SOME OBSERVATIONS ON Pelomyxa carolinensis with
SPECIAL REFERENCE TO MERCURY ORANGE SULFHYDRYL
STAINING

by

Loretta M. McQuillan, B.A.,
University of British Columbia
1957

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

Master of Science

in the Department

of

ZOOLOGY

We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA

1962

In presenting this thesis in partial fulfilment of

the requirements for an advanced degree at the University of

British Columbia, I agree that the Library shall make it freely

available for reference and study. I further agree that permission

for extensive copying of this thesis for scholarly purposes may be

granted by the Head of my Department or by his representatives.

It is understood that copying or publication of this thesis for

financial gain shall not be allowed without my written permission.

Department of _____Zoology_____

The University of British Columbia,
Vancouver 8, Canada.

Date _____Sept. 5/62_____

- iii -

ABSTRACT

Cytochemical studies using 1-(4-chloromercuriphenylazo)-
naphthol-2 (Mercury Orange), a specific reagent for the detection
of sulfhydryl groups, were carried out on the giant multi-nucleated
amoeba, Pelomyxa carolinensis. Distribution of -SH groups was
compared in normal well-fed organisms, starved amoebas and in
sodium arsenite-treated individuals. The hypothesis that spindle
fibre formation during mitosis involves, in part, oxidation of
-SH groups to -SS- bonds and that peripheral nuclear granules play
a role in spindle fibre formation was investigated. Preliminary
investigations on mouse and toad muscle fibres were carried out
for the purpose of confirming Bennett's results as well as to test
the specificity of Bennett's Mercury Orange technique for -SH.

Considerable time was spent in observing the living
amoeba. Included in these observations was feeding behaviour,
reaction to various stimuli and reproduction.

Prior to the -SH studies, the general morphology of
P. carolinensis was investigated in considerable detail using
various fixation and staining techniques. Likewise, mitosis in
this amoeba was thoroughly studied. Synchronous division of the
many nuclei (as observed by Kudo and Schaeffer) was confirmed.
Relationship of the amoeba's contour to the stage of nuclear
division was studied. This relationship proved helpful in the
study of -SH groups in the dividing nucleus since it provided a
means of choosing a specifically desired mitotic stage.

TABLE OF CONTENTS

LIST OF TABLES

ILLUSTRATIONS

INTRODUCTION

Considerable cytochemical investigations of sulfhydryl groups in various tissues have been performed within recent years. Except for nitroprusside, however, such studies have not been carried out on protozoa and there are no reports of any for _Pelomyxa carolinensis._ It was the object of the present work to detect and observe the distribution of sulfhydryl groups, by means of a specific cytochemical reagent, in _Pelomyxa carolinensis._ Conditions were set up whereby -SH distribution could be studied in normal, healthy organisms, as well as in starving and arsenic-poisoned individuals.

In order to assess this amoeba as material for cellular research it was necessary to learn as much as possible about the animal maintained under laboratory conditions, thus considerable time was spent observing living _P. carolinensis._

The histological studies were performed for the purpose of becoming acquainted with the internal structure of the animal in order to facilitate the interpretation and evaluation of existing literature as well as my own histological and cytochemical observations. A special interest was taken in the mitochondria of the animal because of the known correlation between sulfhydryl groups, respiratory enzymes and mitochondria in multicellular organisms.

The mitotic studies of fixed material were necessary before further investigation regarding the possible role of sulfhydryl groups in mitosis.

LITERATURE REVIEW

Nomenclature

Considerable confusion exists regarding the nomenclature of the organism used in this study.

Rösel von Rosenhof discovered an organism in 1755 which he called "der kleine Proteus". In 1758 Linnaeus named it Volvox chaos, then renamed it Chaos proteus in 1767. The majority of zoologists considered Volvox a protozoan, therefore, the binomial name of Rösel's "kleine Proteus" is Chaos chaos L.

Dr. A. A. Schaeffer (1937, 1937a), whose cultures I obtained, discovered Pelomyxa carolinensis Wilson in New Jersey. He believed that P. carolinensis, described and named by Wilson (1900) was Chaos chaos and supplied it to Turtox under this name. Turtox now sells this giant amoeba as Chaos chaos. Therefore, the name Chaos chaos has been generally accepted by many despite the objections of certain workers who have studied Rösel's observations. Mast and Johnson (1931; and Mast, 1938) are two such investigators who concluded that Chaos chaos is not identical with Wilson's P. carolinensis but that it is a myxomycete. Kudo (1946, 1952, 1959) who also examined Rösel's paper in conjunction with his own laboratory investigations on P. carolinensis believes that the facts do not support Schaeffer's assumption. He states that, "P. carolinensis is the valid name of the organism described and named by Wilson (1900) and that Chaos chaos

remains an unidentifiable organism of historical interest."

In the present study, this giant amoeba is referred to as Pelomyxa carolinensis Wilson.

P. carolinensis As An Experimental Animal

The nutrient requirements of P. carolinensis are as yet undetermined, therefore it has not been possible to develop axenic cultures of this organism. Kopac (1959) and Prescott (1959) are attempting to solve this problem.

Until recent electron microscopic studies fewer references pertaining to experimental studies on P. carolinensis appear in the literature as compared with some other protozoans, for example paramecium and Amoeba proteus. No reference to histochemical studies could be found. Most of the experimental work has been in connection with behavior, morphological description and physiological investigation.

Vital staining with Neutral Red reveals cytoplasmic granules known as "neutral red bodies" (Andresen 1942, 1945, 1946). These studies have led to the "vacuome" theory reviewed by MacLennan (1941). It is also supported by earlier workers (Hall, 1930; Hall and Loefer, 1930; Nigrelli and Hall, 1930). On the other hand, Goldacre (1950, 1952) and Torch (1959) believe these particles are an artifact of fixation subsequent to staining with neutral red.

Vital staining with Janus Green B carried out by Mast & Doyle (1935 a,b) and Kassell and Kopak (1953) revealed small particles covering the surface of contractile vacuoles. Studies by Torch (1955) and Pappas (1959) have led these workers to believe that these round or oval-shaped bodies are mitochondria. They have observed the detailed structure of individual mitochondria.

Classical studies on the cytology of P. carolinensis have been carried out by Andresen (1942, 1956); Kudo (1946, 1951); Wilbur (1942, 1945) and Torch (1955, 1959). Recent electron microscopic studies on the internal structure of P. carolinensis have extended the classical studies. Much of what was previously observed has been confirmed with the electron microscope. Some of the assumptions have now been turned into facts whilst others have been proven false.

Electron microscopic studies (Borysko & Roslansky, 1959) show that the plasmalemma of P. carolinensis is a single membrane. Pappas (1959) observed the plasmalemma to be approximately 200 $\overset{o}{A}$ thick. Fine fibrous extensions extend approximately 1700 $\overset{o}{A}$ from the surface. The diameter of these filaments is 40 $\overset{o}{A}$ to 60 $\overset{o}{A}$. Earlier workers (Lehmann et al.,(1956 a,b); Manni (1956) and Brandt (1958)) had described the plasmalemma as a double-layered membrane but it is now believed that the outer layer represents the minute filaments revealed in Pappas' (1959) studies and that these fine filaments may serve as

sites of adsorption of substances from the surrounding environment. The mechanism for such membrane formation proposed by Andresen (1957) is currently being investigated by electron microscopic studies (Landau, 1959).

Cytoplasmic ground substance appears homogeneous under the light microscope. However, studies with electron microscope, centrifugation studies of homogenates, chromatographic separation and surface chemical properties of proteins, indicate that sub-microscopic particulate structures are contained in this ground substance ranging in size from 0.06 to 0.2 microns (Kopac, 1950; Kassel, 1959).

Studies by Kopac (1951) suggest that these submicroscopic components, because of their capacity to undergo fibrillization, may be basically responsible for sol-gel changes involved in amoeboid movement and cytokinesis.

The cytoplasmic inclusions found within P. carolinensis and A. proteus appear to be similar in all respects, with the exception of the nuclei, P. carolinensis, being multinucleate (Torch, 1959). Kudo (1949, 1947, 1954) has described the nuclei and nuclear division in Pelomyxa carolinensis. Hinchy (1937), Schaeffer (1938, 1946) and Short (1945, 1946) have also studied mitosis in this organism.

Electron microscopic studies show a definite double-membrane

structure of the nuclear envelope.

Pores having a diameter of approximately 600 $\overset{o}{A}$ were observed by Pappas (1959) within the nuclear envelope and are believed to be formed when the inner and outer membranes are joined with one another. Since these nuclear pores do not appear to be patent, certain workers feel that they might more appropriately be termed "annuli" (Freider et al, 1956; Rebhun, 1956; Gall, 1956 and Swift, 1956). In the three species of amoebae studied (i.e. A. proteus, H. rysodes and P. carolinensis) the pores occupy 15 to 20 percent of the nuclear surface which is approximately twice that estimated for mammalian cells (Watson, 1955).

Immediately within the nuclear envelope of P. carolinensis is found a loose network of fibrils which are approximately 60 $\overset{o}{A}$ to 70 $\overset{o}{A}$ thick but the depth of this intraperinuclear fibrillar network varies a great deal. Nucleoli are found just within the nuclear envelope whilst within the more central areas of the nucleoplasm are found clusters of helices. At present, studies are in progress concerning the appearance and distribution of these helices during the stages of nuclear division (Pappas, 1959). Electron microscope studies have shown that the peripheral granules (nucleoli) contain small clusters of dense granules (Borysko and Roslansky, 1959).

Torch (1955) carried out a careful study of the mitochondria of P. carolinensis for the purpose of identifying and describing them

as a step toward determining their possible significance.

Borysko & Roslansky (1959) and Pappas (1959) during electron microscopic studies noted a complex internal structure of mitochondria which consists of a system of pleated membranes, numerous small granules and amorphous material. Serial sections indicate that many of the mitochondria are serpentine filaments rather than spheres or rods.

The studies of Torch, as well as that of other workers (Joyet-Lavergne, 1926, 1928, 1929, 1934, 1935; Cowdry & Scott,1928; and Weiss, 1950), indicate that the mitochondria in protozoa may function in cellular respiration.

Torch (1959) described "crystal vacuoles" around food vacuoles which are referred to as "vacuole refractive bodies" and appear as particles possessing darkened rims surrounding a colourless interior. Brown (1930) and Mast and Doyle (1935) believed them to be Golgi material because they give positive results with osmium techniques. But recent studies on centrifuged material and electron microscopic identification of structures other than these bodies, similar to vertebrate Golgi have led to a new interpretation (Cohen, 1957 and Pappas, 1959). Further, MacLennan (1940) has shown that the Golgi element cannot be identified on the basis of osmium reduction alone. Pappas (1959) noted a thick layer of granular material in close association with the limiting membrane of newly formed

food vacuoles. Older food vacuoles do not possess this granular ring of material around them. Because of this close association with newly formed food vacuoles they have been assigned a digestive role. Mast and Doyle (1935 a, b) are of the opinion that these homogeneous spherical bodies contain fatty acids and other lipids.

Refractive bodies dispersed throughout the cytoplasm are described by Mast and Doyle (1935 a). Each is composed of three morphological components: a relatively fluid cortical layer in which lipids and proteins have been demonstrated, an inner brittle shell and an innermost apparently fluid core. These observations were confirmed by Pappas (1954). Heller and Kopac (1955 a, b; 1956) and Pappas (1954) have been able to demonstrate organic phosphate, calcium and magnesium in relatively high concentration, as well as other minerals in lower concentration, in the fluid portion of these particles.

Fat globules present in the cytoplasm were shown by Mast and Doyle (1935 a) to be composed largely of neutral fat. Pappas (1954) confirmed these findings. Pappas also observed in A. proteus that although the glycogen disappeared after 3 days of starvation, as much as one third of the normal content of lipid droplets still remained in the cytoplasm of most of the organisms at the time of their death due to starvation. In centrifuged amoebas (Torch, 1959) the fat droplets locate themselves at the extreme end of the centripetal pole.

During the initial redistribution of the cytoplasmic components the other inclusions (i.e. crystals, food vacuoles, nuclei and mitochondria) begin streaming towards the centripetal pole but the fat droplets remain relatively stationary and clumped. When redistribution of the cytoplasmic components is completed (approximately one hour) the fat globules can be seen circulating en masse within certain individuals.

Crystals were not seen directly with the electron microscope but Pappas (1959) states that vacuoles that had contained crystals could be readily identified by the absence of embedding material in the space that formerly contained the crystals. These negative images corresponded to the two types of crystals present in the living amoebas, namely, platelike and truncated bipyramidal. Mast and Doyle (1935 b) and Wilbur (1945) believed that these crystals originated in food vacuoles and thus represented a food reserve. However, Andresen and Holter (1945) have shown that in P. carolinensis these bodies do not decrease in numbers during starvation. These workers further claim that the crystals arise in the cytoplasm and are found only incidentally in the food vacuoles as a result of coalescence.

The contractile vacuoles as seen by the electron microscope (Pappas 1959) show a vacuolar membrane surrounded by a densely packed layer of small, round vesicles believed to be secretory in function.

These vesicles in turn are surrounded by mitochondria. Lehmann et al

(1946), Bairati and Lehmann (1956) and Pappas and Brandt (1958)

have also carried out studies on these vesicles in an attempt to

determine their function and relationship to the contractile vacuole.

Gatenby et al (1955), Dalton and Felix (1956) and Pollister and

Pollister (1957) discuss the contractile vacuoles with respect to

the possibility of their representing Golgi apparatus.

Studies of the food vacuoles of P. carolinensis carried

out by Borysko & Roslansky (1959) revealed that the lumen of the

vacuole is continuous with several long channels that extend

radially away from the vacuole for long distances. They postulate

that these channels extend to the exterior of the amoeba and thus

provide an escape route for water ingested with food organisms.

Arsenic in Relation to Cell Poisoning via -SH

Ehrlich, as early as 1909 thought the toxicity of

arsenic was due to a reaction with receptor groups present in the

protoplasm. He suggested that the groups involved may be hydroxyl

or sulfhydryl.

Voegtlin (1925) and co-workers (1923, 1925) while studying

the trypaniacidal action of arsenicals in rats, found that arsenicals

must be in the tri-valent form (as in arsenoxide R-As=O) in order

to exert a toxic effect on the cell. When rats were injected with

arsenoxide the number of parasites fell. However, if glutathione,

or some other similar -SH compound, was injected together with
the arsenoxide the toxic effect could be inhibited. Thus,
Voegtlin postulated that the toxicity of arsenoxide on trypanosomes
was due to the blocking of -SH groups in the cell. Since then
more evidence has accumulated along these lines (Szent-Gyorgyi, 1930;
Labes, 1929; Cohen et al, 1931; Rosenthal, 1932; Glahn et al, 1938;
Hitchcock, 1946) and in vitro studies show that the toxic effects
of arsenic are brought about by inhibition of metabolic enzymes -
the pyruvate oxidase system in particular - through the blocking
of -SH groups (Pillae, 1938). Peters (1940, 1949) demonstrated the
inhibition of the pyruvate oxidase system in vivo. He found an
increase in blood pyruvate level at an early stage of arsenic
poisoning (lewisite) which he attributed to the effect of arsenic
on -SH groups. However, glutathione and similar monothiols
could not reverse the effect of lewisite on the pyruvate oxidase system.

Baron (1947 a, b) found that lewisite had the greatest
affinity (amongst the trivalent compounds) for -SH groups and
inhibited all sulfhydryl enzymes except d-amino acid oxidase, yeast
carboxylase and transaminase. Inhibition was reversed by BAL
(British anti-lewisite) in all cases.

Stocken and Thompson (1946, 1948) found that arsenic
combined with the thiol groups of protein in the ratio: 1 arsenic
to 2 -SH groups and hence postulated the "Dithiol Theory" which may

be illustrated as follows:

$$R\text{-}As\text{=}O \ + \ 2 \ R_1 \text{-}SH \ \text{---------}\rightarrow \ R\text{-}As \begin{smallmatrix} \diagup S\text{-}R_1 \\ \diagdown S\text{-}R_1 \end{smallmatrix} \ + \ H_2O$$
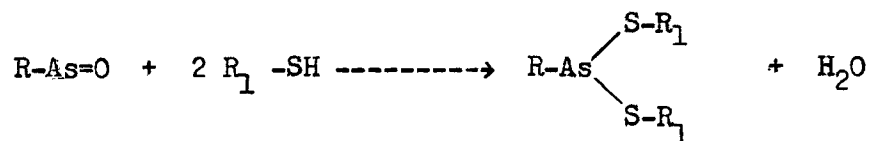
Whittaker (1947) supports this theory and Danielli (1947) states

that BAL reverses the above reaction.

Sulfhydryl Groups

Soluble thiol groups, distributed throughout the cell,

and fixed sulfhydryl groups attached to side chains of proteins,

serve important functions in the regulatory mechanisms of cellular

respiration as well as in the performance of the processes leading

to cell division and growth.

Glutathione, a tripeptide - (γ-glutamylcysteinylglycine) is

the most representative example of such soluble thiol groups.

The biological functions of glutathione have not yet been definitely

established. Functions attributed to this substance date back

to 1888 when it was discovered by deRey Pailhade. It was forgotten

and rediscovered in 1921 by Hopkins. It was first postulated

to be a catalyst for cell respiration. The role of glutathione

in regulation of respiration is the result of its low oxidation-

reduction potential. Glutathione also exerts a protective or

reactivation role towards metabolic enzymes which contain -SH groups

in their protein moeity. Whenever these -SH groups are attacked by

destructive agents, glutathione restores them by either withdraw-
ing heavy metal or by reducing the oxidized sulfhydryl group
(Barron, 1951).

Some workers in the field of cell division believed that
the -SH groups involved belonged to glutathione (Shearer, 1922; Hammett,
1929; Dulzetto, 1931; Rapkine, 1931; Rapkine et al, 1931; Binet and
Weller, 1936; Barron et al, 1948; Infantellina and LaGrutta, 1948;
Bolognari, 1952; Mazia, 1952, 1954 a, b; 1955; Backstrom, 1956;
Stern, 1956). Others felt that both fixed and soluble -SH groups
play a part in cell division - probably in spindle formation as well
as in plasmotomy (Binet et al, 1937; Ghosh, 1937; Vincent, 1937;
Colien, 1938; Williams, 1947; Ecker and Pillemer, 1938; Watkins and
Wormall, 1938).

Still others felt that glutathione does not play the lead-
ing role but a rather more passive one serving as a respiratory
carrier (Mapson and Moustafa, 1956) or as a regulator of the
oxidation-reduction level in the cell (Barron et al, 1948) and it was
therefore felt that the key to the solution of division activity should
be sought for in the sulfhydryl (-SH) disulfide (-SS-) transformation
occurring in the complicated protein systems which are required for
the formation of the spindle apparatus.

The manner in which -SH groups take part in cell division
is poorly understood and the elucidation of this problem is one of

the major tasks of biochemical cytology (Brachet, 1940; 1941; 1957; Mazia and Zimmerman, 1958; Mazia, 1958; 1959; 1960; Zimmerman, 1960), as well as others whose work is referred to below.

Until the work of Saiki (1959) little progress was made in this regard since Rapkine first suggested this function of -SH groups. Saiki employed several methods for quantitative determination of glutathione - among which was the titration method improved by Kuroiwa (1953) as well as the nitroprusside method proposed by Grunert and Phillips (1951). His results showed that glutathione leaves sea urchin eggs within 2 minutes after addition of trichloro-acetic acid, indicating that the positive sulfhydryl tests obtained by the many experiments must be due to protein-bound -SH groups of the egg protoplasm. The work of Neufeld and Mazia (1957) and Mazia (1957) falls in line with this concept, for they also found that neither glutathione nor ascorbic acid fluctuate in dividing sea urchin eggs.

Recent cytochemical investigation of the distribution of free sulfhydryl groups in Clypeaster japonicus by Kawamura and Dan, (1958) and Kawamura (1960) also indicate that theories of cell division in which glutathione is instrumental may not be valid.

These workers, as well as Mazia (1961 a, b), now feel that the sulfur-containing groups need not necessarily be fully oxidized -SS- bonds similar to those found in such stable structures

as hair and vulcanized rubber.

Cytochemical Methods for the Detection of -SH Groups

Cytochemical methods for demonstration of -SH groups rely on the fact that sulfhydryls react with almost all protein reagents (Olcott and Fraenket-Conrat, 1947). That the reaction has occurred is evident upon inspection because of the chromogenic moiety in the reagent. The latter may be an oxidizing agent bearing a reactive halogen group or a reactive heavy metal on its molecule. Such reagents have been sought since the beginning of the century (Glick, 1949; Pearse, 1953, 1960; Barrnett and Seligman, 1952, 1953 a, b; 1954 a, b; Brachet, 1957; Baker, 1958; Mellors, 1959; Casselman, 1959; Davenport, 1960).

Mörner, in 1902, first used the Nitroprusside reaction for identifying cysteine (Mörner, 1921). Others later employed it to demonstrate sulfhydryl groups in plant and animal material (Gola, 1902; Buffe, 1904; Heffter, 1908). Since then it has been used by many workers (Kaye, 1924; Walker, 1925; Joyet-Lavergne, 1928; Percival and Stewart, 1930; Giroud, 1931; Giroud and Bulliard, 1933; Rapkine, 1934; Bourne, 1935; Lison, 1936; Hammett and Chapman, 1938; Serra, 1946). Some of the aforementioned have modified the original technique in various ways. When thiols react with Nitroprusside in the presence of ammonium hydroxide and ammonium sulfate, a purple colour is produced. Nothing is known of

the chemistry of the reaction, however, this technique has been

extensively treated in the literature (Kay, 1924; Walker, 1925;

Giroud and Bulliard, 1933; Fugita and Numata, 1939; Serra and

Lopes, 1945; Mescon and Flesch, 1952; Rudall, 1952; Adams, 1956;

Pearse, 1961).

Golodetz and Unna (1909) used the Ferricyanide method to

demonstrate "reduction sites". Chèvremont and Frederic (1943)

later applied the in vitro ferric ferricyanide reduction test

described by Mason (1930) to the demonstration of thiols in tissue.

The method has since been used successfully by Frederic (1949),

Yao (1949), Hardy (1952) and Rudall (1952), whilst some workers

feel that the need for caution and many controls before safe conclusion

can be reached is a considerable disadvantage (Lillie and Burtner, 1953;

Findlay, 1955). Barron (1951) points out that the inability of

ferricyanide to oxidize all the -SH groups of denatured protein

results in an ill-defined end-point in the colour reaction.

Adams (1956) has carefully considered the histochemical interpretation

of this method.

Bennett (1951) demonstrated thiol groups in certain tissues

by means of a red mercaptide-forming sulfhydryl reagent

(1-[4-chloromercuriphenylazo]-2-naphthol). This reagent is commonly

referred to as Mercury Orange. Bennett and Yphantis (1948)

synthesized Mercury Orange while attempting to develop a quantitative

analytical cytochemical method for measuring sulfhydryl content of

tissue components too small for conventional microchemical procedures.

The technique is a modification of the <u>in vitro</u> phenyl mercuric

chloride (p-chloro-mercuri-benzoic acid) sulfhydryl reaction of

Hellerman, Perkins and Clark (1933). The red-coloured azo

derivative of Hellerman's reagent retains the specificity for

mercaptans displayed by phenyl mercuric chloride and also, because

of its chromogenicity, acts as an optical tag which dilineates the

sites where it is bound to tissue components by mercaptide linkage.

The basic reaction is:

$$R-SH + Cl-Hg-\langle\bigcirc\rangle-N \underset{OH}{=N} \rightleftharpoons R-S-Hg-\langle\bigcirc\rangle-N \underset{HO}{=N} + HCl$$

position of double bonds ?

Protein -	Mercury	Compound responsible
containing	Orange	for Characteristic
Groups		Colour = Chromogen
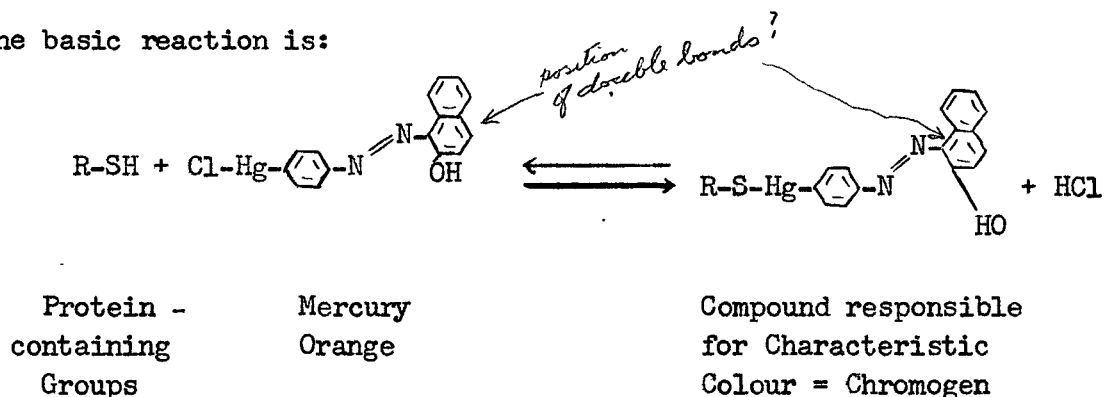
Since Bennett (1951) first used Mercury Orange for

qualitative work certain conditions have come to be appreciated not

previously considered which must be met if all, or most, of the

-SH groups of a tissue are to be demonstrated cytochemically.

Hence, Mercury Orange has not enjoyed the popularity of the Barrnett-

Seligman Method (Barrnett et al, 1955; Ravin et al, 1952; Ashbel and

Seligman, 1949; Seligman et al, 1949). But, when these conditions

are satisfied, Mercury Orange possesses the characteristics of an ideal

cytochemical reagent (Bennett and Watts, 1958). Pearse (1961)

regards both methods as possessing equal usefulness for cytochemical

work. Cafruny et al (1955) observed, from sections which stained

sufficiently to allow photometric determination, that amounts of thiol

in various cell types were comparable by the two methods, and feels

therefore, that the two methods are comparable.

The success of the Barrnett and Seligman Method (1952,

1953 a, b; 1954 a, b) is attributed to the fact that the reagent

(2,2'-dihydroxy-6,6'-dinaphthyl disulfide or DDD) contains a

disulfide group which reacts with sulfhydryl groups and no other

groups at alkaline pH. DDD has been used to demonstrate histo-

chemically both sulfhydryls and disulfides or disulfides alone

(Cafruny et al, 1954, 1955). The reaction is as follows:

(I)
"Reagent"(2,2'-dihydroxy-
6,6' dinaphthyl disulfide)

(II)

(III)
Tetrazotized di-
ortho-anisidine

(IV)
Coloured Product

(I) Reagent (2,2'-dihydroxy-6,6' dinaphthyl disulfide) when used in excess at pH 8.5 reacts with active -SH groups of fixed tissue proteins to form a colourless substance (II) plus by-product (III). The colourless oxidation product (II) is insoluble in both water and ether-alcohol therefore excess reagent (I) as well as by-product (III) can be washed out of the tissue with organic solvents. Subsequent treatment of the tissue with tetrazotized di-orthoanisidine results in the rapid development of a red colour (Monocoupling) or a blue colour (di-coupling) at the site of protein-SH groups (IV). Mono-coupling (red or pink) indicates sparse, widely separated -SH groups whereas di-coupling (blue) indicates a greater concentration of -SH groups.

MATERIALS AND METHODS

Cultivation of Amoebas and Parameciums

Cultures of P. carolinensis and P. multimicronucleatum were set up in November, 1958. The amoebas were maintained by subculturing in syracuse watch glasses twice weekly. Approximately 12 amoebas were placed in thoroughly cleaned[1] watch glasses containing fresh stream water and a 1"-long piece of timothy hay. The timothy hay for this purpose was allowed to mature before picking and then dried. Before use, it was boiled in stream water for 10 minutes to kill any undesirable organisms which might be attached thereon. Before placing the small stick of hay into the watch glass, all of the juice was poured off. The cultures were kept at room temperature in diffuse light. The watch glasses were stacked one on top of the other and the top one covered by a clean inverted watch glass. The amount of water in the glass was not sufficient to wet the bottom of the glass placed above it.

