BIKEN JOURNAL Vol. 11, 225-234, 1968

SHORT COMMUNICATION

OCCURRENCE OF POLY-β-HYDROXYBUTYRIC ACID INCLUSIONS IN *VIBRIO PARAHAEMOLYTICUS* A55

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(Received July 1, 1968)

A polymeric ester of D(−)-β-hydroxybutyric acid (PHBA) is accumulated by a variety of species of bacteria (ASSELINEAU, 1966) as an endogenous reserve of carbon (DAWES and RIBBONS, 1964; DOUDOROFF, 1966). However, the existence of PHBA in members of the genus *Vibrio* has not yet been established.

In 1950, FUJINO *et al.* (1951, 1953) isolated a motile Gram negative rod as a causative agent of "Shirasu" food poisoning. This bacillus took on polar stain with basic dyes. Later this rod bacillus was classified into the genus *Vibrio* and designated as *Vibrio parahaemolyticus* (SAKAZAKI *et al.*, 1963; FUJINO *et al.*, 1965). During studies on the envelope of this organism, it was noted that isolated envelopes held distinct granular bodies which could not be removed by repeated washing with water. These inclusions presumably correspond to refractile and electron-dense spherical bodies, which have about 100–200 mμ diameter and are visible near the polar regions of intact rods. They have strong affinity for phenolized basic dyes and are not decolorized by acid treatment when modified Moeller's acid-fast staining method is applied (TAMURA *et al.*, 1967). These findings suggest that this vibrio might possess PHBA inclusions. The present work was undertaken to see whether these acid-fast, polar inclusions actually consist of PHBA.

Vibrio parahaemolyticus strain A55[1] (serotype 0 : 5, *K* : 15), originally isolated from cases of food poisoning in Yokohama, Japan, by TAKIKAWA (1958) was grown in medium containing 1 % each of casamino acid (Nissan, Nissui Seiyaku Co.), yeast extract (Oriental Yeast Co.), maltose and 3 % NaCl. Before the addition of maltose the medium was adjusted to pH 7.8 with NaOH and sterilized by autoclaving at 120°C for 20 min. Maltose was autoclaved separately and added to the medium after cooling. Flasks of 500 ml capacity containing 200 ml of medium were inoculated with 8 ml of a seed culture grown at 37°C for 16 hr with shaking at 140 strokes/min at an amplitude of

[1] This strain was generously supplied by Dr. G. Omori, Osaka City Institute of Hygiene, Osaka, Japan.

7 cm, and then incubated with shaking under the same conditions. Cells were harvested at the late exponential growth phase (usually 5 hr after inoculation) in a continuous flow centrifuge. They were washed with 3% saline supplemented with 0.01 M $MgCl_2$, and stored at –20°C.

The cells were treated with sodium dodecylsulfate (SDS) by the method of KOLENBRANDER and ENSIGN (1968). SDS was added to 500 ml of a stock cell suspension containing 21.06 g dry weight of cells to a concentration of 1 %. After being stirred at 37°C for a few hours, 1 mg of deoxyribonuclease (Sigma Chemical Co.) was added to reduce the viscosity of the disintegrated cell suspension. The mixture was kept at 37°C for 16 hr with stirring, and then centrifuged at 13,000 *g* for 60 min at room temperature. The pellet was resuspended in 1% SDS and treatment with SDS was repeated. The insoluble residue thus obtained was washed thoroughly with water, and digested with 10 mg of crystalline trypsin (Sigma Chemical Co.) in 100 ml of 0.02 M Tris-HCl buffer (pH 7.0) for 4 hr at 37°C. This digestion was repeated and the pellet obtained by centrifugation was washed and lyophilized (envelope fraction). The dried material (717 mg) was thoroughly extracted with 40 ml of chloroform in a Soxhlet apparatus at 65°C for 16 hr. The chloroform extract was evaporated to dryness and the residue was redissolved in a small amount of hot chloroform. A white flocculant material which formed in the solution on addition of excess ether-ethanol (1: 3, v/v), was collected on a filter paper as a white amorphous material. It was further purified by repeating the above cycle of dissolving it in hot chloroform and precipitating it with ether-ethanol. The final product was washed successively with hot water and hot methanol, dried and kept in a desiccator.

