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THE MECHANISM OF BALLOON CELL FORMATION
IN SN-EHRlich ASCITES TUMOR CELLS

by

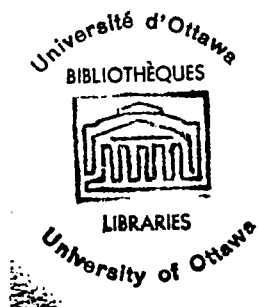
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A Thesis

Submitted to the Faculty of Medicine
of the
University of Ottawa
in Partial Fulfilment of the Requirements
for the Degree
Doctor of Philosophy

Department of Histology and Embryology

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CHAPTER I

INTRODUCTION

A. The Ehrlich Ascites Carcinoma

Experimental cancer research has been aided greatly by the use of transplantable tumors. In 1891, Moreau transplanted for the first time a mammary carcinoma from one mouse to another mouse, with the result that the second mouse developed a tumor microscopically similar to the original (Cowdry 1955). Transplantable tumors may differ in some respects from primary tumors, but they show that malignant characteristics once acquired by the cell may persist indefinitely in their descendants, if transfers are made under favourable conditions.

The development of transplantable tumors in the ascites form was another important step forward. Loewenthal and Jahn experimented with a particular variant of the Ehrlich mouse carcinoma which had descended from a spontaneously arisen mouse mammary carcinoma (Loewenthal and Jahn 1932). By inoculating mice intraperitoneally with cell suspensions of this tumor, Loewenthal and Jahn found that in most mice solid tumors developed, but in some mice a large amount of fluid of a milky or bloody

character was formed in the peritoneal cavity, a condition usually termed ascites (peritonitis carcinomatosa). This ascitic fluid contained a large number of tumor cells, and in mice so affected no solid tumors were found. By inoculating some of the ascitic fluid intraperitoneally into other mice, the tumor could readily be transmitted in the ascites form. After several such transplants it was possible for Loewenthal and Jahn to obtain a successful Ehrlich ascites carcinoma transplant in 100% of the inoculated mice. The tumor cells multiplied freely in the ascitic fluid, and as their number increased the tumor cell population resembled a nearly pure culture.

Besides tumor cells a few "normal exudate cells" are usually found. These may be detached mesothelial cells and white blood cells. If the ascites becomes hemorrhagic large numbers of red blood cells may also be found.

Thus in the ascites tumors there is no complicating stroma made up of blood vessels, nervous and connective-tissue elements, as is the case in solid tumors. Since the cells are in a homogenous suspension, they are all exposed to the same nutritive elements, oxygen tension and toxic substances. In solid tumors on the other hand outer regions of the tumor may have different properties from

inner regions, due to differences in their environment (Caspersson and Santesson 1942).

Besides the Ehrlich ascites carcinoma several other tumors have been transformed into the ascites form, such as the Krebs' mouse carcinoma (Klein and Klein 1951) and several other carcinomas, lymphomas and sarcomas by the same group of investigators (Klein 1951).

The Ehrlich ascites tumor is widely used, and over the years different strains have been developed in various laboratories. Most of the strains are near-tetraploid, but hyperdiploid strains have also been used (Hauschka et al. 1957). It seems that the cell size and growth characteristics of the tumor cells are related to the degree of ploidy of the strain of Ehrlich ascites tumor (Hauschka et al. 1957).

Ehrlich ascites tumor cells have often been used as "model tumor cells" in biochemical, physiological, pharmacological and virological studies, and it was in the course of studies on compounds with antitumor activity, that a morphologically anomalous type of tumor cell was discovered (Tolnai et al. 1962).

B. Balloon Cells of the Ehrlich Ascites Carcinoma

The presence of large balloon cells in the Ehrlich ascites subline used in the present study, was first

discovered by Tolnai (Tolnai et al. 1962), in an attempt to isolate a subcellular transmissible agent from this tumor. When the total ascitic fluid was centrifuged, a white lipid-like material accumulated on top of the supernatant, which upon microscopic observation was seen to consist of an accumulation of large cells. The nucleus was pushed to one part of the cell, and the rest of the cell appeared as a clear area (Tolnai et al. 1963). The density of these large balloon cells was apparently very low, since they did not sediment after centrifuging at 17,000 rpm, but remained in morphologically unaltered form (Tolnai 1964) in the supernatant. When balloon cells were inoculated into mice, ascites developed (Tolnai et al. 1963).

It was therefore of interest to investigate the nature of these balloon cells and their relationship to the smaller tumor cells. Staining smears with orcein showed that all stages of mitosis could be observed in the balloon cells, which indicated that they were cells capable of division (Tolnai 1964). An analysis of their life cycle using the incorporation of tritiated thymidine followed by autoradiography showed that the total generation time as well as the duration of the various phases of the mitotic cycle was approximately the same as the smaller

"regular tumor" cells, found in this tumor (Tolnai 1964).

In a careful analysis of the types of cells that are found in Ehrlich ascites exudates, Seeger described the occurrence of large "Siegelringzelle" (signet ring cells), which probably were the same type of cell as the balloon cells discussed here (Seeger 1937, 1938, 1939). Auler and Hohenadel also reported the presence of large vacuolated cells in the supernatant of Ehrlich ascitic fluid, that had been centrifuged for 10 minutes at 3,000 rpm (Auler and Hohenadel 1938). Gey found large distended and vesiculated cells in an Ehrlich ascites carcinoma strain that had been grown in continuous tissue culture for three years (Gey 1956). After peritoneal passage into mice this cell type persisted, which suggested to the author that a viral contaminant or passenger agent might be present in these cells. More recently similar cells have been described amongst cells of the Ehrlich ascites strain employed by Molomut and his group (Molomut et al. 1964). Similar types of cells have been shown in photographs of other ascites tumor cells, although the authors did not mention their presence (Klein and Klein 1951). It is therefore possible that the occurrence of large balloon cells is a more common phenomenon amongst tumor cells that grow in the ascites form, but that in many cases their

presence has not been noticed.

In none of the references cited above has there been an adequate explanation of the nature of such balloon cells. Vacuolization of cytoplasm is found in many cellular pathological conditions and perhaps vacuolization plays a part in the development of the balloon cells. Therefore vacuolar and vesicular systems of normal cells and cells subjected to adverse environmental conditions will be discussed in the next section.

C. Vacuolar Systems, Vesicular Systems and Lysosomes

Ultrastructural investigations of different cells have revealed a variety of structures, which have been given various morphologically descriptive names such as: vacuoles, vesicles, dense bodies, granules, droplets and lysosomes. Some of these structures are very variable in size and shape, which makes it difficult to decide whether they are cell organelles or cell inclusions (Ham 1965). Organelles are structures within the cytoplasm of cells which perform some special function related to the metabolism or functioning of the cells in which they are present, whereas inclusions do not play such a role. Some inclusions are formed within the cell (e.g. zymogen granules), whereas others are taken into the cell from the environment (Ham 1965).

1. Structures which Arise Through the Participation of the Cell Surface

The uptake of solid particles by certain cells (ameba, macrophages) has long been observed microscopically and this process is known as phagocytosis ($\phi\alpha\gamma\epsilon\iota\nu$ = to eat) (Policard 1964). The uptake of fluid droplets from the medium was first described by Lewis in tissue culture cells and this process was termed pinocytosis ($\pi\iota\nu\epsilon\iota\nu$ = to drink) (Lewis 1937). In phagocytosis the cell throws out large pseudopods, which surround the structure to be engulfed and afterwards the pseudopods fuse with the cell membrane again. In pinocytosis large upward folds, or ruffles of the cell surface trap some fluid from the medium, and then fuse with the cell membrane. A similar type of process has recently been found to occur at the junction of endothelial cells (Fawcett 1965). There does not seem to be much selectivity in this type of pinocytosis although in the ameba it can be induced by cationic agents such as cations, cationic dyes, positively charged proteins (Marshall and Nachmias 1965). Some selectivity appears to occur in phagocytosis, since only certain ciliates will be taken up by the ameba, and macrophages seem to be able to distinguish dead cells from living cells.

Although pinocytosis and phagocytosis seem to

occur in only a few types of cells (such as tissue culture cells, macrophages, amebae), the advent of the electron microscope has shown that many cells take up droplets of submicroscopic size. This type of fluid uptake has been termed micropinocytosis. Under the influence of a suitable inducing agent small depressions are formed in the cell surface, the cell membrane invaginates and small vesicles of about 80 m μ are pinched off.

Two types of micropinocytotic vesicles may be distinguished (Fawcett 1965). The first is a smooth type vesicle, which apparently forms very rapidly and accumulates at the cell surface. Such an arrangement of rows of smooth type micropinocytotic vesicles is often found in endothelial cells (Fawcett 1965). Uptake of fluid that occurs in this way does not seem to be very selective. The second type of vesicle is of a rough or coated type, in which there are tiny bristle-like striations projecting into the cytoplasm, and an amorphous or filamentous coating (believed to be mucopolysaccharide) on the extracellular side. These rough vesicles do not accumulate at the cell surface and many intermediate stages of invagination may be found (Fawcett 1965). Rough-type vesicles have been observed in cells that are engaged in the selective uptake of proteins, such as in the erythroblasts of the guinea

pig (Fawcett 1964), in the Kupffer cell of the hamster (Fawcett 1964) and in the oocytes of the mosquito (Roth and Porter 1964).

Once the vesicles that have formed as a result of phagocytosis, pinocytosis or micropinocytosis, have entered the cytoplasm their walls may disappear and their contents taken up by the cytoplasm, or they may remain as a discrete entity as they move through the cell. Such vesicles have been called phagosomes (Straus 1958) and pinosomes (Rustad 1964). As the phagosomes or pinosomes move to the interior of the cell, their contents are concentrated and digested, so that they become smaller. They also may fuse with each other giving rise to larger vesicles.

In endothelial cells and intestinal cells vesicles move through the cell and may leave at the other side by fusing again with the cell membrane. Such a transport is called cytopempsis (Staubesand 1965), and it allows for the uptake, transport and discharge of large molecules through cells, without actual contact with the cytoplasm, and without being broken down in the process. Cytopempsis may operate in two directions as was shown in endothelial and mesothelial cells by injecting tracer substances into the blood and into the tissue fluid

(Staubesand 1965).

The mechanism of vesicle formation in phagocytosis, pinocytosis and micropinocytosis may be divided into two phases. First there is the initial contact in which the inducing agent (a cationic protein or other cationic agent) neutralizes negative charges on the surface of the cell. The negative charges are due to the presence of a mucopolysaccharide coating on the outer surface of the cell membrane. Such a mucopolysaccharide coating may be quite thick as in the slime coat of the ameba (Marshall and Nachmias 1965) and the extraneous coats of the ova of many invertebrate and vertebrate species, or it may be much thinner as in many somatic cells of higher animals (Fawcett 1965). In the erythroblasts of the normal guinea pig the mucopolysaccharide coating is only formed in local areas of the cell surface. In these areas endogenous ferritin is adsorbed, so that the mucopolysaccharide coating gives a local selectivity to the cell surface (Fawcett 1965).

The second phase in vesicle formation is the response of the cell surface to the initial contact. As a result pseudopods, folds or depressions appear on the cell surface, followed by the formation of a vesicle, which takes the adsorbed material into the cell.

In summary, we may find in the cytoplasm of cells a number of vesicles, that have gained entrance

through the involvement of the cell surface. These are the phagocytotic vesicles (phagosomes), pinocytotic vesicles (pinosomes) and the smooth and rough type micropinocytotic vesicles. Novikoff has pointed out that although there may be some differences between these vesicles, regarding the mechanism of formation, the nature of the material engulfed, and their dimension, the basic feature in all these processes is the same: a certain area of the cell surface encloses a droplet or particle of the surrounding medium, separates from the surface and migrates into the cell (Novikoff 1961). For this reason, and in order to distinguish these activities of the cell surface from "active transport", Novikoff introduced the term cytosis, to include phagocytosis, pinocytosis and micropinocytosis. De Duve, although in agreement with Novikoff's argument, preferred to add the prefix endo for etymological reasons (de Duve 1963), and the term endocytosis is now frequently used when referring to vesicular engulfment by cells.

2. Vesicular Structures Arising in the Interior of the Cell

Some organelles, such as the endoplasmic reticulum, the Golgi apparatus, and the mitochondria may sometimes take on a vesicular appearance.

a. Endoplasmic Reticulum

In cells that are actively engaged in protein synthesis, the cisternae of the endoplasmic reticulum may swell considerably, so that they take on a vesicular appearance. The presence of gamma-globulin has been shown in the dilated cisternae of a mouse plasma-cell tumor, using ferritin bound antibody (Rifkind et al. 1962). Under certain pathological conditions, such as in dietary deficiencies (Hartroft 1963), during cell death (Bessis 1963), as well as in tissues improperly prepared for electron microscopy (Pease 1964), swelling of the endoplasmic reticulum may also occur.

b. Golgi Apparatus

The appearance of the Golgi apparatus varies in different cells, and under different physiological conditions. It is made up of three types of vesicles. The central portion of the Golgi area consists of a series of flattened vesicles, stacked on top of each other, often called lamellae. Closely associated with the lamellae are a number of large vesicles. Depending on the state of activity of the cell, the large vesicles may become very distended. They often appear as empty looking vesicles, but also may contain a denser material. Further away a

large number of much smaller smooth vesicles, of about the same diameter as the cisternae of the endoplasmic reticulum, may be found (Ham 1965).

The function of the Golgi apparatus appears to be twofold. Studies of polarized protein synthesis, storage and discharge in the pancreatic exocrine cell have shown that proteins that have been synthesized in the endoplasmic reticulum are moved to the Golgi regions, where they are aggregated and concentrated into large dense vesicles, often called granules (e.g. zymogen granules), surrounded by a smooth membrane (Warshawsky, Leblond and Droz 1963). It is thought that the small (40 m μ) Golgi vesicles transport the protein to the Golgi region, either by budding off the endoplasmic reticulum, in a process similar to micropinocytosis, or by shuffling back and forth (Caro and Palade 1964).

The second role of the Golgi apparatus is the linking of a carbohydrate moiety to the protein moiety to form glycoproteins and mucopolysaccharides. This was shown to occur in various mucous secreting cells and in chondrocytes of the rat after the injection of tritium labelled glucose (Peterson and Leblond 1964).

c. Mitochondria

Mitochondria are cell organelles that can

readily be recognized in electron micrographs despite their varied appearance in different cells. They are double-membraned vesicular structures, the inner membrane being thrown into several folds, termed cristae. Mitochondria may swell under certain physiological conditions, such as in the presence of thyroxine and other hormones (Lehninger 1964). Enlargement of mitochondria has also been observed in certain pathological conditions, such as in rats fed on a choline deficient diet (Hartroft 1963). In these cases the mitochondria can hardly be recognized: their cristae shorten and are further apart, the inner compartment becomes more transparent to electrons, and the enlarged mitochondria frequently become spherical in shape.

The abovementioned changes in cell organelles are frequently of a transitory nature, depending on the activity of the cell.

d. Lysosomes

Another frequently found vesicular structure, endogenous to the cell, is the lysosome. Lysosomes were first found by de Duve and his group in 1955, as part of the microsome fraction of liver cell homogenates. This fraction contained a large amount of acid phosphatase and other hydrolytic enzymes associated with particles, which were called lysosomes (bodies that lyse). With the

electron microscope the particles of this fraction appeared as small dense structures, about $1.0\ \mu$ in diameter, and of varied internal appearance (de Duve 1963). Soon similar structures were found in electron micrographs of many cell types. The identity of these structures as lysosomes was confirmed by histochemical tests, either at the light microscopic level (Barka and Anderson 1962, Novikoff and Essner 1962) or at the ultrastructural level (Novikoff 1961, 1963; Barka 1964 and Ericsson 1964).

With the electron microscope it was seen that lysosomes as defined by de Duve (a structure containing hydrolytic enzymes, surrounded by a membrane, also termed a "suicide" bag) had an extremely varied appearance (Novikoff and Shin 1964, Ericsson et al. 1965). Lysosomes sometimes contained mitochondria and other cytoplasmic components, almost intact or in various stages of breakdown (Novikoff and Shin 1964, Ericsson et al. 1965). Lysosomes could also contain material exogenous to the cell (Ericsson 1965). Despite their polymorphic appearance the lysosomes all contained acid phosphatase.

The above findings led to the formulation of the lysosome concept as expressed by de Duve and coworkers (de Duve 1963). In this concept the polymorphic lysosomes which contain cell components or endocytosed material, in

various stages of breakdown, are interpreted as active or secondary lysosomes, in which hydrolytic enzymes derived from elsewhere act to digest exogenous and endogenous components. It was postulated that primary or inactive lysosomes were made by the cells themselves. The origin of these primary lysosomes and the enzymes they contained was not known.

i. Primary or inactive lysosomes

It was suggested by Novikoff and his collaborators (Novikoff et al. 1964) that the lysosomal enzymes were synthesized in the rough endoplasmic reticulum, since in a few instances acid phosphatase reaction product was shown to be localized in it. From the site of synthesis the lysosomal enzymes could move directly to the site of activity, or indirectly through the involvement of the Golgi apparatus (Fig. 1) (Novikoff et al. 1964, Bainton and Farquar 1966).

Primary lysosomes are surrounded by a lipoprotein membrane, which prevents the enzyme contents from leaking into the cytoplasm. Storage granule-like primary lysosomes are found in abundance in phagocytic cells, such as polymorphonuclear neutrophil leucocytes (Hirsch and Cohn 1964).

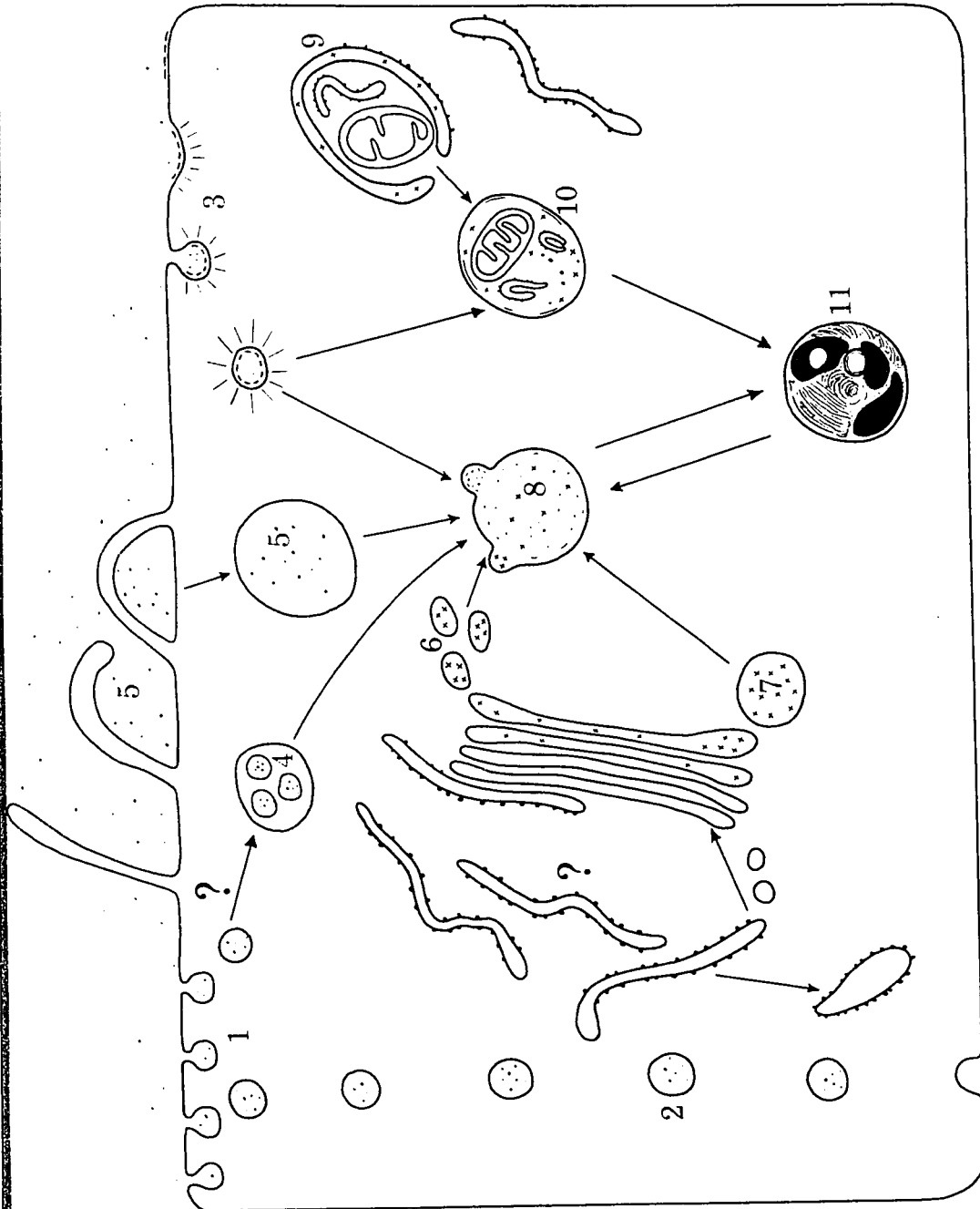
ii. Secondary or active lysosomes

In his discussion of the lysosome concept,

Fig. 1 Diagram showing various types of endocytosis and their relationship to primary and secondary lysosomes.

1. Micropinocytosis by which smooth vesicles are formed.
2. Cytopempsis.
3. Micropinocytosis with rough, coated vesicles.
4. Fusion of smooth or rough vesicles into larger structures (multivesicular bodies).
5. Classical pinocytosis, with microscopical visible droplets.
6. Small primary lysosomes developing from the Golgi apparatus.
7. Large storage granule type lysosomes developing from the Golgi apparatus.
8. Digestive vacuole, which contains ingested materials and lysosomal enzymes.
9. Formation of autophagic vacuoles, by wrapping of membranes around areas of cytoplasm and cytoplasmic components.
10. Autophagic vacuole, to which endocytosed materials may be added.
11. Residual body with myelin figures.

The small crosses in the diagram indicate the presence of lysosomal enzymes.



de Duve (de Duve 1963) distinguished three types of secondary or active lysosomes. He proposed that endocytotic vesicles (phagosomes or pinosomes) containing materials ingested by the cells, would fuse with primary lysosomes to form a digestive vacuole. Primary lysosomes could also fuse with endogenous cell components, resulting in autophagic vacuoles. When the contents of the digestive or autophagic vacuoles were broken down and concentrated, the indigestible compounds that remained would give the vesicles a dense appearance. Such denser secondary lysosomes, frequently containing myelin figures, were called residual bodies.

Although apparent fusion of phagosomes with existing lysosomes to form digestive vacuoles has been observed with the light microscope (Straus 1964), little evidence for actual fusion of phagosomes and primary lysosomes has been seen in electron micrographs (Miller and Palade 1964, Novikoff et al. 1964). However, in rabbit peritoneal leukocytes electron microscopic evidence has indicated actual fusion of "storage granule" type lysosomes with large phagosomes containing bacteria or zymosan granules (Zucker-Franklin and Hirsch 1964).

In the cells of the proximal tubules of rat kidney, phagosomal contents enter pre-existing secondary

lysosomes which had originated as autophagic vacuoles (Ericsson 1965), and these secondary lysosomes may repeatedly be involved in digestive functions. No evidence for primary lysosomes resembling "storage granules" has been found in this tissue (Ericsson 1965, Miller and Palade 1964).

There is little evidence in electron micrographs to show a fusion of primary lysosomes and cell organelles or areas of cytoplasm, to form autophagic vacuoles (Novikoff et al. 1964). Ultrastructural evidence indicates that autophagic vacuoles (or cytosegresomes as they are called by Ericsson et al. 1965) are formed by the wrapping of membranes around organelles or areas of cytoplasm (Novikoff et al. 1964, Ericsson et al. 1965).

Thus the process of primary- and secondary-lysosome formation may vary among different types of cells, and it may depend on endogenous and exogenous factors (Novikoff et al. 1964). Furthermore in certain cells it is difficult to distinguish between autophagic and digestive vacuoles, since digestion of endogenous and exogenous materials is carried out in the same structure (Ericsson 1965). Nevertheless the lysosome concept as expressed by de Duve and his coworkers (de Duve 1963) has greatly aided in the understanding of a wide array of

structures and their physiological significance.

iii. Functional significance of lysosomes

From the time of their discovery, it was thought that lysosomes, because of their content of hydrolytic enzymes, had a role in cell digestion and cell injury. Lysosomes have been found in most vertebrate cells, as well as in invertebrates and other organisms (Weissman 1965), and evidence has accumulated that active lysosomes are compatible with the normal functioning of cells. This is apparently because endogenous and exogenous components are digested within lysosomes (digestive or autophagic vacuoles), segregated from the rest of the cytoplasm by the lysosomal lipoprotein membrane.

The role of lysosomes in the digestion of endocytosed materials has already been mentioned. Indigestible materials such as colloidal suspensions of carbon, thorium dioxide, gold and iron, as well as certain vital dyes have been seen to accumulate in lysosomes, and use has been made of this phenomenon in the identification of lysosomes (Weissman 1965).

The second role of lysosomes is in cell autophagy. This may involve the disposal of worn out organelles or areas of cytoplasm. Autophagic vacuoles often contain mitochondrial remnants, which may indicate frequent

removal of this organelle (Ericsson et al. 1965). The number of autophagic vacuoles greatly increases in cell injury, such as caused by anoxia or toxic compounds, and in this way lysosomes may function as the urban-renewal system of the cell (Swift and Hruban 1964). Under conditions of starvation autophagy of cell components may furthermore be necessary for the mobilization of nutrient resources (Brandes et al. 1964, Swift and Hruban 1964).

A third role of lysosomes is in tissue damage in which lysosomal enzymes are released in the cell sap, an event which usually leads to the death of the cells involved. Such a release of enzymes occurs during traumatic, hemorrhagic and endotoxic shock (Weissman 1965), and it has been observed in virus infected cell cultures (Allison and Malluci 1965).

D. Aims of the Present Work

The primary aim of this work was to investigate the nature of the balloon cells of the Ehrlich ascites carcinoma. This problem was of interest since a survey of the literature revealed that although balloon cells had been observed before, little was known that could account for the unusual appearance of these cells.

It was therefore decided to investigate the

cytological, cytochemical and ultrastructural characteristics of the balloon cells, with special attention to the vacuolar systems, and to study the changes in the above parameters accompanying the development of the balloon cells.

CHAPTER II

EXPERIMENTAL METHODS

1. Procedure for Maintenance of the Ehrlich Ascites Carcinoma

Three sublines of the Ehrlich ascites carcinoma have been maintained. One of the sublines, a near-tetraploid strain, has been maintained for more than 125 generations in this laboratory as a control, since it contained very few, if any, balloon cells. The tumor was transplanted weekly by intraperitoneal inoculation of 0.2 ml ascitic fluid into 6-to 8-week old male Swiss mice of the Connaught strain. Mice thus inoculated had an average survival of 7 days.

Two other sublines of the Ehrlich ascites carcinoma (designated SN-576 and SN-578) were developed from the same tumor (Tolnai et al. 1963) by using 0.2 ml of the high-speed supernatant of the ascitic fluid for transfer. The supernatant for inoculation was prepared by centrifuging 20 to 30 ml of the ascitic fluid twice for 10 min. at 2200 rpm in an International Clinical centrifuge (Model 3001M-7) to remove red blood cells and most tumor cells. The supernatant so obtained was further centrifuged for

one hour at 17,000 rpm in a Servall centrifuge (Model SS-3 superspeed). This centrifugation sedimented all regular and intermediate size tumor cells, but some large balloon cells remained suspended in or on top of the supernatant. Care was taken to avoid the floating balloon cells when removing the supernatant with a Pasteur pipette. The supernatant contained 10^5 to 10^6 balloon cells per ml.

These two sublines have now been maintained for more than 50 generations in this laboratory. The tumors were transplanted at 17 to 21 day intervals into 6- to 8-week old male Swiss mice of the Connaught strain. Average survival of the inoculated mice varied between 18 and 27 days.

2. Separation of Cell Types

The tumor cells, present in the ascitic fluid of the two SN-Ehrlich sublines, were classified according to size and appearance in the following manner: a. regular tumor cells, which were similar to tumor cells found in other Ehrlich ascites tumors; b. balloon cells, in which a large clear area was present in the cytoplasm. Some balloon cells were only slightly larger than the regular tumor cells, whereas others were extremely distended.

An attempt was made to separate the tumor cells into various cell fractions, according to their density (Fig. 3). In this regard the term cell fraction was used to denote a group of cells, within a certain density range, which had been separated from the rest of the tumor cells by centrifugation. The separation into cell fractions was further aided with the use of a 10% ficoll¹ solution.

Ascitic fluid was centrifuged for 10 min. at 2200 rpm, so that the red blood cells and most of the tumor cells were sedimented. Any large balloon cells present remained suspended in or on top of the supernatant (Fig. 2a, Fig. 3).

The large balloon cells were collected from the top of the supernatant as a first cell fraction, designated as blebs. To remove smaller tumor cells that might be trapped, the bleb fraction was mixed with a small amount of supernatant and centrifuged. The bleb fraction was again collected, mixed with 10% ficoll (in 0.9% NaCl) and centrifuged (Fig. 2b).

Two more cell fractions were prepared from the layer of cells that sedimented after centrifugation of the

¹Available from Pharmacia Laboratories Inc., 501 Fifth Avenue, New York 17, N.Y., U.S.A. The ficoll used in these experiments had been kindly supplied by Dr. A. D'Iorio, Department of Biochemistry, Faculty of Medicine, University of Ottawa.

Fig. 2 Separation of the bleb fraction, intermediate fraction and cell fraction of whole ascitic fluid

- a. Whole ascitic fluid, centrifuged for 10 min. at 2200 rpm (SN 576, 17 days post inoculation).

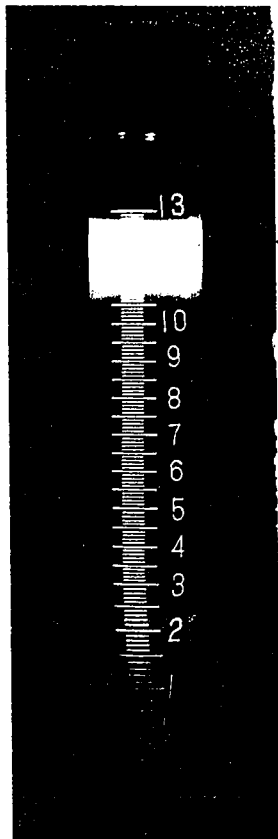
total cell volume	4.5 ml
bleb layer	2.2 ml
intermediate and regular cell layer	2.3 ml
supernatant	8.3 ml

- b. Bleb layer from a, mixed with 10% ficoll, and centrifuged for 10 min. at 2200 rpm.

bleb fraction	1.7 ml
intermediate fraction (on bottom of tube)	0.1 ml

- c. Intermediate and regular cell layer from a, mixed with 10% ficoll, and centrifuged for 10 min. at 2200 rpm.

intermediate fraction	1.2 ml
regular cell fraction	0.9 ml

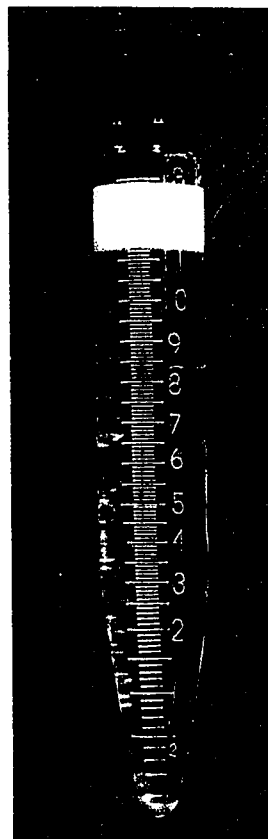


bleb

int.

cell

a



bleb

int.

b



int.

cell

c

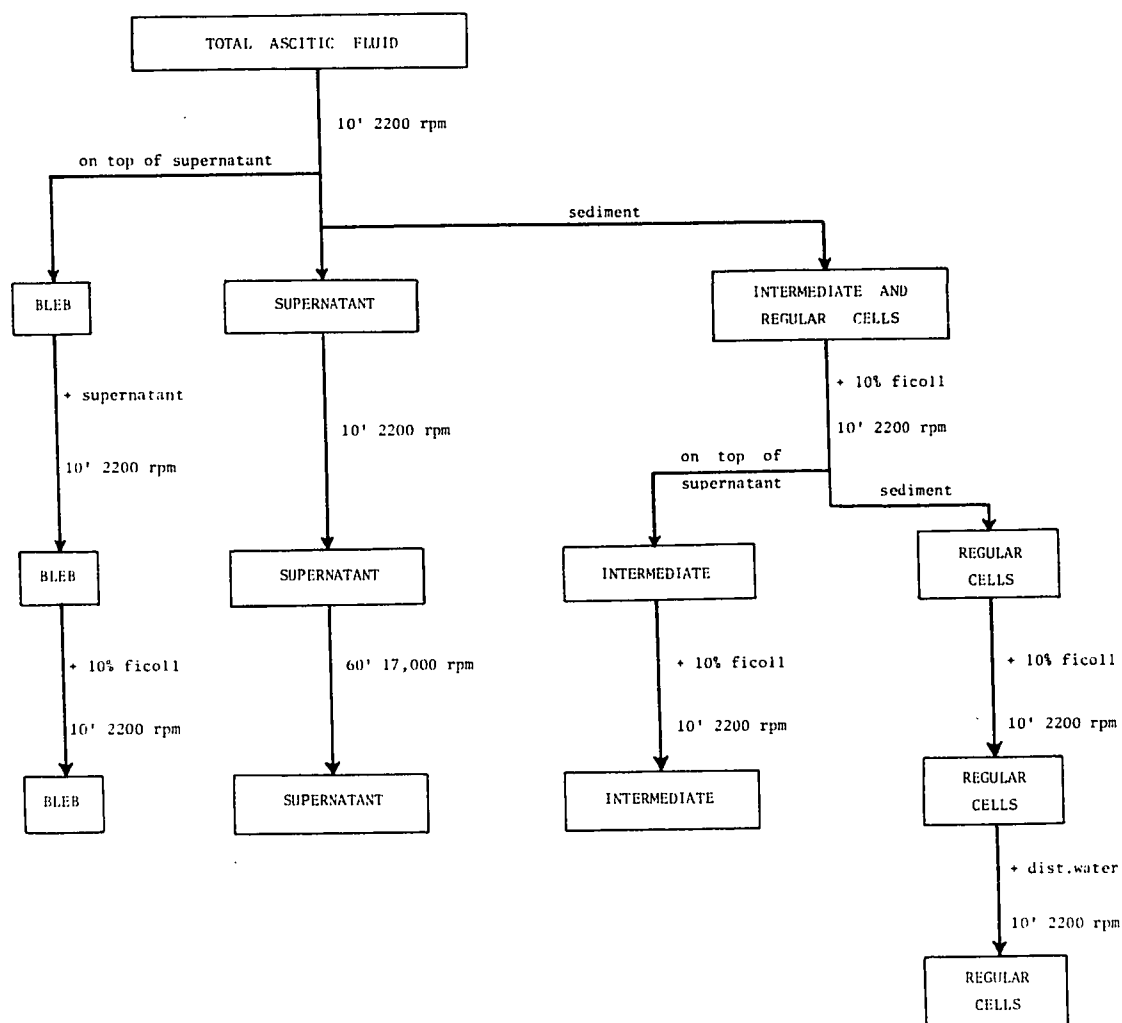


Fig. 3 Diagram indicating the procedure used in the separation of cell fractions from whole ascitic fluid

whole ascitic fluid (Fig. 2a). After mixing the bottom layer with an equal volume of 10% ficoll and centrifuging for 10 min. at 2200 rpm, a large number of cells collected on top of the supernatant (Fig. 2c and Fig. 3). These cells were once more mixed with 10% ficoll, centrifuged at 2200 rpm for 10 min., and the cell fraction thus collected was called the intermediate fraction. The 10% solution of ficoll was selected because at this concentration red blood cells and small tumor cells were sedimented, whereas intermediate-size balloon cells floated on the supernatant.

