(b) Optical microscope - Albert - Nigrosin method.

By over-spreading an Albert stained smear with a thin layer of nigrosin (Fleming 1941) the edge of the bacillus was found to be sharply defined whilst the granules were largely unaffected. (Figs. ii. and iv.).

1(c) Optical microscope - Metachromatic stains.

When stained by Loeffler's methylene blue (Mackie and McCartney 1953a) the bacillus stained blue but the granules were pinkish-violet, i.e. they stained metachromatically. Toluidine blue also gave pinkish-violet granules in a blue

cytoplasm. The acid-fastness of this metachromatic staining was examined by subjecting a methylene blue stained smear to the action of sulphuric acid. It was found that the granules retained their pinkish-violet colour when treated with 1.0 per cent sulphuric acid for 1 minute, but that 5 per cent for 3 minutes or 20 per cent for 1 minute decolorised the granules completely leaving in their place a vacuole. The cytoplasm did not show any increased acid-fastness when compared with the staining properties of the non-volutin containing control cells.

1(d) Optical Microscope - Carbol fuchsin and sulphuric acid.

It was found that when volutin containing smears were stained with carbol fuchsin for 5 minutes there was no differentiation of the granules from the cytoplasm. If, however, the stained smear was treated with 1 per cent sulphuric acid for 1 minute the volutin stood out clearly as dark red granules against the pale cytoplasm. 5 and 20 per cent sulphuric acid for 1 minute and 5 per cent sulphuric acid for 5 minutes did not remove the stain from the granules so the volutin was concluded

to be slightly acid fast.

1(e) Optical Microscope - Modified Ziehl Neelsen method. (Mackie and McCartney 1953b).

This is not a good method for the demonstration of volutin as it entails the heating of the smear and volutin is known to "disappear" on heating so the granules cannot be observed. Also, if the granules did resist the heating process the methylene blue would give rise to the metachromatic reaction and this would lead to a false interpretation of the staining results.

1(f) Optical Microscope - Periodate-Schiff method of Hotchkiss (1948).

Polysaccharide is indicated by this method as red staining areas. Volutin-containing cells had a red staining cytoplasm with clear refractile spaces, which seemed to correspond to the volutin granules.

1(g) Optical Microscope - Gram's iodine.

Neither the brown granules indicating the presence of glycogen nor the blue granules indicating iogen were observed.

1(h) Optical Microscope - Burdon's fat stain (1946).

No black granules indicating the presence of fat were observed.

1(j) Optical Microscope. Lead impregnation method of Bravo Oliva and Piédrola Gil (1951).

The volutin here appeared as black granules in a pink cytoplasm.

2. Phase Contrast Microscope.

When unstained, wet films of volutin-containing organisms were examined by the phase contrast microscope, granules were observed (fig. xiv.) as spherical bodies denser than the protoplasm. In the control organisms, i.e. those grown on buffered media and showing no volutin by Albert's method, no granules were observed by the phase contrast microscope.

3. Electron Microscope.

In a very few cases clear cut granules were observed but in the majority of the organisms the granules were blurred and indistinct. The only really good micrograph is shown. (Fig. xv.).

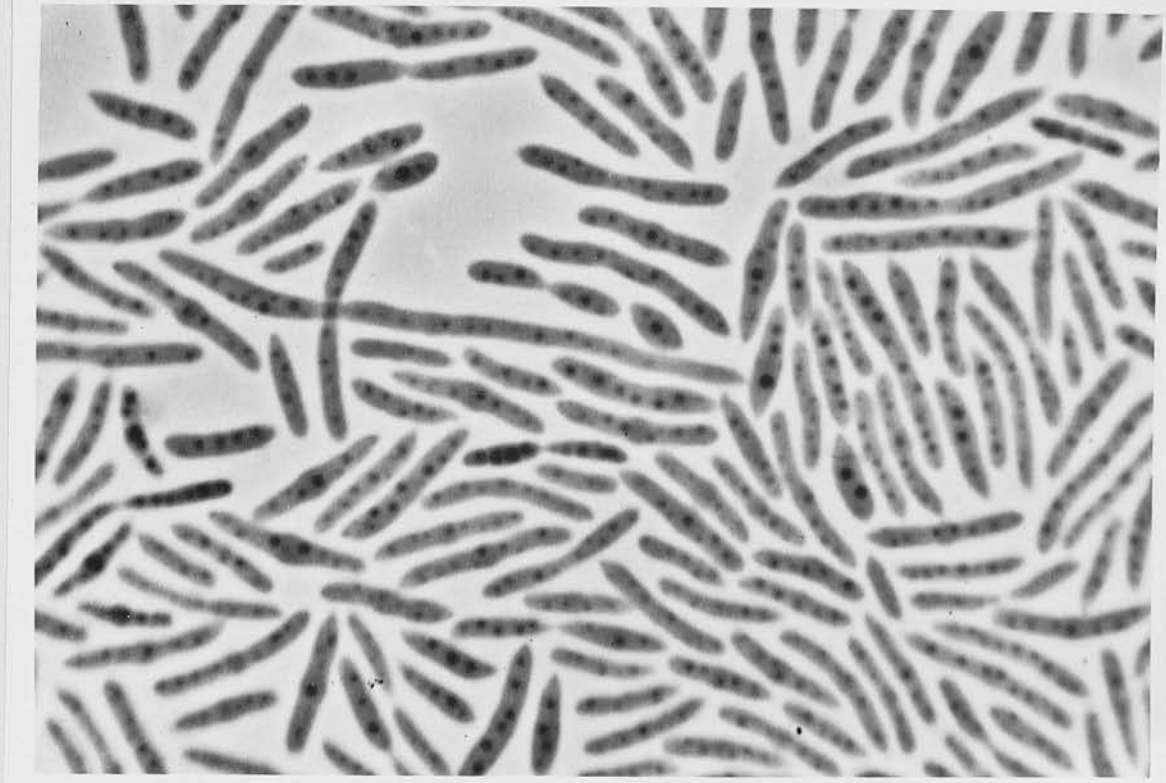


Fig. xiv. Aero. aerogenes A.3 showing volutin due to acidity developed in glucose media - unstained wet film in phase constrast microscope.

Strain grown on 0.01% phosphate, 0.1% glucose, 0.5% ammonium sulphate, salts agar for 24 hours at 35°C. Photograph taken by Dr Duguid. x 3,000

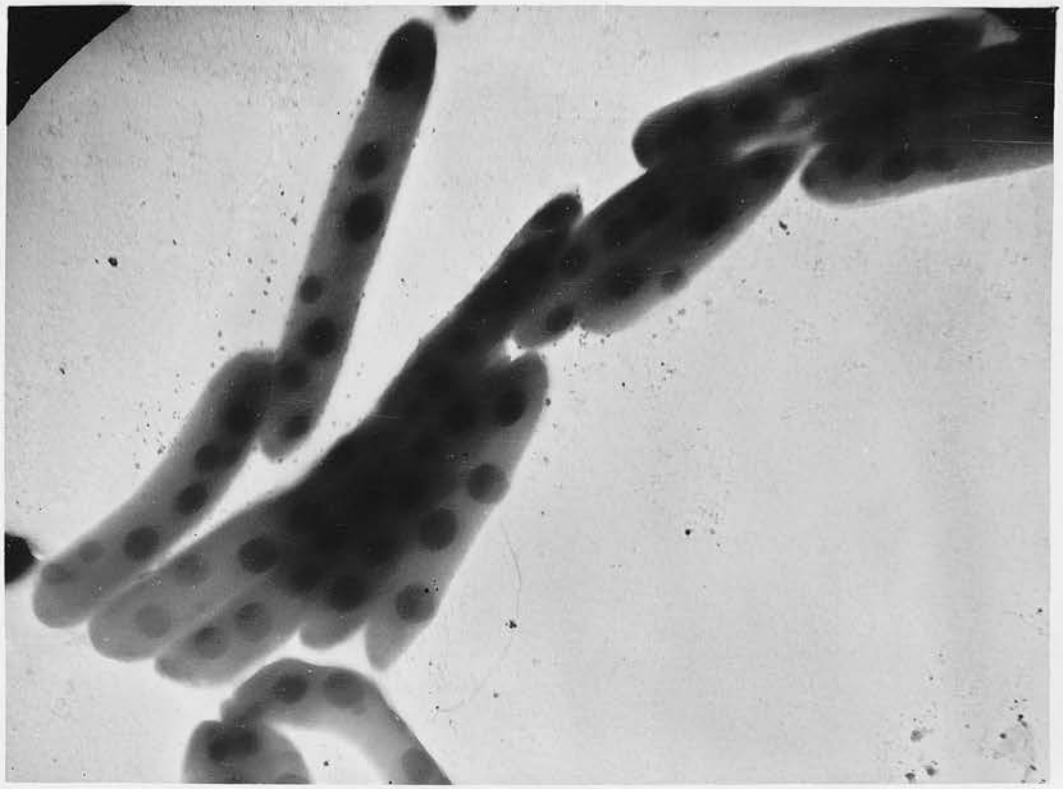


Fig. xv. Aero. aerogenes A.3 showing volutin due to acidity developed in glucose media - unshadowed preparation in electron microscope.

Strain grown on 0.01% phosphate, 0.1% glucose, 0.5% ammonium sulphate, salts agar for 24 hours at 35°C. Photograph taken by Dr Duguid. x 10,000

CHEMICAL OBSERVATIONS ON THE VOLUTIN PRODUCED ON POORLY-BUFFERED SUGAR CONTAINING MEDIA.

To study the ratio of total phosphorus to total nitrogen content of the cultures.

In order that the total phosphorus to total nitrogen ratios for volutin-rich and volutin-free cells might be estimated, the growths from 10 or more 50 ml. plates of 0.01 per cent phosphate media for the volutin-rich cells or 4 or more 50 ml. plates of 0.1 per cent phosphate media for the volutin-free cells were pooled. After the cultures had been examined for the presence or absence of volutin, the growth was washed off in saline, centrifuged, washed in saline and resuspended in a known volume of saline. The resulting opacity of the suspension was measured and the final pH of the media found, suitable aliquots of the suspension being then examined for their total phosphorus and total nitrogen contents. The results are shown in table XV. where the amounts of phosphorus and nitrogen are expressed as mg. per ml. of a suspension whose opacity was 1.

When the medium contained 0.01 per cent phosphate, the pH had fallen to 4.3 after 24 hours growth and much volutin was produced. The

TABLE XV.

Comparison of the phosphorus and nitrogen contents and the ratio of total phosphorus to total nitrogen in volutin-rich cells grown on a poorly-buffered medium containing 0.01 per cent phosphate, 0.1% glucose, 0.5% ammonium sulphate, salts agar at 35°C for 24 hours with volutin-free cells from a similar medium buffered by the presence of 0.1 or 0.2% phosphate or 0.01% phosphate and 0.05% citrate. The phosphorus and nitrogen contents are expressed as mg/ml. of a suspension whose opacity is 1.

% phosphate in medium	Volutin-rich cells			% phosphate in medium	Volutin-free cells.		
	mg P/ml	mg N/ml	P/N		mg P/ml	mg N/ml	P/N
0.01	0.016223	0.04035	0.40	0.1	0.009385	0.03898	0.24
	0.01820	0.03455	0.53		0.008034	0.0385	0.21
	0.01720	0.03681	0.47		0.004109	0.03458	0.19
	0.01455	0.03643	0.48		0.011840	0.04403	0.27
	0.01588	0.03789	0.42		0.01032	0.04964	0.21
	0.01570	0.04423	0.36		0.009012	0.03994	0.23
	0.01460	0.03814	0.38		0.007409	0.04214	0.18
	0.01319	0.04134	0.32		0.008995	0.03856	0.23
	0.01655	0.04218	0.39		0.006005	0.03726	0.16
	0.01765	0.04700	0.38		0.01051	0.04514	0.23
	0.01022	0.03291	0.31	0.2	0.006441	0.0306	0.21
	0.02060	0.05691	0.36	0.01 + 0.05% citrate	0.004453	0.0347	0.13
	0.01610	0.03856	0.42		0.005345	0.03557	0.15
Average	0.01595	0.04074	0.40	Average	0.00809	0.03967	0.20

phosphorus to nitrogen ratio was found to be between 0.31 and 0.53 for 13 estimations, the average being 0.40. The volutin-free cells were grown on 0.1 per cent phosphate medium, the pH of which had not fallen below 6.0 by 24 hours. Here the phosphorus to nitrogen ratio was found to vary from 0.16 - 0.27 for 10 estimations, the average being 0.22. One estimation was carried out with 0.2 per cent phosphate in the medium and the ratio was 0.21. Finally the volutin-free cells were prepared by growth on a medium containing 0.01 per cent phosphate buffered by the presence of 0.05 per cent of citrate. Here the 2 ratios were 0.13 and 0.15 average 0.14. The average ratio for the 13 volutin-free estimations was 0.20.

This would appear to show that the production of volutin by the cells was correlated with the presence of extra phosphorus per unit of nitrogen in the cell. It is of interest to note that the amount of nitrogen in the cells measured as mg. per ml. of suspension with an opacity reading of 1 is the same for both the volutin-rich and volutin-free cells despite their difference in cell length and probably refractility.

The distribution of the phosphorus compounds in the volutin-rich and volutin-free cultures.

The growths from a large number of plates (40 of 0.1 per cent phosphate and 120 of 0.01 per cent phosphate) were washed off in saline, centrifuged, washed and resuspended in a known volume of saline. The pH and presence or absence of volutin was checked and the resulting opacity of the saline suspension found. The phosphorus to nitrogen ratio of the suspension was estimated and, after analysis of the suspension according to the method of Juni et al. (1948), the amount of phosphorus in each fraction was found. The results for 3 volutin-free and 6 volutin-rich cultures are shown in table XVI. where the amount of phosphorus in each fraction is expressed as a percentage. In table XVII. the amount of phosphorus is expressed as µg. phosphorus per mg. nitrogen.

The percentages of phosphorus (table XVI.) found in the same fractions of different estimations varied to some extent, the S2 fraction of both the volutin-rich and volutin-free cultures being one such example. This may have been due to an insufficient washing of the barium precipitate, which was precipitated out by sulphate, resulting

TABLE XVI.

Comparison of the distribution of phosphorus compounds (expressed as percentage of phosphorus) in volutin-free cells grown on 0.1% phosphate, 0.1% glucose, 0.5% ammonium sulphate, salts agar at 35°C for 24 hours with volutin-rich cells from a similar medium which was poorly-buffered due to the presence of only 0.01% phosphate.

% PHOSPHATE IN MEDIUM	FINAL PH OF MEDIUM.	P/N	% RECOVERY OF PHOSPHORUS	T.C.A. SOLUBLE			T.C.A. INSOLUBLE						
				Mg MIXTURE SOLUBLE	Mg. MIXTURE PRECIPITATE		ALCOHOL ETHER SOLUBLE	ALCOHOL / ETHER INSOLUBLE					
					Ba. pH 2.5 SUPERNATANT	Ba pH 2.5 PRECIPITATE		KOH SOLUBLE			KOH INSOLUBLE		
								T.C.A. pH 2.5 SOLUBLE		T.C.A. pH 2.5 INSOLUBLE	10% T.C.A. SOLUBLE		10% T.C.A. INSOLUBLE
								Mg. MIXTURE SUPERNATANT	Mg. MIXTURE PRECIPITATE		Ba pH 3.5 SUPERNATANT	Ba pH 3.5 PRECIPITATE	
				S_2	S_3	R_3	S_4	S_5	R_5	R_6	S_9	R_9	R_8
				VOLUTIN – FREE CELLS									
0.2	6.6	0.21	90.2	20.8	2.5	0.5	4.8	43.4	0.8	1.62	12.4	0	2.5
0.1	6.0	0.16	86.4	14.4	1.4	0.5	6.5	44.5	0.4	4.4	11.2	0	2.9
0.1	6.2	0.23	86.2	9.3	3.2	0.4	8.9	45.4	0	5.5	9.6	0	2.8
				VOLUTIN – RICH CELLS.									
0.01	4.3	0.39	81.2	3.9	1.8	0.4	4.4	40.2	0.9	1.0	1.1	13.6	13.6
0.01	4.3	0.38	90.4	8.6	3.4	2.9	3.0	56.6	0.84	3.9	1.8	7.3	1.4
0.01	4.3	0.24	65.6	13.0	1.2	0.3	3.1	34.5	0.88	5.6	1.4	0.8	1.5
0.01	4.3	0.38	44.2	6.0	4.9	0.5	2.6	48.1	2.9	3.2	1.9	2.4	1.4
0.01	4.3	0.36	96.6	6.5	4.2	1.5	5.1	65.0	2.6	3.8	3.2	0	1.4
0.01	4.3	0.31	83.9	6.5	4.9	0.3	4.4	39.4	1.4	4.5	2.9	15.2	4.4

T.C.A. = TRICHLORACETIC ACID.

TABLE XVII.

Comparison of the distribution of phosphorus compounds (expressed as μg P/mgN) in volutin-free cells grown on 0.1% phosphate, 0.1% glucose, 0.5% ammonium sulphate salts agar at 35°C for 24 hours with volutin-rich cells from a similar medium which was poorly buffered due to the presence of only 0.01% phosphate.