Attempts were also made to extract PHBA, if present, from intact cells by treating cells of the same lot as in the preceding experiment with alkaline hypochlorite by the method used by WILLIAMSON and WILKINSON (1958) for quantative isolation of PHBA from *Bacillus cereus*.

As shown in Table 1, 488 mg of purified material were extracted from 717 mg of the envelope fraction with hot chloroform, corresponding to 2.3 % of the original intact cells (21.06 g). This yield was comparable to that obtained from the intact cells with alkaline hypochlorite. The purified materials isolated both from intact cells and their envelopes were readily soluble in chloroform giving a viscous solution. When the chloroform was evaporated off, the material formed a thin translucent plastic-like film adhering to the surface of the test tube. The material stained well by modified Moeller's method like the granules in intact cells. It gave off white fumes with an irritant odor of crotonic acid when melted by heating. The material was hydrolyzed with alkali and paper chromatographic analysis of the hydrolyzate by the method of BROWN(1950) in *n*-butanol saturated with 1.5 N NH_4OH (ascending technique) showed two spots on spraying with 0.4 % BTB in ethanol. The major spot of R_F 0.10 and the minor spot of R_F 0.30, corresponded to authentic β-hydroxy-

TABLE 1 *Isolation of PHBA from an envelope fraction of Vibrio parahaemolyticus A55*

Material	Dry weight	Weight ratio (%)
Starting material (intact cells)	21.06 g	100
Trypsin-digested residue from SDS-treated cells (envelope fraction)	717 mg	3.4
Material extracted with hot chloroform and precipitated with ether-ethanol (PHBA)	488 mg	2.3
Non-extractable residue	202 mg	1.0

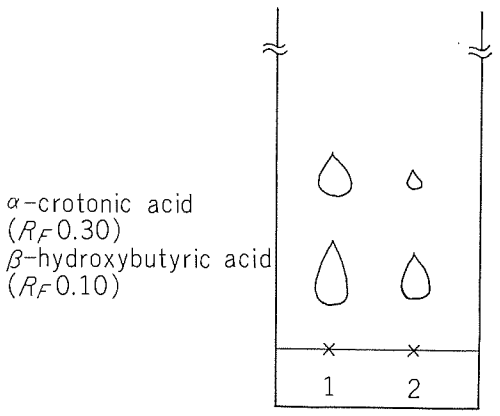


FIGURE 1 Paper chromatogram of hydrolyzate of test material (PHBA) from *Vibrio parahaemolyticus* A55.

Ten mg of sample were hydrolyzed with 5 ml of 0.5 N methanol-KOH in a sealed tube for 3 hr. One-way ascending chromatography was carried out with *n*-butanol saturated with 1.5 N NH_4OH for 12 hr at room temperature. Spots of organic acid were detected with 0.4% BTB solution in ethanol.

1. Mixture of authentic β-hydroxybutyric acid and α-crotonic acid (50 μg each).
2. Hydrolyzed test sample (50 μg).

butyric and crotonic acids, respectively (Fig. 1). Following the method of LAW and SLEPECKY (1961) the material was heated in concentrated sulfuric acid and then the ultraviolet absorption spectrum was examined and found to be identical with that of crotonic acid derived from authentic β-hydroxybutyric acid by heating in the same condition. As shown in Fig. 2, the test and reference materials gave identical spectra with an absorption maximum at 235 mμ. The infrared spectrum of the test material exhibited the absorption bands (Fig. 3) due to -C-CO-O-C- at 1,735, 1,250 and 1,050 cm^{-1}, and those due to $-CH_3$ and $-CH_2-CO-$ at 1,460 and 1,390 cm^{-1} respectively.

Analysis: $(C_4H_6O_2)_n$;
calculated: C, 55.57; H, 7.03
found: C, 55.80; H, 6.94

The contents of nitrogen and phosphorus were 0.5% and <0.01%, respectively.