An adequate supply of parameciums was kept on hand as a constant source of food for the amoebas by subculturing periodically in a timothy hay infusion. The infusion was made up in wide-mouthed 250-cc Erlenmeyer flasks. The flasks were filled to within 5 - 6 cm of the mouth with fresh stream water into which was placed approximately ten 1-inch sticks of timothy hay. The flask was

---

1 Distilled water and nitric acid (conc.) 1:1, rinsed several times and dried thoroughly.

covered with a clean inverted 100-cc beaker and the mixture
allowed to boil for 15 minutes. After cooling, it was innoculated
with approximately 10 cc of a heavy paramecium culture. Within one
week to ten days, this culture was ready to use for feeding the amoebas.

Considerable time was necessary before growth in these
cultures became luxuriant and it seemed to me that by increasing the
amount of timothy hay, it should be possible to develop thicker
cultures. But, this did not prove to be the case. An excessive
amount of hay had a deliterious effect upon the organisms. Rather
than a heavy culture developing, the parameciums failed to multiply
appreciably and eventually the infusion became cloudy and contained
many dead organisms. The problem, therefore, of providing sufficient
food for the amoebas was handled by maintaining a sufficient number
of individual flasks of cultures. More hay and water could be
added from time to time and, in this manner, cultures were kept in
good condition for months.

The parameciums were washed before adding them to the
amoeba cultures. A small amount of cotton was placed within a
15-cc conical centrifuge tube and pushed down just beyond the bend;
stream water was poured in till it reached the top of the cotton
and the remainder of the tube was filled with paramecium culture (Figure 1).

Paramecium
culture

Cotton
wool

Fresh stream
water
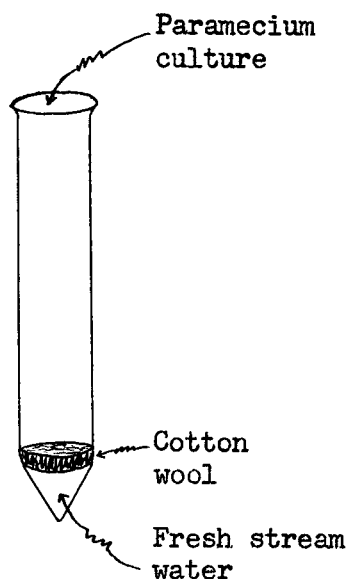
Figure 1 - Method of washing
parameciums before feeding to
P. carolinensis. A small amount
of cotton was placed just beyond
the bend of a 15-cc centrifuge
tube. Stream water was poured in
to top of the cotton and remainder
of tube was filled with paramecium
culture. Tubes were spun in a hand
centrifuge.

The tubes were spun in a hand centrifuge (50 turns of the handle

during 2 minutes). If spun too rapidly, the parameciums sent out

many trichocysts and died. If spun too slowly, they did not

pass through the cotton and therefore failed to become concentrated

in the fresh water beneath. When the organisms had been brought

down, it was possible to invert the tube without the cotton plug

becoming displaced and the old culture fluid, containing only debris,

could be poured off. When the cotton was carefully removed, by means

of a flamed glass rod, the parameciums were sufficiently clean to

be fed to the amoebas.

During routine cultivation of P. carolinensis, 500 to 800

parameciums were added to each watch glass. Five or six watch

glasses of amoebas were sufficient to keep on hand for carrying out

various studies. During studies on mitosis the feeding procedure

described above, but without removing amoebas from watch glasses, was repeated approximately 4 to 6 hours before the organisms were required. Some water was removed and fresh stream water added. This ensured richly dividing material. To obtain desired stages for experimental purposes the cultures were scanned at intervals during the day and individuals selected according to body form.

Cytological Studies

1. ### Routine Histological Staining

   a) #### Vital Staining

   At the commencement of this study, vital staining was employed in an attempt to observe the living amoebas in more detail. For example, the nuclei in the living organisms were not visible under the stereoscopic binocular microscope nor under low power of the light microscope. High power could not be used without disturbing the animals (i.e. the watch glass could not be placed under the high power objective and it was necessary to transfer the amoebas to a depressed microscope slide).

   The first concentration of Neutral Red used was 1:1,000. It proved too strong but the results were interesting.

   A 1:100,000 dilution of Neutral Red and a 1:200,000 of Janus Green B (in culture media) was next tried. It is

generally stated that concentrations of this order are
non-toxic, however, it seems reasonable that normal physiology
must be disrupted to a certain extent by "vital" stains
since cells exposed to such staining are eventually killed.
Thus, after a few trials, vital staining was abandoned
for methods such as phase contrast and water mounts.

b) Permanent Histological Preparations

Routine histological techniques, known to demonstrate
particular components of the animal cell were modified in
order to render them suitable for the morphological
investigation and mitotic studies performed on
P. carolinensis (McClung, 1950; Gatenby & Beams, 1951;
Ford, 1958; Baker, 1958; Davenport, 1960; Armed Forces
Inst. Tech., 1960; Pearse, 1953 and 1961).

The various fixatives used were Bouin's, Schaudinn's,
Osmium tetroxide, Formol Saline, Trichloroacetic Acid,
Zeigwalner's and Carnoy. Attempts at freezing the
amoebas before staining were made using dry ice and liquid
air.

Ehrlich's and Heidenhain's iron haematoxalin and
Grenacher's Borax carmine were employed for nuclear studies.

Formalin calcium fixation with Sudan Black stain was

used to demonstrate lipids.

For mitochondria, Regaud's Method with Regaud as well as neutral formalin fixation was used. Staining was carried out using Janus Green B, Regaud's Method for mitochondria, Heidenhain's Iron Haematoxylin (Champy Fixative) and Altman's Acid Fuchsin.

In making permanent histological preparations several methods of handling the amoebas were employed.

A modification of Kirby's (1950) Method was used for the first whole mounts that were prepared. The amoebas were allowed to become attached to the bottom of a syracuse watch glass, then most of the water was pipetted off before pouring hot ($65^{o}C$) Bouin's Fluid onto the amoebas. The first alcohol in the hydration series was poured in with the Bouin's. The animals were then taken through a series of alcohols (85% - 50%). During this procedure the amoebas were pipetted individually and the alcohol was contained in black glass stenders which showed up the white amoebas well. The amoebas in the staining solution could not be seen with the naked eye but when placed on the stage of a dissecting microscope the light passing through the glass from the bottom allowed them to be observed sufficiently well to be handled. After differentiating, the animals were

cleared in clove oil, terpineol, oil of wintergreen or
lilac oil and mounted in Permount.

The above manner of handling the material was adequate
for observing characteristic "body" shapes, pseudopods and
some cytoplasmic inclusions (i.e. droplets, crystals,
vacuoles). However, because of the thickness of the
preparations, it was not satisfactory for demonstrating nuclear
structure nor for observing mitotic nuclei. Thus, for
visualization of the nucleus, both during interphase as
well as during the various stages of mitosis, the amoebas
were fixed in the shape in which they happened to be at the
time. Certain fixatives (formol saline, Schaudinn's, osmium
tetroxide and trichloroacetic acid) had an instantaneous
effect without being heated. Following fixation, the amoebas
were handled by means of pipettes, individually, as
described in the previous method. Subsequent to staining they
were embedded in small wax blocks, sectioned at 4 u, 5 u
and 10 u, mounted on slides which were very clean and
therefore required no egg albumin nor PSA. They were
then cleared in xylene and mounted in Permount. Ball-
point pen lines were drawn beneath the very tiny sections in
order to facilitate finding them under the microscope.

Since sectioned material provides preparations of uniform

thickness it seemed necessary to employ it for purposes
of comparing intensity of Mercury Orange staining in
various stages of mitosis.   However, I discovered that the
Mercury Orange staining of the cytoplasm was poor. (This is
in part due to the fact that Mercury Orange does not produce
an intense colour in thin preparations.   Although, in the
past this was considered to be a disadvantage, it is now
believed that this property, rather, indicates the
specificity of the reagent (Pearse, 1961).   Therefore,
in addition to serial sections I also prepared a large
number of squashes for mitotic as well as cytochemical study.

Squashes were prepared as follows.   One amoeba was
placed on a slide in a small drop of culture fluid.   Strips
of filter paper were used to absorb most of this water.
Dropping fixative directly onto the amoeba and then lowering
the coverglass damaged the preparation considerably since
this resulted in fixation prior to flattening of the animal.
Therefore, a method adopted from bacteriological study
techniques was employed.   A drop of fixative was placed on
the coverglass which was inverted and lowered onto the amoeba.
Such simultaneous fixation and flattening caused no
apparent damage to the internal structures.   In many
instances the outer limiting membrane of the animal remained
intact.   For nuclear observations there was no particular

advantage in maintaining an intact membrane, therefore a

slight amount of pressure was applied to the coverglass to further

flatten the amoeba, while still maintaining intact nuclei.

Once the correct degree of flattening was obtained,

the coverglass was floated off with whichever fluid the

particular method required. With the coverglass floated

off, the amoeba remained attached to the slide (or in many

instances to the coverglass) and the latter could then

be handled in the usual manner during dehydration, staining,

clearing and mounting procedures.

2.   Cytochemical Methods for Demonstration of -SH Groups

In selecting a suitable cytochemical reagent, certain

criteria had to be satisfied by the chromogenic agent:

(1) The reagent should be specific for thiol groups. It

must combine with thiols so as to demonstrate their site

but should not react with any other groups in the cell

or tissue, or if capable of such binding, convenient methods

should be available for detaching the molecules bound non-

specifically without removing any from the thiol groups.

(2) The reagent should be monofunctional. Each molecule

should possess only one group through which the molecule

might be bound to a tissue component.

(3) The equilibrium constants of the binding reaction should favour strongly the bound state.

(4) It should have a relatively small molecular size, so as to minimize steric hindrace to binding.

(5) The reaction should not be affected by any of the histological procedures necessary for the preparation of permanent microscopic slides.

(6) The colour should be sufficiently intense to be seen microscopically.

(7) The colour should remain for a considerable time in order to obtain a stable preparation which can be used as a permanent record of -SH content of the cell.

(8) The reagent should not destroy the cytological quality of the preparation.

(9) It should be available in very pure form.

a) Preliminary Experiments with Mercury Orange

 Bennett's Reagent, Mercury Orange, (1-(4-chloromercuri-
-phenylazo)-2-Naphthol) was believed to satisfy all of the
above criteria. Nos. (2) to (9) were apparent from the
literature and the chemical structure. However, I felt

it desirable to prove No. (1) in our laboratory prior

to using it in this study.   Preliminary experiments were

performed, therefore, on muscle tissue in order to

test the specificity of the reagent and to confirm the results

of Bennett (1951).   Toad and mouse muscle fibres fixed

24 hours in 5% Trichloroacetic Acid, washed and dehydrated,

were teased and immersed in the Mercury Orange reagent

(a saturated solution in butanol containing 6 mgs/litre).

Mouse muscle fibres were left in the solution for 7

hours and toad fibres for 24 hours.   Then they were washed

in butanol 24 hours, cleared in xylene and mounted in

Permount.   These served as the controls.   The experimental

group were fixed similarly but prior to immersion in

Mercury Orage they were left in 1%, 5% and 10% sodium

arsenite ($NaAsO_3$) for 24 hours.

b)   <u>Application of Mercury Orange to P. carolinensis</u>

   After confirming Bennett's experiments as above,

techniques were developed for adapting this method to

<u>P. carolinensis</u>.   Mercury Orange was used in the same

concentrations and solvent as for preliminary muscle

experiments (i.e. 6 mgm/litre in butanol).   Amoebas

taken directly from healthy cultures were fixed with 5%

TCA, washed in distilled water, dehydrated and rinsed in

butanol, then allowed to remain in Mercury Orange reagent for

varying periods of time (i.e. 3, 4, 8, 24 and 48 hours).
They were washed in pure solvent 24 hours, cleared in clove
oil, oil of wintergreen or turpineol and mounted in
Permount on clean slides with no mounting media. Some
of the amoebas were subjected to sodium arsenite both in
the living state and subsequent to TCA fixation, but prior
to Mercury Orange staining. Mercury Orange was also
applied to "starving" individuals.

The method chosen for the application of arsenic was
the direct immersion of the specimen into the test solution.
This is a common procedure employed for application of a
chemical agent to the living cell for studying its effect
but some of the problems involved should be borne in mind.
They are: (1) modification of culture media;
(2) diffusion of substance into the organism, and
(3) chemical solubility of the test substance. The chemical
solubility of arsenic was a problem at the outset because
I chose As_2O_3 (Arsenic Tri-Oxide). To overcome this, 1 gram
of As_2O_3 was added to 10 ml of glycerine. It was hoped
that glycerine would be sufficiently inert to have a
minimum deleterious effect upon the physiology of the
amoeba. The mixture was heated until clear. After cooling,
90 ml of stream water were added to it giving a 1% stock
solution. This method was not satisfactory because the

maximum concentration of arsenic which could be used was 1% and it was desired to try concentrations of 1% and greater since concentrations below 1% did not affect the intensity of the -SH stain. Increasing the proportion of glycerine was not desirable since the results would probably be less significant if a great quantity of extraneous substance was introduced along with the arsenic. The soluble sodium salt, sodium arsenite $(NaAsO_3)$ was therefore tried on living material in concentrations of 0.01 gm %, 0.5 gm % and 1.0 gm %. Although some changes were observed at concentrations of less than 1.0 gm %, death of the organisms did not occur - nor was there any difference between these amoebas and untreated ones with respect to sulfhydryl staining. On the other hand, concentrations of 1.0 gm % and above caused disintegration of the membrane and the animals could not be used for cytochemical studies. Thus, it was decided to use 1.0 gm %, 5.0 gm % and 10.0 gm % solutions of sodium arsenite on fixed material, as a means of verifying the specificity of Mercury Orange and to determine whether arsenic has an affinity for -SH in protozoa as has been found in in vitro studies.

In applying Bennett's Mercury Orange method, some sections were prepared as well as many squashes.

For the study of changes in -SH content of P. carolinensis

during nuclear division, the same procedures of feeding

and selection of individual amoebas as described for

making permanent histological preparations of mitotic stages

were used but the cytochemical reagent was used (as described

above) in place of routine stains.

PLATE I
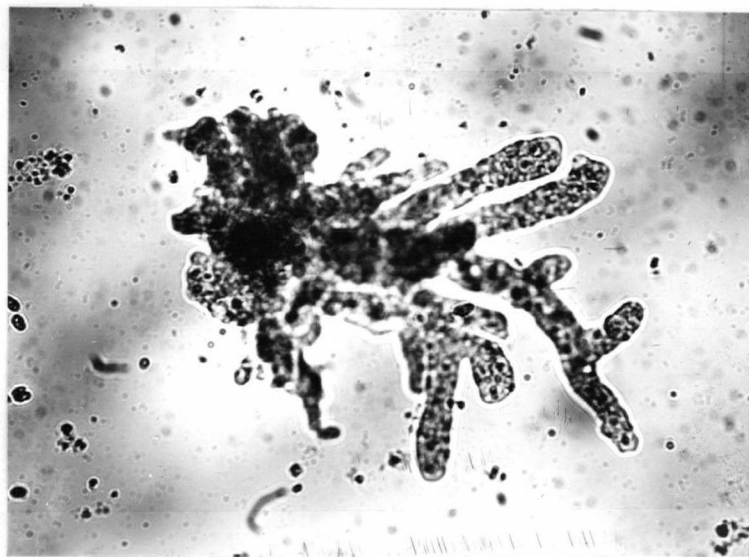
Figure 2 - Photomicrograph of healthy, normal
living P.carolinensis placed in clean watch
glass containing fresh stream water immediately
begins to send out pseudopodia and attaches
itself to the substratum.   X 95
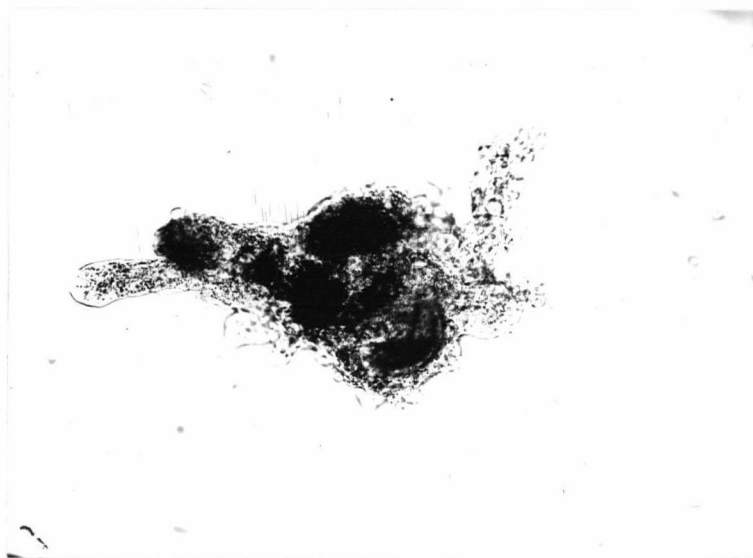
Figure 3 - Once attached to the substratum
P. carolinensis begins ingesting food organisms.
Recently ingested paramecium in food vacuole is
seen here. Note the thin, delicate-appearing
membrane of the amoeba compared with the dense,
thick, ciliated pellicle of the entrapped para-
mecium.   X 95

OBSERVATIONS & RESULTS

I. LIVING MATERIAL

General Behaviour

Feeding

Unless undergoing mitosis, healthy, normal, living

P. carolinensis became attached to the bottom of the glass

containing fresh stream water (Figure 2). The amoebas

attached themselves whether or not parameciums were added,

provided the glassware was meticulously cleaned and dried

prior to adding fresh water. If food was withheld

indefinitely, however, they became detached once more.

Likewise, when the food organisms were depleted or when

environmental conditions were not optimum the amoebas

became detached once more.

P. carolinensis is a voracious feeder. Almost immediately

following attachment to the substratum it began ingesting

food organisms. Frequently an individual amoeba was seen

to contain several parameciums at one time. Each paramecium

was contained in a separate food vacuole (Figure 3) usually,

but two parameciums in one food vacuole have been observed.

When feeding on smaller protozoa (Chilomonas, Menoidium,

Chilodon and Colpitium) one food vacuole contained many

PLATE II
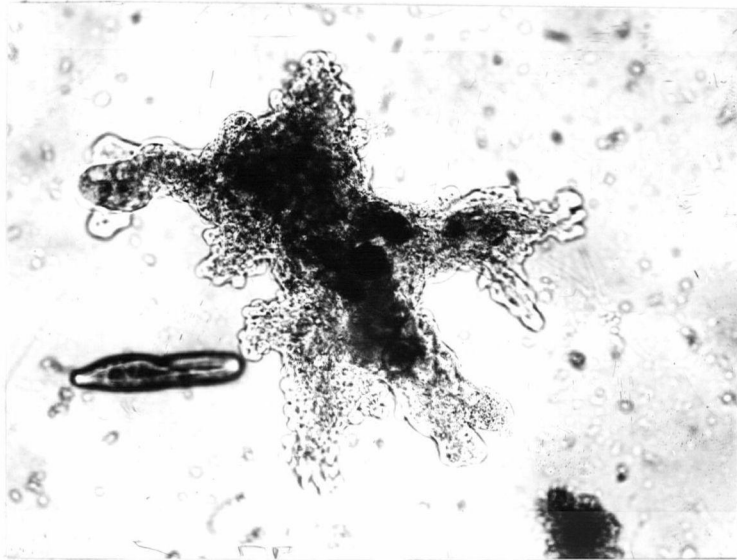
Figure 4 - Photomicrograph illustrates the
apparent attraction of paramecium to the amoeba.
This phenomenon seems to be a significant factor
in feeding of P. carolinensis.   X 95
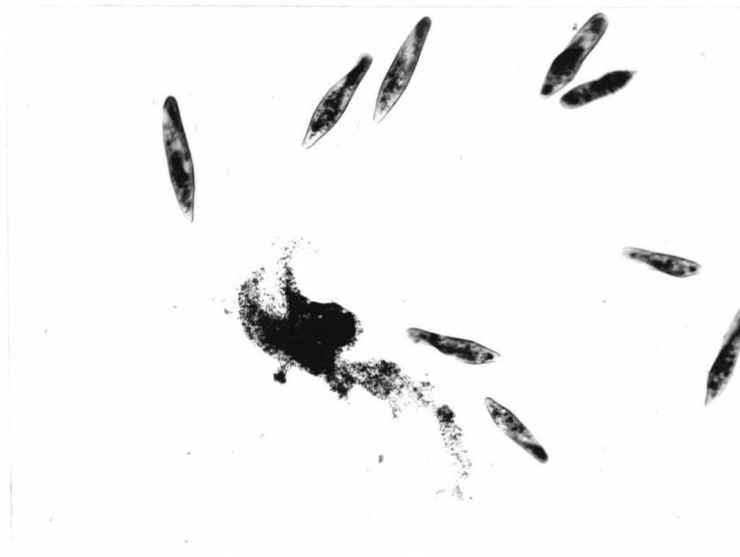
Figure 5 - Parameciums have just been added to
the watch glasses containing fresh stream water,
a stick of boiled timothy hay and P. carolinensis.
The characteristic exploratory behaviour of the
parameciums is evident.   X 40

organisms but the vacuole remained approximately the same size as those which were formed about one paramecium. Thus, there appears to be an average size that a food vacuole attains.

Although the amoebas move by means of pseudopodia, this movement is too slow to be a significant factor in the pursuit of food. Rather, it was observed that the parameciums are attracted to the amoebas (Figure 4). Parameciums, when first put into a watch glass containing P. carolinensis, swam around exploring various bits of organic material in the culture. In this respect their behavior was similar whether they encountered bits of timothy hay or an amoeba (Figure 5).

All parameciums coming in contact with a groping pseudopod were not ingested. Whether or not ingestion took place depended on a co-ordination of events. The paramecium usually crawled under a part of the amoeba and remained sufficiently long for the amoeba to move its pseudopodia about in a position such that cytoplasm flowed over top of the paramecium completing the vacuole (Figure 6). The paramecium usually did not move away until the bottom of the vacuole began filling in. Once the vacuole was formed the previously almost stationary paramecium began frantic movements

PLATE III
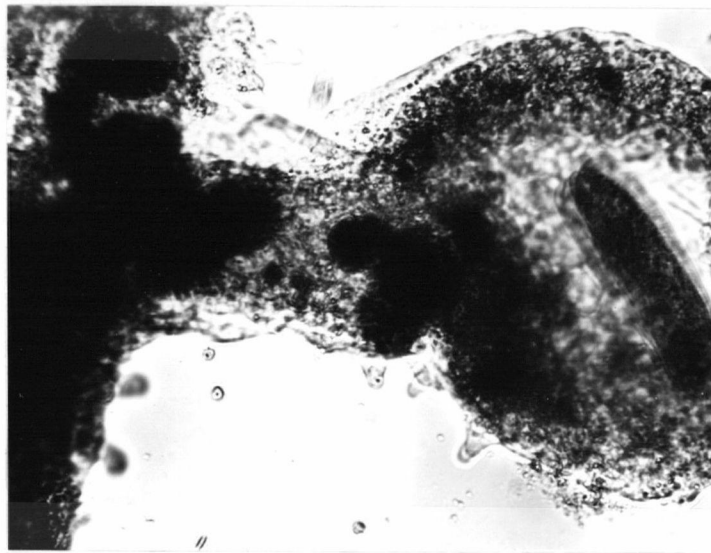
Figure 6 - Newly formed food vacuole. Photo-
micrograph of living material was taken soon
after pseudopodia had surrounded the paramec-
ium, the cytoplasm had flowed over the top,
and the "floor" of the food vacuole was form-
ing.   The paramecium was stationary at this
time as indicated by the clarity of cilia and
the fact that it was not yet swimming in a
curled position as seen in Figure 3 (i.e. a
later stage of food vacuole).   X 200

which continued until it was swimming continuously round
and round in the vacuole in a curled position corresponding
to the round shape of the food vacuole.

An ingested paramecium circled continuously within the
new food vacuole for approximately 15 minutes before
slowing down. Eventually it became less motile and
by 2 to 6 hours was usually in pieces. Upon ingestion of
a paramecium, small dark granules in the cytoplasm were
seen streaming towards the point of ingestion. On formation
of the new food vacuole these granules "lined" its periphery.
As digestion of the paramecium progressed the granules
diminished in size and by approximately 3 hours could
not be detected.

Body Form

Relation to Physiological State

Degenerating amoebas undergo alterations of body
form from flat, attached, with surface wrinkles,
indicating health and normal function (Figure 2), to
a perfect sphere which precedes "death".

Of a less permanent nature is the change in form
when it is irritated. When disturbed it tends to
round up.

Club-shaped organisms were often seen in older

PLATE IV
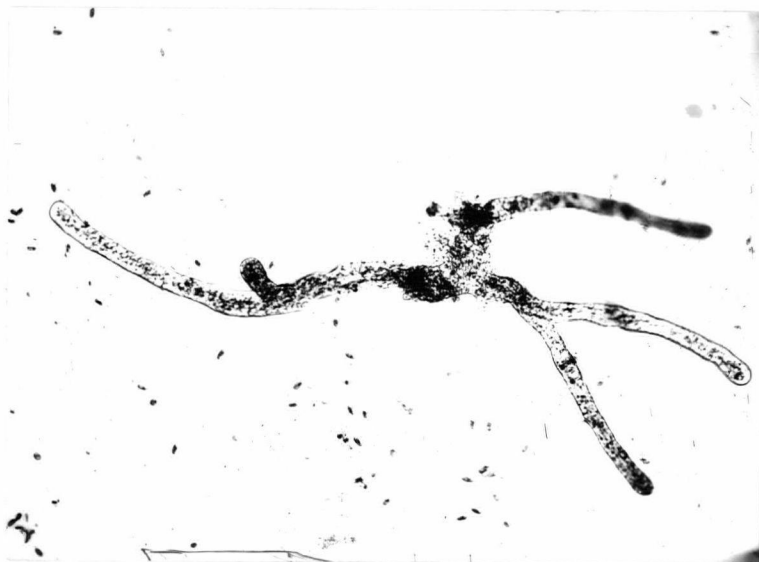
Figure 7 - Photomicrograph of "star-shaped" living P. carolinensis. Individuals of this shape were numerous when food was scarce or absent.   X 45
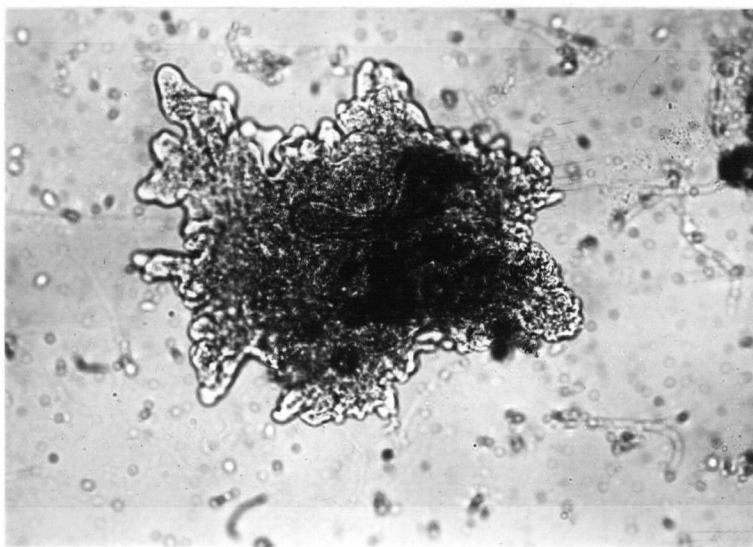
Figure 8 - Living P. carolinensis which has ingested food and is beginning the process of digestion assumes a more compact form. X 95

cultures where food was becoming scarce.

Star-shaped amoebas (Figure 7) were numerous
when little food remained (i.e. 10 to 20 hours after
addition of parameciums) as well as immediately upon
transferring organisms from old, barren cultures into
fresh cool stream water before any food was given
(and before attachment to the substratum).

Amoebas which had ingested food and were beginning
the process of digestion assumed a compact form, more or
less irregular in outline but tending towards a
spherical shape with many short pseudopodia firmly
attached to the substratum (Figure 3 and Figure 8).

The type of food organisms influenced the shape
of P. carolinensis. When feeding on very small protozoa
it was more regular in shape with more rounded contours
than when feeding on larger organisms such as parameciums.
(Figure 9).