% PHOSPHATE IN MEDIUM	FINAL PH OF MEDIUM	P/N	% RECOVERY OF PHOSPHORUS	FRACTION.									
				S_2	S_3	R_3	S_4	S_4	R_4	R_6	S_9	R_9	R_8
				VOLUTIN- FREE CELLS.									
0.2	6.6	0.21	90.2	44	5.4	1.0	10.1	91.6	1.4	3.4	26.1	0	5.3
0.1	6.0	0.16	86.4	23.4	2.4	0.8	10.5	41.2	1.1	4.6	18.1	0	4.6
0.1	6.2	0.23	86.2	21.8	4.5	1.6	20.8	104.1	0	12.4	22.1	0	6.5
				VOLUTIN- RICH CELLS.									
0.01	4.3	0.39	81.2	16.5	4.0	2.9	14.0	154.6	3.6	3.9	0.4	53.2	53.2
0.01	4.3	0.38	90.4	32.3	13.4	10.9	11.1	212.2	3.3	14.4	6.4	24.5	6.5
0.01	4.3	0.24	65.6	34.8	3.2	0.4	8.3	101.3	2.3	15.0	4.6	2.1	3.9
0.01	4.3	0.38	44.2	23.4	4.0	1.8	9.9	189.4	11.1	12.4	4.4	9.1	6.5
0.01	4.3	0.36	96.6	23.4	25.9	5.4	18.1	233.6	9.3	13.1	11.5	0	6.1
0.01	4.3	0.31	83.9	20.5	15.4	0.8	13.4	124	4.6	13.8	9.0	48.0	13.8

in a loss of phosphate as barium phosphate. Despite the variations, however, some general trends can be found.

The percentage of phosphorus in S2, which is free nucleotide according to Mitchell (1949), S4-phospholipid, and the unidentified fraction S9 are all reduced in the volutin-rich cells as compared with the volutin-free ones. This may have resulted from a blocking of an enzyme system which built up complex organic phosphorus compounds from relatively simple ones, due to the acidity developed during growth. The nucleo-phosphorus fractions (S7 ribo- and R6 deoxyribo-) are practically identical in both the volutin-rich and volutin-free cultures. The inorganic acid-soluble fraction S3 is increased but this is probably due to an increase in contamination with phosphate from the medium. The pyrophosphate and phosphoprotein fration R7 is increased about 3 times in the volutin-rich cultures and this may have been due to the piling up of phosphate compounds when the enzyme system was blocked. The unidentified fraction R8, and the acid-soluble metaphosphate fraction R3 both appear to show increases in the volutin-rich

cultures but due to the variability of the results too much stress cannot be laid on these results. The fraction which shows the greatest increase is the acid-insoluble fraction R9. In the volutin-free cells no precipitate was given with the barium at pH 3.5 but with the volutin-rich cells, in all cases but one, a phosphorus-containing precipitate was given. The amount of phosphorus in the R9 fraction of the volutin-rich cells varied, but in three cases quite a considerable amount was found.

As the only fraction showing any considerable change in phosphorus content was R9 this might point to the correlation between the production of volutin and the presence of extra phosphorus in the cell. Juni et al. (1948) stated that this fraction from yeasts was metachromatic in nature but unfortunately the amount of phosphorus obtained in a fractionation of Aero. aerogenes was insufficently concentrated to carry out a metachromatic reaction.

In considering the absolute amounts of phosphorus in each fraction (table XVII.) it will be seen that the actual amount of phosphorus in fractions S2, S4 and R8 are about the same in volutin-rich as in volutin-free cultures. S3, R3,

R7 and R6 are slightly increased and S9 is decreased. The biggest difference is, however, found in the two fractions S7 and R9. The S7 represents the ribonucleic acid phosphorus and R9 the acid-insoluble metaphosphate, but as the R9 fraction represents as increase from zero it is the greater and therefore this presence of acid-insoluble metaphosphate may be correlated with the presence of volutin.

(b) THE INFLUENCE OF A DEFICIENCY IN ONE OF THE COMPONENTS OF THE MEDIUM ON THE PRODUCTION OF VOLUTIN.

METHODS.

Strain:- The observations were all made using the Aero. aerogenes strain A3.

Medium:- In order to obtain media deficient in nitrogen and phosphate the amounts of these components were greatly reduced. 100 mls. of the nitrogen deficient medium contained:-

1.0gm.	Phosphate. (1 part KH_2PO_4 to 3 parts NaH_2PO_4 giving a pH of 7.3).
0.001gm.	Ammonium sulphate.
0.1gm.	Glucose.
0.2gm.	Sodium chloride.
0.1gm.	Potassium sulphate.
0.01gm.	Magnesium sulphate.
0.0001gm.	Calcium chloride.
0.0001gm.	Ferrous sulphate.
2gm.	Washed agar.

100mls. of the phosphate deficient medium contained:-

0.0001gm.	Phosphate (1 part KH_2PO_4 to 3 parts Na_2HPO_4 giving a pH of 7.3).

0.5gm. Ammonium sulphate.
0.1gm. Glucose.
0.2gm. Sodium chloride.
0.1gm. Potassium sulphate.
0.01gm. Magnesium sulphate.
0.0001gm. Calcium chloride.
0.0001gm. Ferrous sulphate.
2gm. Washed agar.

In order to obtain a sulphur deficient medium, all the sulphates were replaced by chlorides. 100mls. of the sulphur deficient medium contained:-

1.0gm. Phosphate (1 part KH_2PO_4 to 3 parts Na_2HPO_4 giving a pH of 7.3).
0.5gm. Ammonium chloride.
0.1gm. Glucose.
0.2gm. Sodium chloride.
0.1gm. Potassium chloride.
0.01gm. Magnesium chloride.
0.0001gm. Calcium chloride.
0.0001gm. Ferric chloride.
2gm. Washed agar.

The medium which was considered to be deficient in carbon and energy source, as it would support a

larger growth if a larger amount of sugar were added to it, had the following nutrients in 100mls.

1.0gm.	Phosphate (1 part KH_2PO_4 to 3 parts Na_2HPO_4 giving a pH of 7.3)
0.5gm.	Ammonium sulphate.
0.1gm.	Glucose.
0.2gm.	Sodium chloride.
0.1gm.	Potassium sulphate.
0.01gm.	Magnesium sulphate.
0.0001gm.	Calcium chloride.
0.0001gm.	Ferrous sulphate.
2gm.	Washed agar.

All the chemicals were A.R., the agar was washed in the usual manner and the medium was sterilised by steaming for 1 hour. This latter medium was also used as the "Second Medium" as it contained sufficient nitrogen, phosphate and sulphur for the full utilisation of the sugar.

Incubation:- The nitrogen, sulphur and carbon and energy deficient media were poured in 50ml. amounts into Petri dishes, inoculated and incubated as in Section A. When the phosphate deficient medium was being studied it had to be buffered by either the

addition of "citrate" or by the use of a bocarbonate-carbon dioxide buffering mixture. The medium buffered by "citrate" was poured into Petri dishes in 50 ml. amounts and treated as above while the bicarbonate was added to 25 mls. of media in a 120 ml. bottle which was incubated in a sealed tank containing the additional carbon dioxide.

Estimation of the pH, amount of growth and volutin production.

This was carried out in the same manner as in Section A.

Estimation of the total phosphorus and total nitrogen content and the distribution of the phosphorus compounds in nitrogen-deficient cells.

In order to obtain a good yield of nitrogen-deficient cells Aero. aerogenes A3R was cultured in liquid synthetic media five litres of which contained the following nutrients.

50 gm.	Phosphate mixture pH 7.3 (3 parts Na_2HPO_4 to 1 part KH_2PO_4).
0.05 gm.	Ammonium sulphate.
10 gm.	Glucose.
10 gm.	Sodium chloride.
5 gm.	Potassium sulphate.

0.5 gm. Magnesium sulphate.
0.005 gm. Calcium chloride.
0.005 gm. Ferrous sulphate.
0.005 gm. Manganous sulphate.
0.005 gm. Cobalt nitrate.

All the chemicals were A.R. and the medium was sterilised by steaming for 1½ hours.

The medium was inoculated with a saline suspension of A3R and the flask placed in a 37°C water bath for 24 hours. In order to give sufficient aeration, air was bubbled in through a sterile cotton wool filter.

The growth was partially spun down in the Sharples super centrifuge but as this did not remove all the media, the spinning was completed in an angle head centrifuge. The residue was washed twice in saline and analysed as by Juni et al. (1948). The phosphorus and nitrogen contents were estimated as in Section A.

Aero. aerogenes A3R was used in this case as it reacted to a deficiency of nitrogen in the same way as the capsulate Aero. aerogenes A3 but due to its lack of capsules it could be spun down much more easily.

Influence of nitrogen, phosphate, sulphur and carbon and energy deficiency on volutin production.

The influence of deficiencies in the nitrogen, phosphate, sulphur and carbon and energy contents of the media on the production of volutin by Aero. aerogenes A3 was studied and the results are shown in table XVIII. As in the case of the carbon and energy deficient media the growth was maximal for the amount of glucose in the medium and all the different types of media contained the same amount of sugar this amount was regarded as full growth for this experiment. It will be seen that the amount of growth as expressed as an opacity measurement, for the nitrogen, phosphate and sulphur deficient media was less than that of the full growth although that obtained on the sulphur deficient medium was almost equal to full growth. The capsules of the sulphur-deficient cells were very large and this would tend to increase the opacity slightly but it would not account for the large growth. As no sulphur was added to the medium, there must have been a good deal of contamination sulphur from the glucose and even more from the inoculum. The nitrogen, sulphur and carbon and energy-deficient cultures

TABLE XVIII.

The influence of deficiencies in the nitrogen, phosphate, sulphur and carbon and energy sources on the growth, pH and volutin production by Aero.aerogenes A3 when grown on a buffered, glucose containing medium at 35°C.

TIME HOURS	MEDIUM DEFICIENT IN							
	NITROGEN SOURCE		PHOSPHATE SOURCE		SULPHUR SOURCE		CARBON AND ENERGY SOURCE	
	pH	VOL.	pH	VOL	pH	VOL	pH	VOL.
0	6.9	–	7.3	–	7.2	–	7.2	–
6		–					7.2	–
16		+					7.1	–
24	6.9	+	6.8	–	7.1	++	7.1	–
48	6.9	++	6.5	–	7.1	+	7.0	–
72	6.9	–	6.6	–	7.1	–	7.0	–
OPACITY	0.112		0.316		0.636		0.760	

VOL. = AMOUNT OF VOLUTIN PRODUCED.

were buffered by the addition of 1.0 per cent phosphate and the phosphate-deficient culture was buffered by the addition of "citrate" so there was no question of a drop in pH causing the formation of acid volutin. Volutin was found to be produced in the later stages of culture on both the nitrogen and sulphur deficient media but not on the phosphate or carbon and energy deficient media. The nitrogen-deficient volutin was produced by 24 hours; rose to a maximum by 48 hours (see fig. xvi.) and disappeared by 72 hours. The sulphur-deficient volutin was maximum at 24 hours (see figs. xvii.-xix.) but again fell away to nothing at 72 hours. The nitrogen, sulphur and phosphate-deficient cells all showed the "nuclear" appearance typical of deficient cells while those cells which were deficient in carbon and energy source did not lose their cytoplasmic stainability and therefore appeared to be quite normal cytologically.

Influence of transferring the nitrogen, phosphate, sulphur and carbon and energy deficient cells to a "Second Medium".

The 24-hour volutin-containing cells from the nitrogen and sulphur-deficient media and the 48 hour non-volutin containing cells from the phosphate-

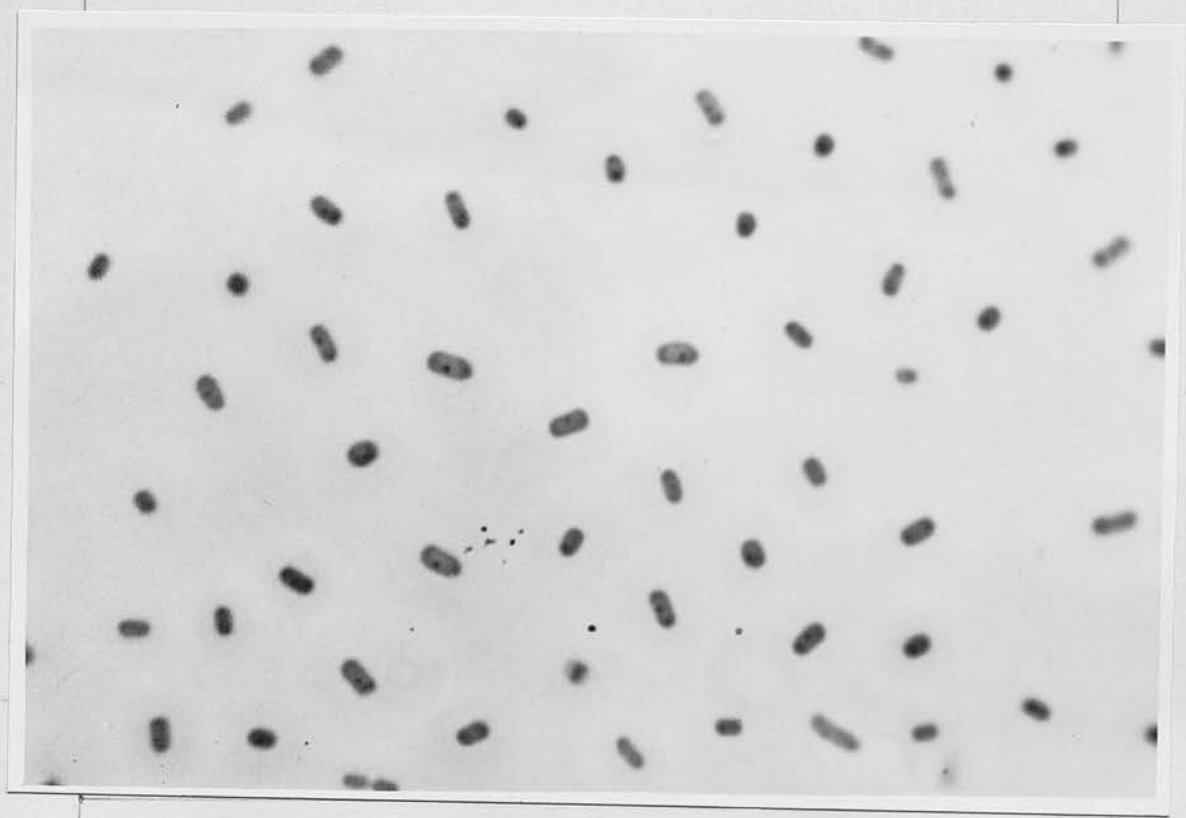


Fig. xvi. Aero. aerogenes A.3 showing volutin due to nitrogen deficiency - unstained wet film in phase contrast microscope.

Strain grown on 1.0% phosphate, 0.1% sucrose, 0.001% ammonium sulphate, salts agar for 48 hours at 35°C. Photographed by Dr Duguid. x 3,000

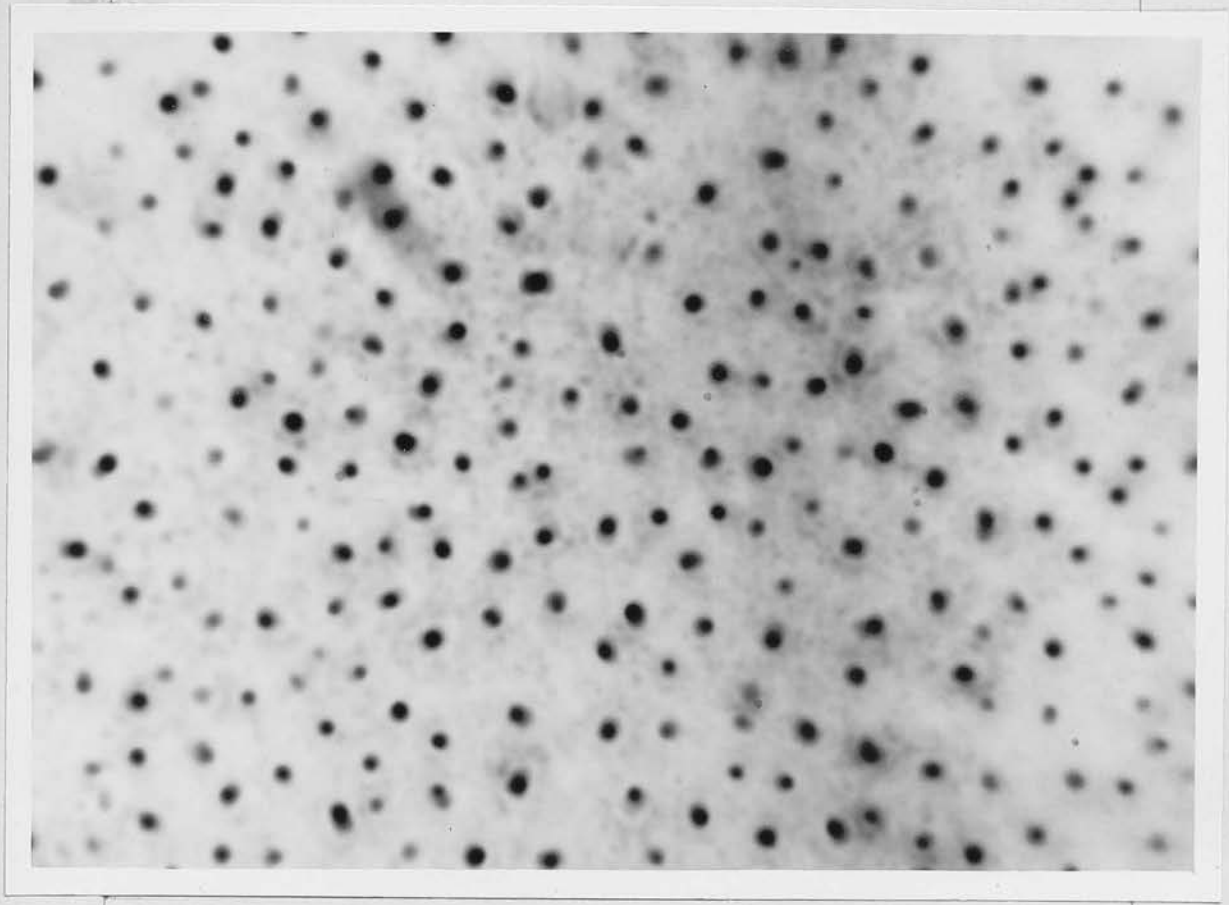


Fig. xvii. Aero. aerogenes A.3 showing volutin due to sulphur deficiency - Albert's stain.

