

# INTRACELLULAR STRUCTURES IN CAULOBACTER VIBRIOIDES<sup>1</sup>

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In a recent communication (Gruła *et al.*, 1954), it was demonstrated that inorganic phosphate was involved in the genesis of the electron-opaque granules observed in *Caulobacter vibrioides*. However, it was not shown whether the phosphate was involved in the synthesis of ribonucleic acid, deoxyribonucleic acid, or metaphosphate. Each of the three compounds has been reported as the main constituent in what appear to be similar granules in several different organisms. Hartman and Liu (1954) implicated ribonucleoprotein in yeast cytoplasmic cell granules. Knaysi *et al.* (1950) observed that deoxyribonuclease digestion decreased the staining potential of such granules in an avian strain of *Mycobacterium tuberculosis*. König and Winkler (1948) reported the presence of calcium phosphate and nucleic acid in *Spirillum volutans*, whereas Ruska *et al.* (1952) implicated metaphosphate in *Mycobacteria*.

This paper reports further studies on the chemical nature of the granules and vacuoles of *C. vibrioides*.

## MATERIALS AND METHODS

Two semisynthetic media were employed. The "A" medium (minimal phosphate) contained 1 g casein hydrolyzate<sup>2</sup> (enzymatic), 2 mg riboflavin, 2 mg FeSO<sub>4</sub>·7H<sub>2</sub>O, and 2 g agar per 100 ml of distilled water. The "B" medium (phosphate enriched) contained 1 g casein hydrolyzate, 2 mg riboflavin, 2 mg FeSO<sub>4</sub>·7H<sub>2</sub>O, 100 mg K<sub>2</sub>HPO<sub>4</sub>, 100 mg KH<sub>2</sub>PO<sub>4</sub>, and 2 g agar per 100 ml of distilled water.

Cells transferred from nutrient agar slants were grown at 30 C for 72 to 80 hours on the "B" medium before extraction and digestion since

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<sup>2</sup> Nutritional Biochemical Corporation, Cleveland, Ohio.

older cells appeared to have larger and better developed granules. For synthesis reactions regarding the granule and vacuole in enriched phosphate-sugar solutions, the cells were grown at 30 C for 18 to 24 hours on the "A" medium. Cells for extraction and digestion were harvested by two washes in double distilled water whereas an 0.85 per cent solution of NaCl was substituted as the wash medium for cells incubated in the phosphate-sugar solution.

The phosphate-sugar solution consisted of 100 mg K<sub>2</sub>HPO<sub>4</sub>, 100 mg KH<sub>2</sub>PO<sub>4</sub>, and 200 mg glucose per 100 ml of water. Aeration in this solution was accomplished in small Erlenmeyer flasks by shaking the bacterial suspensions throughout the incubation period (25 C) on a Burrell wrist-action shaker at a setting of 2.

Crystalline ribonuclease,<sup>3</sup> deoxyribonuclease,<sup>3</sup> and trypsin<sup>3</sup> were made up to 0.1 per cent solution using double distilled water. The deoxyribonuclease was diluted 1-1 with 0.15 M MgSO<sub>4</sub>·7H<sub>2</sub>O prior to use. All digestions with these enzymes were performed by covering glass slides, or collodion-covered, electron-microscope grids on glass slides, containing the fixed cells with the enzyme solution, and incubating at 37 C for one hour. Fixation of cells prior to extraction was accomplished by immersion in 70 per cent ethyl alcohol for 18 hours in the refrigerator (4 to 6 C) or exposure to the vapors of OsO<sub>4</sub> (one per cent solution) for 3 minutes. Cells fixed in alcohol were washed by immersion in double distilled water for 5 minutes prior to further processing.

Extraction of fixed cells using perchloric acid or trichloroacetic acid was carried out by the following procedure: Washed cells were placed on glass slides or collodion covered grids on glass slides and permitted to air dry. After fixation, they were immersed immediately in cold 10 per cent perchloric acid or 10 per cent trichloroacetic

<sup>3</sup> Worthington Biochemical Sales Co., Freehold, New Jersey.

acid and kept in the refrigerator for 18 to 24 hours for extraction. Washing after extraction was accomplished by permitting the cells to remain in distilled water for 10 minutes (without agitation), after which they were air dried.