The accumulation of excess waste products seemed to
cause death to the amoebas more rapidly than did lack
of food. If food was added periodically without changing
or adding fresh water, the amoebas soon died in spite
of the presence of adequate food. If unhealthy,
detached amoebas, tending towards the smooth spherical
shape (which signifies degeneration) were removed from

PLATE V
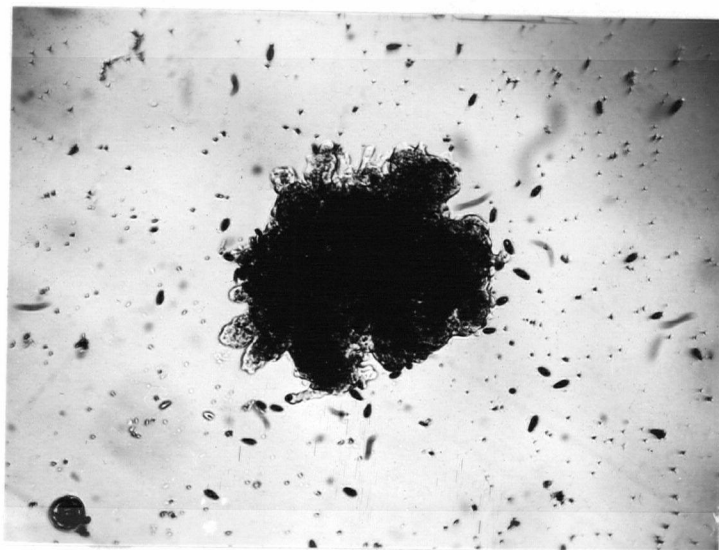
(a)

(b)

Figure 9 - (a) and (b) are photomicrographs of
living amoebas to illustrate the compact regular
form assumed when feeding on small protozoa.

X 110

their environment and placed into fresh water they
immediately spread out and assumed a healthy appearance.
The pH of the media in which degenerating individuals
were numerous was nearer to alkaline (6.5) than was the
pH (5.2) of culture media containing healthy organisms
undergoing growth and reproduction.

Amoebas put into fresh water without added food
organisms gradually decreased in size and became a great
deal more transparent. Within 8 to 9 weeks the
membrane disintegrated and the small amount of cytoplasm
remaining was released. In contrast to this, when an
amoeba is exposed to injurious chemicals or other toxic
conditions, the membrane breaks down rapidly and the
contents are released explosively.

Correlation of Body Form with Mitotic Events

There was another unattached, roughly spherical form,
with numerous wart-like pseudopodia which was not due to
unfavourable environment. This morphological change
occurred, as a rule, in amoebas which had been feeding for
some time under healthy environmental conditions. Visible
cytoplasmic streaming stopped, or decreased, as did ingestion.
But, often one still observed parameciums within food vacuoles
of these amoebas.

Amoebas possessing the shape shown in Figure 10 contained
early prophase nuclei. This stage was difficult to detect

PLATE VI

Body Form
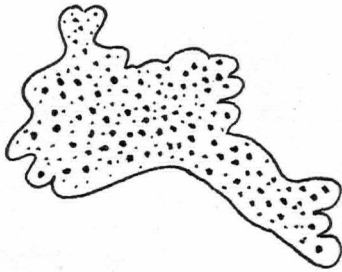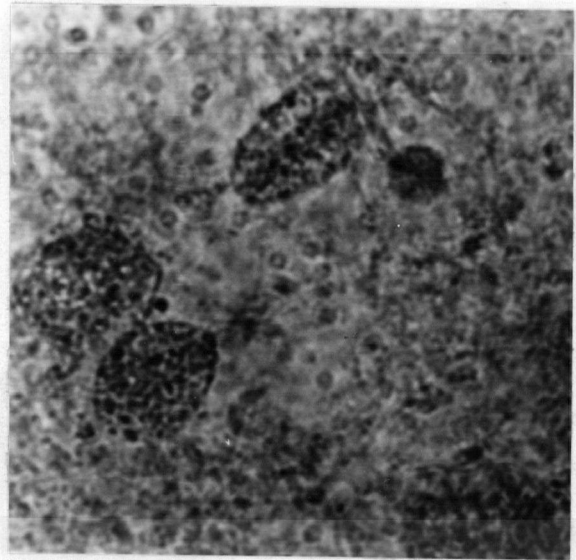
Nuclear Stage

X 1250

(a) Early Prophase
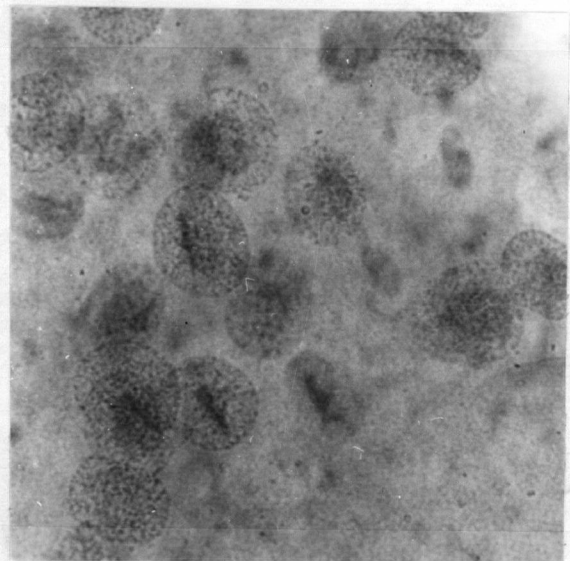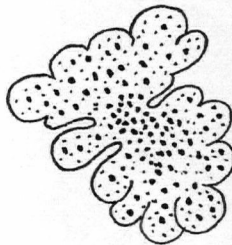
X 500

(b) Late Prophase

Figure 10 - Amoebas having the shapes shown diagramatically
on the left contained prophase nuclei. Nuclear stages are
shown in photomicrographs of histological preparations on
the right.

PLATE VII
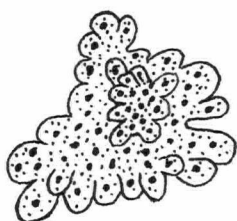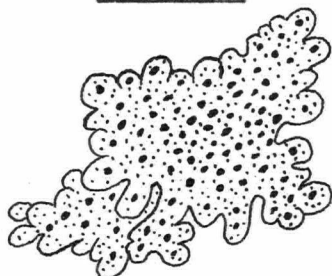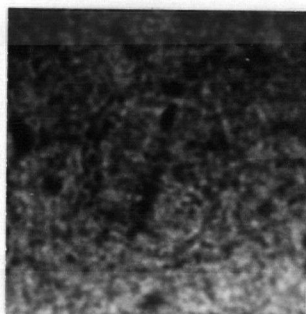
Body Form	Nuclear Stage	
		(a) Late prophase to early metaphase. X 1500
		(b) Early metaphase. X 800
		(c) Early metaphase. Later than (b). X 950
		(d) More advanced metaphase. X950
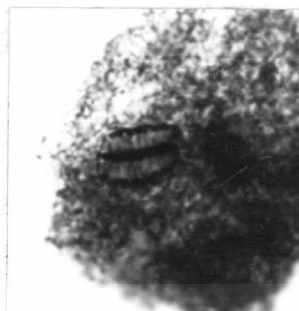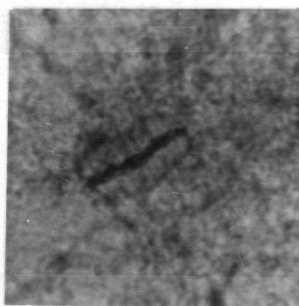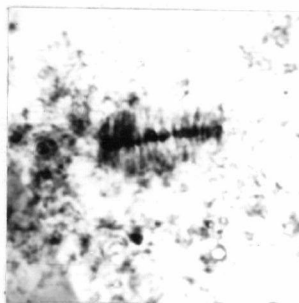
Figure 11 - Amoebas having the shapes shown diagramatically on the left contained metaphase nuclei. Nuclear stages are shown in photomicrographs of histological preparations on the right.

PLATE VIII

Body Form   Nuclear Stage
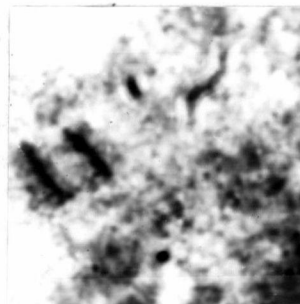
X 1000

(a) Early Anaphase
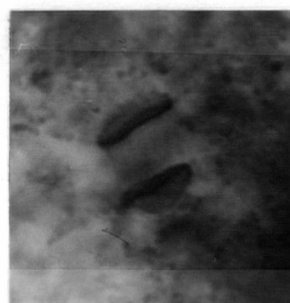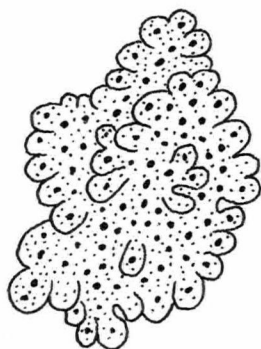
X 1500

(b) Later Anaphase
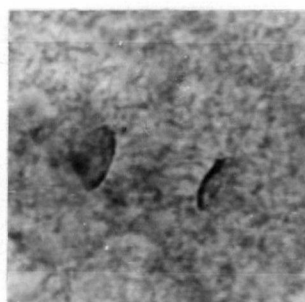
X 1200

(c) Late Anaphase

Figure 12 - (a) The most symmetrically spherical and
compact form ("berry form") contains early to mid-anaphase
nuclei. Very little activity was observed in the pseudo-
podia during this stage. (b) and (c) Pseudopodia began to
change shape and enlarge during late anaphase.

PLATE IX

Body Form   Nuclear Stage
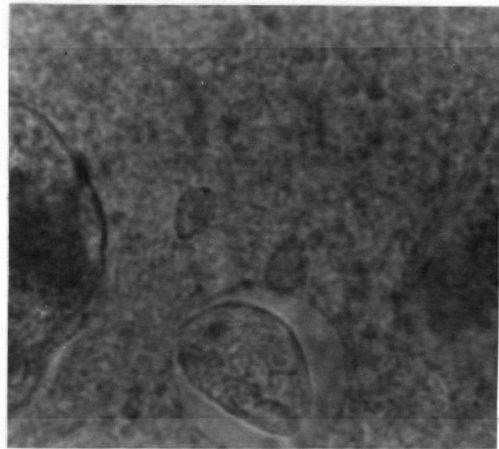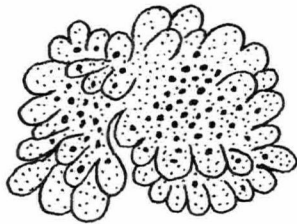
Figure 13 - Telophase. The plates are progressing towards
interphase nuclei. Body shape resembles late anaphase.

                                                    X 1200
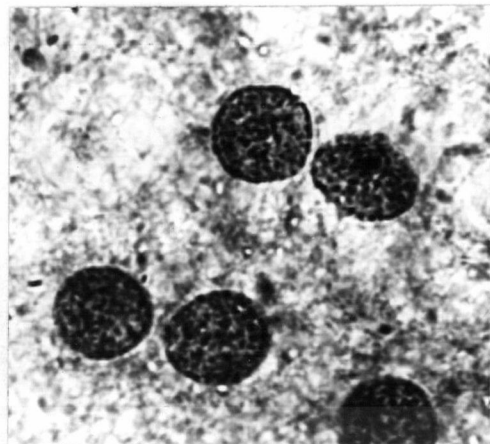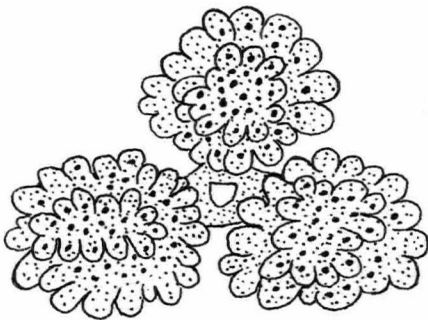
Figure 14 - Interphase. Constriction of the body with cyto-
plasmic bridges indicates interphase nuclear stage has been
reached. Plasmotomy usually follows the above sequence of
events but occasionally the constrictions disappear and the
amoeba settles down to feed once more.   X 800

with certainty since the shape differed little from elongated forms in which interphase nuclei occur. The body was irregular in outline and the amoeba was fairly spread out. Pseudopodia were rounded and rather short; their size and shape changed slowly. Cytoplasmic streaming was slow and directed towards the centre of the body.

Body form of an amoeba containing late prophase nuclei is shown in Figure 10b. The organism continued to contract and exhibited many short rounded pseudopodia until it became compactly rounded with short, round pseudopodia extending in all directions.

Figure 11 illustrates the shape assumed when the nucleus was undergoing metaphase. The body was contracted still further and very little change could be detected in the numerous, short, round pseudopodia.

The most symmetrically spherical and compact form was exhibited during early anaphase as seen in Figure 12a. It had the appearance of a blackberry.

During late anaphase (Figure 12b and c) pseudopodia began to change shape and enlarge. Increased cytoplasmic activity was noted.

Telophase resembled late anaphase (Figure 13). The body form in which young interphase nuclei occurred was similar to
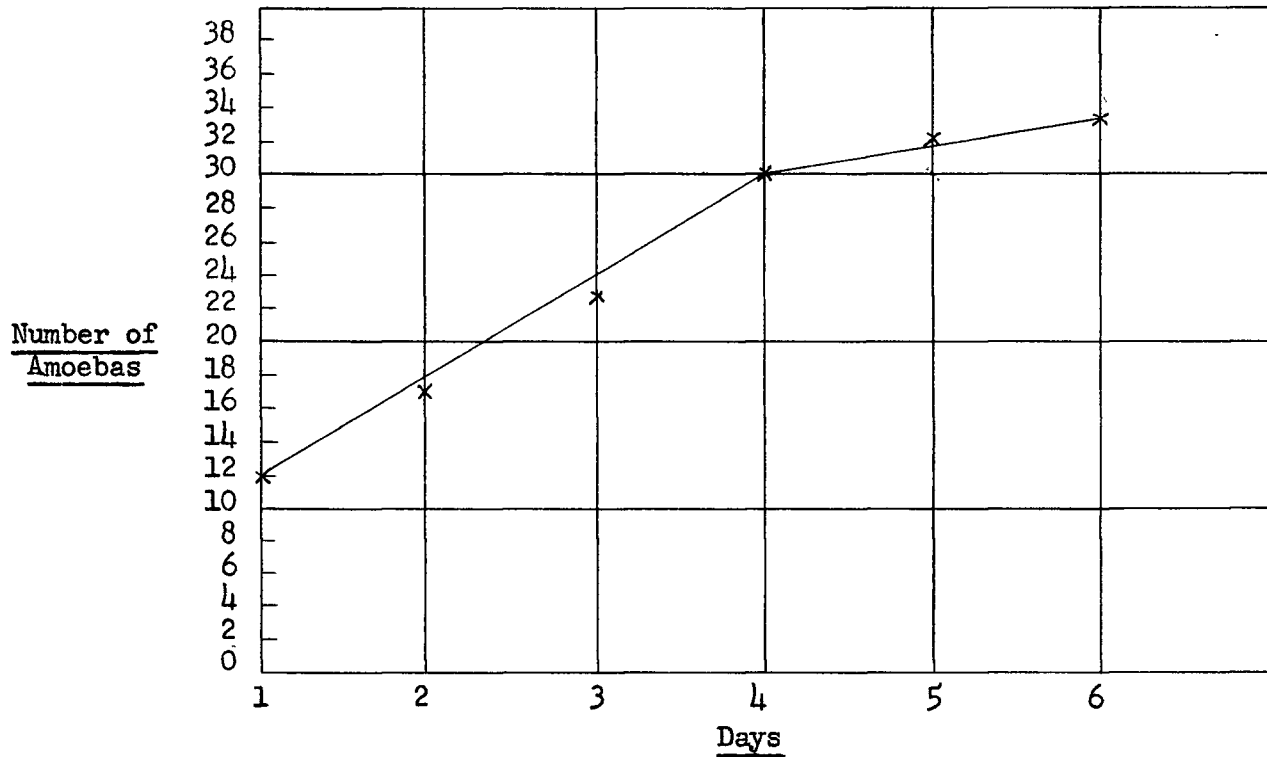
Figure 15 - Within 24 hours, approximately one-quarter of the amoebas underwent plasmotomy into two or three individuals. By the second day, another one-quarter divided, followed by another one-fifth or one-sixth by the third day. After the third day, unless fresh food was added, very few amoebas divided and the remaining individuals assumed an unhealthy appearance.

late anaphase and telophase (Figure 14). In addition, constriction of the body became evident and cytoplasm could be seen streaming back and forth through one or more bridges. Plasmotomy into 2 to 6 daughters usually followed the above observed sequence of events. However, this was not always the case. At times such an individual would settle down and begin feeding once more, then some time later the above events would be repeated before plasmotomy finally occurred.

Conditions which favour Reproduction and Frequency of Mitosis

The nuclear details of dividing amoebas were best observed and recorded by making permanent histological preparations. However, studies of living organisms lead to observations relating to frequency of nuclear division and conditions favouring mitosis and plasmotomy.

No periodicity in division was found. Regardless of the time of day, if 50 to 100 parameciums per amoeba (and fresh water) were added to the culture, the animals went about feeding for approximately 4 to 6 hours, after which time pre-plasmotic individuals (undergoing nuclear division) appeared in abundance. Figure 15 represents the general trend in reproduction of P. carolinensis and is the result of 33 months of observation. Within 24 hours, approximately one-quarter of the amoebas underwent plasmotomy into 2 or 3

individuals. Plasmotomy into 4 to 6 daughters was observed on rare occasions. By the second day another quarter divided, followed by a further one-fifth or one-sixth by the third day. Within three days, therefore, there was generally a two to three-fold increase in organisms and often the yield was greater. After the third day, unless fresh food was added, very few amoebas divided and the remaining individuals assumed an unhealthy appearance.

Division appeared to be related to size of the amoeba in that the interphase period was longer in smaller animals. Body size of parent was not an apparent factor in determining the number of daughters into which it would divide.

Duration of Various Stages of Nuclear Division

These times could not be determined for undisturbed organisms since it was impossible to place the culture dishes under sufficiently high power to observe the nuclei. Therefore, the data appearing in Table 1 applies to organisms which, because of their characteristic shape were believed to be undergoing mitosis. They were removed from the culture glass to microscopic slides for observation as described in Materials and Methods (Page 21)

Table No. 1

AVERAGE DURATION OF MITOTIC STAGES

Mitotic Stage of Nucleus		Average Time Taken
Early Prophase	(Figure 10a)	45 minutes
Late prophase to metaphase	(Figure 10b-11)	8 minutes
Metaphase	(Figure 11)	A few seconds
Anaphase	(Figure 12)	5 minutes
Late anaphase through telophase to interphase	(Figure 12c, through 13 to 14)	30 to 45 min.

Plasmotomy was usually complete within 15 to 25 minutes after the young interphase nuclei appeared. When plasmotomy resulted in more than two amoebas, the event required slightly longer.

The distribution of nuclei amongst the daughters was random, but the variation in size of newly divided amoebas was not great.

II. HISTOLOGICAL STUDIES

Vital Staining

When placed in a concentration of Neutral Red of 1:1,000 a large vacuole began to form at one end of the amoeba. This

was later pinched off. The process was repeated several times during the time the organism remained in the staining solution. Eventually the entire amoeba ruptured and disintegrated. As a rule, when animals were transferred from the stain to fresh water during the vacuole-forming stages existing vacuoles were pinched off and the amoeba appeared normal once more, with respect to form and function. In concentrations of 1:100,000 no immediate toxic effects were observed. The material within food vacuoles stained bright red after 15 to 20 minutes whilst the fluid was coloured pale reddish orange (pH 5 - 6). There also appeared vacuoles of various sizes (2 - 10 u in diameter) which contained small bright red stained inclusions. The vacuolar fluid was not stained. The number of granules contained within each vacuole varied and did not appear to be correlated with the size of the vacuole. The granules showed very definite Brownian movement. After 3 or 4 days in the staining solution, large vacuoles containing these granules almost completely filled the amoebas, resulting in the occlusion of all other cytoplasmic inclusions. Finally the amoebas began to degenerate.

In material stained with Janus Green B, large spherical structures believed to be contractile vacuoles covered with small, oval-shaped and round particles were observed. These

PLATE X
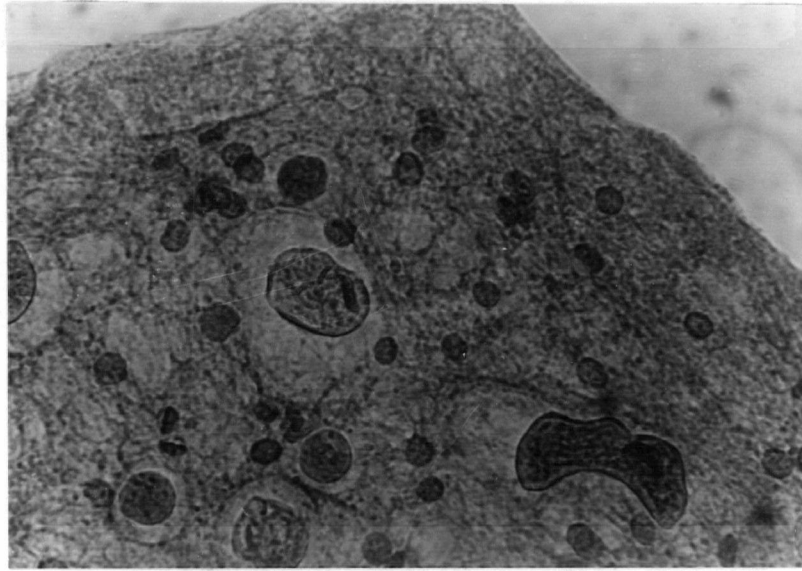
Figure 16 - Schaudinn's Fixation. Note the thin, delicate outer limiting membrane of the amoeba compared with the thick, dense, ciliated pellicle of the ingested paramecium.   X 400
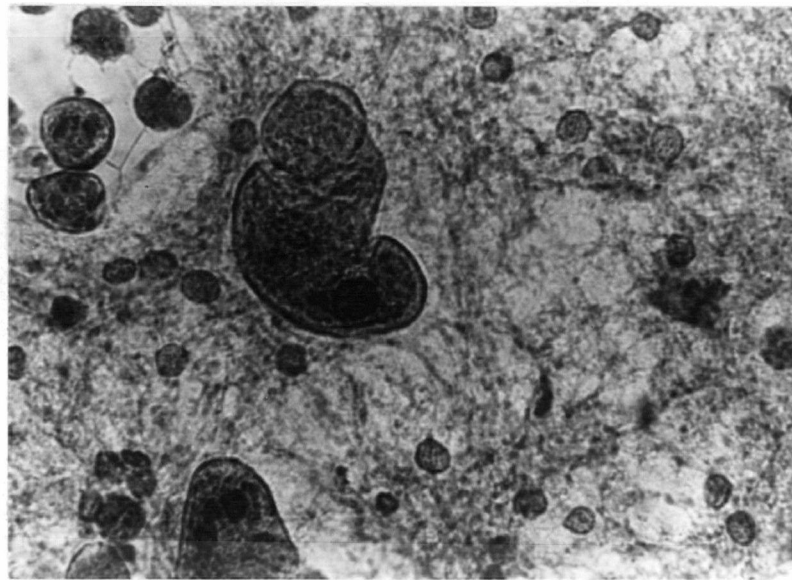
Figure 17 - Schaudinn's Fixation. Appearance of ground substance is somewhat "stringier" than Osmium Tetroxide-fixed and frozen material (Figures 19 and 21) but resembles the latter two methods of fixation more closely than does Bouin's.
                                                    X 400

are further discussed and illustrated below.

Permanent Histological Preparations

Material fixed with Schaudinn's, TCA and Osmium
Tetroxide was superior to that fixed with Bouin's. Using
frozen material as a criterion, Osmium Tetroxide caused
the least post-fixation changes in the cytoplasm.
Heidenhain's Iron Haematoxalin staining gave a somewhat
clear picture of nuclear detail than did Grenacher's
Borax Carmine, but the latter was satisfactory for making
quick preparations for nuclear counts or other studies
where detailed observation was not necessary. For such
purposes Schaudinn's fixation was used since it eliminated
the extreme caution necessary when working with the highly
toxic Osmium Tetroxide.

Figures 16 to 38 illustrate some morphological details
of P. carolinensis observed in the histological material.

Morphology

Plasmalemma

Studies with the light microscope revealed little
regarding the structure of the outer limiting membrane. It
is apparent from Figures 16 and 18b however, that it is a
relatively thin, simple membrane compared with the thick,
dense, double, ciliated pellicle of the entrapped paramecium.

PLATE XI
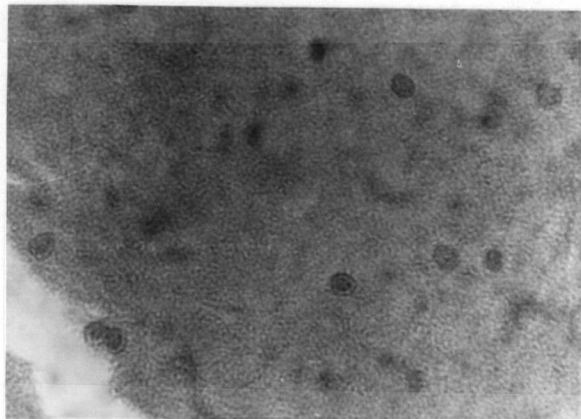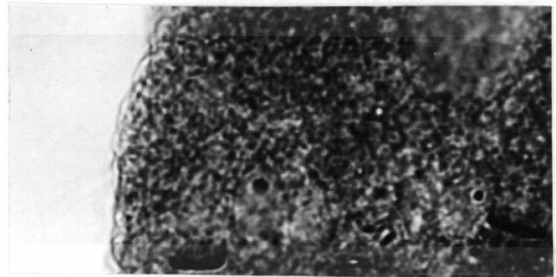
(a)   X 400   (b)   X 1000

Figure 18 - Bouin's Fixation. (a) Cytoplasm appears more dense and
fine-grained than is the case with other fixatives tried. Magnifica-
tion is the same as Figures 16, 17, 19, 20 and 21. (b) Higher
magnification. Note the plasmalemma and clear area between the
latter and main body of the cytoplasm.
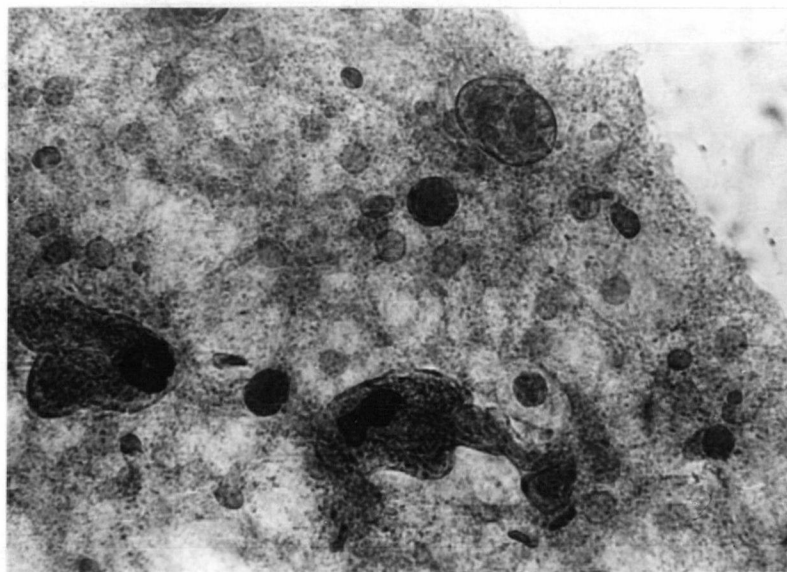
Figure 19 - Osmium tetroxide fixation. Ground substance has a
loose appearance. Small granules may be seen adhering to the
ground substance. The similarity to frozen material (Figure 21)
is striking.   X 400

PLATE XII
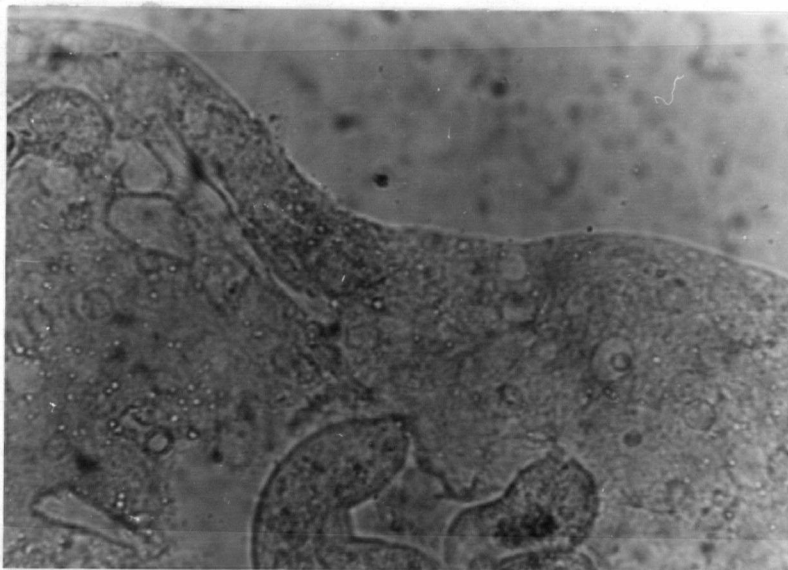
Figure 20 - Trichloroacetic acid fixation. X 400
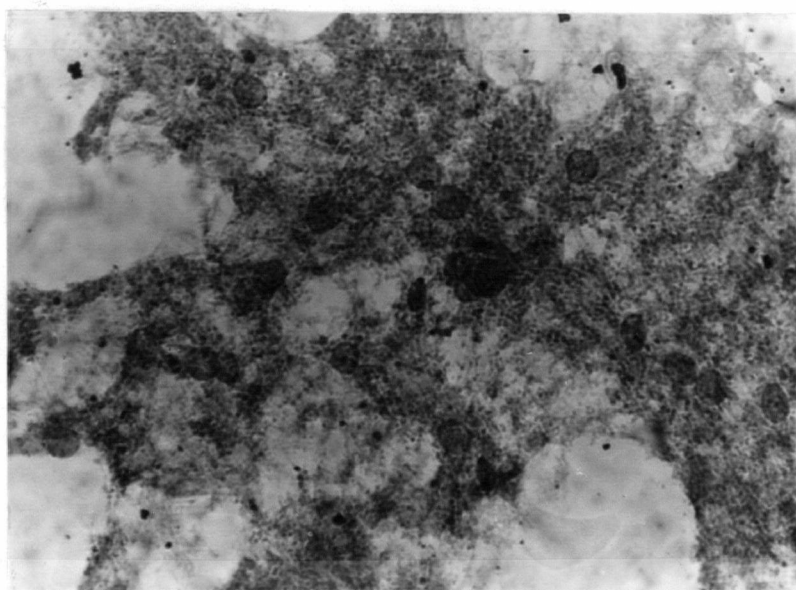
Figure 21 - Frozen preparation closely resembles
living material.   X 400

Photomicrographs of living material (Figures 3, 4, 6, 8)
also illustrate this comparison.   The clear area between
plasmalemma and main body of cytoplasm is illustrated -
especially in the advancing end of pseudopodia (Figures 3, 4,
6, 7, 8).