Strain grown on 1.0% phosphate, 0.1% sucrose, 0.1% ammonium chloride, salts agar for 24 hours at 35°C. (All the sulphates were replaced by chlorides here). x 3,000

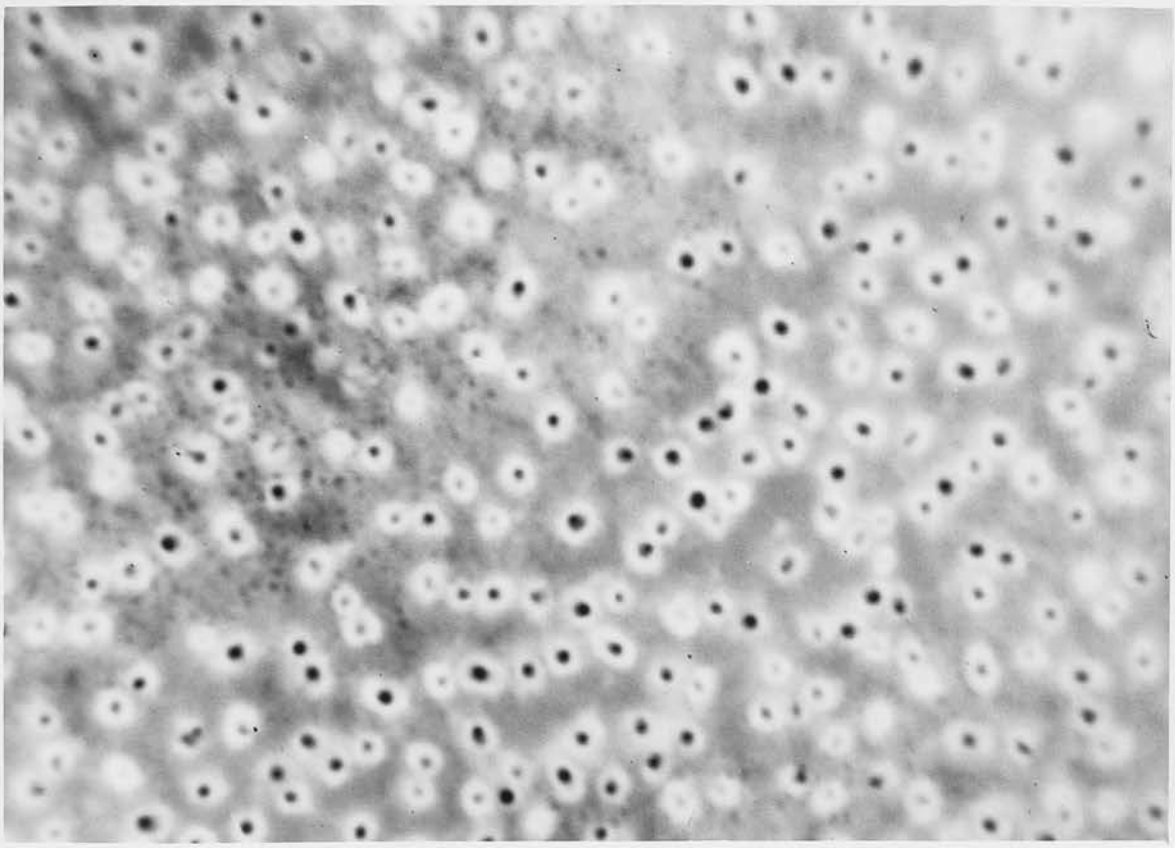


Fig. xviii. Aero. aerogenes A.3 showing volutin due to sulphur deficiency - Albert - Nigrosin stain.

Strain grown on 1.0% phosphate, 0.1% sucrose, 0.1% ammonium chloride, sulphate-free salts agar for 24 hours at 35°C. x 3,000

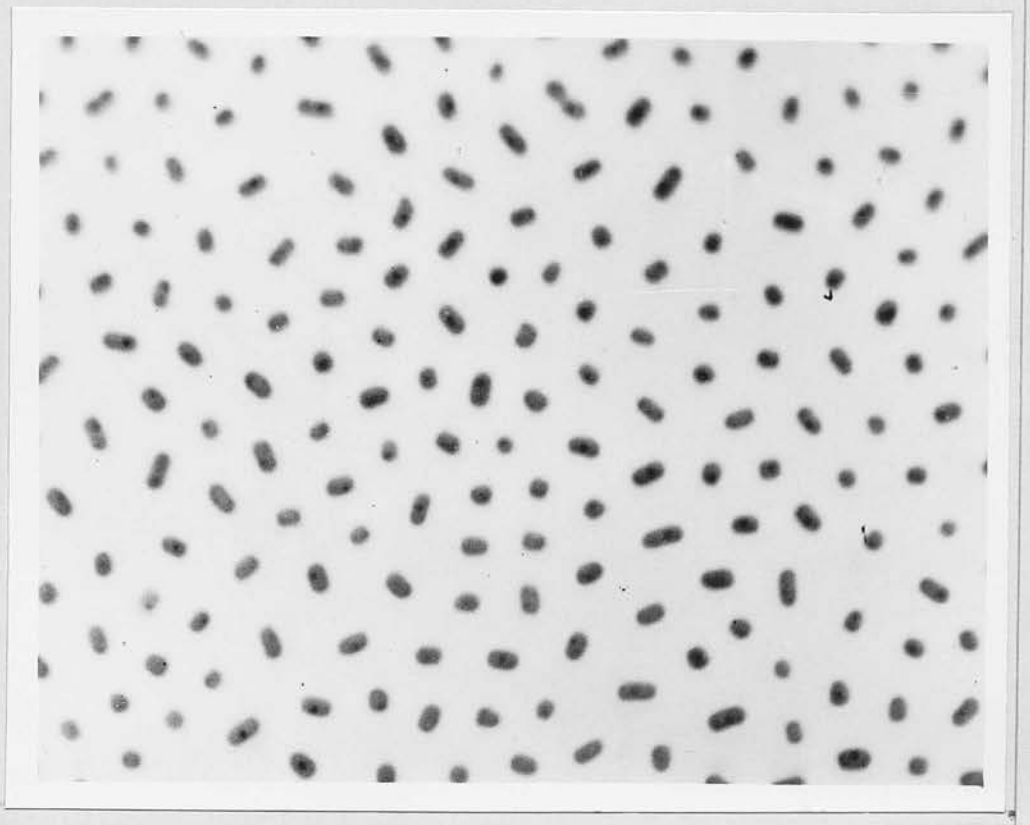


Fig. xix. Aero. aerogenes A.3 showing volutin due to sulphur deficiency - unstained wet film in phase contrast microscope.

Strain grown on 1.0% phosphate, 0.1% sucrose, 0.1% ammonium chloride, sulphate-free salts agar for 24 hours at 35°C. Photographed by Dr Duguid.

x 3,000

deficient and carbon and energy deficient media were sub-cultured on a "Second Medium" and the results are shown in table XIX. The volutin content of the nitrogen and sulphur-deficient cells did not appear to increase or to decrease markedly until 1 hour after transfer when it began to decrease. At 2 hours, the cytoplasm had become slightly stainable and by 4 hours the volutin had completely disappeared leaving normal cells. The initial and final pH of the "Second Medium" lay between 6.7 and 7.2 and the growth on the "Second Medium" was maximal. This means that the transfer of nitrogen and sulphur-deficient cells to a complete medium results in a disappearance of the volutin when growth occurs.

On the other hand, when the phosphate-deficient cells which did not contain volutin were transferred to the complete medium volutin was produced in ¼ hour, rose to a maximum at 2 hours and gradually fell away to none at 24 hours in parallel with an increasing amount of stainability of the cytoplasm. This phenomenon will be dealt with more fully in the following section.

The transfer of the carbon and energy deficient-cells to a "Second Medium" did not cause any apparent cytological change and no volutin was formed.

TABLE XIX.

The effect of transferring 24 hr. cultures of nitrogen and sulphur deficient organisms and 48 hr. phosphate and carbon and energy deficient cultures of Aero. aerogenes A3 to a complete medium at 35°C.

TIME ON "SECOND MEDIUM" HOURS.	"FIRST MEDIUM DEFICIENT IN							
	NITROGEN SOURCE		PHOSPHATE SOURCE		SULPHUR SOURCE		CARBON AND ENERGY SOURCE	
	C.S.	VOL.	C.S.	VOL.	C.S.	VOL.	C.S.	VOL.
0	⊥	++	⊥	−	−	++	+	−
1/4	⊥	++	−	+	−	+⊥	+	−
1	⊥	++	−	++	−	+⊥	+	−
2	+	+	−	+++	⊥	+	+	−
4	+	−	+	+	+	−	+	−
8	+	−	+	⊥	+	−	+	−
24	+	−	+	−	+	−	+	−
OPACITY "FIRST MEDIUM"	0.152		0.158		0.542		0.832	
INITIAL pH " FIRST MEDIUM "	4.2		Ca 4.5		4.1		Ca 4.5	
FINAL pH "FIRST MEDIUM"	6.8		6.4		6.9		6.8	
FINAL pH "SECOND MEDIUM"	4.0		4.1		4.0		6.8	

VOL. = AMOUNT OF VOLUTIN PRODUCED.

C.S. = CYTOPLASMIC STAIN ABILITY.

CYTOLOGICAL OBSERVATIONS ON NITROGEN- AND SULPHUR-DEFICIENT VOLUTIN.

1(a) Optical microscope - Albert's method (modified by Laybourn 1924).

The cells produced in both cases were nuclear i.e. their cytoplasm did not stain. They consisted of a central body surrounded by a colorless area in turn surrounded by an amorphous capsular material. When the cell contained volutin the central granule stained black. The capsular material assumed a purplish-brown colour. (See fig. xvii.).

1(b) Optical microscope - Albert - Nigrosin method.

By overspreading the Albert stained smear with nigrosin the capsular material was more sharply defined. (See fig. xviii.).

1(c) Optical microscope - Metachromatic stains.

In both cases, the capsule stained a purplish violet and the granules a dark blue with Loeffler's methylene blue. On treatment with 1 per cent sulphuric acid for 1 minute the capsules are destained leaving only either pale blue or differentiated dark and pale blue granules. The application of 20 per cent sulphuric acid for 1 minute completely destained the whole smear in the case of the nitrogen-deficient cells but with the sulphur-deficient cells the

the granules remained a very pale blue.

1(d) Optical microscope - Carbol fuchsin and sulphuric acid.

The carbol fuchsin gave a reddish-purple colour to the granules and a lighter red colour to the capsules. On treatment with 1 per cent sulphuric acid for 1 minute only the granules retained the stain.

1(e) Optical microscope - Periodate-Schiff method of Hotchkiss (1948)

No red staining appeared in the volutin granules.

1(f) Optical microscope - Gram's iodine.

No brown or blue granules indicating the presence of glycogen or iogen were observed.

1(g) Optical microscope - Burdon's fat stain (1946).

No black granules indicating the presence of fat were observed.

2. Phase Contrast Microscope.

When unstained, wet films of the volutin containing cells were examined by the Phase Contrast microscope, granules were observed (see figs. xvi. and xix.). In the control i.e. non volutin containing organisms no granules were observed.

3. Electron Microscope.

When unshadowed preparations were examined by the electron microscope, granules were observed in those cultures which were deficient in nitrogen and which showed volutin by Albert's stain.

CHEMICAL OBSERVATIONS ON NITROGEN-DEFICIENT CULTURES.

The distribution of the Phosphorus compounds in a nitrogen-deficient, volutin-containing culture (expressed as percentage of phosphorus) is shown in table XX together with the average distribution of the phosphorus compounds in phosphate-deficient and phosphate-sufficient cells. It will be seen that the pH did not fall below 6.9 in the nitrogen-deficient culture so that the presence of volutin could not have been due to a pH effect. The phosphorus to nitrogen ratio was 0.24 which is about 3 times that of the phosphate-deficient cells and 1½ times that of phosphate-sufficient cells neither of which contained volutin. The distribution of the phosphorus throughout the various fractions was similar to that of the phosphate-deficient and phosphate-sufficient cultures no difference being more than four fold except in the case of R9 (acid insoluble metophosphate) which showed a ten fold increase over that of the phosphate-deficient cells and a six fold increase over that of the phosphate-sufficient cells. The increase in the phosphorus content of the acid insoluble metaphosphate fraction

TABLE XX.

Comparison of the percentage distribution of phosphorus in the various fractions of a nitrogen-deficient, volutin-containing culture with that of a phosphate-deficient and a phosphate-sufficient non-volutin containing culture.

FRACTION	NITROGEN DEFICIENT CELLS.	PHOSPHATE DEFICIENT CELLS.	PHOSPHATE SUFFICIENT CELLS
S_2	12.58	14.53	5.6
S_3	8.61	2.30	4.2
R_3	0.52	0.46	0.9
S_4	3.89	6.96	5.9
S_5	44.95	43.45	44.8
R_5	0.40	0.54	0.8
R_6	8.04	9.24	8.4
S_9	5.50	2.92	6.3
R_9	3.38	0.31	0.5
R_8	1.29	2.14	1.9
TOTAL	89.16	83.18	99.3
P/N	0.24	0.086	0.15
av. pH.	6.9	6.4	6.9

PHOSPHATE DEFICIENT ≡ PHOSPHATE STARVED.

may therefore be connected with the presence of the volutin in the nitrogen-deficient culture.

(c) VOLUTIN PRODUCTION IN PHOSPHATE-STARVED CELLS ON TRANSFERENCE TO A PHOSPHATE-CONTAINING MEDIUM. (PHOSPHATE-STARVED=PHOSPHATE-DEFICIENT).

Strain:- Aero. aerogenes A3 was used for the most of the experiments. A few other strains were tested for their ability to form volutin under similar conditions.

Medium:- To obtain phosphate-starved cells only a small amount of phosphate was added to the "First Medium" 100 mls. of which contained various amounts of Phosphate (1 part KH_2PO_4 to 3 parts Na_2HPO_4 giving a pH of 7.3)

0.1gm. Glucose.
0.5gm. Ammonium sulphate.
0.2gm. Sodium chloride.
0.1gm. Potassium sulphate.
0.01gm. Magnesium sulphate.
0.0001gm. Calcium chloride.
0.0001gm. Ferrous sulphate.
2gm. Washed Agar.

All the chemicals were A.R; the agar was washed as before and the medium was sterilised by steaming for an hour.

In addition to the above nutrients a buffer was added.

This was either in the form of citric acid - sodium citrate mixture or a sodium bicarbonate - carbon dioxide system. In the first case the acid fermentation products of the sugar were balanced by the alkali released on metabolism of the "citrate". The citric acid - sodium citrate buffer was prepared by mixing 40mls. 25 per cent anhydrous citric acid with 15.2mls. 10N. sodium hydroxide. This gave a 1 per cent "citrate" buffer of pH 6.5 when included in one litre of the medium. It was found, however, that the final pH of the medium containing glucose and "citrate" was not constant for all the different phosphate concentrations employed so further experiments were carried out using the sodium bicarbonate - carbon dioxide buffering system, 0.3 per cent sodium bicarbonate being incorporated in the medium and 20 per cent carbon dioxide added to the air of a sealed tank containing the cultures.

The "Second Medium" contained 1.0 per cent phosphate (pH 7.3) and so did not require any further buffering agent. Otherwise it was the same as the "First Medium".

In order to obtain a very pure "Second Medium" a liquid medium was employed. The composition of this medium differed only in that it contained 0.1

per cent of ammonium sulphate instead of 0.5 per cent as in the solid media, but as 0.1 per cent ammonium sulphate was more than sufficient for complete growth this did not cause any discrepancies between the results from solid and liquid media.

Incubation:- When the "First Medium" contained "citrate" as buffer, 50 mls. of the medium were contained in a Petri dish; the plate inoculated with two loopfuls of a light saline suspension of the organism from an agar slope; three bent pins placed over the edge of the Petri dish thus holding the lid raised 1mm. in order to allow free aeration. The plate was incubated at 35°C for two days.

When the bicarbonate buffer was used, 25mls. of the "First Medium" were contained in a 120ml. flat bottle giving as large a surface as possible. The agar was inoculated with 0.5ml. of a saline suspension of standard opacity (approximately 10^7 per ml.). The open bottles were then placed in a glass tank or an anaerobic jar allowing more than one litre of air to each bottle. The calculated amount of marble chips and 2N hydrochloric acid to give 20 per cent carbon dioxide was placed in a bottle with a glass delivery tube inside the tank and the tank sealed with adhesive tape. The tank was incubated

at 35°C for two days.

The phosphate-starved cells were transferred from this "First Medium" to 25 mls. of a "Second Medium" contained in a Petri dish. A large loopful of the growth from the "First Medium" was evenly stroked over the surface of the agar and the plate incubated at 35°C.

