STUDIES ON THE HEAVY SPHERICAL (REFRACTIVE) BODIES OF FRESHWATER AMOEBAE. I. MORPHOLOGY AND REGENERATION OF HSBS IN CHAOS CAROLINENSE.

by

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The heavy spherical bodies of *Amoeba* and *Chaos* are refractive organelles, varying in size from ca. 10 µm in diameter, down to the limit of resolution of the light microscope. These inclusions have many characteristics in common with the phosphate-rich, »volutin« granules of other unicellular organisms, but differ from the latter in being constantly present in healthy growing amoebae, while in other organisms they appear only under conditions of nutritional imbalance. The fine structure of the HSBs of *Amoeba* and *Chaos* show that the organelles are membrane-bound, with an electron translucent periphery, and an electron dense inner core which sublimes in the electron beam, but which is stabilized by lead staining. During centrifugation in vivo, the HSBs collect at the centrifugal pole of the amoebae; under favourable conditions, a HSB-sack is formed at the heavy pole, and can be excised, leaving amoebae, normal in other respects, but containing only ca. 5% of the normal complement of HSBs. Using this method for obtaining practically HSB-free amoebae, the regeneration of HSBs was studied in *Chaos carolinense*, by determining the number and size distribution of HSBs in a standard volume in fixed, whole mounts by phase contrast microscopy. It was found that although the number of HSBs per standard volume in both fed and starved amoebae returned to that found in control, unoperated, *Chaos* by 7 days after operation, the size distribution did not return to normal until ca. 50 days after excision of the HSBs, indicating that HSBs originate from precursors in the cytoplasm. Operated amoebae showed normal feeding and locomotory behaviour, and their division rate was similar to that of control amoebae. However, HSB-free amoebae were more prone to rupture at an air-water interface, presumably owing to their lower specific gravity.

CONTENTS

1. INTRODUCTION

To the refractive, dense, spherical inclusion bodies of the large freshwater amoebae of the genera *Amoeba* and *Chaos,* various functions and structures have been ascribed, as indicated by the nomenclature devised for them: albuminous globules (64), corps luisants (48), Eiweisskügelchen (51, 60), excretion spheres (49), nutritive spheres (57, 58, 54), refractive bodies (36, 40, 41, 62), Golgi bodies (47), heavy spherical bodies (2). They have also been considered to be cysts (57), reproductive bodies (61, 11, 57) or parasites (43). The term Glanzkörper, originally used by GREEFF 1874 (24) for the glycogen bodies of *Pelomyxa palustris,* has also been used for the heavy spherical bodies of the genera *Amoeba* and *Chaos* (39, 35). MAST and DOYLE (41) stress that the different authors using these divergent terms probably did not recognize the inclusions which they were studying from the descriptions of other authors.

Although the heavy spherical bodies (HSBs*) are easily recognized by light or phase contrast microscopy in living amoebae, no detailed electron microscopic studies have so far been published, apart from a brief mention by HAYWARD (28) and a reference to unpublished observations by CHAPMAN-ANDRESEN and NILSSON, cited by ANDRESEN (4).

Several authors (38, 63, 3) have ascribed variations in the number of HSBs to the species of prey organism, but no detailed studies relating cell growth, and division rate, during a given diet, to the number and size of the inclusions have yet been made.

Data so far accumulated on HSBs show that they are the heaviest organelles in the cell (41, 54, 2), soluble in water (31), present a two-phase appearance in the light microscope, are partial-

* Footnote: The abbreviation »HSBs« will be used to designate the heavy spherical bodies throughout this paper.

ly fluid and compressible (40), contain peripherally lipoprotein (40, 10, 47) and, in the inner core, Mg and Ca (30, 31). Recent studies on *Amoeba proteus* using electron probe analysis (19) have shown that Mg, Ca, K, and P are present throughout the HSBs.

The origin of the HSBs is still obscure; MAST and DOYLE (41) suggested that they were formed in food vacuoles, which divided to form smaller vacuoles containing the bodies, while ANDRESEN and HOLTER (5) considered that they might originate in the cytoplasm from an accumulation of waste products.

Functions attributed to the HSBs include reserve nutrition (57, 58, 54), reproductive bodies (57, 11, 61), Golgi bodies (9, 55, 47), storage of calcium (19), and a significant contribution to the specific gravity of the cell (present paper). In the view of the high phosphate content of HSBs (14) they may be comparable to the phosphate rich, so-called volutin or metachromatic granules of other unicellular organisms, to which functions as phosphate reserve, regulator of phosphate economy, or phosphagen, i.e. energy storage (52) have been ascribed. As regards the fate of the HSBs, there is no evidence that they are excreted from healthy amoebae, but in starved cells they may enter debris vacuoles, together with other organelles and be extruded by defecation (5).

The recent interest in calcium storage and the suggestion that calcium storage could be a function of the HSBs of amoebae (19) indicate that additional data on the HSBs in amoebae would be of value in comparative studies on calcium-phosphate rich inclusions shown to be present in a wide range of organisms. The present paper of which a short abstract has been published (14) gives quantitative data on regeneration of HSBs in *Chaos carolinense*, and will be followed by a paper on the content of phosphate, calcium and magnesium in these inclusions.

2. MATERIAL AND METHODS

2.1. The amoebae.

Chaos carolinense, strain B, from the Biological Supply House, Chicago, was kept in modified Pringsheim's solution, and fed daily on *Tetrahymena*, according to the method used in previous studies (13). For some experiments amoebae from mixed cultures with *Colpidium* sp. and *Paramecium caudatum* as food organisms and kept in Chalkley's solution were also used.

The two inorganic culture media (modified Pringsheim's and Chalkley's solutions (13)) differ considerably in total salt concentration and ionic strength, although the molar ratios calcium: phosphate (ca 7:1) are similar; the total concentration of these two ions in Pringsheim's solution is about 20 times higher than that in Chalkley's solution. The Ca and P contents of the two media correspond to 0.85 mM, 0.043 mM Ca, and 0.112 mM, 0.0063 mM P for Pringsheim's and Chalkely's solutions respectively.

2.2 Procedure for centrifugation

Centrifugation of amoebae was carried out as described previously (2, 16, 15) using a Harvey Type centrifuge microscope, manufactured by Struers K/S, Copenhagen. The amoebae were deprived of food for 3-4 days before centrifugation, since the presence of many food vacuoles disturbs the layering of other inclusion bodies, and results in a less satisfactory shape for operation. Large amoebae were selected from the starved cultures.

Since the aim of most of these experiments was to excise the HSBs from the cells, without removing other inclusion bodies, it was desirable to obtain amoebae in which these organelles had collected into a sack at the centrifugal pole, the sack being attached to the main body of the cell by a thin bridge of cytoplasm. This desirable shape was often attained when the amoebae were »massaged« in the inorganic culture medium at room temperature (by sucking up and down in a braking pipette), and placed in the plastic centrifugation chamber at room temperature; the amoebae were then rapidly transferred into the centrifuge in the cold room (ca 5° C). Centrifugation for 15-20 minutes at 3000 r.p.m. (relative centrifugal force ca 1000 x G) followed by 5 minutes at 4000 r.p.m. (ca 1430 xG) usually produced the most suitably shaped amoebae for separation of the HSB-sacks.

After centrifugation, the amoebae were removed from the centrifuge chamber, rapidly washed through 4 transfers of cold Pringsheim's

solution, and those of suitable shape were operated immediately with a fine glass needle. The separated parts were placed at room temperature, and then examined *in vivo* at ca 200 x magnification to check the success of the procedure. Only those amoebae, in which it could be seen that very few HSBs remained in the main portion of the cell, were used for the experiments.

2.3. Preparation of amoebae for measuring and counting HSBs

Amoebae were fixed for an hour at room temperature in 96% ethyl alcohol, followed by transfer to 90% for 30 minutes, and to 70% for 18-20 hrs.; after dehydration through graded series of ethyl alcohol, they were cleared and mounted in cedar wood oil.

This procedure results in dissolution of the crystals (triuret (12)) and also of the lipid droplets, which intefere with the identification of the HSBs, owing to their refractive properties.

HSBs in whole mounts were counted and measured using Reichert phase contrast optics, 40 x objective, and a Patterson globe and circle ocular micrometer, in a counting area of 0.0056 mm^2. In each amoeba, 5 areas were counted, through a thickness of 60 µm, checked by the fine screw of the stage, thus giving the number of bodies in a volume of 0.00168 mm^3 (»standard« volume).

Starved, operated amoebae were fixed at intervals of 1-12 days after cutting, while operated amoebae, fed daily on *Tetrahymena* were fixed from 1 to 65 days after cutting. Control, non-operated amoebae, taken at the start of the experiment from the same cultures, and starved or fed as the experimental amoebae, were fixed at the same intervals. In two experiments, additional control amoebae, which had been centrifuged, but not cut, were also included.

In one experiment, amoebae were sectioned at 10 µm after embedding in paraffin, and stained for 2 minutes with toluidine blue (0.5% aqueous), rapidly rinsed and passed quickly through a graded series of ethyl alcohol, and mounted in Euparal. The same ocular micrometer was employed for counting the bodies, and the number in 30 areas of each amoeba were counted to correspond to the same volume as used for counting of HSBs in the unstained material.