Cytoplasmic Ground Substance

There were variations in the appearance of the ground
substance depending upon the fixative employed.   For example,
Bouin's Fluid (Figure 18a) gave a more dense, fine-grained
picture than Schaudinn's (Figures 16 and 17), TCA (Figure 20)
or Osmium tetroxide (Figure 19).   The latter three resulted
in a looser appearance with small granules adhering to the
ground substance similar to frozen material (Figure 21).
It is interesting to compare the fixed material described
above with the living cytoplasm (Figure 6).

Cytoplasmic Inclusions

Mitochondria, vacuole refractive bodies, fat droplets,
glycogen (carbohydrate), crystals, contractile vacuoles, food
vacuoles and nuclei were observed.

Mitochondria - Rod and oval-shaped dark particles observed
in living animals under high dry (X450) and oil immersion
(X940) were thought to be mitochondria.   Other cytoplasmic
inclusions which were approximately the same size, resembled
these particles.   On more careful observation, however, a

PLATE XIII
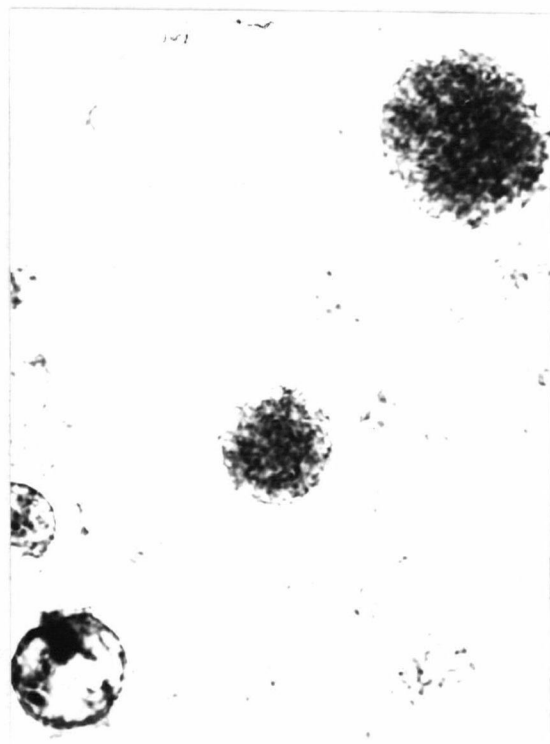
Figure 22 - Characteristic accumulation of mitochondria around contractile vacuoles. Regaud's Method for mitochondria.   X 850
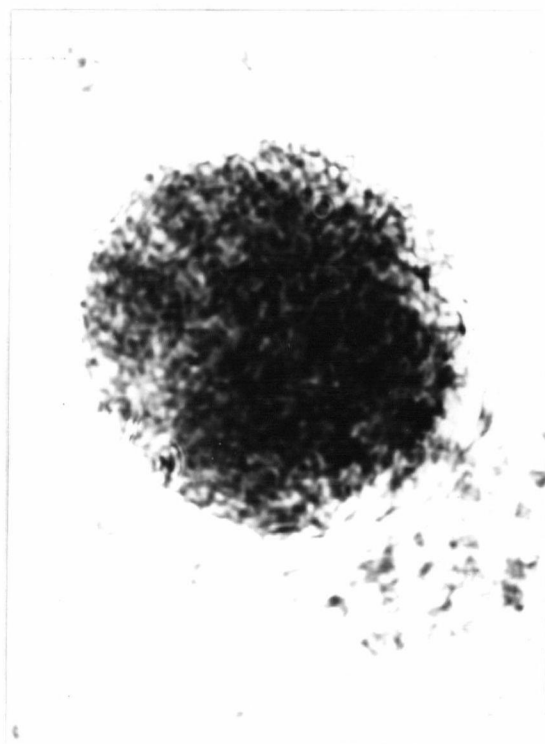
Figure 23 - Higher magnification of figure 22.   X 1825
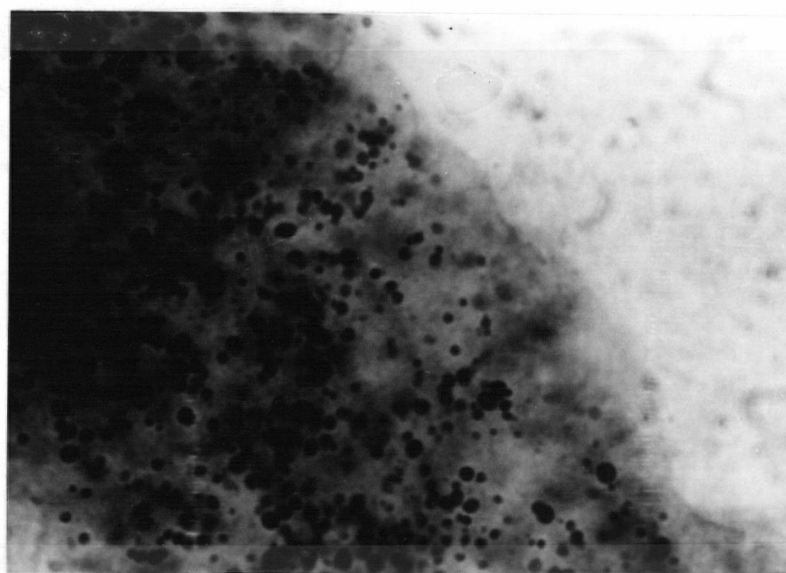
Figure 24 - Fat droplets in P. carolinensis are very numerous as seen in this Sudan Black Formol-Calcium preparation.   X 400

difference in the refractability between the two types was
detected. The latter were highly refractile and appeared
bluish-green by transmitted light, whereas those believed to
be mitochondria had a refractive index similar to that of the
cytoplasmic ground substance and appeared dull. Despite
this distinction, it was deemed necessary to employ histological
methods specific for mitochondria and thus confirm that the
particles were indeed mitochondria. Furthermore, permanent
preparations were desired for record purposes.

Although many of the mitochondria were rod-shaped in living
amoebas, they appeared round (approximately 1.5 to 2.5 u in
diameter) in fixed material. With Regaud's method, the
particles appeared dark grey or black within the lighter grey
cytoplasm. Although distributed fairly evenly throughout the
cell, their characteristic accumulation in a single layer
around the contractile vacuoles was quite apparent (Figures 22
and 23).

A similar picture to that described above resulted with
Heidenhain's Iron Haematoxylin when Champy fixation was used.
With osmium chromium fixation, however, the mitochondria stained
intensely black. Although this blackening was desirable for
observing histological detail, it resulted, in this case, in
difficulty in distinguishing the mitochondria from other
inclusions of similar size and shape.

Fat Droplets - These inclusions were clearly demonstrated

PLATE XIV
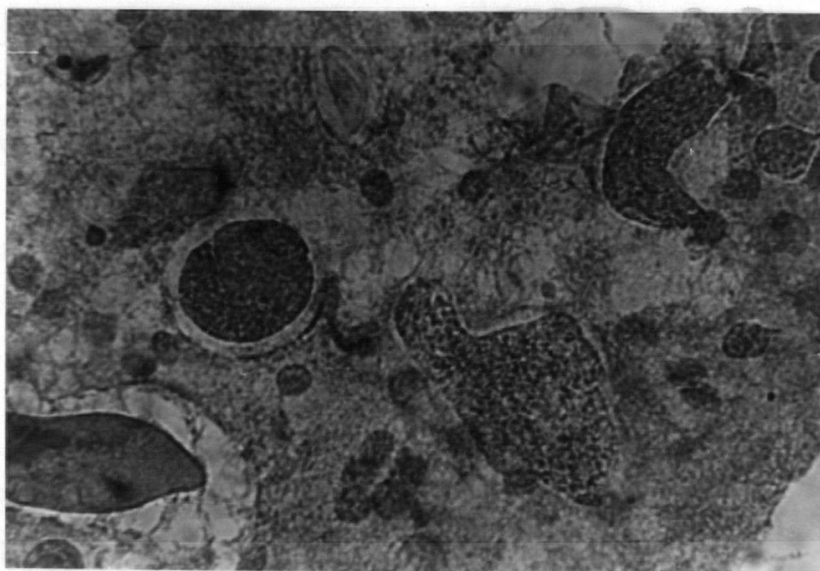
(a)   X 400
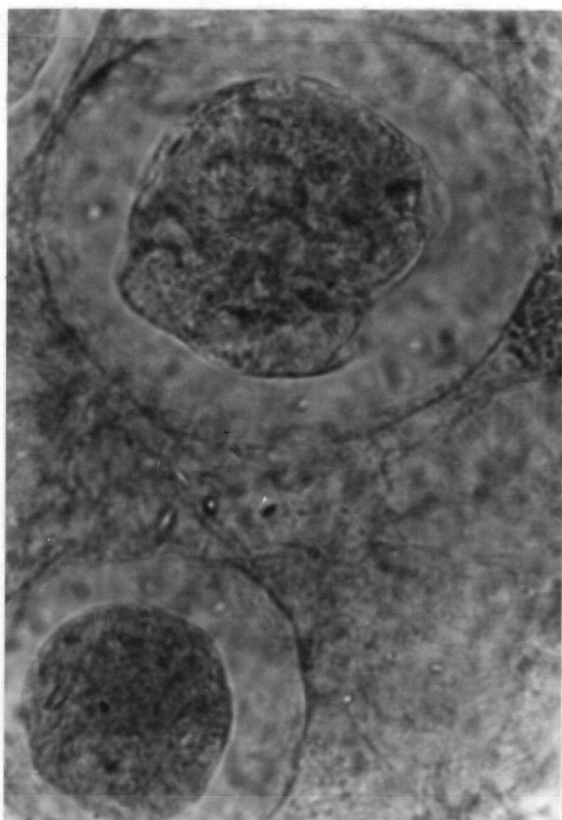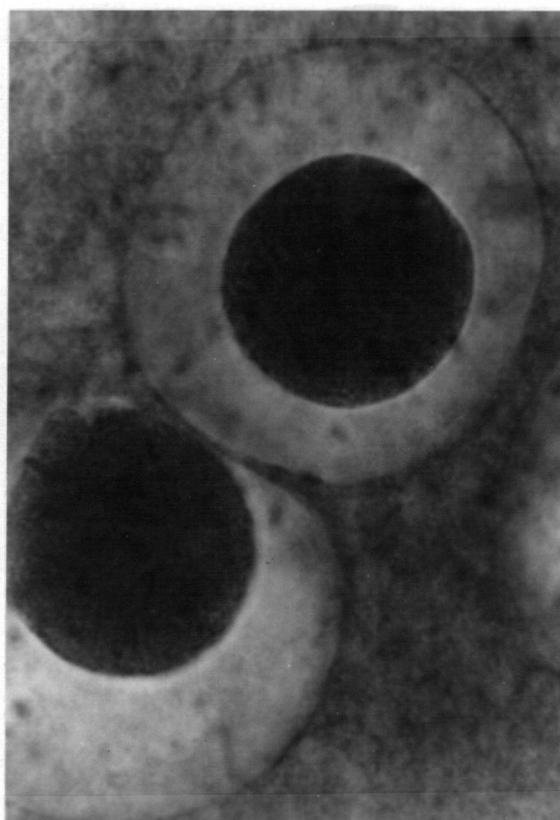
(b) X1200

(c) X1200

Figure 25 - Small, dark granules similar to those described in living P. carolinensis are seen forming a narrow rim around the periphery of food vacuoles. (a) and (b) were fixed in Schaudinn's (c) was fixed in Bouin's. Staining in all three figures was with Grenacher's Borax Carmine.

by the Sudan Black formol-calcium technique (Figure 24).

Glycogen (carbohydrates) - Since existing histological
procedures for detection of "glycogen" are not specific, any
carbohydrate material in the food vacuoles as well as in the
cytoplasm took the stain when Best's Carmine and Periodic-acid
Schiff was used. As might be suspected, less carbohydrate was
present in starving individuals.

Contractile Vacuoles - These could be readily identified
in the histological preparations because of the surrounding
layer of mitochondria, but little else regarding structure
or function could be determined.

Food Vacuoles - Small, dark granules, similar to those
described in living amoebas, were also seen forming a narrow
rim around the periphery of young food vacuoles in fixed material
(Figure 25).

Around the older food vacuoles, small, empty vesicles
were seen. In starved individuals there remained only a
few older vacuoles filled with solid-appearing particles
(Figure 44). The appearance of food vacuoles in both normal
and starved amoebas is discussed later under Mercury Orange
studies (page 56). The membrane of the food vacuoles appeared
identical to those surrounding the amoeba itself as well as
those surrounding small or large cytoplasmic vesicles
(Figures 16a and 18b).

Crystals - These particles, seen both in living (Figure 26)

PLATE XV
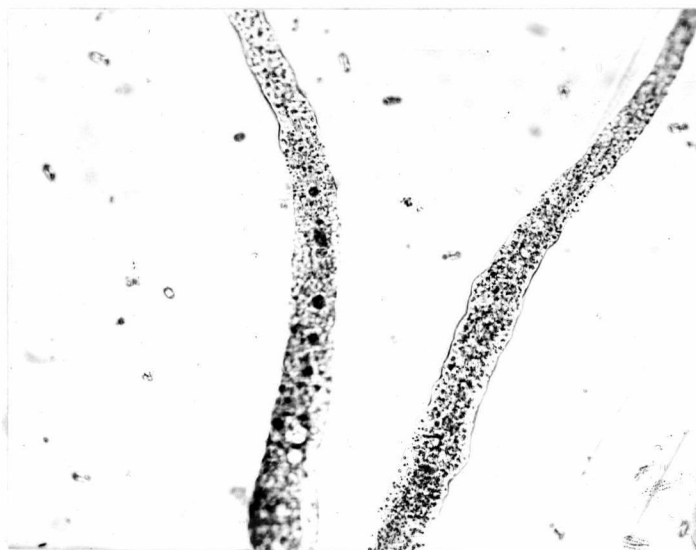
Figure 26 - Crystals seen in living Pelomyxa
carolinensis.   X 200
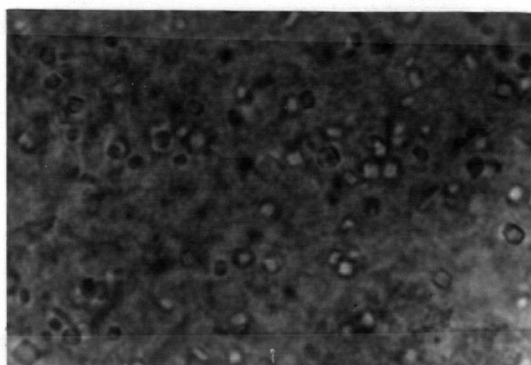
Figure 27 - Crystals seen in Schaudinn's-fixed
material. Size and shape of these crystals is
remarkably uniform.   X 1400

PLATE XVI
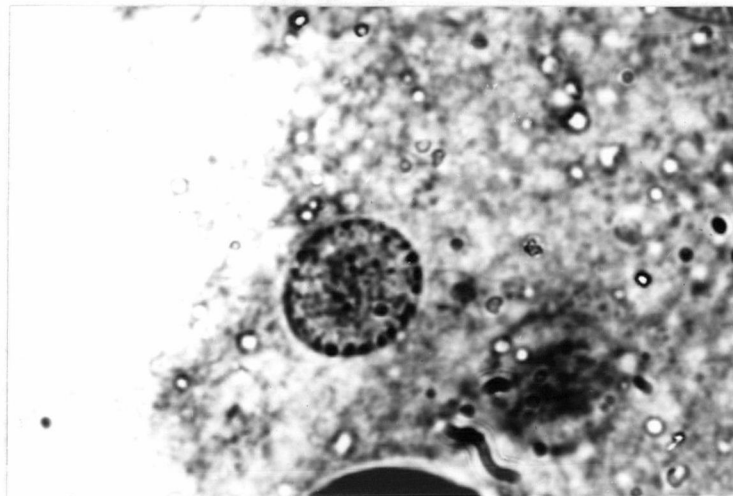
Figure 28 - Large peripheral granules are
characteristic of the interphase nucleus. A
few granules are also seen distributed through-
out the nucleoplasm. Granules stained intensely
with Heidenhain's Iron Haematoxylin and with
Mercury Orange.   X 1500
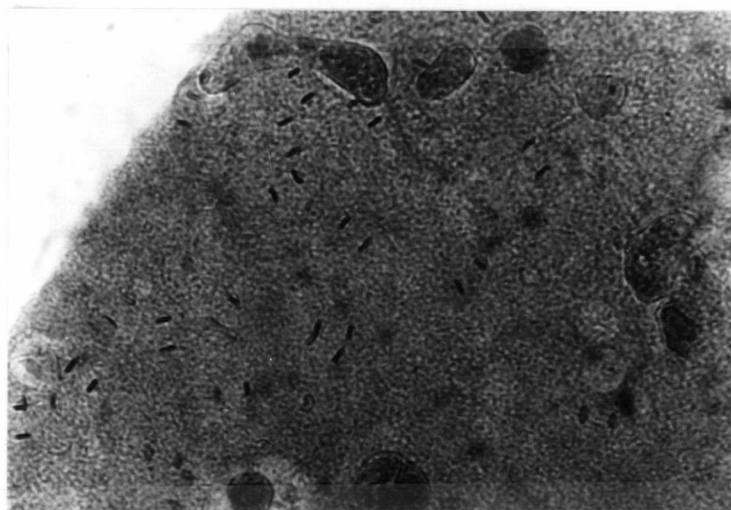
Figure 29 - Nuclear division in P. carolinensis
is mitotic. With few exceptions, nuclei under-
going mitosis do so synchronously. This amazing
uniformity of timing is well illustrated in
anaphase where plates are the same distance apart.
                                        X 400

and fixed materials (Figure 27) were better appreciated
when observed by phase contrast which showed their
crystalline shape, colour and texture very well.

Nuclei - The results of nuclear counts made on 50
amoebas showed that the nuclei contained in any one individual
may vary between 75 to 150.   The vesicular-shaped nucleus
of P. carolinensis was approximately 24 x 10 μ in diameter.
The interphase nucleus was characterized by large granules
within it closely applied to the nuclear membrane.   In
cross-section, these granules appeared as a ring around the
periphery (Figure 28).   A few of these granules were also
distributed throughout the nucleoplasm.   Heidenhain's
Iron Haematoxalin stained the peripheral granules intensely
black whilst the chromatin appeared less intense (Figure 30).
The same was true of Mercury Orange Staining.   This is
discussed later (page 59) and illustrated in Table 3 and
Figure 39.

Nuclear Division

Nuclear division in P. carolinensis was mitotic.   Except
for minor variations on rare occasions, all of the nuclei
underwent the same stage of mitosis synchronously.   This
amazing uniformity of timing is well illustrated in anaphase
(Figure 29) where the plates are the same distance apart.
In figure 30 are seen 3 nuclei which were slightly more advanced

PLATE XVII

Figure 30 - Three nuclei in this photomicrograph
are in late prophase whilst the others are still
in interphase. This phenomenon was very rarely
observed. Note the well-staining peripheral
granules in most nuclei. Heidenhain's Iron
Haematoxylin stain.   X 400
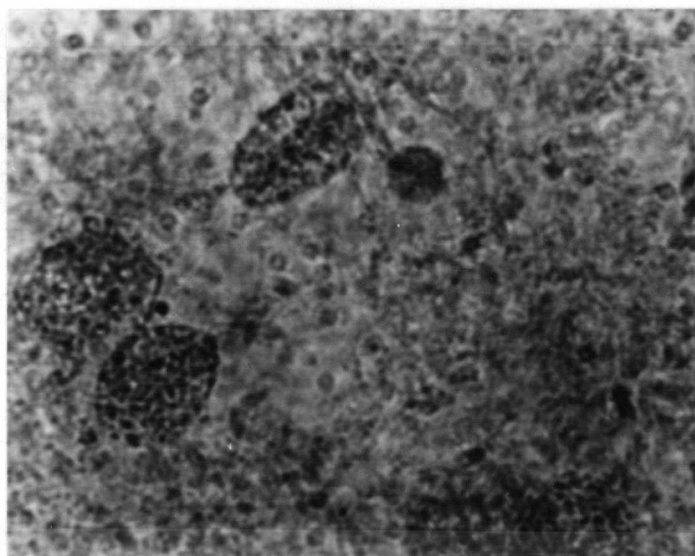
Figure 31 - Early prophase. The beginning of
prophase is characterized by migration of the
peripheral granules towards the central area.
Schaudinn's fixation and Grenacher's Borax
Carmine staining.   X 1250

PLATE XVIII
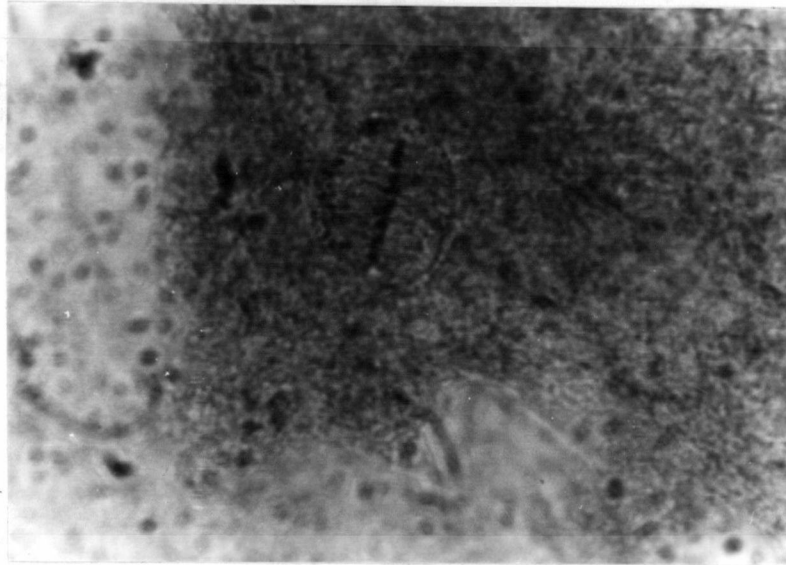
Figure 32 - Late prophase. Some peripheral granules remain close to the nuclear membrane. Schaudinn's fixation and Grenacher's Borax Carmine staining.   X 1500
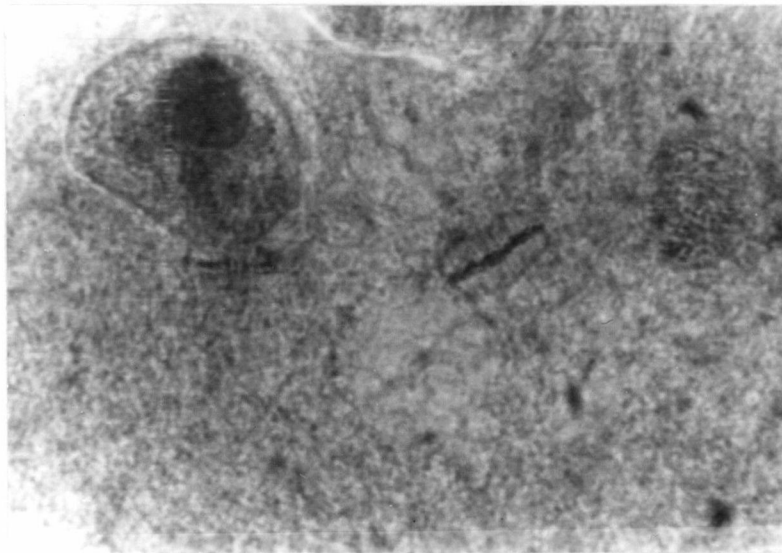
Figure 33 - Early metaphase. Peripheral granules become smaller whilst spindle fibres in close connection with them become more conspicuous. Nuclear membrane is still present. Schaudinn's Fixation and Grenacher's Borax Carmine.   X 950

PLATE XIX
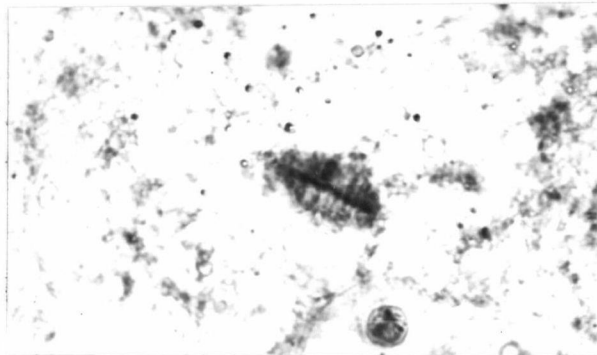
Figure 34 - Metaphase has progressed further than Figure 33.
Peripheral granules and nuclear membrane are not seen.
Schaudinn's fixation and Grenacher's Borax Carmine Staining.
X 950
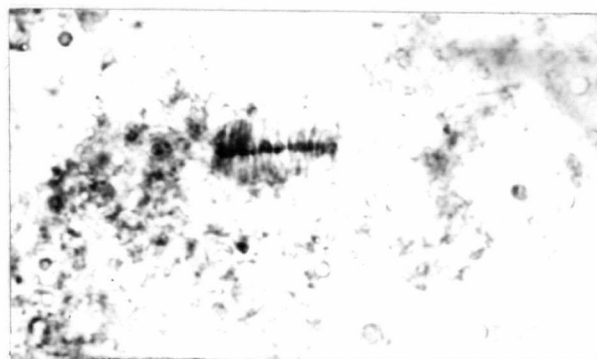
Figure 35 - As metaphase progresses and the nuclear membrane
disappears the shape of the mitotic figure changes from ovoid
towards rectangular. Schaudinn's fixation and Grenacher's
Borax Carmine staining.   X 950
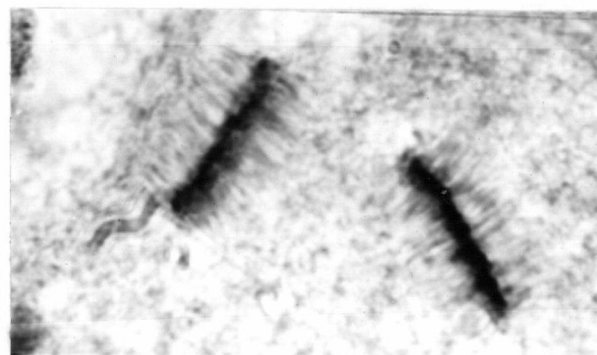
Figure 36 - Late metaphase. Note rectangular shape of mitotic
figure, complete lack of peripheral granules and nuclear membrane.
Schaudinn's fixation and Grenacher's Borax Carmine staining.
X 1750

than the rest. They had progressed to prophase (Figure 31) slightly before the others.