When bulk extraction was attempted, 90 to 100 mg (dry weight) of washed cells were resuspended in cold 70 per cent ethyl alcohol and placed in the refrigerator for 18 hours. Then they were centrifuged at refrigeration temperatures, drained, and resuspended in cold 10 per cent perchloric acid and stored in the refrigerator for 18 to 24 hours, after which they were spun out again and the supernate analyzed for inorganic phosphate by the method of Lowry and Lopez (1946).

When Loeffler's methylene blue was employed, all preparations were stained 3 to 5 minutes, washed, and immediately counterstained with 0.5 per cent safranin for 10 seconds. They were washed then and blot dried. The granule and vacuole both stained dark blue whereas the cytoplasm stained pink.

When the metaphosphate stain was used (Lindegren, 1951), air-dried, and heat-fixed, cells were stained 3 to 5 minutes, washed, blot-dried, and counter-stained with 0.5 per cent safranin for 30 seconds. The granules stained an intense purple-blue color whereas the cytoplasm was pink.

Collodion films were prepared by placing 14 drops of a 10 to 40 (vol/vol) mixture of collodion and amyl acetate on a distilled water surface (surface area, 78 in<sup>2</sup>). The Philips and the RCA-EMU-2D type electron microscopes were both used. All preparations were shadowed with chromium.

#### RESULTS

To determine whether the granule contained either ribonucleic acid or desoxyribonucleic acid, perchloric acid extraction was used since it can extract selectively ribonucleic acid when used cold and desoxyribonucleic acid when heated (Cassel, 1950). Preparations of extracted cells stained with Loeffler's methylene blue showed that the granules were absent. To determine the extent of the extraction, fixation and extraction were performed directly on electron microscope grids. This technique showed that the granules were extracted completely (figure 3). Controls observed after alcohol fixation showed that the granules were still present (figure 2). Although

the alcohol did not extract the granule or make it less opaque, it was noted that volatilization under electron bombardment occurred more readily in some preparations after this treatment (compare figures 1 and 2). Cells fixed with OsO<sub>4</sub> did not show this effect. The granules also were extracted completely by cold trichloroacetic acid.

Although the granules were extracted by cold perchloric acid and trichloroacetic acid, it did not necessarily mean that the material removed was ribonucleic acid and nothing else. The procedure did demonstrate that desoxyribonucleic acid was not present. Mann (1944) used cold 10 per cent trichloroacetic acid to extract metaphosphate from *Aspergillus niger*. Further, these granules stained very well with the metaphosphate stain.

Therefore, to double check the chemical nature of the granules, ribonuclease, desoxyribonuclease, and trypsin digestion were employed. Staining with Loeffler's methylene blue as well as electron microscopic observations showed that neither ribonucleic acid nor desoxyribonucleic acid was present. Trypsin digestion appeared to decrease the affinity for the stain slightly; however, *no* change could be observed with the electron microscope.

Polymetaphosphate is unstable and will break down at low pH values (Ingelman, 1947). Also, inorganic phosphate precipitates are generally known to be acid soluble. Therefore, the Lowry-Lopez technique was used to check for the presence of inorganic phosphate in the supernate of cold perchloric acid extracted cells. It was found that approximately two per cent, as compared to dry weight of the cells, was extracted as inorganic phosphate.

Therefore, it would appear that the extracted material which is responsible for the electron opaqueness and the basophilic staining characteristics of the granule is metaphosphate or some other form of inorganic phosphate. Apparently, the granules also contain some fat since they stain lightly with Sudan Black B.

*Structural synthesis in phosphate-sugar solutions.* Wiame (1946) reported that phosphate starved yeast cells took up phosphate very well. Therefore, to increase the size of the granules in the absence of a nitrogen source, 24 hour cells from the "A" medium which showed very poor granule development (figure 4) were suspended in the phosphate-sugar solution and placed on a shaker.

Periodic checks, using Loeffler's methylene

blue staining, showed that numerous, large darkly-stained bodies were present after 4 hours' incubation and that they increased in size and number throughout an 18 hour period. Electron microscope examination showed that the cells had not only synthesized rather large granules but also contained big vacuolated structures (figure 5). This type of structure was not new to this organism (Bowers *et al.*, 1954; Grula *et al.*, 1954). However, in this case, different nutritional circumstances evoked its synthesis.