In one experiment, large, uncentrifuged *Chaos* were cut into three pieces of as equal size as possible, fixed as described above and mounted in cedar wood oil. One third of each amoeba was left unstained, another third was stained with toluidine blue, and the third part was stained with lead at pH 3.5 according to EBEL COLAS and MULLER (22). Centrifuged *Chaos*, fixed in the same way, were sectioned at 10 µm, orientated through the long axis. Sections containing all layers of inclusion bodies from each amoeba were stained either with toluidine blue, or lead at pH 3.5, or left unstained for phase contrast microscopy.

3. MORPHOLOGY

The presence of HSBs has been noted in all the described species of the genera *Amoeba* and *Chaos* (4) and also in *Thecamoeba striata* (47), although these characteristic organelles are not included in the table of comparative cytoplasmic data compiled by BOVEE and JAHN (7). No mention has, however, been made of similar organelles in descriptions of the small freshwater »Limax« amoebae, *Acanthamoeba castellanii* (8), *Hartmanella astronyxis, Naegleria gruberi* and *Schizopyrenus* (58) and *Hyalodiscus simplex* (65).

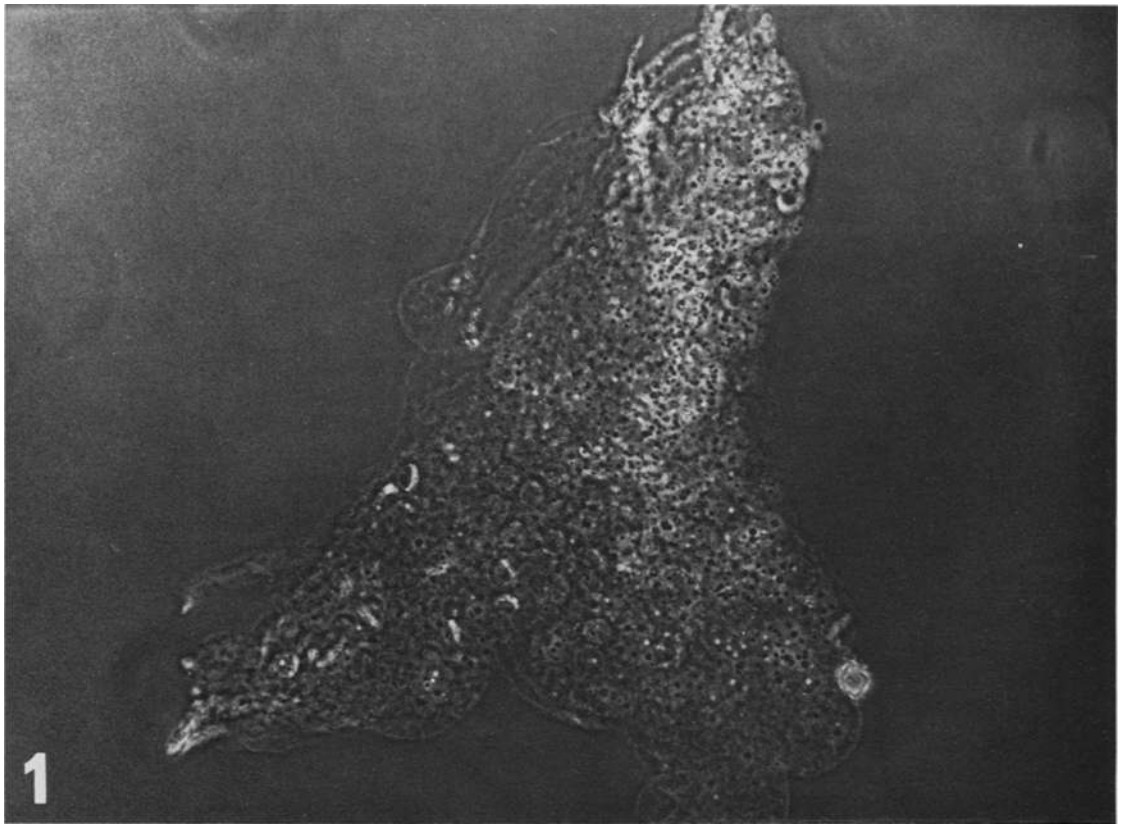
The number of HSBs per amoeba may vary considerably: in *Amoeba proteus*, MAST and DOYLE (41) give 0-40 per amoeba (size 2-10 µm), and PAPPAS (47) noted 4-40. For *Chaos* PAPPAS (47) found up to 200, ANDRESEN and HOLTER

All photographs, with the exception of Plate V. fig. 13, are of Chaos carolinense.

Plate I

Figure 1. Distribution of HSBs in uncentrifuged amoebae. Fixation, 96% ethyl alcohol, whole mount in cedar wood oil, unstained preparation. Phase contrast optics. Only few HSBs can be distinguished, as the thickness of the preparation prevents clear resolution with phase contrast optics. Magn. 250 x.

Figure 2. As fig. 1, but stained with toluidine blue, normal light optics. HSBs are clearly seen, but no other inclusion bodies are visualized. Magn. 250 x.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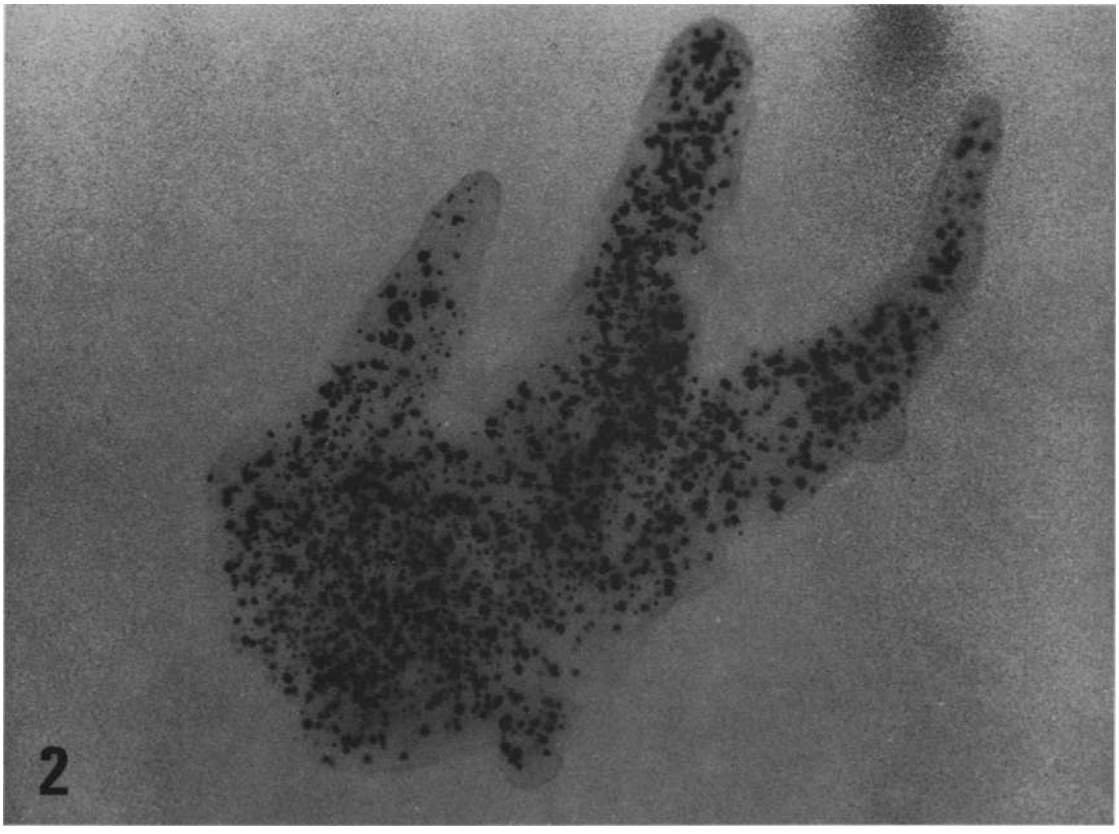
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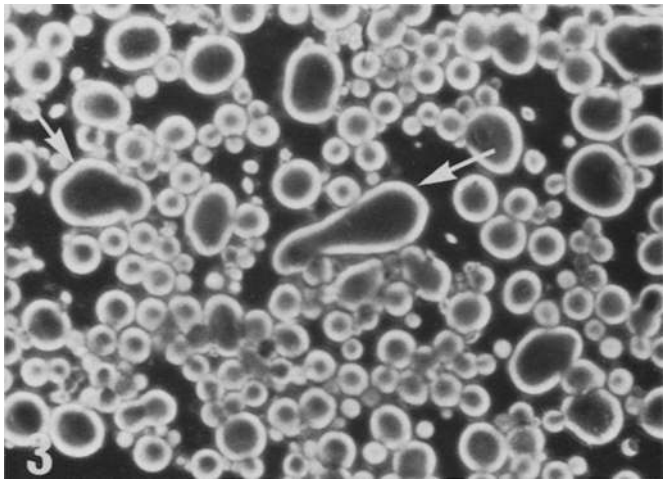
(5), 2800 (amoeba volume 0.035 µl), while the values found in the present paper are ca 1.5-4 times higher.

The following description applies to *Amoeba proteus* and to *Chaos carolinense,* and is taken from papers by MAST and DOYLE (40, 41) and by ANDRESEN (3), as well as from the author's own observations. In living healthy amoebae the HSBs are not found among the small inclusion bodies at the tip of the streaming pseudopodia, but are otherwise randomly distributed throughout the amoeba. They occupy ca 0.3% of the volume in *Chaos* (5). In unhealthy or starved specimens, when streaming is slow or absent, the HSBs collect at the underside of the amoebae, giving a »pseudocentrifuged« appearance, as first noted by WILSON (64). In starved *Chaos,* the number per unit volume of cytoplasm remains rather constant; HSBs may be collected and extruded in debris vacuoles together with other organelles, such as crystals (5).