Enlargement of the nucleus prior to mitosis, as described in the literature, was not observed.

The beginning of prophase was characterized by migration of the large achromatic peripheral granules towards the central area in which chromatin granules and filaments are distributed (Figure 31). Some peripheral granules, however, remained close to the nuclear membrane, until late prophase (Figure 32). As prophase progressed towards early metaphase the achromatin granules became smaller and less distinctly outlined whilst spindle fibres in close connection with them became more and more conspicuous (Figure 33). The fibres were mainly oriented at right angles to the discoid mass of chromatin. At this stage the fibres usually did not reach the nuclear membrane but extended as far as the diminishing peripheral granules.

The nuclear membrane, although thinner, was visible well on into late prophase and very early metaphase (Figures 32 and 33) but disappeared during metaphase. Coincident with the disappearance of the nuclear membrane, the shape of the metaphase figure changed from ovoid to rectangular. This transition is depicted in Figures 34, 35, and 36. The complete lack of peripheral granules was observed in the rectangular figure (Figure 36).

PLATE XX
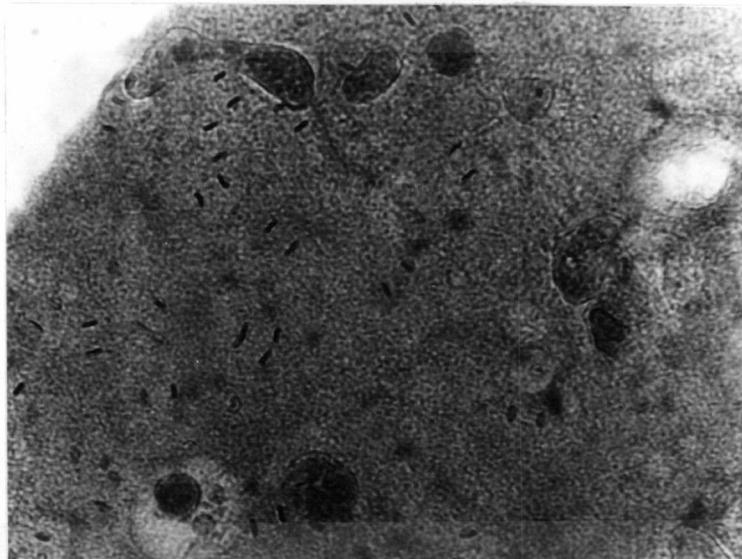
(a)   X 400
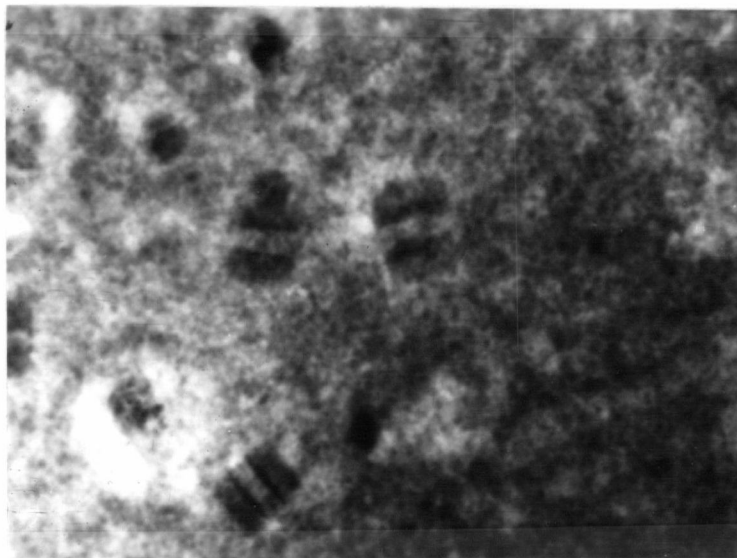
(b)   X 1000

Figure 37 – Anaphase. (a) Anaphase discs pull apart in many directions at once. (b) Polar fibres end bluntly during early anaphase and chromatin plates are arranged parallel to each other.

PLATE XXI
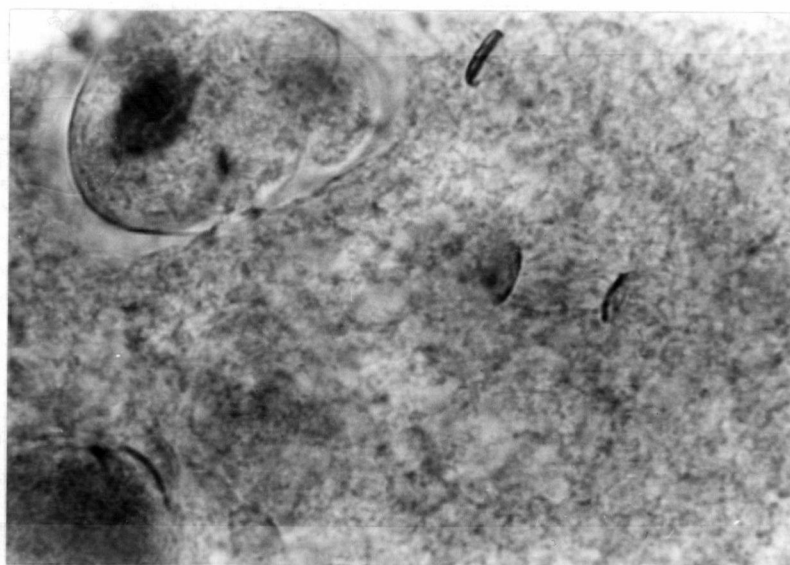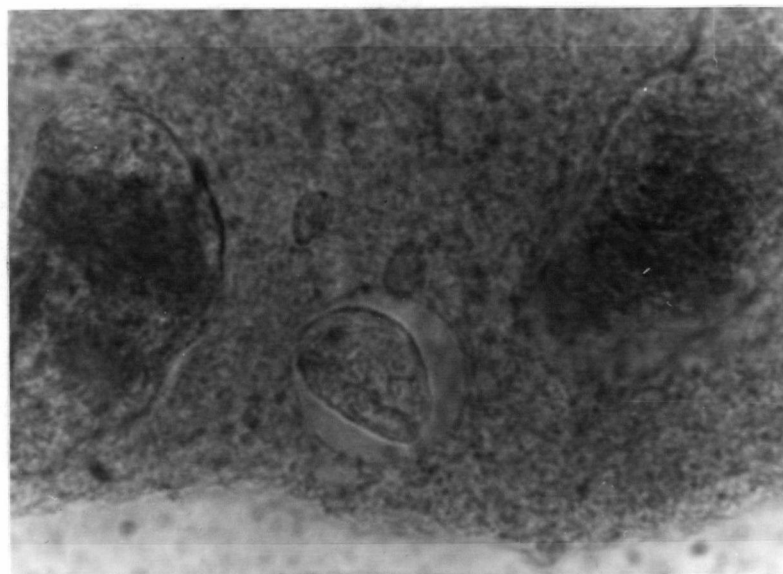
(a)   X 1200

(b)   X 1200

Figure 38 - Late anaphase to telophase. (a) and (b) As the
plates pull further apart they appear arched and the polar
fibres converge towards a point. As telophase is reached
the interphase fibres begin breaking up into granules.
Newly formed nuclei are triangular-shaped at first, then
oval-shaped, and finally discoid-shaped.

It was interesting to note that the anaphase discs pulled apart in many directions at once (Figure 37a). Although individual chromosomes were too small to be observed singly, much less detail was seen in the chromatin plate during anaphase than at any of the previous stages. Compare metaphase (Figures 33 to 36) with anaphase (Figures 12b, 29, 37 and 38). This was true of all fixatives and stains tried. In the anaphase photo-micrographs the chromosomes often appear as a fused dense mass. As the 2 anaphase plates pulled apart, spindle fibres appeared between them and the previously existing (outer) spindle fibres became the so-called polar fibres. These polar fibres ended bluntly during early anaphase (Figures 12a and 37b) but from late anaphase through telophase the fibres converged towards a point (Figures 12b, 13 and 38). During early anaphase the chromatin plates were arranged parallel to each other (Figures 12a and 37b) but as the plates pulled further apart they became arched (Figures 13 and 38a). As telophase was reached the inter-plate fibres began breaking up into granules. After separating, each plate became surrounded by a delicate membrane. These newly-formed nuclei were irregular in outline (Figures 13, 14 and 38b) triangular-shaped at first, then oval-shaped; and finally they assumed the discoid shape of the interphase nucleus.

As the young nuclei were growing larger the chromatin

granules and filaments became less conspicuous whilst the

peripheral achromatic granules grew larger and more

numerous until the characteristic appearance of the mature

interphase nucleus was reached.

III. CYTOCHEMICAL STUDIES

Preliminary Experiments with Mercury Orange on Toad and Mouse Muscle.

Untreated muscle tissues stained reddish-orange whilst

arsenic-treated fibres remained colourless when subjected to the

sulfhydryl reagent.

Effects of Sodium Arsenite on Living Amoebas

Results of experimental work which was repeated every day

for one week appear in Table 2 (a).

Table No. 2 (a)

RESULTS OF TREATING 12 LIVING AMOEBAS WITH $NaAsO_3$ FOLLOWED BY
MERCURY ORANGE STAINING

Conc.$NaAsO_3$ (gms%)	Time Exposed	Motility	Shape	Results with M.O. staining (24 hrs.)
0.01	12:00 a.m. to 3:00 p.m.	Decreased at end of 3 hrs.	Normal	Same as untreated (++++)

Table No. 2 (a) (cont'd)

RESULTS OF TREATING 12 LIVING AMOEBAS WITH NaAsO$_3$ FOLLOWED BY
MERCURY ORANGE STAINING
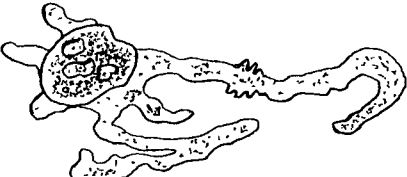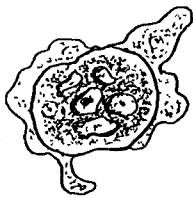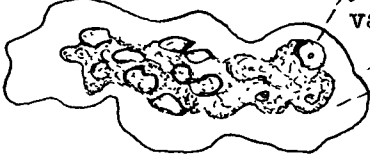
Conc.NaAsO$_3$ (gms%)	Time Exposed	Motility	Shape	Results with M.O. staining (24 hrs.)
0.5	12:00 a.m.	Immediate increase in cytoplasmic streaming.	Pseudopodia began to elongate and assumed bizzare shape.	
	12:20 p.m.	Animal darker in centre as cytoplasm streamed in this direction (similar to mechanical irritation).	Tending towards a spherical shape.	
	1:00 p.m.	All the amoebas were well attached to sub-stratum.	Normal	Same as untreated (++++)

Table No. 2 (a) (cont'd)

RESULTS OF TREATING 12 LIVING AMOEBAS WITH NaAsO₃ FOLLOWED BY
MERCURY ORANGE STAINING

Conc.NaAsO₃ (gms%)	Time Exposed	Motility	Shape	Results with M.O. staining (24 hrs.)
1.0	12:00 a.m.	Immediately became immobile.		
	12:10 p.m.	Cytoplasm streaming began.	Normal The few pseudopodia assumed bizzare shapes.	
	12:35 p.m.		Yellowish vacuoles formed throughout the cytoplasm. Broad, clear area between cytoplasmic inclusions and plasmalemma.	
	1:05 p.m.		Disintegration of outer limiting membrane and contents of animal flowed out.	Could not be stained.

PLATE XXII
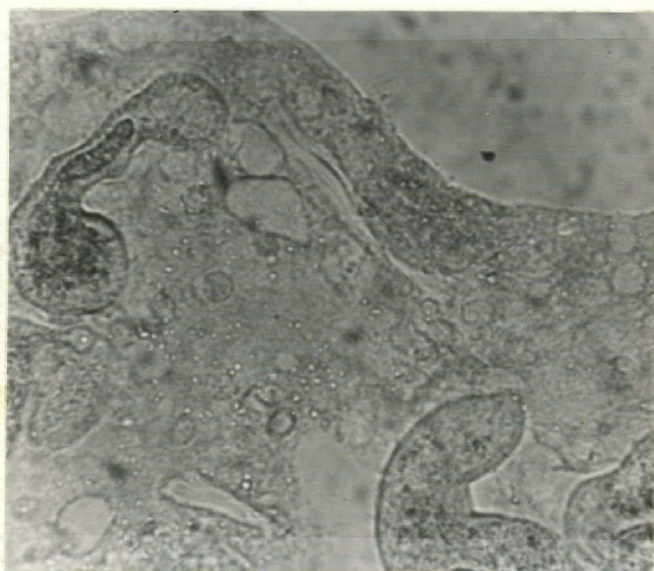
(a) X 400
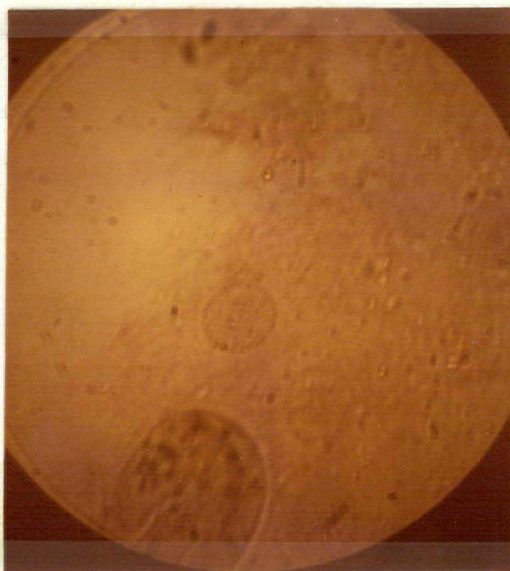
(b) X 800

Figure 39 - Normal, well-fed P. carolinensis stained with M.O.
to demonstrate -SH distribution.
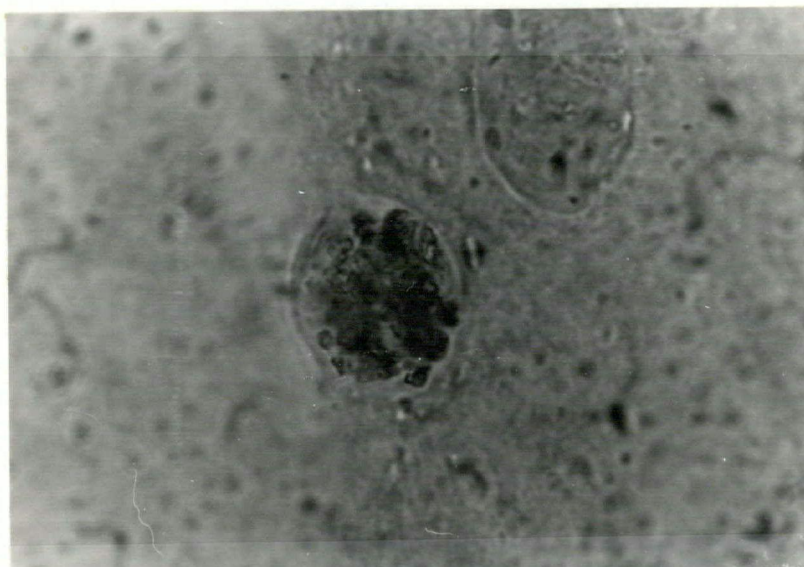
Figure 40 - Small, round food vacuole packed with crystals.
TCA fixation and Mercury Orange Staining.   X 1400

Application of the Mercury Orange Method to P. carolinensis

Untreated amoebas were coloured orange (4+); those treated

with 1 gm% concentrations of arsenic (24 hours) were faintly

tinted (+); those immersed in 5 gm% solutions (24 hours) showed

only a trace of colour; whilst those subjected to 10 gm% arsenic

(24 hours) remained completely colourless (Table 2 (b)).

Table No. 2 (b)

RESULTS OF MERCURY ORANGE STAINING OF P. carolinensis TREATED
WITH SODIUM ARSENITE (NaAsO$_3$) PRIOR TO TRICHLOROACETIC ACID
FIXATION

Conc. of NaAsO$_3$	Time Exposed	Color Intensity (M.O. Stain)
Non-treated	0 hours	++++
1 gm%	24 hours	+
5 gm%	24 hours	±
10 gm%	24 hours	0

Using the intensity of colour produced as a criterion,

sulfhydryl groups were found uniformly distributed throughout

the entire animal but their concentration was greater in some

areas. These results (for normal, well-fed amoebas) appear in

Figure 39 and Table 3.

PLATE XXIII
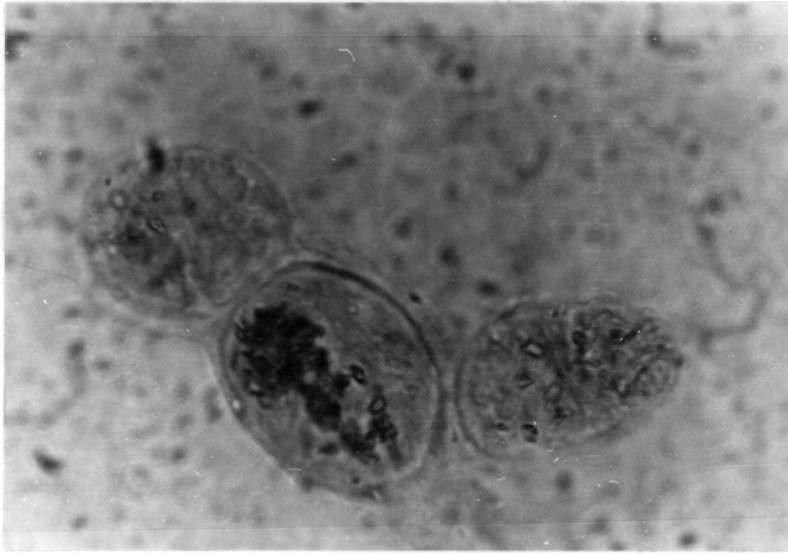
Figure 41 - In this preparation the food vacuoles contain-
ing crystals are so close to the surface of the amoeba as
to lead one to believe they are being extruded. Trichloro-
acetic acid fixation and Mercury Orange staining. X 1400
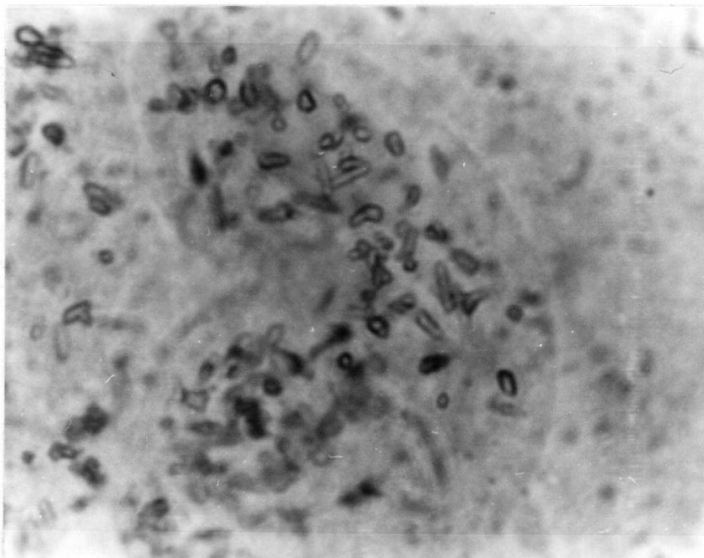
Figure 42 - Crystals seen in a larger food
vacule appear to be limited to the para-
mecium whose outline can still be seen
within a food vacuole. Majority of crystals
are rod-shaped. T.C.A. fixation and M.O.
staining. X 1400
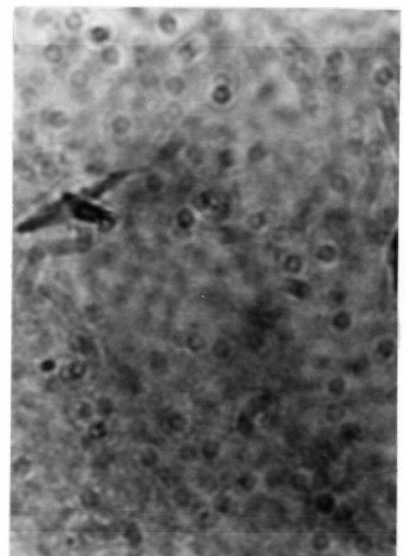
Figure 43 - Crystals
scattered about in the
cytoplasm are smaller
and tend to have a
square shape. T.C.A.
fixation and M.O. stain.
X 1400

PLATE XXIV
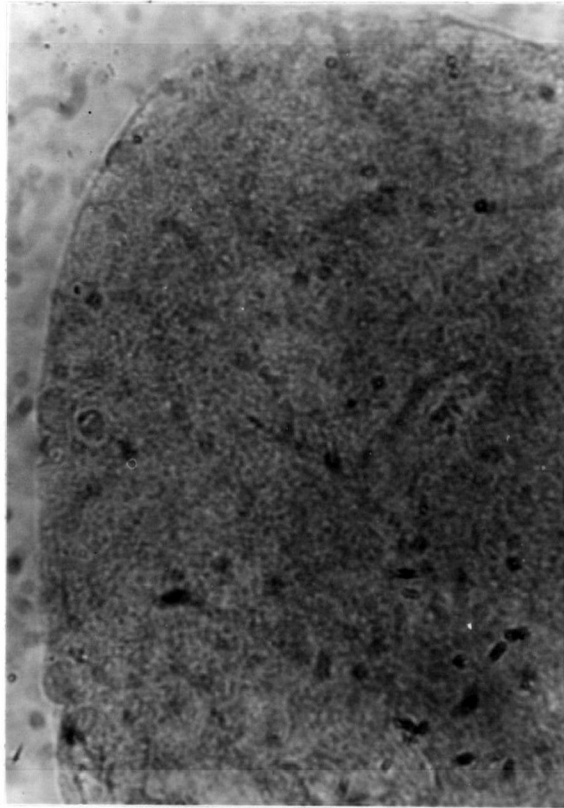
Figure 44 - Starved <u>Pelomyxa carolinensis</u> stained for
<u>sulfhydryl</u> groups by Bennett's Mercury Orange method.
Much structural detail is seen but ground substance is
more intensely stained compared with normal well-fed
individuals illustrated in Figure 39.   X 400

Table No. 3

MERCURY ORANGE STAINING OF Pelomyxa carolinensis

	Colour Intensity	
	Normal, Well-fed (Figure 39)	Starving (Figure 44)
Plasmalemma (appears as a very narrow line inside of which is a clear area, with the cytoplasm innermost.)	+++	++
Ground substance	++	++++
Cytoplasmic crystals		
Interior	0	0
Rims	++++	++++
Interphase Nuclei		
Nuclear membrane	+++	+++
Peripheral granules	++++	++++
Chromatin	+++	+++
Nucleoplasm	++	+++
Contractile vacuoles		
Interior	+	+
Rims	++++	++
Food Vacuoles		
Food inclusions	++++	Only a few
Crystals* - Interior	0	small vacuoles
- Rims	++++	filled with
- Fluid	0	crystals re- mained.

*Some smaller food vacuoles, perfectly round and only

slightly larger than the nuclei were literally "stuffed" with

these rectangular, square, and round-shaped crystals (Figure 40).
These smaller food vacuoles were similar to the larger ones in
every respect except for the numerous crystals and small, round
size of the latter. Similar crystals appeared within the larger,
variously shaped food vacuoles but in fewer numbers (Figures 41
and 42).

The crystals described above appeared considerably larger
than those scattered about in the cytoplasm (Figure 43). These
crystals appeared to be inside the food vacuole and not merely
adhering to the outside for they were never seen on the border-
line of the vacuole nor were they apparent on nuclei (Figures 39,
40 and 42). In some preparations these vacuoles stuffed with
crystals were so close to the surface of the amoeba as to lead
one to believe they were being extruded (Figures 40 and 41).
Although these crystals were distributed as described above in
material stained by other methods, they were never so clearly
visible as in Mercury Orange stained individuals. (Compare
Figure 39, TCA fixation and Mercury Orange stain with Figure 19,
Osmium Tetroxide fixation and Grenacher's Borax Carmine Staining.)

Results for starved amoebas appear in Figure 44 and Table 3,
on page 56. Much less structural detail was observed but the
ground substance was more densely stained and granular in starved
organisms. Food vacuoles were rarely seen and those present were
exceedingly small. There were fewer contractile vacuoles, their

rims were paler and they contained crystals only. Nuclei showed up more distinctly in the starved preparations, but peripheral granules were considerably smaller.

Mitotic Nuclei Stained with Mercury Orange

The results of Mercury Orange staining of amoebas undergoing mitosis appear in Table 4 below.

Table No. 4

MERCURY ORANGE STAINING (24 hours) OF DIVIDING NUCLEI in P. carolinensis

Mitotic Stage	Colour Intensity				
	Nuclear Membrane	Peripheral Granules	Nucleo-plasm	Chromatin	Spindle Fibres
Interphase (Figure 45)	+++	++++	++	+++	0^{**}
Prophase (Figure 46)	++	++	++	+++	0^{**}
Metaphase (Figure 47)	Early + Late _	Early + Late -	Early + 0^{*}	+++(+)	++
Anaphase (Figure 48)	-	-	0^{*}	++++	Polar: +++(+) Inter-disc: ++

* No membrane, therefore, nucleoplasm appears to blend with the rest of the cytoplasm.