When a liquid "Second Medium" was employed the phosphate-starved cells were grown on the surface of sterile 1 x 2 cm. strips of cellophane. These strips were placed on the agar and the surface well dried before inoculation with a light saline suspension of the organism from an agar slope culture. The plate of "First Medium" was incubated as above (the bicarbonate-carbon dioxide buffer being used). After two days growth at 35°C the strips were removed with sterile forceps and submerged in sterile saline to free the growth of any traces of the "First Medium". The strips were then transferred into a second dish of sterile saline and finally into the liquid medium contained in 5.5 cm. Petri dishes in 10 ml. amounts. These Petri dishes were then incubated at 35°C. If the liquid "Second Medium" had to be buffered with bicarbonate carbon dioxide mixture it was treated as the "First Medium" - see above.

Method for measuring the pH, amount of growth and volutin production.

This was carried out in the same manner as in Section A. When the liquid "Second Medium" was employed the cellophane strips were drawn across the slides thus giving a smear.

Method for the total and viable counts.

A loopful of the growth was suspended in a few ccs. of broth giving a uniform suspension, one ml. of which was then used to make ten fold dilutions in nutrient broth. These dilutions were performed in duplicate and 1 ml. of each was put in a Petri dish. Cooled, melted agar was added to the plates with mixing and the plates incubated at 35°C for two days when the number of colonies was counted and the viable count obtained. The total count was obtained in the usual manner.

Method for the estimation of the total nitrogen content of the cultures.

The growth from one 50 ml. agar plate was washed off in 50mls. of saline. 45 mls. of this were spun down, the residue washed once in saline and finally suspended in 25 mls. saline. After the resulting opacity had been measured, the nitrogen content of a suitable aliquot was estimated as in Section A.

Method for the estimation of the distribution of the phosphorus compounds, in the phosphate - starved cells.

As the number of plates which would be required to produce a sufficiently large growth of phosphate-starved cells for chemical analysis was so great, the cultures for such an experiment were grown in 5 litre amounts of liquid media, which contained the following nutrients:-

50 gm.	Citric acid.
29 gm.	Sodium hydroxide.
25 gm.	Ammonium sulphate.
20 gm.	Glucose.
10. gm..	Sodium chloride.
5 gm.	Potassium sulphate.
0.5 gm.	Magnesium sulphate.
0.005 gm.	Ferrous sulphate.
0.005 gm.	Calcium chloride.
0.005 gm.	Manganous sulphate.
0.005 gm.	Cobalt nitrate.
0.1 gm.	Glutamic acid.
0.1 gm.	Phosphate mixture pH 7.3 (3 parts Na_2HPO_4 to 1 part KH_2PO_4).

The pH of the medium was adjusted to 6.6 to 7.0 and the presence of the "citrate" was sufficient to

hold the pH above 6.5 for 24 hours growth at 37°C. The manganous and cobalt salts were added to the liquid medium because traces of these elements are normally found in agar. As the medium had to be aerated by bubbling in air through a sterile cotton wool filter, the glutamic acid was added to counteract any loss of carbon dioxide in the early stages of growth and in addition the air was not bubbled through until the culture had been incubated for 6 hours. For phosphate-starved cultures the growth was treated in the same manner as the nitrogen-deficient cultures in Section B except that here a nitrogen-salts mixture was used as the wash solution. For the production of volutin containing cells, the phosphate-starved cells were suspended in the 0.1 per cent nitrogen-salts solution after centrifuging in the angle head centrifuge, 0.1 per cent glucose and 0.1 per cent phosphate (pH 7.3) added and the flask placed in a 37°C water bath. (The culture was again aerated during the incubation). The culture was removed from the water bath after the required length of time, cooled and centrifuged. It was then washed twice in cold 0.1 per cent nitrogen-salts solution and treated as the nitrogen-deficient cells in Section B. For the production

of the control, phosphate-sufficient, non-volutin containing cells A3R was again cultured in liquid media but this time the medium contained 50 gms. of phosphate mixture pH 7.3 and only 5 grams of glucose. The phosphate was sufficient to buffer the medium so that no additional buffer was required. Such cells were also transferred to a phosphate containing "Second Medium" and were treated in the same manner as the phosphate-starved cells.

As in Section B the non-capsulate Aero. aerogenes A3R was used since it reacted to a deficiency of phosphate in like manner to the capsulate parent strain A3 but A3R could be spun down much more easily.

RESULTS.

The optimum incubation period for the production of viable phosphate-starved cells.

Initial experiments were carried out to determine the optimum period of incubation for the production of still viable, phosphate-starved cells and the results are shown in table XXI. The phosphate-starved cells grown on the medium containing only 0.0001 per cent phosphate were found to be about 100 per cent viable at 1 day and again at 2 days, but only about 70 per cent viable at 3 days. Since the amount of growth, as measured by the total count and the total nitrogen content was greater at 2 days than at 1 day on this phosphate poor medium it was decided to use the 2 day culture for the production of phosphate-starved cells. Phosphate-sated cells grown on a medium containing 0.1 per cent phosphate were also found to be 100 per cent viable at 2 days.

The influence of the phosphate content of the "First Medium" on volutin production.

Volutin production on the transference of cells from a "First Medium" containing various amounts of phosphate to a "Second Medium" containing a large amount of phosphate was studied. The results are shown in table XXII. In this case the "First Medium" contained glucose and was buffered with the

TABLE XXI.

The influence of the incubation period on the total count, viability and nitrogen content of phosphate-deficient* cells of Aero.aerogenes A3 grown on 0.0001% or 0.1% phosphate, 0.1% glucose, 0.5% ammonium sulphate, salts agar buffered with 0.3% sodium bicarbonate and 20% carbon dioxide at 35°C for 1 - 4 days.

TIME DAYS	MEDIUM CONTAINING 0.0001% PHOSPHATE		
	TOTAL COUNT	PERCENTAGE VIABILITY	TOTAL NITROGEN CONTENT
1	5×10^9	> 100	0.22mg.
2	4.4×10^9	92	0.39mg.
3	4.8×10^9	40	0.32mg.
4	4.1×10^9	6	0.38mg.

TIME DAYS	MEDIUM CONTAINING 0.1% PHOSPHATE		
	TOTAL COUNT	PERCENTAGE VIABILITY	TOTAL NITROGEN CONTENT
1	46.8×10^9	100	1.3mg.
2	46.0×10^9	100	1.3mg.
3	42.0×10^9	100	

* PHOSPHATE-DEFICIENT ≡ PHOSPHATE-STARVED.

TABLE XXII.

The effect on volutin production of the transference of Aero.aerogenes A3 cells from a glucose medium containing various concentrations of phosphate; buffered by means of a bicarbonate-carbon dioxide mixture after 2 days growth at 35°C to an identical medium containing 1% phosphate which acted as buffer.

TIME ON "SECOND MEDIUM"	PERCENTAGE pH 7·3 PHOSPHATE IN "FIRST MEDIUM"															
	0·00001		0·0001		0·0003		0·001		0·003		0·01		0·1		1·0	
HOURS	VOL.	C.S.	VOL.	C.S.	VOL.	C.S.	VOL.	C.S.	VOL.	C.S.	VOL.	C.S.	VOL.	C.S.	VOL.	C.S.
0	-	-	-	⊥	-	-	-	+	-	+	-	+	-	+	-	+
1/20	-	-	⊥	⊥	+	-	-	+	-	+	-	+	-	+	-	+
1/4	+	-	+	⊥	++	-	⊥	+	-	+	-	+	-	+	-	+
1	++	-	++	-	+++	-	+	+	-	+	-	+	-	+	-	+
2	+++	⊥	+++	-	+++	+	+	+	-	+	-	+	-	+	-	+
4	+	+	+	+	+	+	⊥	+	-	+	-	+	-	+	-	+
8	⊥	+	⊥	+	⊥	+	-	+	-	+	-	+	-	+	-	+
24	-	+	-	+	⊥	+	-	+	-	+	-	+	-	+	-	+
OPACITY "FIRST MEDIUM"	0·088		0·158		0·508		0·892		0·968		0·852		0·820		0·832	
INITIAL pH "FIRST MEDIUM"	ca 4·5		ca 4·5		ca 4·5		ca 4·5		ca 4·5		ca 4·5		ca 4·5		ca 4·5	
FINAL pH "FIRST MEDIUM"	6·8		6·4		6·8		6·8		6·8		6·4		6·8		6·8	
FINAL pH "SECOND MEDIUM"	4·1		4·1		4·1		4·0		6·8		6·9		6·8		6·8	

VOL. = AMOUNT OF VOLUTIN PRODUCED.

C.S. = CYTOPLASMIC STAINABILITY.

sodium bicarbonate - carbon dioxide buffer and culture on it was continued for 2 days. The "Second Medium" was used in plates of 25ml. amounts. The amounts of growth on the "First Medium" containing 0.001 per cent phosphate or more were maximal and nearly constant (opacities 0.77-0.89); on the media containing less phosphate (i.e. 0.0003, 0.0001 and 0.00001 per cent) the amount of growth was greatly reduced, the reduction being in proportion to the reduction in phosphate. Thus it was concluded that the cells grown on media with 0.0003 per cent phosphate or less were phosphate-starved and those grown on media with 0.001 per cent phosphate or more were phosphate-sated. The phosphate-sated cells from a "First Medium" with 0.001 per cent phosphate, or more, did not produce any volutin on transfer to the "Second Medium". On the other hand, the phosphate-starved cells from a "First Medium" with 0.0003 per cent phosphate or less produced much volutin on transfer (see figs. xx. and xxi.). These cells produced a little volutin within 3 minutes after transfer; the volutin content rose to a maximum in 2 hours and disappeared in the following few hours so that none remained at 24 hours. The initial pH of the "First Medium" was calculated to

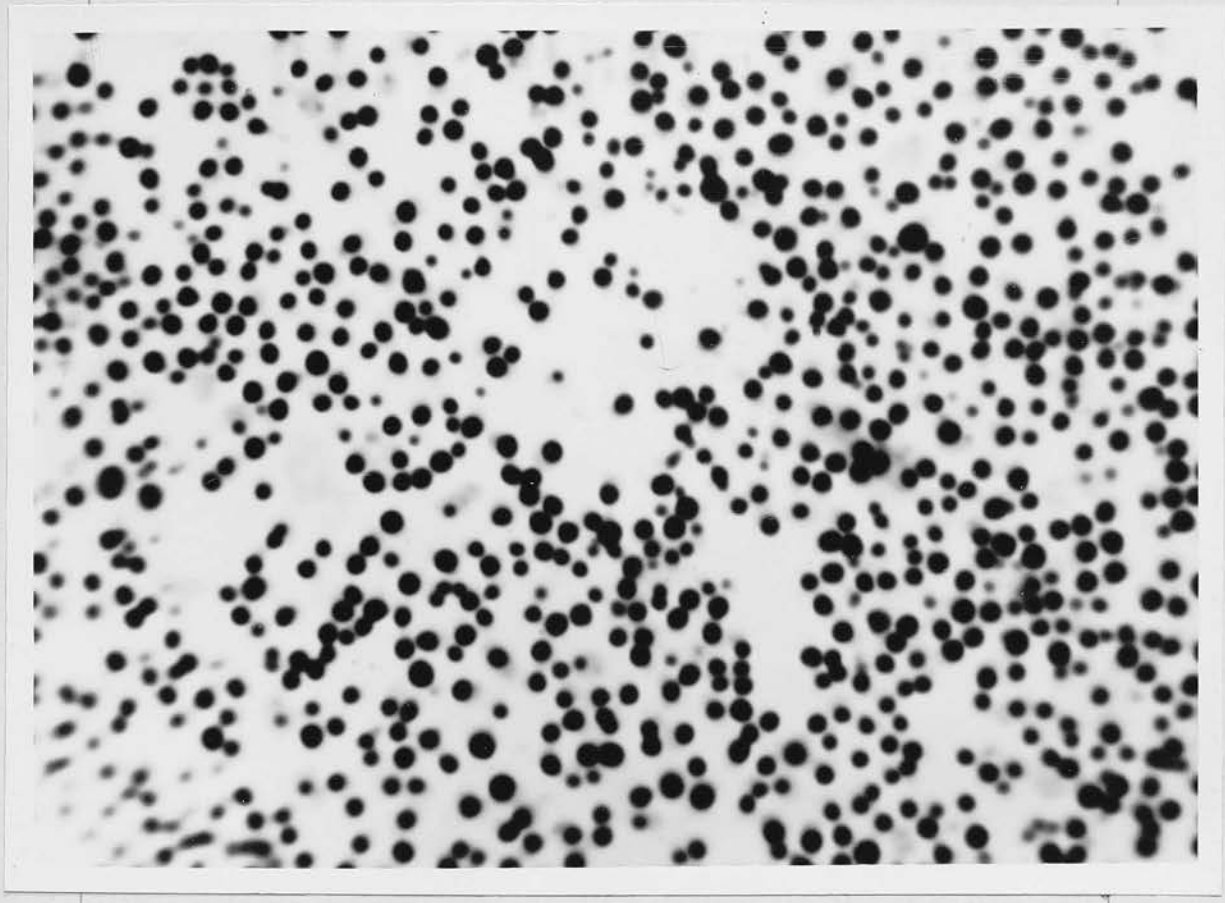


Fig. xx. Aero. aerogenes A.3 showing volutin due to addition of phosphate to phosphate-starved cells - Albert's stain.

Strain grown on 0.0001% phosphate, 0.1% glucose, 0.5% ammonium sulphate, salts agar buffered with 0.3% bicarbonate and 20% carbon dioxide for 48 hours at 35°C and sub-cultured on a similar medium containing 1.0% phosphate for 2 hours at 35°C x 3,000

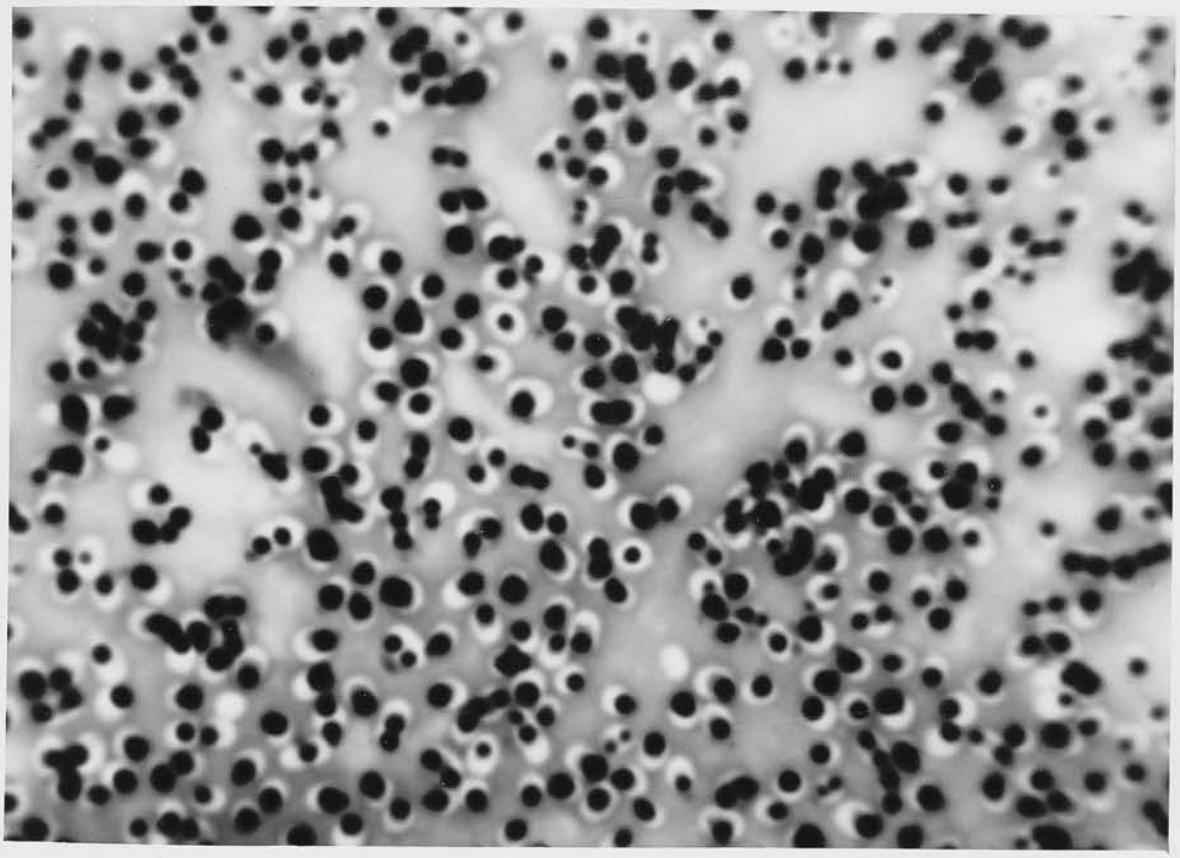


Fig. xxi. Aero. aerogenes A.3 showing volutin due to addition of phosphate to phosphate-starved cells - Albert - Nigrosin stain.