The vacuole stained intensely with Sudan Black B. Enzyme digestion showed that the affinity for Loeffler's methylene blue was removed entirely by ribonuclease digestion but was not affected by desoxyribonuclease treatment. Only a slight decrease in Loeffler's methylene blue staining was noted after trypsin digestion.

Therefore, the structure is a fat vacuole containing large amounts of fat and ribonucleic acid and some protein. It appears that the basophilic character of the vacuole is due to ribonucleic acid and protein.

The synthesis of the large amounts of ribonucleic acid located in the fat vacuoles in the absence of a known supply of nitrogen and the occurrence of relatively good growth on Ashby's nitrogen-free medium indicates that this organism may be capable of fixing atmospheric nitrogen.

*Oxidation-reduction indicator dye reactions.* The O/R indicator dyes, 2,3,5 triphenyltetrazolium chloride and Nadi reagents, have been used as indicators of localized enzyme accumulation in the bacterial granules (mitochondria) by Mudd *et al.* (1951). However, reports by several in-

vestigators (Tynen and Underhill, 1949; Zweifach *et al.*, 1951; Shelton and Schneider, 1952) have shown that fat solubility affects the distribution of reduced tetrazolium *in situ*.

Cells from the "A" medium (18 to 24 hours) were harvested, washed, and incubated 8 hours in the phosphate-sugar solution for vacuole synthesis, after which time one of the O/R indicator dyes was added. With tetrazolium, the suspension became pink in 10 minutes and was red at one hour. Examination with both the optical and electron microscopes showed that the reduced dye (red formazan) was present in the fat vacuoles (figure 6). After one hour of exposure to the Nadi reagents, the oxidized dye (blue indo-phenol) was also present in the fat vacuoles. If these results were interpreted according to the viewpoint of Mudd and his co-workers, then these fat vacuoles could also be the sites for localized accumulations of enzymes (mitochondria).

However, to determine whether enzyme action alone was responsible for the *localization* of the dyes in the fat vacuoles, 12 hour cells were taken from control flasks (no dye) and killed by fixation for 3 minutes in the vapors of OsO<sub>4</sub>. These cells then were stained with chemically reduced tetrazolium or autooxidized Nadi reagents for varying periods of time (5 to 45 minutes). Examination showed that the dyes were deposited again in the fat vacuole.

*Further nutritional factors relating to granule synthesis.* During the course of the investigation relating to digestion and extraction of the granules, the organism had been grown routinely for 72 to 80 hours since older cells appeared to have larger and better developed granules. The slants

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*Figure 1.* 72 hour cells grown on the "B" medium.\*

*Figure 2.* 72 hour cells grown on the "B" medium after fixation in cold ethyl alcohol for 18 hours. The granules are still electron opaque although some have been volatilized in this preparation.

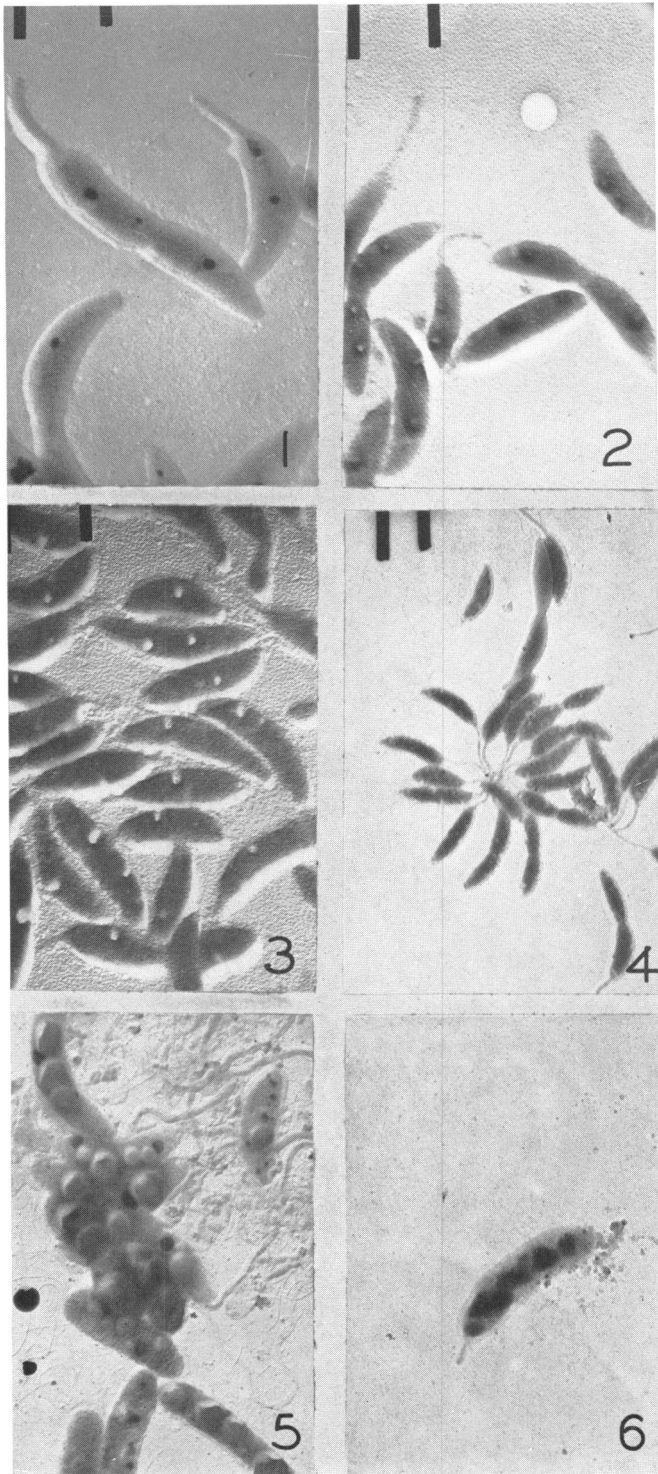
*Figure 3.* 72 hour cells grown on the "B" medium after extraction with cold perchloric acid. The granules have been completely extracted.