** No spindles yet formed.

PLATE XXV
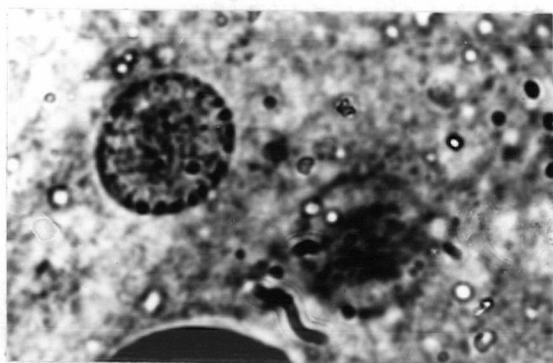
Figure 45 - Interphase nucleus stained with Mercury Orange. Intensely stained peripheral granules indicates abundant -SH groups in these structures.
X1500
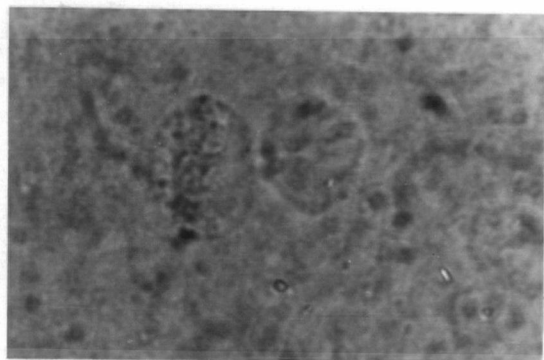
Figure 46 - Prophase nucleus stained with Mercury Orange. Most of the peripheral granules have migrated to the central portion. Prophase nuclei were difficult to detect in these preparations since there was not much contrast between the cytoplasm and the nuclei.
X12500
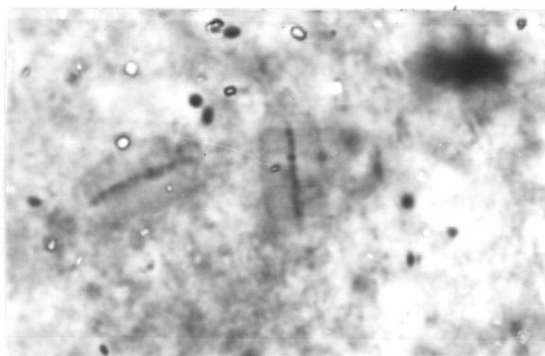
Figure 47 - Metaphase nuclei stained with Mercury Orange. Were it not for the chromosomes, these nuclei would be difficult to detect.
X1000
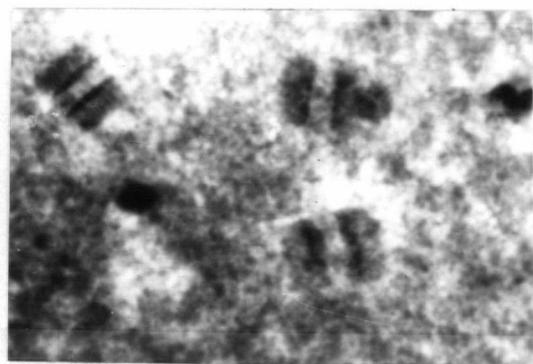
Figure 48 - Anaphase nuclei stained with Mercury Orange. Polar fibres and chromosomes are well seen. Interplate fibres are faintly stained - approximately the same intensity as metaphase spindle fibres.
X1000

Interphase nuclei were easily detectable because they were outlined by intensely stained peripheral granules (Figures 39 and 45). Once the peripheral granules had migrated to the centre of the nucleus they did not stain as intensely with Mercury Orange. No granules could be detected by late metaphase and anaphase (Figure 48). Staining of the nuclear membrane was similar to that of the peripheral granules. (Note that by late metaphase and anaphase no nuclear membrane is visible (Figure 48)). Mercury Orange staining of the nucleoplasm of interphase and prophase nuclei was of approximately the same intensity (Figures 45 and 46). In stages where the nuclear membrane was absent, the nucleoplasm could not be detected with certainty. Chromatin staining was approximately the same in interphase and prophase nuclei (Figures 45 and 46). As chromosomes became visible, staining was more intense, with the chromosomes appearing darker in anaphase (Figure 48) than in metaphase (Figure 47). Spindle fibres were never observed in interphase or prophase (Figures 45 and 46). Metaphase and interdisc spindle fibres were faintly stained whilst polar fibres in anaphase were deeply stained (Figures 47 and 48).

DISCUSSION

I. LIVING MATERIAL

General Behaviour

Feeding

The explanation of why parameciums (when added to

a watch glass containing fresh culture media and P. carolinensis)

swim towards the latter, was not revealed by this study, nor

have I found any reference to this phenomenon in the

literature. Whether it is due to a "stickiness", or

to ionic phenomena of the amoeba's outer membrane, or to

the fact that parameciums feed on bacteria in the vicinity

of the amoeba is not known.

Kudo (1959) observed parameciums feeding on bacteria

around amoebas, and Roslansky (1959) obtained electron

photomicrographs of ingested bacteria in paramecium food

vacuoles. (The rod-shaped particles which I observed

in food vacuoles could be bacteria since they resemble the

latter in size and shape and show up extremely well with

-SH Mercury Orange staining. However, specific bacterial

staining was not carried out, hence I have no positive

evidence to indicate that these are indeed bacteria.)

The explanation that parameciums are feeding on bacteria does not account for the apparent struggle to extract themselves voluntarily. However, the apparent struggle may merely be a sluggishness due to abundant food in the vicinity, since parameciums also exhibit a sluggishness around bits of timothy hay.

It is known that many cells have an outer coating of mucoprotein which has an important function in pinocytosis (Halter, 1959; Robertson, 1960). Pinocytosis and ingestion are considered similar phenomena, therefore, the amoeba might well have a "sticky" property which explains why parameciums require some effort to extract themselves from the vicinity of the amoeba. deRobertis et al (1957) states that the cytoplasmic matrix of the amoeba is slightly acidic (pH approximately 6.8) but he makes no reference to the charges on the animal's surface. Parameciums have been observed to move toward the negative pole if placed in an electrical field of direct current. Elliott (1952) attributes this to the fact that externally the paramecium is positively charged but does not suggest the nature of this charge.

Before the answer to the above observation can be found, it will no doubt be necessary to determine the

molecular structure of the cell membrane, generally, and

of Paramecium multimicronucleatum and Pelomyxa carolinensis

in particular. Although several theories concerning the

cell membrane exist, Ponder (1961), in an extensive treatise

on the cell membrane, indicates that he is not convinced

about structure - or even the necessary existence - of

the cell membrane as it is generally described. He feels

that "many of the conclusions regarding the cell membrane

are based on pre-existing ideas, on unallowable simplifications,

as well as on a disregard of both physical chemistry and

the results of experiments on the cells themselves."

The observations regarding ingestion and formation of

food vacuoles raised these questions:

(1) Why do parameciums remain stationary while food vacuoles

   are formed about them; then commence very definite

   movement?

(2) How does the amoeba sense the presence of the paramecium

   and begin deliberate movements directed at surrounding

   the food with its pseudopodia?

Regarding the first question, the reason for the

paramecium remaining stationary while the food vacuole forms

about it is not likely to be known until the explanation

for the apparent attraction of parameciums to amoebas is found.

Regarding the second part of the first question, there is
ample evidence that a change in pH occurs inside the food
vacuole (Mast, 1942). This relatively sudden change in
the environment could conceivably have a stimulatory effect
on the paramecium for it is known that protozoa are
sensitive to pH changes in the surrounding media.
Parameciums tend to avoid an alkaline environment and to
seek an acidic environment (Elliott, 1952).

Mast (1942) investigated the cause of death of living
organisms after ingestion and concluded that it was due to
a decrease of oxygen in the food vacuoles and not to the
acidic environment. He believes the oxygen decrease is
due to respiration by the entrapped food organism and the
diffusion of oxygen out into the cytoplasm as well as
to decreased vacuolar fluid volume.

Regarding the second question above, since ingestion
(phagocytosis) is a process very similar to pinocytosis, the
essential features of which are similar in all cells,
pseudopod formation can be considered analagous to the
formation of a recess extending toward the inside of the cell
by the sliding along of the membrane once a particle,
molecule, or ion has been absorbed on the cell surface
(Bennett, 1956). However, the mechanisms underlying this

membrane sliding is not well understood and until more

knowledge is acquired regarding the phenomena of pinocytosis

and phagocytosis this question cannot be satisfactorily answered.

It is not yet known how the pinocytosed fluid or

phagocytosed organisms become assimilated into the cytoplasm.

Bennett, 1956, postulated that the vacuolar membrane

disintegrates under the influence of cytoplasmic enzymes

while others (Prescott, Chapman-Andresen and Holter)

feel that the permeability properties of the vacuolar

membrane are involved.

Regarding the disintegration of the membrane, it

has never actually been seen to disappear by the electron

microscope (Holter, 1959 a, b; 1961). Further evidence

against regarding membrane disintegration as the sole

explanation of assimilation of food into the cytoplasm is

found in the work of Andresen and others (1952) who

carried out studies on the digestion of P. carolinensis

using Carbon[14] - labelled food organisms. They demonstrated

that the rapid spread of radio-active glucose throughout

the cytoplasm was probably due to membrane permeability

rather than to membrane disintegration because radio-activity

during subsequent feeding on unlabelled food revealed a

period of reduced activity but with persistence of activity

as long as 700 to 800 hours. Similar studies carried out

by Cohen recently (1959) on Amoeba proteus, but using

colloidal gold, confirmed Andresen's conclusions that food

derivatives persist in vacuoles for some time after the

original feeding. The plasmalemma of P. carolinensis is

notoriously impermeable to many, especially high molecular

substances. Chapman-Andresen and Holter (1955) found the

cell membrane of this amoeba was almost completely impermeable

to glucose. Since the vacuolar membrane originally was

part of the cell surface and, according to Brandt (1958)

and Marshall et al (1959), preserves the morphological

characteristics of the plasmalemma, a permeability theory of

assimilation of phagocytosed (or pinocytosed) material should

suggest a mechanism for altering the permeability of

the plasmalemma once it has become the food vacuole wall.

The small, dark granules which I noted streaming towards

the point of ingestion (page 37) and later lining food

vacuoles (pages 37 and 48) might conceivably be associated

with assimilation of food vacuole contents. I believe these

granules are identical with those forming the compact layer

seen by Pappas (1959) and those studied by Mast and

Doyle (1935 a, b). My observations coincide with Horning's

(1933) who noted that the incorporated food is brought into

intimate contact with some "chondriosomes". He further

states that later a vacuole forms enclosing the food

together with adjacent "chondriosomes". However, since the

vacuolar membrane is in fact what was plasmalemma prior to

phagocytosis, all the granules would have to enter the vacuole

at the same point through a narrow opening just before the

two free ends join to form a vacuole. I did not observe

this and I also found it difficult to determine, from

the photomicrographs (Figure 25), whether these granules

are inside or on the outside of the membrane. Horner

observed the diminishing size and eventual disappearance

of the "chondriosomes" as digestion proceeds. This, I also

observed to be so. deRobertis (1957) believes that the

events described above indicate a hydrolytic type of enzyme

action in connection with digestion. The close relationship

of the "chondriosomes" to the food vacuoles, therefore,

would seem to support the permeability hypothesis of food

assimilation.

Body Form

Body form is a reliable indicator of the physiological

state of an amoeba at any given time.

The spherical form seems to be the result of gradual

withdrawal, in all directions, from injury and therefore

occurs when an unfavourable environment envelops the amoeba.

Club-shaped individuals, I believe, indicate that the organism is concentrating its efforts in one direction only in an attempt to reach food or more favourable environment existing some distance away. This agrees with Kudo (1951).

Formation of elongated pseudopodia in all directions as seen in star-shaped organisms where little food remained probably indicates that the environment was not yet sufficiently unfavourable to cause withdrawal of pseudopodia and the amoebas were searching food in all directions. Assumption of a star-shape when removed from old, barren cultures and placed in fresh water might be explained by the fact that no inhibitory influence now prevented pseudopod formation (as when the sphere-shape occurs) hence the amoeba begin seeking food in all directions.

Regarding the more immediate cause of death of amoebas in old cultures, changes in pH of the environment would be expected to have an immediate effect compared with starvation which is a gradual process. That is, the organisms can store food materials but when the internal milieu is disrupted, degeneration occurs fairly rapidly.

The gradual fading away of "starving" amoebas appears to reflect a gradual lack of building materials for normal function and structure much as wasting in multicellular

organisms possessing a negative nitrogen balance. In contrast
to this slowly acting (weeks) nutritional effect, the
disintegration of organisms exposed to deliterious chemical
agents follows rapid (minutes, hours) membrane disruption
which can be observed as it is occurring.

Correlation of Body Form with Mitotic Events

Body forms described in Results (page 35) indicated that
the nuclei were undergoing mitosis, a fact which was most useful
in studying -SH distribution in dividing amoebas since one
could choose a desired stage of mitosis with great accuracy.
Before dividing, Amoeba proteus has also been observed to round
up into a spherical shape with short, blunt pseudopodia all
over its surface (Schaeffer, 1946).

Conditions which favour Reproduction and Frequency of Mitosis

The observations regarding periodicity and frequency of mitosis,
as well as a study of conditions favouring division were of
assistance in obtaining material for later experimental work on
-SH groups.

The occurrence and frequency of nuclear division and
plasmotomy was correlated with food supply only. Although
division appeared to be related to size of the amoeba, this
can be interpreted as an aspect of adequate food. Kudo (1949)

believes that, as the number of nuclei in the parent increases, the number of resulting daughters also increases. Perhaps this explains why at times plasmotomy did not follow mitoses of the nuclei, and that often when plasmotomy was deferred until mitosis was repeated, the amoeba divided into more than 2 daughters.

The fact that the variation in size of newly divided amoebas was not great correlates with Prescott's (1959) findings concerning Amoeba proteus. He weighed newly divided amoebas and found that weight variations were insignificant except when division occurred in strong light.

The finding that distribution of nuclei amongst daughter amoebas is random agrees with Kudo (1949) who carried out careful statistical tests in this connection and found that any equal distribution was not significant.

II. HISTOLOGICAL STUDIES

Vital Staining

The formation and pinching off of vacuoles is probably a means of eliminating toxic substances (in this case Neutral Red). This is in agreement with Kassel and Kopac (1953) who exposed organisms to toxic materials. It is, therefore, inferred that the "vital" stain behaved as an injurious chemical material.

The red-stained vacuolar inclusions observed in amoebas exposed to 1:100,000 concentrations of stain are believed analagous to the "neutral red bodies" described by Andresen (1942, 1945 and 1946) and others. Regarding the question as to whether these structures are normal cellular inclusions or whether they represent a response to the stain, I agree with Torch (1959) and others, who believe they are an artifact of vital staining, since they were not seen in any other material. Torch (1959) offers a possible explanation regarding the nature of these particles. He notes that identical structures are formed during staining with other vital dyes (1959 paper refers to unpublished data). Since these structures appear to be lipid in nature he believes they may represent a protective mechanism against the toxicity of the stain. That is, the amoeba might utilize lipid to form an insoluble complex with the dye, thus removing the dye from the cytoplasm. This could explain why neutral red bodies occur only in organisms exposed to dilute solutions, for higher concentrations kill the organisms before the bodies are formed.

The bodies seen covering the contractile vacuoles in organisms stained by Janus Green B are similar to those described by Mast and Doyle (1935, a, b) and Kassel and Kopac (1953). I believe these particles are mitochondria

since Janus Green B is a stain for mitochondria. Studies carried out by Torch (1955) and confirmed by recent electron microscope studies (Pappas, 1959) further support this opinion. Mitochondria in P. carolinensis are further discussed under permanent histological preparations.

Permanent Histological Preparations

That Osmium Tetroxide fixation gave a picture closely resembling frozen material is in keeping with the findings of workers who have studied the action of various fixatives on cultured cells (Bang and Gey, 1959; Palade, 1952; Greider et al, 1958; Borysko and Roslansky, 1959). The fixatives were evaluated by comparing the living cell under phase contrast with the fixed cell under the electron microscope. Best results were obtained with Osmium Tetroxide.

Morphology

Plasmalemma

Although the light microscope is of little assistance in revealing the structure of the outer limiting membrane, this structure has been extensively studied by the electronmicroscope and is described in detail by the workers referred to in this literature review.

Cytoplasmic Ground Substance

Since material fixed in Schaudinn's, Osmium Tetroxide

and trichloroacetic acid closely resembles frozen
preparations it is felt that the former three fixatives
give a picture nearer the living state than does Bouin's.

Cytoplasmic Inclusions

Mitochondria - Transformation of mitochondria from
rod-shaped in living organisms to round-shaped in fixed
material is an occurrence sufficiently invariable to warrant
Torch's (1955) regarding it as a criterion of death of the
amoeba.   Torch (1955) found that in all cases Janus Green B
was concentrated in eccentric granules or crescentic areas
within the mitochondria.   This is interesting in view of
recent work on the chemistry of Janus Green B staining.
Lazarow and Cooperstein (1953); Cooperstein, et al (1953);
Cooperstein and Lazarow (1953) and Showacre (1953) have related
colouration by Janus Green B to the presence of cytochrome
oxidase within the mitochondria.   If this is so, the stained
granules or crescentic areas within the mitochondria of
P. carolinensis possibly indicate localization of cytochrome
oxidase.

The characteristic accumulation of mitochondria around
contractile vacuoles leads to speculations regarding the
function of mitochondria in P. carolinensis.   Most of the
knowledge concerning the function of mitochondria was derived
from vertebrate studies.   Vertebrate mitochondria apparently

contain all the enzymes necessary for the respiration of the cell (Lindberg and Ernster, 1954). Whilst the biochemistry of protozoan mitochondria is virtually unknown it is reasonable to assume that their enzymatic complement does not differ markedly from that of vertebrates. On the basis of morphological studies, Torch (1955) believes that mitochondria in P. carolinensis possibly function in one or more of the following processes:

1) accumulation and transport of fluids;

2) accumulation and transport of waste products;

3) digestion;

4) respiration.

No morphological evidence for the first two possibilities exists since there is no difference between the granules surrounding contractile vacuoles and those freely circulating. Likewise, morphological evidence for a digestive function is limited. However, the meagre amount of work done concerning function of protozoan mitochondria points to a probable function concerned with cellular respiration (Joyet-Lavergne, 1926, 1928, 1929, 1934, 1935; Cowdry & Scott, 1928; Weiss, 1950). If this is so, their accumulation in areas of high energy expenditure such as contractile and food vacuoles is understandable.

Contractile Vacuoles - Although these vacuoles could be readily identified in our histological preparations because

of the surrounding layer of mitochondria, little else

regarding structure or function could be determined.

Electron microscopic studies of Pappas and Brandt (1958)

and Pappas (1959) show that the contractile vacuole is

surrounded by a densely packed layer (2 u thick) of small

vesicles which in turn are surrounded by a layer of

mitochondria. Pappas estimates the thickness of the

contractile vacuolar membrane to be 70 $\overset{o}{A}$.

Food Vacuoles - The narrow rim of dense small particles

probably represents the granular layer of material characteristically

found around young food vacuoles observed by the electron

microscope (Pappas, 1959). The fact that they are seen

around newer food vacuoles, together with my own observations

of their migration towards the site of ingestion, suggest

they are concerned with digestion. The empty vesicles

around older food vacuoles are identical with the vacuole

refractive bodies described by Torch (1959). Since they

surround older food vacuoles and contain crystals it is

possible that they are concerned with waste products. The

crystals contained within older food vacuoles, likewise are

believed to represent wastes. Because the latter crystals

were observed so clearly after Mercury Orange staining,

they are further discussed under "Application of Mercury

Orange Method to P. carolinensis (page 84).

Crystals - In the past, several workers have speculated
on the functional significance of these cytoplasmic crystals.
Mast and Doyle (1935a) and Wilber (1945) believe they
originate within food vacuoles and hence represent a food
reserve. I found that the quantity of these crystals does
not decrease in starving P. carolinensis. This agrees
with the observation of Andresen and Holter (1945).
Further, both these workers and Torch (1955) maintain that
the crystals arise in the cytoplasm. The fact that
expulsion of crystals is seen in both well-fed and starving
individuals suggests that they might be metabolic wastes.
Torch (1955) believed they represent an accessory mechanism
for excreting nitrogen. At the time that I observed these
crystals, no decisive data could be found concerning their
nature. Recent data, however, shows conclusively that they
are new nitrogen excretion products (Griffin 1959, 1960;
Grunbaum et al, 1959). Griffin has positively identified
the platelike crystals as carbonyldiurea on the basis of
evidence from microanalysis, X-ray diffraction pattern,
infrared absorption spectra and petrographic analysis.
Grunbaum et al suggest that the bipyramids are not
carbonyldiurea but a related substance. Allen (1961) feels
that both substances are probably breakdown products of
purine metabolism through allantoic acid.

Nuclei - Although the number of nuclei is usually
described as "several hundred", I found fewer (75 to 150).
However, it is possible that more were present but not
seen because of the difficulty in observing all planes of
the amoeba at once.

I believe the granules found at the periphery of the
interphase nucleus represent nucleoli and are the same
as those described in the electron microscopic studies of
Borysko and Roslansky (1959) and Brandt and Pappas (1959).
Chalkley, as early as 1936, found that these peripheral
granules were basophilic but contained no desoxyribonucleic
acid. Their lack of DNA is revealed by the fact that they
are Feulgen negative. . Their basophilia is evident by their
staining reactions with acidified Methyl Green, Geimsa
(ultra-marine blue), Grenacher's Borax Carmine, Crystal Violet
and Heidenhain's Iron Haematoxylin (dense black granules
with the latter stain whilst chromatin stains paler).
Electron micrographs of the nucleoli reveal them to be packed
with tiny granules which resemble the ribosomes of the
cytoplasm. The nucleoli, in fact are known to be rich in
ribonucleic acid and appear to be active centres of protein
and RNA synthesis (Brachet, 1961). Certain workers estimate
the protein content of nucleoli to be between 70% to 85% (Vincent
and Huxley, 1954; Nurnberger et al, 1952). Boltus (1954)

found a high content of nucleoside phosphorylase and the DPN-synthesizing enzyme in isolated echinoderm nucleoli. Although nucleoli are not generally believed to contribute to cellular mitosis, the behaviour of these peripheral granules during mitosis suggests that they perform some function in nuclear division, as discussed under mitotic nuclei below.

Nuclear Division

Synchronous mitotic nuclear division in P. carolinensis was first reported by Schaeffer (1937). Short (1945, 1946) and Kudo (1949) observed, as I did, that on rare occasions a few nuclei appeared in more or less advanced stages. This difference, I found was very slight but significant. For example, Figure 30 shows most nuclei in interphase with a few in late prophase. The differences in distance between anaphase plates as noted by Kudo probably does not constitute reliable evidence in this respect since this could be an artifact - i.e. cytoplasm could have been stretched slightly during handling.

Regarding the increase in size of nucleus (up to six times as noted by Schaeffer) which I did not observe, a possible suggestion might be accumulation of additional materials from the cytoplasm for energy or building

materials necessary to produce two nuclei from one.
Electron microscopic studies definitely reveal pores in the
nuclear membrane of this organism (Pappas, 1959). In
P. carolinensis the pores occupy 15 to 20 percent of the nuclear
surface which is approximately twice that estimated for
mammalian cells (Watson, 1955).

Observations of the behaviour of the peripheral
granules during mitosis led me to agree with Kudo who believes
that the peripheral granules contribute, in part, to the
formation of the mitotic apparatus. Mitochondria in
close proximity to the nuclear membrane and peripheral
granules has been observed by Brandt and Pappas (1959).
These workers feel that the outer membranes of mitochondria
and nuclei are continuous during and shortly after nuclear
division in P. carolinensis. This lends support to
Swanson's (1960) statement that, although the function of the
nucleolus other than to manufacture proteins is unknown, the
fact that it disappears during cell division leads him to
suspect that it may be involved in passing genetic information
and materials from nucleus to cytoplasm. In view of the
intense staining with Mercury Orange of P. carolinensis'
nucleoli, indicating a rich protein sulfhydryl content, it is
possible that they represent a means of transfer in the
opposite direction as well (i.e. of materials from cytoplasm

to nucleolus to mitotic apparatus). Thus, if the theory
that -SH groups contribute to spindle formation is correct
(i.e. by oxidation to -SS- or by some other as yet unknown
bonding) then these peripheral granules might be the source
of such -SH groups. The movement of the peripheral
granules towards the central area of the nucleus as
prophase begins; the spindle fibres appearing at first
to end in the granules; the decrease in size and number
of granules concomitant with the appearance of spindle fibres
during late prophase and early metaphase and their absence
in metaphase and anaphase when the spindle apparatus is
fully formed also lends support to this theory. Schaeffer
(1937) noted small granules, perhaps from the disintegrating
interplate fibres during telophase, coalescing to form the
larger peripheral granules of the mature nucleus. These
are also seen in Figure 13 of this study.

I believe that the change from oval (in prophase) to
rectangular shape assumed by late metaphase is related to the
disappearance of the nuclear membrane. The two seem to
occur simultaneously. The more indistinct the membrane becomes
the more nearly perfectly rectangular becomes the shape of the
figure. Perhaps all the spindle fibres can now attain the
same length since they are not restricted by the membrane.

The significance of nuclear membrane disappearance is

of interest but to date remains obscure. The answer is unlikely to be found until the membrane's detailed structure is known. Connections between the nuclear membrane and cytoplasmic membrane are evident and observations on the formation of the nuclear membrane at telophase suggest that the membrane is a specialized cytoplasmic structure (Mirsky and Osawa, 1961). Yasuzumi (1959) is of the opinion that it has its origin in the cytoplasmic membrane system. Another picture of nuclear membrane formation at telophase in Yoshida sarcoma cells is provided by Barer et al (1959). It shows vesicles (indistinguishable from the cytoplasmic membrane system) surrounding the chromosomes at telophase which eventually fuse to form a complete nuclear membrane. Lafontaine (1958) also describes membranes around the telophase chromosomes seen in electron micrographs of other cells. The close proximity of these vesicles (apparently parts of the nuclear membrane) to the chromosomes suggests that nuclear membrane breakdown during mitosis could provide structural materials, enzymes, or energy for mitotic apparatus formation. Schaeffer (1937) states that the anaphase discs are pulled apart by protoplasmic streaming. However, since the plates in P. carolinensis separate in many directions simultaneously it would appear that some other force is involved. Kudo (1949) suggests that the interdisc and polar fibres might be responsible for this movement.

Mazia (1961) feels that the poles are pushed apart by the

growth of the interdisc fibres. Although this may be

descriptively correct, (and is the most likely explanation

of the observations in this study) it remains to be explained

how the growth of the interdisc spindle is translated into

an actual movement of the discs. The electron microscope

studies of Porter, Bernard and Dehaven (Mazia, 1961) show

that the morphology of the interdisc and polar fibres is

similar - but - the latter must lengthen and the former must

shorten when the poles move apart. Thus, it seems

reasonable that biochemical (and physical) alterations

within the interplate fibres are involved. This correlates

well with my hypothesis that there is a difference between

polar and interplate fibres since Mercury Orange staining

is exceedingly faint in the latter compared with the former.

In this study I made no attempt to count chromosomes

and thus did not employ special methods for the purpose. As

yet no satisfactory method seems to have been employed for

this purpose and the results have been extremely variable.

For example, Schaeffer (1937) quotes a figure of 2,500;

Short (1945, 1956) quotes a figure of 300 and Kudo (1949)

believes the number of chromosomes in this organism

is approximately 100.

That doubling of the metaphase plate to form two anaphase

plates is the result of fission of chromosomes seems plausible (Figure 37).

Enhanced fusion of chromosomes during anaphase was also observed by other workers (Schaeffer, 1937). It is not possible, by the methods used in this study, to determine whether this enhanced fusion is actual (i.e. chromosomes fused due to physical proximity or chemical alteration) or apparent (i.e. failure of resolution of individual chromosomes due to fixation and staining techniques).

Regarding the bluntly-ending polar fibres, a similar multi-polar appearance of the spindle fibres was observed by Dawson and co-workers (1935) who considered it "due solely to fixation". However, I (and Kudo, 1949) have observed this broad termination of the spindle apparatus in late prophase, metaphase and early anaphase in living organisms under phase contrast.

Nowhere in the literature have I found any explanation of why the polar fibres eventually terminate at a common point. I believe the arching of the anaphase plates occurs as the result of the convergence of polar fibres.