Strain grown on 0.0001% phosphate, 0.1% glucose, 0.5% ammonium sulphate, salts agar buffered with 0.3% bicarbonate and 20% carbon dioxide for 48 hours at 35°C and sub-cultured on a similar medium containing 1% phosphate for 2 hours at 35°C x 3,000

be 7.5; the final pH in all cases was found to be about 6.7 or 6.8. The initial pH of the "Second Medium" was 7.3 and its final pH lay between 6.8 and 7.1, so that volutin production could not have been the result of acidity. Initially the cells were observed to consist of a green granule surrounded by a clear unstained area which in turn was surrounded by a purplish-brown amorphous material. (Albert's stain). From this it was concluded that the cytoplasm was unstainable. On transfer of the cells, to a phosphate-rich medium the green granules became black due to the deposition of volutin but the cytoplasm remained unstained until about 4 hours after the transfer. This, it will be noted, corresponded to the time when the volutin disappeared from the cells. So that the disappearance of the volutin was accompanied with a return of the staining properties of the cytoplasm.

Similar results were obtained from further experiments which were made under slightly different conditions of culture. Table XXIII. shows the results of transferring the phosphate-starved cells to a solid-and-liquid type of "Second Medium". In this case a loopful of the phosphate-starved cells were emulsified in 0.5ml. of the liquid "Second

TABLE XXIII.

The influence on volutin production by Aero. aerogenes A3 cells of transfer from a glucose medium containing various concentrations of phosphate buffered by bicarbonate-carbon dioxide mixture after 2 days growth at 35° C to an identical medium containing 1% phosphate which acted as buffer. In this case the "Second Medium" was in the liquid-solid form.

Time on "Second Medium" Hours	Percentage pH 7·3 phosphate in "First Medium"															
	0·00001		0·0001		0·0003		0·001		0·003		0·01		0·1		1·0	
	Vol.	CS	Vol	CS	Vol	CS	Vol	CS	Vol	CS	Vol	CS	Vol	CS	Vol	CS
0	—	—	—	—	—	—	—	+	—	+	—	+	—	+	—	+
1/8	+	—	+	—	+	+	—	+	—	+	—	+	—	+	—	+
1/4	++	—	++	—	++	+	⊥	+	—	+	—	+	—	+	—	+
1/2	++	—	++	—	++	+	—	+	—	+	—	+	—	+	—	+
1	++	⊥	++	—	++	+	+	+	—	+	—	+	—	+	—	+
2	++	—	+++	+	+++	+	—	+	—	+	—	+	—	+	—	+
4	++	+	+++	+	+++	+	⊥	+	—	+	—	+	—	+	—	+
8	+	+	+	+	+	+	—	+	—	+	—	+	—	+	—	+
24	⊥	+	+	+	⊥	+	—	+	—	+	—	+	—	+	—	+
Opacity "First Medium"	0·020		0·096		0·346		0·892		0·992		0·968		1·080		0·840	
Initial pH "First Medium"	Ca 4·5		Ca 4·5		Ca 4·5		Ca 4·3		Ca 4·5		Ca 4·5		Ca 4·5		Ca 4·5	
Final pH "First Medium"	4·0		4·0		6·8		6·9		6·9		6·9		6·8		6·8	
Final pH "Second Medium"	4·2		4·2		4·2		4·2		4·2		4·2		4·2		4·2	

Vol. = amount of volutin produced.

C.S. = cytoplasmic stainability.

Medium" which was then superimposed on 10ml. of a comparable solid "Second Medium" - the idea being that as the nutrients were used up in the liquid, fresh ones would diffuse from the solid medium. Table XXIV. shows the results for the transfer of 3 day growths on a "First Medium" containing glucose and buffered by the addition of "citrate"; to a "Second Medium" (in plates), again volutin was produced by those cultures whose growths had been reduced by phosphate deficienty in the "First Medium". The pHs of the "First Medium" in this case were not nearly so constant varying between 6.6 and 7.5 initially and from 5.2 to 7.7 finally.

When the organism was cultured on a sucrose medium buffered by "citrate" incubation was continued for 3 days at 35°C before the cells were transferred to a "Second Medium". The results are shown in tables XXV. - XXVIII. The percentage of "citrate" ranges from 0.1 to 1.0 per cent but in all cases we find that the cells which were grown on a medium which curtailed growth, produced volutin on transfer. (The liquid-solid form of the "Second Medium" was used in these cases). It was noted that the final pH of the "First Medium" tended to vary a good deal and often went to the alkaline side, presumably due

TABLE XXIV.

The influence on the volutin production by Aero. aerogenes A3 of transfer from a glucose medium buffered by 0.1% citrate and containing various amounts of phosphate after 3 days growth at 35°C to a similar medium containing 1% phosphate which acted as buffer.

TIME ON "SECOND MEDIUM" HOURS	PERCENTAGE pH 7.3 PHOSPHATE IN "FIRST MEDIUM"															
	0.00001		0.0001		0.0003		0.001		0.003		0.01		0.1		1.0	
	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS
0	−	−	−	−	−	−	−	⊥	−	+	−	+	−	+	−	+
1/8	++	−	++	−	++	−	⊥	+	+	+	−	+	−	+	−	+
1/4	++	−	++	−	++	−	++	−	−	+	−	+	−	+	−	+
1/2	++	−	++	−	++	−	⊥	+	−	+	−	+	−	+	−	+
1	++	−	++	−	++	−	⊥	+	−	+	−	+	−	+	−	+
2	++	+	++	+	++	+	−	+	−	+	−	+	−	+	−	+
4	++	+	++	+	++	+	−	+	−	+	−	+	−	+	−	+
8	⊥	+	⊥	+	⊥	+	−	+	−	+	−	+	−	+	−	+
24	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+
OPACITY "FIRST MEDIUM"	0.046		0.156		0.424		0.806		0.956		1.136		1.086		1.194	
INITIAL pH "FIRST MEDIUM"	6.6		6.6		6.6		6.6		6.7		6.9		7.4		7.5	
FINAL pH "FIRST MEDIUM"	5.2		5.9		6.8		7.6		7.7		7.7		7.7		7.5	
FINAL pH "SECOND MEDIUM"	7.2		7.2		7.2		7.2		7.2		7.2		7.2		7.2	

VOL. = AMOUNT OF VOLUTIN PRODUCED

C.S. = CYTOPLASMIC STAINABILITY

TABLE XXV.

The influence on volutin production by Aero. aerogenes A3 of transfer of cells from a sucrose medium buffered by 0.1% citrate and containing various amounts of phosphate after 3 days growth at 35°C to a similar medium containing 1% phosphate which acted as buffer.

TIME ON "SECOND MEDIUM" HOURS	PERCENTAGE pH 7.3 PHOSPHATE IN "FIRST MEDIUM"													
	0.0001		0.0003		0.001		0.003		0.01		0.1		1.0	
	VOL.	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS
0	−	−	−	−	−	⊥	−	+	−	⊥	−	+	−	+
¼	++	−	++	−	+	+	⊥	+	−	+	−	+	−	+
1	++	−	++	−	+	+	+	+	⊥	+	−	+	−	+
2	++	+	++	⊥	++	+	+	+	−	+	−	+	−	+
4	+	+	+	+	+	+	−	+	−	+	−	+	−	+
24	−	+	⊥	+	⊥	+	−	+	−	+	−	+	−	+
OPACITY "FIRST MEDIUM"	0.144		0.264		0.30		0.576		0.404		0.412		0.454	
INITIAL pH "FIRST MEDIUM"	6.5		6.5		6.5		6.6		6.5		7.1		6.5	
FINAL pH "FIRST MEDIUM"	7.2		7.3		7.4		7.9		8.0		7.6		6.5	

VOL. = AMOUNT OF VOLUTIN PRODUCED

C.S. = CYTOPLASMIC STAINABILITY.

TABLE XXVI.

The influence on volutin production by Aero. aerogenes A3 of transfer of cells from a sucrose medium buffered by 0.1% citrate and containing various concentrations of phosphate after 3 days growth at 35°C to a similar medium containing 1% phosphate which acted as buffer.

TIME ON "SECOND MEDIUM" HOURS	PERCENTAGE pH 7.3 PHOSPHATE IN "FIRST MEDIUM"													
	0.0001		0.0003		0.001		0.003		0.01		0.1		1.0	
	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS
0	−	−	−	−	−	−	−	⊥	−	+	−	+	−	+
¼	++	−	+	−	+	−	+	+	−	+	−	+	−	+
1	++	−	++	−	++	−	+	+	⊥	+	−	+	−	+
2	++	−	++	−	++	−	+	−	⊥	+	−	+	−	+
4	++	+	++	+	+	+	−	+	−	+	−	+	−	+
24	−	+	−	+	−	+	−	+	−	+	−	+	−	+
OPACITY "FIRST MEDIUM"	0.228		0.406		0.496		0.884		0.930		1.492		1.556	
INITIAL pH "FIRST MEDIUM"	7.4		7.3		7.3		7.4		7.4		7.4		7.4	
FINAL pH "FIRST MEDIUM"	6.9		7.2		7.5		8.0		8.0		8.0		8.0	

VOL. = AMOUNT OF VOLUTIN PRODUCED.

C.S. = CYTOPLASMIC STAINABILITY.

TABLE XXVII.

The influence on volutin production by Aero. aerogenes A3 of transfer of cells from a sucrose medium containing 0.2% citrate as buffer and various concentrations of phosphate after 3 days growth at 35°C to a similar medium containing 1% phosphate which acted as buffer.

TIME ON "SECOND MEDIUM" HOURS	PERCENTAGE pH 7.3 PHOSPHATE IN "FIRST MEDIUM"													
	0.0001		0.0003		0.001		0.003		0.01		0.1		1.0	
	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS
0	—	—	?	—	—	—	—	—	—	+	—	+	—	+
¼	++	—	++	—	++	—	+	—	—	+	—	+	—	+
1	++	—	++	—	++	—	++	—	—	+	—	+	—	+
2	+++	—	+++	—	++	—	++	—	—	+	—	+	—	+
4	+++	—	++	—	++	+	+	+	—	+	—	+	—	+
24	—	+	—	+	—	+	—	+	—	+	—	+	—	+
OPACITY "FIRST MEDIUM"	0.204		0.324		0.446		0.714		1.062		1.198		1.288	
INITIAL pH "FIRST MEDIUM"	6.2		6.2		6.2		6.2		6.2		6.4		6.5	
FINAL pH "FIRST MEDIUM"	4.0		6.4		6.6		8.6		8.6		8.3		4.0	

VOL. = AMOUNT OF VOLUTIN PRODUCED.

C.S. = CYTOPLASMIC STAIN ABILITY.

TABLE XXVIII.

The influence on volutin production by Aero. aerogenes A3 cells of transfer from a sucrose medium containing 1.0% citrate as buffer and various concentrations of phosphate after 3 days growth at 35°C to a similar medium containing 1% phosphate which acted as buffer.

TIME ON "SECOND MEDIUM" HOURS	PERCENTAGE pH 7.3 PHOSPHATE IN THE "FIRST MEDIUM."											
	0.0001		0.0002		0.0004		0.001		0.01		1.0	
	VOL.	CS	VOL	CS	VOL.	CS	VOL	CS	VOL	CS	VOL	CS
0	−	−	−	−	−	−	−	+	−	+	−	+
¼	++	−	+ +	−	+ +	−	⊥	+	−	+	−	+
1	++	−	+ +	−	+ +	−	+	+	−	+	−	+
2	+++	−	+ +	−	+++	−	+	+	−	+	−	+
4	+++	⊥	+ +	−	+ +	−	+	+	−	+	−	+
24	−	+	−	+	−	+	−	+	−	+	−	+
OPACITY "FIRST MEDIUM"	0.088		0.162		0.284		0.654		1.006		1.428	
INITIAL pH "FIRST MEDIUM"	6.8		6.8		6.8		6.8		6.8		6.4	
FINAL pH "FIRST MEDIUM"	6.4		6.4		7.0		8.0		8.8		6.9	

VOL. = AMOUNT OF VOLUTIN PRODUCED

C.S. = CYTOPLASMIC STAINABILITY.

to the utilisation of the "citrate."

The amount of phosphate required in the "Second Medium" for volutin production.

The amount of phosphate necessary in the "Second Medium" for the production of volutin was studied both with sucrose and with glucose. Table XXIX. shows the results obtained when the cells were initially grown for 3 days on a sucrose and "citrate" medium containing 0.0001 per cent phosphate. The cells were transferred to a "Second Medium" containing the same nutrients, but with various amounts of phosphate. It will be seen that volutin was produced in all cases but most when the phosphate concentration was 0.01 per cent or greater. When there was less than 0.001 per cent phosphate in the "Second Medium" the cytoplasmic stainability did not become fully developed so it would appear that the cells still remained phosphate-starved to a certain extent, although not sufficiently so to prevent volutin synthesis. It will also be noted that the pH tends to fall at the lower levels of phosphate concentrations although not sufficiently to give a secondary formation of acid volutin.

When the "Second Medium" contained glucose instead of sucrose no buffer was added so that the

TABLE XXIX.

The influence of the amount of phosphate in the "Second Medium" on the volutin production by Aero.aerogenes A3 cells on transfer from a sucrose medium containing 1.0% citrate as buffer and 0.0001% phosphate after 3 days growth at 35°C to a similar medium with various concentrations of phosphate. The initial and final pHs of the "First Medium" were 6.8 and 6.6 respectively.

TIME ON "SECOND MEDIUM" HOURS.	PERCENTAGE pH 7.3 PHOSPHATE IN THE "SECOND MEDIUM."											
	NONE		0.0001		0.001		0.01		0.1		1.0	
	VOL.	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS
0	−	−	−	−	−	−	−	−	−	−	−	−
1/4	⊥	−	++	−	+	−	+	−	++	−	+	−
1	⊥	−	++	−	+	−	++	−	++	−	++	−
2	+	−	++	−	++	−	++	−	++	−	++	−
4	+	−	++	−	+	−	++	−	++	+	++	+
24	−	⊥	⊥	−	⊥	⊥	+	+	⊥	+	−	+
FINAL pH "SECOND MEDIUM"	6.1		6.6		4.1		4.0		4.3		4.3	

VOL. = AMOUNT OF VOLUTIN PRODUCED.

C.S. = CYTOPLASMIC STAINABILITY.

pH fell to 4.7. Thus the possibility of the acidity volutin being formed complicated the results.

As it was doubtful that the solid media was completely free of phosphate a liquid "Second Medium" was also employed. The omission of phosphate, however, left the medium unbuffered so 0.03 per cent sodium bicarbonate was added to the medium and the plates were incubated in a sealed tank containing 2.5 per cent carbon dioxide for 2 hours. Table XXX. shows the results obtained when the cells which were initially grown for 2 days at 35°C on a glucose medium containing 0.0001 per cent phosphate and buffered with bicarbonate-carbon dioxide mixture, were transferred to a liquid "Second Medium" containing various amounts of phosphate. It will be seen that with 0.01 per cent phosphate maximum volutin production was obtained but that with decreasing amounts of phosphate the volutin formation fell away till no volutin was produced when no phosphate was added to the medium. The pHs of all the media lay between 6.8 and 7.0 so there was no question of acid volutin being formed. It would, therefore, appear that with liquid media 0.01 per cent phosphate was required for maximum volutin production and that in the case of the solid media there must have been

TABLE XXX.

The influence of the amount of phosphate in the "Second Medium" on the volutin production by Aero. aerogenes A3 cells on transfer from a glucose medium containing 0.0001% phosphate and buffered with bicarbonate/carbon dioxide mixture after 2 days growth at 35°C to a liquid "Second Medium" of similar composition and buffered in the same manner.

Percentage KH2PO4 added to "Second Medium"	Volutin production	Final pH "Second Medium"
None	−	4.0
0.0001	⊥	4.0
0.001	+	4.0
0.01	+ + +	6.8

sufficient contamination phosphate to give volutin production with less than 0.01 per cent added phosphate. Phosphate would, therefore, appear to be necessary for the synthesis of volutin.

The influence of the pH of the "Second Medium" on the production of volutin.

The effect of the pH of the "Second Medium" on the production of volutin was studied and the results are shown in tables XXXI. and XXXII. In the experiment shown in table XXXI. the energy sources were sucrose and "citrate", which also acted as the buffering system. Phosphate-starved cells were taken from a 3 day growth on a "First Medium" containing 0.0001 per cent phosphate, and transferred to a "Second Medium" which was identical with that of the "First Medium" except that it contained 1.0 per cent of phosphate mixtures of different pH. It will be seen that volutin was produced in all cases but most at pHs 6 and 7. The results obtained when the cells were grown on a "First Medium" containing glucose and buffered with bicarbonate-carbon dioxide mixture are shown in table XXXII. The "Second Medium" was again identical except that it was buffered at various pHs by 1 per cent phosphate. Here again the best production of

TABLE XXXI.

The influence of the pH of the "Second Medium" on volutin production by Aero.aerogenes A3 cells on transfer from a sucrose medium containing 1.0% citrate as buffer and 0.0001% phosphate after 3 days growth at 35°C to a similar "Second Medium" containing 1% phosphate of different pH.

TIME ON "SECOND MEDIUM" HOURS.	INITIAL pH OF "SECOND MEDIUM"							
	5.2		6.1		6.9		7.8	
	VOL.	C.S.	VOL.	C.S.	VOL.	C.S.	VOL.	C.S.
0	−	−	−	−	−	−	−	−
¼	+	−	+	−	+	−	++	−
1	++	−	++	−	+	−	++	−
2	+	+	++	−	++	−	++	±
4	++	−	++	−	++	+	+	+
24	+	+	±	+	±	+	±	+
FINAL pH "SECOND MEDIUM"	4.8		6.1		6.7		7.1	
INITIAL pH "FIRST MEDIUM"	6.8		6.8		6.8		6.8	
FINAL pH "FIRST MEDIUM"	6.6		6.6		6.6		6.6	

VOL. = AMOUNT OF VOLUTIN PRODUCED

C.S. = CYTOPLASMIC STAINABILITY.