*Figure 4.* 21 hour cells grown on the "A" medium. The granules are either not present or greatly reduced in size.

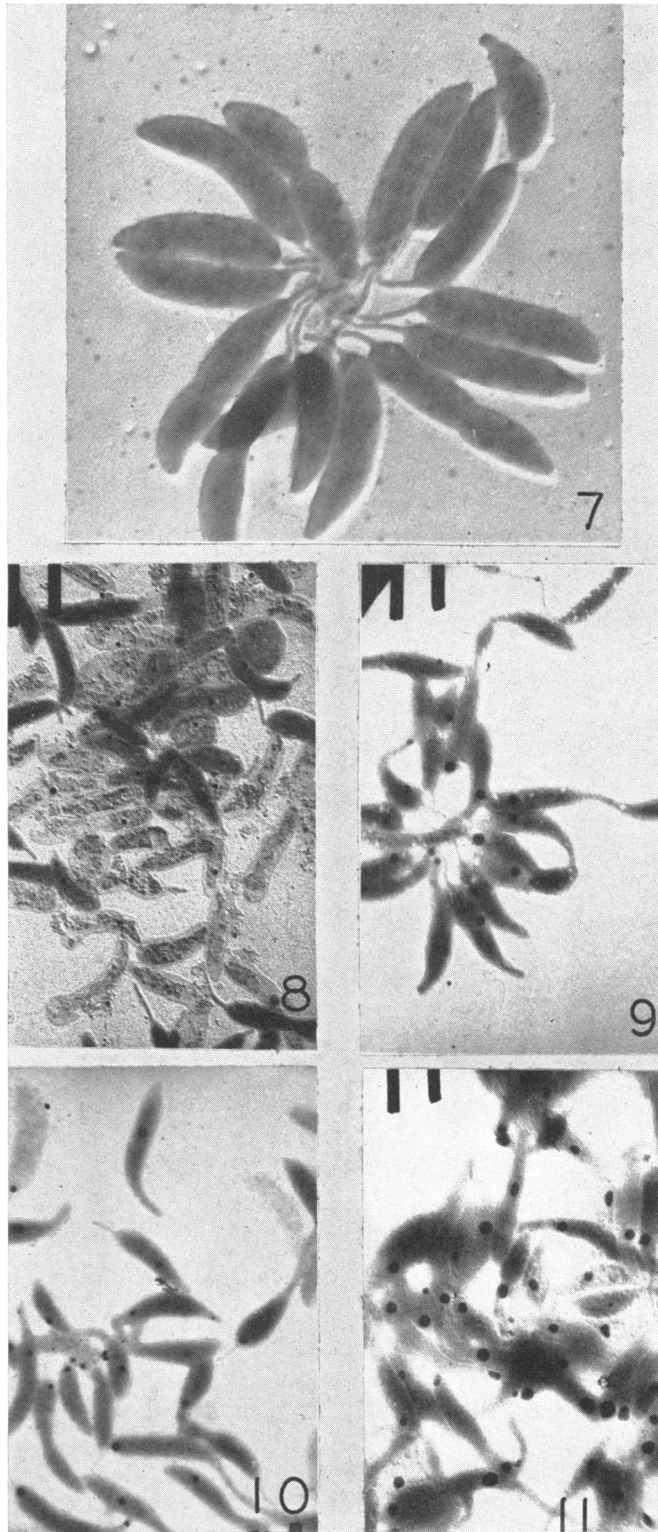
*Figure 5.* 24 hour cells from the "A" medium after incubation in the phosphate-sugar solution for 12 hours. The granules have increased in size (compare to figure 4) and are pressed in by the fat vacuoles.  $\times 10,000$ .