During centrifugation of the amoebae *in vivo* the HSBs collect at the centrifugal pole, as first shown by MAST and DOYLE (41), a property advantageous for the study of their regeneration.



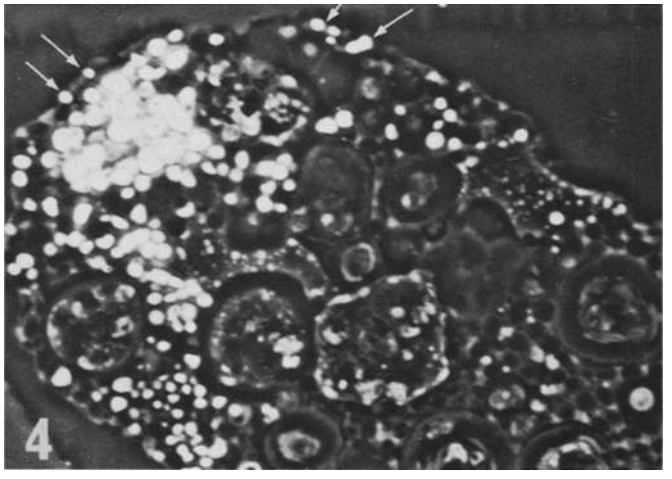


Plate II

Details of HSB-sacks excised from amoebae centrifuged *in vivo.* Figure 3. Contents of HSB-sack, cut open with glass needle, unfixed, unstained, phase contrast optics. Note fused HSBs at arrows. Magn. 880 x.

Figure 4. 1 µm section of OsO_4-fixed, Epon embedded HSB-sack, stained with lead. HSBs at arrows. Magn. 530 x.

3.1. Light microscopy

The HSBs can be observed in living amoebae by normal light and phase contrast microscopy, by reason of their refractive properties. In unstained whole mounts of amoebae, fixed in ethyl alcohol, they are readily identified, as the only other refractive spherical organelles are the lipid droplets, which are dissolved during fixation and dehydration. Owing to the refractive properties of the HSBs, photographs by phase contrast microscopy of whole mounts, as used for measuring and counting these inclusions (see section 4.1.), do not show the distribution clearly, while toluidine blue stained whole mounts give a good representation of the distribution, but not of the size of the HSBs (see section 4.1. and plate I).

The pronounced refractivity of the HSBs of *Amoeba* and *Chaos* has resulted in confusion of these inclusions with the glycogen bodies of *Pelomyxa palustris,* designated »Glanskörper« by GREEFF (24). In MAST's description (37) of *Pelomyxa palustris,* the glycogen bodies of this species are called »refractive bodies«, a designation which this author also uses for the HBSs of *Amoeba proteus,* while MAST and DOYLE (39) use the term »Glanzkörper« for the HSBs of this species. In the recent monograph on large freshwater amoebae (The Biology of Amoeba, ed. K. W. JEON (7)), BOVEE amd JAHN do not clearly define these inclusions in *Amoeba, Chaos* and *Pelomyxa.*

No limiting membrane to the HSBs can be discerned, but some, especially the large bodies,

have a two-phase appearance, like a fried egg. The HSBs are compressible, and may fuse on compression; centrifugal forces may also cause fusion, as illustrated in Plate II, fig. 3, a rather pure preparation of unfixed HSBs from an excised HSB-sack of *Chaos*. The diameter of unfixed HSBs varies from ca 10 µm down to the limit of resolution of the light microscope, as can be seen in Plate II, fig. 4, a 1 µm thick section of an osmium fixed, Epon embedded sack, stained with lead.

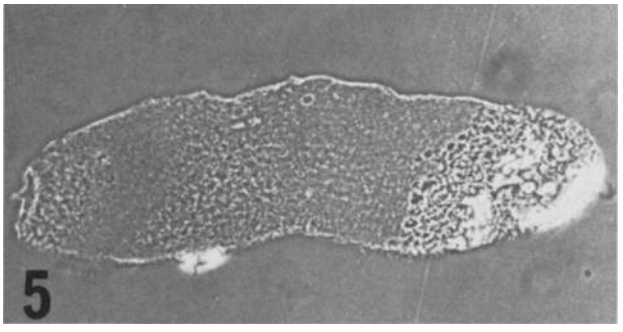
In amoebae sectioned after embedding in paraffin, it is not possible to distinguish the smallest HSBs in unstained sections, but they are clearly visible after staining with toluidine blue or lead nitrate at pH 3.5, although the latter method may result in dissolution of some bodies (see Plates III and IV). These plates illustrate the identification of the organelles in normal and centrifuged *Chaos*.

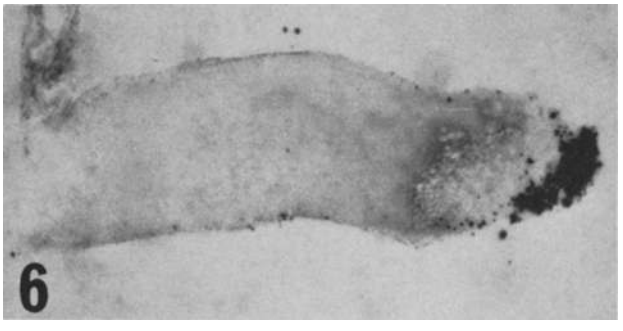
BYRNE (10) studied staining of HSBs *in vivo* in *Amoeba proteus* and found that, of the 11 acidic and 19 basic dyes tested, only Nile blue, neutral violet and neutral red were effective. In this species, staining of HSBs by the latter dye was also reported by MAST (36), MAST and DOYLE (39, 40, 41), SINGH (54), PAPPAS (47). ANDRESEN (2) found neutral red negative in *Chaos;* however, certain fluorescent atebrine derivatives stain these inclusions *in vivo* in *Chaos* (CHAPMAN-ANDRESEN unpub.) and after staining the HSBs are collected in vacuoles and excreted.

3.2. Electron microscopy

A detailed description of the fine structure of HSBs has not so far been published in the many fine structural studies on the large freshwater amoebae. GREIDER et al. (25) could not identify the HSBs of *Amoeba proteus* although they looked for them, and concluded »it is possible that internal material was not preserved, leaving them in the realm of unidentified bodies«. The »heavy vacuoles« described by DANIELS (20) in centrifuged *Amoeba proteus* and by DANIELS and ROTH (21) for *Chaos,* may correspond to HSBs, of which the contents are lacking.

HAYWARD's (28) note on these inclusions in *Amoeba proteus,* and a reference given by ANDRESEN (4) to unpublished data by CHAPMAN-ANDRESEN and NILSSON, are the only descriptions. One reason for this lack of identification may be the instability of Osmium-fixed HSBs in the electron beam; the contents sublime, leaving only a hole within the embedding medium, and often irregular spots





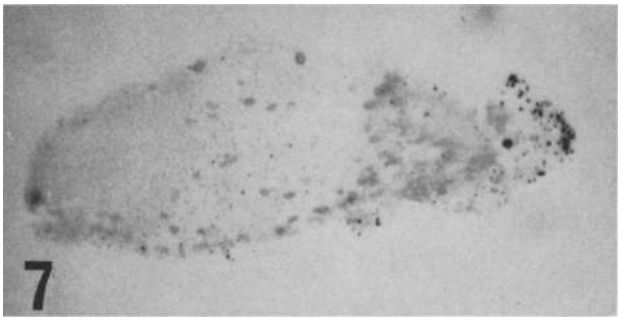


Plate III

Identification of HSBs in amoebae centrifuged *in vivo*. 10 µm sections from one amoeba, centrifugal pole to the right.

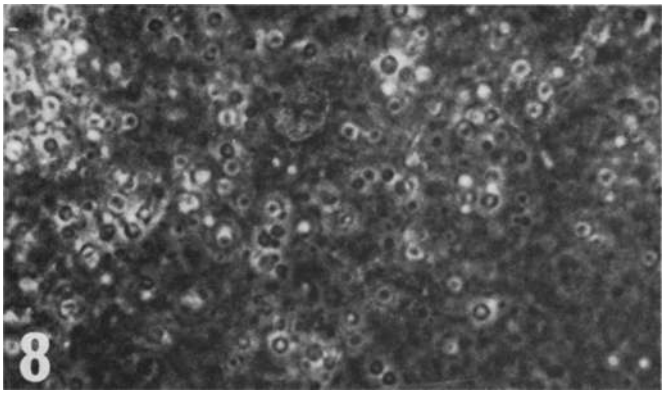
Figure 5. Unstained, phase contrast optics, the HSBs appear white.

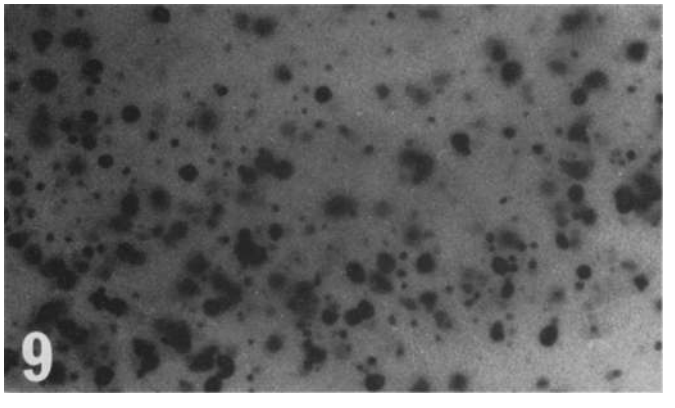
Figure 6. Stained with toluidine blue, normal light optics, the HSBs appear black.