The layer of cells that sedimented in 10% ficoll (Fig. 2c and Fig. 3) consisted of small tumor cells and red blood cells. This layer of cells was once more mixed with 10% ficoll, and centrifuged for 10 min., to remove any tumor cells that belonged to the intermediate fraction. In order to hemolyze the red blood cells, the layer of cells was mixed for a short time with an equal volume of distilled water (Fig. 3). The sediment that was collected after centrifugation at 2200 rpm was designated as the regular cell fraction.

In many experiments all cell fractions were washed three times with Hanks' physiological saline solution (Hanks and Wallace 1949) to remove supernatant, ficoll and hemoglobin. After each washing the cells were

sedimented by centrifuging at 2200 rpm for 5 min.

The supernatant from the first centrifugation of the whole ascitic fluid was centrifuged once more at 2200 rpm for 10 min. (Fig. 3), and at 17,000 rpm for one hour afterwards.

3. Cell Count and Cell Size Measurements

Cell counts of the various cell fractions were made with a Spencer-Neubauer hemacytometer, using a 40x objective lens. The cell fractions were diluted with 0.05% nigrosin (in physiological saline) to about 0.5×10^6 cells per ml for counting. For each sample the number of cells in 10 large squares (1 mm^2) were counted. Nigrosin was used to stain the dead cells which could not exclude this dye (Kaltenbach et al. 1958).

The diameter of the cells was measured with a micrometer at the same time that the cells were counted in the hemacytometer. The cells were grouped according to their diameter, and the number of cells in each group was recorded. Because the distance between the upper and lower surface in the hemacytometer is 0.1 mm, and the diameter of the cells was in general less than 100 μ , it could be assumed that the cells remained spherical. Thus from the diameter of the cells the volume per cell could

be calculated.

The distribution of cell size, the average volume per cell and the total volume occupied by the cells could therefore be calculated for each cell fraction.

As a check on the average volume per cell, the total volume of cells in a diluted cell fraction of known cell count was measured with a hematocrit tube (Type Wintrobe), which was centrifuged for 10 min. at 3300 rpm.

4. Population Dynamics

The development of the various cell types, as related to time after inoculation, was determined in a large scale experiment. Fifty mice were inoculated with 0.2×10^6 balloon cells contained in 0.2 ml supernatant. The initial weight of the mice varied between 35 and 40 gm, and the mice were kept in separate cages.

The increase in weight was used as a general guide to the development of the tumor. Beginning on the eighth day after inoculation, one to three mice were killed at varying intervals. The bulk of the ascitic fluid was removed with a syringe by intraperitoneal puncture, and the remaining portion of the fluid was removed after open dissection.

The total volume of ascitic fluid per mouse was measured, and a total cell count as well as a count of the cell-size distribution was made. The volume of the three cell fractions, obtained after centrifugation at 2200 rpm and separation with 10% ficoll, was also measured. However at early stages of tumor development (8 to 11 days after inoculation) the volume of ascitic fluid present was not sufficient to separate the three fractions by centrifugation.

5. Density and Viscosity Measurements

The approximate density of the three cell fractions and of the supernatant was measured at various times after inoculation by a drop-method. A series of ficoll solutions (in 0.9% NaCl) ranging from 1% to 20%, at 1% intervals, was placed in 8-ml centrifuge tubes. Small, uniform drops of the suspension to be measured were dropped into the ficoll solution from about 2 cm above the surface. The behavior of the drop for the first 10 sec. was noted. If it sank, its density was greater than that of the ficoll solution, and if it rose its density was less. The density of the ficoll solutions was measured with a pycnometer at 25°C. The density of some fractions was also determined by a drop-method in a series of copper sulfate solutions (Gradwohl 1963).

The relative viscosity of the supernatant was determined using an ordinary 1 ml volumetric pipette, on which two marks had been made, one below and one above the bulb. The time-interval in which the meniscus travelled from the first mark to the second mark was measured with a stopwatch. Always the same pipette was used. The purpose of this measurement was to see if the viscosity of the supernatant changed with time after inoculation.

The relative viscosity of the supernatant, as compared to the viscosity of distilled water at 25°C, was calculated from the equation:

$$\frac{\eta_{\text{sup.}}}{\eta_{\text{H}_2\text{O}}} = \frac{d_{\text{sup.}} \times t_{\text{sup.}}}{d_{\text{H}_2\text{O}} \times t_{\text{H}_2\text{O}}} \quad (1)$$

(Daniels, Mathews and Williams 1941) in which the viscosity of water ($\eta_{\text{H}_2\text{O}}$) was taken as 1, the density of the supernatant was measured, and the time of flow of equal volumes of supernatant (t_{sup}) and water ($t_{\text{H}_2\text{O}}$) were measured.

6. Analytical Methods

a. Determination of protein

Dilutions of the various cell fractions were

made with Hanks' physiological saline solution in such a way, that the final solution contained 20 to 150 gamma of protein per ml. The cell concentration in these suspensions was too low to allow for an accurate determination of the cell count, which therefore was established from a more concentrated suspension.

Protein was determined according to the method of Lowry (Lowry et al. 1951) as modified by Oyama (Oyama and Eagle 1956), and the unknowns were analysed in quadruplicate. A series of bovine albumin¹ solutions, containing 20 to 160 gamma of protein, was used as a standard. Optical density readings were taken with a Bausch and Lomb Spectronic-20 spectrophotometer at a wavelength of 660 mμ.

From the protein concentration and the cell count per ml, the protein content per cell was calculated.

b. Determination of wet weight and dry weight

The wet weight and dry weight per cell were determined in two experiments, and the data were compared to the volume and protein content per cell of the same cell fractions. In both experiments the ascitic fluid of 8 mice, inoculated 18 days previously with SN-578,

¹Crystalline 2x, obtained from Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A.

was combined in a 250 ml centrifuge tube. The various layers were separated and washed in the usual way, but this time an International Preparative (Model BE-50) centrifuge, with a 250 ml head, was used.

Five to 10 ml of the packed cell fractions were measured into small beakers, and the wet weight was determined. The beakers were placed in a vacuum desiccator and the packed cells were dried in vacuo over concentrated sulfuric acid. To improve drying a container with sodium hydroxide pellets was also placed in the desiccator. It took about 8 to 14 days to reach constant weight.

The volume of the packed cell fractions thus measured was subject to a large error, since always some cells stuck to the inside of the pipette. It was however possible to calculate the initial volume from the initial wet weight and the known density of each layer.

The cell count, cell volume and protein determinations were made from a suspension of the packed cell fractions, diluted 1:25 with Hanks' physiological saline solution. There was again a systematic error in the measurement of the one ml packed cells, and it was assumed that this error was of the same magnitude and direction as in the determination of the wet and dry weight.

Therefore the error in the measured volume of packed cells was disregarded in the calculations of the wet weight per cell and dry weight per cell.

c. Determination of carbohydrate

The carbohydrate content of diluted cell fractions and of acetone powder preparations of cell fractions was determined. The three cell fractions were prepared in the usual way, except that glucose was omitted from the Hanks' physiological saline solution.

The acetone powder was prepared by adding dropwise 4 volumes of acetone to a cell suspension, consisting of one volume packed cells and one volume Hanks' solution. The resulting suspension was briefly centrifuged at 1500 rpm, and the supernatant was removed. The precipitate was washed a few times in acetone and collected on a Büchner filter. The powder was dried overnight at room temperature and weighed.

i. Total carbohydrate

Total carbohydrate was measured with an orcinol¹-sulfuric acid reagent (Miller et al. 1961), which was prepared fresh daily.

¹The commercially available orcinol (Matheson Coleman and Bell, Norwood, Ohio, U.S.A.) was too colored, and it was therefore recrystallized from chloroform.

Samples of the diluted cell fractions or of acetone powder were prepared in such a way that 1 ml of test solution contained about 0.15 mg of monosaccharide. A series of glucose solutions containing 0.05 mg to 0.25 mg per ml was used as a standard. The absorption spectrum was measured with a Bausch and Lomb Spectronic-20 spectrophotometer, by taking readings between 420 m μ and 600 m μ at 20 μ intervals. Absorption spectra were similarly prepared for the unknowns, and the relative concentration of carbohydrate as monosaccharide was determined.

ii. Chromatography

In order to identify the sugars present, acid hydrolysates prepared from acetone powder were analysed by paper chromatography. About 40 mg acetone powder was weighed into a 5 ml ampoule. The mixture was hydrolysed for 10 hrs. in 1.5 ml of 1N H₂SO₄ at 100°C in a boiling waterbath. After hydrolysis was completed, the hydrolysate was adjusted to pH 6.0 with 1N barium hydroxide. This mixture was centrifuged and the clear supernatant was used for chromatography.

Samples of 25 μ l were spotted on Whatman #1 chromatography sheets, measuring 36 by 36 cm. As controls 10 μ l samples of the following sugars were used: glucose,

galactose, glucuronic acid, galacturonic acid, lactose, N-acetyl glucosamine, d-glucosamine-HCl and fucose¹. These sugars were also used as internal standards, by hydrolysing them together with the acetone powder.

The chromatograms were run in butanol-acetic acid-water (4:1:5 v/v), by multiple ascending development, making three 12 hour runs. The chromatograms were sprayed either with ammoniacal silver nitrate (Block et al. 1958, p 178), or with aniline phthalate (Block et al. 1958, p. 181), and RF values were determined.

7. Preparation for Light Microscopy

a. Cytochemical methods

Smears of the various fractions were usually prepared directly without washing with Hanks' solution. The smears were made on acid cleaned slides, and were left to dry for 10 to 30 min. Depending on the type of stain to be used the smears were fixed in one of the following fixatives:

¹Glucose was obtained from Nichols Chemical Co. Ltd., Montreal; lactose was obtained from British Drug Houses Ltd., Poole, England; d-glucosamine-HCl was obtained from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.; all other sugars were obtained from Bios Laboratories, Inc., New York, N.Y., U.S.A.

acid alcohol	acetic acid:abs.alcohol,1:3.	10 min.
neutral formol	40% formaldehyde solution was saturated over magnesium carbonate for several days; from this a 10% solution in distilled water was prepared.	15 min.
Carnoy	glacial acetic acid 10 ml absolute alcohol 60 ml chloroform 30 ml	10 min.

i. Lipids

Two methods were used to test for the presence of lipids. Smears fixed in neutral formol were stained with oil red O (Pearse 1960, p 854), with which neutral fats show up as bright red droplets. In the osmium tetroxide method for unsaturated lipids (Wigglesworth 1957) the smears were fixed for 8 hrs. in 1% osmium tetroxide at pH 7.2. The smears were rinsed in distilled water and placed overnight in a concentrated ethyl gallate¹ solution. After dehydration through alcohol and clearing in xylol the smears were mounted in Permount.

ii. Carbohydrates

The periodic acid-Schiff (PAS) reaction according to the method of Hotchkiss-McManus (Gomori 1952) was used to demonstrate the presence of polysaccharides, neutral mucopolysaccharides and mucoproteins. The alcian blue

¹obtained from Fluka Chemische Fabrik, Buchs, Switzerland.

method (A.F.I.P. 1960), without a counterstain, was used for acid mucopolysaccharides. In some cases staining with alcian blue was followed by staining with PAS.

iii. Nucleic acids

To demonstrate nucleic acids, smears fixed with acid alcohol were stained with methyl green - pyronin Y according to the Kurnick modification (Pearse 1960, p. 826). In control sections RNA was digested for one hour at 37°C with a 1 mg/ml solution of crystalline ribonuclease¹. As a second method for nucleic acids the azure B method (Flax and Himes 1952) was used.

iv. Total protein

The distribution of total protein was evaluated by staining with 1% aqueous fast green adjusted to pH 2.0 with 1N HCl (Woodard et al. 1961).

b. Cytological methods

i. Alpha radiography

Kodak spectroscopic plates (type 649-0 measuring 1" by 3") were coated with 1% collodion, under a yellow safe light (Kodak filter Wratten OA). A small drop of cell suspension was placed in the central portion of the

¹obtained from Worthington Biochemical Corporation, Freehold, N.J., U.S.A.

slides and was brushed out with a wet camel hair brush. The slides were air dried for 15 min. with the aid of a fan.

The slides were exposed to a Polonium²¹⁰ source¹, according to the method of Bélanger (Bélanger and Bélanger 1959). A source of low strength (0.75 mcurie) was used, so that the slides were exposed for three days.

After exposure the collodion film was dissolved in acetone (2 min.), which also removed the cells. The slides were hydrated through an alcohol series, developed in Kodak D-19 developer for 5 min. at 68°C, rinsed in distilled water for 1 min., fixed in Kodak fixer with hardener for 10 min., washed in running tap-water for 30 min., dried and mounted.

ii. Uptake of vital dyes

The uptake of the vital dye, neutral red, was studied by injecting the dye intraperitoneally at an approximate concentration of 0.01 gm per ml ascitic fluid, using a 0.1% solution of neutral red in Hanks' physiological saline. Ascitic fluid was removed at 15 min., 1 hr., 2 hrs. and 4 hrs. after injection, and the cells, diluted with Hanks' solution, were observed in a hemacytometer.

¹obtained from U.S. Radium Corporation,
Morristown, N.J., U.S.A.

iii. Mitotic index

Cells in mitosis were studied by the aceto-orcein method (Ritter 1958). The mitotic index of each cell fraction was determined by counting the number of dividing cells among 1000 tumor cells. Permanent orcein preparations were obtained by leaving the stained wet slides, that were covered with a coverslip, for 48 hrs. in Coplin jars containing absolute alcohol vapor. The Coplin jars were sealed with vaseline. After 48 hrs. the coverslips were removed and the slides were quickly taken through two changes of 95% alcohol, two changes of absolute alcohol, and dipped for 5 sec. in 0.3% collodion solution (in ether:absolute alcohol 1:1). The slides were drained while standing upright, dried for 30 min., and mounted in Permout.

8. Preparation for Electron Microscopy

Ascitic fluid, collected at various times after inoculation, was separated into the three cell fractions by centrifuging it for 10 min. It was desirable to fix the cells as soon as possible, so that further separation with ficoll was not attempted. Prior to fixing the regular cell fraction was washed in Hanks' physiological saline solution, but the intermediate- and bleb-fractions

were not washed.

All cell fractions, placed in 12 ml centrifuge tubes, were fixed in the cold in 1% isotonic osmium tetroxide (Zetterqvist 1956) for 70 min., since preliminary experiments had shown that this length of time gave the best results. To ensure proper fixation the initial fixative was replaced after 5 min. with fresh fixative.

After fixation the cells were centrifuged for 2 min. at 1000 rpm, the supernatant fixative was removed, and the cells were washed for 15 min. in Tyrode's buffer. Dehydration was in a graded acetone series: 30%, 50%, 75% and 90% for 15 min. each, 100% acetone for 30 min. and 100% acetone for 60 min. During dehydration the samples were shaken frequently by hand, and after each step the cells were centrifuged at 1000 rpm for 2 min. There was a tendency for the cells to clump in 100% acetone. Dehydration was followed by infiltration and embedding in vestopal W using procedures recommended by the manufacturer¹. The blocks were hardened for 48 to 60 hrs. at 60°C, and were tested for the desired degree of hardness.

At the time of embedding, some cells with embedding medium were placed on microscope slides, and

¹Martin Jaeger, Vesenz/Geneve, Switzerland.

were covered with a coverslip. These slides were hardened for the same length of time at 60°C as were the blocks. They were used as light-microscopic controls, to indicate the degree of purity of the fractions, the stage of development and size of the balloon cells, and the degree of damage the cells suffered during preparation.

Light gold to gold colored (500-900 Å thick) sections were cut on a Porter Blum microtome. Frequently 1 μ sections were cut afterwards for light-microscopic observations. The latter sections were stained with a tribasic stain, containing toluidine blue, basic fuchsin and malachite green (Grimley 1964), or with toluidine blue alone.

Thin sections were placed on copper grids (75/300, 200 or 300 mesh). These grids were usually coated and covered with a thin formvar film made from 0.15 to 0.5% formvar solutions in ethylene dichloride. Sometimes grids without supporting film were used in order to improve contrast.

The sections were stained with 1% uranyl acetate at room temperature. The time of staining varied with the thickness of the section: 10 min. for light gold sections and 5 min. for dark gold sections. Staining with uranyl acetate was followed by staining with lead hydroxide

(Karnovsky 1961, procedure A): 5 min. for light gold sections and 2 min. for dark gold sections. Besides the method of Karnovsky, the lead citrate method of Reynolds (Reynolds 1963) using a 1:5 dilution, was used in some later experiments, and both methods gave good results. Lead staining or uranyl acetate staining alone was not satisfactory, resulting in pale sections with poor contrast.

The sections were observed with a Phillips E.M.-100 electron microscope. The images were recorded on 35 mm unperforated roll film (Kodak fine grain positive film P-426). Exposure was approximately 2 sec. depending on illumination. The negatives were developed at 68°C in D-19 developer for 5 min., rinsed in tap water for 30 sec., and fixed in Kodak fixer for 10 min. Prints were made on Kodabromide paper.

CHAPTER III

RESULTS

A. Population Dynamics

Previous experiments had shown that the proportion of balloon cells varied during SN-Ehrlich ascites tumor development, and that the bleb fraction, which appeared at about the 14th day after inoculation, had disappeared again in older mice. Therefore, a detailed study of the development of the various cell types, as related to tumor growth, would contribute to an understanding of the balloon cells.

Most of the results described in this section on population dynamics were obtained from one experiment involving 50 mice. The results of this experiment were compared to data from smaller groups of mice and to general maintenance data.

Although all the mice developed an ascites tumor in some (3 out of 50) tumor development was greatly delayed, and therefore no data were obtained from these mice. In the course of the experiment 25 mice were killed for experimental purposes. The remaining 22 mice died as a result of the tumor, and these mice were used for survival data.

1. Gross Characteristics of Tumor Development

a. Increase in weight

There was no increase in the weight of the mice during the first 7 days after inoculation of 0.2×10^6 balloon cells (Fig. 4), but after this initial lag period the average increase in weight per mouse was 2.6 gm per day until day 20. No significant increase in weight occurred after this date.

In one case the increase in weight was as much as 44 gm (day 19) in a mouse that initially weighed 40 gm.

In Fig. 4 and in other figures relating to the experiment on population dynamics, the mean deviation from the mean is indicated for each day. The number of mice used on each day is given in Table I (page 50).

b. Increase in ascitic fluid volume

The increase in the volume of ascitic fluid (Fig. 5) closely followed the increase in weight, with no detectable accumulation during the first 7 days after inoculation. After day 8 ascitic fluid accumulated at a rate of approximately 3 ml per day until day 20, when fluid accumulation stopped.

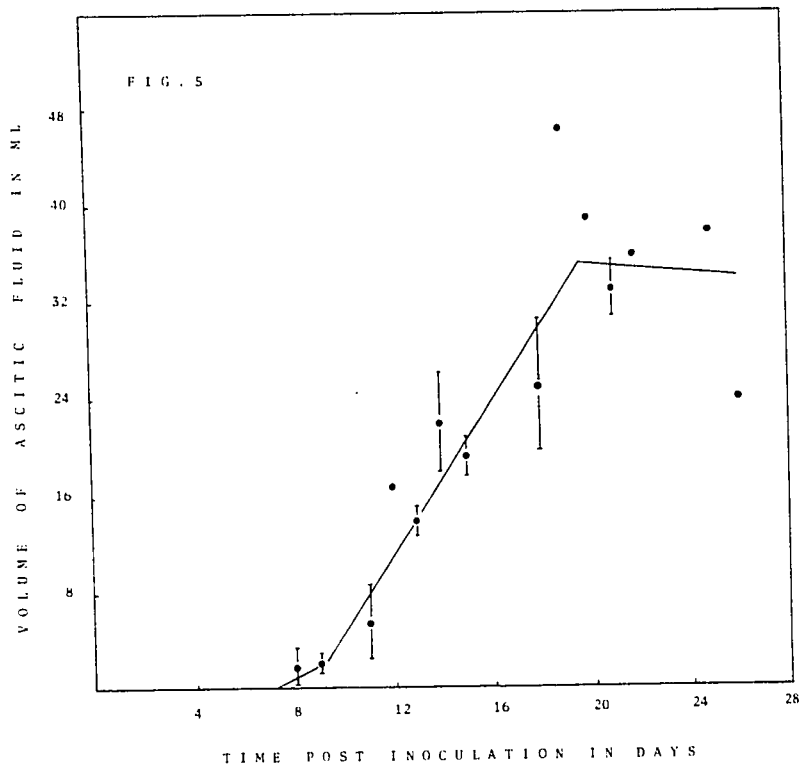
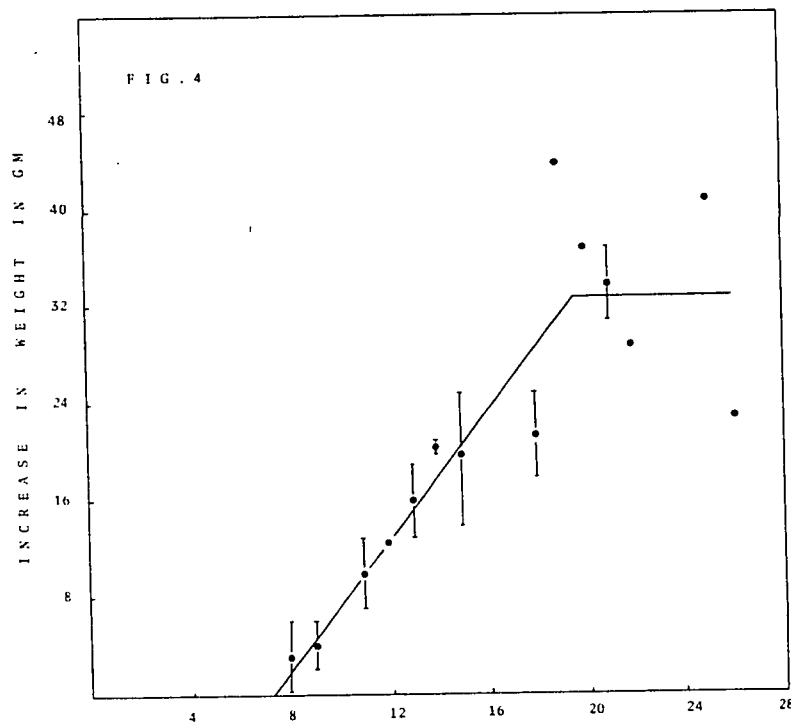
c. Survival

Median survival of inoculated mice was 22.5 days (Fig. 6) which corresponded with the survival time of mice used for maintenance of the tumor.

Fig. 4 The increase in weight of 25 experimental mice as a function of time.

In all figures relating to the experiment on population dynamics the mean deviation from the mean is indicated by vertical bars. The number of animals used each day is given in Table I.

Fig. 5 The increase in ascitic fluid volume of 25 experimental mice as a function of time.



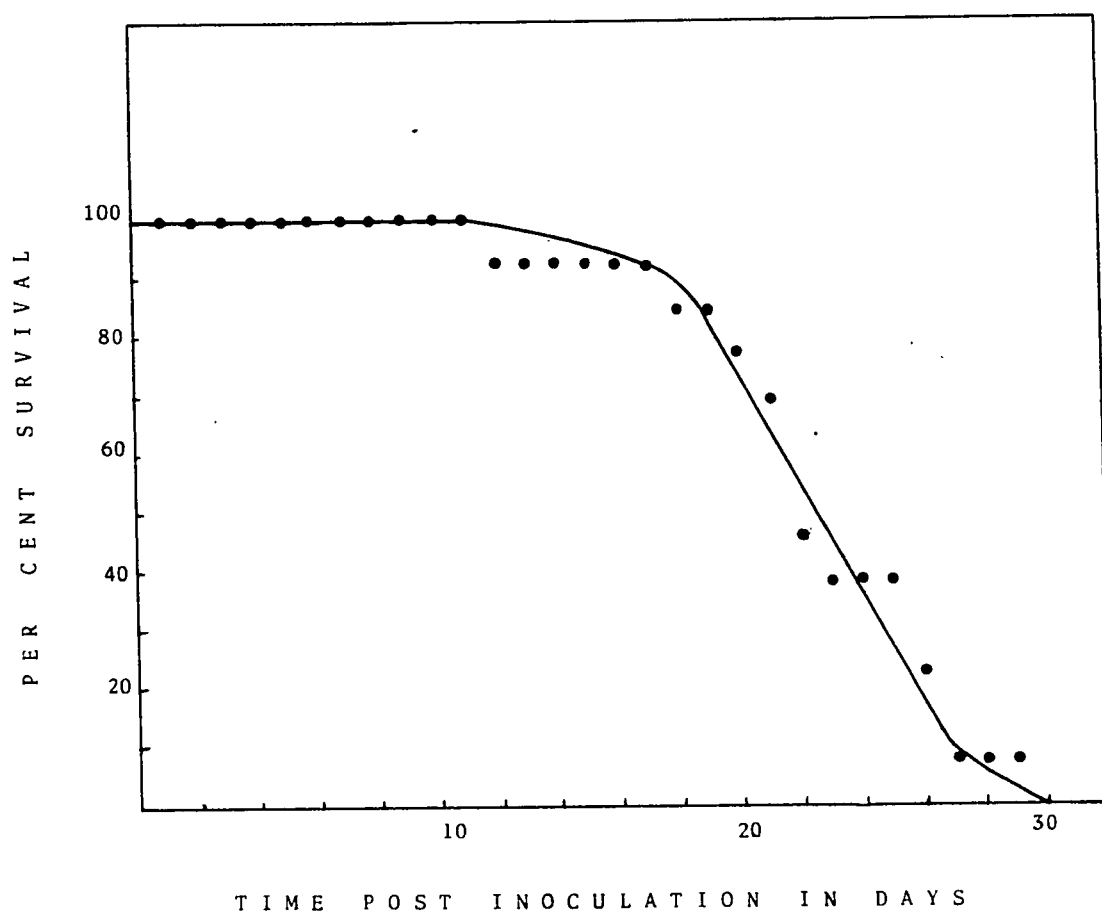


Fig.6 Percentage survival of inoculated mice as function of time

2. Comparison of Cell Count and Tumor Cell Mass During Tumor Development

a. Total cell count per mouse

Eight days after inoculation of 0.2×10^6 balloon cells there were 150×10^6 tumor cells per mouse (Table I, Fig. 7), so that the average generation¹ time during this period was approximately 20 hrs. The actual average generation time may have been even less if some of the inoculated cells did not divide, or if there was a short lag period after inoculation.

The number of tumor cells per mouse reached a peak at 12 days after inoculation, with 1200×10^6 cells per mouse (Table I). As can be seen in Fig. 7, the rate of cell division from 8 to 13 days after inoculation was less than during the first 8 days after inoculation. The generation time during this second period was approximately 40 hrs.

The total number of tumor cells per mouse dropped rapidly after 13 days, reaching 460×10^6 cells at 19 days and 360×10^6 cells at the end of the experiment (Table I, Fig. 7). For the first 18 days after inoculation the number of non-viable tumor cells was less than 10%, as shown with a dye exclusion test (Fig. 8). This number increased to 45% at the end of the experiment.

¹Calculated from $N_t = N_0 \times 2^{\frac{t}{G \cdot T}}$, where N_t is the number of tumor cells at time t and N_0 is the number of tumor cells inoculated.

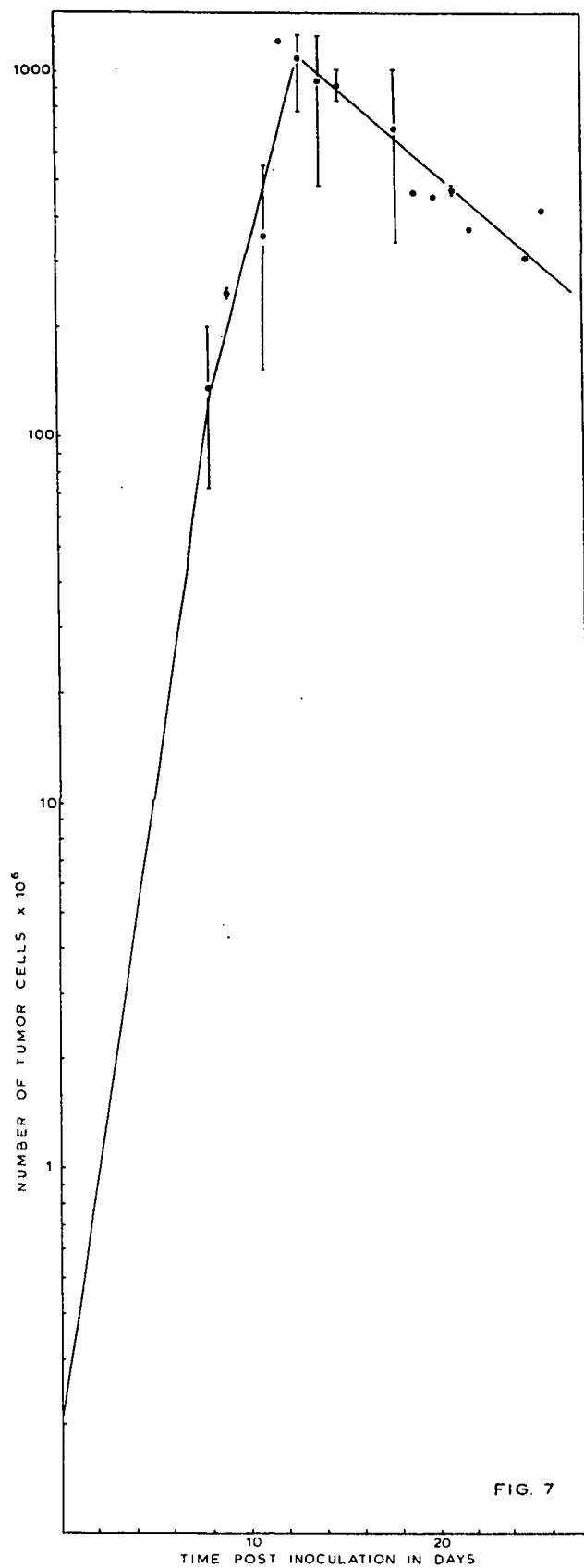
Table I
Total Tumor Cell Count at Various Times After Inoculation

days post inoculation		volume of ascitic fluid in ml	total cell count	
			per mouse x 10 ⁻⁶	per ml ascitic fluid x 10 ⁻⁶
8	(3) ^a	1.7 ± 1.6 ^b	148 ± 63	153 ± 63
9	(2)	2.2 ± 0.8	249 ± 9	128 ± 42
11	(3)	5.5 ± 3.2	355 ± 295	75 ± 20
12	(1)	16.9	1227	73
13	(2)	13.8 ± 1.2	1061 ± 330	78 ± 14
14	(2)	22.1 ± 4.1	948 ± 476	40 ± 14
15	(3)	19.2 ± 1.9	906 ± 91	47.3 ± 0.3
18	(2)	24.8 ± 5.4	701 ± 362	26 ± 9
19/20	(2)	42.7 ± 3.7	458 ± 6	10.8 ± 0.8
21	(2)	33.1 ± 2.6	471 ± 10	14.0 ± 1.9
22	(1)	36.2	373	10.3
25/26	(2)	30.8 ± 6.8	363 ± 54	12.2 ± 2.5

^aFigures in brackets indicate the number of mice used each day.