TABLE XXXII.

The influence of the pH of the "Second Medium" on volutin production by Aero. aerogenes A3 cells on transfer from a glucose medium buffered by bicarbonate-carbon dioxide mixture and containing 0.0001% phosphate after 2 days growth at 35°C to a similar "Second Medium" containing 1.0% phosphate at different pH (this acted as buffer).

TIME ON "SECOND MEDIUM" HOURS.	INITIAL pH OF "SECOND MEDIUM"							
	4.6		6.0		7.0		8.2	
	VOL.	C.S.	VOL.	C.S.	VOL.	C.S.	VOL.	C.S.
0	−	−	−	−	−	−	−	−
¼	+	−	+	−	++	−	+	−
1	+	−	++	−	++	−	+	−
2	++	−	++	−	++	−	++	−
4	++	−	++	−	+++	−	++	−
24	+++	+	+	+	⊥	+	⊥	+
FINAL pH "SECOND MEDIUM"	4.6		6.0		6.9		7.4	
INITIAL pH "FIRST MEDIUM"	Ca 7.5		Ca 7.5		ca 7.5		Ca 7.5	
FINAL pH "FIRST MEDIUM"	6.8		6.8		6.8		6.8	

VOL. = AMOUNT OF VOLUTIN PRODUCED.

C.S. = CYTOPLASMIC STAINABILITY.

volutin was given when the pH was 6 or 7. At pH 5 acid forms were produced so that the results were more difficult to interpret. Wiame and Lefèbvre (1946) reported that the production of volutin was inhibited if the pH of the "Second Medium" was above 8.4 but when phosphate-starved cells of Aero. aerogenes were transferred to a liquid "Second Medium" of pH 8.6 for 2 hours at 35°C there was no appreciable reduction in the amount of volutin produced. (The pH of 8.6 was obtained by adding 0.6 per cent bicarbonate to the medium which contained 0.01 per cent phosphate and incubating the plate in a sealed tank containing 3 per cent carbon dioxide.)

The influence on volutin production of the omission of each of the components of the "Second Medium".

Phosphate-starved cells were transferred from a glucose medium containing 0.0001 per cent phosphate and buffered with bicarbonate-carbon dioxide mixture after 2 days growth at 35°C to a series of "Second Media" in each of which one component had been omitted. The results are shown in table XXXIII. It will be seen that the omission of glucose from the "Second Medium" caused a great reduction in the volutin production whereas the omission of the nitrogen source did not appear to have any initial

TABLE XXXIII.

The influence of the glucose and nitrogen source in the "Second Medium" on volutin production by Aero.aerogenes A3 on transfer of cells from a "First Medium" containing 0.0001% or 0.1% phosphate, 0.1% glucose, 0.5% ammonium sulphate, salts agar and buffered by bicarbonate - carbon dioxide after 2 days growth at 35°C to "Second Media" deficient in either glucose or nitrogen source.

TIME ON "SECOND MEDIUM" HOURS.	PERCENTAGE pH 7.3 PHOSPHATE IN "FIRST MEDIUM."											
	0.0001		0.1		0.0001		0.1		0.0001		0.1	
	COMPOSITION OF "SECOND MEDIUM"											
	P N Sa				P G Sa				P N G Sa			
	VOL.	C.S.	VOL	C.S.	VOL	C.S.	VOL	C.S.	VOL	C.S.	VOL	C.S.
0	−	−	−	+	−	−	−	+	−	−	−	+
1/4	−	−	−	+	+	−	−	+	++	−	−	+
1	−	−	−	+	++	−	−	+	+++	−	−	+
2	⊥	−	−	+	+++	−	−	+	+++	−	−	+
4	⊥	−	−	+	++	⊥	−	+	+	+	−	+
8	⊥	−	−	+	+	−	−	−	⊥	+	−	+
24	⊥	−	−	+	+	−	+	−	−	+	−	+
OPACITY "FIRST MEDIUM"	0.236		0.948		0.228		0.948		0.180		0.824	
INITIAL pH "FIRST MEDIUM"	ca 7.5		ca 7.5		ca 7.5		ca 7.5		ca 7.5		ca 7.5	
FINAL pH "FIRST MEDIUM"	6.4		6.8		6.9		6.8		6.9		6.8	
FINAL pH "SECOND MEDIUM"	7.3		7.2		7.3		7.3		7.0		7.0	
OPACITY "SECOND MEDIUM"	0.02		0.024		0.068		0.148		0.608		0.80	

VOL. = AMOUNT OF VOLUTIN PRODUCED.

C.S. = CYTOPLASMIC STAINABILITY.

P = 1% PHOSPHATE pH 7.3

N = 0.5% AMMONIUM SULPHATE

G = 0.1% GLUCOSE

effect on the production of volutin which was maximal in 2 hours in both the presence and absence of ammonium sulphate. When, however, incubation of the phosphate-starved cells on the nitrogen deficient medium was continued for 24 hours it was found that the volutin was still present. Normally in the complete medium the volutin production rises to a maximum at 2 hours, thereafter falling away till there is no trace of volutin after 24 hours sub-culture. This would appear to show that a deficiency in the nitrogen source either induced a fresh production of volutin or inhibited the utilisation of that formed on the transfer of the phosphate-starved cells to a phosphate containing medium. When the phosphate-sufficient cells were transferred to a phosphate containing medium they also produced volutin after 24 hours incubation thus it would seem that the deficiency of nitrogen source induced the volutin production. (See Section B).

As it was suspected that the solid media might be contaminated with the substances which were being studied, a liquid "Second Medium" was also employed. This has already been described with reference to phosphate in a previous section where it was found

that 0.01 per cent phosphate was required for the maximal production of volutin. When glucose was completely omitted from the liquid "Second Medium" no volutin was produced but the inclusion of increasing amounts of glucose in the "Second Medium" (table XXXIV.) was found to stimulate the formation of increasing amounts of volutin till with the addition of 0.1 per cent glucose maximum volutin production was obtained. Therefore glucose is required for the formation of volutin. On solid media the omission of the ammonium sulphate did not appear to have any effect initially but when the ammonium sulphate was completely omitted from a liquid "Second Medium" the volutin production was slightly reduced. The addition of 0.0002 per cent ammonium sulphate, however, restored maximal volutin production so that only a very little nitrogen source was required. (Table XXXV.).

The various cations and anions were also omitted in turn and it was found that the omission of both potassium and magnesium reduced the amount of volutin produced but that the presence of 0.001 per cent potassium sulphate or magnesium sulphate restored the volutin production to its normal level. From the results in table XXXVI. it will be seen that the

TABLE XXXIV.

The influence of the amount of glucose in the "Second Medium" on the volutin production by Aero. aerogenes A3 cells on transfer from a glucose medium containing 0.0001% phosphate and buffered by a bicarbonate/carbon dioxide mixture after 2 days growth at 35°C to a liquid "Second Medium" buffered by 1% phosphate for 2 hours.

Percentage Glucose added to "Second Medium"	Volutin production	Final pH "Second Medium"
None	−	7.2
0.001	±	7.1
0.01	+	7.1
0.1	+ + +	7.1

TABLE XXXV.

The influence of the amount of nitrogen source in the "Second Medium" on the volutin production by Aero.aerogenes A3 cells on transfer from a glucose medium containing 0.0001% phosphate and buffered with bicarbonate/carbon dioxide mixture after 2 days growth at 35°C to a liquid "Second Medium" buffered with 1% phosphate for 2 hours.

Percentage Ammonium Sulphate added to Second Medium	Volutin Production	Final pH Second Medium
None	+ +	4.1
0.0002	+ + +	4.2
0.002	+ + +	4.2

TABLE XXXVI.

The influence of the various cations and anions in the "Second Medium" on the production of volutin by Aero.aerogenes A3 cells on transfer from a glucose medium containing 0.0001% phosphate and buffered by bicarbonate - carbon dioxide mixture after two days growth at 35°C to a liquid "Second Medium" buffered by 1% phosphate for 2 hours at 35°C.

Amount added to 100mls "Second Medium"	Potassium Sulphate		Magnesium Sulphate		Iron		Calcium		Sulphur	
	Vol.	Final pH	Vol	Final pH	Vol	Final pH	Vol.	Final pH	Vol.	Final pH
None	+	7.2	+	7.2	+++	7.2	+++	7.2	+++	7.2
0.0001gm.	++	7.2	++	7.2						
0.001gm.	+++	7.2	+++	7.2						
0.01gm.	+++	7.2	+++	7.2						
0.1gm.	+++	7.2								

Vol. = Amount of volutin produced.

omission of calcium, sulphur and iron did not appear to have any appreciable effect on the volutin production but as it cannot be stated with certainty that these media were completely free from contamination, no definite conclusions as to the effect of these three ions can be reached.

Influence of various energy sources in the "Second Medium" on the volutin production.

"Citrate" and various organic compounds occuring in the glycolysis cycle were introduced into the "Second Medium" in place of the energy source, glucose, and the results are shown in table XXXVII. The cells were grown for 2 days on a "First Medium" containing glucose, bicarbonate-carbon dioxide buffer and either 0.0001 per cent phosphate for the phosphate-starved cells or 0.1 per cent phosphate for the phosphate-sated cells to serve as controls. (In no case did these latter produce volutin on the "Second Medium"). When pyruvic acid was the energy source the amount of volutin produced was comparable with that produced on glucose; calcium hexose-di-phosphate and sodium lactate gave a little less and calcium glycerophosphate and "citrate" much less. Sodium acetate gave quite a good yield of volutin in the first few hours but the cells appeared to die

TABLE XXXVII.

Comparison of the effect of different energy sources in the "Second Medium" on volutin production by Aero.aerogenes A3 on transfer of the cells from a "First Medium" containing 0.0001% or 0.1% phosphate, 0.1% glucose, 0.5% ammonium sulphate, salts agar and buffered by bicarbonate-carbon dioxide mixture after 2 days growth at 35°C to a "Second Medium" containing 1.0% phosphate, 0.5% ammonium sulphate, salts agar together with 0.1% of the energy source.

TIME ON "SECOND MEDIUM" HOURS	PERCENTAGE pH 7.3 PHOSPHATE IN "FIRST MEDIUM"																							
	0.0001		0.1		0.0001		0.1		0.0001		0.1		0.0001		0.1		0.0001		0.1		0.0001		0.1	
	ENERGY SOURCE IN THE "SECOND MEDIUM"																							
	CITRATE				HEXOSE-DI-PHOSPHATE				CALCIUM GLYCERO PHOSPHATE.				PYRUVIC ACID				SODIUM LACTATE				SODIUM ACETATE			
	VOL.	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	C.S.
0	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	+
1/4	⊥	-	-	+	+	-	-	+	-	-	-	+	++	-	-	+	++	-	-	+	++	-	-	+
1	+	-	-	+	++	-	-	+	+	-	-	+	++	-	-	+	++	-	-	+	++	-	-	+
2	+	-	-	+	++	-	-	+	+	-	-	+	+++	-	-	+	++	-	-	+	⊥	-	-	+
4	+	-	-	+	++	+	-	+	+	+	-	+	+	+	-	+	+	-	-	+	⊥	-	-	+
8	+	+	-	+	++	+	-	+	+	+	-	+	-	+	-	+	⊥	+	-	+	⊥	⊥/-	-	+
24	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	⊥/-	-	+
OPACITY "FIRST MEDIUM"	0.180		0.824		0.20		1.068		0.20		1.068		0.144		0.668		0.144		0.668		0.144		0.668	
INITIAL pH "FIRST MEDIUM."	Ca 4.5		Ca 4.5		Ca 4.5		Ca 4.5		Ca 4.5		Ca 4.5		Ca 4.5		Ca 4.5		Ca 4.5		Ca 4.5		Ca 4.5		Ca 4.5	
FINAL pH "FIRST MEDIUM."	6.9		6.8		6.9		6.8		6.9		6.8		6.9		6.9		6.9		6.9		6.9		6.9	
FINAL pH "SECOND MEDIUM"	4.4		4.5		6.9		6.9		6.9		6.9		6.4		6.4		4.1		4.1		4.1		4.1	

VOL. = AMOUNT OF VOLUTIN PRODUCED

C.S. = CYTOPLASMIC STAINABILITY.

off after about two hours.

Since the hexose-di-phosphate and the glycerophosphate were calcium salts, the possibility that the calcium might be influencing the volutin production was investigated by adding the appropriate amount of calcium to a "Second Medium" containing glucose. The presence of the calcium made no difference to the volutin production. The calcium glycerophosphate was B.P.C. and not an A.R. reagent.

The inability of the phosphate-starved cells from a glucose medium to form a large amount of volutin on a "Second Medium" containing "citrate" as the energy source may well have been due to the fact that the cells were not adapted to utilise "citrate".

The influence on volutin production of the inclusion of sodium azide in the "Second Medium".

The two day phosphate-starved cells were transferred from a glucose medium containing 0.0001 per cent phosphate and buffered by bicarbonate-carbon dioxide mixture to a liquid "Second Medium" containing 0.01 per cent phosphate and varying amounts of sodium azide, the results being shown in table XXXVIII. Only 0.01 per cent phosphate was added so that the occurrence of fermentation could be detected by a drop in the pH. It will be seen

TABLE XXXVIII.

The influence of sodium azide in the "Second Medium" on the production of volutin by Aero. aerogenes A3 cells on transfer from a glucose medium containing 0.0001 per cent phosphate and buffered with bicarbonate - carbon dioxide mixture after 2 days at 35°C to a liquid "Second Medium" containing 0.01% phosphate for 4 hours at 35°C.

Amount of Sodium Azide present in "Second Medium"	Volutin Production 2 hours	Volutin Production 4 hours	Initial pH "Second Medium"	pH "Second Medium" after 4 hours.
None	+ + +	+ + +	4.1	4.6
2.5×10^{-4} M	+ + ±	+ + +	6.9	6.5
2.5×10^{-3} M	+ +	+ +	4.1	6.5
2.5×10^{-2} M	+	+	4.3	4.1
2.5×10^{-1} M	±	+	4.4	4.4

that when no azide was added to the medium much volutin was produced and the pH fell from 7.1 to 4.6 in 4 hours. The inclusion of increasing amounts of sodium azide caused a reduction in the amount of volutin produced together with a reduction in the amount of fermentation taking place as measured by the drop in pH. This would appear to show that the reduction of the amount of volutin production was merely due to the reduction in the fermentation of the glucose.

The ability of other bacterial strains and species to form volutin under the same conditions.

Various other bacterial strains and species were examined for their ability to produce volutin on transfer of 2 day cultures from a phosphate-poor to a phosphate-rich medium. The reactions were controlled by transferring 2 day old cells from a phosphate-rich medium to a phosphate-rich medium. The glucose, bicarbonate - carbon dioxide medium was used throughout as the "First Medium" with the inclusion of either 0.0001 or 0.1 per cent phosphate. The "Second Medium" contained glucose and was buffered by 1.0 per cent phosphate. Tables XXXIX. - XLI. show the results obtained. Aero. aerogenes strains A1, A3R, N5939 and N8172 and Klebsiella pneumoniae

TABLE XXXIX.

Comparison of the volutin production by other strains of Aero. aerogenes on transfer of cells from a glucose containing "First Medium" buffered by bicarbonate-carbon dioxide mixture with either 0.0001% or 0.1% phosphate after 2 days growth at 35°C to a similar "Second Medium" containing 1.0% phosphate which acted as a buffer.

TIME ON "SECOND MEDIUM" HOURS	ORGANISM.																			
	AERO. AEROGENES A1.				AERO. AEROGENES A3R				AERO. AEROGENES 3301.				AERO. AEROGENES N8164				AERO. AERO GENES N8192			
	PERCENTAGE pH 7.3 PHOSPHATE IN "FIRST MEDIUM"																			
	0.0001		0.1		0.0001		0.1		0.0001		0.1		0.0001		0.1		0.0001		0.1	
	Vol.	C.S.	Vol.	C.S.	Vol.	C.S.	Vol.	C.S.	Vol.	C.S.	Vol.	C.S.	Vol.	C.S.	Vol.	C.S.	Vol.	C.S.	Vol.	C.S.
0	−	−	−	+	−	−	−	+	−	−	−	+	−	?	−	+	−	−	−	+
1/4	++	−	−	+	++	−	−	+	+	−	−	+	+	?	−	+	++	−	−	+
1	++	−	−	+	++	−	−	+	+	−	−	+	++	?	−	+	+++	−	−	+
2	+++	−	−	+	+++	−	−	+	++	−	−	+	++	?	−	+	+++	−	−	+
4	++	+	−	+	+++	+	−	+	+	+	−	+	+	⊥	−	+	+	+	−	+
8	+	+	−	+	+	+	−	+	⊥	+	−	+	⊥	+	−	+	⊥	+	−	+
24	−	+	−	+	⊥	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+
OPACITY "FIRST MEDIUM."	0.288		0.400		0.342		1.052		0.112		0.608		0.152		0.768		0.140		0.936	
INITIAL pH "FIRST MEDIUM."	ca 7.5		ca 7.5		ca 7.5		ca 7.5		ca 7.5		ca 7.5		ca 7.5		ca 7.5		ca 7.5		ca 7.5	
FINAL pH "FIRST MEDIUM."	6.8		6.8		6.8		6.8		6.7		6.9		6.8		6.9		6.8		6.8	
FINAL pH "SECOND MEDIUM."	7.0		7.0		7.0		7.0		7.0		7.0		7.2		7.2		7.0		7.0	
TIME WHEN GROWTH WAS OBSERVED ON "SECOND MEDIUM."	8 HOURS		4 HOURS		8 HOURS		4 HOURS		8 HOURS		4 HOURS		8 HOURS		4 HOURS		4 HOURS		4 HOURS	

Vol. = AMOUNT OF VOLUTIN PRODUCED

C.S. = CYTOPLASMIC STAINABILITY.