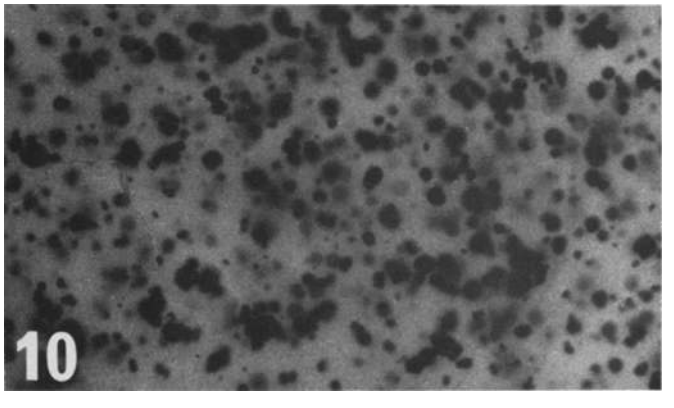
Figure 7. Stained with lead at pH 3.5, normal light optics, the HSBs appear black. Magn. 150 x.

Plate IV

Identification of HSBs by three methods. One amoeba was cut into 3 pieces before fixation in 96% ethyl alcohol.
The 3 pieces were treated as follows:
Figure 8. Unstained phase contrast optics, some refractile HSBs with typical halos are in focus.
Figure 9. Stained with toluidine blue, the small HSBs are well stained, but the larger bodies are not completely spherical.
Figure 10. Stained with lead at pH 3.5. Both small and large bodies are well stained. Magn. 540 x.







of electron dense material, some distance from the hole. Staining with lead salts stabilises the bodies, and indicates that the central core, but not the periphery, consists of electron opaque material (see Plate V, fig. 12).

In our first preparations of *Amoba proteus,* centrifuged *in vivo* (CHAPMAN-ANDRESEN and NILSSON unpub.), fixed in OsO_4 and embedded in methacrylate, we identified the HSBs at the centrifugal pole as electron dense brittle bodies, which often fell out of the sections during mounting, but which were stable. Using Epon embedding, HAYWARD (28) found membrane-bound HSBs with a dense central core, which sublimed in the electron beam (see Plate V, fig. 13). In *Chaos carolinense,* fixed in OsO_4, and embedded in Epon, after *in vivo* centrifugation, the larger HSBs appear distorted in shape, owing to the sublimation of the central dense core, leaving large holes within the translucent peripheral zone; in these preparations the limiting membrane is not readily seen (Plate V, fig. 11). However, small HSBs, of ca 0.3 μm diameter are more stable, contain a relatively larger dense core, and a relatively more narrow translucent periphery than the larger bodies, and the limiting membrane can be clearly seen (Plate V, fig. 14). This fig. also shows part of a large HSB, and of a debris vacuole containing HSBs.

Staining with lead results in stabilization of the core, which remains within the membrane, but

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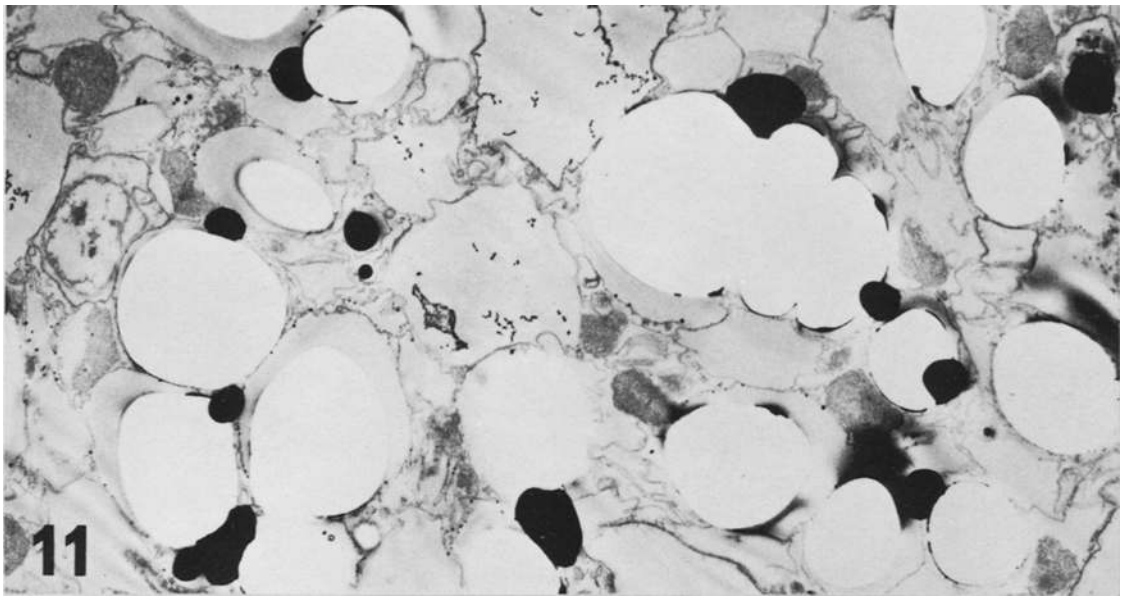
Plate V
(next page)

Electron micrographs of HSBs of *Chaos* and *Amoeba.*
Figure 11. Part of HSB sack from *Chaos* centrifuged *in vivo*, showing holes from which electron opaque material has sublimed to condense outside the area of the HSB. Magn. 4270 x.
Figure 12. As figure 11, but stained with lead, the electron opaque core remain *in situ,* but the peripheral translucent area ruptures. Magn. 12600 x.
Figure 13. Single HSB of *Amoeba proteus,* showing dense core subliming, and clear membrane bounding the peripheral translucent zone, the spherical body (upper right) is a lipid droplet. Magn. 10000 x.
Figure 14. A small HSB from HSB-sack of *Chaos,* showing the large central opaque core, and narrow peripheral zone, surrounded by a membrane. Part of large HSB (at right) and part of a debris vacuole, containing HSBs (at lower edge). Magn. 12600 x.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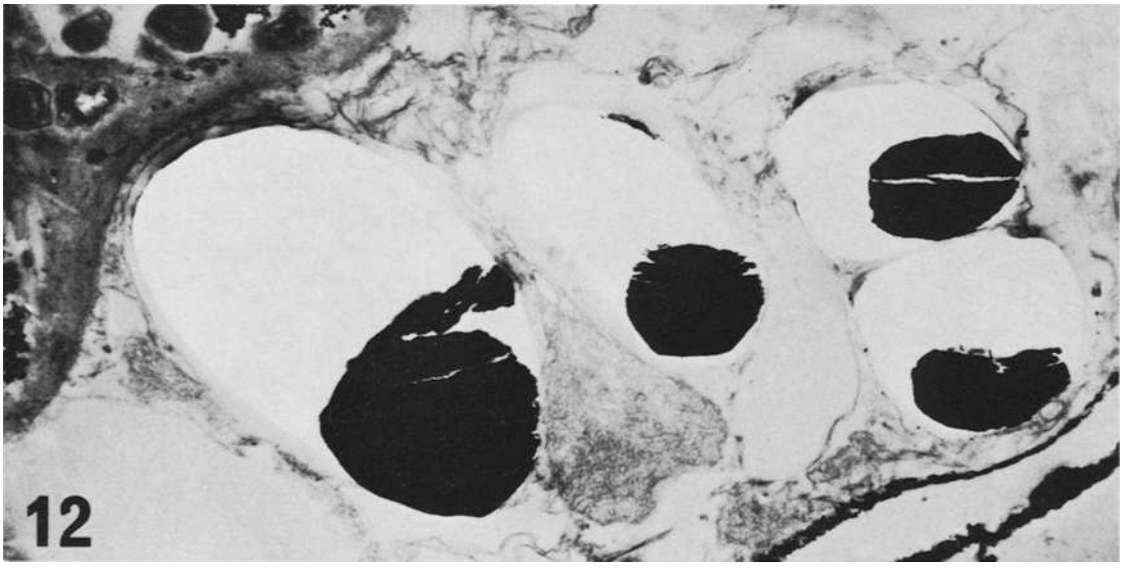
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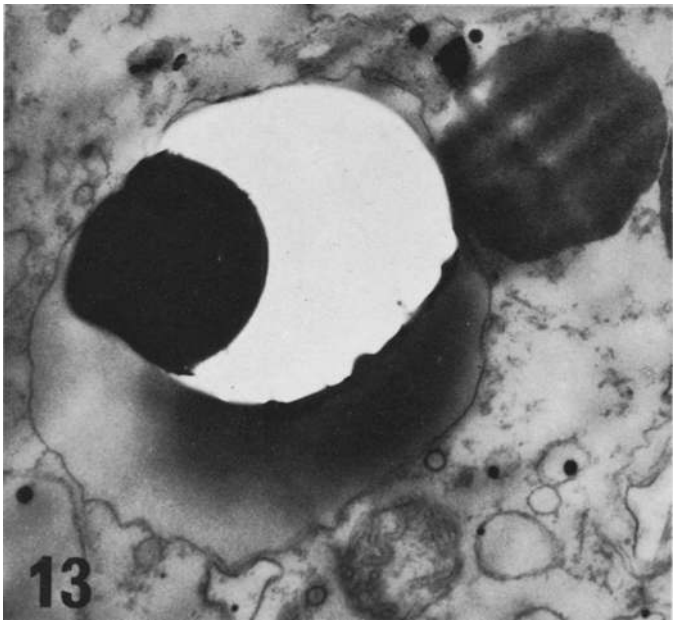
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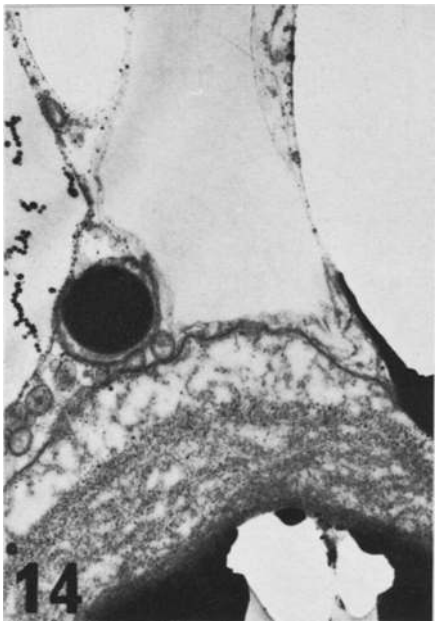
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the brittle cores tear the translucent peripheral zone (Plate V, fig. 12).