*Figure 6.* Deposition of reduced 2,3,5 triphenyltetrazolium chloride inside of the cell and vacuoles of *Caulobacter vibrioides*.  $\times 10,000$ .

\* Magnification of all figures except 5, 6, and 7 is denoted by the micron markers. Distance between the markers is 1 micron.



*Figures 1-6*



*Figures 7-11*

used in that phase of the study had all come from one batch of the "B" medium. Because many of the cells did not synthesize granules in this medium and in order to induce better granule synthesis, a new batch of the "B" medium was made up in which the phosphate level was increased. Cells grown on this medium also showed poor granular growth—even after extended periods of incubation (90 to 120 hours). Regardless of the phosphate level (up to 5 mg per ml of medium), good granule synthesis did not occur. Because this situation had never occurred in a phosphate enriched medium, the reason for such behavior was not understood.

Since adjustment of the phosphate levels did not produce good granule growth, inorganic nutritional factors were tested even though it had been established previously that iron, alone, was able to satisfy the divalent mineral requirement for this organism in a completely synthetic medium (Gruha *et al.*, 1954). The compounds tested included  $MgSO_4 \cdot 7H_2O$ ,  $MnSO_4 \cdot H_2O$ ,  $ZnSO_4$ ,  $CaSO_4$ , and  $FeSO_4 \cdot 7H_2O$  at a concentration of 10 mg per 100 ml of the "B" medium.

The results were very striking. Good granule growth occurred within 24 hours if either Mn or Zn was present in the medium. Good granular growth did not occur when Mg, Ca, or Fe alone was used. Furthermore, combinations of Mn and Zn; Mg and Zn; Mg, Mn, and Zn permitted good development of the granules in 24 hours and at 72 hours; the size and number of the granules were considered excellent (figures 7-11).

This was surprising since Mn and Zn are toxic to this organism when it is grown in a completely synthetic medium wherein ammonium sulfate is the sole source of nitrogen (Gruha *et al.*, 1954). Good growth of the organism occurred in the presence of all of these ions; however, Mn did inhibit growth slightly even in the presence of casamino acids. This inhibition was most apparent during the first 24 hours of incubation. Why ions, which can be shown to be toxic, can promote such excellent growth of the granules in the presence of excess phosphate is not apparent at this time.

Cells grown on the "A" medium continued to show poor granule development either in the presence or absence of Mn and Zn (figures 7 and 8).

#### DISCUSSION

Apparently, the judicious employment of nutritional requirements can be an excellent adjunct in cytological procedures since it has been shown that two different intracellular structures can be manipulated at will with slight variations in technique. Because these cells appear to grow equally well with or without either type body, it appears logical to designate them as "accessory" structures. We believe them to be "volutin" and "fat vacuoles" (Gruha *et al.*, 1954).

#### SUMMARY

The size and presence of two types of intracellular bodies in *Caulobacter vibrioides* have been demonstrated to be nutritionally controlled.

The electron-opaque granules (volutin) appear to be composed of metaphosphate or another form of inorganic phosphate, some fat, and possibly small amounts of protein. They can be extracted completely from the cells by either cold perchloric or trichloroacetic acid. They give a positive staining reaction with the Lindegren metaphosphate stain. Their presence and size in cells are related to the phosphate concentration of the growth medium in the presence of an energy source and specific divalent ions (Mn and Zn). Older cells possess larger granules. Their basophilic nature does not depend on either ribonucleic acid or desoxyribonucleic acid.