^bThe mean and the median deviation from the mean were calculated separately for each of the three sets of data. Therefore the product of the mean volume of ascitic fluid and the mean cell count per ml ascitic fluid was not always the same as the mean cell count per mouse.

Fig. 7 Logarithmic plot indicating the change in total tumor cell count per mouse as a function of time.



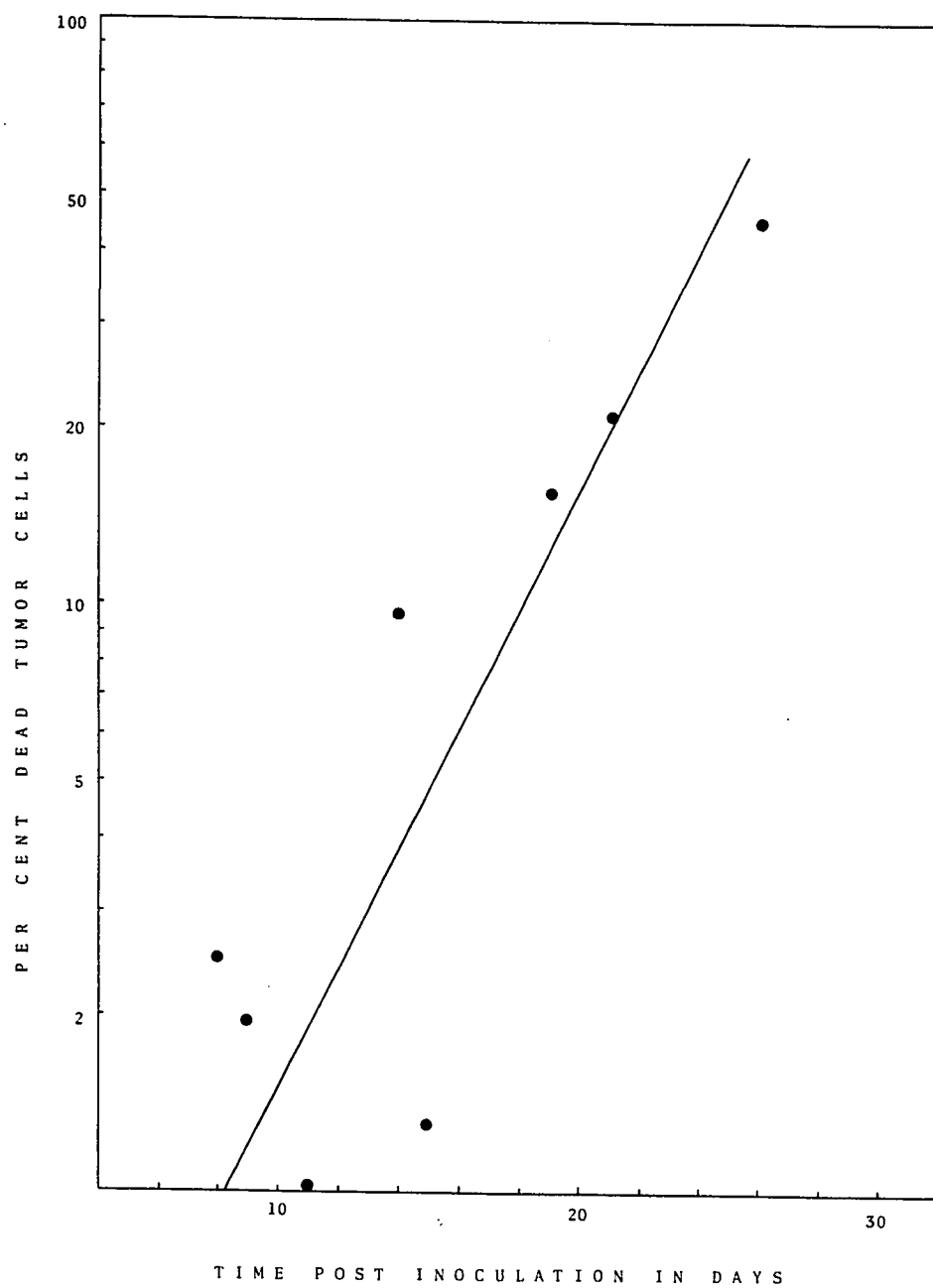


Fig.8 Logarithmic plot indicating the percentage of dead tumor cells at various times after inoculation

b. Total tumor cell mass per mouse

The total volume of tumor cells (total tumor cell mass) per mouse, as measured after centrifugation of ascitic fluid, increased at about the same rate as the volume of ascitic fluid until day 12; at this time the total tumor cell mass measured 9.1 ml per mouse (Table II, Fig. 9a). The total tumor cell mass increased to 10.7 ml at day 15, changing little thereafter. However at the end of the experiment the total tumor cell mass had decreased to 5.7 ml per mouse (Table II, Fig. 9a).

Fig. 9b shows corresponding values of five groups of mice from experiments conducted during a 6-month period. In these experiments the size of the inoculum varied, containing generally less than 0.2×10^6 balloon cells, and the maximum tumor cell mass of 11 ml was reached at 18 days after inoculation.

From the total tumor cell mass and the total tumor cell count the average volume per cell was calculated (Fig. 10). This increased gradually from $5.7 \times 10^3 \mu^3$ at day 8 to $24 \times 10^3 \mu^3$ at 19 and 20 days after inoculation, but decreased thereafter.

c. Cell count and tumor cell mass per ml ascitic fluid

The volume of ascitic fluid and the total number of tumor cells increased at approximately the same rate from 8 to 13 days after inoculation (Fig. 11). During

Table II

Total Tumor Cell Mass at Various Times After Inoculation

days post inoculation		total tumor cell mass	
		per mouse in ml	per ml ascitic fluid in ml
11	(3) ^a	3.4 $\pm$ 0.1 ^b	0.44 $\pm$ 0.02 ^b
12	(1)	9.1	0.54
13	(2)	7.8 $\pm$ 0.9	0.56 $\pm$ 0.02
14	(2)	7.7 $\pm$ 1.7	0.34 $\pm$ 0.02
15	(3)	10.7 $\pm$ 0.5	0.56 $\pm$ 0.03
18	(2)	9.8 $\pm$ 2.8	0.39 $\pm$ 0.03
19/20	(2)	11.1 $\pm$ 0.1	0.26 $\pm$ 0.02
21	(2)	10.3 $\pm$ 1.3	0.32 $\pm$ 0.06
22	(1)	14.0	0.39
25/26	(2)	5.7 $\pm$ 0.8	0.20 $\pm$ 0.06

^aFigures in brackets indicate the number of mice used each day.

^bThe mean and the mean deviation around the mean are presented.

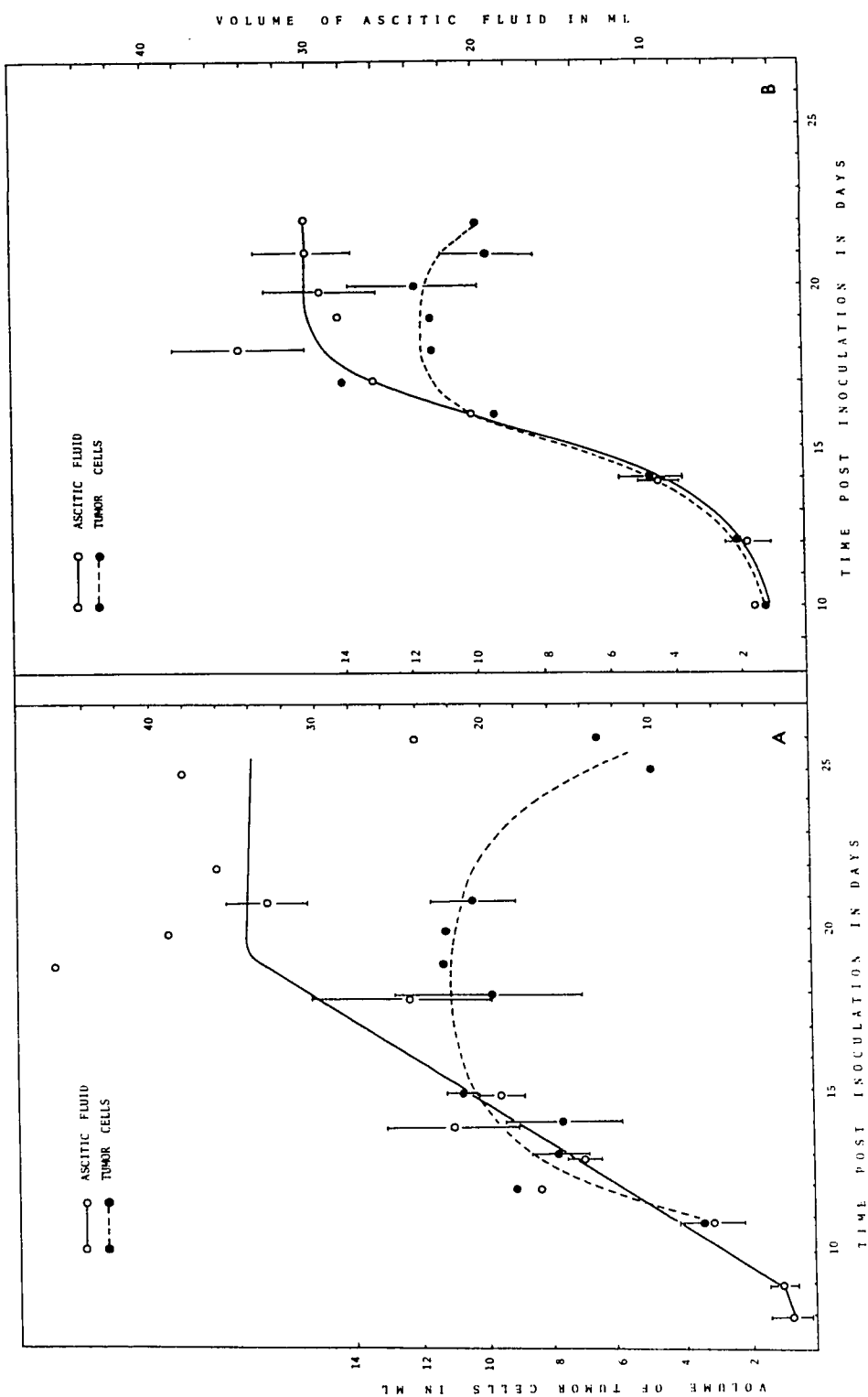


Fig. 9 Volume of tumor cells and volume of ascitic fluid per mouse as a function of time.
 a. 25 experimental mice; b. values from five groups of mice of experiments conducted over
 a 6-month period

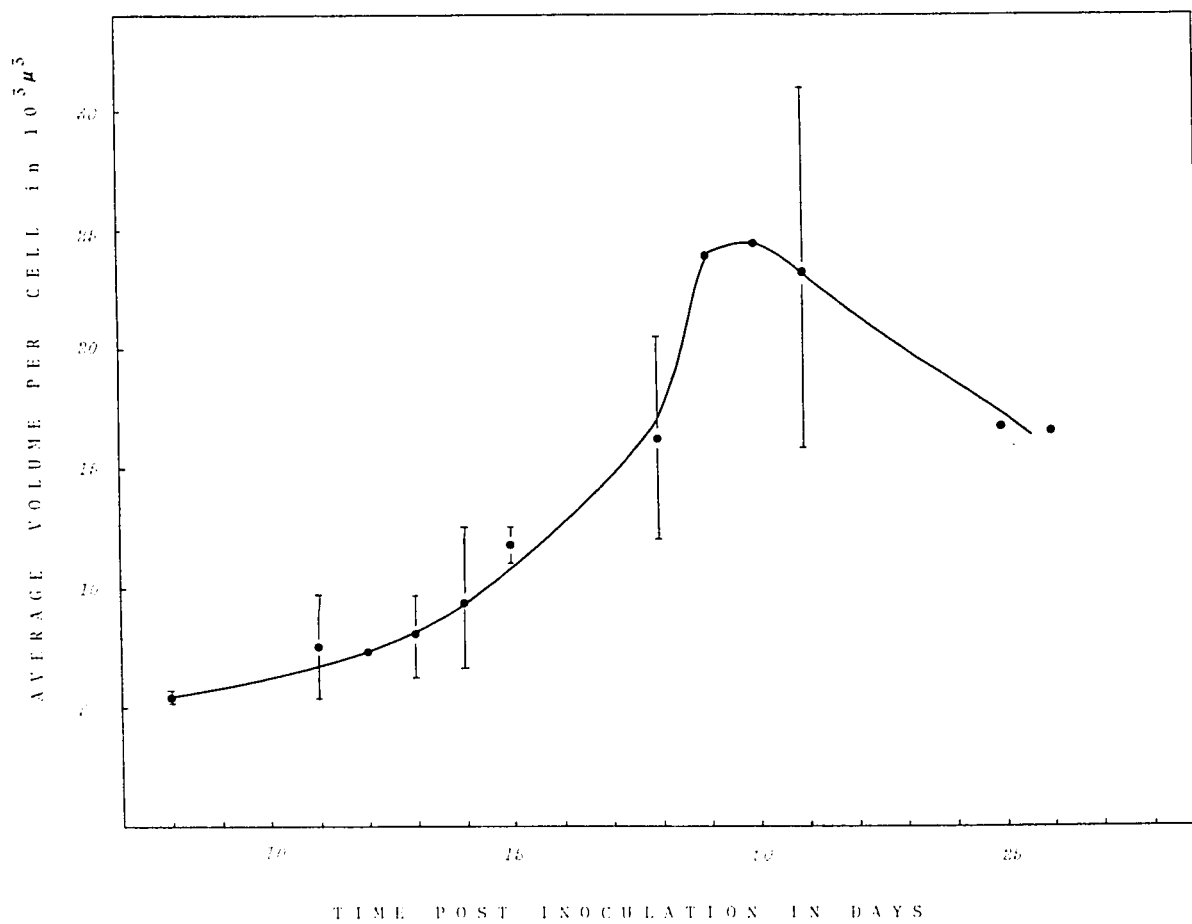


Fig.10 Average volume per cell in whole ascitic fluid as a function of time

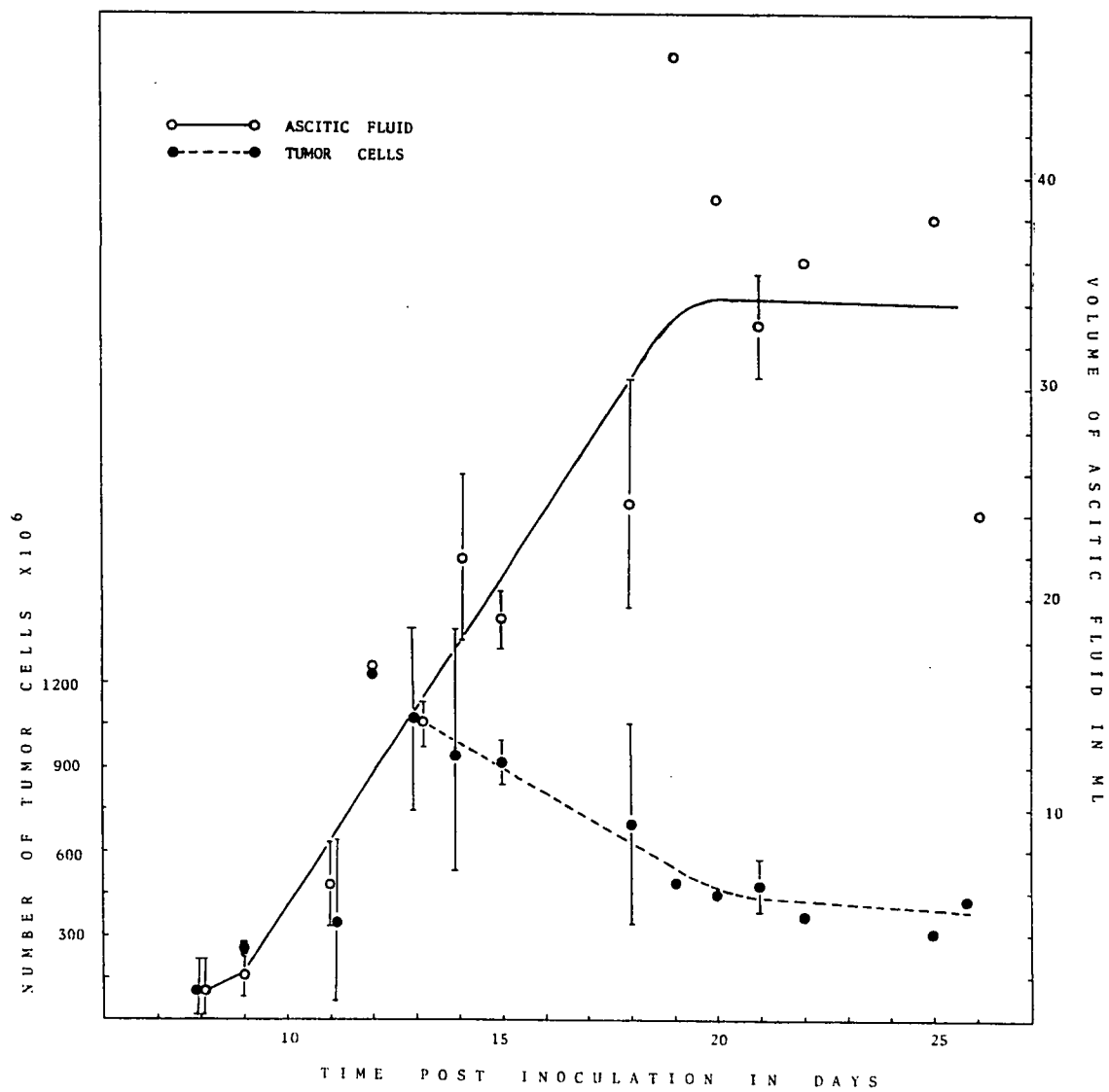


Fig.11 Total number of tumor cells and total volume of ascitic fluid per mouse as a function of time

this period the cell concentration was greater than 70×10^6 tumor cells per ml ascitic fluid (Table I). However from day 13 to day 20 ascitic fluid continued to accumulate, whereas the total number of tumor cells per mouse decreased, so that at 19 and 20 days after inoculation the cell concentration had decreased to 11×10^6 tumor cells per ml ascitic fluid (Table I). No further ascitic fluid accumulated after day 20, and the cell concentration remained approximately the same until the mice died.

The total tumor cell mass per ml ascitic fluid also decreased after day 13, but this decrease was more gradual than the drop in cell concentration (Table II). The volume of tumor cells per ml ascitic fluid was 0.54 ml at day 12, 0.26 ml at day 19 and 0.20 ml at the end of the experiment.

A similar decline in tumor cell mass per ml ascitic fluid was found in five groups of mice from experiments conducted during a 6-month period.

3. Distribution of Cell Types and Cell Fractions in Whole Ascitic Fluid

a. Identification of cell types

In order to classify the various cell types, the cells were grouped according to diameter (Table III). The diameter of the regular cells ranged from 13μ to 25μ with an average of 17μ . The small balloon cells with an average

Table III
Cell Diameter and Cell Volume of Various Cell Types

cell type	micrometer reading	diameter		volume	
		range in μ	midpoint in μ	range μ^3 $\times 10^{-3}$	average μ^3 $\times 10^{-3}$
regular cells	12-22	13-25	17 ^a	1.15- 7.56	2.57 ^b
small balloon cells	15-20	17-22	20 ^a	2.57- 5.58	4.19 ^b
interm. balloon cells	20-30	22-34	28	5.58- 20.6	11.50 ^c
large balloon cells	30-40	34-45	40	20.6 - 47.7	33.5
extra large balloon cells	40-60	45-67	56	47.7 -157.5	92.0

^aMean diameter

^bThe mean diameter was used in the calculation of the average volume of the regular cells and the small balloon cells

^cThe midpoint of each diameter range was used in the calculation of the average volume of the cells in the other groups

diameter of 20 μ were almost of the same size as the regular tumor cells, but were distinguished because of the presence of a small clear area in them. The larger balloon cells were divided in three arbitrary groups: intermediate balloon cells, large balloon cells, and extra large balloon cells, with micrometer readings of 20-30 (22-34 μ), 30-40 (34-45 μ), and 40-60 (45-67 μ) respectively.

b. Distribution of cell types in cell fractions

To ascertain the degree of separation of the three cell fractions (regular cell, intermediate and bleb), the cell type distribution in each fraction and in the supernatant from the first centrifugation of the ascitic fluid was determined. Table IV gives the distribution of cell types in cell fractions of ascitic fluid collected at 15 days post inoculation. The bleb fraction contained mainly large and extra large balloon cells, the intermediate fraction contained mainly intermediate balloon cells, and the regular cell fraction contained regular cells and small balloon cells.

c. Comparison of the distribution of cell types in whole ascitic fluid with the distribution of cell fractions in whole ascitic fluid

To facilitate graphic representation the small and intermediate balloon cells were treated as one group, and the large and extra large balloon cells as another group (Fig. 12). This procedure also aided in comparing trends

Table IV
Example of the Distribution of Cell Types in Various Cell
Fractions and in the Supernatant^a

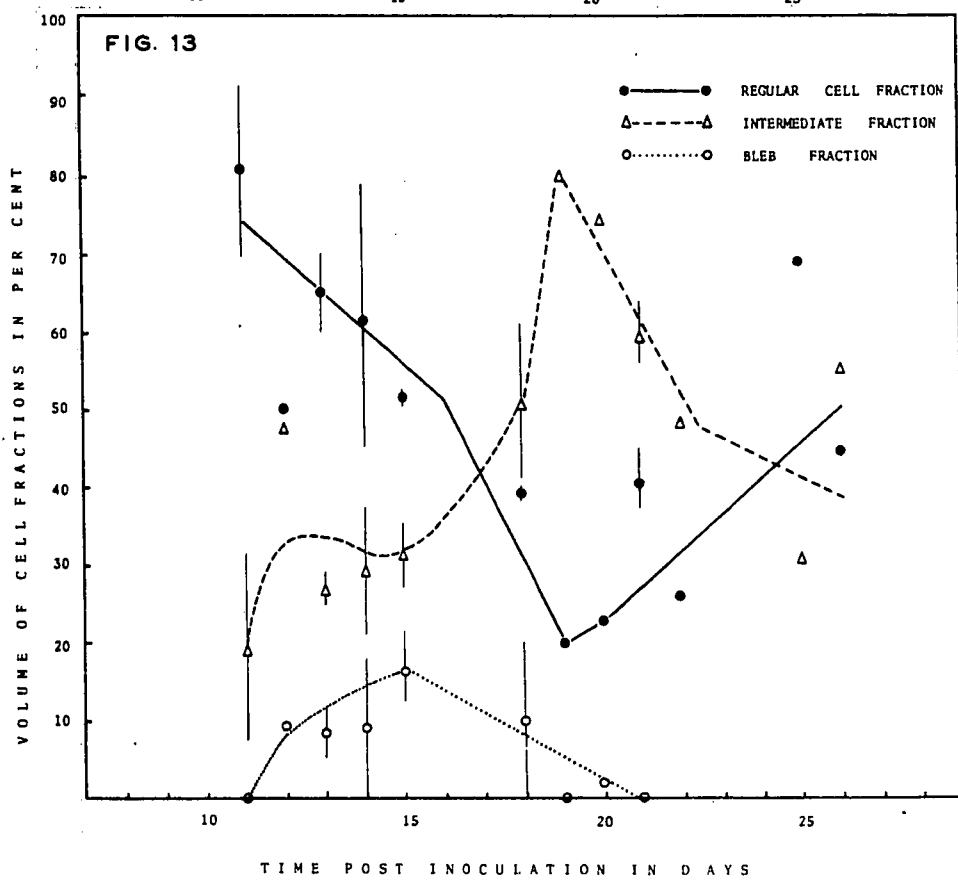
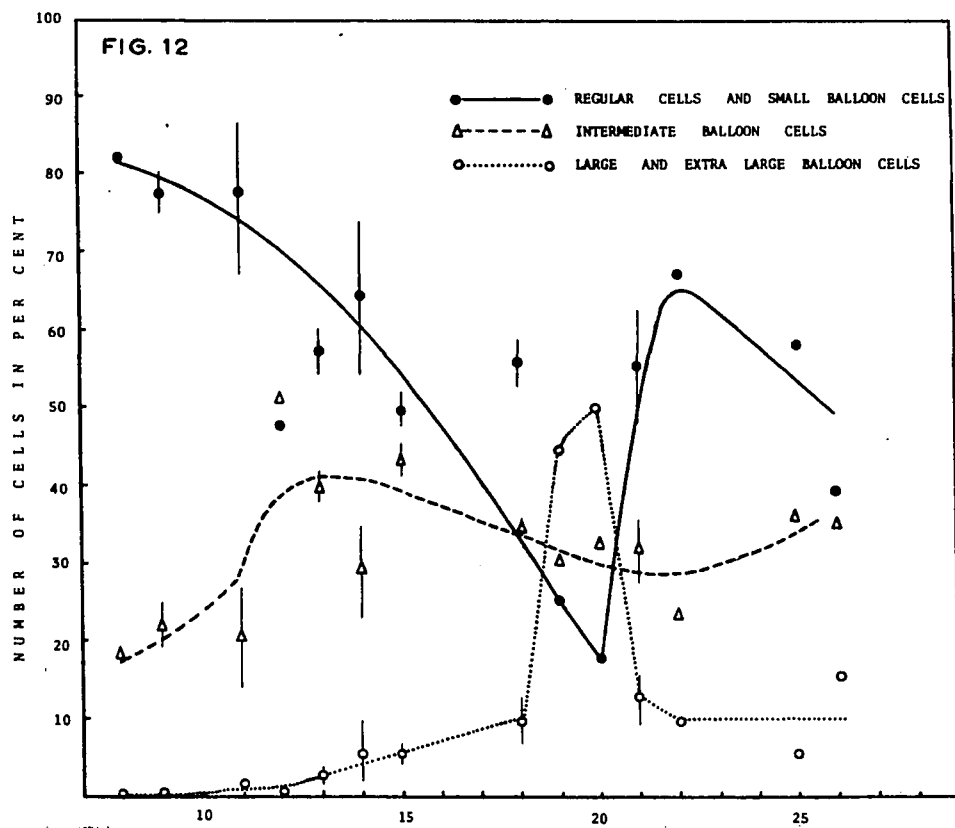
cell fraction	regular cells %	small balloon cells %	interm. balloon cells %	large balloon cells %	extra large balloon cells %	average volume per cell ^b μ^3 $\times 10^{-3}$
regular cell	67	31	2	-	-	3.25
intermediate	4	6	82	8	-	12.5
bleb	-	-	1	74	25	47.9
supernatant	2	1	8	80	9	36.1

^aFrom ascitic fluid (SN-578) collected at 15 days post inoculation.

^bCalculated from the average volume per cell for each cell type (Table III).

Fig. 12 Distribution of cell types in whole ascitic fluid of 25 experimental mice as a function of time. In this figure 100% represents the total number of tumor cells per mouse.

Fig. 13 Distribution of cell fractions in whole ascitic fluid of 25 experimental mice as a function of time. The volume of cell fractions was measured after separation with 10% ficoll. In this figure 100% represents the total volume of tumor cells per mouse.



in the distribution of cell types in whole ascitic fluid (Fig. 12) with trends in the distribution of cell fractions in whole ascitic fluid (Fig. 13). The regular cells were compared to the regular cell fraction, the small and intermediate balloon cells were compared to the intermediate fraction and the large and extra large balloon cells were compared to the bleb fraction. The distribution of cell types was expressed as a percentage of the total tumor cell number and the distribution of cell fractions was expressed as a percentage of the total volume of tumor cells.

i. Distribution of cell types in whole ascitic fluid

At 8 days after inoculation 82% of all tumor cells were regular cells, and the rest of the cells were either small or intermediate balloon cells (Fig. 12). As ascitic fluid accumulated the percentage of regular cells decreased, reaching their lowest value, 21%, at 19 and 20 days after inoculation. At the terminal stage of the experiment the percentage of regular tumor cells had increased again to 49%.

The percentage of small and intermediate balloon cells increased from 18% at day 8 to 40% at day 13, and from then on there was little change (Fig. 12).

From 8 to 18 days after inoculation the percentage of large and extra large balloon cells was small (Fig. 12). Their contribution to the total cell count increased to

47% at day 19 and 20, and decreased rapidly again to 12% at the end of the experiment.

ii. Distribution of cell fractions in whole ascitic fluid

Parallel changes were seen in the distribution of cell fractions. At 11 days after inoculation the regular cell fraction accounted for 81% of the total tumor cell mass, the intermediate fraction making up the other 19% (Fig. 13). The contribution of the regular cell fraction to the total tumor cell mass decreased to 21.5% at day 19 and 20, but increased again to 57% at day 25 and 26.

The contribution of the intermediate fraction to the total tumor cell mass increased from 19% at day 11 to 31% at day 15 (Fig. 13). The percentage volume of the intermediate fraction continued to increase however, reaching a peak value of 77% at day 19 and 20, and decreasing to 43% at day 25 and 26.

The changes during tumor development in the contribution of the bleb fraction to the total tumor cell mass (Fig. 13) did not correspond to the changes in the distribution of large and extra large balloon cells (Fig. 12). At 12 days after inoculation the bleb fraction accounted for 9% of the total tumor cell mass. The contribution of the bleb fraction increased to 17% at day 15, but at day 20 the bleb fraction had almost disappeared.

Fig. 14 indicates the distribution of cell fractions in five groups of mice from experiments conducted during a 6-month period.

d. Determination of average volume per cell in cell fractions

The average volume per cell in each cell fraction was calculated from the distribution of cell types in the cell fractions (Table IV) and from the average volume per cell for each cell type (Table III). Results for the average volume per cell in the three cell fractions (regular cell, intermediate and bleb) and in the supernatant of ascitic fluid collected at 15 days post inoculation are indicated in the last column of Table IV.

Fig. 15 indicates the change in the average volume per cell in each of the three cell fractions, as related to the development of the tumor. In the regular cell fraction the average volume per cell was approximately $4 \times 10^3 \mu^3$ throughout the experiment, except from day 19 to day 21, when the volume per cell increased about twofold.