4. REGENERATION OF HEAVY SPHERICAL BODIES IN CHAOS

As first shown by MAST and DOYLE (41) in *Amoeba proteus*, centrifugation *in vivo* under suitable conditions results in the accumulation of the HSBs at the centrifugal pole of the cell; excision of this pole can readily be achieved with a fine glass needle, giving an amoeba with normal motility and behaviour. These authors followed the fed or starved operated amoebae for some days, and deduced from their experiments that regeneration in fed cells is rapid, within a few days, while in starved cells the residual HSBs degenerate.

SINGH (54) notes that when *A. proteus* is centrifuged at high speed, the HSBs are thrown out, and that they reappear within ca 14 days.

In the large amoeba *Chaos carolinense*, with many hundreds of HSBs, a similar technique of centrifugation *in vivo* was employed to obtain amoebae with very few residual HSBs, in order to reassess the process of their regeneration under conditions of normal nutrition and during starvation.

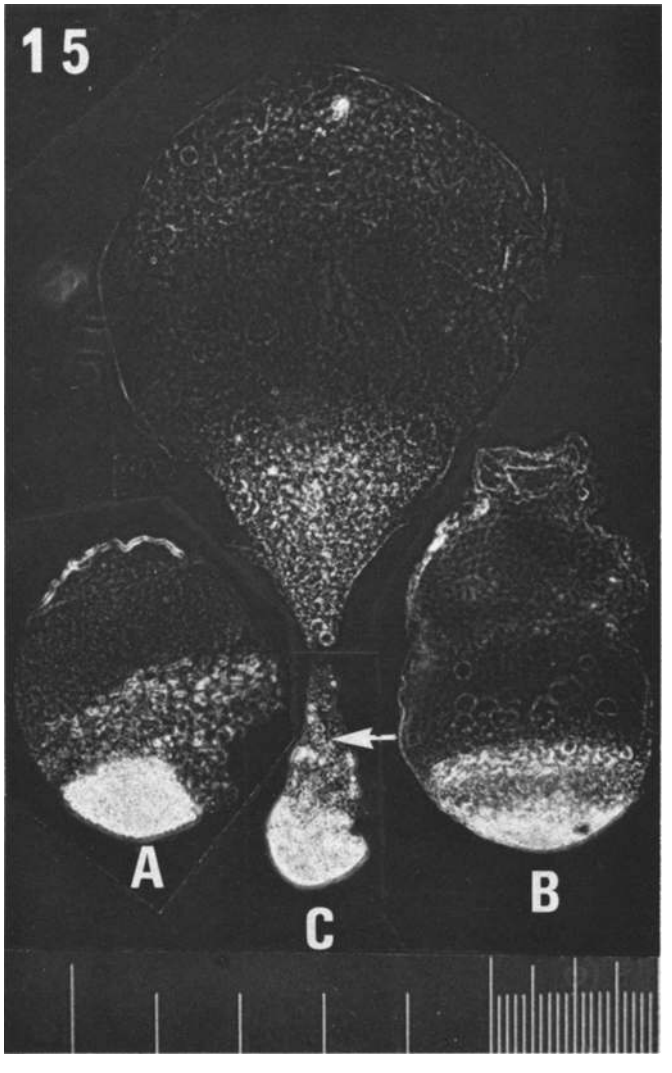

Plate VI

Figure 15. Whole mounts of *Chaos carolinense* centrifuged *in vivo*, fixed in 96% ethyl alcohol, mounted in cedar wood oil. Phase contrast optics. The scale shows 100 μm and 10 μm.
A. Spherical shape B. Elongated shape
C. Elongated shape with HSB-sack, as used for regneration experiments. Excision of HSB-sack is made at arrow.

4.1. Results of regeneration experiments

As mentioned in section 2.2. only amoebae which attain a certain shape during centrifugation *in vivo* can be used for regeneration experiments. Plate VI illustrates the characteristic shapes of well centrifuged *Chaos*, and C is a typical example of the amoebae used for these experiments.

In the eight experiments performed, a total of 381 amoebae were centrifuged; the number which were satisfactorily centrifuged, cut, and

Table I

Yield of operated amoebae, expressed as percentage of total number of centrifuged amoebae, which were satisfactory in shape, survived cutting, showed on *in vivo* examination very few remaining HSBs and showed normal locomotion on day following operation.

Expt. nr.	no. amoebae centrifuged	no. used for experiments	% age yield
I	46	18	39.1
II	75	35	46.7
III	30	7	23.3
IV	60	14	23.3
V	30	11	36.7
VI	20	11	55.0
VII	72	47	65.3
VIII	48	34	70.8
Total	381	177	mean 46.5

used for the experiments was 177 or 46.5% (see table I).

The majority of the amoebae which appeared normal immediately following operation and recovery were also able to ingest *Tetrahymena* on the day following operation. The main loss of amoebae during the experiments occured during the first few days; owing to the relatively low density of the HSB-free amoebae, they were more difficult to handle, and were sometimes lost through breakage at the air-water interface during washing.

The growth rates of the operated amoebae, in the experiments which were carried to 14 days or more (in Expt. VII and VIII to 65 days) were very similar to those of the non-operated controls, and in two experiments in which centrifuged, but not cut, amoebae were used as an additional control, growth rate was also the same. The growth rates for experiments VII and VIII are shown in Table II, for different

Table II

Expt. no.	Period, days after start of experiment	Growth rate, as percentage of number at start of each period	
		Experimental	Control
VII	1 - 6	190	170
	21 - 29	260	162
	30 - 38	162	154
VIII	1 - 12	227	232
	13 - 30	224	282
	31 - 60	245	207

periods, expressed as percentage increase in number of amoebae during each period.

The growth rates of the control amoebae are lower than those usually found in mass cultures of *Chaos* fed daily on *Tetrahymena*, but the small number of amoebae and the small culture vessels may have affected the growth rate; only at the end of experiment VII did the number of amoebae approach what could be called a mass culture, with a corresponding growth rate. Growth in experiment VIII was influenced by a mitochondrial abnormality, which affected both experimental and control amoebae.

For the reasons given in section 4.1. phase contrast photographs of unstained whole mounts of *Chaos* do not show the HSBs clearly, hence no illustrations can usefully be given to show distribution of HSBs during regeneration. However, although toluidine blue staining does not give satisfactory preparations for counting and measuring the HSBs, photographs of such preparations illustrate generally the progress of

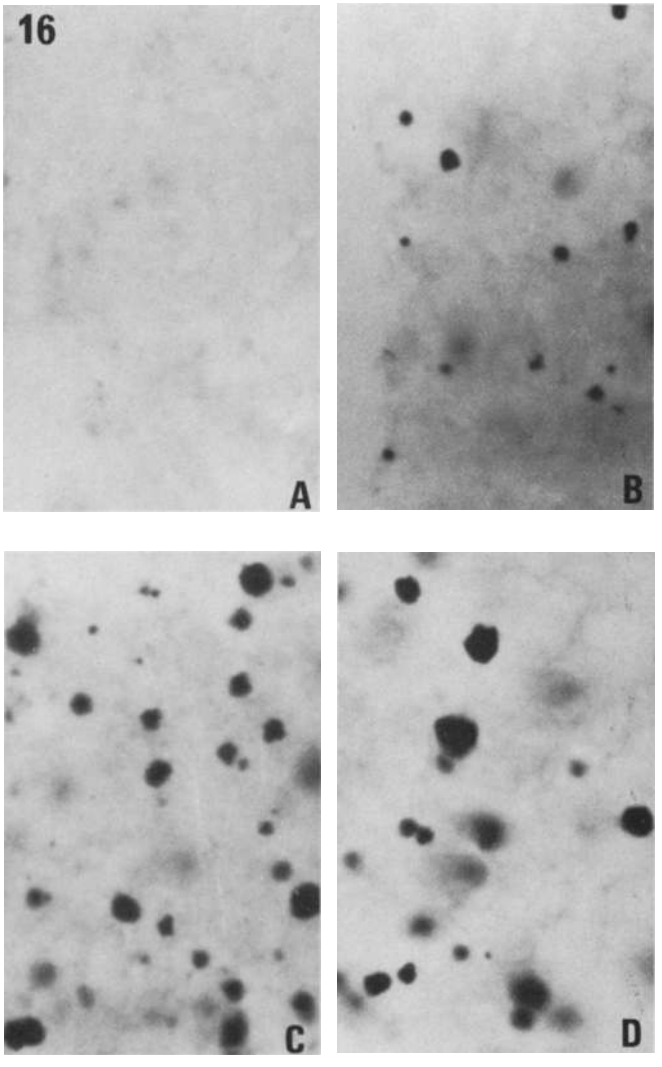

Plate VII

Regeneration of HSBs in *Chaos carolinense*. 10 µm sections of amoebae fixed in 96% ethyl alcohol, and stained with toluidine blue.