TABLE XL.

Comparison of the volutin production by Aero. cloacae, Aero. aerogenes, K. pneumoniae and Ser. marcescens strains on transfer of cells from a glucose containing "First Medium" buffered by bicarbonate-carbon dioxide mixture with either 0.0001% or 0.1% phosphate after 2 days growth at 35°C to a similar "Second Medium" containing 1% phosphate which acted as the buffer.

TIME ON "SECOND MEDIUM" HOURS.	ORGANISM.																			
	AERO. CLOACAE N 5936				AERO. CLOACAE N5920.				AERO. AEROGENES N5939				K. PNEUMONIAE N5054				SER. MARCESCENS. 2			
	PERCENTAGE pH 7.3 PHOSPHATE IN "FIRST MEDIUM."																			
	0.0001		0.1		0.0001		0.1		0.0001		0.1		0.0001		0.1		0.0001		0.1	
	VOL.	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS
0	⊥	+	—	+	—	—	—	+	—	—	—	+	—	—	—	+	—	—	—	+
1/4	⊥	+	—	+	⊥	—	—	+	+	—	—	+	++	—	—	+	—	—	—	+
1	+	?	—	+	+	—	—	+	++	—	—	+	+++	—	—	+	⊥	—	—	+
2	⊥	+	—	+	++	—	—	+	+++	—	—	+	+++	—	—	+	+	—	—	+
4	⊥	+	—	+	⊥	+	—	+	⊥	+	—	+	+	+	—	+	+	+	—	+
8	⊥	+	—	+	⊥	+	—	+	⊥	+	—	+	⊥	+	—	+	—	+	—	+
24	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—	+
OPACITY "FIRST MEDIUM"	0.048		0.508		0.132		0.542		0.156		0.428		0.100		0.608		0.156		0.608	
INITIAL pH "FIRST MEDIUM"	ca 7.5		ca 7.5		ca 7.5		ca 7.5		ca 7.5		ca 7.5		ca 7.5		ca 7.5		ca 7.5		ca 7.5	
FINAL pH "FIRST MEDIUM"	6.9		6.8		6.8		6.8		6.8		6.7		6.9		6.7		6.8		6.7	
FINAL pH "SECOND MEDIUM"	7.0		6.8		6.9		6.9		7.1		7.1		7.0		7.0		7.0		7.0	
TIME WHEN GROWTH WAS OBSERVED ON "SECOND MEDIUM"	8-24 HOURS		8 HOURS		8 HOURS		8 HOURS		8 HOURS		4 HOURS		8 HOURS		4 HOURS		8 HOURS		4 HOURS	

VOL. = AMOUNT OF VOLUTIN PRODUCED

C.S. = CYTOPLASMIC STAINABILITY.

TABLE XLI.

Comparison of the volutin production by various Esch.coli strains on transfer of cells from a glucose containing "First Medium" buffered by bicarbonate-carbon dioxide mixture with either 0.0001% or 0.1% phosphate after 2 days growth at 35°C to a similar "Second Medium" containing 1% phosphate which acted as a buffer.

TIME ON "SECOND MEDIUM" HOURS.	ORGANISM																	
	ESCH. COLI A55				ESCH. COLI A93				ESCH. COLI A100				ESCH. COLI A101				ESCH. COLI. A102.			
	PERCENTAGE pH 7·3 PHOSPHATE IN "FIRST MEDIUM"																	
	0·0001		0·1		0·0001		0·1		0·0001		0·1		0·0001		0·1		0·0001		0·1	
	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS	VOL	CS
0	−	?	−	+	−	?	−	+	−	?	−	+	−	?	−	+	−	?	−	+
1/4	+	?	−	+	−	?	−	+	−	?	−	+	⊥	?	−	+	−	?	−	+
1	+	?	−	+	+	?	−	+	−	?	−	+	+	?	−	+	+	?	−	+
2	+	?	−	+	+	?	−	+	⊥	?	−	+	+	?	−	+	+	?	−	+
4	+	+	−	+	⊥	+	−	+	⊥	+	−	+	+	⊥	−	+	⊥	+	−	+
8	⊥	+	−	+	−	+	−	+	−	+	−	+	+	+	−	+	−	+	−	+
24	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+
OPACITY FIRST MEDIUM	0·108		0·956		0·152		1·024		0·056		0·916		0·080		1·004		0·156		0·842	
INITIAL pH "FIRST MEDIUM"	ca 7·5		ca 7·5		ca 7·5		ca 7·5		ca 7·5		ca 7·5		ca 7·5		ca 7·5		ca 7·5		ca 7·5	
FINAL pH "FIRST MEDIUM"	6·9		6·8		6·4		6·4		6·9		6·8		6·9		6·4		6·8		6·8	
FINAL pH "SECOND MEDIUM"	7·0		7·0		7·1		7·0		7·2		7·2		7·1		7·2		7·1		7·1	
GROWTH OBSERVED ON "SECOND MEDIUM" (TIME)	8 HOURS		4 HOURS		8 HOURS		4 HOURS		8 HOURS		4 HOURS		78 HOURS		4 HOURS		8 HOURS		4 HOURS.	

VOL. = AMOUNT OF VOLUTIN PRODUCED

C.S. = CYTOPLASMIC STAINABILITY.

N5054 produced as much volutin as A3. Aero. aerogenes strain N8167, D301 and Aero. cloacae strain N5920 and N5936 produced less while Esch. coli strains and Ser. marcescens strains produced hardly any. Some of the Bacillus genus were also studied but due to the normal production of volutin by these organisms on phosphate rich media the results could not be interpreted easily.

CYTOLOGICAL OBSERVATIONS ON THE VOLUTIN PRODUCED WHEN PHOSPHATE-STARVED CELLS WERE TRANSFERRED TO A PHOSPHATE-RICH MEDIUM.

1(a) Optical microscope. Albert's method. (Modified by Laybourn 1924).

The cells in this case were found to consist of a black staining volutin granule surrounded by a colourless area which in turn was surrounded by a purplish-brown amorphous mass - presumably capsule. (See fig. xx.).

1(b) Optical microscope. Albert - Nigrosin method.

By over-spreading the Albert stained smear with a thin layer of nigrosin the organism becomes more clearly outlined. (See fig. xxi.).

1(c) Optical microscope. Metachromatic staining.

By staining the volutin containing cells with Loeffler's methylene blue the volutin assumed a bluish-violet colour and the capsule a pinkish-violet colour. The granules were found to resist the action of 1.0 per cent sulphuric acid for 1 minute but were completely destained by 20 per cent acid in 1 minute or by 5 per cent acid in 5 minutes.

1(d) Optical microscope. Carbol fuchsin and sulphuric acid.

After the smear had been stained with carbol

fuchsin for 5 minutes the volutin appeared as purplish-red granules in an unstained cytoplasm surrounded by a pale red line - presumably cell wall or capsular material. Treatment of the stained smear with 1 per cent sulphuric acid for 1 minute resulted in a slight loss of stain on the granules and the appearance of purplish-red polar granules but when 20 per cent sulphuric acid was applied for 1 minute the smear was completely destained.

1(e) Optical microscope. Periodate method of Hotchkiss (1948).

No red granules were seen so it was concluded that the volutin did not contain an insoluble polysaccharide.

1(f) Optical microscope. Gram's iodine.

No brown or blue granules indicating the presence of glycogen or iogen were observed.

1(g) Optical microscope. Burdon's fat stain.

No black granules were seen therefore volutin did not contain an appreciable amount of lipid.

2. Phase Contrast Microscope.

When unstained wet films of volutin containing cells were examined in the phase contrast microscope, the volutin could be clearly seen as dark granules

embedded in a paler cytoplasm.

3. Electron microscope.

When unshadowed preparations of volutin containing cells were examined in the electron microscope, the granules were found to stand out clearly as in fig. xxii.

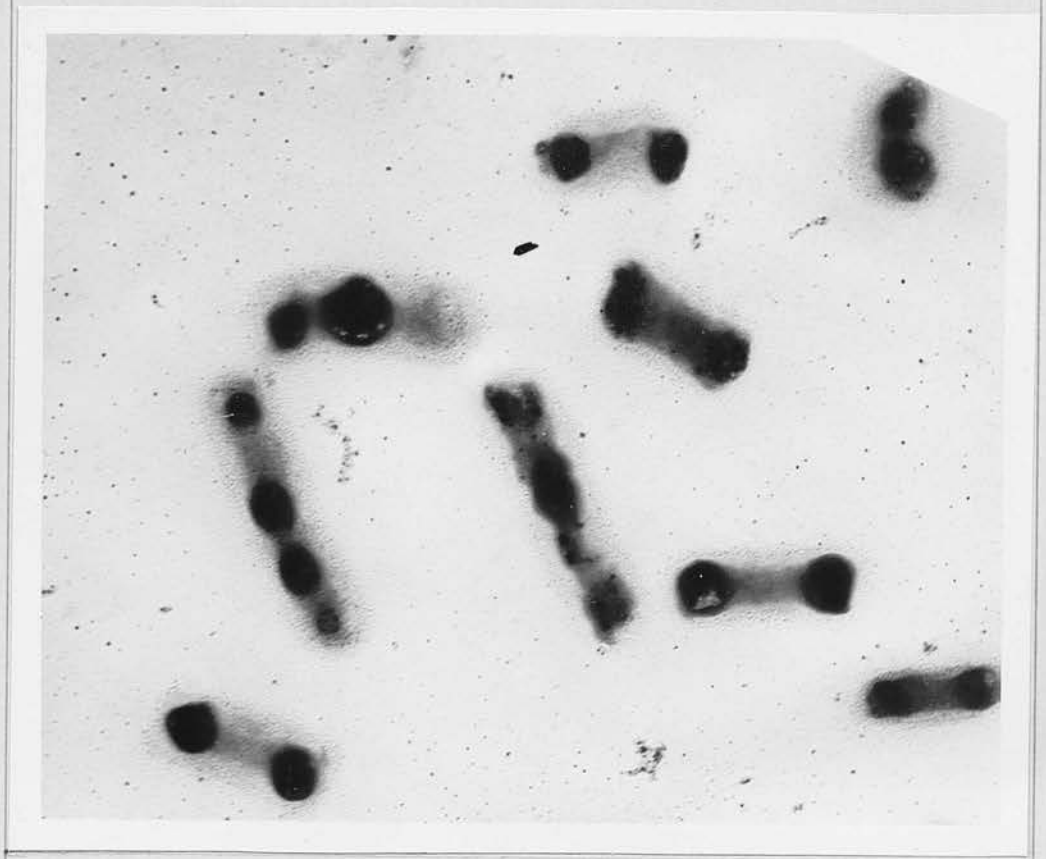


Fig. xxii. Aero. aerogenes A.3 showing volutin due to addition of phosphate to phosphate-starved cells - unshadowed preparation in electron microscope.

Strain grown on 0.0001% phosphate, 0.1% glucose, 0.5% ammonium sulphate, salts agar buffered with 0.3% bicarbonate and 20% carbon dioxide for 48 hours at 35°C and sub-cultured on a similar medium containing 1.0% phosphate for 2 hours at 35°C. x 24,000

CHEMICAL OBSERVATIONS ON THE PHOSPHATE STARVED CULTURES.

Four different types of cells were examined viz. (i) phosphate-starved cells, (ii) phosphate-starved cells transferred to phosphate containing medium i.e. volutin containing cells, (iii) phosphate-sufficient cells and (iv) phosphate-sufficient cells transferred to a phosphate containing medium. The phosphate-sufficient cells did not produce any volutin on transfer to a phosphate containing medium but they were examined to determine if any of the fractions increased significantly on sub-culture of such cells in the stationary phase on a fresh medium. No such increases were, however, found.

The phosphorus to nitrogen ratio for the phosphate-starved cells was found to be very low being about 0.08 (table XLII.) but when such cells were subcultured on phosphate containing media much volutin was produced in half an hour and the ratio rose in this time to 0.18 which was the normal level for phosphate-sufficient cultures. This doubling of the amount of phosphorus in the volutin-containing cells is reflected in an approximate doubling of all the fractions except R9 which shows a much larger

TABLE XLII

Comparison of the distribution of the phosphate compounds (expressed as μgP/mgN) in phosphate-deficient* and phosphate-sufficient cells and in such cells after sub-culture on a phosphate containing "Second Medium".

% Phosphorus Recovered	P/N	Final pH "First Medium"	Time on "Second Medium" Minutes.	Volutin Production.	Fraction. S_2	S_3	R_3	S_4	S_7	R_7	R_6	S_9	R_9	R_8
Phosphate-deficient cells.														
80·22	0·088	6·8	–	–	13·2	1·9	0·5	8·3	37·28	0·49	6·1	1·8	0·3	1·4
89·40	0·090	6·4	–	–	13·4	1·4	0·5	6·4	39·8	0·4	11·2	3·4	0·4	2·4
80·02	0·093	6·8	–	–	10·11	2·09	0·18	3·3	32·31	0·31	6·14	2·1	0·13	0·13
Phosphate-deficient cells sub-cultured on a phosphate containing "Second Medium."														
82·51	0·13	6·6	15	+	16·63	4·19	1·16	8·40	55·14	0·64	10·44	2·49	1·20	2·44
90·51	0·18	6·6	30	++	22·44	3·64	1·42	11·21	86·36	0·91	14·14	4·88	15·22	3·23
88·96	0·14	6·4	60	++	25·44	9·41	0·44	13·12	45·28	0·44	10·46	4·08	6·54	3·55
44·85	0·20	6·6	180	+	28·32	4·91	1·34	15·21	44·21	0·59	19·40	5·63	0·54	4·44
Phosphate-sufficient cells.														
64·03	0·16	6·9	–	–	11·88	2·14	0·35	4·49	43·19	1·04	10·48	1·84	1·33	1·63
44·10	0·14	6·9	–	–	4·3	9·44	0·65	6·84	52·28	0·45	11·65	12·13	0·84	3·2
93·40	0·14	6·9	–	–	4·34	4·24	2·41	13·45	90·03	2·01	18·04	15·18	0·39	4·02
Phosphate-sufficient cells sub-cultured on a phosphate containing "Second Medium."														
48·15	0·15	6·9	30	–	9·12	3·5	0·25	8·89	60·34	0·49	14·41	8·13	0·31	2·99
84·02	0·15	6·9	30	–	5·26	10·45	2·83	11·14	66·28	0·81	15·28	9·44	1·15	3·53
89·41	0·20	6·9	120	–	12·99	10·44	1·91	14·24	95·13	0·14	21·84	11·48	0·22	4·46

* Phosphate deficient ≡ phosphate starved.

increase viz. forty-fold (table XLII). So that the production of volutin may well be correlated with the presence of an increased amount of phosphorus in the fraction R9. It is of interest to note that the transfer of the phosphate-sufficient cells to a fresh phosphate medium for two hours caused the phosphorus to nitrogen ratio to rise to 0.20 i.e. higher than that given on the transfer of the phosphate-starved cells to a fresh phosphate medium for half an hour but the amount of phosphorus in the R9 fraction of the transferred phosphate-sufficient cultures was only one seventieth of that of the phosphate-starved cultures.

The correlation of the presence of volutin with the increase of the phosphorus content of R9 was substantiated by the fact that the R9 phosphorus content rose from 1.2 μgP/mgN after 15 minutes transfer to 15.22 μgP/mgN at 30 minutes and thereafter fell away to 6.54 μgP/mgN at 60 minutes and 0.57 μgP /mgN at 180 minutes. This followed the same pattern as the volutin production which again rose to a maximum at 30 minutes and thereafter fell away to practically none at 180 minutes.

This correlation of the presence of volutin with

the increase in the amount of phosphorus in fraction R9 is also well illustrated when the amount of phosphorus in each fraction is expressed as a percentage. (Table XLIII.). Here again the only fraction which showed a marked change was R9. It rose from 0.32 per cent in the phosphate-starved cells to 8.4 per cent in the same type of cells after 30 minutes sub-culture on a phosphate containing medium. This increase in the amount of phosphorus in R9 was accompanied by the appearance of volutin. So the production of volutin may well be correlated with this increase in the phosphorus content of the acid insoluble metaphosphate fraction R9.

TABLE XLIII.

Comparison of the distribution of the phosphate compounds (expressed as percentage phosphorus) in phosphate-deficient* and phosphate-sufficient cells and in such cells after sub-culture on a phosphate-containing "Second Medium".