In the other two fractions the average volume per cell increased with time after inoculation. This increase was very pronounced in the bleb fraction, where the volume per cell was about $26 \times 10^3 \mu^3$ at day 12, and $72 \times 10^3 \mu^3$ at day 18. The average volume per cell in the intermediate fraction increased from $9.2 \times 10^3 \mu^3$ at 12 days to about $34 \times 10^3 \mu^3$ at 19 days and from then on

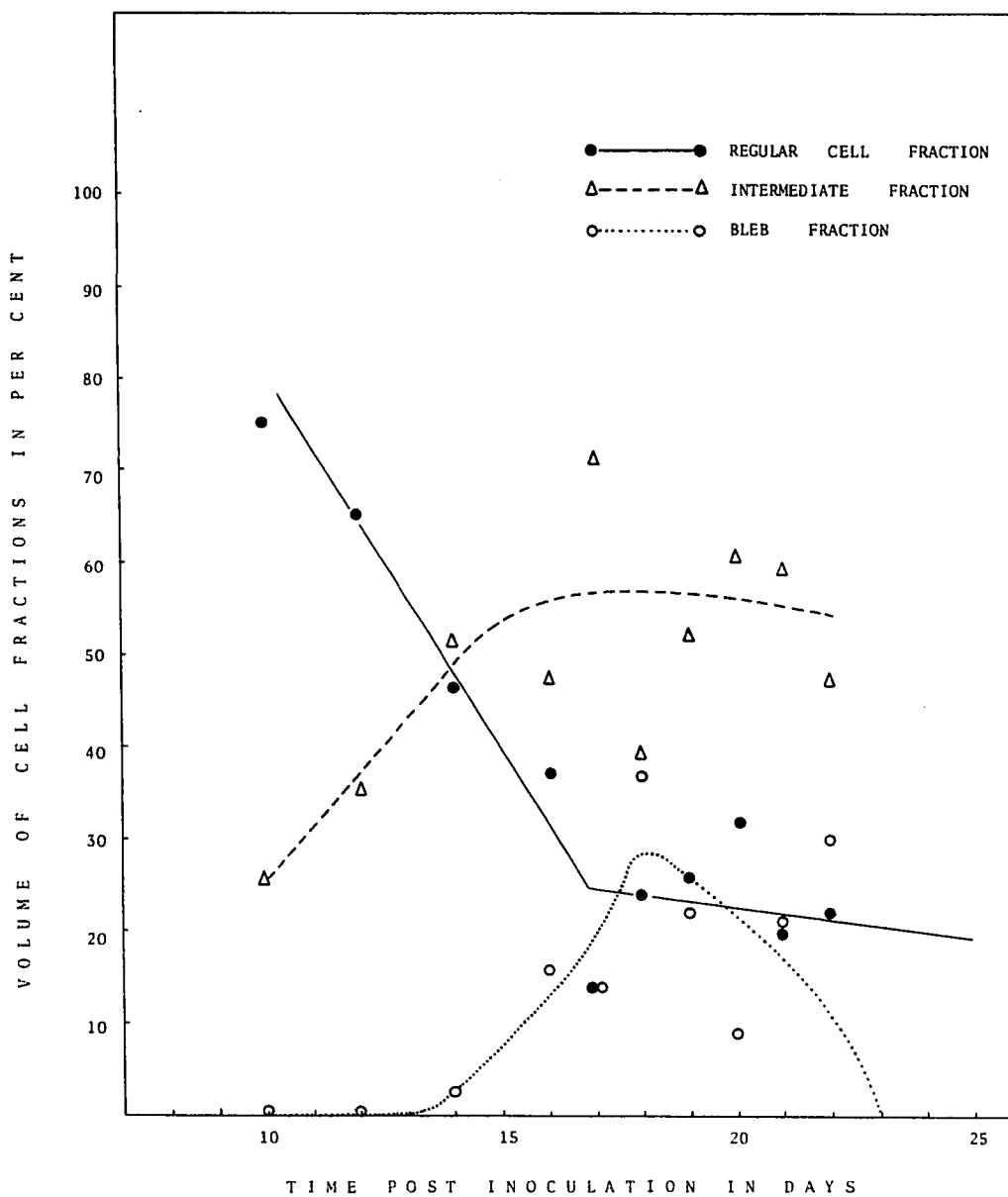


Fig.14 Distribution of cell fractions in whole ascitic fluid as a function of time. Values from 5 groups of mice of experiments conducted during a 6-month period. The procedure for measuring the volume of the cell fractions was the same as in Fig.13.

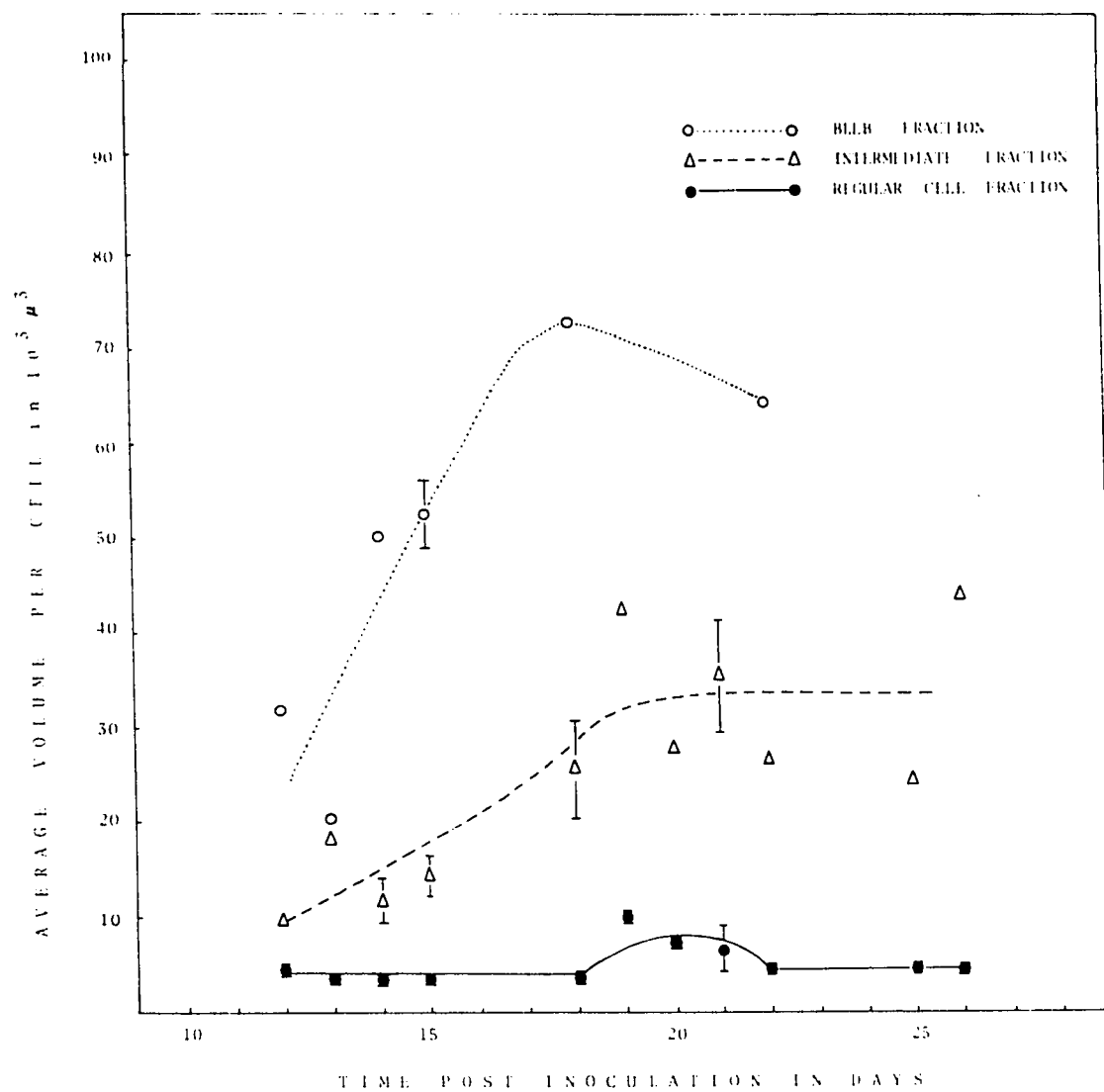


Fig.15 Average volume per cell in cell fractions as a function of time

there was little change.

The average volume per cell of cells that were suspended in the supernatant, although not indicated in Fig. 15, was intermediate between the average volume per cell in the bleb fraction and in the intermediate fraction (Table IV).

4. Changes in the Mitotic Index

The mitotic index served as an indication of the ability of the tumor cells to divide. In whole ascitic fluid the mitotic index was 5.4 ± 0.7 % from 8 to 13 days post inoculation (Fig. 16a), but this value dropped gradually to 1.7% at day 26. The number of cells in prophase and in metaphase was about equal, except towards the end of the third week when the proportion of cells in metaphase increased slightly.

In the regular cell fraction the mitotic index remained at 5.0 ± 0.9 % until day 21, after which it dropped rapidly (Fig. 16b). During the third week of tumor development the proportion of cells in metaphase was greatly increased, perhaps because of an arrest of mitosis at this stage.

The mitotic index in the intermediate fraction was 6.1 ± 0.5 % until day 13, and decreased rapidly to 0.5% at the end of the experiment (Fig. 16c).

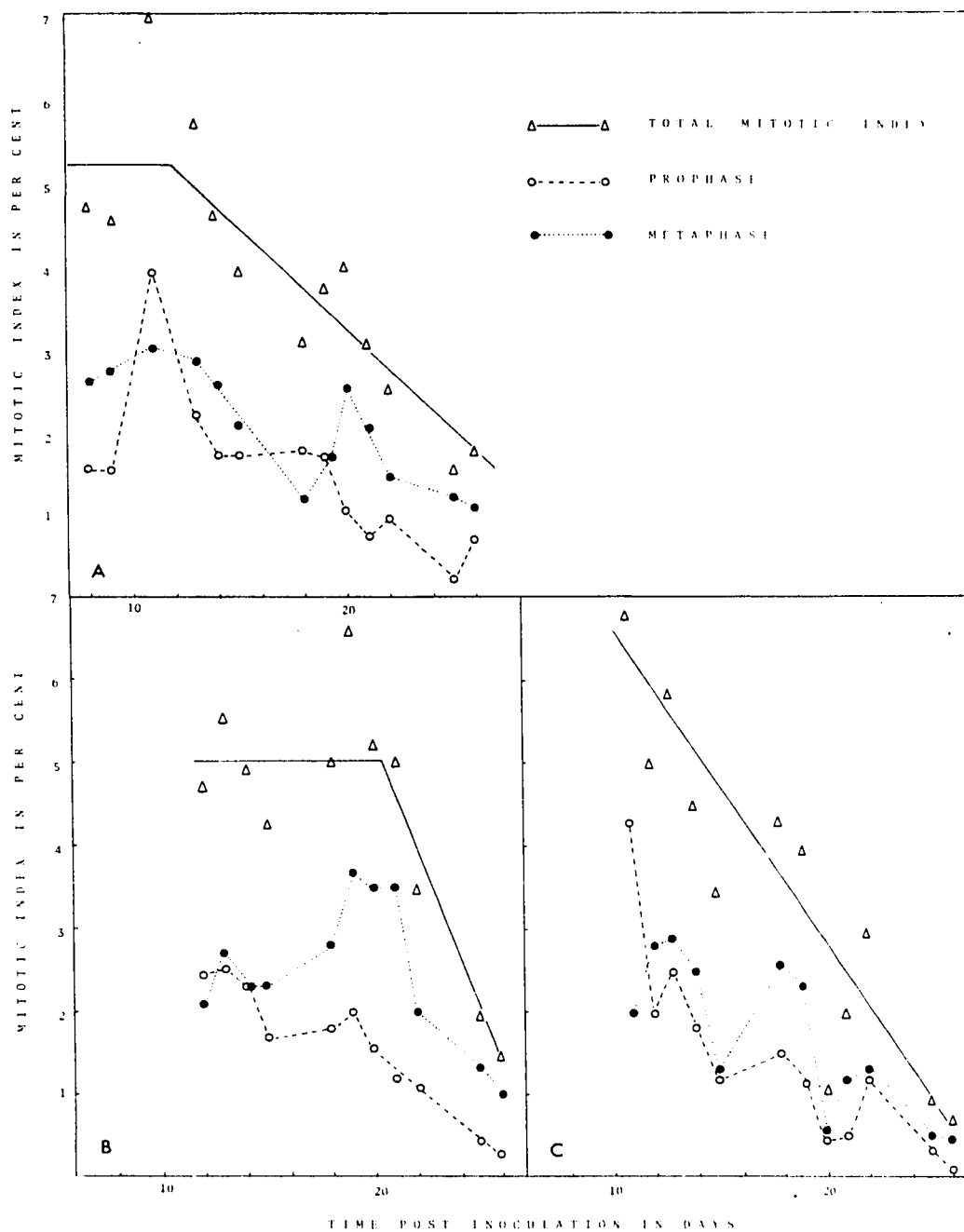


Fig.16 Change in mitotic index and in distribution of mitotic figures during tumor growth.
a. whole ascitic fluid; b. regular cell fraction; c. intermediate fraction.
The bleb fraction was omitted because too few points were available.

In the bleb fraction the values for the mitotic index were similar to those of the intermediate fraction. Since bleb fractions were only available during a short period in tumor development, the data are not presented.

B. Physical and Chemical Investigations

1. Density and Viscosity of the Various Cell Fractions and of the Supernatant

The approximate densities of the various cell fractions¹, as measured in a series of ficoll solutions, are shown in Fig. 17. The density of whole ascitic fluid was 1.033 gm/ml at 8 days after inoculation, decreasing to 1.014 gm/ml at day 19 and to 1.012 gm/ml at the end of the experiment.

Throughout the experiment the density of the regular cell fraction was approximately 1.042 gm/ml. The density of the intermediate fraction decreased from 1.028 gm/ml at 13 days to 1.020 gm/ml at day 19 and remained unchanged thereafter. During the first 18 days after inoculation the supernatant had a density of 1.013 gm/ml, which decreased to 1.010 gm/ml at the end of the experiment. It was difficult to determine the density of the bleb

¹Same group of mice as was used in the experiment on population dynamics.

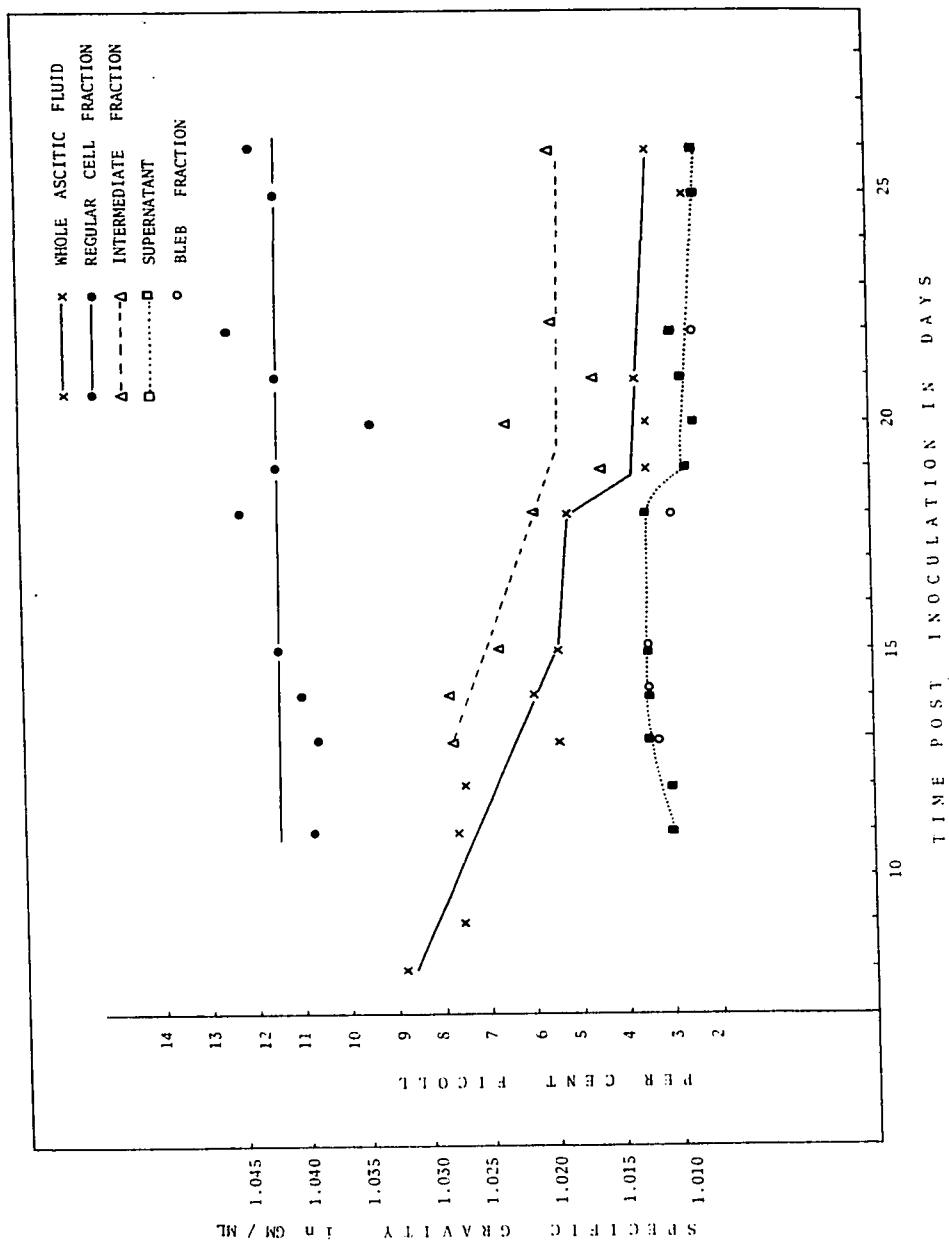


Fig.17 Density of whole ascitic fluid and of various cell fractions as a function of time

fraction, since it was often contaminated with supernatant. Nevertheless the density of the bleb fraction was either the same, or somewhat less than the density of the supernatant.

The difference in cell density between cell fractions was statistically significant¹ ($P < 0.01$), and could be related to the difference in average volume per cell in the cell fractions. As shown in Fig. 18, the density of the cell fractions was inversely proportional to the logarithm of the average volume per cell in the cell fractions, a relationship which could be expressed by the equation

$$d = (1.132 \pm 0.014) - (0.0253 \pm 0.009) \log V \quad (2)$$

If it was assumed that in the balloon cells the volume (a) and density (x) of the true cytoplasm² and nucleoplasm combined was the same as in the regular cells, then the density of the balloon vesicle (y) could be calculated from

¹Tested with an analysis of variance for two variables (Dixon and Massey 1951, p 127). P is the level of significance. The difference in density between days was not significant ($P > 0.1$).

²The term "true cytoplasm" refers to the cytoplasmic rim surrounding the clear area (balloon vesicle) of the balloon cells.

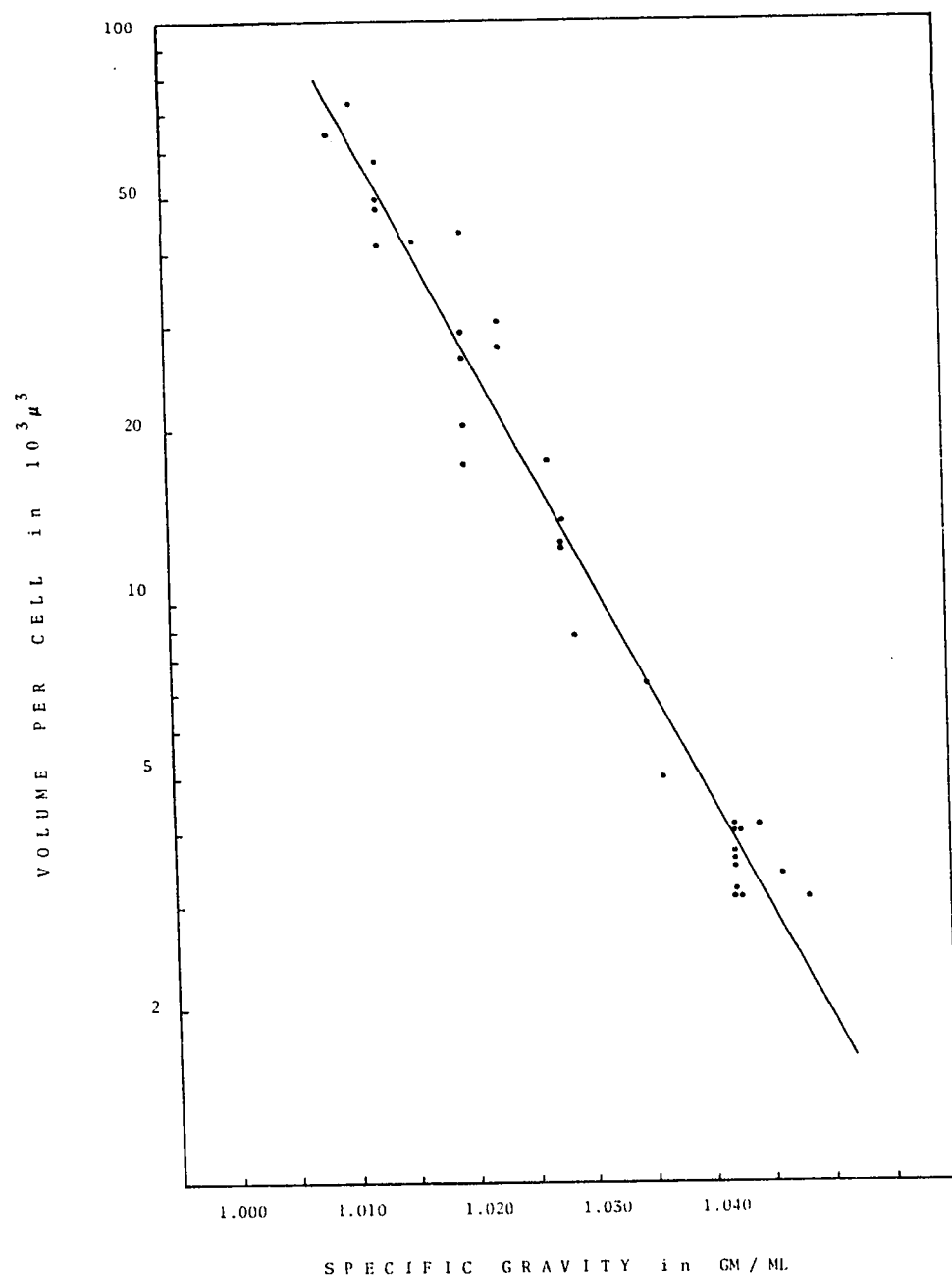


Fig.18 Logarithmic plot indicating the relationship between average volume per cell and density of cell fractions

$$d_b = \frac{ax + (V-a)y}{V} \quad \text{or,} \quad (3)$$

$$y = \frac{(d_b \cdot V) - ax}{V-a} \quad (4)$$

in which d_b is the density of the balloon cell and V is the total volume of the balloon cell. This calculation showed that the density of the balloon vesicle dropped from 1.028 gm/ml in the small balloon cells to 1.009 gm/ml in the large balloon cells (Table V). It should be pointed out that these were maximum values. If in the balloon cells the volume of true cytoplasm and nucleus had increased, the density of the balloon vesicle would be correspondingly lowered.

The relative viscosity of the supernatant, as compared to the viscosity of distilled water, was 1.27 on day 11, 1.12 on day 13 and 1.08 at the end of the experiment (Fig. 19).

Table V
Calculation of the Density of Balloon Vesicles^a

cell density ^b (d _b) gm/ml	cell volume (V) μ^3 x 10 ⁻³	volume of balloon vesicle (V-a) μ^3 x 10 ⁻³	density of balloon vesicle (y) gm/ml
1.042	4	0	0
1.035	8	4	1.028
1.028	14.5	10.5	1.023
1.020	29	25	1.016
1.011	65	61	1.009

^aIt was assumed that the true cytoplasm and nucleoplasm combined had a volume of 4000 μ^3 and a density of 1.042.

^bData from Fig. 17.

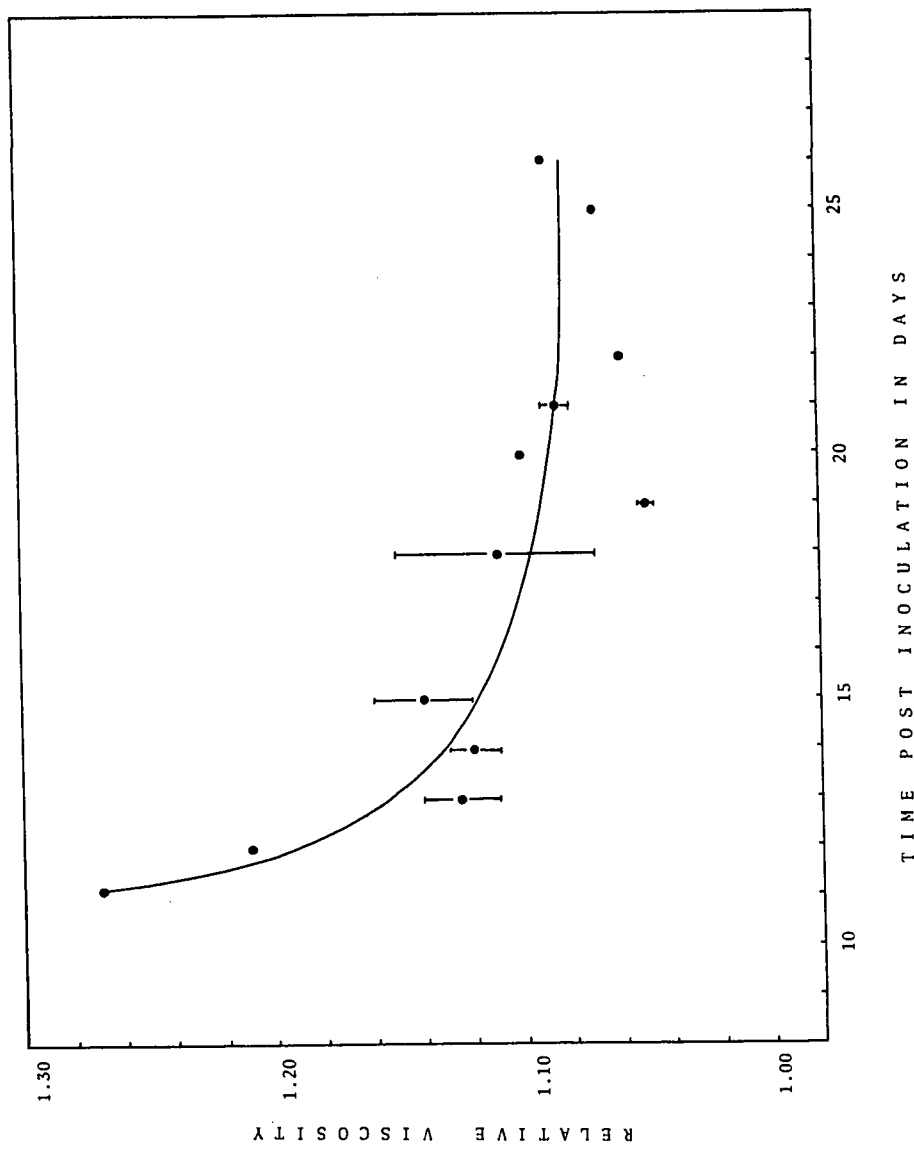


Fig.19 Change in relative viscosity of the supernatant during tumor development.
The viscosity of distilled water at the same temperature was taken as 1.00 .

2. Determination of Protein in Cell Fractions and Supernatant

The protein content per cell was different for the various cell fractions, and always increased with time after inoculation (Fig. 20)¹. In the regular cell fraction the protein content per cell increased from $(4.3 \pm 0.1) \cdot 10^{-7}$ mg at day 12 to $(7.8 \pm 0.7) \cdot 10^{-7}$ mg at day 22. During the same period the protein content per cell in the intermediate fraction increased from $(7.2 \pm 0.6) \cdot 10^{-7}$ mg to $(15.7 \pm 0.5) \cdot 10^{-7}$ mg. There was no bleb fraction at day 12, but from 14 days to 22 days after inoculation the protein content of the bleb fraction increased from $(13.2 \pm 1.0) \cdot 10^{-7}$ mg to $(19.9 \pm 1.8) \cdot 10^{-7}$ mg.

In order to relate protein content to cell size the average volume per cell in each cell fraction was determined from the cell distribution in the cell fractions (Table VI).

The increase in protein content per cell was not as great as the increase in cell volume so that the protein concentration per cell decreased (Fig. 21)². The protein concentration was highest in the regular cell fraction. In the intermediate fraction the protein concentration

¹The difference between cell fractions and days were both statistically significant ($P < 0.01$ and $P < 0.05$ respectively).

²The difference in protein concentration between the three cell fractions was statistically significant ($P < 0.01$).

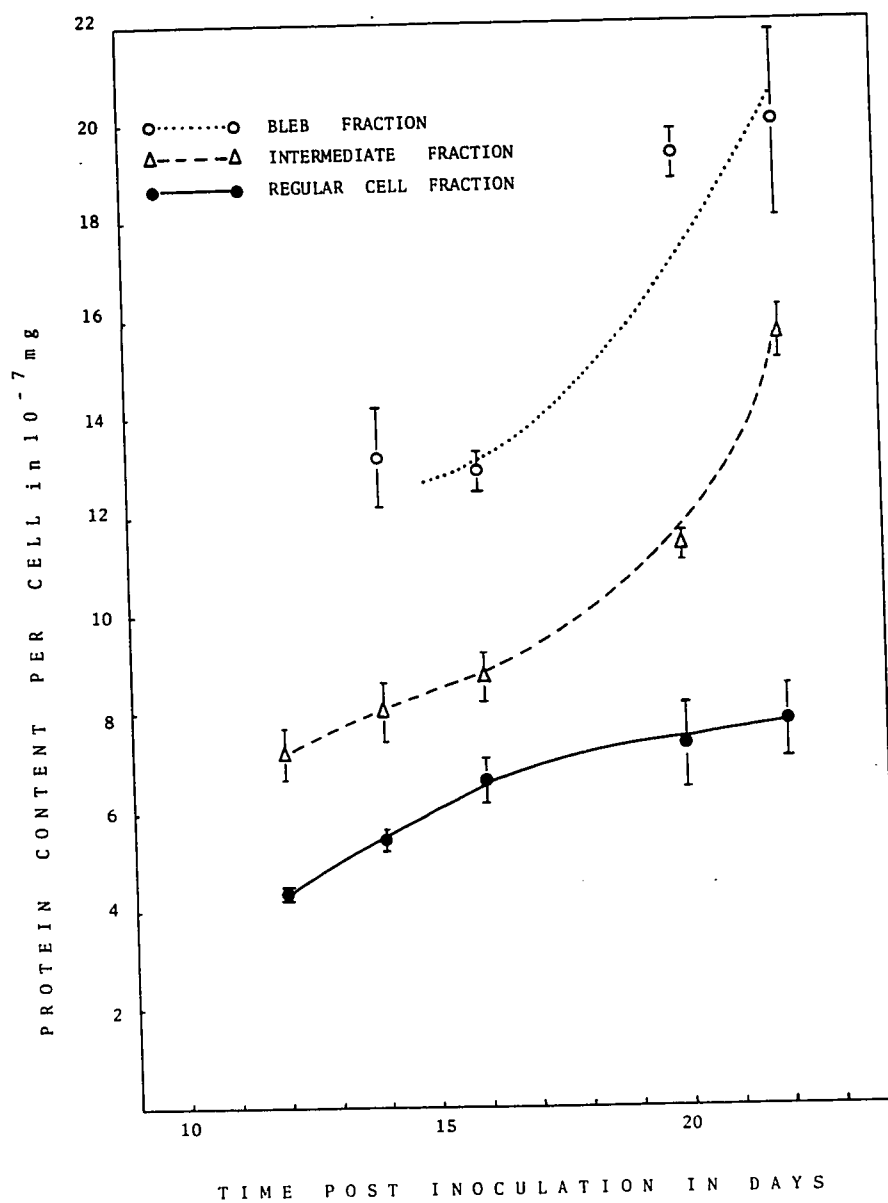


Fig.20 Protein content per cell in cell fractions as a function of time. The mean deviation from the mean of 4 to 5 replicate determinations on the same animal is indicated by vertical bars.