Experiment VII. A, B, C amoebae fixed at following times after excision of HSB-sack: A, 1 day. B, 21 days. C, 51 days. D, non-operated control, fixed 51 days after start of experiment. Magn. 1280 x.

regeneration, as can be seen in Plate VII, which shows results from experiment VII: part of the cytoplasm of *Chaos*, 1 day (no HSBs), 21 days (a few small HSBs) and 51 days (numerous HSBs of all sizes) after excision of the HSB-sacks, as compared with a control non-operated amoeba 51 days after the start of the experiment (numerous HSBs of all sizes).

4.2. Counting data

Collection of the HSBs by centrifugation *in vivo* and excision of the HSBs-sack does not, of course, result in a perfectly HSB-free amoeba. However, all amoebae were inspected *in vivo* in a chamber after recovery from the manipulations, and only those in which very few HSBs were observed, were used for the ex-

Table III

Comparison of counts on HSBs in unstained, whole mounts, and in toluidine blue stained 10 μm sections. - Experiment VII

Days after start of experiment	Total no. of HSBs per counting volume ± SEM		Total no. of HSBs per counting volume corrected to "living volume"	
	whole mounts	sections	whole mounts[1])	sections[2])
21 Exptl.	846 ± 64	1243 ± 61	423 ± 32	236 ± 12
41 Exptl.	598 ± 27	1581 ± 37	299 ± 14	300 ± 7
51 Exptl.	582 ± 14	1453 ± 52	291 ± 7	276 ± 10
51 Control	512 ± 27	1219 ± 41	256 ± 14	232 ± 8

correction factors: 1) shrinkage to 50% of original volume
2) shrinkage to 19% of original volume

Table IV

Comparison of size distribution of HSBs in unstained whole mounts, and in toluidine blue stained 10 μm sections. Experiment VII

Days after start of experiment	Preparation	size distribution of HSBs as %age of total number				
		<1μm	1-2.5	2.6-4.4	4.5-6.6	>6.6μm
21 Exptl.	whole mounts	57.1 ± 5.4	34.0 ± 2.8	7.0 ± 0.8	1.8 ± 0.4	0.1 ± 0.1
	sections	26.7 ± 2.5	47.6 ± 4.2	20.2 ± 1.1	4.9 ± 0.3	0.6 ± 0.2
41 Exptl.	whole mounts	26.6 ± 1.6	42.1 ± 2.3	24.0 ± 2.3	6.5 ± 0.9	0.8 ± 0.3
	sections	15.6 ± 1.3	43.5 ± 2.1	27.1 ± 1.2	10.9 ± 1.3	2.9 ± 0.3
51 Exptl.	whole mounts	24.5 ± 2.3	46.3 ± 5.7	20.6 ± 1.4	6.8 ± 0.7	1.8 ± 0.5
	sections	13.1 ± 1.5	32.6 ± 2.2	33.4 ± 1.8	16.0 ± 1.4	4.9 ± 0.7
51 Control	whole mounts	25.6 ± 2.2	48.4 ± 3.1	19.0 ± 1.9	5.8 ± 0.8	1.2 ± 0.4
	sections	6.9 ± 1.1	35.0 ± 1.5	39.8 ± 3.2	14.1 ± 1.1	4.2 ± 0.5

Figure 1. Regeneration of HSBs in amoebae fed daily on *Tetrahymena* after operation.
The wide columns represent the mean, total number of HSBs counted in the standard counting volume of 0.00168 μl (ordinate on right), at the times (days after operation) indicated on the abscissa. The 5 narrow columns show the percentage distribution of 5 size groups of HSBs (ordinate on left), size increasing from left to right and representing following sizes of HSBs: <1, 1-2.5, 2.6-4.4, 4.5-6.6, >6.6μm. In all amoebae groups, the smallest size group of HSBs is present, but the largest are not represented in all the experimental groups, for further explanation, see text p. 204.
Control amoebae were non-operated amoebae from same batch, and fed as the experimental amoebae. Each group of counts represents mean values for groups of 3-9 amoebae (see text p. 204). The same numbers of experimental and control amoebae were counted at each time.

periments. Counts on the number of HSBs remaining in amoebae fixed on the day after operation, as compared with the number in control, non-operated cells (9 amoebae counted, by the procedure described in section 2.3.) showed mean values for the operated amoebae of 40.5, and controls 764.1 (per standard counting volume of 0.00168 µl) indicating that ca 5% of the HSBs remained in the operated cells at the start of the experimental period.

These residual HSBs, which are not collected at

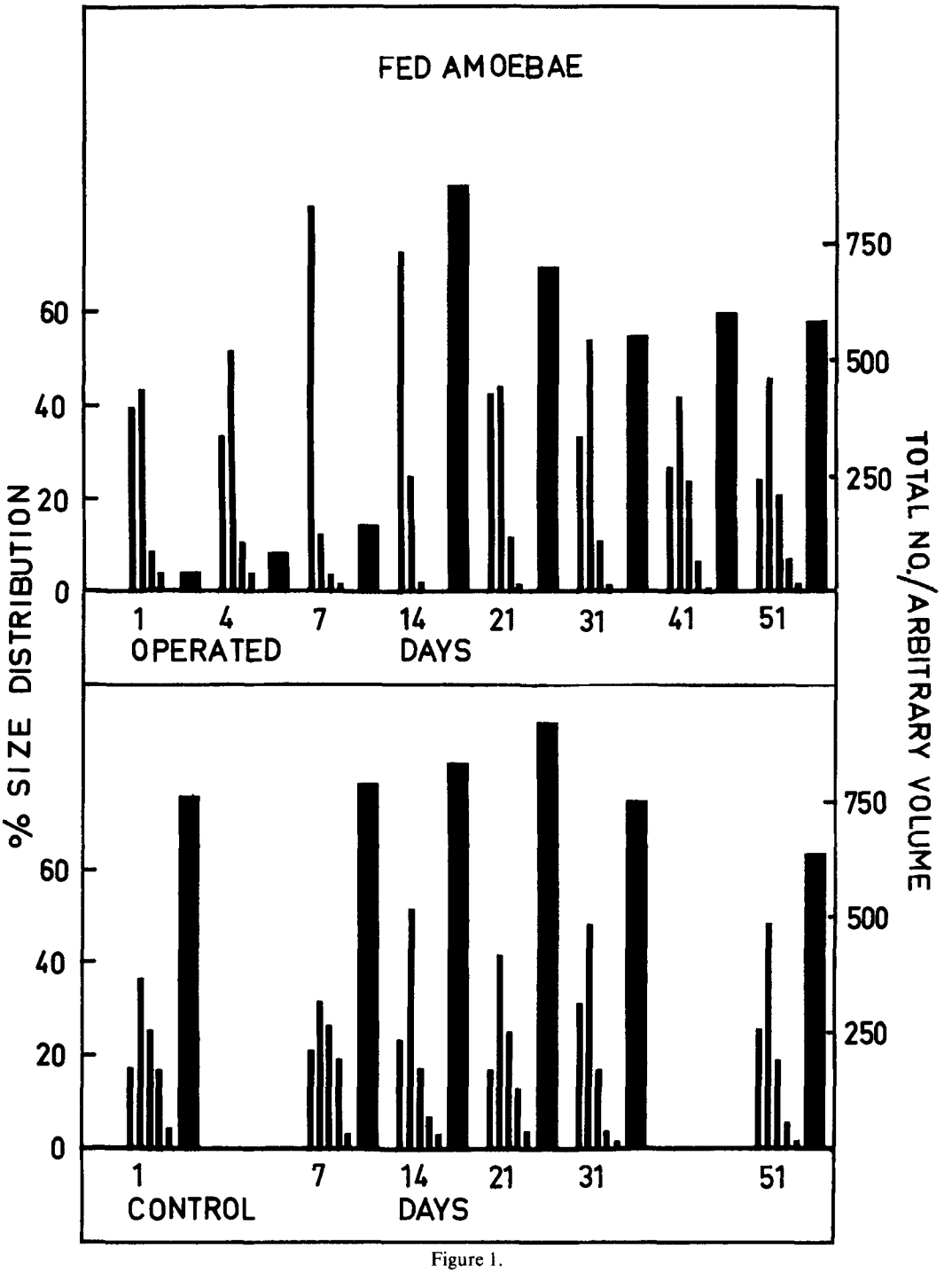

Figure 1.

the heavy pole, are often trapped at the periphery of the amoebae during centrifugation, and can be seen in preparations of centrifuged amoebae; these HSBs are often of small size, as can be seen in Plate III, which shows sections of centrifuged *Chaos*, unstained, and stained with toluidine blue, and with lead at pH 3.5.