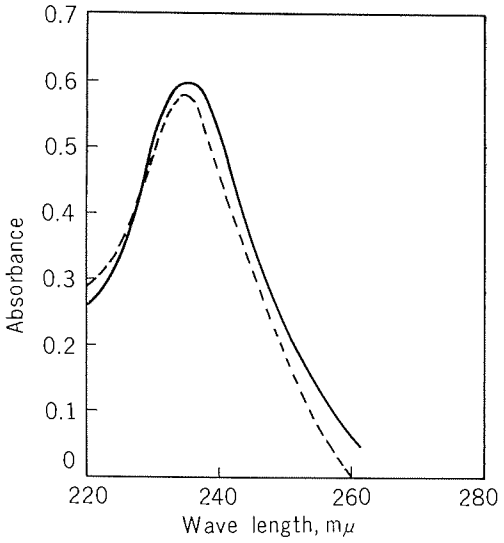


FIGURE 2 Ultraviolet spectra of test material (PHBA) and of authentic β-hydroxybutyric acid, after heating in concentrated H_2SO_4.
Solid line: test material.
Dotted line: authentic sample.

All these findings indicate that the test material is PHBA with the structure presented in Fig. 4. Thus, the presence of PHBA in *Vibrio parahaemolyticus* A55 was established.

Electron microscopic examination with a JEM 120 electron microscope (Japan Electron Optics Laboratory Co.) showed that after SDS treatment and trypsinization, the residue (envelope fraction) of *Vibrio parahaemolyticus* A55 retained a thin membranous structure with an electron dense granule (Fig. 5). Most of the granules were in clumps and each granule was wrapped up by very thin membranes (Fig. 7 and 8). Extraction of the envelope fraction with hot chloroform seemed to remove these granules (Fig. 6). From these findings, together with the fact that the chloroform extractable portion of the envelope fraction consisted almost entirely of PHBA, it was concluded that PHBA was probably a major constituent of the granules.

Evidence has been obtained that the membranous structures left after extraction with

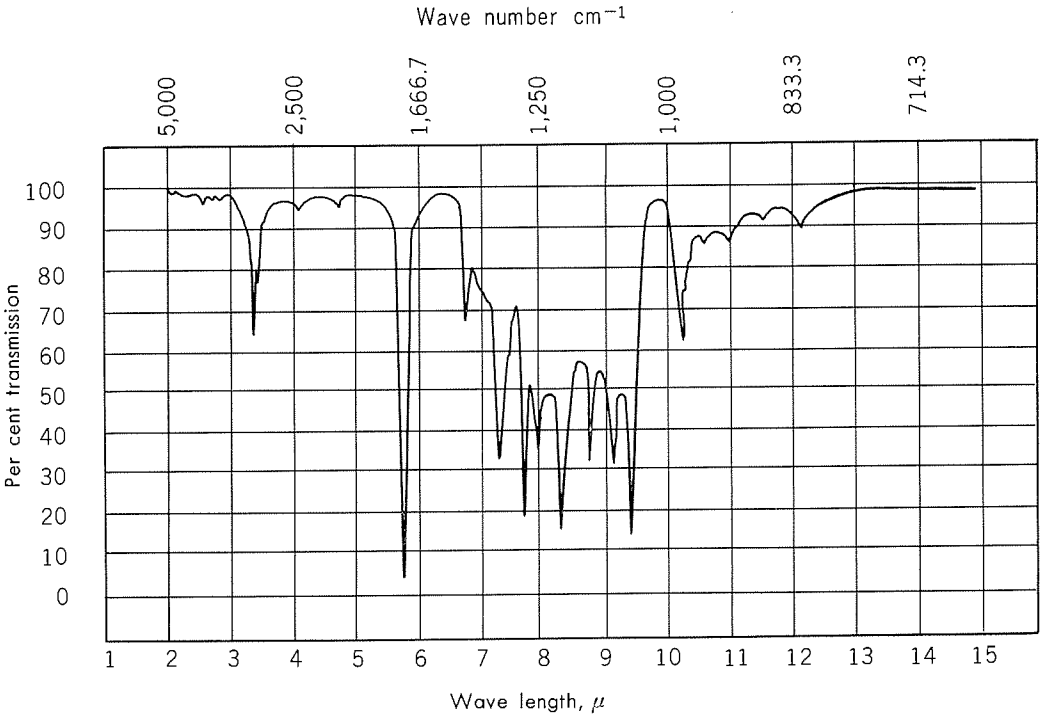


FIGURE 3 Infrared absorption spectrum of test material (PHBA).