Table VI
Average Volume per Cell in Cell Fractions^a used for
Protein Determination

time post inoculation in days	average volume per cell in		
	regular cell fraction	intermediate fraction	bleb fraction
	μ^3 $\times 10^{-3}$	μ^3 $\times 10^{-3}$	μ^3 $\times 10^{-3}$
12	2.9	6.6	-
14	4.4	11.7	17.4
16	3.0	15.6	33.3
20	6.1	16.1	44.9
22	7.3	25.0	65.0

^aSn-578

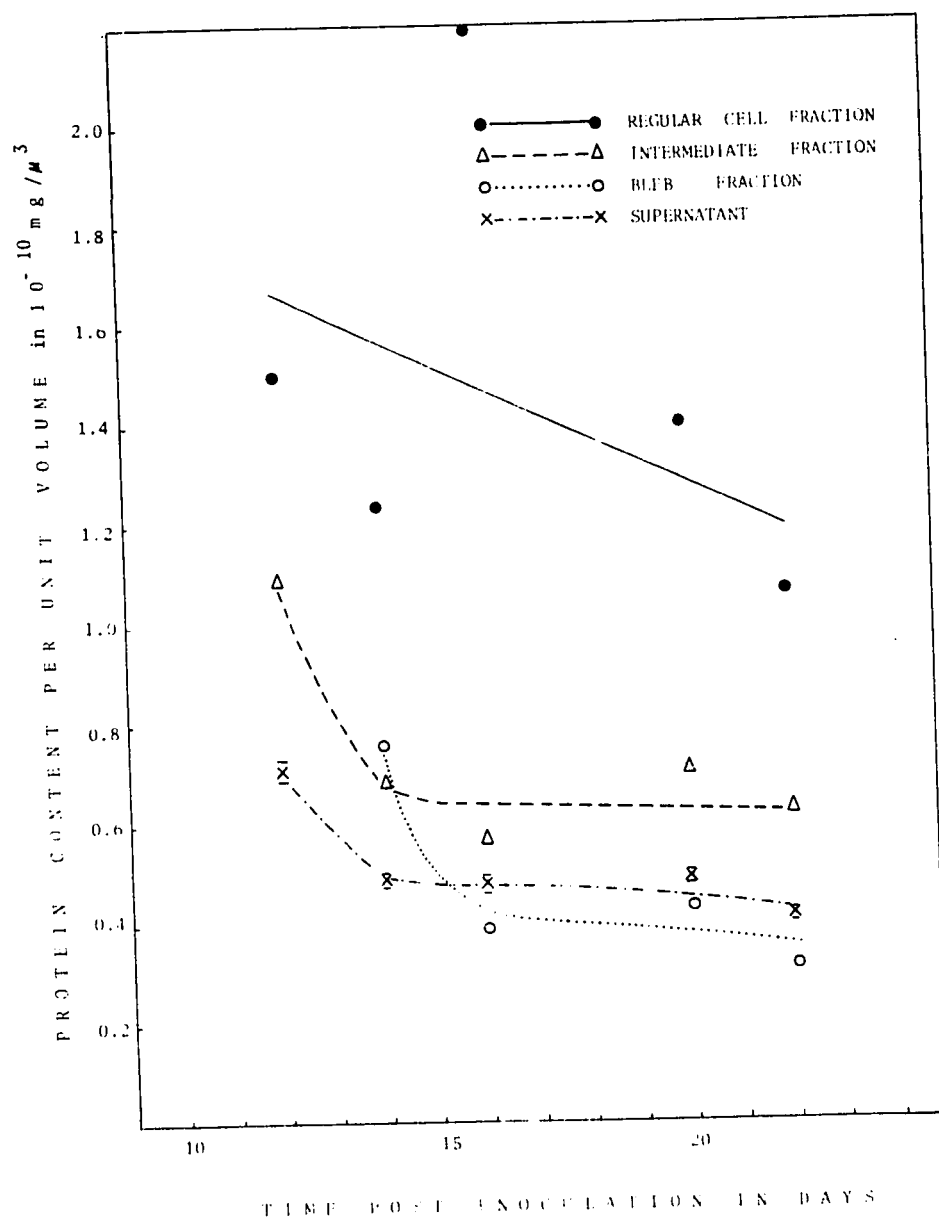


Fig. 21 Protein content per unit volume in cell fractions and supernatant as a function of time

decreased from $1.09 \times 10^{-10} \text{ mg}/\mu^3$ at day 12 to $0.68 \times 10^{-10} \text{ mg}/\mu^3$ at day 14, but after this time little change occurred. The protein concentration in the bleb fraction similarly decreased, from $0.76 \times 10^{-10} \text{ mg}/\mu^3$ at day 14 to $0.39 \times 10^{-10} \text{ mg}/\mu^3$ at day 16, and remained fairly constant thereafter.

In the supernatant the protein concentration decreased from $(0.71 \pm 0.02) \cdot 10^{-10} \text{ mg}/\mu^3$ at day 12 to $(0.49 \pm 0.02) \cdot 10^{-10} \text{ mg}/\mu^3$ at day 14. Further decrease to a value of $(0.41 \pm 0.01) \cdot 10^{-10} \text{ mg}/\mu^3$ occurred between days 20 and 22.

The protein concentration per cell was inversely proportional to the average volume per cell (V) (Fig. 21a), and this relationship could be expressed by the equation:

$$p = (4.77 \pm 0.54) - (0.96 \pm 0.07) \log V \quad (5)$$

where (p) is the protein concentration in $10^{-10} \text{ mg}/\mu^3$. Using the above equation and equation (2), relating cell density to cell volume, the protein concentration could be related to cell density by the equation:

$$p = 38.1 (d - 1.007). \quad (6)$$

Using similar assumptions as were made for the calculation of the density of the balloon vesicle, the protein concentration (y) of the balloon vesicle could be

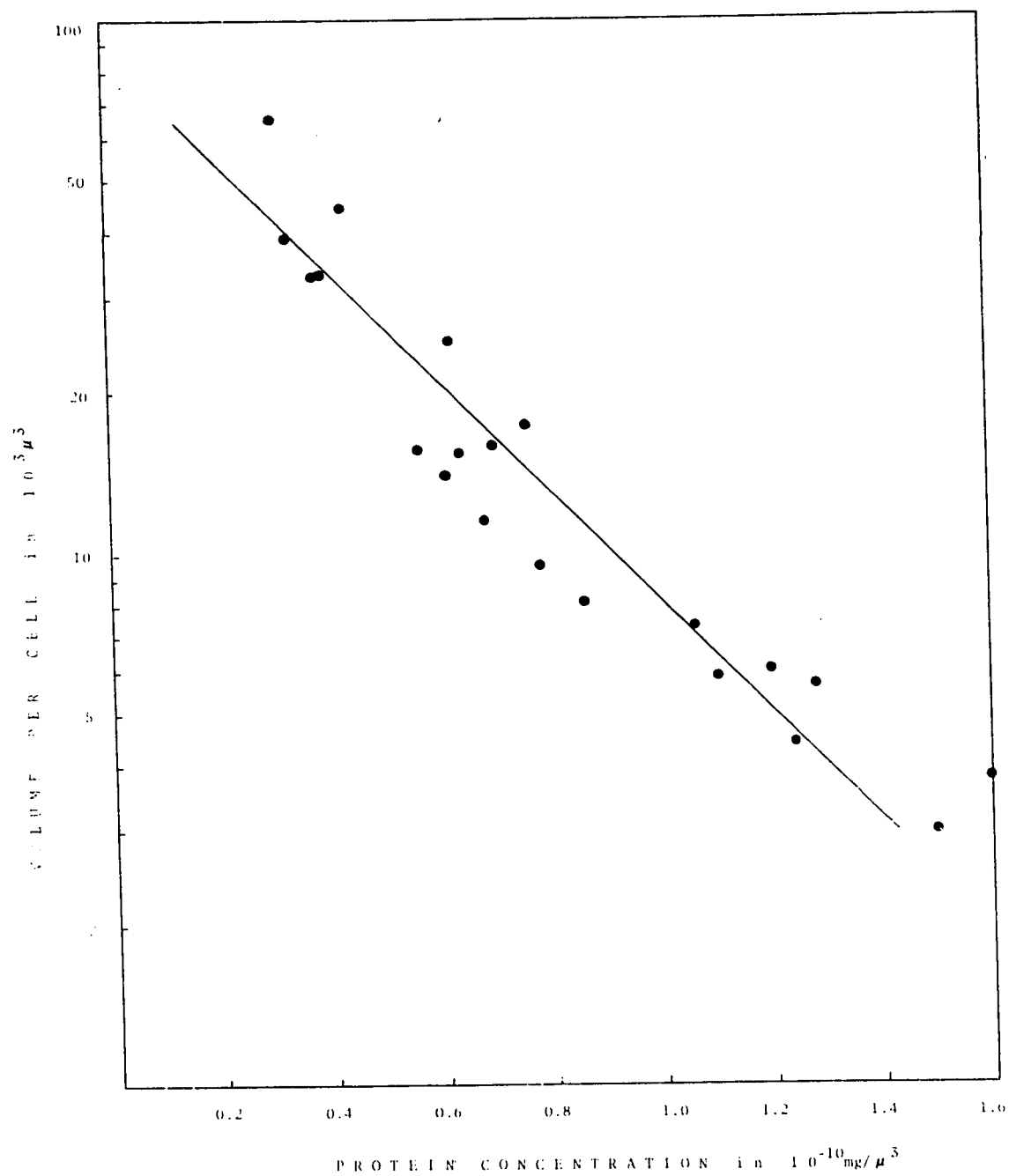


Fig.21 a logarithmic plot indicating the relationship between average volume per cell and protein concentration of cell fractions

calculated from the formula:

$$y = \frac{pV - ax}{V-a}, \quad (7)$$

in which (p) is the protein concentration of the balloon cell, (V) is the total volume of the balloon cell, (pV) is the protein content of the balloon cell and (ax) is the protein content of the regular tumor cells of the same day.

Amongst the small and intermediate balloon cells there was a wide fluctuation in the protein concentration of the balloon vesicle (Table VII). The average value $(0.39 \pm 0.22) \cdot 10^{-10} \text{ mg}/\mu^3$ was not significantly different ($P = 0.10$) from the protein concentration in the balloon vesicle of the large balloon cells, $(0.22 \pm 0.05) \cdot 10^{-10} \text{ mg}/\mu^3$, but there was less fluctuation amongst the latter type of cells.

3. Determination of Dry Weight and Wet Weight of Cell Fractions

The dry weight and wet weight per cell were determined and compared with the protein content per cell and the average volume per cell. The results of two separate experiments showed good agreement (Table VIII), but only the results of the first experiment, which was conducted in duplicate, will be cited in the text.

On the 18th day after inoculation the percentage

Table VII
Calculation of the Protein Concentration of Balloon Vesicles^a

balloon cell volume (V) μ^3 $\times 10^{-3}$	regular cell volume (a) μ^3 $\times 10^{-3}$	protein content of		protein concentration of balloon vesicle (y) mg/μ^3 $\times 10^{10}$
		balloon cell (pV) mg $\times 10^7$	regular cell (ax) mg $\times 10^7$	
6.5	3	7.2	4.4	0.80
12	4	8.0	5.4	0.32
14	6	8.6	7.3	0.16
16	6	9.9	7.3	0.26
16	3	8.7	6.6	0.16
16	6	11.4	7.4	0.40
17	4	13.2	5.4	0.60
25	7	15.6	7.8	0.43
33	3	12.8	6.6	0.21
33	6	12.8	7.3	0.20
39	6	13.0	7.3	0.17
44	6	18.4	7.4	0.29
65	7	20.0	7.8	0.21

^aData from Table VI and Fig. 21; some data from Table VIII are also included.

Table VIII
Wet Weight and Dry Weight in Cell Fractions^a Compared to Protein
Content and Average Volume per Cell

parameter determined	units	bleb fraction ^b	intermediate fraction	cell fraction
wet weight per ml packed cells	in mg	940 (930) ^c	844 ± 12 (859)	915 ± 2 (959)
wet volume per ml packed cells ^d	in μ l	929 (922)	826 ± 12 (840)	878 ± 2 (920)
dry weight per ml packed cells	in mg	39.3 ± 0.8 (35.8)	58.0 ± 0.4 (60.4)	103.7 ± 0.5 (91.4)
per cent dry weight	in %	4.2 ± 0.1 (3.8)	6.9 ± 0.1 (7.0)	11.3 ± 0.1 (9.5)
cell concentration per ml packed cells	in 10^6	16.6 ± 0.15 (18.5 ± 0.2)	39.3 ± 0.6 (43.8 ± 0.5)	86.6 ± 1.0 (148.1 ± 1.5)
wet weight per cell	in 10^{-6} mg	56.8 ± 0.8 (50.5 ± 0.5)	21.5 ± 0.6 (19.6 ± 0.2)	10.6 ± 0.1 (6.5 ± 0.1)
dry weight per cell	in 10^{-6} mg	2.37 ± 0.07 (1.88) ⁻	1.48 ± 0.03 (1.37) ⁻	1.20 ± 0.02 (0.62) ⁻
volume per cell from cell distribution	in $10^3 \mu^3$	33.3 ± 1.6 (38.6)	15.5 ± 0.5 (14.0)	5.7 (6.1)
from hematocrit of 1:25 diluted suspension	in $10^3 \mu^3$	30	17.8	8.1
protein content per cell	in 10^{-6} mg	1.28 ± 0.12 (1.30) ⁻	0.99 ± 0.03 (0.86) ⁻	0.73 ± 0.02

^aThe ascitic fluid (SN-578) was collected at 18 days post inoculation

^bExcept for the first two rows, the difference between cell fractions was always significant ($P < 0.05$)

^cThe numbers in brackets refer to a second experiment, in which ascitic fluid was also collected at 18 days post inoculation

^dCalculated from wet weight per ml and density of cell fractions

dry weight compared to wet weight was 4.2 ± 0.1 % in the bleb fraction, 6.9 ± 0.1 % in the intermediate fraction, and 11.3 ± 0.1 % in the regular cell fraction. In order to calculate the dry weight and wet weight per cell the cell concentration was determined in each cell fraction (Table VIII).

The wet weight per cell was highest in the bleb fraction, $(56.8 \pm 0.8) \cdot 10^{-6}$ mg, and lowest in the cell fraction, $(10.6 \pm 0.15) \cdot 10^{-6}$ mg (Table VIII). Taking the density of the cell fractions into account, the results for the wet weight per cell were somewhat high when compared to the average volume per cell of the same fractions (Table VIII). Part of the discrepancy was probably due to a small amount of contamination with physiological saline in the packed cells, which would increase the total wet weight of the packed cell fractions.

Although the percentage dry weight was lowest in the bleb fraction, the dry weight per cell was the highest in this fraction, $(2.37 \pm 0.07) \cdot 10^{-6}$ mg. In the intermediate fraction the value obtained was $(1.48 \pm 0.03) \cdot 10^{-6}$ mg per cell, and in the cell fraction $(1.20 \pm 0.02) \cdot 10^{-6}$ mg. When these data were compared with the protein content per cell in the same fractions, it was seen that in each case about 65% of the dry weight could be accounted for as protein.

4. Determination of Carbohydrate

a. Total carbohydrate

In order to establish the applicability of the orcinol-sulfuric acid method (Miller 1961) for the determination of total carbohydrate content (as monosaccharides) in ascites cells, the absorption spectra of a monosaccharide (glucose) and a disaccharide (lactose) were compared. Both sugars had been dried over concentrated sulfuric acid.

Fig. 22 shows that the absorption spectra of the two sugars differed. Both sugars had a relatively flat portion between 480 m μ and 500 m μ , but whereas in glucose absorption at 500 m μ was slightly lower than at 480 m μ , the reverse was true for lactose. Furthermore the optical density readings at which the flattened portion was reached varied between the two sugars.

The orcinol method could therefore not be used to determine the absolute monosaccharide content of ascites cells, unless the sugars that were present and the proportion in which they occurred was known (Brückner 1955).

However applying the orcinol method to tumor cells showed that the absorption spectra for the different cell fractions were very similar (Fig. 23). The orcinol method could therefore be used to make a comparison of the relative carbohydrate content in the various cell fractions.

The relative carbohydrate content (expressed as

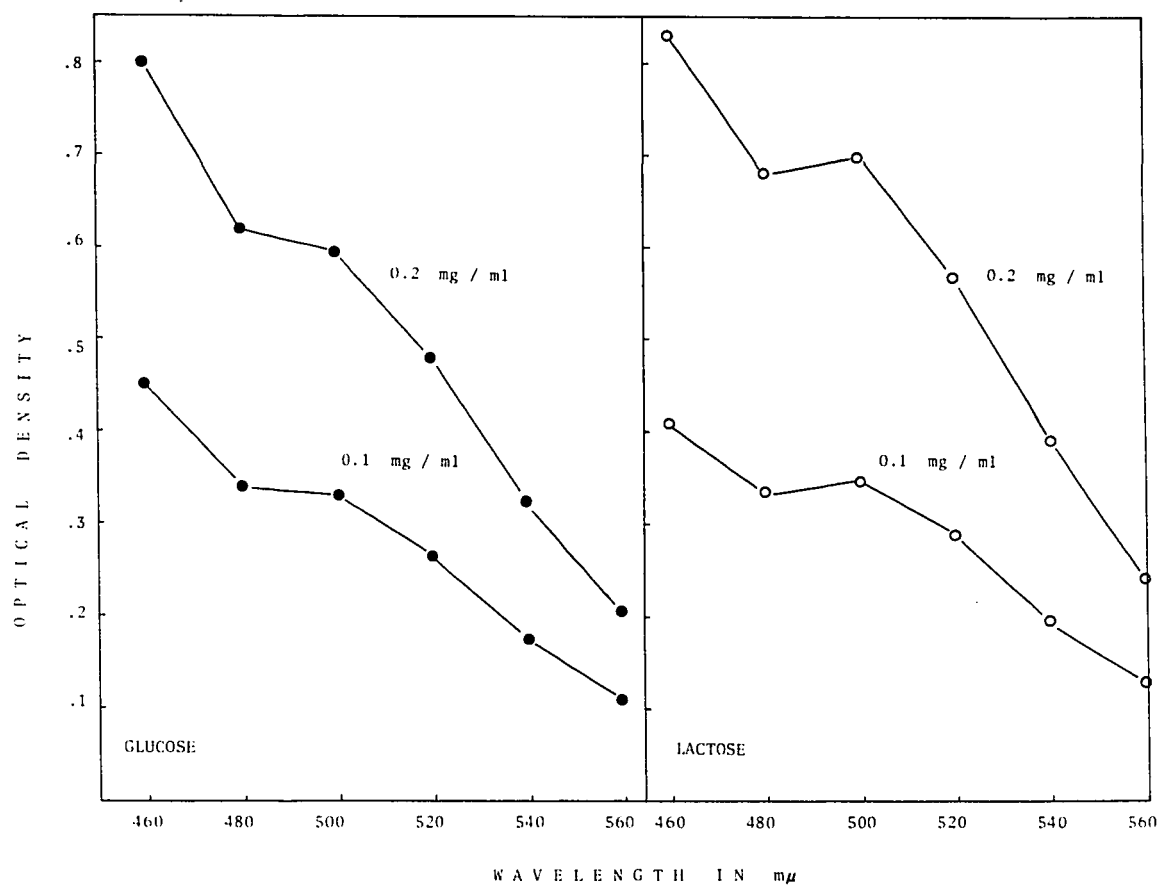


Fig.22 Comparison of absorption spectra of glucose and lactose, using orcinol-sulfuric acid reagent (Miller 1961) for the determination of carbohydrate

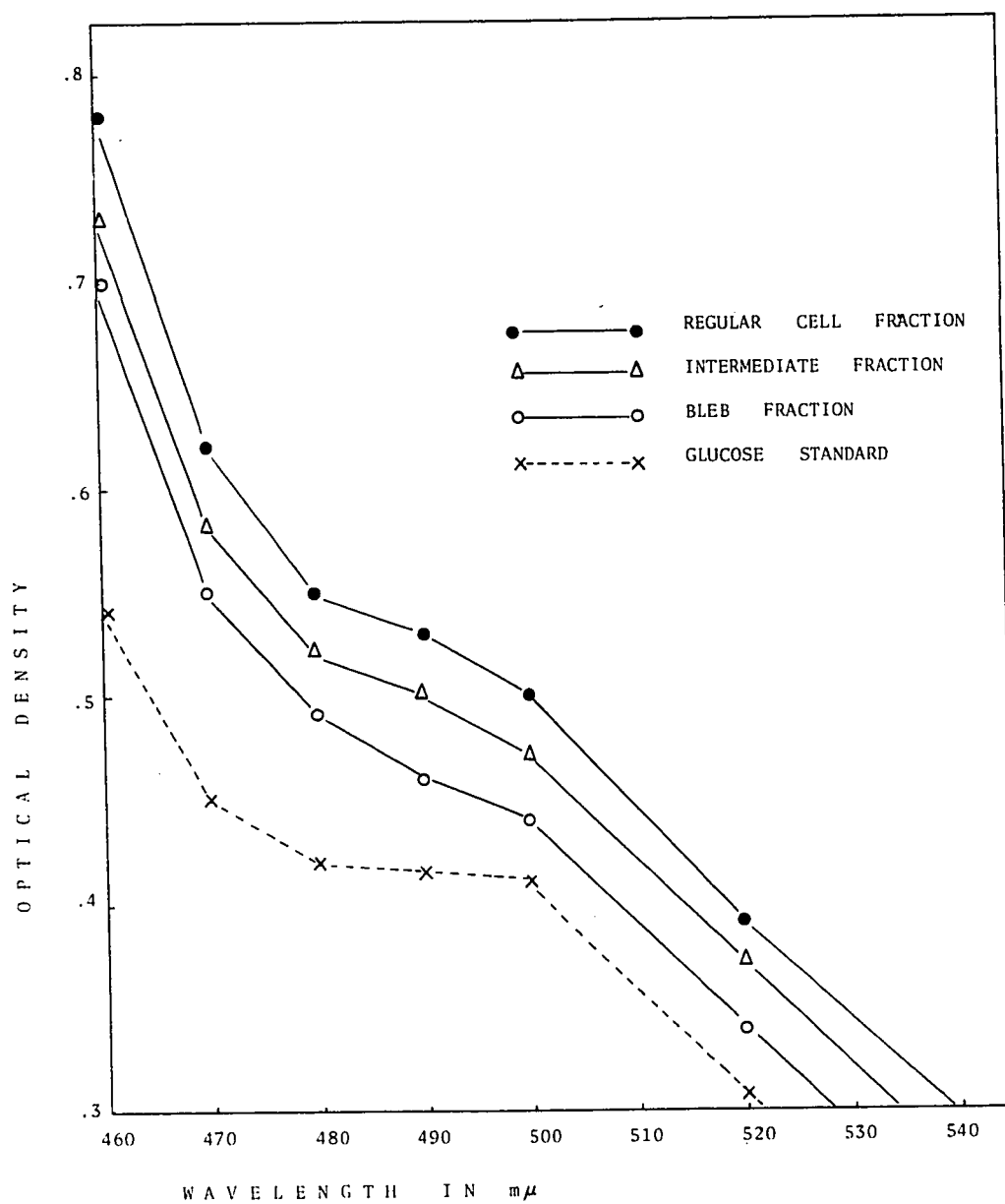


Fig. 23 Comparison of absorption spectra of various cell fractions using orcinol-sulfuric acid reagent for the determination of carbohydrate. The absorption spectrum of glucose is also shown.

monosaccharide) of acetone powder that had been prepared from the three cell fractions is shown in Table IX. The acetone powder was prepared from pooled ascitic fluid of the same mice as were used for the experiment tabulated in Table VIII. The differences between the carbohydrate content of the three acetone powders were not significant (Table IX, column 2). The carbohydrate content per cell was highest in the bleb fraction, and lowest in the regular cell fraction (Table IX, column 5), whereas the carbohydrate concentration was lowest in the bleb fraction and highest in the regular cell fraction (Table IX, column 7).

Table X gives the relative carbohydrate content (expressed as monosaccharide) in the three cell fractions and in the supernatant of two mice, which had been inoculated 17 days previously. This time no acetone powder was prepared, but cell suspensions were used directly.

In the first mouse the carbohydrate content per cell was 2.6×10^{-8} mg, 4.5×10^{-8} mg and 6.7×10^{-8} mg for the cell-, intermediate- and bleb-fraction respectively. In the second mouse these values in the same order of sequence were 4.1×10^{-8} mg, 6.3×10^{-8} mg and 10.0×10^{-8} mg. The difference between the results of the two mice could probably be accounted for by the fact that the tumor in the first mouse was at an earlier stage of development,

Table IX
Carbohydrate Content of Acetone Powder from Various Cell Fractions^a

cell fraction	carbohydrate per mg acetone powder ^b mg x 10 ³	acetone powder per ml packed cells mg	cell count per ml packed cells x 10 ⁻⁶	carbohydrate per cell mg x 10 ⁸	average volume per cell μ^3 x 10 ⁻³	carbohydrate concentration mg/ μ^3 x 10 ¹¹
bleb	44 ± 1	23.3 ± 0.6	16.6 ± 0.15	6.17 ± 0.36	33.3 ± 1.6	0.18 ± 0.01
intermed.	50 ± 2	46.5 ± 1.4	39.3 ± 0.6	5.92 ± 0.48	15.5 ± 0.5	0.38 ± 0.04
cells	45 ± 2	76.5 ± 2.0	86.6 ± 1.0	3.98 ± 0.25	5.71	0.68 ± 0.04

^aSN-578, 18 days after inoculation

^bSpectrophotometer readings were taken at a wavelength of 500 m μ

Table X
Carbohydrate Content of Various Cell Fractions^a

cell fraction	carbohydrate per ml packed cells ^b mg	cell count per ml packed cells x 10 ⁻⁶	carbohydrate per cell mg x 10 ⁸	average volume per cell ^c μ^3 x 10 ⁻³	carbohydrate per unit volume mg/ μ^3 x 10 ¹¹
No. 1 mouse					
bleb	2.54 ± 0.04	38.0	6.74	13.0	0.52
int.	2.38 ± 0.13	54.1	4.53	12.7	0.36
cell	3.03 ± 0.09	113.7	2.56	4.3	0.59
sup.					0.123 ± 0.001
No. 2 mouse					
bleb	1.71 ± 0.12	17.5	10.0	21.2	0.47
int.	2.61 ± 0.06	41.2	6.3	14.1	0.45
cell	4.60 ± 0.14	107.5	4.09	6.7	0.61
sup.					0.127 ± 0.002

^aSN-578, 17 days after inoculation

^bSpectrophotometer readings were taken at 480 mμ

^cFrom cell count distribution

as was indicated by the smaller size of the tumor cells (Table X, column 5).

The average carbohydrate concentration in the cell fractions of the two mice was $(0.60 \pm 0.04) \cdot 10^{-11}$ mg/ μ^3 in the regular cell fraction, $(0.40 \pm 0.04) \cdot 10^{-11}$ mg/ μ^3 in the intermediate fraction and $(0.49 \pm 0.05) \cdot 10^{-11}$ mg/ μ^3 in the bleb fraction. The difference between the intermediate fraction and the bleb fraction was not significant ($P > 0.1$). The average carbohydrate concentration of the supernatant was $(0.125 \pm 0.002) \cdot 10^{-11}$ mg/ μ^3 .

There was generally good agreement between the results presented in Tables IX and X. However in the bleb fraction the carbohydrate concentration was higher when carbohydrates were determined in a cell suspension than when first an acetone powder was prepared. Part of this discrepancy was also due to the smaller volume of the balloon cells in the bleb fractions of Table X.

b. Chromatography

Multiple ascending paper chromatography of the acid hydrolysates prepared from acetone powder revealed no difference in composition between the various cell fractions (Table XI). In all cell fractions a fast moving sugar was present, which was tentatively identified as fucose. When fucose was added to the acetone powder prior to hydrolysis, it gave a spot that overlapped with the

Table XI

R.F.-Values of Carbohydrates in Acetone-powder Hydrolysates of Cell Fractions and R.F.-Values of Carbohydrate Standards^a

carbohydrate	R.F. value	
	hydrolysate	direct
cell fractions		
fucose	0.17-0.18	
glucose or galactose	0.11-0.12	
unidentified	0.05	
standards		
fucose	0.17-0.19	0.17-0.19
galactose	0.10-0.12	
glucose	0.11-0.13	0.11-0.13
glucuronic acid	0.10-0.11	0.18-0.20
galacturonic acid	0.09-0.12	0.10-0.13
D-glucosamine-HCl	0.10-0.11	0.06-0.11
N-acetyl glucosamine	0.08-0.10	0.16-0.17
lactose		0.06

^aThe R.F. values were obtained after multiple ascending development in butanol-acetic acid-water (4:1:5 V/V).

unknown. Other sugars that were known to have RF values in the neighborhood of the unknown (glucuronic acid, N-acetyl glucosamine) were also added as an internal standard, but upon acid hydrolysis they were broken down in such a way that the fast moving component disappeared.

A second spot present in all cell fractions had an RF value closely corresponding to glucose and galactose. Sometimes a third spot with an RF value less than lactose was found. This component was not identified.

C. Light Microscopic and Electron Microscopic Observations

1. Cytochemical Staining

a. Lipids

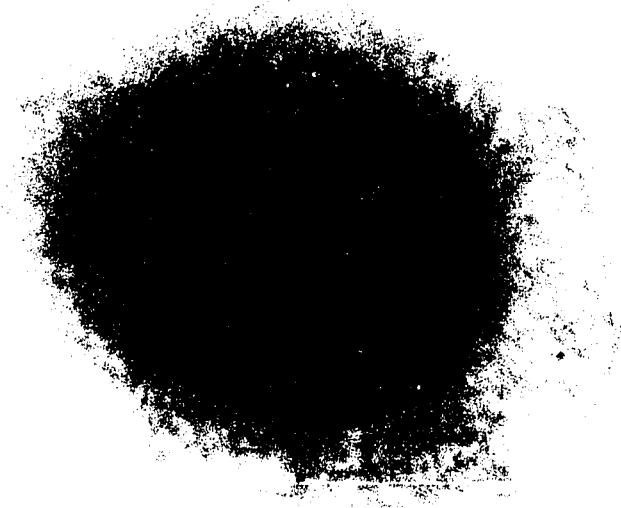
The large clear areas (balloon vesicles) of the balloon cells were not stained with either osmium tetroxide or with oil red O. However both methods for lipids revealed the presence of lipid droplets, located mainly around the nucleus of the balloon cells (Figs. 24 and 25). Sometimes lipid droplets were also found in the regular tumor cells.

b. Carbohydrates

There was no evidence for the presence of carbohydrate in the balloon vesicles using either the PAS method or the alcian blue method. There was however a

Fig. 24 Large balloon cell fixed and stained with osmium tetroxide - ethyl gallate. Note the perinuclear lipid droplets. 1462 x.

Fig. 25 Balloon cells stained with oil red O. Location of red stained lipid droplets is the same as in Fig. 24. 1462 x.



24



25

thin rim of PAS-positive material around the lipid droplets, so that the highly refractile lipid droplets may at least partially consist of glycolipids. With the alcian blue method (without the use of a counterstain) the outline of the cells was blue, indicating the presence of acid mucopolysaccharides. No other portions of the balloon cells were stained with this dye.

c. Nucleic acids

The methyl green-pyronin method and the azure-B method gave similar results for the distribution of RNA and DNA. Large amounts of RNA were present in the cytoplasm of regular tumor cells and the small and intermediate balloon cells. In the balloon cells the RNA was located in a cytoplasmic rim close to the nucleus, as well as in the nucleoli. This RNA could be removed by treating the sections with ribonuclease. The nucleoli of the large balloon cells stained strongly for RNA, but the staining of the cytoplasm was pale.

d. Total protein

Staining with fast green at low pH showed that the protein concentration of the balloon cells was highest in the nucleus and in the rim of cytoplasm surrounding the nucleus. The clear area of the balloon cells was not stained.

2. Light Microscopic Cytology

a. Thin sections

Good cytological detail could be seen in 1- μ thick sections, cut from vestopal embedded material. Fig. 26 shows several regular tumor cells and two small balloon cells. In the small balloon cell, in the upper part of the photograph, 3 small balloon vesicles were present (although only 2 are shown in this section). In the other small balloon cell the balloon vesicle had a fuzzy border, which was probably due to the presence of microvilli.

b. Alpharadiography

Alpharadiographs of balloon cells showed that the nucleus and small rim of outer cytoplasm absorbed alpha particles, thereby preventing blackening of the emulsion at those sites (Fig. 27). The clear vesicle of the balloon cells did not stop alpha particles to an appreciable extent, since the emulsion was as much blackened as the background. The dry mass of the clear vesicle must therefore be very low compared to the mass of the nucleus and small rim of outer cytoplasm.

c. Neutral red uptake

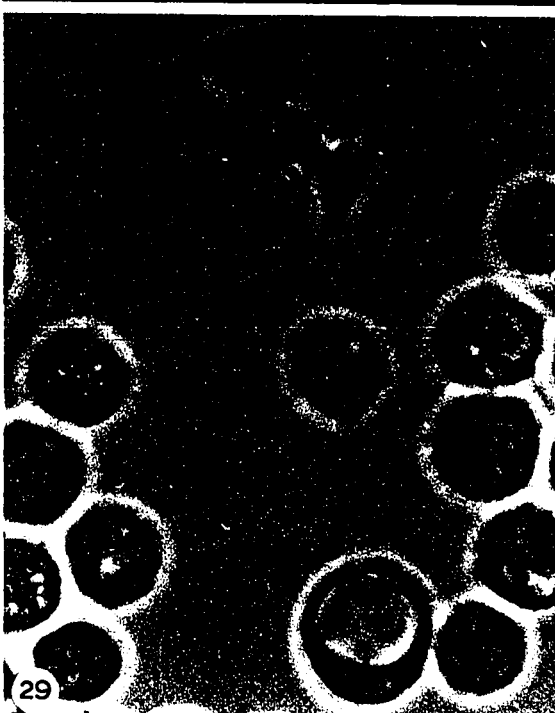
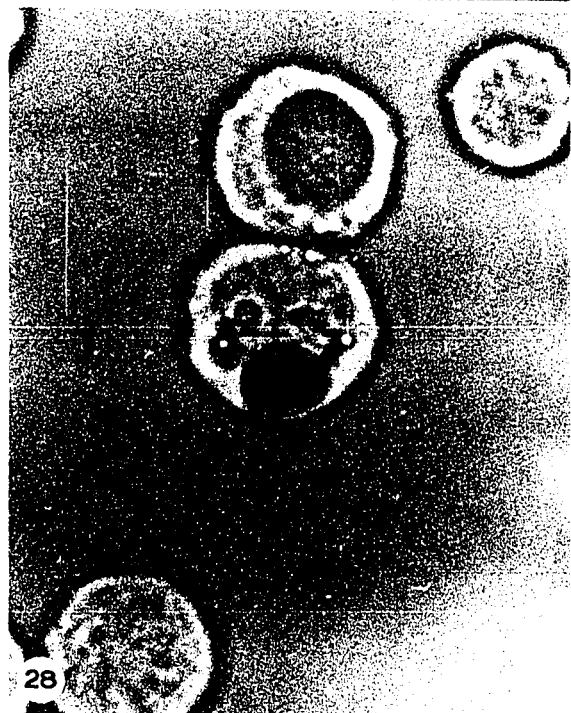
Neutral red was injected into the peritoneal cavity of mice bearing the Ehrlich ascites tumor. Within 5 to 10 min. less than 1% of the tumor cells had taken up

Fig. 26 Section through regular tumor cells and small balloon cells, which were fixed in osmic acid and embedded in vestopal. Section thickness was 1 μ ; toluidine blue-malachite green-basic fuchsin. 1100 x.