III. CYTOCHEMICAL STUDIES

Preliminary Experiments with Mercury Orange on Toad and Mouse
Muscle

The reddish-orange colour of the untreated muscle fibres

observed after Mercury Orange staining confirms Bennett's (1951)

results which he attributes to the following reaction between

sulfhydryl groups and the chlori-mercuri group of the Mercury

Orange reagent:

R-SH + Cl-Hg-◯-N◯◯OH ⇌ R-S-Hg-◯-N◯◯OH + HCl   (I)

Protein   Mercury Orange   Compound responsible
containing   Reagent   for characteristic
-SH groups   orange-red colour
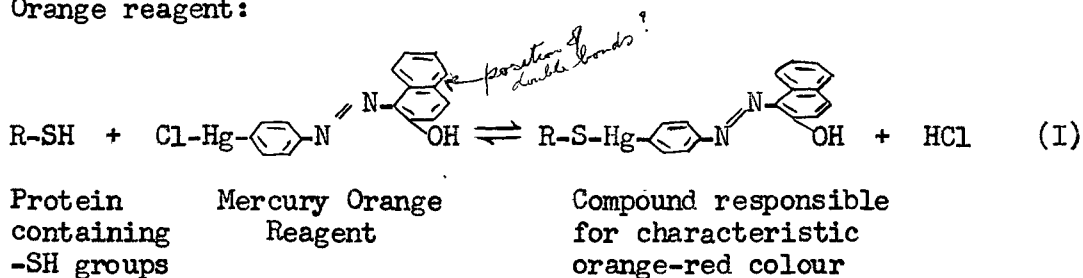
The observation that muscle fibres which were treated

with arsenic before Mercury Orange staining failed to become

coloured is believed to be due to the binding of the -SH

groups by the arsenical according to the Dithiol Theory

(Stacken and Thompson, 1946, 1948) as follows:

$$R\text{-As=O} + 2\ R_1\text{-SH} \longrightarrow R\text{-As}\begin{matrix} \diagup S\text{-}R_1 \\ \diagdown S\text{-}R_1 \end{matrix} + H_2O \qquad (II)$$

Trivalent   Protein con-
Arsenical   taining -SH   Sulfhydryl groups
                 groups   bound to arsenical
                                         (Dithioarsenite)
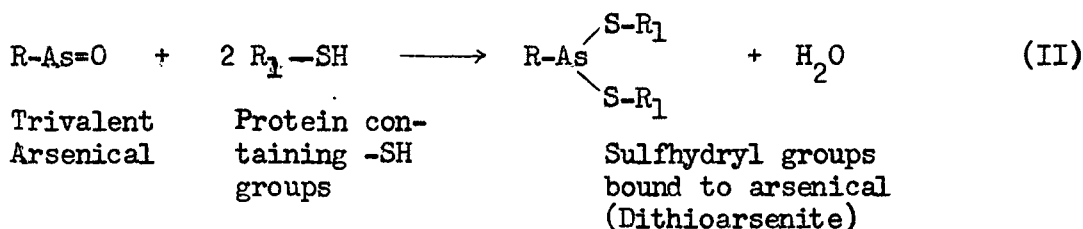
By thus blocking the -SH groups, reaction between the latter

and the chlori-mercuri groups of the Mercury Orange reagent was

prevented.   These experimental results indicate the specificity

of Mercury Orange for -SH groups.

Effects of Sodium Arsenic Tri-oxide on the Living Amoeba

The immediate increase in cytoplasmic streaming noted in organisms which were immersed in an 0.5 gm% solution of arsenic trioxide is likely a protective reaction, whereby the animal is attempting to escape the effects of the toxic environment. However, if one wishes to analyze this behaviour more closely, it is necessary to consider the animal's defence mechanisms in the light of possible phenomena which are taking place at a molecular or ionic level.

For example, the rate of cellular metabolism is presumably increased in order to produce the visible increase in cytoplasmic movement. The increase in metabolism could, in part, be a reflection of increased rate of enzyme production.

Since it is known that the toxic substance (namely arsenic) to which the amoeba was exposed combines with -SH groups, the postulated increase in enzyme production would effectively replace those enzymes which are inactivated as the result of binding by arsenic of -SH groups on the enzyme's active sites.

Application of the Mercury Orange Method to P. carolinensis

There was a direct relationship between intensity of colour imparted by Mercury Orange and concentration of arsenic to which the amoeba was exposed (Table 2b). That is, the greater the concentration of arsenic the less intense was the staining.

Since it is known that arsenic (in vitro studies) combines with
-SH groups, the results suggest that arsenic combined with the
-SH groups of P. carolinensis thus inhibiting the combination of
Mercury Orange and -SH taking place. This resulted in less
intense staining with Mercury Orange in the arsenic treated
amoebas as compared with the controls.

The fact that the experimental results of Mercury Orange
staining are qualitative must be borne in mind in their
interpretation (Table 3). However, the differences in
concentration noted in the various areas are in keeping with some
experiments on -SH content in A. proteus (Heller, 1959) and it
seems reasonable that a close similarity in this respect exists
between the two amoebas. For example, the plasmalemma of
A. proteus contains mucopolysaccharides and proteins. The latter
probably contain -SH groups. Cytoplasmic ground substance of
A. proteus contains -SH proteins that are low in concentration
compared with nucleolar concentration. In Pelomyxa carolinensis
I also found that the ground substance contained less -SH (2 plus)
than did the peripheral granules of the nucleus (4 plus). The
mitochondria of A. proteus contain sulfhydryl-containing proteins
and the rims of contractile vacuoles in Pelomyxa were 3 plus
for -SH groups. This, together with the fact that mitochondria
surround these contractile vacuoles would suggest that mitochondria
in P. carolinensis also contain -SH since the mitochondria

surrounding contractile vacuoles and those loose in the cytoplasm seem comparable in every way.

That the number of crystals in older food vacuoles is greater than in young food vacuoles suggests they may be wastes or storage material. It is interesting that these crystals show up very distinctly in the Mercury Orange stained amoebas. This seems to be due to the rims being coloured (indicating -SH superficially) thus outlining their structure clearly. Apart from the surface, these crystals probably do not contain -SH in any appreciable amount since the inner portion is colourless in the -SH treated amoebas. Although the size and shape of the food vacuole crystals differed considerably from the cytoplasmic crystals of the amoeba it was interesting that the cytoplasmic crystals, known to be nitrogen excretion products, were also coloured on their surface by the Mercury Orange reagent. (although the intensity of the colour reaction of the cytoplasmic crystals was much paler).

Sulfhydryl distribution in starved individuals differed from that in normal amoebas in that, in the former, the cytoplasmic ground substance was more intensely stained, whereas the rim surrounding the contractile vacuoles was less intensely stained. These results correspond to Heller's (1959) findings in A. proteus. He observed more densely staining cytoplasm and decreased numbers of mitochondria in starving amoebas. The marked decrease in the

number of contractile vacuoles is in keeping with what one would

expect as the result of a diminished ingestion of food organisms.

The lack of structural detail, likewise, might conceivably

be attributed to decreased "raw materials". Perhaps the

increased intensity of staining of the nuclei in the starved

organisms is due to increased staining of the nucleoplasm

corresponding to the darker cytoplasm.

Mitotic Nuclei stained with Mercury Orange

The intense staining of the interphase peripheral granules

may be due to the fact that they represent material (containing

-SH) which is to be incorporated into the spindle apparatus

during mitosis. This is in line with the belief of other workers

that the nucleoli contribute to spindle fibre formation (Kudo, 1946;

Schaeffer, 1937).

If the theory that oxidation of -SH groups to form -SS- bonds

is in part responsible for spindle formation, one would expect

anaphase spindle fibres should be the least densely coloured with

Mercury Orange staining since presumably the greatest quantity of

-SH groups would be oxidized to form -SS- bonds. This was found

to be the case with respect to interdisc fibres which were poorly

stained. However, polar fibres were very well stained and

metaphase fibres were faintly stained. These results suggest that:

(1) the interdisc fibres contain many -SS- bonds (or, at

any rate, are poor in -SH groups)

(2) polar fibres are in fact rich in -SH, and

(3) metaphase spindle fibres contain appreciable amounts of -SH groups.

The fact that the -SH content differs between polar fibres and interdisc fibres may be related to the fact that interdisc fibres are lengthening and polar fibres are shortening during anaphase (Taylor, 1959). According to electron microscopic studies, as the fibres become shorter or longer they do not become thicker nor thinner, nor do they become less straight. Therefore, it is postulated (Mazia, 1961) that the "elongation" might be actually growth in one dimension (the addition of molecules) and "contraction" is in fact shortening due to removal of molecules.

It is quite likely that chemical groups (and/or bonds) other than -SH are also involved in mitotic apparatus formation and although -SS- bonds in fact appear to be significant in spindle formation, they are not likely to be the sole factor. Other workers are also of this opinion. For example, Mazia (1959) at first stressed -SS- bonds but more recently (1961 a, b) attention has been turned to other (poorly understood) bonds involving protein sulfhydryl. Gross (1960) provides convincing evidence of a major role of hydrogen bonds.

The following, highly speculative, explanation is suggested as an explanation of the cytochemical investigations in this study. Since peripheral granules are deeply stained with the

Figure 49(a)
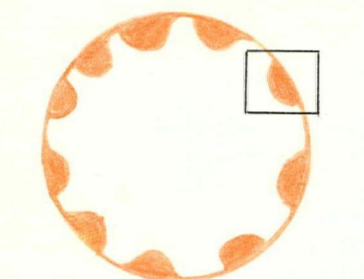
+ Mercury Orange +

(1) Interphase nucleus
showing peripheral
granules.

Inset of (1) - A hypothetical
peripheral granule containing
abundant sulfhydryl groups.

Peripheral granules
stain 4 plus, indica-
ting rich -SH content.

Figure 49 (b)
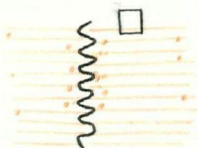
+ Mercury Orange +

(2) Majority of peripheral
granules have migrated
centrally. Faintly
coloured spindle fibres.
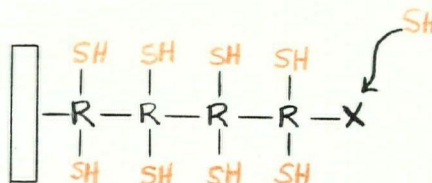
Inset of (2) - A hypothetical
single metaphase fibre. -SH
groups from peripheral
granules, which migrate
centrally, contribute to
mitotic apparatus. -SH
attaches at "free" ends so
that fibre grows in length
but not in thickness.
Fibres can shorten by reverse
reaction.

Metaphase fibres
stain 2 plus,
indicating some -SH
groups are present.

Figure 49 (c)

$$
\begin{array}{ccccc}
S-S-S-S-S \\
| \quad | \quad | \quad | \quad | \\
-R-R-R-R-R- \\
| \quad | \quad | \quad | \quad | \\
S-S-S-S-S
\end{array}
$$

+ Mercury Orange +
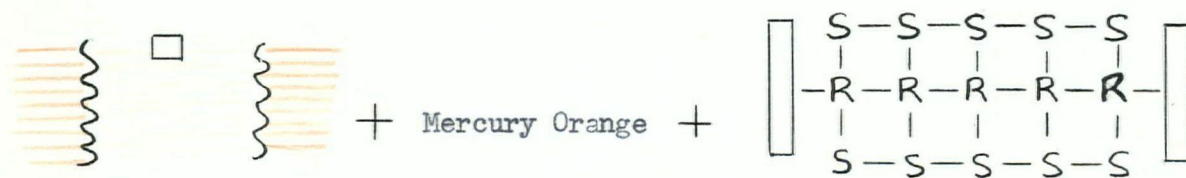
(3) Interdisc spindle
fibres poorly stained.
Polar fibres very
intensely stained.

Inset of (3) - A hypothetical
single interdisc anaphase
fibre. -SH groups have been
oxidized to form -SS- bonds
(or some other form of high
energy sulfur bond). No free
ends on this fibre, therefore
it must grow by cleavage of
internal bonds with addition
of chemical groups. In this
process of cleavage and re-
formation of bonds, chemical
energy to push discs apart is
generated - fibres elongate
but do not thicken. Polar
fibres are actually the meta-
phase fibres (2) above, there-
fore probably structurally
analagous - but contraction
or increased accessibility of
-SH groups results in more
intense staining.

Polar fibres stain
3 plus. Interdisc
fibres stain 1 plus.

sulfhydryl reagent they are doubtlessly rich in -SH groups
(Figure 49 a). Their close proximity to the nuclear membrane
during interphase might be related to synthetic processes
taking place involving raw materials obtained from the cytoplasm.
When these granules migrate centrally, sulfhydryl groups are
made available to assist in mitotic apparatus formation, but
these -SH groups are not yet oxidized to -SS- bonds
- therefore metaphase spindles stain faintly with Mercury Orange
(Figure 49 b).

As interdisc fibres are formed and elongate, -SH groups
are oxidized to -SS- bonds and therefore Mercury Orange staining
of these fibres is very faint (Figure 49 c). On the other hand,
the polar fibres being structurally analagous to the metaphase
fibres, contain -SH groups and therefore stain well with Mercury
Orange. The reason for the intense staining may be due to
increased accessibility of -SH groups (i.e. soon to become
dissociated from the mitotic apparatus) or to the fact that the
fibres are "contracting" hence -SH groups are closer together
than they were in metaphase.

It is felt that the molecular structure of spindle fibres
is in reality much more complicated than postulated in Figure 49.
For example, neither large protein molecules nor metal ions
have been incorporated into the hypothetical structure of a
spindle fibre postulated here.

SUMMARY

1.   Although P. carolinensis is a highly specialized structure,

I believe it is a fairly good representative of the living cell,

and its use as research material for cellular studies is justified.

When one considers working with tissue cultures, whether fibroblasts

or white blood cells, each is a specialized cell and hence it is

difficult to find a single organism or individual mammalian cell

which represents all cells.

2.   Feeding occurs only when the organism is attached to the

substratum.   The latter must be clean and the environment

"healthy".   Feeding habits are of a passive nature in that

movement of the amoeba from place to place is not the most

significant factor in obtaining food.   Rather, food organisms

advance towards the amoeba.   The explanation of this behaviour

is as yet undetermined.   The parameciums remain sluggish until the

food vacuole is completely formed, then commence violent movement.

        Small dark granules surround newly formed food vacuoles but

"empty" vesicles surround older food vacuoles.   Older food vacuoles

contain masses of crystals which are seen clearly with

Mercury Orange staining.

3.   Environmental conditions (pH) appear to be of greater

significance in maintaining a healthy state in P. carolinensis

than does the amount of food.

4.   Body form is correlated with physiological state as well as mitotic events.

5.   The most satisfactory fixative for histological studies was Osmium Tetroxide but Schaudinn's and TCA were also satisfactory.

6.   Results of this work confirmed that arsenic combines with sulfhydryl groups and therefore inhibits metabolic enzymes. Using activity (i.e. cytoplasmic movement) as a criterion, low concentrations had a stimulatory effect and this is believed to indicate that metabolism is temporarily stimulated at the molecular level, in an attempt to cope with destruction of necessary substances. Effects of arsenic in these low concentrations were reversible. Arsenic in concentrations sufficient to cause death invariably damaged the membrane of P. carolinensis resulting in complete dissemination of its contents into the surrounding environment.

7.   Frequency of mitosis is apparently related primarily to food supply.

All of the nuclei contained in a given amoeba undergo mitosis synchronously except on rare occasions and then the difference is slight.

Peripheral granules in the nucleus stain intensely with Mercury Orange and appear to give rise to spindle fibres.

8. Mitochondria characteristically surround contractile vacuoles and contain -SH groups. The intensity of colour of the mitochondria diminishes in starved individuals stained with Mercury Orange.

9. The Mercury Orange method for the detection of -SH groups worked well for the material in this study. Mercury Orange as used here is believed to react with free, "masked" and "sluggish" -SH groups indiscriminately. Variation in the distribution of -SH groups was observed in the various structures of P.carolinensis. Sulfhydryl distribution in starving individuals differed from that of normal, well-fed animals. Crystals in food vacuoles showed up very clearly with Mercury Orange stain indicating considerable -SH content.

10. Oxidation of -SH groups, the main source of which may be peripheral granules, to -SS- bonds is believed to contribute, in part, to the formation of mitotic spindles in P.carolinensis. Metaphase and polar (anaphase) fibres are believed to be structurally similar. They are, however, believed to differ structurally (chemically and/or physically) from interdisc anaphase fibres.

BIBLIOGRAPHY

Adams, C.W.M.
        1956   Histochem. Cytochem. 4:23.

Allen, R.D.
        1961   Amoeboid movement. In: The Cell. Biochem. Physiol.
                    Morphol. 2:135. (Editors: Brachet and Mirsky).
                    Academic Press, N.Y.

Andresen, N.
        1942   Cytoplasmic components in the amoeba Chaos chaos Linne.
                    Compt. Rend. trav. Lab Carlsberg, Ser. Chim. 24:139.

Andresen, N.
        1945   Coalescence between vacuoles during vital staining with
                    neutral red of Chaos chaos L. Compt. rend. trav. Lab
                    Carlsberg, Ser. Chim. 25:147.

Andresen, N.
        1946   Cytoplasmic changes during neutral red staining of the
                    amoeba Chaos diffluens (A. proteus. ibid) 25:169.

Andresen, N.
        1956   Cytological investigations on the giant amoeba
                    (Chaos chaos). Compt. rend. trav. Lab Carsberg, Ser.
                    Chim. 29:435.

Andresen, N.
        1957   Labile colloidal complexes of the cytoplasm. J. Cellular
                    Comp. Physiol. 49:221.

Andresen, N. and Holter, H.
        1945   Cytoplasmic changes during starvation of the amoeba
                    Chaos chaos L. Compt. rend. trav. Lab Carlsberg,
                    Ser. Chim. 25:107.

Andresen, N., Chapman-Andresen, C. and Holter, H.
        1952   Compt. rend. trav. Lab Carlsberg, Ser. Chim. 28:189.

Armed Forces Instit. of Technology.
        1960   Manual of Histologic and Special Staining Techniques.
                    2nd Ed.

Ashbel, R. and Seligman, A.M.
        1949   A new reagent for the histochemical demonstration of
                    active carbonyl groups. Endocrinol. 44:565.

Bachstrom, S.
        1956   Experimental Cell Research 11:322.

Bairati, A. and Lehman, F.E.
        1956   Structural and chemical properties of the contractile
                    vacuole of A. proteus. Protoplasma 45:525.

Baker, J.R.
    1958   Principles of biological microtechnique. John Wiley
                and Sons, Inc., N.Y.

Bang, F.B. and Gey, G.O. et al.
    1951   The validity of cellular structures as seen with the
                electron microscope IX Meeting of E.M.S.A.

Barer, R., Joseph, S., and Meek, G.A.
    1959   Exptl. Cell Research 18:179.

Barrnett, R.J. and Seligman, A.M.
    1952   Histochemical demonstration of protein-bound -SH groups.
                Science 116:323.

Barrnett, R.J.
    1953   Histochemical demonstration of the sites of protein-
                bound -SH and disulfide groups. Abstract Anat.
                Record 115:280.

Barrnett, R.J. and Seligman, A.M.
    1953a   The histochemical demonstration of protein-bound -SH
                groups. J. Nat. Cancer Inst. 13:905.

Barrnett, R.J. and Seligman, A.M.
    1953b   Investigation of the histochemical localization of
                disulfides (Abstract). J. Histo. and Cyto. 1:392.

Barrnett, R.J. and Seligman, A.M.
    1954a   Histochemical experiments on sulfhydryls and
                disulfides. In: A symposium on Glutathione. Academic
                Press, N.Y. page 89.

Barrnett, R.J. and Seligman, A.M.
    1954b   Histochemical demonstration of -SH and -SS- groups of
                protein. J. Nat. Cancer Inst. 14:769.

Barrnett, R.J., Tsou, K.C. and Seligman, A.M.
    1955   Further histochemical characterization of protein-
                bound -SH groups; The use of naphthol-containing,
                mercaptide-forming and alkylating compounds as
                reagents. J. Cyto. and Histo. 3:406.

Barron, E.S.G.
    1951   Thiol groups of biological importance. In: Recent
                Advances in Enzymology. Interscience Publishers, Inc.
                N.Y. 11:201.

Barron, E.S.G. and Kalnitsky, G.
    1947   The inhibition of succinoxidase by heavy metals and
                its reactivation with dithiols. Biochem. J. 41:346.

Barron, E.S.G. and Singer, T.P.
    1945   Sulfhydryl enzymes in carbohydrate metabolism.
            J. Biol. Chem. 151:221.

Barron, E.S.G., Miller, Z.B., and Kalnitsky, G.
    1947a   The oxidation of dithiols. Biochem. J. 41:62.

Barron, E.S.G., Miller, Z.B., Bartlett, G.R., Meyer, J. and Singer, T.P.
    1947b   Reactivation by dithiols of enzymes, inhibited by
            lewisite. Biochem. J. 41:69.

Barron, E.S.G., Nelson, L. and Ardao, M.I.
    1948   J. Gen. Physiol. 32:179.

Baumberger, J.P.
    1941   Am. J. Physiol. 133:206.

Beckett, E. and Bourne, G.H.
    1958   Some histochemical observations on normal and diseased
            human skeletal muscle. J. Histo. and Cyto. 6:13.

Bennett, H.S.
    1951   The demonstration of thiol groups in certain tissues
            by means of a new colored sulfhydryl reagent. Anat.
            Rec. 110:231.

Bennett, H.S.
    1956   J. Biophys. Biochem. Cytol. 2(4) part 2 (Suppl.):99.

Bennett, H.S. and Watts, R.M.
    1958   The cytochemical demonstration and measurement of -SH
            groups by azo-aryl mercaptide coupling with special
            reference to Mercury Orange. In: General cytochemical
            methods. Edited by Danielli, J.F. 1:375. Academic
            Press Inc. N.Y.

Bennett, H.S. and Yphantis, D.A.
    1948   1-(4-chloromercuriphenylazo)-naphthol-2. J. Am. Chem.
            Soc. 70:3522.

Binet, L. and Weller, G.
    1936   Bull. Soc. chim. Biol. 18:358.

Binet, L., Joulmes, C. and Weller, G.
    1937   Compt. rend. 204:1761.

Biology of the Amoeba
        June/59 Annals of N.Y. Academy of Sci. <u>78</u>:Art. 2:401-704.

Board, F.A.
        1951   J. Cellular Comp. Physiol. <u>38</u>:377.

Bolognari, A.
        1954   Aspetti dell' ovagenesi di <u>Aplysia depilans</u> L.
                (Moll. Gast. Opis). Boll. Zool. 21:185.

Bolognari, A.
        1952   Arch. Sci. Biol. Italy. <u>36</u>:40.

Boltus, E.
        1954   Biochim. et Biophys. Acta <u>15</u>:263.

Borysko, E. and Roslansky, J.
        1959   Methods for correlated optical and electron microscopic
                study of amoeba. In: Annals of N.Y. Acad. Sci.<u>78</u>(2):432.

Bourne, G.
        1935   Mitochondria, Golgi apparatus and vitamins. Australian
                J. Exptl. Biol. Med. Sci. <u>13</u>:238.

Brachet, J.
        1940   La localization de l'acide thymonucleique pendant
                l'oogenese et la maturation chez les amphibians.
                Arch. Biol. <u>51</u>:151.

Brachet, J.
        1950   Chemical Embryology. Interscience Publishers Inc. N.Y.

Brachet, J.
        1957   Biochemical Cytology. Academic Press Inc. N.Y.

Brachet, J.
        1961   The Living Cell. Sci Amer.   205(3):51.

Brandt, P.W.
        1958   A study of the mechanism of pinocytosis. Exptl. Cell
                Research <u>15</u>:300.

Brandt, P.W. and Pappas, G.D.
        1959   J. Biophys. Biochem. Cytol. <u>6</u>:91.

Brown, V.E.
        1930   The Golgi apparatus of <u>A. proteus</u>. Biol. Bull. <u>30</u>:240.

Buffa, E.
        1904   Sur une combinaison sulfuree des tissues animaux.
                J. de Physiol. et de pathol. gen <u>6</u>:645.

Cafruny, E.J., DiStefano, H.S. and Farah, A.
        1954   Effects of mercural diuretics on the -SH content of kidney
                cells. Fed. Proc. Amer. Soc. for Expl. Biol. <u>13</u>:340.

Cafruny, E.J., DiStephano, H.S. and Farah, A.
    1955   A cytometric determination of protein-bound -SH groups.
            J. Histochem. and Cyto. 3:354.

Calkins, G.N.
    1941   Protozoa in Biol. Research. Columbia Univ. Press.

Cameron, G.R.
    1951   The morphology of the normal cell. In: Path. of the
            Cell, pages 56-76. Charles C. Thomas, Springfield, Ill.

Casselman, W.G.B.
    1959   Histochemical Technique. John Wiley and Sons, Inc., N.Y.

Chalkley, H.W.
    1936   J. Morphol. 60:13.

Chapman-Andresen, C. and Holter, H.
    1955   Exptl. Cell Research Suppl. 3:52.

Chevremont, M. and Frederic, J.
    1943   Une nouvelle methode histochimique de mise en evidence
            des substances a fonction sulfhydryle. Arch. Biol.
            Liege 54:589.

Claude, A.
    1948   Studies on Cells: Morphology, chemical constitution
            and distribution of biochemical functions. Harvey
            Lectures 43:121. Springfield, Ill.

Cochran, W.G. and Cox, G.M.
    1957   Experimental design. John Wiley and Sons, Inc., N.Y.
            2nd Ed.

Cohen, A., King, H. and Strangeways, W.I.
    1931 . J. Chem. Soc.Pt 2:3043.

Cohen, A.I.
    1957   Electron microscopic observations on A. proteus
            in growth and inanition. J. Biophys. Biochem.
            Cytol. 3:859.

Cohen, A.I.
    1959   Physiological and morphological observations on
            amoebae. Annals N.Y. Acad. of Sci. 78(2):609.

Colien, F.E.
    1938   J. Lab. Clin. Med. 24:245.

Cooperstein, S.J., Lazarow, A. and Patterson, J.W.
    1953   Studies on the mechanism of Janus Green B. Staining
            of mitochondria II. Reactions and properties of
            Janus Green B and its derivatives. Exptl. Cell Res. 5:69.

Cooperstein, S.J., and Lazarow, A.
    1953   Studies on the mechanism of Janus Green B staining
            of mitochondria III. Reduction of Janus Green B by
            isolated enzyme systems. Exp. Cell Research 5:82.

Cowdry, C.V. and Scott, G.H.
    1928   Etude cytologiques sur le paludisme III. Mitochondries,
            granules colorable au rouge neutre et appareil de Golgi.
            Arch. inst. Pasteur Afr. N. 17:233.

Dalton, J.A. and Felix, M.D.
    1956   A comparative study of the Golgi complex. J. Biophys.
            Biochem, Cytol. 2(Suppl):79.

Danielli, J.F.
    1957   Cytochemistry: a critical approach. Academic Press.

Danielli, J.F., Danielli, M., Fraser, J.B., Mitchell, P.D., Owen, L.N.,
            and Shaw, G.
    1947   BAL-Intrav: A new nontoxic thiol for intravenous
            injection in arsenical poisoning. Bioch. J. 41:325.

Davenport, H.A.
    1960   Histological and Histochemical Techniques. W.B. Saunders
            Co., Phaladelphia (London.)

Davson, H.   The permeability of natural membranes, page 254.
    1943   Cambridge and N.Y.

deRey Pailhade, J.
    1888   Compt. rend. 106:1683 and 107:43.

deRobertis, E.D.P., Nowinski, W.W., and Saez, F.A.
    1957   General Cytology. W.B. Saunders, Philadelphia.

Dulzetto, F.
    1931   Arch. Biol. 41:221.

Ecker, E.E. and Pillemer, L.
    1938   Proc. Soc. Exptl. Biol. Med. 38:316.

Eagle, H. and Doak, G.O.
    1951   The biological activity of arsenobenzenes in relation
            to their structure. Pharmacol. Rev. 3:107.

Eagle, H, Hogan, R.B. and Doak, G.O.
    1944   The therapeutic efficacy of phenyl arsenoxides in mouse
            and rabbit trypanosomiasis. Pub. Health Rep. 59(PartI):765.

Ehrlich, P.
    1909   Ber. 42:17.

Elliott, A.M.
    1952   Zoology. Appelton Century Crofts, Inc. N.Y.

Engelhardt, V.A.
    1942   Yale J. Biol. Med. 15:21.

Faure-Fremiet, E.
    1910   Etude sur les mitochondries des protozoaires et des
              cellules sexuelles. ·Arch. Anat. Micr. 11:458.

Findlay, G.H.
    1955   J. Histo. and Cyto. 3:331.

Ford, Peter
    1958   Advanced Histological Technique. Scholar's Library, N.Y.

Frederic, J.
    1949   Arch. Biol. Liege 60:79.

Fugita, A. and Numata, I.
    1939   Biochem. Z. 300:249.

Gall, J.G.
    1956   Small granules in the amphibian oocyte and their
              relationship to RNA. J. Biophys. Biochem. Cytol.
              2(Suppl.):393.

Gatenby, J.B. and Beams, H.W. (Editors)
    1951   The Microtomists' Vade-Mecum (Bolles Lee). The
              Blakiston Company, Pa.

Gatenby, J.B., Dalton, A.J. and Felix, M.D.
    1955   The contractile vacuole of parazoa and protozoa and the
              Golgi apparatus. Nature 167:301.

Ghosh, B. and Guba, B.C.
    1937   Science and Culture 3:243.