hot chloroform (non-extractable residue) had amino acids and amino sugars characteristic of cell wall peptidoglycan: the non-extractable residue contained 1.0, 1.7, 0.9, 1.2 and 1.2 molar ratio of glutamic acid, alanine, α,ε-diaminopimelic acid, glucosamine and muramic acid, respectively. Details of experiments on this will be reported elsewhere.

In general, PHBA inclusions in bacterial cells have been regarded as Sudan black B stainable 'lipid granules'. Isolated and purified PHBA itself is not sudanophilic but stains with basic dyes, while PHBA inclusions in intact cells scarcely stain with basic dyes without pretreatment. This discrepancy was explained by supposing that the PHBA in the inclusions was surrounded by a sudanophilic lipid coat (WILLIAMSON and WILKINSON, 1958; YONEDA and KONDO, 1959; KONDO, 1960). This coat dose not seem to be permeable to basic dyes. Thus in a report on *Bacillus cereus*, YONEDA and KONDO recommended pretreatment of test specimens with acetone and ether-ethanol to remove the lipid layer, to enhance the acid-fast stainability by modified Moeller's method. In the present work the inclusions of *Vibrio parahaemolyticus* A55, did not stain with Sudan black B by the technique of BURDON (1946), but they stained well by modified Moeller's method without any pretreatment. These findings suggest that the PHBA inclusions in this organism had no, or only an incomplete lipid layer covering the granules. Further studies are required on the stainability of the granules of this organism grown under various culture conditions and on the granules of other strains of *Vibrio parahaemolyticus*.

PHBA was not readily extractable from dried intact cells with hot chloroform without pre-

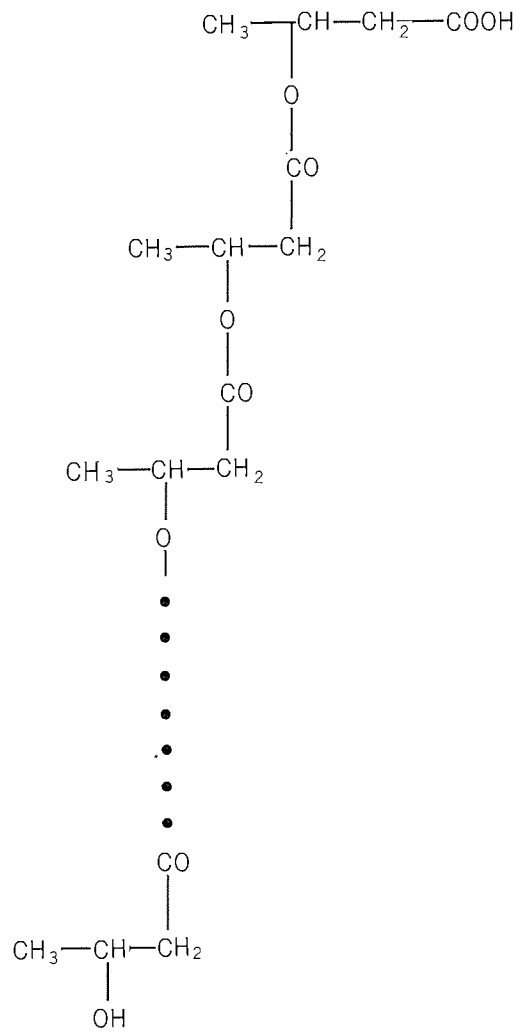


FIGURE 4 Possible structure of PHBA

treatment of the cells. Moreover ultrasonic disruption of cells did not improve the yield which was less than 0.1%. Pretreatment with SDS or alkaline hypochlorite, on the other hand, greatly increased the extraction of PHBA. Although these treatments may remove something which prevents extraction of PHBA, no experimental support for this has not yet been obtained.