% Phosphorus Recovered.	P/N	Final pH "First Medium"	Time on "Second Medium" (minutes)	Volutin Production	Fraction S_2	S_3	R_3	S_4	S_5	R_5	R_6	S_9	R_9	R_8
Phosphate-deficient cells.														
80·22	0·088	6·8	–	–	14·68	2·11	0·55	9·24	42·34	0·5	6·81	2·05	0·36	1·55
89·40	0·090	6·4	–	–	14·95	1·90	0·58	7·1	44·31	0·78	12·48	3·80	0·40	3·10
80·02	0·078	6·8	–	–	13·96	2·88	0·25	4·54	44·58	0·43	8·52	2·90	0·18	1·78
Phosphate-deficient cells sub-cultured on a phosphate containing "Second Medium."														
82·51	0·13	6·6	15	+	13·29	3·36	0·92	6·97	44·12	0·53	8·36	2·0	0·96	2·0
90·51	0·18	6·6	30	++	12·8	2·0	0·88	6·17	47·50	0·50	7·78	2·69	8·34	1·82
88·96	0·14	6·4	60	+±	15·14	5·6	0·44	7·78	44·74	0·44	6·4	2·42	3·89	2·11
77·85	0·20	6·6	180	±	13·42	3·83	0·66	7·34	37·41	0·29	9·4	2·43	0·24	2·14
Phosphate-sufficient cells.														
64·03	0·16	6·9	–	–	7·15	1·29	0·21	4·69	44·03	0·62	6·31	1·11	0·64	0·98
77·10	0·14	6·9	–	–	5·34	7·12	0·48	5·02	38·23	0·55	8·53	8·88	0·61	2·34
93·40	0·14	6·9	–	–	4·24	4·2	1·98	7·48	52·20	1·14	10·44	8·48	0·22	2·33
Phosphate-sufficient cells subcultured on a phosphate containing "Second Medium."														
78·14	0·15	6·9	30	–	6·56	2·52	0·18	6·38	43·39	0·57	10·36	5·85	0·22	2·14
84·02	0·18	6·9	30	–	3·51	7·14	1·89	7·05	44·19	0·54	10·04	6·50	0·77	2·36
89·41	0·20	6·9	120	–	6·64	5·54	0·98	7·33	48·84	0·73	11·23	5·89	0·10	2·40

* Phosphate deficient ≡ phosphate starved.

VOLUTIN PRODUCTION DUE TO THE PRESENCE OF ANTIBIOTICS.

Another example of volutin production due to adverse conditions of culture has been described by Bringmann (1951-52). He found that the presence of streptomycin and chloromycetin in the medium induced the formation of the "Karyoide" forms which appear to be volutin granules in the coliform organisms. The action of various Antibiotics on Aero. aerogenes A3 was studied and it was found that chloromycetin and terramycin induced the production of volutin but as it was a most transient property no definite conclusions could be reached.

DISCUSSION.

Volutin was found to be produced by Aero. aerogenes under three different conditions of culture (1) by allowing the organism to grow on a poorly buffered phosphate containing medium, (2) by growing the organism in a nitrogen-deficient or sulphur-deficient medium, (3) by transferring phosphate-starved cells to a medium containing phosphate. In all these Aero.aerogenes cultures the volutin stained metachromatically with methylene blue, suggesting that the granules were similar to the classical volutin granules found in C.diphtheriae and Sp.volutans. The resemblance included also the degree of acid-fastness. The granules produced by Aero.aerogenes, when stained with methylene blue and then subjected to acid treatment, were found to be slightly acid-fast; they resisted the action of 1.0 per cent sulphuric acid for 1 minute, but were destained with 20 per cent sulphuric acid. The fact that the volutin could be seen in the organisms in the unstained wet films by the phase contrast microscope showed that the granules are not artefacts caused by staining as asserted by Bisset (1950).

Volutin produced due to acid conditions developed during growth.

When a phosphate mixture (pH 7.3) was the only source of buffer in a glucose containing medium and the phosphate concentration lay between 0.03 and 0.001 per cent the pH of the medium fell to 4.3 during growth and much volutin was produced. If, however, the phosphate concentration was greater than 0.03 per cent the pH did not fall below 6.0 and no volutin was produced. Therefore the volutin production would appear to depend upon either the decreased phosphate concentration or the acid condition developing during growth or a combination of both.

To study the effect of the decreased phosphate concentration alone, buffer was added to the glucose medium containing 0.01 per cent phosphate. The addition of 0.03 per cent bicarbonate or 0.05 per cent "citrate" held the pH above 6.0 and no volutin was produced. This showed that the acid conditions developed during growth were the more important factor in inducing volutin production. This conclusion is supported by the fact that the substitution of 1.0 per cent acid phosphate

(KH_2PO_4 pH 5.2) for the 1.0 per cent phosphate mixture pH 7.3 allowed the pH of the medium to fall to 4.5 and volutin was again produced.

The growth as measured by the opacity was 0.24 on the unbuffered medium with 0.01 per cent phosphate but the addition of 0.05 per cent "citrate" to the same medium supported a growth of opacity 0.778. This showed that there was an excess supply of phosphate in the unbuffered medium when the volutin was produced. Similarly an excess of glucose appeared to be present in the 0.01 per cent phosphate medium as the growth was increased from 0.24 to 0.86 by addition of 1.0 per cent phosphate buffer to medium with the same 0.1 per cent glucose supply.

The presence of the extra phosphorus and extra glucose may have been important in the production of the volutin because it was found that no volutin was produced if the concentration of phosphate was below 0.0003 per cent. The cells from such media when stained with methyl violet had a "nuclear" appearance (Duguid, 1948), so it was concluded that they were phosphate-starved. This would appear to show that a supply of phosphate was required for the production of volutin.

The substitution of other energy sources e.g. "citrate" or pyruvate for glucose or sucrose in a medium (1.0 per cent acid phosphate) which normally supported volutin production gave little or no volutin so an easily metabolisable energy source such as glucose would appear to be necessary for volutin production.

As an energy source was required for the production of volutin, it would seem that volutin was a metabolic product and not a cytological artefact concurrently dependent on low pH. This is supported by the evidence that 24 hour volutin-containing cells from a medium of pH 4.5 did not lose their volutin granules immediately on transfer to a neutral medium, but only after a few hours when growth and cell division had commenced.

As volutin appeared to contain phosphorus, the amount of phosphorus per unit of nitrogen was measured for both volutin-rich and volutin-free cells. The ratio of total phosphorus to total nitrogen of the volutin-rich cells was almost twice that of the volutin-free cells so volutin granules would appear to be rich in phosphorus. Both types of cells were then fractionated and the phosphorus content of each fraction estimated. It was found that

for nine of the ten fractions of the volutin-rich cells the phosphorus content was almost twice that of the corresponding fractions of volutin-free cells, while the one remaining fraction, R9, was only present in the volutin-containing cells so that the production of volutin may be correlated with the presence of phosphorus in the fraction R9.

Volutin production due to deficiency in one component of the medium.

When Aero. aerogenes was grown on a medium with 1.0 per cent phosphate, 0.1 per cent glucose, salts and a limited supply (0.001 per cent) of ammonium sulphate, the cells produced volutin after 18 hours incubation at 35°C. This rose to a maximum at 24 - 48 hours and had largely disappeared by 72 hours. The cells here were not elongated, as in the acid cultures, but were of the "nuclear" type indicating the deficiency of nitrogen. The growth (0.112) was less than that on the medium containing 1.0 per cent phosphate, and 0.1 per cent glucose and 0.5 per cent ammonium sulphate (0.760) so that there would be an excess of both phosphate and of carbon and energy source but a deficiency of nitrogen. The findings thus show that volutin is produced when growth is

limited by a deficiency of nitrogen in the presence of excess phosphate and carbon and energy source.

Similarly if all the sulphates in the synthetic medium containing 1.0 per cent phosphate and 0.1 per cent glucose were replaced by chlorides the growth of Aero.aerogenes A3 was reduced (0.636) and volutin was produced after 24 hours incubation. Such cells were found to be "nuclear" in appearance indicating the sulphur-deficiency. Again there was no question of pH inducing the production of volutin as the pH of the medium never fell below 7.0. As in the case of the nitrogen-deficient cells there was an excess of both phosphate and carbon and energy source. The findings thus show that volutin is produced when growth is limited by deficiency of sulphur in the presence of excess phosphate and carbon and energy source.

When the amount of phosphate in a buffered medium was reduced to 0.0003 per cent the growth was reduced to 0.508 as compared with 0.832 when 1.0 per cent phosphate was present in the medium. The cells which were therefore considered to be phosphorus-starved were found to be "nuclear" in appearance. However they did not contain any volutin. The findings thus show that volutin

is not produced when growth is stopped by a deficiency of phosphate even though an excess of carbon and energy source is present.

Likewise when Aero.aerogenes A3 was grown on a medium containing 1.0 per cent phosphate and 0.1 per cent glucose no volutin was produced. In this case the cells were not "nuclear" in appearance but were regarded as carbon and energy-deficient as if the sugar content of the medium were increased to 0.5 per cent, the amount of growth was increased (Duguid and Wilkinson 1953) indicating that 0.1 per cent sugar did not support the full growth possible as regards the other nutrient factors present. These findings thus show that volutin is not produced when growth is limited by a deficiency in the carbon and energy source.

From the above conclusions it would appear that both phosphate and carbon and energy source were necessary for the production of volutin and that volutin is produced when growth is limited by exhaustion of some other nutrient factor.

The transfer of the nitrogen and sulphur-deficient cells to a complete "Second Medium" resulted in a loss of the volutin only after cell division had occurred - apparently metabolism was

required for the utilisation of the volutin. Transfer of the carbon and energy-deficient cells to a complete "Second Medium" did not have any visible cytological effect on the cells, but similar transfer of the phosphate-starved cells resulted in the production of much volutin after 15 minutes incubation.

Volutin production on subculture of phosphate-starved cells to a phosphate containing "Second Medium".

The transfer of phosphate-starved, non-volutin containing cells to a phosphate containing "Second Medium" resulted in abundant volutin production within a short time. Volutin was visible in 3 minutes and rose to a maximum at 2 hours. Subsequently it disappeared, little remaining at 8 hours. This disappearance of the volutin appeared to be correlated with the return of the normal staining reaction of the cells and the occurrence of cell division.

When the amount of the phosphate supply required for this production of volutin was studied, it was found that the total absence of phosphate in the "Second Medium" was paralleled by a total failure of volutin production but that the addition of increasing amounts of phosphate caused increasing

amounts of volutin to be synthesised. The maximum volutin production was reached when the "Second Medium" contained 0.01 per cent phosphate. These findings thus show that phosphate is required for volutin production.

Both in the poorly-buffered media and in the nitrogen- and sulphur-deficient media, an excess of carbon and energy source was found necessary for the production of volutin. Similarly it was found necessary for the volutin production in phosphate-starved cells. When glucose was entirely omitted from the "Second Medium" it was found that no volutin was produced. The inclusion of increasing amounts of glucose caused increasing amounts of volutin to be produced; the maximum being reached when the glucose content of the medium was 0.1 per cent. These findings show that a carbon and energy source is required for the production of volutin. This suggests that the production of volutin is an endothermic reaction and that volutin is an exothermic compound. When glucose was replaced by the various components of the glycolysis cycle, most of the compounds were found to be as good a source of energy for volutin production as glucose. However, when "citrate" replaced the glucose, the

volutin production was not so good; possibly previous adaptation of the strain to "citrate" was required.

The importance of the other nutrients in the "Second Medium" was also studied and it was found that the complete omission of the nitrogen source from a liquid "Second Medium" slightly reduced the volutin production; very little nitrogen source (0.0002 per cent ammonium sulphate) was, however, required to restore volutin production to its normal level. Thus very little nitrogen appears to be required for volutin production. When either potassium or magnesium were completely omitted from the "Second Medium" the volutin production was greatly reduced but the addition of 0.001 per cent of the sulphates of these metals in both cases again restored the volutin production to its normal level. Therefore potassium and magnesium ions are required for the production of volutin.

When the ratio of total phosphorus to total nitrogen content of the phosphate-starved cells was compared with that of similar cells which had been subcultured for 30 minutes on a phosphate containing medium it was found that the ratio had risen from 0.08 to 0.18. This doubling of the phosphorus to

nitrogen ratio was paralleled in all the fractions except R9 which showed a forty-fold increase. As the phosphate-starved cells had produced much volutin on subculture in the phosphate containing medium and the phosphorus content of the fraction R9 had increased to such a marked extent after the same length of time, the presence of the volutin might be correlated with the increase in the R9 substance. This was substantiated by the fact that the phosphorus content of the fraction R9 followed the same pattern as the volutin production in that they both rose to a maximum in 30 minutes after transfer and fell away to almost nothing by 180 minutes. Also the subculture of phosphate-sufficient cells on a phosphate containing medium did not result in volutin formation nor did the phosphorus content of R9 show any significant increase. According to Juni et al. (1948) the corresponding fraction of volutin containing yeast cells is metaphosphate in nature. The findings therefore suggest that volutin consists of metaphosphate.

Summarising, volutin would appear to be an exothermic compound which contains phosphorus probably metaphosphate, and which only accumulates in microscopically observable amounts when the

growth of Aero.aerogenes is curtailed due to adverse cultural conditions such as acidity or to deficiencies in the growth medium other than in the phosphate and carbon and energy source. The fact that volutin is only detected under adverse conditions of culture may therefore mean that it is a normal metabolic product which the cell usually utilises as fast as it is synthesised.

SUMMARY.

1. Aero.aerogenes A3, an organism which does not produce volutin under normal conditions of culture, was found to produce volutin under three different special conditions namely (1) at the acid reaction developed during growth on a poorly-buffered sugar containing medium, (2) by growth on a medium deficient in either its nitrogen or sulphur source and (3) on transfer of phosphate-starved cells from a "First Medium" deficient in phosphate to a "Second Medium" rich in phosphate.

The volutin produced under all three cultural conditions was found to react metachromatically with methylene blue - the characteristic property of the volutin found in C.diphtheriae and Sp.volutans. Thus the volutin of Aero.aerogenes was thought to be of the same nature as the classical volutin. The volutin granules of Aero.aerogenes were also observed in unstained, wet films by the phase contrast microscope, demonstrating that the granules were not staining artefacts.

2(a) When Aero.aerogenes A3 was grown aerobically at 35°C on a 0.1 per cent glucose medium the growth was maximal when the phosphate content was 0,1 per cent or greater; the pH did not fall below 6.1 and no

volutin was produced. When the phosphate content of the medium was between 0.001 and 0.03 per cent, and no other buffer was present, the pH fell to 4.5 or less, and much volutin was produced in 24 hours. This fall in pH of the medium was found to be the determining factor for the volutin production since (1) when "citrate" or bicarbonate buffer was added to a medium containing 0.01 per cent phosphate the pH did not fall below 6.0 and volutin production was prevented, and (2) when 1.0 per cent pH 5.2 acid phosphate (KH_2PO_4) was substituted for 1.0 per cent pH 7.3 phosphate mixture the pH fell to 4.5 and abundant volutin was produced.

(b) When the phosphate content of the unbuffered medium was less than 0.0003 per cent, the amount of growth was reduced even further and the pH again fell to 4.5 or less but no volutin was produced. Such cells were found to be "nuclear" indicating that they were phosphate-starved. This showed that a certain amount of phosphate was necessary for the production of volutin.

(c) The substitution of various other energy sources which did not cause such a large drop in the pH of the medium resulted in a smaller volutin production. This together with the fact that

volutin only disappeared with the occurrence of cell division would show that volutin was a metabolic product.

3(a) When the organisms were grown at 35°C on a nitrogen or sulphur-deficient medium buffered at neutral pH "nuclear" volutin-containing cells were produced in 24 hours but a deficiency in the phosphate or carbon and energy source did not induce a similar production of volutin.

(b) The subculture of such nitrogen- and sulphur-deficient cells on a fresh medium resulted in the loss of the volutin only after a few hours when cell division had occurred. The transfer of the "nuclear" non-volutin containing phosphate-starved cells to a fresh phosphate containing medium resulted in abundant volutin production.

4(a) When Aero.aerogenes was grown on a series of buffered media containing 0.1 per cent glucose and various amounts of phosphate, it was found that the cells were "nuclear" in appearance and the growth was curtailed on media containing 0.0003 per cent phosphate or less. Only these phosphate-starved produced volutin on transfer to a fresh medium containing phosphate. This volutin production was apparent after 3 minutes subculture, rose to a

maximum at 2 hours and disappeared mainly within 8 hours. The disappearance of the volutin again appeared to be accompanied with the return of the normal staining reaction to the cells and with the occurrence of cell division.

(b) If no phosphate were added to the "Second Medium" no volutin was produced on transfer of phosphate-starved cells. Therefore phosphate was required for the production of volutin.

(c) Likewise the total omission of glucose from the "Second Medium" caused a complete absence of the volutin production but other components of the glycolysis cycle were found to support volutin production in the "Second Medium". Therefore glucose or another carbon and energy source was required in the "Second Medium" for the production of volutin.

(d) The presence of ammonium sulphate, potassium and magnesium ions was found to be required for maximal volutin production.

5. As phosphate seemed to be required for the production of volutin the ratio of phosphorus to nitrogen was determined for the volutin-free and volutin-rich cells produced under the different cultural conditions. In all cases it was found

that the ratio of total phosphorus to total nitrogen for the volutin-rich cells was almost twice that of the volutin-free cells. When the cells were fractionated and the phosphorus content of each fraction estimated, nine of the ten fractions of the volutin-rich cells were found to contain double the amount of phosphorus present in the corresponding fraction of the volutin-free cells while the remaining fraction R9 of the volutin-rich cells showed a forty-fold increase over that of the volutin-free cells. In the phosphate-starved cells which were transferred to the phosphate containing medium the phosphorus content of the fraction R9 was found to follow the same pattern as the volutin production in that it rose to a maximum in 30 minutes and thereafter fell away to practically none at 180 minutes.

6. Volutin in Aero.aerogenes appears to be a normal metabolic product which contains phosphorus - probably metaphosphate and which is only observed when some enzyme systems are blocked due to growth under adverse cultural conditions.

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