Giroud, A.
    1931   Protoplasma 12:23.

Giroud, A. and Bulliard, H.
    1933   Reaction des substances a fonction sulfhydryle.
              Protoplasma 19:281.

Glahn, W.C. et al.
    1938   Effect of certain arsenates on the liver. Arch. Path.
              24:488.

Glick, D.
    1949   Techniques of Histo. and Cyto-chemistry. Interscience,
                New York.

Godeaux, J.
    1944   Bull. Soc. Roy. Sci. Liege 13:219.

Godeaux, J.
    1946   Compt. rend. soc. biol. 140:678.

Gola, G.
    1902   Lo zolfo e i suoi composti nell' economia delle
                piante, Malpighia. 16:368.   -

Goldacre, R.J.
    1952   The folding and unfolding of protein molecules as a
                basis of osmotic work. Intern. Rev. Cytol. 1:135.

Goldacre, R.J. and Larch, I.J.
    1950   Folding and unfolding of protein molecules in relation
                to cytoplasmic streaming, amoeboid movement and osmotic
                work. Nature 166:497.

Goldetz, L. and Unna, P.G.
    1909   Monotsschrift. prakt. Dermatol. 48:149.

Gomori, G.
    1949-50   Pitfalls in histochemistry. In: Ann. New York
                Academy Sci. 50:968.

Gomori, G.
    1952   Microscopic Histochemistry: Principles and Practice.
                Chicago Univ. Press.

Gomori, G.
    1956   Histochemical methods for protein-bound -SH and -SS-
                groups. J. of Microscopical Science. 97:1.

Goodman, L.S. and Gilman, A.
    1956   The Pharmacological Basis of Therapeutics. 2nd Ed.
                The MacMillan Co. N.Y.

Gray, P.
    1958   Handbook of Basic Microtechnique. 2nd Ed. McGraw-Hill
                Book Co. Inc. N.Y. (Toronto, London).

Gray, P.
    1954   The Microtomists' Formulary and Guide. The Blakeston
                Press Co. Inc. N.Y.

Greider, M.H., Kostir, W.J. and Frajola, W.J.
    1956   Electron Microscopy of the nuclear membrane of A. proteus.
              J. Biophys. Biochem. Cytol. 2(Suppl.):445.

Greider, M.H., Kostir, W.J. and Frajola, W.J.
    1958   Electron microscopy of A. proteus. J. Protozool. 5(2):139.

Gross, Paul, R. and Spindel, W.
    1960   Heavy water inhibition of cell division. An approach
              to mechanism. In: Annals of N.Y. Acad. of Sci.
              90(Art.2):500.

Grunert, R.R. and Phillips, P.H.
    1951   Arch. Biochem. 30:217.

Gurr, E.
    1956   Manual of Med. and Biolog. Staining Tech.

Guthwin, H. and Kopac, M.J.
    1959   Microscopic enzyme chemistry of carboxylic esterases
              in amoeba. Annals of N.Y. acad. of Sci. 78(2):538.

Hall, R.P.   Protozoology. Prentice-Hall, N.Y.
    1953

Hall, R.P.
    1930   Cytoplasmic inclusions of trichamoeba and their reaction
              to vital dyes and to osmic and silver impregnation.
              J. Morphol. 49:139.

Hall, R.P. and Loefer, J.B.
    1930   Studies on Euglypha I. Cytoplasmic inclusions of
              Euglypha alveolata. Arch. Protistenk. 72:365.

Hammett, F.S.
    1929   Protoplasma. 7:297.

Hammett, F.S. and Chapman, S.S.
    1938   The quantitative unrealiability of the nitroprusside
              test for -SH and -SS-. J. Lab. Clin. Med. 24:293.

Hardy, M.H.
    1952   Am. J. Anat. 90:285.

Harris, L.J.
    1923   Proc. Roy. Soc. London. B94:426.

Heffter, A.
        1908   Med. Naturwise. Arch. 1:81.

Heilbrunn, L.V.
        1952   An outline of General Physiology. pages 367 to 380.
                    Saunders, Philadelphia, Pa.

Heilbrunn, L.V.
        1956   The dynamics of living protoplasm. Academic Press, Inc. N.Y.

Heller, I.M. and Kopak, M.J.
        1955a   Exptl. Cell Research 8:62.

Heller, I.M. and Kopak, M.J.
        1955b   Exptl. Cell Research 8:563.

Heller, I.M. and Kopak, M.J.
        1956   Exptl. Cell Research 11:206.

Hellerman, L., Perkins, M.E. and Clark, W.M.
        1933   Proc. Natl. Acad. Sci. U.S. 19:855.

Hitchcock, P.
        1946   The effect of dithiols and other enzyme inhibitors
                    on blood vessels. J. Pharmacol. and Exper. Therap.
                    87:55.

Hoffman, J.G.
        1957   Life and death of cells. Hanover House Books,
                    Garden City, N.Y.

Holter, H.
        1959a   Problems of pinocytosis with special regard to
                    amoebae. Annals N.Y. Acad. of Sci. 78(2):524.

Holter, H.
        1959b   Pinocytosis. In: International Review of Cytology. 8:481

Holter, H.
        1961   How things get into cells. Sci. Amer. 205(3):167.

Holter, H. and Doyle, W.L.
        1938   Uber die lokalisation der amylase in amoeben. Compt.
                    rend. trav Lab. Carlsberg, Ser. Chim. 22:219.

Hopkins, F.G.
     1921   Biochem. J. 15:286.

Hopkins, F.G.
     1925   Glutathione, Its influence in the oxidation of fats
               and proteins. Bioch. J. 19:787.

Horning, E.S.
     1926   Observations on mitochondria. Australian J. Exptl.
               Biol. Med. Sci. 3:149.

Horning, E.S.
     1928   Studies on the behavior of mitochondria within the
               living cell. Australian J. Exptl. Biol. Med. Sci. 5:143.

Horning, E.S.
     1933   Enzymatic function of mitochondria. Erg d. Enzymforsch
               2:336.

Houssay, B.A.
     1950   Am. J. Med. Sci. 219:353.

Hughes, W.L.
     1949   Protein mercaptides. Cold Spring Harbor Symposia on
               Quant. Biol. 14:79.

Infantellina, F. and LaGrutta, G.
     1948   Arch. Sci. Biol. Italy. 32:85.

Jacobs, M.H. and Carson, S.A.
     1934   Biol. Bull. 67:325.

Jahn, T.L.
     19   How to Know the Protozoa. Wm. E. Brown Co. Dubuque,
               Iowa.

Jeener, R.
     1947   Experientia. 3:243.

Joyet-Lavergne, P.
     1926   Reserches sur le cytoplasm des sporozoaires. Arch.
               d'Anat. Micr. 22:1.

Joyet-Lavergne, P.
     1928   Le pouvoir oxydo-reducteur du chondriome des Gregarines
               et les procedes de recherches du chondriome. Compt. Rend.
               Soc. Biol. 98:567.

Joyet-Lavergne, P.
    1929   Glutathion et chondriome, Protoplasma 6:84.

Joyet-Lavergne, P.
    1934   Une theorie nouvelle sur le mecanisme des oxydo-
            reductions intracellulaires. Compt. Rend. Acad.
            Sci. Paris. 199:1159.

Joyet-Lavergne, P.
    1935   Recherches sur la catalyse des oxydo-reductions dans
            la cellule vivante. Protoplasma. 23:50.

Joyet-Lavergne, P.
    1938   Compt. Rend. Soc. Biol. 128:59.

Kassel, R.
    1959   Particulates of amoebae. In: Annals of N.Y. Acad.
            Sci. 78(2):421.

Kassel, R. and Kopac, M.J.
    1953   Experimental approaches to the evaluation of
            fractionation media. I The action of ions on the
            protoplasm of A. proteus and P. carolinensis. J. Exptl.
            Zool. 124:279.

Kawamura, N. and Dan, K.
    1958   A cytochemical study of the -SH groups of Sea Urchin
            eggs during first cleavage. Jour. Biophys. Biochem.
            Cytol. 4:615.

Kawamura, N.
    1960   Exptl. Cell Research. 20:127.

Kaye, M.
    1924   Biochem. J. 18:1289.

Kirby, H.
    1950   Materials and methods for the study of the protozoa.
            University of Calif. Press., Berkeley and Los Angeles.

Knisely, M.H.
    1936   A method of illuminating living structures for micro-
            scopic study. Anat. Rec. 64:499.

Kopac, M.J.
    1950   The surface chemical properties of cytoplasmic proteins.
            Ann. N.Y. Acad. Sci. 50(8):870.

Kopac, M.J.
    1951   Probable ultrastructure involved in cell division.
            Ann. N.Y. Acad. Sci. 51(8):151.

Kopac, M.J.
    1959.   Research on the amoeba in 2158 A.D. In: Annals N.Y.
             Acad. Sci. 78(2):696.

Kudo, R.R.
    1954   Protozoology. 4th Ed. C.C. Thomas, Springfield, Ill.

Kudo,R.R.
    1946   Pelomyxa carolinensis Wilson. I. General observations
             on the Illinois stock. J. Morphol. 78:317.

Kudo, R.R.
    1947   Pelomyxa carolinensis Wilson. II. Nuclear division
             and plasmotomy. J. Morphol. 80:93.

Kudo, R.R.
    1949   Pelomyxa carolinensis Wilson. III. Further observations
             on plasmotomy. J. Morphol. 85:163.

Kudo, R.R.
    1951   Observations on Pelomyxa carolinensis. J. Morphol. 88:145.

Kudo, R.R.
    1952   The genus Pelomyxa. Trans. Am. Microscop. Soc. 71:108.

Kudo, R.R.
    1959   Pelomyxa and related organisms. In: Annals N.Y. Acad.
             Sci. 78(2):474.

Kuroiwa, Y.
    1953   J. Agr. Chem. Soc. Japan. 27:473.

Labes, R.
    1929   Uber die pharmakologische bedeutung der chemischen
             reaktionen zwischen arseniger saure und thiol verbindungen.
             Arch Exp. Path. Pharm. 141:148.

Ladman, A.M. and Barrnett, R.J.
    1954   Endocrinology. 54:355.

Lafontaine, J.G.
    1958   J. Biophys. Biochem. Cytol. 4:777.

Landau, J.V.
    1959   Sol-gel transformation in amoebae. II. Physical
             studies and cell division. Annals N.Y. Acad. Sci.
             78(2):487.

Lazarow, A.
    1945   The chemical structure of cytoplasm as investigated
             in Professor Bensley's laboratory during the past
             ten years. Biol. Symposia. 10:9.

Lazarow, A.
    1949   Physiol. Revs. 29:48.

Lazarow, A. and Cooperstein, S.T.
    1953   Studies on the mechanism of Janus Green B staining of
            mitochondria - Review of the Literature. Expt. Cell
            Research 5:56.

LeFevre, P.G.
    1948   J. Gen. Physiol. 31:505.

Lehmann, F.E., Manni, E. and Bairati, A.
    1956a   Der feinbau von plasmalemma und kontraktiler vakuole
            bei A. proteus in schnitt und fragment-proparaten.
            Rev. suisse Zool. 63:246.

Lehmann, F.E., Manni, E. and Geiger, W.
    1956b   Der schichtenbau des plasmalemmas von A. proteus in
            elektronenmikros-kopischen schnittbild. Naturwiss 43:91.

Li, C.H. and Evans, H.M.
    1948   Chemistry of anterior pituitary hormones. In: The
            Hormones. Academic Press, Inc. N.Y. (Editors: Pincus,G.
            and Thimann, K.V.).

Lillie, R.D. and Burtner, H.J.
    1953   J. Histochem. 1:87.

Lindberg, O. and Ernster, L.
    1954   Chemistry and physiology of mitochondria and microsomes.
            Protoplasmatologia. 3A,4:1.

Linderstrom-Lang, K. and Jacobsen, C.F.
    1940   Compt. Rend. trav. Lab Carlsberg. Ser Chim. 23:289.

Linneaus, K.
    1758   Systema Naturae.   10th Ed. Brit. Museum, Nat. Hist.
            London, England.

Linneaus, K.
    1767   Systema Naturae.   12th Ed. Brit. Museum, Nat. Hist.
            London, England.

Lison, L.
    1953   Histochimie et cytochimie animales principes et methodes.
            2nd Ed. Gauthier-Villars, Paris.

Lyons, R.N.
    1945   Nature. 155:633.

MacLennan, R.F.
    1940   A quantitative study of osmic acid impregnation in
            protozoa. Trans. Am. Microscop. Soc. 59:149.

MacLennan, R.F.
    1941   Cytoplasmic inclusions. In: Protozoa in Biological
            Research. page 111. G.N. Calkins Editor. Columbia
            Univ. Press., N.Y.

Mapson, L.M. and Moustafa, E.M.
    1956   Biochem. J. 62:248.

Marshal, J.M., Jr., Schumaker, V.N. and Brandt, P.W.
    1959   Pinocytosis in amoebae. Annals N.Y. Acad. of Sci.
            78(2):515.

Mason, H.L.
    1930   J. Biol. Chem. 86:623.

Mast, S.O.
    1938   Amoeba and Pelomyxa vs. Chaos. Turtox News. 16:56.

Mast, S.O.
    1942   The hydrogen ion concentration of the content of the
            food vacuoles and the cytoplasm in amoeba and other
            phenomena concerned with food vacuoles. Biol. Bull.
            Woods Hole. 83:173.

Mast, S.O. and Doyle, W.L.
    1935a   Structure, origin and function of cytoplasmic constituents
            in A. proteus. I. Structure. Arch. Protistenk.
            86:155.

Mast, S.O. and Doyle, W.L.
    1935b   Structure, origin and function of cytoplasmic constituents
            in A. proteus with special reference to mitochondria and
            Golgi substance. II. Origin and function based on
            experimental evidence. Effect of centrifuging on
            A. proteus. Arch. Protistenk. 86:278.

Mast, S.O. and Hahnert, W.F.
    1935   Feeding, starvation and digestion in A. proteus. Leidig
            Physiol. Zool. 8:255.

Mast, S.O. and Johnson, P.L.
    1931   Concerning the scientific name of the common large
            amoeba usually designated Amoeba proteus. Leidig.
            Arch. Protistenk. 75:14.

Mazia, D.
1954a   Glutathione.   page 209.   Academic Press, N.Y.

Mazia, D.
1954b   Proc. Natl. Acad. Sci. U.S.   40:521.

Mazia, D.
1952   Physiol of the Cell Nucleus.   Modern Trends in Physiol.
         and Biochem.   (E.G. Barron, Ed.).   Academic Press, N.Y.
         77:122.

Mazia, D.
1955   Symposia Soc. Exptl. Biol.   9:335.

Mazia, D.
1957   The Chemical Basis of Heredity.   page 169.   The Johns
         Hopkins Press, Baltimore.

Mazia, D.
1958   Exptl. Cell Research   14:486.

Mazia, D.
1959   The role of thiol groups in the structure and function
         of the mitotic apparatus.   In: Sulfur in Proteins:367.
         R. Benesch, et al Editors.   Academic Press, N.Y.

Mazia, D.
1960   The analysis of cell reproduction.   In: Annals of
         N.Y. Acad. of Sci.   90(2):345.

Mazia, D.
1961a   Mitosis and the physiology of cell division.   In: The Cell
         3:77.   Academic Press, N.Y.

Mazia, D.
1961b   How cells divide.   Sci. American.   205(3):100.

Mazia, D. and Dan, K.
1952   Proc. Nat. Acad. Sci. U.S.   38:826.

McClung, C.E.
1950   Handbook of Microscopical Technique.   3rd Ed.   Hoeber, N.Y.

Mellors, R.C.   (Editor).
1959   Analytical Cytology - Methods for studying cellular form
         and function.   2nd Ed.   McGraw-Hill, Toronto.

Mescon, H. and Flesch, P.
1952   J. Invest. Dermatol.   18:261.

Mirsky, A.E. and Anson, M.L.
    1936   -SH and -SS- groups of proteins. II. The relation
                between the number of -SH and -SS- groups and quantity
                of insoluble protein in denaturation and in reversal
                of denaturation. III. -SH groups of native proteins,
                hemoglobin and the proteins of the crystalline lens.
                The reducing groups of proteins. J. Gen. Physiol. 19:427,
                439, 451.

Mirsky, A.E. and Pauling, L.
    1936   Proc. Natl. Acad. Sci. U.S. 22:439.

Mirsky, A.E. and Osawa, S.
    1961   The Interphase Nucleus. In: The Cell. 2:677 Acad. Press
                Inc.

Mörner, K.A.H.
    1921   Biochem. J. 15:285.

Neufeld, E.F. and Mazia, D.
    1957   Exptl. Cell Research 13:622.

Nickerson, W.J. and Falcone, G.
    1956   Science 124:318.

Nickerson, W.J. and Falcone, G.
    1956   Science 124:722.

Nigrelli, R.F. and Hall, R.P.
    1930   Osmiophilic and Neutral Red stainable inclusions of
                Arcella. Trans. Am. Microscop. Soc. 49:18.

Nurnberger, J., Engtröm, A. and Lindstrom, B.
    1952   J. Cellular Comp. Physiol. 39:215.

Olcott, H.S. and Fraenket-Conrat, H.
    1947   Specific group reagents for proteins. Chem. Revs. 41:151.

Pace, D.M. and Belda, W.H.
    1944   The effects of Potassium cyanide, potassium arsenite
                and ethyl urethane on respiration in P. carolinensis.
                Biol. Bull. 87:138.

Pace, D.M. and Kimura, T.E.
    1946   Relation between metabolic activity and cyanide
                inhibition in P. carolinensis (Wilson) Proc. Soc.
                Expl. Biol. Med. 62:223.

Pace, D.M. and McCashland, B.W.
    1951   Effects of low concentrations of cyanide on growth
                and respiration in P. carolinensis (Wilson). Proc. Soc.
                Expl. Biol. Med. 76:165.

Pace, D.M. and Frost, B.L.
    1952   The effects of ethyl alcohol on growth and respiration
            in P. carolinensis. Biol. Bull 103:97.

Palade, G.E.
    1952   A study of fixation for electron microscopy.
            J. Exp. Med. 95:285.

Pappas, G.D.
    1954   Structural and Cytochemical Studies of the cytoplasm
            in the family Amoebidae. Ohio J. Sci. 54:195.

Pappas, G.D. and Brandt, P.W.
    1958   The Fine Structure of the contractile Vacuoles in Amoeba.
            J. Biophys. Biochem. Cytol. 4:485.

Pappas, G.D.
    1959   Electron Microscope Studies on Amoebae. Annals of N.Y.
            Acad. of Sci. 78(2):448.

Pearse, E.A.G.
    1953   Histochemistry, Theoretical and Applied.
            J.A. Churchill Ltd., London.

Pearse, E.A.G.
    1961   Histochemistry, Theoretical and Applied.
            J.A. Churchill Ltd., London.

Percival, G.A. and Stewart, C.P.
    1930   Brit. J. Dermatol. Syphilis 42:215.

Peters, R.A.
    1949   The study of enzymes in relation to selective toxicity
            in animal tissue. Soc. for Expl. Biol. Symp. 3:36.

Peters, R.A., Sinclair, H.M. and Thompson, R.H.S.
    1940   An analysis of the inhibition of pyruvate oxidation
            by arsenicals in relation to the enzyme theory of
            vesication. Biochem. J. 40:516.

Pillai, R.K.
    1938   Action of Arsenate in Glycolysis. Biochem. J. 32:1961.

Prescott, D.M.
    1959   Microtechniques in Amoebae studies. Ann. N.Y. Acad.
            Sci. 78(2):655.

Pollister, A.W. and Pollister, P.F.
    1957   The structure of the Golgi apparatus. Intern. Rev.
            cytol. 6:85.

Ponder, E.
    1961   The Cell Membrane and its Properties. In: The Cell 2:1
            Ed. Brachet and Mirsky. Acad. Press, N.Y. and London.

Prosser, C.L.
    1950   Comparative Animal Physiol. p. 630-639.
            Saunders, Philadelphia, P.A.

Rao, H.S.M.
    1928   Studies on Soil Protozoa. II. The function of mitochondria in
            some soil protozoa. J. Indian Inst. Sci. 11A:117.

Rapkine, L.
    1931   Ann. Physiol. Physicochim Bio. 7:382.

Rapkine, L.
    1934   In: Langeron, Precis de Microscopie, Masson, Paris.

Rapkine, L., Chatton, E., Lwoff, A.
    1931   Compt. Rend, Soc. Biol. 106:626.

Raven, C. P.
    1958   Morphogenesis - The analysis of Molluscan Development.
            Pergamon Press, Los Angeles.

Ravin, H.A., Tsow, K.C. and Seligman, A.M.J.
    1951   Biol. Chem. 191:843.

Rebkun, L.I.
    1956   Electron Microscopy of Basophilic Structure of
            some invertebrate oocytes. I. Periodic lamellae and
            the nuclear envelope. J. Biophys. Biochem. Cytol. 2:93.

Robertson, J. D.
    1960   The Molecular Structure and Contact Relationships of
            Cell Membranes in Progress in Biophysics and
            Biophysical Chemistry. 10:348.

Rollo, I.M., Williamson, J. and Lourie, E.M.
    1949   Studies on the Chemotherapy of Melaminyl Arsenicals
            and Antimonials in Laboratory Trypanosome Infections.
            Ann. Trop. Med. 43:194.

Rösel von Rosenhof, A.J.
    1755   Der kliene Proteus. Monatlichhorausgegebenen
            Insectin-Belistigung 3:622.

Rosenthal, S.M.
    1932   Action of arsenic upon the fixed sulphydryl
            groups of proteins. Pub. Health Rep. 47:241.

Roughton, F.J.W. and Clark, A.M.
    1950   Carbonic anhydrase. In: The Enzymes. Vol. 1. pp. 1250-1265.
            Edited by Sumner, J.B. and Myrback, K. Acad. Press, N.Y.

Rudall, K.M.
    1952   The proteins of the mammalian epidermis. Advances
            in protein chem. 7:253.

Saiki, H. and Dan, K.
    1959   Studies on sulfhydryl groups during cell division
            of sea urchin egg. Exper. Cell Res. 16:24.

Schaeffer, A.A.
    1937   Morphology behaviour and reproduction in Type A and
            Type B of chaos chaos Linnaeus, the giant multinuclear
            amoeba of Rösel, Biol. Bull. 73:355.

Schaeffer, A.A.
    1937a   Rediscovery of the giant amoeba of Rösel, Chaos chaos
            Linnaeus, 1767. Turtox News 15:114.

Schaeffer, A.A.
    1938a   Biol. Bull. Woods' Hole 91:224.

Schaeffer, A.A.
    1938b   Significance of 3-daughter divisions in the giant
            amoeba. Turtox News 16:96.

Schaeffer, A.A.
    1946   Formation of the nuclear membrane and other mitotic
            events in Chaos spp. (observations during life).
            Biol. Bull Woods' Hole 91:224.

Seligman, A.M. et al.
    1949   Ann. Surg. 130:333.

Seligman, A.M., Tsou, K.C. and Barrnett, R.J.
    1954   A new histochemical method for demonstration of protein
            bound sulfhydryl. J. Histo and Cytochem. 2:484.

Serra, J.A.
    1946   Histochemical tests for proteins. Stain Tech. 21:(1):5.

Serra, J.A. and Lopes, A.
    1945   Queiroz Donnees pour une cytophysiologie du nucleole.
            I. L'activite nucleolaire pendant la croisance de
            l'oocyte chez les Helicidal. Portug. Acta Biol. 1:51.

Shearer, C.
    1922   Proc. Roy. Soc. London B93:213.

Short, R.B.
    1945   Spindle Twisting in the giant amoeba. Science 102:484.

Short, R.B.
    1946   Observations on the giant amoeba, Amoeba carolinensis
             (Wilson, 1900). Biol. Bull. 90:8.

Showacre, J.L.
    1953   A critical study of Janus Green B coloration as
             a tool for characterizing mitochondria. J. Natl. Ca.
             Inst. 13:829.

Singer, T.
    1943   Enzyme systems containing active sulfhydryl groups.
             The role of glutathione. Science 97:356.

Smith E.L.
    1951   Proteolytic enzymes. In The Enzymes Vol. 1. pp. 793-872.
             (Ed. Sumner, J.B. and Myrback, K.) Acad. Press, N.Y.

Spector, W.S.
             Handbook of Toxicology. Vol. 1,1956. Vol.2,1957.
             Philadelphia.

Stern, H.
    1956   Science 124:1292.

Stern, H.
    1957   J. Biophys. Biochem. Cytol. 4:157.

Stocken, L.A. and Thompson, R.H.S.
    1946   Arsenic and thiol excretion in animals after treatment
             of lewisite burns. Biochem. J. 40:548.

Stocken, L.A. and Thompson, R.H.S.
    1925.   Reactions of British antilewisite with arsenic and
             other metals in living systems. Physiol. Rev. 5:63.

Straub, F.B. and Feuer, G.
    1950   Biochim. et Biophys. Acta 4:455.

Swanson, C.P.
    1960   The cell. Foundation of modern Biology Series.
             Prentice-Hall, Inc., New Jersey.

Swift, H.
    1956   The Fine Structure of annulate lamellae. J. Biophys.
             Biochem. Cytol. 2(Suppl):331.

Szent-Gyorgyi, A.
    1930   The action of arsenite on tissue respiration.
             Biochem. J. 24:1723.

Tatum, A.L.
    1937   Some relationships between structure and function of
                organic arsenicals in experimental chemotherapy.
                Science 85:54. No.2193.

Taylor, E.W.
    1959   Dynamics of spindle formation and its inhibition by
                chemicals. J. Biophys. Biochem. Cytol. 6:193.

Thompson, R.H.S.
    1948   Biochem. Soc. Symposia. 2:28.

Torch, R.
    1955   Cytological studies on Pelomyxa carolinensis with
                special reference to the mitochondria. J. Protozool.
                2:(No.3):167.

Torch, R.
    1959   The cytology of Pelomyxa. Annals of N.Y. Acad. of
                Sci. 78:(2):407.

Vincent, H.
    1937   Compt. Rend. 204:1693.

Vincent, W.S. and Huxley, A.H.
    1954   Biol. Bull. 107:290.

Voegtlin, C., Dyer, H.A. and Leonard, C.S.
    1923   On the mechanism of action of arsenic upon protoplasm.
                Pub. Health Rep. 38:1882. Part II.

Voegtlin, C.
    1925   The Pharmacology of arsphenamine (Salvarsan) and
                related arsenicals. Physiol. Rev. 5:63.

Voegtlin, C., and Dyer, H.A. and Leonard, C.S.
    1925   On the specificity of the so-called arsenic receptor
                in the higher animals. J. Pharm. exp. Ther. 24:297.

Walker, E.
    1925   A colour reaction for disulfides. Biochem. j. 19:1085.

Watkins, W.M. and Wormall, A.
    1948   Nature 162:535.

Watson, M.L.
    1955   The nuclear envelope. Its structure and relationship
                to cytoplasmic membranes. J. Biophys. Biochem. 1:257.

Weatherall, M.
    1954   Drugs and porphyrin metabolism. Pharmacological
                Reviews 6:145.

Weisz, P.B.
    1950   The mitochondrial nature of the pigmented granules in
            stentor and blepharisma. J. Morphol. 86:177.

Whittaker, V.P.
    1947   An experimental investigation of the "ring hypothesis"
            of arsenical toxicity. Biochem. J. 41:56.

Wilbur, C.G.
    1942   The cytology of Pelomyxa carolinensis. Trans. Am.
            Micr. Soc. 61:227.

Wilbur, C.G.
    1945   Origin and function of the protoplasmic constituents
            in Pelomyxa carolinensis. Biol. Bull. 88:207.

Williams, R.R.
    1947   J. Immunol. 55:161.

Wilson, E.G.
    1952   An Introduction to Scientific Research. Pure Science
            Publications.

Wilson, H.V.
    1900   Notes on a species of Pelomyxa. Am. Naturalist. 34:535.

Yao, T.
    1949   Quart. J. Microscop. Sci. 90:401.

Yasuzumi, G.
    1959   Z. Zellforch. u. mikroscop. Anat. 59:110.

Zimmerman, A.M.
    1959   Effects of selected chemical agents on amoebae.
            Annals of N.Y. Acad. Sci. 78(2):631.