The method of extraction of PHBA from the envelope fraction obtained by SDS-trypsin treatment of intact cells seems better than the conventional method of treating intact cells with alkaline hypochlorite for the following reasons. First, the former method gave pure material almost free from contaminating material so further purification was hardly necessary. Second, SDS-trypsin treatment is much milder than alkaline hypochlorite treatment and, so undegraded PHBA could be isolated. It is unknown whether the SDS-trypsin-hot chloroform method is applicable on other bacterial species as an easy and efficient method of extraction of PHBA, and this requires study.

FORSYTH, HAYWARD and ROBERTS (1958) and HAYWARD (1959) reported the presence of PHBA in bacteria isolated from soil and river water. However, there are no sufficient descriptions in these papers indicating that any of the bacteria examined by them belongs to the genus *Vibrio*. In this connection it would be pointed out that the IAMS subcommittee on taxonomy of *Vibrio* agrees upon that only bacteria producing acidity from dextrose by the Embden-Mayerhof glycolytic pathway should included into this genus (FEELEY, 1966). Therefore, *Vibrio parahaemolyticus* seems to be the first identified vibrio in which the presence of PHBA as inclusions was established.

The ability to synthesize PHBA may serve as an useful taxonomic criterion for classification of vibriones, as it dose in the primary subdivision of the genus *Pseudomonas* (STANIER *et al.* 1959). A screening survey for PHBA in other related vibriones is now in progress.

The authors are grateful to Dr. C. Kawasaki, Faculty of Pharmaceutical Sciences, Osaka University, and Dr. M. Yoneda, Research Institute for Microbial Diseases, Osaka University, for their helpful discussion. They also thank Mr. T. Nishihara and other members of the Faculty of Pharmaceutical Sciences for analytical studies. Their thanks are also due to Dr. K. Kato and the staff of the Department of Microbiology, Osaka University Dental School, for useful suggestions. The taxonomical characteristics of genus *Vibrio* was discussed with Dr. J. Yasuda, the Curator of the Culture Collection, Research Institute for Microbial Diseases.

FIGURE 5 Electron micrograph of an envelope fraction, a trypsinized insoluble residue isolated from *Vibrio parahaemolyticus* A55 cells after SDS treatment. A granule in a membranous structure is seen. Shadowed at an angle of 25° with Pt-Pd.

FIGURE 6 Electron micrograph of an envelope fraction after extraction with hot chloroform.
The membranous structure is free from granules. Shadowed as in Fig. 5.