Fig. 27 Alpharadiograph of balloon cells. 112 x.

Fig. 28 Uptake of neutral red into the balloon vesicle of a small balloon cell (arrow). 1300 x.

Fig. 29 Uptake of neutral red into one vesicle of a large balloon cell (arrow). The larger adjacent vesicles of the same cell did not take up the dye. 972 x.



the dye. The dye was rapidly excreted, so that if ascitic fluid was removed from the mice at 1 to 2 hrs. after injection no evidence of neutral red uptake was found. The dye remained however for several hours in tumor cells that had been removed from the mice 5 to 10 min. after injection and were kept at room temperature.

In cells that had taken up neutral red, the dye was frequently located in the clear vesicle of the small balloon cells (Fig. 28). In intermediate balloon cells compartmentalization of the balloon vesicle was frequently seen, one compartment taking up neutral red while an adjacent compartment remained colorless (Fig. 29). In large balloon cells the balloon vesicle was never colored red by the dye, but often small red vesicles were seen in the outer rim of cytoplasm.

3. Ultrastructural Observations

a. Regular tumor cells

In the early stages of tumor development (10 days after inoculation) the regular tumor cells had many of the characteristics of normal free floating cells. The tumor cells were spherical in shape, with the nucleus placed centrally or slightly to the side (Fig. 30). The nucleus was somewhat lobed, and several nucleoli were usually present.

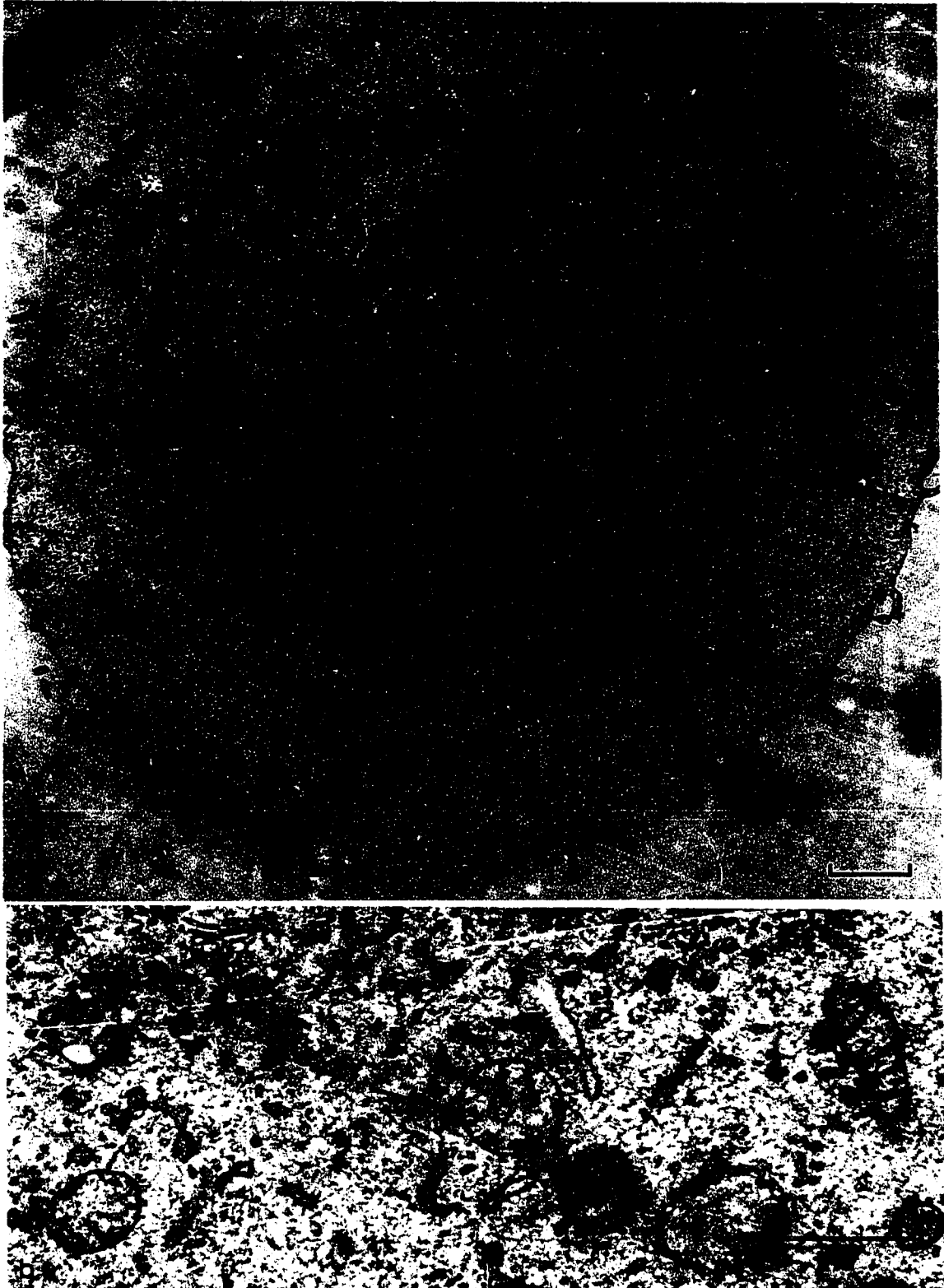
Fig. 30 a. Section through a regular tumor cell, collected at 10 days post inoculation. The nucleus has prominent nucleoli, and most of the chromatin is loosely dispersed. The cell has an extensive Golgi apparatus. Pale mitochondria with few cristae are distributed throughout the cytoplasm. In the central area of the cell, which is enlarged in b, small pale vesicles that contain a dense 300 Å core are seen and are frequently associated with mitochondria (single arrow). There are few strands of endoplasmic reticulum, and most of the ribosomes are dispersed freely throughout the cytoplasm. Irregularly oriented microvilli are found at the cell surface.

a. 13,200 x.

b. 32,370 x.

Approximate cell volume $741 \mu^3$

Unless indicated otherwise, the bar shown in all the following electron micrographs represents 1 μ .



Throughout the cytoplasm were pale and slender mitochondria with few cristae. Most of the ribosomes appeared to be free, but some were associated with endoplasmic reticulum. An abundance of small vesicles was seen in the Golgi region, and similar vesicles were sometimes arranged in a circle. A number of isolated small vesicles, containing a dense 300 Å core, were also seen in the Golgi region, and were frequently associated with mitochondria (Fig. 30b-arrow). Lysosomes were not conspicuous and no fat droplets were seen. The outer portion of the cytoplasm (ectoplasm) was sometimes devoid of organelles (Fig. 30).

Associated with the cell membrane was a large number of irregularly oriented microvilli, about 0.5 μ in length and about 0.09 μ in diameter. These were not upward folds of cytoplasm, since circular cross-sections were frequently observed. Sometimes the microvilli appeared to entrap droplets of fluid (Fig. 30-double arrow), as if engaged in pinocytosis.

At 17 days after inoculation the appearance of the regular tumor cells was about the same, except that many cytoplasmic evaginations projected into the nucleus (Fig. 31). Surrounding these evaginations was much condensed chromatin. The nucleoli were well differentiated and stained pale gray. A number of secondary

Fig. 31 Section through a regular tumor cell collected at 17 days post inoculation. Many evaginations of the cytoplasm extend into the nucleus. Much of the chromatin is condensed at the nuclear membrane, surrounding cytoplasmic invaginations, or around the well differentiated nucleoli. A few lysosomes are seen in the central part of the cell and in the lower right corner (arrows).
8,775 x.

Approximate cell volume: $2,070 \mu^3$



lysosomes could be observed. A similar appearance of the regular tumor cells was seen at day 21 (Fig. 32). Here the secondary lysosomes were even more prominent, and in the lower part of the cell a fat droplet could be seen.

b. Balloon cells

In samples obtained at 14 days after inoculation one or more large vesicles (3 to 15 μ in diameter) were observed in the small and intermediate balloon cells (Figs. 33 to 39). These large vesicles were called balloon vesicles. Irregularly oriented microvilli projected into the lumen of the balloon vesicles. The contents of the balloon vesicles was electron transparent. Dispersed throughout the vesicle were thin strands of an electron opaque material.

In the cells with the larger balloon vesicles (Figs. 38 and 39) the nucleus was pushed to the periphery of the cell, and frequently deep cytoplasmic evaginations projected into the nucleoplasm. The rim of true cytoplasm surrounding the balloon vesicle was sometimes very thin (Fig. 37-arrow).

The true cytoplasm of the small balloon cells was similar to that of the regular tumor cells. It contained about the same number of mitochondria, which were similar in shape and size to those of the regular tumor cells. No obvious change was observed in the

Fig. 32 Section through a regular tumor cell collected at 21 days post inoculation. The appearance of the nucleus is the same as in Fig. 31. Note the abundance of lysosomes. The arrow indicates a fat droplet. 14,022 x.



Fig. 33 Small balloon cell collected at 14 days post inoculation in which a small balloon vesicle, about 3 μ in diameter, can be observed. Several slender microvilli project into the lumen of the electron transparent balloon vesicle. Note clusters of lysosomes at the lower left and the upper right of the photograph. Three prominent nucleoli and numerous cytoplasmic invaginations are seen in the nucleus. Small vesicles with a dense 300 $\text{\AA}$ core are indicated by arrows. 11,000 x.

Approximate cell volume:	960 μ^3
Approximate vesicular volume:	12 μ^3
Volume of true cytoplasm and nucleoplasm:	948 μ^3



Fig. 34 a. Small balloon cell collected at 14 days post inoculation, in which two clear balloon vesicles, each about $4\ \mu$ in diameter, can be observed. 10,340 x.

b. The enlarged portion shows an area of invagination of the cell surface, closely associated with the upper balloon vesicle. 20,680 x.

Approximate cell volume:	$1,507\ \mu^3$
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Approximate total vesicular volume:	$66\ \mu^3$
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Volume of true cytoplasm and nucleoplasm:	$1,441\ \mu^3$
---	----------------



Fig. 35 Small balloon cell collected at 14 days post inoculation, in which three balloon vesicles ranging from 2 μ to 4 μ in diameter can be observed. Four fat droplets are seen in the upper right corner. The single arrow and the inset indicate vesicles surrounded by a dense, 300 Å-thick membrane. The double arrow points at a small vesicle with a dense 300 Å core. 9,350 x.

Inset: 25,500 x.

Approximate cell volume: 1,597 μ^3

Approximate total vesicular volume: 55 μ^3

Volume of true cytoplasm and nucleoplasm: 1,542 μ^3

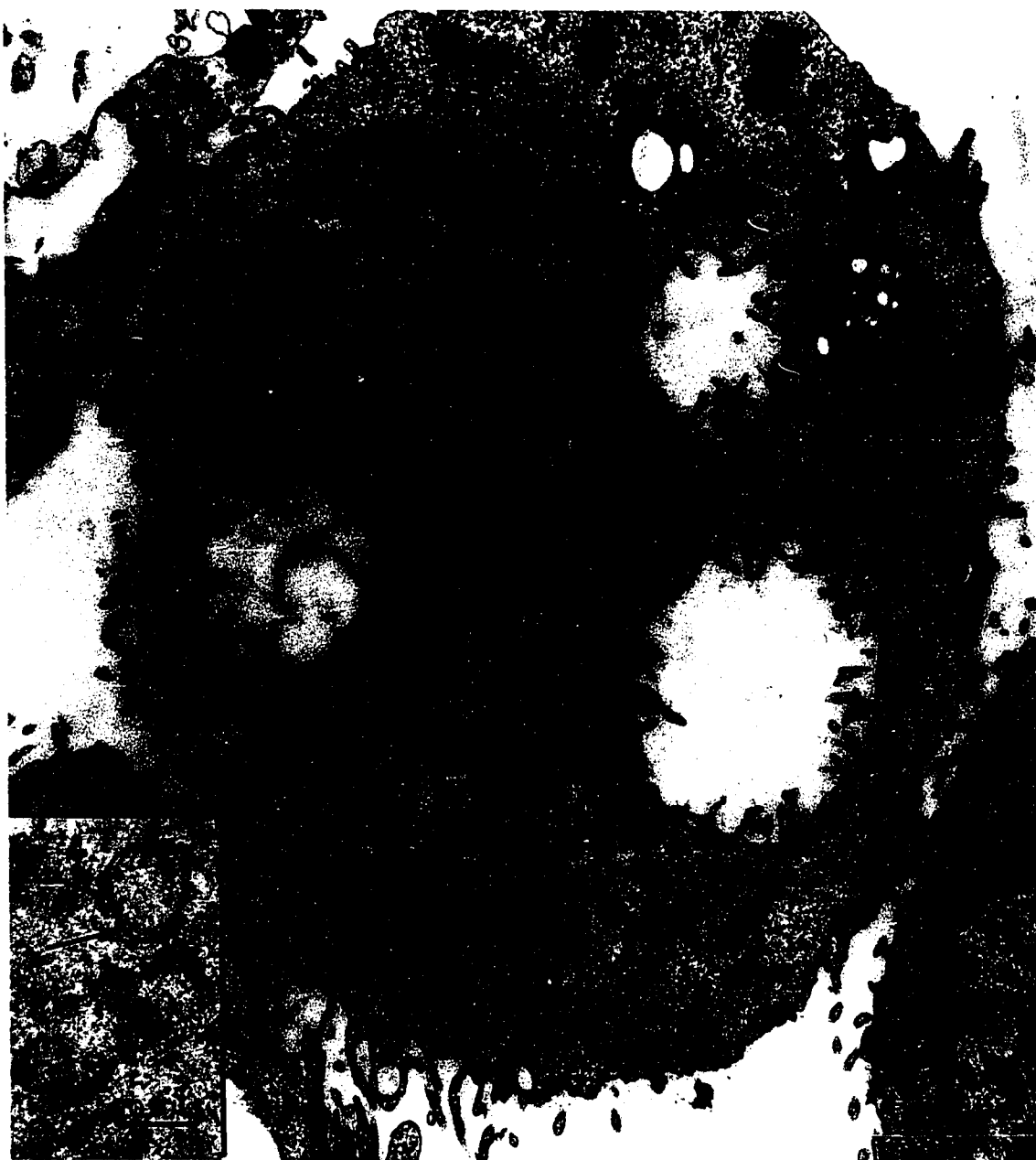


Fig. 36 a. Small balloon cell collected at 14 days post inoculation, showing a balloon vesicle of about $5\ \mu$ in diameter. The arrows indicate small vesicles, probably formed by micro-pinocytosis. 13,570 x.

b. The enlarged portion shows several strands of rough endoplasmic reticulum that lose their ribosomes on approaching the balloon vesicle (arrow). Several small vesicles are found in the same area. 33,250 x.

Approximate cell volume:	$749\ \mu^3$
Approximate vesicular volume:	$77\ \mu^3$
Volume of true cytoplasm and nucleoplasm:	$672\ \mu^3$



Fig. 37 Intermediate balloon cell collected at 14 days post inoculation, in which an enlarged balloon vesicle of about 8 μ in diameter can be seen. Note the small lobe of the nucleus below the major part of the nucleus. Where the arrow points the rim of true cytoplasm surrounding the balloon vesicle is about 600 $\text{\AA}$. 10,280 x.

Approximate cell volume:	796 μ^3
Approximate vesicular volume:	270 μ^3
Volume of true cytoplasm and nucleoplasm:	526 μ^3



Fig. 38 Intermediate balloon cell, collected at 14 days post inoculation, in which two enlarged balloon vesicles, separated by a thin cytoplasmic partition are observed. Note the extremely lobed nucleus, which has been pushed to the periphery of the cell by the expanding balloon vesicle. The arrow points at a branching mitochondrion. 8,691 x.

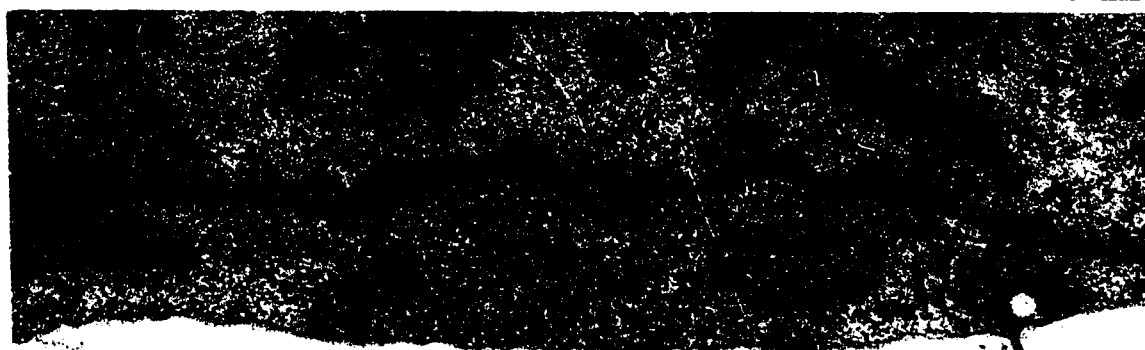
Approximate cell volume:	$2,316 \mu^3$
Approximate vesicular volume:	$605 \mu^3$
Volume of true cytoplasm and nucleoplasm:	$1,711 \mu^3$



Fig. 39 a. Intermediate balloon cell, collected at 14 days post inoculation, in which one large balloon vesicle, 14 μ in diameter, can be seen. 8,064 x.

b. The enlarged portion shows an abundance of small (rough) vesicles between the nucleus and the balloon vesicle, and in cytoplasmic evaginations into the nucleus (arrow). 18,480 x.

Approximate cell volume:	2,832 μ^3
Approximate vesicular volume:	906 μ^3
Volume of true cytoplasm and nucleoplasm:	1,926 μ^3



b

distribution of free ribosomes and endoplasmic reticulum. The Golgi area often contained innumerable small vesicles, closely packed together, and often located between the nucleus and the large balloon vesicle (Fig. 39). The small vesicles were also found in cytoplasmic evaginations into the nucleus (Fig. 39b). Small vesicles with a dense 300 Å core were also observed (Fig. 33-arrow, Fig. 35-arrow). Sometimes pale vesicles enveloped in one thin and one thick membrane were observed (Fig. 35-arrow and insert).

At 17 days after inoculation many tumor cells contained vesicles of the same size and appearance as those described for day 14 (Fig. 40). In the cell shown in Fig. 40 there were deep invaginations of the cell surface in the rim of true cytoplasm surrounding the balloon vesicle (Fig. 41b). In these areas there were also some pinocytotic vesicles (Fig. 41b-arrow). Many secondary lysosomes were present some of which seemed to surround portions of cytoplasm (Fig. 41a-arrow). Lysosomes were also found in the rim of true cytoplasm surrounding the balloon vesicle (Fig. 42c). Fig. 42a shows a section of a secondary lysosome in which a partially digested mitochondrion could be recognized. Some lysosomes were divided into two parts, with a thin membrane in between (Fig. 42b). It seemed that such lysosomes were actively engaged in the segregation and incorporation of small areas of cytoplasm into the lysosome structure.

Fig. 40 Intermediate balloon cell collected at 17 days post inoculation. Large, well differentiated nucleoli and condensed chromatin are observed in the nucleus. Many lysosomes are found in the cytoplasm, and are shown at higher magnification in Fig. 41 a. In the thin rim of true cytoplasm are several deep invaginations of the outer cell surface, which are shown in more detail in Fig. 41 b. 7,578 x.

Approximate cell volume:	2,608 μ^3
Approximate vesicular volume:	689 μ^3
Volume of true cytoplasm and nucleoplasm:	1,919 μ^3



Fig. 41 The areas indicated in Fig. 40 are shown at higher magnification.

- a. Area to show the lysosomes in greater detail.
The arrow indicates a lysosome that seems to surround a portion of cytoplasm. 27,300 x.
- b. Several areas of invagination in the rim of true cytoplasm are seen in this photograph.
The arrow indicates a pinocytotic vesicle.
27,300 x.

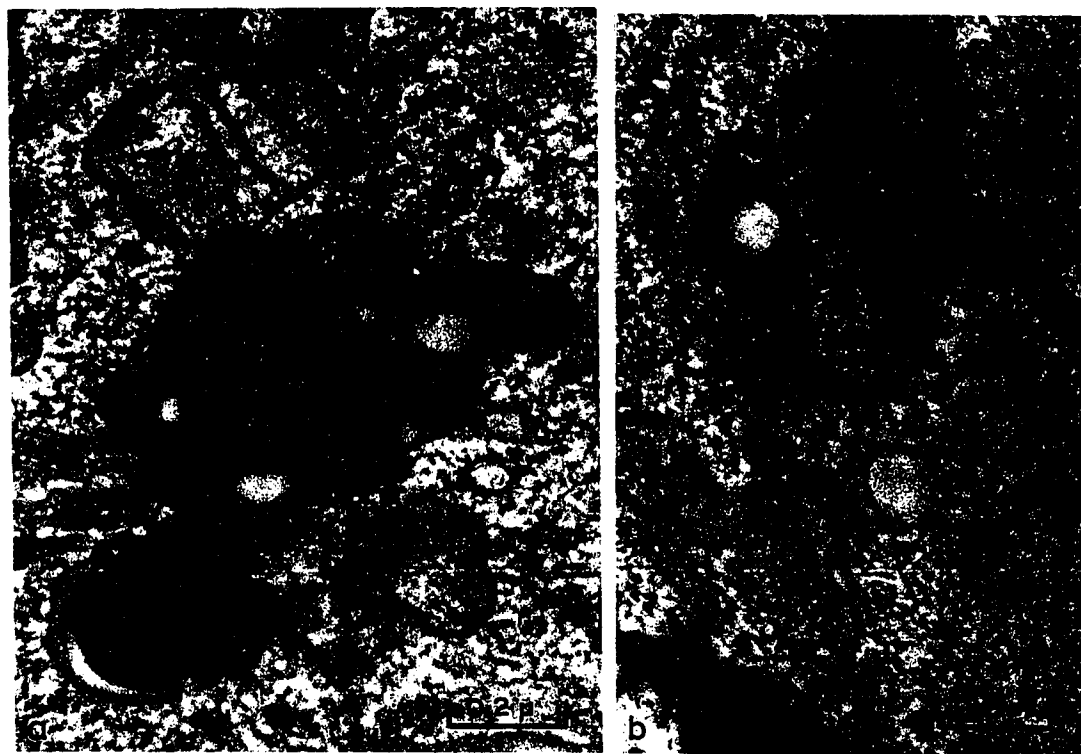


a



b

- Fig. 42
- a. Lysosome from a tumor cell collected at 21 days post inoculation. In the central portion of the lysosome is a structure resembling a partially digested mitochondrion. 74,750 x.
 - b. Lysosome from a tumor cell collected at 21 days post inoculation. The lysosome seems to be in the process of wrapping itself around a small portion of cytoplasm. 74,750 x.
 - c. Lysosome in the rim of true cytoplasm surrounding a balloon vesicle (b.v.) collected at 17 days post inoculation. 126,000 x.



b.v.



Fig. 43 shows a large balloon cell, collected at day 21, in which the balloon vesicle was about 30 μ in diameter. The general appearance of the true cytoplasm and nucleoplasm was the same as in previous cells.

From electron micrographs it was calculated that the combined volume of true cytoplasm and nucleoplasm in balloon cells was the same or slightly greater than the total volume of the regular tumor cells (Table XII).

c. Vesicle formation

In the preceding section the general features of the different types of tumor cells were described. It was of importance to find out how the large electron transparent vesicle of the balloon cells was formed. If it was assumed that the balloon cells developed from the regular tumor cells, some indication of the initial stages of balloon vesicle formation should be found in the regular tumor cells. Furthermore, if the balloon vesicle was formed from cytoplasmic components, such as for instance the Golgi region, these components would be expected to show the first changes.

Examination of sections of regular tumor cells, cut through the nucleus, showed no evidence for the presence of early balloon vesicles in the Golgi area. Such early balloon vesicles were seen in sections that were cut near the surface of the cells. A section of a regular tumor cell, that did not pass through the nucleus, showed several

Fig. 43 Large balloon cell collected at 21 days post inoculation, in which a large balloon vesicle, about 30 μ in diameter, is present. 4,940 x.

Approximate cell volume:	15,447 μ^3
Approximate vesicular volume:	13,403 μ^3
Volume of true cytoplasm and nucleoplasm:	2,044 μ^3



Table XII

Calculations from Electron Micrographs of the Approximate
Volume of the True Cytoplasm and Nucleoplasm in Regular
Tumor Cells and Balloon Cells

cell type	total cell volume in μ^3	volume of balloon vesicles in μ^3	volume of true cytoplasm and nucleoplasm in μ^3
regular tumor cell	1,223 ^a $\pm$ 421 ^b		1,223 $\pm$ 421 ^b
	960	12	948
small	1,507	66	1,441
balloon	1,597	55	1,542
cell	749	77	672
	735	4	731
	796	270	526
intermed.	2,316	605	1,711
balloon	2,832	907	1,926
cell	2,608	689	1,919
large balloon cell	15,447	13,402	2,044

^aThe total cell volumes are less than expected from hematocrit values, due to a shrinkage of about 30% in the vestopal embedded samples.

^bAverage of six determinations.

small vesicles lined by microvilli (Fig. 44). It seemed that these vesicles originated from invaginations of the cell surface (canaliculi) (Fig. 44 - lower right corner). Fig. 45 was a slightly deeper cut section of a different tumor cell, showing the tip of the nucleus, and a cross-section of a small canaliculus close to the cell surface (arrow). These two figures, and other photographs not shown, therefore seemed to indicate that the large vesicles found in balloon cells arose from small canalicular invaginations of the cell surface, that subsequently separated from the cell surface and then enlarged (Fig. 46). The continuity of the outer cell membrane was apparently re-established in the process.

d. Vesicle enlargement

Since the balloon vesicle probably originated through activities of the cell surface, it was possible that similar activities contributed to the enlargement of the balloon vesicle. The presence of deep invaginations of the cell surface and the formation of secondary balloon vesicles in cells that already possessed one large balloon vesicle was often observed (Fig. 47). The secondary balloon vesicles were probably formed in the same way as the initial balloon vesicle: through an invagination of the cell surface, that subsequently separated, and enlarged.

A small secondary vesicle with the appearance of

Fig. 44 Small tumor cell collected at 21 days post inoculation. This photograph is interpreted as a section through the surface of the cell, so that the nucleus is not shown (see insert). The arrows indicate areas of invagination of the cell surface, which resemble small intracellular canaliculi. Several small balloon vesicles, about $1.5\ \mu$ in diameter, are seen close by. 10,762 x.

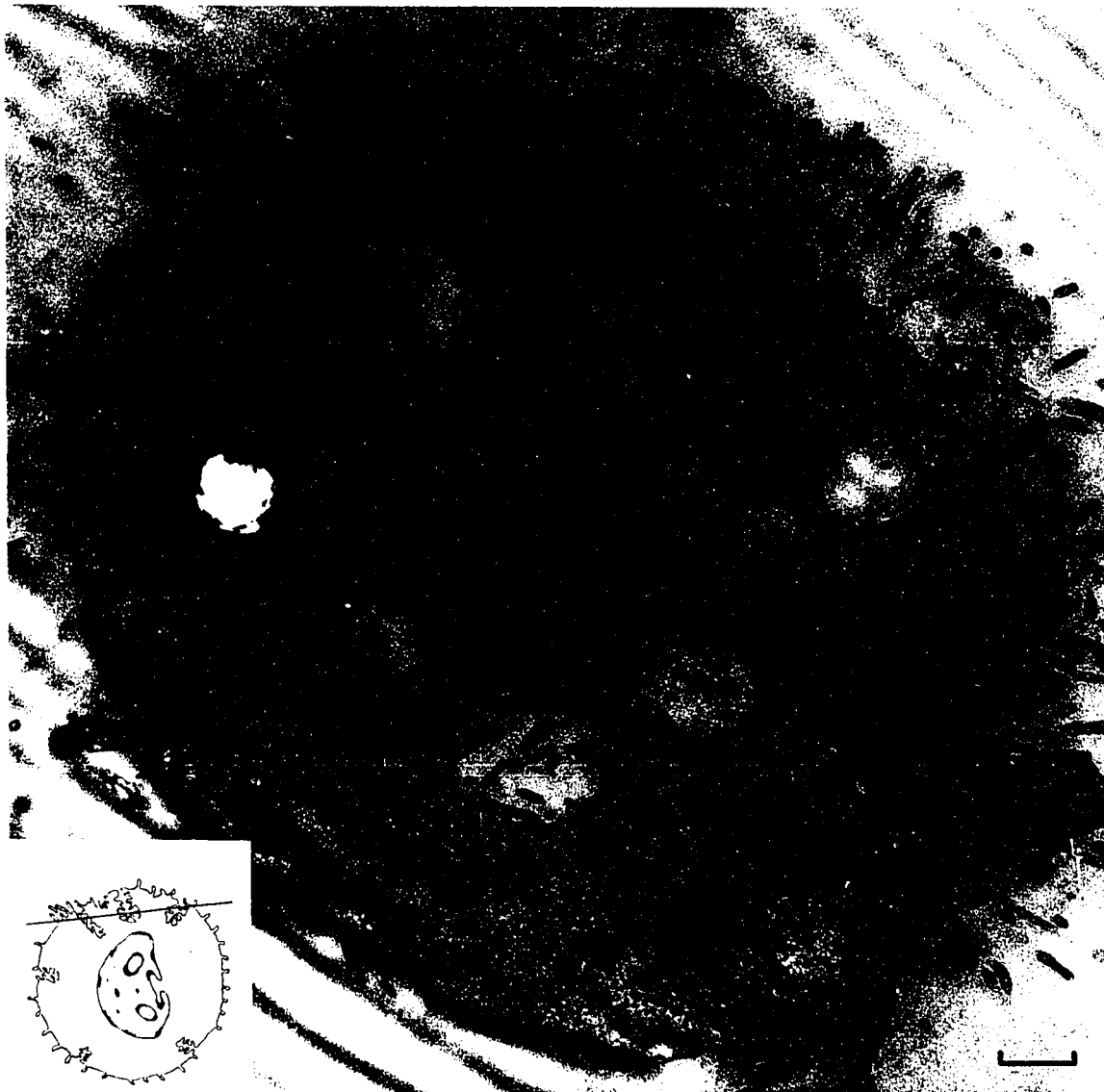


Fig. 45 Small tumor cell collected at 21 days post inoculation. The section passes through the tip of the nucleus and through one small balloon vesicle. The arrow indicates a small canaliculus in cross-section. 10,760 x.

Approximate cell volume: $735 \mu^3$

Approximate total vesicular volume: $4 \mu^3$

Volume of true cytoplasm and nucleoplasm: $731 \mu^3$



Fig. 46 Schematic representation indicating the mechanism by which balloon vesicles of the Ehrlich ascites tumor cells are probably formed.

- a. Invagination of the cell surface of a regular tumor cell.
- b. Invaginated area closes off to form a small vesicle, and the continuity of the outer cell membrane is re-established.
- c. Invaginated vesicle enlarges, resulting in a balloon vesicle.

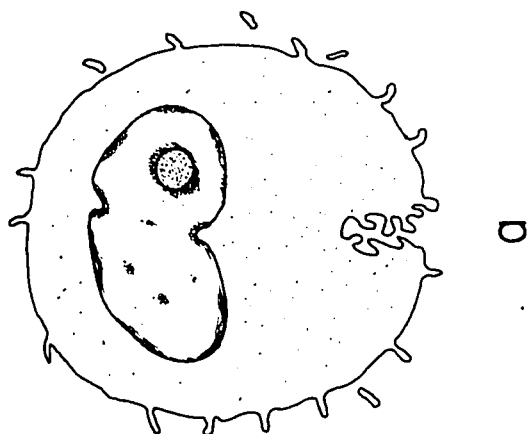
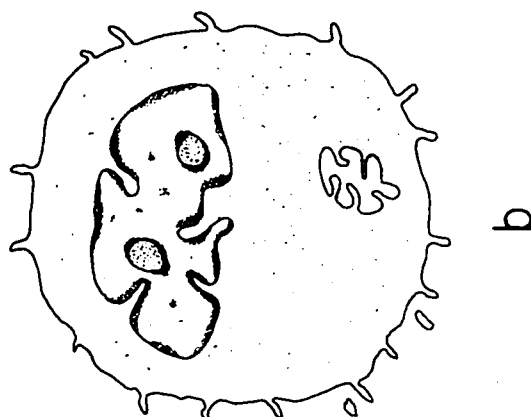
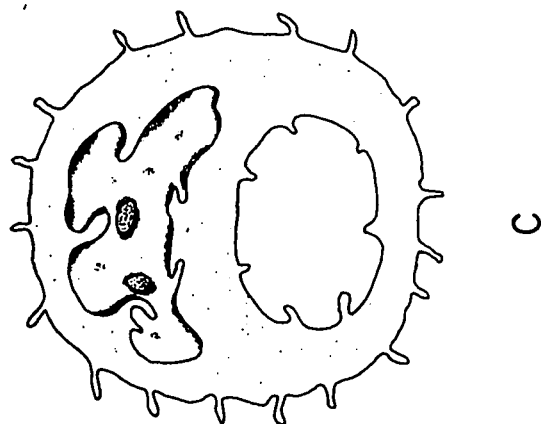


Fig. 47 Portion of an intermediate balloon cell collected at 21 days post inoculation. A sketch of the whole cell indicates the area of the cell shown. At the upper right (single arrow) is a deep secondary invagination (intracellular canaliculus), which communicates with the outside medium. The area at the upper left (double arrow) is interpreted as a cross section through an intracellular canaliculus. At right is a portion of a secondary balloon vesicle. 27,300 x.



an intracellular canaliculus was seen between the nucleus and the balloon vesicle of Fig. 48. It could not be determined whether this secondary vesicle was in contact with the medium outside the cell or with the contents of the balloon vesicle. A third possibility was that the vesicle was isolated.