Transparent structures (fat vacuoles) are synthesized in phosphate deficient cells after incubation in a phosphate-sugar solution. They contain large amounts of fat and ribonucleic acid and small amounts of protein. Their basophilic nature is due to ribonucleic acid and protein.

The O/R indicator dyes, 2,3,5 triphenyl-tetrazolium chloride and Nadi reagents, are found localized in the fat vacuoles in both live and fixed cells in their respective reduced and oxidized states.

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Figure 7. 24 hour cells from the "A" medium plus Mn.  $\times 12,500$ .

Figure 8. 72 hour cells from the "A" medium plus Mn.

Figure 9. 72 hour cells from the "B" medium plus Zn.

Figure 10. 24 hour cells from the "B" medium plus Mn.

Figure 11. 72 hour cells from the "B" medium plus Mn.

## REFERENCES

- BOWERS, L., WEAVER, R. H., GRULA, E. A., AND EDWARDS, O. F. 1954 Studies on a strain of *Caulobacter* from water. I. Isolation and identification as *C. vibrioides* Henrici and Johnson with emended description. *J. Bacteriol.*, **68**, 194-200.
- CASSEL, W. A. 1950 The use of perchloric acid in bacterial cytology. *J. Bacteriol.*, **59**, 185-187.
- GRULA, E. A., WEAVER, R. H., AND EDWARDS, O. F. 1954 Studies on a strain of *Caulobacter* from water. II. Nutrition, with implications for cytology. *J. Bacteriol.*, **68**, 201-206.
- HARTMAN, P. E., AND LIU, C. 1954 Comparative cytology of wild type *Saccharomyces* and a respirationally deficient mutant. *J. Bacteriol.*, **67**, 77-85.
- INGELMAN, B. 1947 Isolation of a phosphorus-rich substance of high molecular weight from *Aspergillus niger*. Preliminary communication. *Acta Chem. Scand.*, **1**, 776-777.
- KNAYSI, G., HILLIER, J., AND FABRICANT, C. 1950 The cytology of an avian strain of *Mycobacterium tuberculosis* studied with the electron and light microscopes. *J. Bacteriol.*, **60**, 423-447.
- KONIG, H., AND WINKLER, A. 1948 Über Einschlüsse in Bakterien und ihre Veränderung im Elektronmikroskop. *Naturwissenschaften*, **35**, 136-144.
- LINDEGREN, C. C. 1951 The relation of metaphosphate formation to cell division in yeasts. *Exptl. Cell Research*, **2**, 275-278.
- LOWRY, O. H., AND LOPEZ, J. A. 1946 The determination of inorganic phosphate in the presence of labile phosphate esters. *J. Biol. Chem.*, **162**, 421-428.
- MANN, T. 1944 Studies on the metabolism of mould fungi. II. Isolation of pyro and metaphosphate from *A. niger*. *Biochem. J. (London)*, **38**, 345-351.
- MUDD, S., WINTERSCHIED, L. C., DELAMATER, E. D., AND HENDERSON, H. J. 1951 Evidence suggesting that the granules of mycobacteria are mitochondria. *J. Bacteriol.*, **62**, 459-475.
- RUSKA, H., BRINGMANN, G., NECKEL, I., AND SCHUSTER, G. 1952 Über die Entwicklung sowie den morphologischen und zytochemischen Aufbau U. M. avian (Chester). *Z. wiss. Mikroskop.*, **60**, 425-447.
- SHELTON, E., AND SCHNEIDER, W. C. 1952 On the usefulness of tetrazolium salts as histochemical indicators of dehydrogenase activity. *Anat. Record*, **112**, 61-82.
- TYNEN, J., AND UNDERHILL, S. W. F. 1949 Triphenyltetrazolium chloride as a reagent for the histological demonstration of subcutaneous fat. *Nature*, **164**, 236.
- WIAME, J. M. 1946 Basophilia and phosphorus metabolism in yeast. *Bull. soc. chim. biol.*, **28**, 552-556.
- ZWEIFACH, B. W., BLACK, M. M., AND SHORR, E. 1951 Evaluation of tetrazolium as a histochemical index of adrenal cortical activity. *Proc. Soc. Exptl. Biol. Med.*, **76**, 446-454.