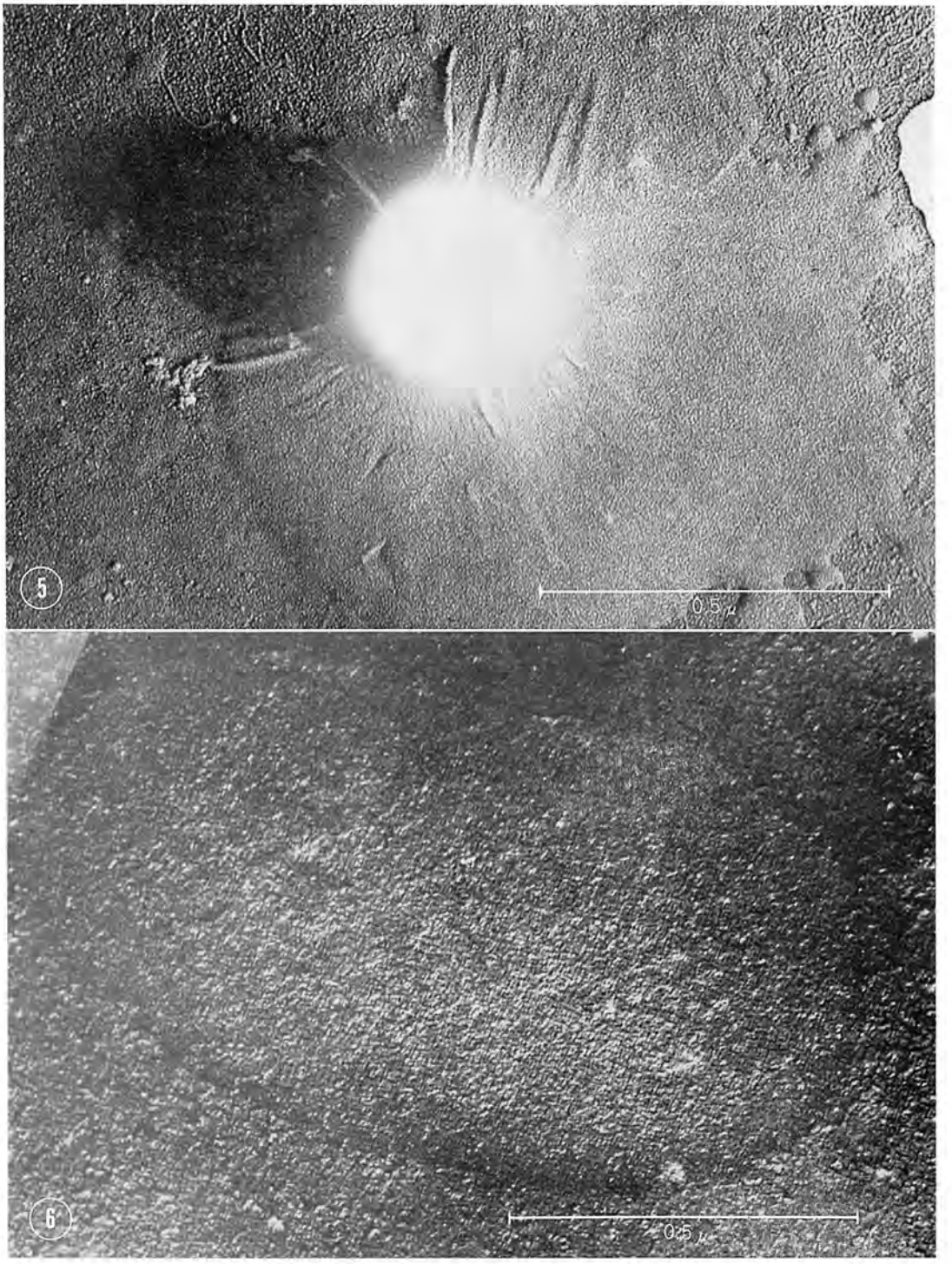
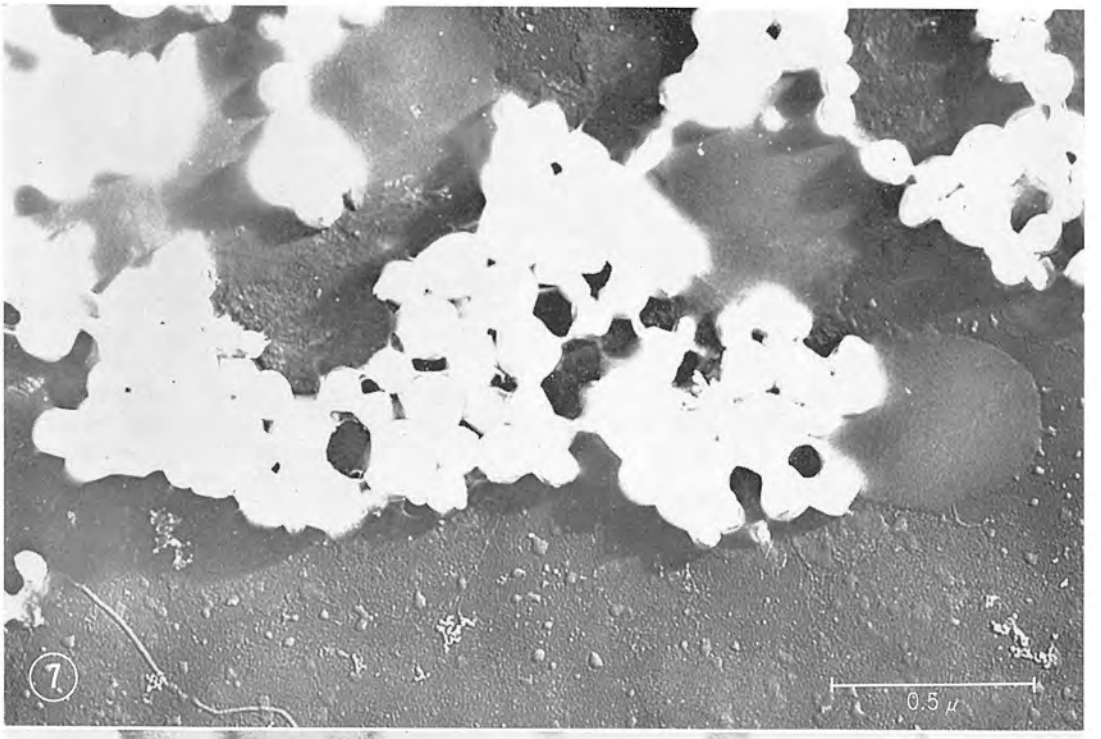