Several interesting phenomena that could be of importance in vesicle enlargement were observed in sections of a large balloon cell shown in Fig. 50. The sections passed through a large invagination of the cell surface in the rim of true cytoplasm bordering the balloon vesicle. To investigate this area more thoroughly, photographs of several serial sections were taken. The sections showed that the invagination was probably in the shape of a cup, in which the thin base was in close contact with the large balloon vesicle (Fig. 50-d). As far as could be seen from these photographs, there was no direct contact between the medium outside the cell and the contents of the balloon vesicle. In another portion of the same balloon cell was a similar invagination, but this time it appeared to be opening into the balloon vesicle, being closed off from the external medium (Fig. 51 a,b).

A small infolding of the cytoplasm, close to a balloon vesicle was also observed in Fig. 34 (inset). Sometimes the infoldings of the cell surface involved a

Fig. 48 Portion of an intermediate balloon cell collected at 17 days post inoculation. The area indicated by the arrow is interpreted as a cross-section through an intracellular canaliculus.
B.V. = balloon vesicle. 10,930 x.

Fig. 49 Small balloon cell collected at 17 days post inoculation. An extensive invagination of the cell surface is seen at the upper right. A very thin partition (arrow) separates the left balloon vesicle from the external medium. 9,450 x.

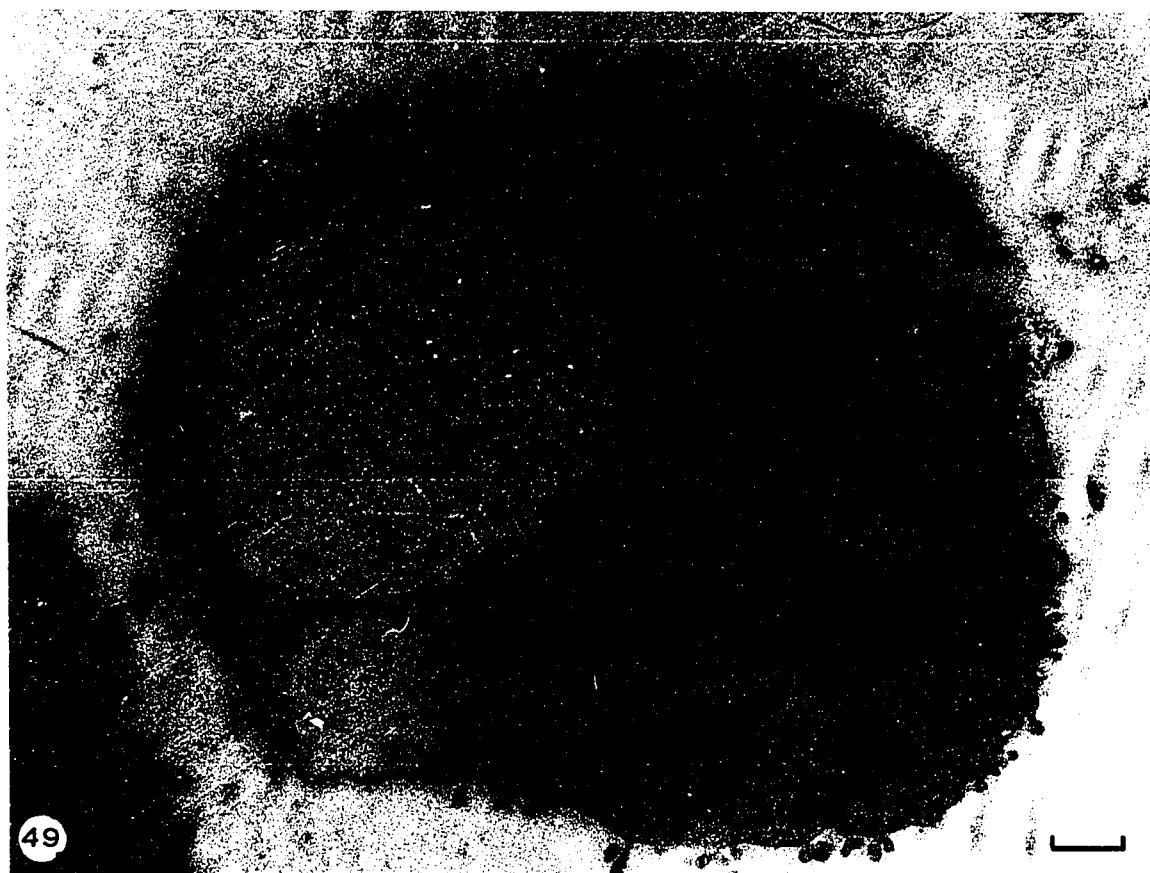


Fig. 50 A series of sections from a large balloon cell, collected at 21 days post inoculation. These sections pass through a large invagination of the cell surface present in the thin rim of true cytoplasm bordering the balloon vesicle. In each case the external medium is on the left side of the photograph, and the balloon vesicle is on the right side. In sections d and e only a very thin rim of true cytoplasm separates the external medium from the contents of the balloon vesicle.

- a. Section 1 26,670 x.
- b. Section 2 26,670 x.
- c. Section 3 26,670 x.
- d. Section 7 43,830 x.
- e. Section 8 43,830 x.

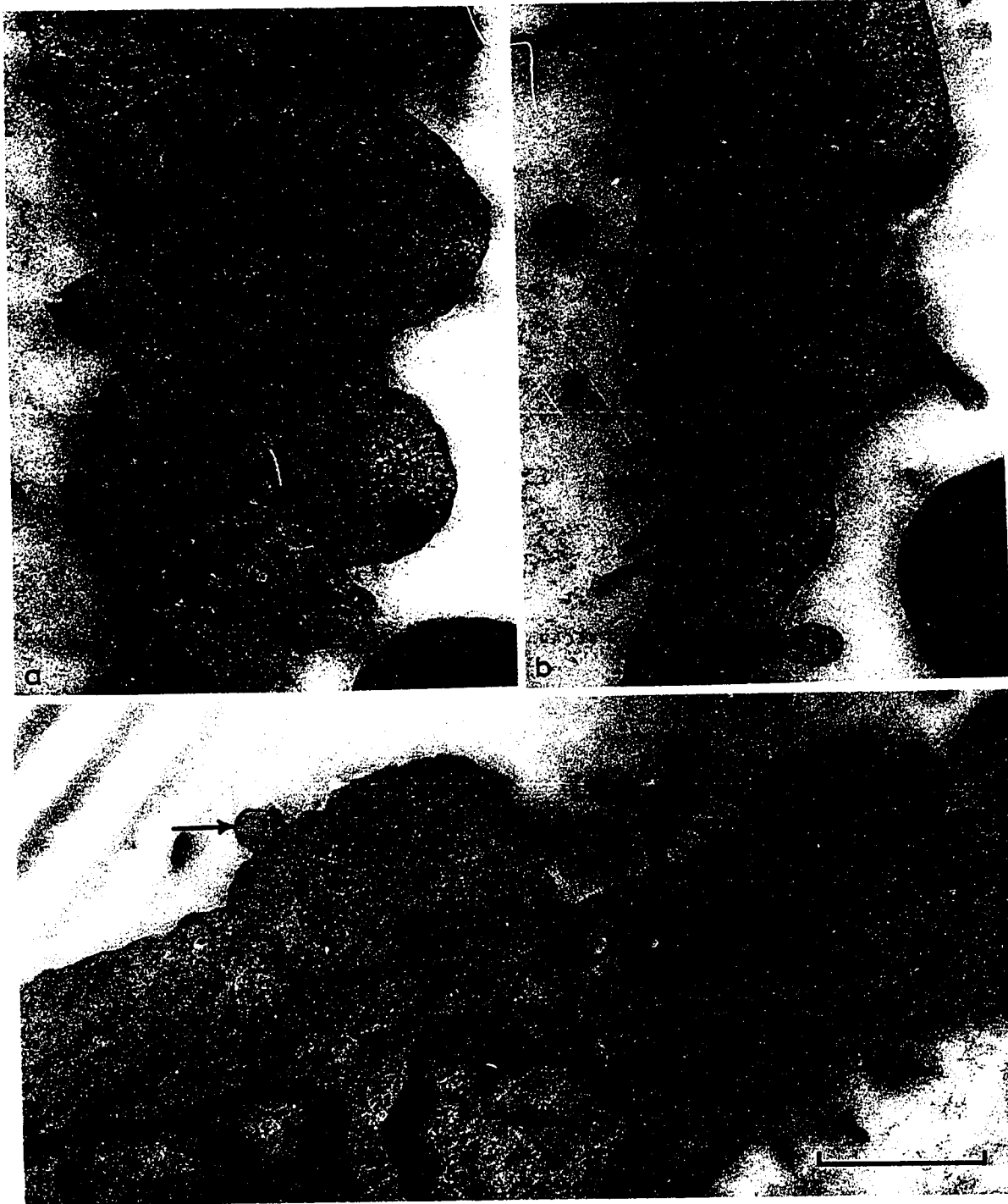
The intervening sections, section 4, 5 and 6, were obscured by a grid wire.



Fig. 51 Sections from the same balloon cell as shown in Fig. 50. a and b are from section 1 and section 3 respectively. These two sections pass through a second area of infolding, which this time appears to be opening into the balloon vesicle (see b). In the upper left of b is a dense vesicle surrounded by two membranes.

c. A portion from section 3, to show the presence of pinocytosis droplets (arrows). Similar droplets can also be seen in Fig. 51-b.

a, b and c 26,670 x.



large area of the cell (Fig. 49 and 53), but in Fig. 49 a thin partition (arrow) remained, to separate the contents of the balloon vesicle from the external medium.

Fig. 54 summarizes the above observations regarding the suggested mechanism of vesicle enlargement.

Associated with the outer cell surface of the balloon cells (Fig. 52) were sometimes found dense structures (a) (0.2 to 0.4 μ in diameter), the nature of which was not known. Similar structures (b) were also found in association with the membrane of the balloon vesicle, and inside the true cytoplasm (c), surrounded by a second membrane. A comparable arrangement of like structures was also found in other cells (Figs. 34 and 45).

Other mechanisms such as classical pinocytosis and micropinocytosis might be involved in the enlargement of the balloon vesicle. Small vesicles that could have been formed by micropinocytosis were occasionally seen and were usually located close to the cell surface (Figs. 36 and 53). Pinocytotic vesicles were seen more frequently (Figs. 41-b, 51-b, 51-c, 52), particularly during the later stages of tumor development.

In some older tumor cells the Golgi apparatus appeared to be distended with dense structures, resembling storage granules, and large, electron transparent vesicles (Fig. 55). Using serial sections, it could be seen that

Fig. 52 Enlarged portion of section 7 of the balloon cell shown in Figs. 50 and 51. This area shows large structures (a), often attached to the cell surface by a thin stem. Similar structures are found attached to the membrane of the balloon vesicle (b), and in the true cytoplasm, where they are surrounded by a second membrane (c). Pinocytotic vesicles are indicated by arrows. Part of the balloon vesicle is on the left side of the photograph. 35,180 x.

B.V.

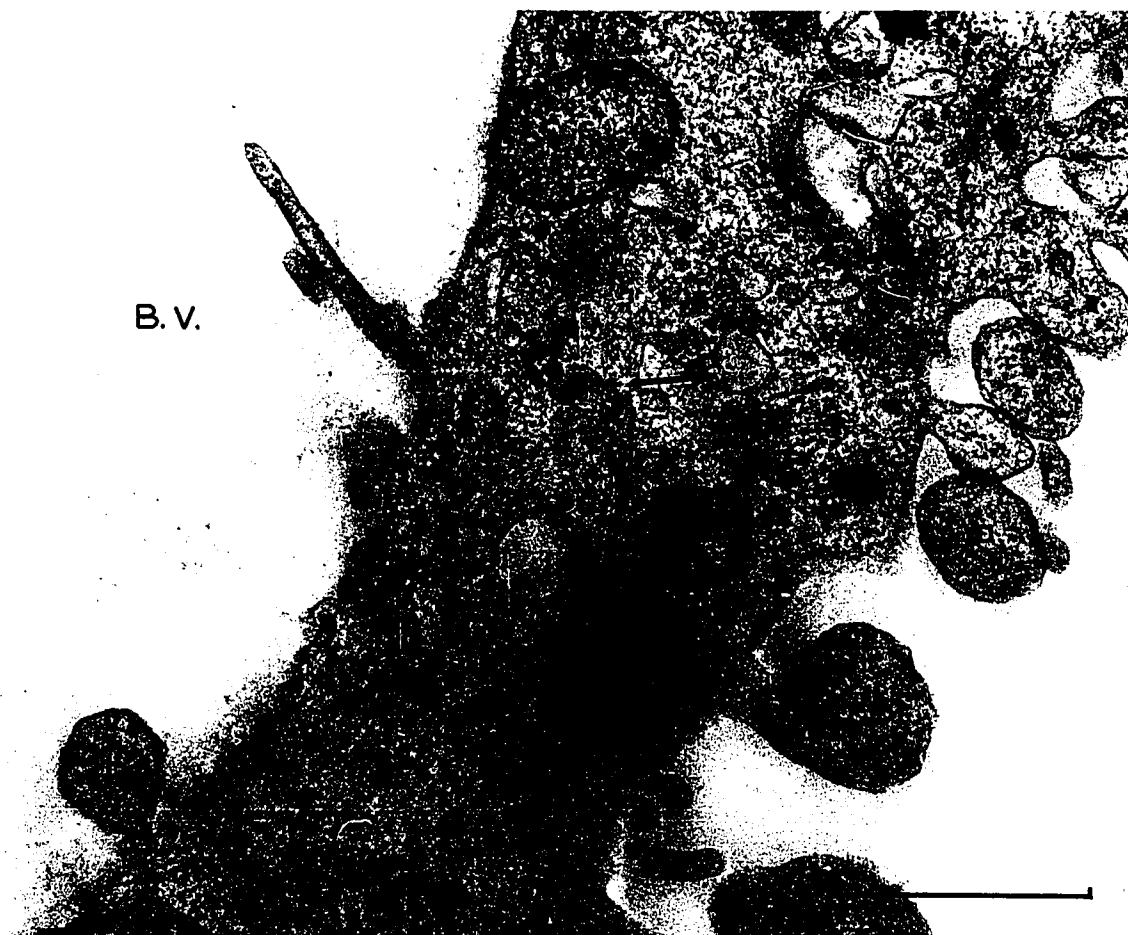


Fig. 53 Section through a small balloon cell,
collected at 14 days post inoculation, in
which one balloon vesicle, about 5 μ in
diameter can be observed. The arrows indicate
small vesicles that have probably been formed
by micropinocytosis. The lower portion of the
cell shows what may be a large infolding of
the cell surface. 12,950 x.

Approximate cell volume:	927 μ^3
Approximate vesicular volume:	50 μ^3
Volume of true cytoplasm and nucleoplasm:	877 μ^3



Fig. 54 A schematic representation indicating one of the possible mechanisms by which balloon vesicle enlargement can take place.

- a. Formation of secondary balloon vesicles by invagination of the cell surface.
- b. The secondary balloon vesicle has separated from the cell surface and the outer cell membrane has been re-established.
- c. The secondary balloon vesicle joins with the large balloon vesicle.

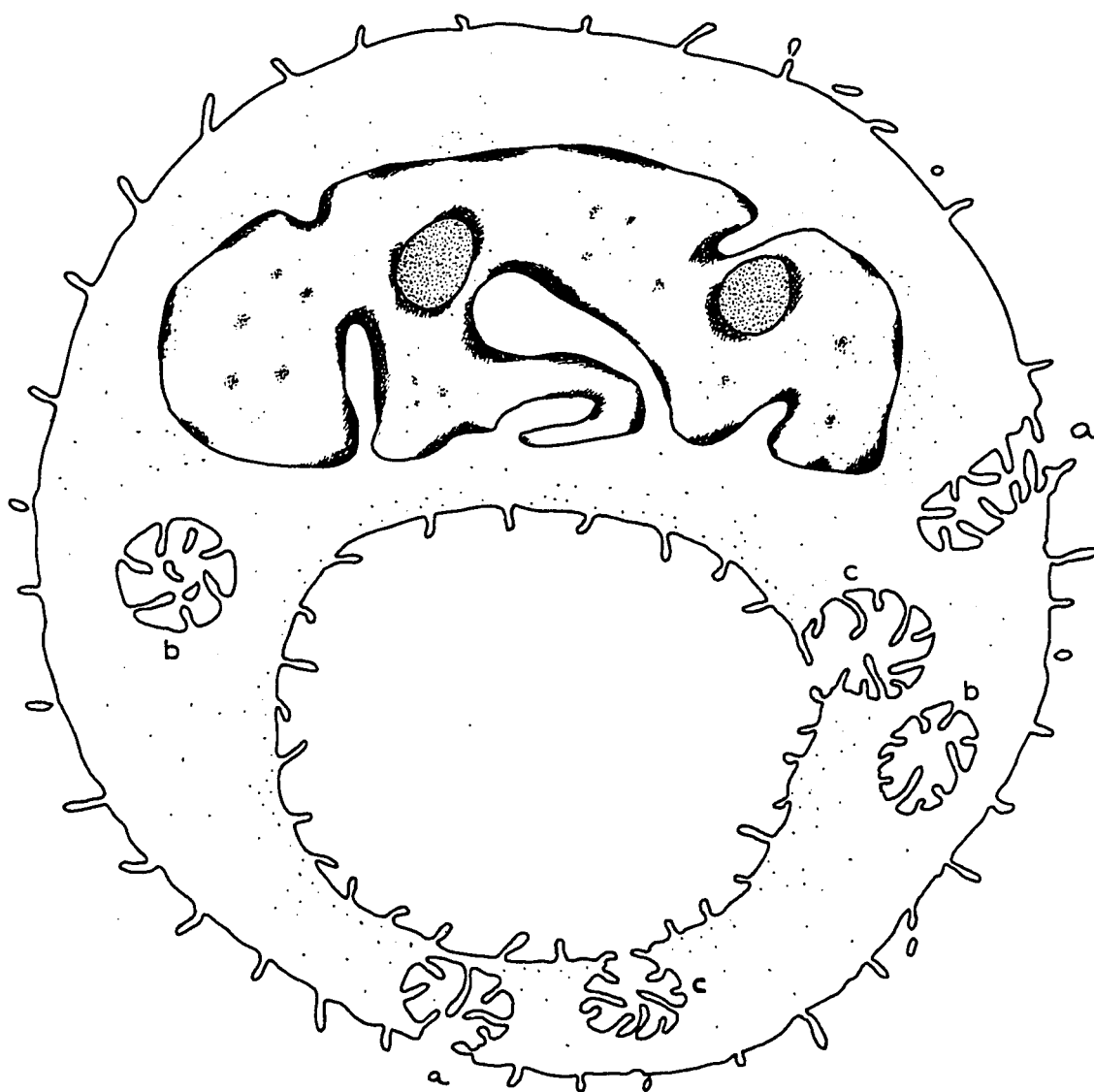
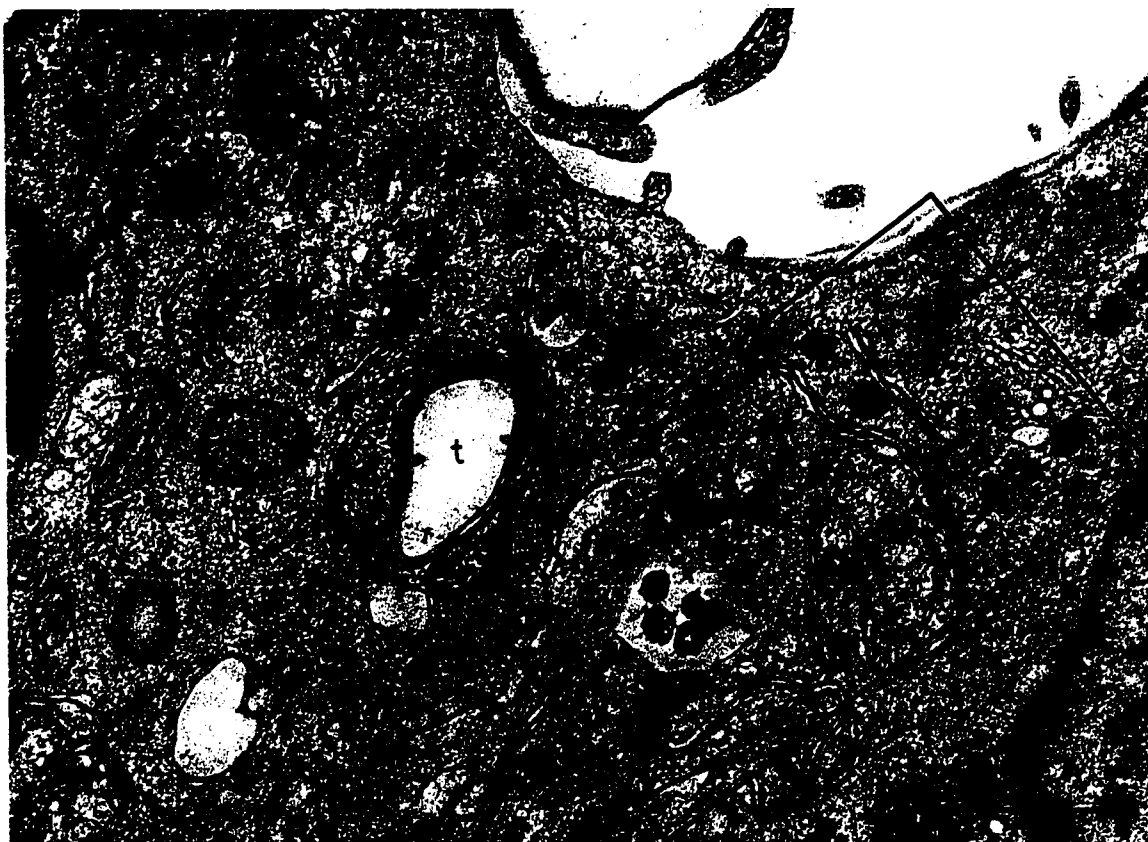


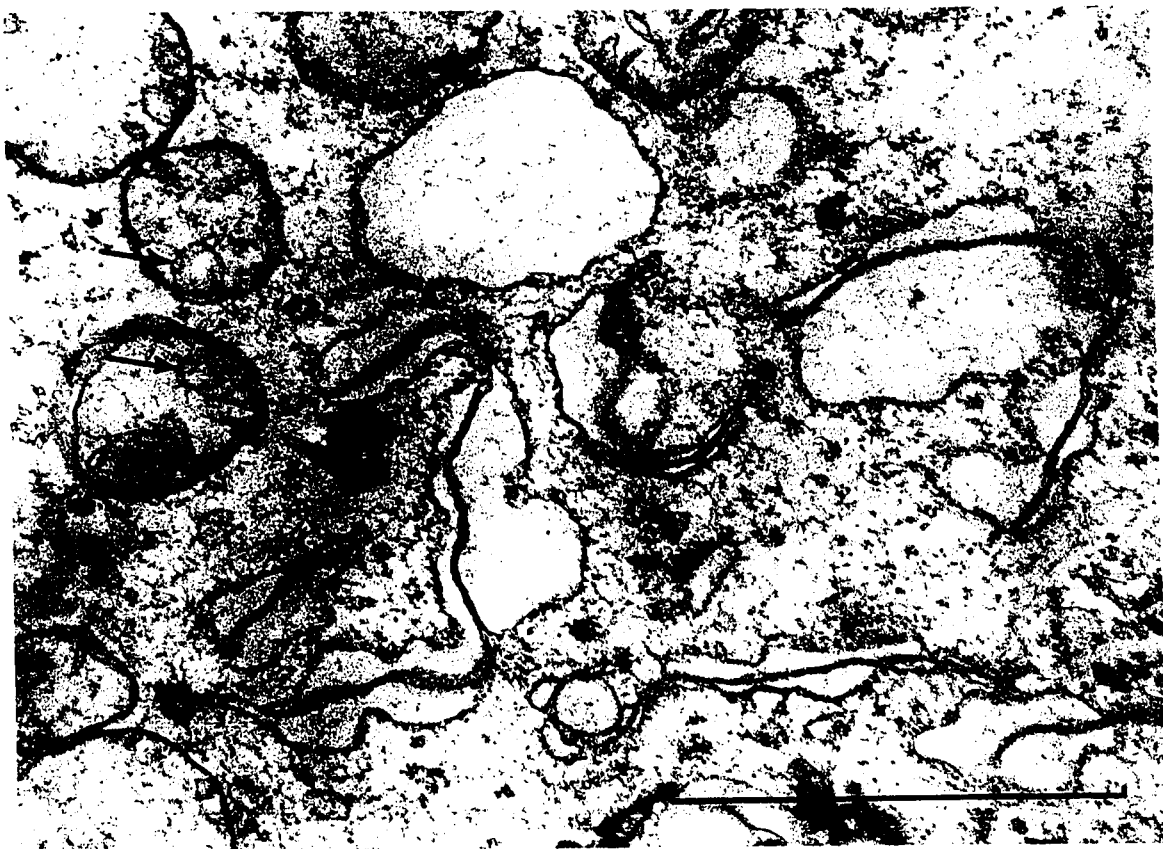
Fig. 55

- a. Part of a tumor cell collected at 21 days post inoculation. At left is a portion of the Golgi apparatus, with one large electron transparent vesicle (t), and two denser vesicles (d). A peculiar membrane component can be observed in the cytoplasm of the cell. 28,030 x.
- b. A higher magnification of the area indicated in a shows that the membrane component consists of two membranes, each about $60 \text{ \AA}$ thick, separated by a space of $40 \text{ \AA}$. On either side of this double membrane is a small clear space, the width of which varies. The clear space is separated from the rest of the cytoplasm by a membrane. A similar group of membranes, perhaps at an earlier stage of formation was observed in the lower part of Fig. 46. 50,960 x.



the electron transparent vesicles fused with larger vesicles. Peculiar membrane structures that seemed to have been formed by the endoplasmic reticulum were also found in older tumor cells (Figs. 55 and 56).

Fig. 56 Portion of a tumor cell collected at 17 days post inoculation. The cell shows signs of degeneration, as is indicated by the swollen mitochondria (arrows), which have swollen cristae. Many pale vesicles are present in the cell, and in some vesicles the same double membrane component, as was observed in Fig. 55, can be seen. 60,000 x.



CHAPTER IV

DISCUSSION

A. Population Dynamics

1. General Growth Characteristics

The Ehrlich ascites subline used in this research differed greatly in general growth characteristics from other Ehrlich ascites tumor lines. Mean survival was 21 days whereas in other sublines values of 14 days (Klein and Révész 1953), 12 and 17 days (Hauschka et al. 1957) have been found. The longer survival may have been partially due to the small size of inocula (2×10^5 cells per mouse), since in other Ehrlich ascites sublines survival increases when the size of the inoculum is less than 1×10^6 cells per mouse (Lettre 1941 and Hauschka et al. 1957).

The subline differed furthermore from other Ehrlich ascites tumors in that extremely large volumes of ascitic fluid (up to 45 ml) accumulated, which accounted for about 50% of the weight of the mouse. In other sublines values of 1 to 8 ml (Goldberg et al. 1950), 9 ml (Hauschka et al. 1957) and 11 ml (Lettre 1941) have been found at 50% mortality. But despite the accumulation of a larger volume of ascitic fluid, the mice were able to survive longer than usual.

The maximum number of tumor cells was reached at about 12 days post inoculation and was 0.9×10^9 to 1.3×10^9 cells per mouse, which is in good agreement with data on other Ehrlich ascites tumors. Hauschka and co-workers reported 1.04×10^9 cells for their hypertetraploid strain and 2.47×10^9 cells for their hyperdiploid strain (Hauschka et al. 1957). Other values are 1.90×10^9 for a hypotetraploid strain (Baserga 1963) and 1.5×10^9 to 2.0×10^9 cells for a tetraploid strain (Klein and Révész 1953). In the present study the maximum value was reached when the average cell size was $8,000 \mu^3$, so that the total tumor cell mass per mouse was 9 ml. The total tumor cell mass was therefore larger than in other Ehrlich ascites tumor lines where the "killing cell mass" is about 3 ml, or 10% of the initial weight of the mice (Klein and Révész 1953, Hauschka et al. 1957). It seems therefore that in the present case there was no inverse relationship between the larger size of the tumor cells and the maximum number of tumor cells per mouse, such as had been found by Hauschka (Hauschka et al. 1957). This may be because the total volume of "true cytoplasm" in the balloon cells was not much larger than in regular tumor cells.

After reaching a maximum, the number of tumor cells per mouse declined to 458×10^6 cells at 19 days, although ascitic fluid continued to accumulate. At the

same time the average volume of the ascites cells increased to a maximum value of $24,000 \mu^3$, indicating during this period an inverse relationship between tumor cell size and total number of tumor cells.. The total tumor cell mass therefore did not change much between day 12 and day 19. Although a decline in concentration of tumor cells has been found in other Ehrlich ascites sublines, no decrease in the total number of tumor cells per mouse has been reported (Klein and Révész 1953). Because the number of non-viable cells increased with time after inoculation and the mitotic index decreased, it appears that the tumor cells were removed by death, but were not replaced fast enough by newly divided cells, so that the total tumor cell population decreased.

Taking the above mentioned characteristics into account, it was possible to divide tumor development in the SN-Ehrlich ascites tumor in four stages:

Stage I lasted for 7 days. During this period the generation time was about 18 hrs. The number of tumor cells increased exponentially, but there was little accumulation of ascitic fluid.

During Stage II the generation time became increasingly longer, and ascitic fluid accumulated rapidly. There were few balloon cells. This stage ended at about 12 days post inoculation, when the maximum number of tumor

cells had been reached.

Ascitic fluid continued to accumulate during Stage III, but the cell concentration and the total number of tumor cells decreased. The average volume per cell increased greatly, so that the total tumor cell mass remained about the same. Survival of the mice remained high. Stage III ended at 19 to 20 days post inoculation.

Stage IV was the terminal stage, during which no further ascitic fluid accumulation took place. The average volume per cell decreased, resulting in a decrease of total tumor cell mass. The number of non-viable tumor cells increased. Apparently the nutrient resources of the hosts had been depleted and the mice died.

2. Change in Distribution of Cell Types and Cell Fractions in Whole Ascitic Fluid

A study of the distribution of cell types during development of the SN-Ehrlich ascites tumor revealed that during Stage I and the beginning of Stage II, when there was little ascitic fluid, the majority of the tumor cells were of the regular type although only large balloon cells had been inoculated. Some tumor cells contained a small clear vesicle, the size of which was usually not larger than the size of the nucleus, and these cells were called small balloon cells. This indicated that the presence of a balloon vesicle may be a reversible phenomenon, influenced

by the environmental conditions of the surrounding ascitic fluid.

The average diameter of the regular tumor cells was $17\ \mu$ which is similar to the values of $15\ \mu$ to $18\ \mu$ that have been reported for other Ehrlich ascites tumor cells (Klein and Révész 1953, Lindström 1955, Hauschka et al. 1957).

During Stage II and III the proportion of balloon cells increased and the balloon cells increased in size, reaching their maximum size and number at the end of Stage III. At this time the large balloon cells made up almost 80% of the total tumor cell population. The presence of large balloon cells, up to $50\ \mu$ in diameter, has also been reported in other Ehrlich ascites sublines, but the proportion of balloon cells was less than 10% of the total tumor cell population (Molomut et al. 1964).

At every stage there was a wide range in the distribution of cell size, and most cell types were present. These observations supported the idea that the balloon cells developed from the regular tumor cells. The progress in the formation of the large balloon cells was slow and occurred very gradually, as if the cells were adapting themselves to a gradually changing environment, in which more and more ascitic fluid was present.