The most satisfactory, although very tedious, method for estimating the number and size distribution of the HSBs proved to be counting and measuring the organelles in whole mounts in cedar wood oil, using phase contrast optics. Staining with toluidine blue was not satisfactory for this purpose, as it proved impossible to obtain even staining and colour intensity in HSBs of all size groups; when the largest organelles were well stained, the smallest could not be detected, hence the latter would be omitted from the measurements.

Tables III and IV give comparative data on HSBs using these two methods; sections in which the small bodies were well stained were chosen for this comparison. The total number of HSBs counted per unit volume is higher in the sectioned and stained material; this could be due to visualization of HSBs smaller than those discernible in unstained material; however, a difference in the shrinkage of the cell during preparation (embedding in paraffin and sectioning) could also be expected, especially as stretching of the sections could not be done at too high a temperature, as this results in leaching of stainable material from the HSBs. When counting volumes are corrected for shrinkage, according to the values given by BAKER (6), the total number of HSBs found by the two methods are in reasonable agreement (Table III). However, the size distributions found by the two methods are clearly different (Table IV); staining with toluidine blue shifts the observed size towards higher values in all amoebae, the percentage of the smallest HSBs being appreciably higher, and that of the largest HSBs being appreciably lower in unstained material.

The combined data on counts of HSBs from the eight experiments on unstained whole mounts of amoebae are summarized graphically in fig. 1 for amoebae fed daily on *Tetrahymena* from the first day after operation, and in fig. 2 for amoebae starved after excision of the HSBs. Control values for non-operated amoebae from the same cultures are also included in these figures. The data for day 1 are identical in figs. 1 and 2, as samples were taken for fixation before feeding, on the first day.

As previously mentioned, excision of the HSB-sacks from centrifuged amoebae does not result in a completely HSB-free cell, ca 5% of the HSBs are retained, hence the initial values for the counts of both starved and fed amoebae are not zero. The figures show that the total number of HSBs per unit counting volume in fed amoebae returns to that found in control amoebae by 14 days (see wide columns in fig. 1). However, the size distribution (narrow columns in fig. 1) first becomes similar to that of control cells by ca 50 days after operation. Starved cells could only be followed for 12 days; since the amoebae had previously been starved for 3-4 days before centrifugation, this correponds to a period of 15-16 days of deprivation of food. Fig. 2 shows no significant difference between the number and size distribution of the HSBs in fed and starved operated amoebae. It should be noted that in all control amoebae, all five of the size groups of HSBs are represented; the next smallest size (1-2.5 μm) is always most numerous, and the largest size (6.7 μm) is least frequent. In the experimental groups, both fed and starved, the three smallest size groups (1, 1-2.5, 2.6-4.4 μm) are always present; however, in the experimental, fed amoebae, at 41 and 51 days after operation, the two largest size groups (4.5-6.6, >6.7 μm) have reappeared in sufficient numbers to be included in the diagram.

It is clear from these data that although the number of HSBs returns rather rapidly, i.e. within about 7 days for both starved and fed amoebae, to that found in controls, subjected to the same nutritional conditions, the growth of the HSBs to give a size distribution similar to that of control amoebae is a slow process, taking up to at least 50 days for the fed amoebae, and not attainable in amoebae starved after operation.

In the pooled data presented in figs. 1 and 2, each set of columns represents mean values for

three amoebae from each experiment. However, for days 30, 41 and 51, only results from experiment VII are given, as the other experiments were concluded earlier.

The relatively small number of amoebae, which for practical reasons could be included in the data, makes the results qualitative rather than quantitative and precludes the use of statistical analysis for testing the significance of the results.

Some conclusions as to significance can, however, be drawn from the data; as regards

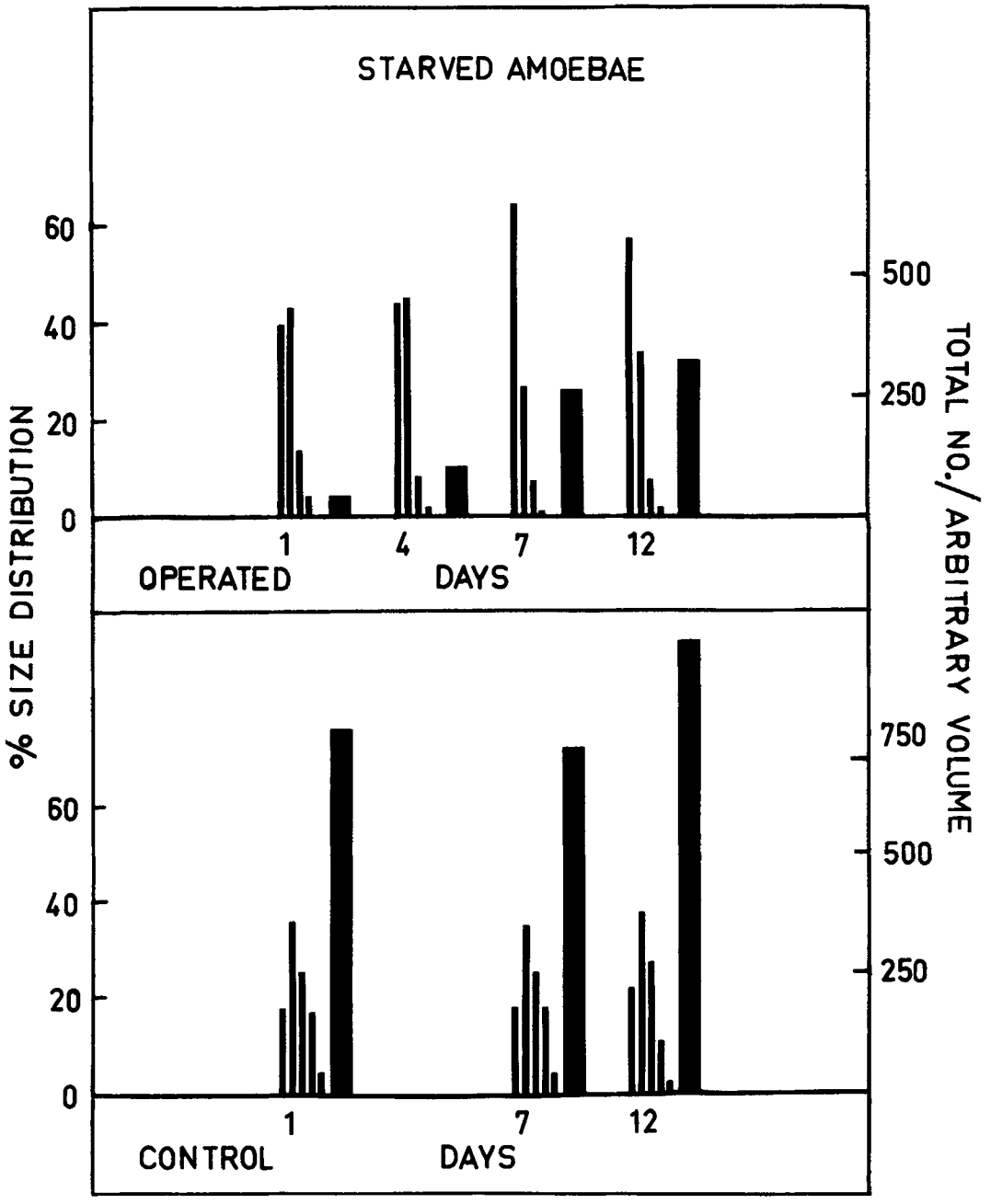

Figure 2. Regeneration of HSBs in amoebae starved after operation. The data are presented in the same way as in fig. 1.

variations in control material, the mean value for total HSBs per standard counting volume is 761 ± 40 for all 36 non-operated controls (both fed and starved), range 470 to 1315. The mean value for all fed controls is 742 ± 77.3, n = 15, range 470 to 1315, and for all starved controls, 775 ± 43, n = 21, range 532 to 1180. Thus there is clearly no significant difference between the mean values for control fed and control starved amoebae.

The data presented in figs. 1 and 2 do not give any indication of the variations between different amoebae; these are shown for a typical experiment (number VII) in Table V, in which the mean number of five size groups of HSBs per standard counting volume are given together with the standard error of the mean, for five counts on each of 3 amoebae (15 counts total), for 5 experimental and 3 control groups. The total number per standard volume is also given. These values are also expressed as percentages of each size group represented.

5. DISCUSSION

Many unicellular organisms (e.g. bacteria, yeast, bluegreen algae, Protozoa) contain spherical, refractive inclusion bodies, known as volutin, metachromatic, refractive or polyphosphate granules. The characteristic light microscopic morphology of these granules is shared by the HSBs of *Amoeba* and *Chaos*; their appearance in the electron microscope is similar to that described for granules in the bluegreen algae *Nostoc* (32) and *Plectonema* (53) and in the bacterium *Micrococcus lysodeikticus* (23): unstained granules sublime in the electron

Table V

Errors (S.E.M.) of size distribution counts in Experiment VII.