5
0.5 μ
6
0.5 μ

FIGURE 7 and 8 Electron micrographs of an envelope fraction.
Granules were assembled in clumps and wrapped by membranous structures.
Shadowed as in Fig. 5.


7
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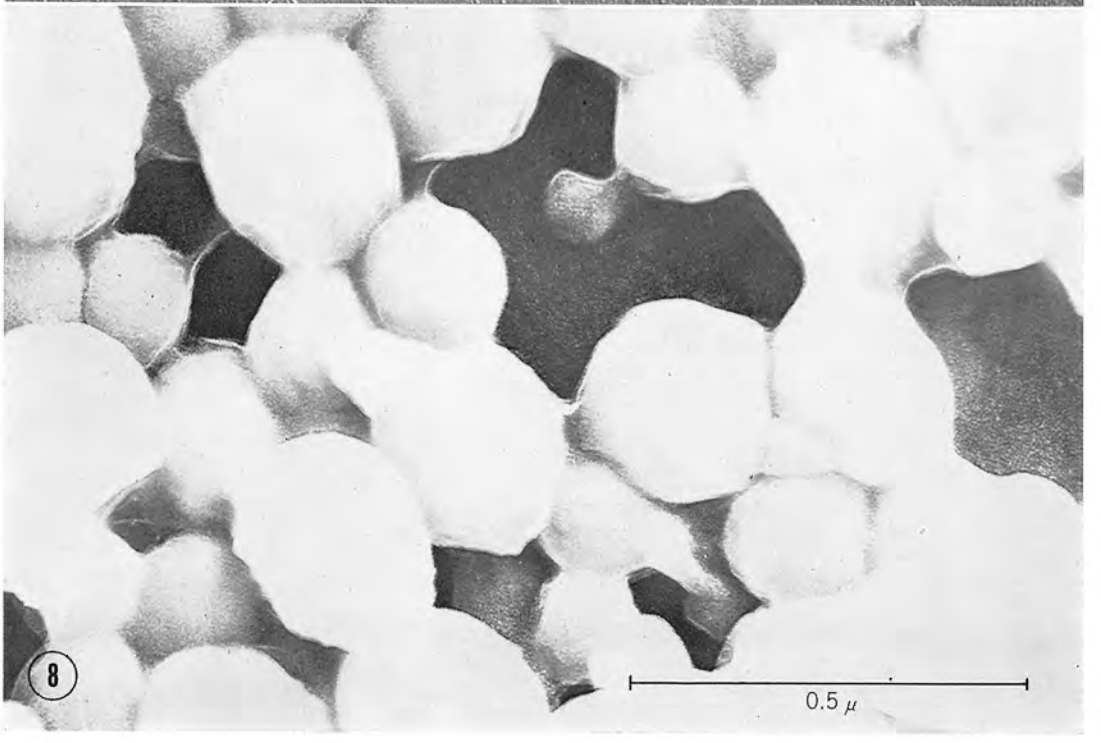
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0.5 μ

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