During the course of this work a method was developed by which the tumor cells could be separated in

various cell fractions according to their density and size. Since there was a gradual transition in the size and density of the tumor cells, the separation was only approximate and several overlapping cell types could be found in each cell fraction. The separation of the various cell types into cell fractions was used as an alternative method of studying changes in the balloon cell population during tumor development, and was also useful for making analytical comparisons between the various cell types.

Although the separation of tumor cells into cell fractions was approximate, the distribution of cell fractions in whole ascitic fluid showed in general good agreement with the distribution of cell types in whole ascitic fluid.

However, a discrepancy was found in the distribution of the intermediate fraction and the bleb fraction. The proportion of small and intermediate balloon cells remained approximately 35% after day 12. The intermediate fraction had a similar value until day 18 and then suddenly increased to 77% at day 19 and 20. On day 19 and 20 the bleb fraction was very small, whereas the large and extra large balloon cells accounted for about 47% of all tumor cells. The reason for this discrepancy seemed to be that at day 19 the density and viscosity of the ascitic exudate had decreased to such an extent that the large balloon cells

no longer floated, but became incorporated in the intermediate fraction. That more and more large balloon cells became part of the intermediate fraction, instead of the bleb fraction, was also reflected in the increase of the average volume per cell in the intermediate fraction. Intermediate balloon cells which floated on the ascitic exudate on day 12, proved to be too heavy on day 19. At this time only the largest balloon cells had a specific gravity that was low enough for the cells to float, so that the average volume per cell in the bleb fraction also increased.

B. Cytological and Cytochemical Investigations of Balloon Cells

1. Ultrastructure of the Ehrlich Ascites Balloon Cells

a. General morphology

Except for the presence of one or more large vesicles (balloon vesicles) the fine structure of the balloon cells in the Ehrlich ascites tumor was similar to that of the regular tumor cells and other Ehrlich ascites cells (Selby et al. 1956, Yasuzumi and Sugihara 1958, Herdson and Kaltenbach 1965). The balloon vesicle, measuring up to 30 μ in diameter, displaced the nucleus to the periphery of the cell, giving the large balloon cell the appearance of a signet-ring cell.

Signet-ring cells are commonly found in adipose tissue, but in adipose cells the stored lipid leaves a ragged outline (Porter and Bonneville 1964) due to the absence of a limiting membrane (Wasserman and McDonald 1963,

Fawcett 1966). On the other hand, except for the presence of microvilli, the large vesicles of the Ehrlich ascites balloon cells had a smooth outline, which in general appearance bore a great resemblance to the outer cell membrane.

In tissues fixed with osmium tetroxide alone, lipid droplets often have a high electron density, due to their content of unsaturated fatty acids, which greatly reduce osmium tetroxide during fixation (Fawcett 1966). Compared to the rest of the cytoplasm of the balloon cells, the contents of the balloon vesicle had a very low electron density, so that lipid was not one of its major constituents. Fine filamentous strands of material that could be strands of protein or peptides, were sparsely distributed throughout the balloon vesicle, giving the vesicular contents the same appearance as cavities containing tissue fluid, capillary lumens containing blood (Fawcett 1966), or plant cell vacuoles (Whaley et al. 1964).

The occasional presence of large clear vacuoles, 14 μ in diameter, have been described in electron micrographs of other Ehrlich ascites sublines, and it was considered by the authors that the vacuolization represented a degenerative change, since in the same cells the mitochondria appeared degenerated (Selby et al. 1956). In the present material, cells that showed mitochondrial swelling and degeneration were only occasionally found among the balloon

cells and regular tumor cells. The nucleus and the remaining cytoplasm ("true cytoplasm") of the balloon cells also had the same general appearance as the "healthy" regular tumor cells, so that the presence of balloon vesicles was not accompanied by any obvious degenerative changes. Furthermore the total volume of the nucleus and true cytoplasm of the balloon cells was not decreased compared to the regular tumor cells, so that a gradual lysis of the true cytoplasm did not seem to be a likely cause of balloon cell formation.

b. Formation of balloon cells

Since many intermediate stages between the regular tumor cells and the large balloon cells were observed, the electron microscopic findings confirmed the belief, based on light microscopic findings (Tolnai 1964), that the balloon cells developed from regular tumor cells by a gradual transition. In a combined light- and electron-microscopic investigation of effusions associated with malignant disease, degenerated mesothelial cells have been observed, that had the appearance of signet-ring cells, and that bore some resemblance to the balloon cells (Luse and Reagan 1956). Others have also pointed out difficulties in distinguishing tumor cells from degenerated mesothelial cells (Ceelen 1964). Since both the true cytoplasm and the nucleus were so similar in appearance in the balloon cells and in the regular tumor cells, it seemed however very unlikely that the balloon cells of the Ehrlich ascites tumor

developed from mesothelial cells.

Several of the electron micrographs presented indicated that the small balloon vesicles may have originated as a large invagination of the cell surface. These invaginated areas resembled the intracellular canaliculi seen in the parietal cells of the fundic stomach (Helander 1962), although they did not extend quite as deeply into the tumor cells. The similarity in appearance (except for the size of the lumen) of cross sections through the earliest invaginations and through the balloon vesicles, suggested that the balloon vesicles resulted from a vesiculation of the original invagination, which enlarged. In this respect the invaginated areas differed from the canaliculi of the parietal cells, where the intracellular canaliculi remained narrow and in contact with the lumen of the fundic gland (Helander 1962).

The invaginated area comprised about 4 to 8 μ^2 , whereas in classical pinocytosis the area is only 1 μ^2 (Lewis 1937, Staubesand 1965). The size and manner of invagination corresponded more closely to the extensive invaginations of the cell surface that occur in the pinocytosing ameba Chaos chaos (Holter 1959). In this organism pinocytosis channels are formed, which vary in diameter, depending on the type of solutes in the medium. The larger channels greatly resemble the foodcups that are formed, when the ameba is engaged in the phagocytosis of

of paramecia (Holter 1959).

In the ameba much energy seems to be required for the invagination of the cell surface, since large accumulations of mitochondria have been found surrounding the invaginated area (Chapman-Andresen and Nilsson 1960). In the Ehrlich ascites cells the distribution of mitochondria surrounding the invaginated area was however the same as in other parts of the tumor cells.

No dense particles were found in the invaginated areas, so that the tumor cells were apparently engaged in a type of fluid engulfment that resembled channel pinocytosis of the ameba. The reason for this extensive fluid engulfment in the Ehrlich ascites cells was not clear. It could be a mechanism for uptake of cell nutrients. It could also be a passive response of the cells, evoked by anoxia or the presence of large volumes of ascitic exudate. The invaginations were not caused by a low pH of the surrounding medium (Holter 1964), since the pH of the ascitic exudate was in the normal range (7.0).

c. Enlargement of small balloon vesicles

The further fate of the small balloon vesicles differed in an important way from normal endocytosis. As they moved to the interior of the cell, the vesicles increased in size, giving the appearance of intracellular cysts. Several electron micrographs indicated that the

balloon vesicles probably enlarged by fusing with small secondary balloon vesicles. Secondary balloon vesicles could form in the neighborhood of the nucleus, or in the thin rim of true cytoplasm surrounding the balloon vesicle. It seemed that the secondary vesicles separated from the cell surface, with the outer membrane being re-established, before they fused with the large balloon vesicle.

It was also possible that the balloon vesicle increased in size by incorporating pinocytotic and micropinocytotic vesicles. Micropinocytotic vesicles were however infrequently encountered, while pinocytotic vesicles were seen more often, especially during the later stages of tumor development. The electron microscopic findings did not exclude the possibility of other methods of fluid transfer, such as a molecular transport of fluid from the exterior of the cell to the balloon vesicle in response to an osmotic gradient (Tanaka 1964, Manery 1966 a and b).

The cyst-like appearance of the balloon vesicle suggested that the vesicle might be storing materials synthesized by the cells themselves. If this was so then it would be expected that the endoplasmic reticulum would be greatly enlarged, whereas it was only sparsely developed. It would furthermore be expected that small vesicles containing the newly synthesized secretion in transport would be present. In some balloon cells indeed many small vesicles

were found in the Golgi area between the nucleus and the large balloon vesicle, which therefore indicated that perhaps some of the contents of the balloon vesicle was synthesized by the cell itself.

Intracellular cysts have also been observed in the mucous membrane of the nasal epithelium in normal rats (Stockinger 1963). The cysts were largest at the base of the cells (up to 14 μ in diameter) and into their lumen projected cilia, microvilli and cytopila. Stockinger believed that the cysts originated from an insinking of the cell surface, and that they increased in size by taking up cytoplasm or storing secretion products.

Polymorphic secondary lysosomes were frequently observed in the balloon cells and regular tumor cells. Since no acid phosphatase reaction was carried out on the cells used for electron microscopy, it could not be established whether primary lysosomes, either as "storage granules", (Hirsh and Cohn 1964), or as small Golgi vesicles (Moe et al. 1965) were present. Sometimes a gradation in size and complexity was seen amongst the lysosomes, with the smaller lysosomes being nearer the Golgi region.

Although the balloon vesicle could be considered as a large pinosome, fusion of the lysosomes with the balloon vesicle to form a digestive vacuole (de Duve 1963) was not observed. Therefore, no morphological evidence was obtained for the involvement of lysosomes in the digestion

of the contents of the balloon vesicle. The presence of lysosomes could be due to general pathologic phenomena caused by anoxia, the presence of toxic compounds in the ascitic exudate, or starvation (Brandes et al. 1964, Swift and Hruban 1964). Occasionally it appeared as if small areas of cytoplasm were incorporated directly into lysosomes, although other investigators have seen little evidence of such a fusion in other cells (Novikoff et al. 1964).

In older tumor cells long membranous structures were sometimes observed that seemed to be involved in a compartmentalization of the cytoplasm. These membranous structures seemed to have been derived from the endoplasmic reticulum. This observation agrees with results obtained by Ericsson, who has shown that in the cells of the proximal tubules of rat kidney, membranes involved in the formation of autophagic vacuoles were probably derived from the endoplasmic reticulum, which might also synthesize digestive enzymes directly into the vacuoles (Ericsson 1965). In carbon starved *Euglena* on the other hand similar membranes appeared to have been derived from the Golgi apparatus, which in these cells was hypertrophied and contained large amounts of acid phosphatase (Brandes et al. 1964).

2. Light Microscopy

Staining by a variety of histochemical methods failed to show the presence of any of the major organic

constituents in the balloon vesicle (Tolnai 1964). Although the balloon cells resembled the cells of a mucous cell adenocarcinoma (AFIP 1960), such as found in the Krukenberg tumor (Robbins 1962), the contents of the balloon vesicle could not be stained by the PAS method. Thus the balloon vesicle did not contain protein, carbohydrate or lipid in sufficient concentration to be detected by the above methods.

The regular tumor cells and small balloon cells contained large amounts of RNA, as is often the case in tumor cells (Caspersson and Santesson 1942). Goldberg and co-workers reported an RNA/DNA ratio of 1.94 for Ehrlich ascites cells, whereas in normal exudate cells the ratio was 0.59 (Goldberg et al. 1950). In the large balloon cells the large nucleoli stained strongly for RNA, but the cytoplasm was stained much paler than that of the regular tumor cells. It was not determined whether this pale staining was due to a loss of ribosomes or due to a dilution effect caused by the increase in size of the large balloon cells. In considering the RNA staining, nucleolar size and cell volume, the regular tumor cells resembled "A"-type tumor cells and the large balloon cells resembled "B"-type tumor cells as defined by Caspersson and Santesson (Caspersson and Santesson 1942).

The lack of cytochemical staining of the balloon vesicle indicated the presence of low molecular weight

substances, such as simple carbohydrates, peptides, amino acids or electrolytes. These compounds would diffuse readily from the cells during fixation and staining, and would therefore escape undetected. The low molecular weight substances could osmotically attract large amounts of water, which would result in a low density of the vesicular contents. That the balloon vesicle had a low density was confirmed by electron micrographs and by alpha radiographs. Therefore the balloon vesicles resembled somewhat the large vacuoles found in vacuolar (hypokalemic) nephrosis of the kidney (Robbins 1962). Such vacuoles do not contain fat, glycogen or mucous, but are apparently filled with tissue fluid.

3. Cytochemistry

The data for protein concentration, carbohydrate concentration, density and dry weight of regular tumor cells and various sized balloon cells showed a close correlation.

The density of the balloon cells was lower than that of the regular tumor cells, and this was apparently due to the low specific gravity of the balloon vesicle. Although the protein content of the balloon cells had increased over the protein content of the regular tumor cells, the protein concentration of the balloon cells was decreased and it was calculated that this decrease must have been caused by a low protein concentration of the

balloon vesicle. In the calculations of the density and protein concentration of the balloon vesicle it was assumed that the combined volume of true cytoplasm and nucleus was the same, whether a balloon vesicle was present or not. Estimates from electron micrographs showed that this assumption was mostly correct.

Except for the initial stages of tumor development, the protein concentration of the supernatant was about 4.8%, which was similar to values of 3.0% to 5.4% for other Ehrlich ascites tumors (Christensen and Riggs 1951).

The analytical results were consistent with the hypothesis that the regular tumor cells engulf large volumes of ascitic exudate, thereby taking up protein and carbohydrate. Direct uptake of plasma proteins by tumor cells may occur in malignant cells, as was shown in neoplastic liver cells (Cohen et al. 1961). In the small and intermediate balloon cells the protein concentration and density of the balloon vesicle were as high or higher than the corresponding values for the ascitic exudate, so that initially some concentration of proteins may have taken place. As the balloon cells increased in size the density and protein concentration of the balloon vesicles decreased to values below those of the ascitic exudate, and eventually the combined volume of the nucleus and true cytoplasm was too small, in relation to the total balloon cell volume, and

hence the cells floated.

It seems likely that the proteins that had initially been engulfed were broken down into low molecular weight breakdown products. In the ameba the permeability of the membrane lining the pinosome increases and low molecular weight breakdown products, followed by some water, diffuse from the pinocytotic vacuoles into the cytoplasm. In this way the osmotic pressure of the food vacuole in the ameba does not increase significantly, and the vacuole does not swell (Giese 1962). In Ehrlich ascites cells galactose metabolism is defective (Kalckar 1965) and this has a profound effect on the cell membrane. It is therefore possible that in the balloon cells the membrane surrounding the balloon vesicle had not become permeable to low molecular weight breakdown products, so that the osmotic pressure in the balloon vesicle was increased and water was attracted. A lack of available energy or of enzymes necessary for trans membrane transport may also have been responsible for the accumulation of low molecular weight products in the balloon vesicle.

SUMMARY

1. The mechanism of balloon cell formation in the SN-Ehrlich ascites cells was investigated.
2. During early stages of tumor development mainly regular tumor cells that had developed from inoculated balloon cells, were present. The time of appearance of large balloon cells in the tumor cell population coincided with the accumulation of large volumes of ascitic fluid.
3. Electron micrographs indicated that the balloon vesicle originated as a large invagination of the cell surface, which subsequently closed off to form a small balloon vesicle. In this process the continuity of the outer cell membrane was re-established.
4. Enlargement of the balloon vesicle seemed to occur through fusion with secondary balloon vesicles that had been formed by the same mechanism.
5. The presence of a balloon vesicle in the tumor cells was not accompanied by degenerative changes in the remaining cytoplasm or nucleus.
6. None of the major organic substances were present in sufficient amounts in the balloon vesicle to be detected by cytochemical methods. Electron micrographs and alpha-radiographs showed furthermore that the dry mass of the balloon vesicles was low, indicating a high water content.

7. It was postulated that the invaginated material was broken down inside the balloon vesicle, but that the resulting low molecular weight breakdown products were unable to diffuse from the vesicle into the cytoplasm of the balloon cells. The osmotic pressure inside the balloon vesicle would therefore increase, and water would accumulate to such an extent that the protein concentration and the density of the balloon vesicle would fall below the corresponding values for the ascitic exudate.

BIBLIOGRAPHY

A.F.I.P. Manual of histologic and special staining technics. Armed Forces Institute of Pathology. 2nd ed., McGraw Hill, Toronto. 1960.

Allison, A.C., and Malluci, L. Histochemical studies of lysosomes and lysosomal enzymes in virus infected cell cultures. J. Exp. Med. 121, 463 (1965).

Auler, H., and Hohenadel, B. Untersuchungen am Mäuse-ascitescarcinom. Z. Krebsforsch. 48, 149 (1938).

Bainton, D.F., and Farquar, M.G. Origin of granules in polymorphonuclear leucocytes. J. Cell Biol. 28, 277 (1966).

Barka, T. Electron histochemical localization of acid phosphatase activity in the small intestine of mouse. J. Histochem. Cytochem. 12, 229 (1964).

Barka, T. and Anderson, P.J. Histochemical methods for acid phosphatase using hexazonium pararosanilin as coupler. J. Histochem. Cytochem. 10, 741 (1962).

Baserga, R. Mitotic cycle of ascites tumor cells. Arch. Path. (Chicago) 75, 156 (1963).

Bélanger, L.F. and Bélanger, C. Alpharadiography: A simple method for determination of mass concentration in cells and tissues. J. Biophys. Biochem. Cyt. 6, 197 (1959).

Bessis, M. Studies on cell agony and cell death. In Ciba Foundation Symposium: Cellular Injury, 1963. Edited by A.V.S. de Reuck and J. Knight. Churchill, London. 1964.

Block, R.J., Durrum, E.L. and Zweig, G. Paper chromatography and paper electrophoresis. 2nd ed., Academic Press, New York. 1958.

Brandes, D., Buetow, D., Bertini, F. and Malkoff, D.B. Role of lysosomes in cellular lytic processes. 1. Effect of carbon starvation in E. gracilis. Exp. Mol. Pathol. 3, 583 (1964).

Brückner, J. Estimation of monosaccharides by the orcinol-sulfuric acid reaction. *Biochem. J.* 60, 200 (1955).

Caro, L.G. and Palade, G.E. Protein synthesis, storage and discharge in the pancreatic exocrine cell. An autoradiographic study. *J. Cell Biol.* 20, 473 (1964).

Caspersson, T. and Santesson, L. Studies on protein metabolism in the cells of epithelial tumors. *Acta Radiol.* 46 (1942).

Ceelen, G.H. The cytologic diagnosis of ascitic fluid. *Acta Cytol. (Balt)* 8, 175 (1964).

Chapman-Andresen, C. and Nilsson, J.R. Electron micrographs of pinocytosis channels in amoeba proteus. *Exp. Cell Res.* 19, 631 (1960).

Christensen, H.N. and Riggs, T.R. Concentrative uptake of amino acids by the Ehrlich mouse carcinoma cell. *J. Biol. Chem.* 194, 57 (1952).

Cohen, S., Beiser, S.M. and Hsu, K.C. Immunohistochemical study of the uptake of serum proteins by neoplastic liver cells. *Cancer Res.* 21, 1510 (1961).

Cowdry, E.V. *Cancer Cells*. Saunders, Philadelphia. 1955.

Daniels, F., Mathews, J.H. and Williams, J.W. *Experimental physical chemistry*. 3rd ed., McGraw Hill, New York. 1941.

Dixon, W.J. and Massey, F.J. *Introduction to statistical analysis*. McGraw-Hill, New York. 1951.

Duve, C. de. General properties of lysosomes. The lysosome concept. In *Ciba Foundation Symposium: Lysosomes*. Edited by A.V.S. de Reuck and M. Cameron. Churchill, London. 1963.

Duve, C. de. Role of lysosomes in general pathology. In *Ciba Foundation Symposium. Cellular injury 1963*. Edited by A.V.S. de Reuck and J. Knight. Churchill, London. 1964.

Ericsson, J.L.E. An electron microscopic and histochemical study of lysosomes in the proximal tubules of rat kidney. *J. Histochem. Cytochem.* 12, 8 (1964).

Ericsson, J.L.E. Transport and digestion of hemoglobin in the proximal tubule. II. Electron microscopy. *Lab. Invest.* 14, 16 (1965).

Ericsson, J.L.E., Trump, B.F. and Weibel, J. Electron microscopic studies of the proximal tubule of rat kidney. II. Cytosegresomes and cytosomes: Their relationship to each other and to the lysosome concept. *Lab. Invest.* 14, 1341 (1965).

Fawcett, D.W. Local specialization of the plasmalemma in micropinocytosis vesicles of erythroblasts. *Anat. Rec.* 148, 369 (1964).

Fawcett, D.W. Surface specializations of absorbing cells. *J. Histochem. Cytochem.* 13, 75 (1965).

Fawcett, D.W. *The Cell*. Saunders, Philadelphia. 1966.

Flax, M.H. and Himes, M.H. Microspectrophotometric analysis of metachromatic staining of nucleic acids. *Physiol. Zool.* (Chicago) 25, 297 (1952).

Gey, G.O. Some aspects of the constitution and behavior of normal and malignant cells maintained in continuous culture. *Harvey Lect., Series L*, 156 (1956).

Giese, A.C. *Cell Physiology*. Saunders, Philadelphia. 1962.

Goldberg, L., Klein, E. and Klein, G. The nucleic acid content of mouse ascites tumor cells. *Exp. Cell Res.* 1, 543 (1950).

Gomori, G. *Microscopic Histochemistry*, pp 50-69. University of Chicago Press, Chicago. 1952.

Gradwohl, R.B.H. *Clinical laboratory methods and diagnosis*. Edited by S. Fraenkel and S. Reitman. 6th ed., Vol. 2, p III15. Mosby, Saint Louis. 1963.

Grimley, P.M. A tribasic stain for thin sections of plastic-embedded, OsO₄ - fixed tissues. *Stain Technol.* 39, 229 (1964).

Ham, A.W. *Histology*. 5th ed., Lippincott, Montreal. 1965.

Hanks, J.H. and Wallace, R.E. Relation of oxygen and temperature in the preservation of tissues by refrigeration. *Proc. Soc. Exp. Biol. Med.* 71, 196 (1949).

Hartroft, W.S. Electron microscopy of liver and kidney cells in dietary deficiencies. In *Ciba Foundation Symposium: Cellular Injury, 1963*. Edited by A.V.S. de Reuck and J. Knight. Churchill, London. 1964.

Hauschka, T.S., Grinell, S.T., Révész, L. and Klein, G. Quantitative studies on the multiplication of neoplastic cells in vivo. IV. Influence of doubled chromosome number on growth rate and final population size. J. Natl. Cancer Inst. 19, 13 (1957).

Helander, H.F. Ultrastructure of fundus glands of the mouse gastric mucosa. J. Ultrastruct. Res. Suppl. 4 (1962).

Herdson, P.B. and Kaltenbach, J.P. Fine structural changes in hypotonically treated Ehrlich ascites cells. Exp. Cell Res. 42, 74 (1966).

Hirsch, J.G. and Cohn, Z.A. Digestive and autolytic functions of lysosomes in phagocytic cells. Fed. Proc. 23, 1023 (1964).

Holter, H. Problems of pinocytosis, with special regard to amoeba. Ann. N.Y. Acad. Sci. 78, 524 (1959).

Holter, H. Membrane in correlation with pinocytosis. In Intracellular membrane structure. Edited by S. Seno and E.V. Cowdry. Chugoku, Okayama. 1964.

Kalckar, H.M. Galactose metabolism and cell sociology. Science 150, 305 (1965).

Kaltenbach, J.P., Kaltenbach, M.H. and Lyons, W.B. Nigrosin as dye for differentiating live and dead ascites cells. Exp. Cell Res. 15, 112 (1958).

Karnovsky, M.J. Simple methods for "staining with lead" at high pH in electron microscopy. J. Cell Biol. 11, 729 (1961).

Klein, G. Use of the Ehrlich ascites tumor of mice for quantitative studies on the growth and biochemistry of neoplastic cells. Cancer 3, 1052 (1950).

Klein, G. Comparative studies of mouse tumors with respect to their capacity for growth as "Ascites Tumors" and their average nucleic acid content per cell. Exp. Cell Res. 2, 518 (1951).

Klein, G. and Klein, E. The transformation of a solid transplantable mouse carcinoma into an ascites tumor. Cancer Res. 11, 466 (1951).

Klein, G. and Révész, L. Quantitative studies on the multiplication of neoplastic cells in vivo. I. Growth curves of the Ehrlich and MClM ascites tumors. J. Natl. Cancer Inst. 14, 229 (1953).

Lehninger, A.L. The Mitochondrion. Benjamin, New York. 1964.

Lettre, H. Einige Beobachtungen über das Wachstum des Mäuse-Ascites-Tumors und seine Beeinflussung. Hoppe-Seyler Z. Physiol. Chem. 268, 59 (1941).

Lewis, W.H. Pinocytosis by malignant cells. Amer. J. Cancer 29, 666 (1937).

Lindström, B. Roentgen absorption spectrophotometry in quantitative cytochemistry. Acta Radiol. Suppl. 125, 129 (1955).

Loewenthal, H. and Jahn, G. Übertragungsversuche mit carcinomatöser Mäuse-Ascitesflüssigkeit und ihr Verhalten gegen physikalische und chemische Einwirkungen. Z. Krebsforsch. 37, 30 (1932).

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265 (1951).

Luse, S.A. and Reagan, J.W.. Histocytologic and electron microscopic study of effusions associated with malignant disease. Ann. N.Y. Acad. Sci. 63, 1331 (1956).

Manery, J.F. Connective tissue electrolytes. Fed. Proc. 25, 1799 (1966a).

Manery, J.F. Effects of Ca ions on membranes. Fed. Proc. 25, 1804 (1966b).

Marshall, J.M. and Nachmias, V.T. Cell Surface and pinocytosis. J. Histochem. Cytochem. 13, 92 (1965).

Miller, F. and Palade, G.E. Lytic activities in renal protein absorption droplets. An electron microscopical cytochemical study. J. Cell Biol. 23, 519 (1964).

Miller, G.L., Slater, R., Birzgalis, R. and Blum, R. Application of different colorimetric tests to cello-dextrins. Anal. Biochem. 2, 521 (1961).

Moe, H., Rostgaard, J. and Behnke, O. On the morphology and origin of virgin lysosomes in the intestinal epithelium of the rat. J. Ultrastruct. Res. 12, 396 (1965).

Molomut, N., Padnos, M., Satory, V. and Gross, L. Survival of Ehrlich ascites carcinoma cells at high ultracentrifugal forces. Nature 203, 740 (1964).

Novikoff, A.B. Lysosomes and related particles. In *The Cell*. Edited by J. Brachet and A.E. Mirsky, Vol. II, p 447. Academic Press, New York. 1961.

Novikoff, A.B. Lysosomes in the physiology and pathology of cells. In *Ciba Foundation Symposium: Lysosomes*. Edited by A.V.S. de Reuck and M.P. Cameron. Churchill, London. 1963.

Novikoff, A.B. and Essner, E. Cytolysosomes and mitochondria degeneration. *J. Cell Biol.* 15, 140 (1962).

Novikoff, A.B., Essner, E. and Quintana, N. Golgi apparatus and lysosomes. *Fed. Proc.* 23, 1010 (1964).

Novikoff, A.B. and Shin, W.Y. The endoplasmic reticulum in the Golgi zone and its relations to microbodies, Golgi apparatus and autophagic vacuoles in rat liver cells. *J. Microscopie* 3, 187 (1964).

Oyama, V.I. and Eagle, H. Measurement of cell growth in tissue culture with a phenol reagent (Folin-Ciocalteu). *Proc. Soc. Exp. Biol. Med.* 91, 305 (1956).

Pearse, A.G.E. *Histochemistry*. 2nd ed., Churchill, London. 1960.

Pease, D.C. *Histological techniques for electron microscopy*. 2nd ed., Academic Press, New York. 1964.

Peterson, M. and Leblond, C.P. Synthesis of complex carbohydrates in the Golgi region as shown by radioautography after injection of labelled glucose. *J. Cell Biol.* 21, 143 (1964).

Policard, A. *Cellules vivantes et populations cellulaires*. Masson, Paris. 1964.

Porter, K.R. and Bonneville, M.A. *An introduction to the fine structure of cells and tissues*. Lea and Febiger, Philadelphia. 1964.

Reynolds, E.S. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* 17, 208 (1963).

Rifkind, R.A., Morgan, C. and Harvet, M.R. The ferritin conjugated antibody technique. In *Fifth International Congress for Electron Microscopy*. Academic Press, New York. 1962.

Ritter, H.B. A simple permanent aceto-orcein stain for free cells, cell cultures and homogenates. *Stain Technol.* 33, 167 (1958).

Robbins, S.L. Textbook of pathology with clinical applications. Saunders, Philadelphia. 1962.

Roth, T.F. and Porter, K.R. Yolk protein uptake in the oocyte of the mosquito *Aedes aegypti*, L. *J. Cell Biol.* 20, 313 (1964).

Rustad, R.C. The physiology of pinocytosis. *Recent Progr. Surface Sci.* 2, 353 (1964).

Seeger, P.G. Untersuchungen am Tumoraszites der Maus. I. Vitalfärbbarkeit der aszites Zellen. *Arch. Exp. Zellforsch.* 20, 280 (1937).

Seeger, P.G. Untersuchungen am Tumoraszites der Maus. II. Mikrochemische Untersuchung. *Arch. Exp. Zellforsch.* 21, 309 (1938).

Seeger, P.G. Die Unterschiede zwischen normalen Exsudatzellen und Tumoraszites Zellen hinsichtlich ihres Vitalfärberischen. *Arch. Exp. Zellforsch.* 22, 356 (1939).

Selby, C.C., Biesele, J.J. and Grey, C.E. Electron microscope studies of ascites tumor cells. *Ann. N.Y. Acad. Sci.* 63, Art. 5, 748 (1956).

Staubesand, J. Cytopempsis. In Funktionelle und morphologische Organisation der Zelle. II. Sekretion und Exkretion. Springer, Berlin. 1965.

Stöckinger, L. Die Ultrastruktur des Flimmerepithels des Nasen-septums der Ratte. *Z. Zellforsch.* 59, 443 (1963).

Straube, R.L. Fluid accumulation during initial stages of ascites tumor growth. *Cancer Res.* 18, 57 (1958).

Straus, W. Colorimetric analysis with N,N-dimethyl-p-phenylenediamine of the uptake of intravenously injected horseradish peroxidase by various tissues of the rat. *J. Biophys. Biochem. Cyt.* 4, 541 (1958).

Straus, W. Occurrence of phagosomes and phagolysosomes in different segments of nephron in relation to reabsorption, transport and digestion of intravenously injected horseradish peroxidase. *J. Cell Biol.* 21, 295 (1964).

Swift, H. and Hruban, Z. Focal degradation as a biological process. *Fed. Proc.* 23, 1026 (1964).

Tanaka, H. Electron microscope studies on the neutral red vacuoles. In *Intracellular membrane structure*. Edited by S. Seno and E.V. Cowdry. Chugoku, Okayama. 1964.

Tolnai, S. Cytological studies of "Balloon Cells" occurring in the Ehrlich ascites carcinoma. *Can. J. Biochem.* 42, 1375 (1964).

Tolnai, S. An analysis of the life cycle of Ehrlich ascites cells. *Lab. Invest.* 14, 701 (1965).

Tolnai, S., Scantland, R. and Morgan, J.F. A subcellular transmissible agent from Ehrlich carcinoma cells producing ascites tumors in mice. *Nature* 193, 591 (1962).

Tolnai, S., Scantland, R. and Morgan, J.F. A subcellular transmissible agent from Ehrlich carcinoma cells producing ascites tumors in mice. *Can. Cancer Conf.* 5, 175 (1963).

Warshawsky, H., Leblond, C.P. and Droz, B. Synthesis and migration of proteins in the cells of exocrine pancreas as revealed by specific activity determination from radioautographs. *J. Cell Biol.* 16, 1 (1963).

Wasserman, F. and McDonald, T.F. Electron microscopic study of adipose tissue (fat organs) with special reference to the transport of lipids between blood and fat cells. *Z. Zellforsch.* 59, 326 (1963).

Weissman, G. Lysosomes. *New Eng. J. Med.* 273, 1084 and 1143 (1965).

Whaley, W.G., Kephart, J.E. and Mollenhauer, H.H. The dynamics of cytoplasmic membranes during development. In *Cellular membranes in development*. 22nd Symposium of the Society for the Study of Development and Growth. Edited by M. Locke. Academic Press, New York. 1964.

Wigglesworth, V.B. The use of osmium in the fixation and staining of tissues. *Proc. Roy. Soc. (Biol.)* 147, 185 (1957).

Woodard, J.W., Rasch, E. and Swift, H. Nucleic acid and protein metabolism during the mitotic cycle in *Vicia faba*. *J. Biophys. Biochem. Cyt.* 9, 445 (1961).

Yasuzumi, G. and Sugihara, R. A comparative electron microscopic study on Ehrlich ascites cells, Yoshida sarcoma cells, and human cancerous peritonitis ascites cells. *Cancer Res.* 18, 1167 (1958).

Zetterquist, H. The ultrastructural organisation of the columnar absorbing cells of the mouse jejunum. Karolinska Institute Monograph, Karolinska Institute, Stockholm. 1956.

Zucker-Franklin, D. and Hirsch, J.G. Electron microscope studies on the degranulation of rabbit peritoneal leucocytes during phagocytosis. *J. Exp. Med.* 120, 569 (1964).