Exptl. days		Size distribution of HSBs (μm) ± S.E.M. <1	1-2.5	2.6-4.4	4.5-6.6	>6.6	Total
1	a)	4.5 ± 0.9	3.9 ± 1.1	1.6 ± 0.6	0.1 ± 0.1	0	10.1 ± 2.4
	b)	44.6 ± 8.8	38.6 ± 10.8	15.8 ± 5.9	1.0 ± 1.0	0	100.0 ± 23.8
21	a)	96.7 ± 9.2	57.5 ± 4.7	11.8 ± 1.3	3.1 ± 0.6	0.1 ± 0.1	169.2 ± 12.8
	b)	57.2 ± 5.4	34.0 ± 2.8	6.9 ± 0.8	1.8 ± 0.4	0.1 ± 0.1	100.0 ± 7.6
30	a)	36.8 ± 2.1	59.6 ± 3.4	11.7 ± 1.0	1.7 ± 0.3	0.3 ± 0.1	110.0 ± 5.2
	b)	33.4 ± 1.9	54.2 ± 3.1	10.6 ± 0.9	1.5 ± 0.3	0.3 ± 0.1	100.0 ± 4.7
41	a)	31.9 ± 1.9	50.3 ± 2.8	28.7 ± 2.7	7.7 ± 1.1	0.9 ± 0.3	119.5 ± 5.3
	b)	26.7 ± 1.6	42.1 ± 2.3	24.0 ± 2.3	6.4 ± 0.9	0.8 ± 0.3	100.0 ± 4.4
51	a)	28.5 ± 2.7	53.9 ± 6.6	24.0 ± 1.6	7.9 ± 0.8	2.1 ± 0.6	116.4 ± 11.0
	b)	24.5 ± 2.3	46.3 ± 5.7	20.6 ± 1.4	6.8 ± 0.7	1.8 ± 0.5	100.0 ± 9.4
controls							
1	a)	38.4 ± 2.7	67.6 ± 5.4	42.7 ± 3.0	14.9 ± 1.3	4.4 ± 0.8	168.0 ± 6.0
	b)	22.9 ± 1.6	40.3 ± 3.2	25.4 ± 1.8	8.8 ± 0.8	2.6 ± 0.5	100.0 ± 3.6
30	a)	46.1 ± 2.9	74.3 ± 5.8	24.4 ± 2.4	4.9 ± 0.9	2.5 ± 0.5	152.1 ± 6.1
	b)	30.3 ± 1.9	48.9 ± 3.8	16.0 ± 1.6	3.2 ± 0.6	1.6 ± 0.3	100.0 ± 4.0
51	a)	26.3 ± 2.2	49.5 ± 3.2	19.5 ± 1.9	5.9 ± 0.8	1.2 ± 0.4	102.3 ± 5.5
	b)	25.7 ± 2.2	48.4 ± 3.1	19.0 ± 1.9	5.7 ± 0.8	1.2 ± 0.4	100.0 ± 5.4

a) mean number of HSBs of each size class counted in volume of 0.000336 μl; five such volumes are counted in each amoeba, and the values are means of three amoebae.

b) values from a) expressed as percentages of the total number per 0.000336 μl.

beam, leaving holes, and spots of electron opaque material may be found some distance from the holes; the electron opaque material is stabilized by lead staining. In a recent review on the inclusion bodies of Prokaryotes, SHIVELEY (52) includes polyphosphate granules among the nonmembrane-enclosed inclusions; however, limiting membranes have been reported for the phosphate-rich granules of the bluegreen alga *Plectonema* (53). In unicellular Eukaryotes, the presence of a limiting membrane around HSBs was reported by HAYWARD (28) for *Amoeba proteus* (see Plate V, fig. 13), and was also found in *Chaos carolinense* (see Plate V, fig. 14). From a consideration of their properties, COLEMAN et al. (18) assumed that the refractive granules of *Tetrahymena* were membrane-bound, and this assumption has recently been shown by NILSSON (45) to be correct.

In at least one respect, the HSBs of *Amoeba* and *Chaos* differ from the refractive granules so far described of other unicellular organisms, as they are constantly present in the trophic form, regardless of the nutritional state of the amoebae. The data presented in figs. 1 and 2, and the mean values found for control fed and starved *Chaos* (section 4.2.) indicate that the number of HSBs per unit volume of cytoplasm is rather constant, confirming the earlier data of ANDRESEN and HOLTER (5) for this species. In *Tetrahymena,* NILSSON and FORER (46) found that the appearance of the refractive inclusions is cyclic;no granules were present during the logarithmic phase of growth, but when cultures passed into the stationary phase, or were subjected to starvation, granule formation commenced. The refractive granules of bluegreen algae are induced by excess phosphate in the medium, together with continuous illumination (32, 33), and in yeast, when phosphate is added to starved cells (50). In some bacteria, large amounts of polyphosphate accumulate when cell growth ceases (i.e. during the stationary growth phase) (26), or under conditions of nutritional imbalance (27). In other bacteria, granules accumulate during the logarithmic growth, but disappear during the stationary phase (23).

Variations in the number and size of HSBs in amoebae have been ascribed to the species of ciliate used as food organism (38, 63, 3), but so far there are no data which relate food organisms and growth rate with the content, in the inorganic culture medium, of the ions found in the HSBs. Preliminary data (CHAPMAN-ANDRESEN unpub.) comparing the influence of Pringsheim's medium (high calcium and phosphate) with Chalkley's medium (low calcium and phosphate) on the number and size of HSBs in *Chaos* fed daily with *Tetrahymena,* indicate poor growth and few HSBs in Chalkley's medium, although this medium is satisfactory for *Chaos* in cultures fed with *Paramecium caudatum* and *Colpidium* sp. when the food organisms multiply with the amoebae in the same culture dish, but with a lower growth rate and a higher number of HSBs, than in the *Tetrahymena* fed cultures. These experiments are being continued, and will be reported in the second paper in this series. Although more data are needed, there is no indication that HSBs in amoebae are present only under conditions of nutritional imbalance, but rather that they are normal constituents of healthy, growing organisms.

The regeneration experiments (section 4) show that when the HSBs are excised, the number per unit volume returns to that found in control amoebae within 7 days, which corresponds to about one generation time under the conditions used in these experiments (see Table II). However, the normal size distribution of HSBs was not restored until about 50 days after excision, corresponding to about 6 generation times. In *Tetrahymena,* the refractive granules appear within one generation time under unfavourable conditions (46).

Since HSBs of all sizes are present in the excised HSB-sacks, and small HSBs are the first to reappear after excision, it must be assumed that the bodies originate *de novo* in the cytoplasm; the experiments now in progress on the fine structure of regenerating HSBs may show that initiation of new HSBs is more rapid than can be detected at the light microscopic level.

Recent fine structural studies by NILSSON (45) show that the refractive granules of *Tetrahymena* are morphologically heterogeneous

organelles, the different types perhaps representing stages in their development. Studies on bluegreen algae (33) indicate, as the initial stage of the polyphosphate granules, a fibrous matrix, which becomes infiltrated with polyphosphates. More recent data (34) on the granules of *Plectonema boryanum* indicate a more complex initiation, with five possible sites, dependent on conditions in the medium and within the cell. Since the regeneration studies on *Chaos* indicate that the HSBs develop from small nuclei in the cytoplasm, it might be expected that the smallest size which was detected in the electron microscope (Plate V, fig. 14) is a »young« HSB; this type has a relatively large electron dense core, and a narrow electron translucent periphery. The smallest type of HSB detected in *Amoeba proteus* by COLEMAN et al. (19) had a metal to phosphorus ratio differing from that of the larger HSBs, and was of the same size as the smallest type detected by electron microscopy, hence might indicate an early stage in development.

A possible contribution to the initiation of HSBs might be found in the small (ca 500-1000 Å diameter) dense bodies, which are often present on the cytoplasmic aspect of the plasmalemma, and to which a function as possible precursor of membrane material has been attributed by SZUBINSKA (56). It was suggested by MERCER (44) that these electron opaque bodies, which were changed in the electron beam to vesiculated bodies, might be phosphates, resembling volutin bodies of bacteria. This suggestion is interesting in view of the high phosphate content found in the surface layers of *Amoeba* sp. (1), and the presence, in electron micrographs of the surface layers from which the chemical analyses were made, of numerous small dense bodies. The attachment of these bodies to the plasmalemma appears to be constant, and the bodies apparently glide beneath the plasmalemma under the influence of centrifugal forces, since they are collected at the centripetal pole of *Amoeba proteus* centrifuged *in vivo* (CHAPMAN-ANDRESEN and NILSSON unpub.). In view of the known lipid content of the HSBs (47, 10) and of NILSSON's (45) recent observaions on the presence of lipid in the refractive granules of *Tetrahymena*, a possible connection between the small dense bodies and the HSBs may be considered.

Of the functions which may be attributed to the HSBs of amoebae, the observation that HSB-free *Chaos* are more fragile than normal, HSB-containing ameobae, in that they are more liable to be ruptured at an air-water interface, owing to their lower specific gravity, may be of survival significance. A detailed discussion of the chemical composition of the HSBs, as compared to the refractive granules of other unicellular oganisms, will be deferred to the next paper in this series (CHAPMAN-ANDRESEN in preparation). It is, however, evident that the high concentration of phosphate (up to 50% of total cellular P (14)) and the presence of calcium and magnesium (19, 30) within the HSBs, indicate sequestration of these ions within the membrane-bound HSBs, which may function as a reserve of ions essential to metabolic processes, as previously suggested by COLEMAN et al. (17, 18, 19) or serve to maintain physiological concentrations of the ions within the cell. The question of the relative roles of the different consitutents of HSBs, including lipids (10, 17), as essential components or as binding complexes, remains open.

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