STUDIES ON THE SUBCELLULAR DISTRIBUTION OF ACID PHOSPHATASE

STUDIES ON THE SUBCELLULAR DISTRIBUTION OF ACID PHOSPHATASE

By

ARTHUR JAMES ZINTEL, B.Sc.

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

October 1963

MASTER OF SCIENCE (1963)
(Chemistry)

HAMILTON COLLEGE
McMASTER UNIVERSITY
Hamilton, Ontario.

TITLE: Studies on the Subcellular Distribution of Acid Phosphatase

AUTHOR: Arthur James Zintel, B.Sc. (Acadia University)

SUPERVISOR: Professor D. R. McCalla

NUMBER OF PAGES: iv, 63

SCOPE AND CONTENTS:

Preliminary experiments indicated that lysosomes are present in rat liver and onion embryos. A differential centrifugation study was made of the intracellular distribution of acid phosphatase in pea embryo tissue in an attempt to show that this enzyme is enclosed by a membrane forming granules similar to the lysosomes of hepatic tissue. The results reveal that acid phosphatase is soluble under the conditions employed, but it is believed that this may well have resulted from excessive damage to the subcellular bodies during homogenization.

ACKNOWLEDGMENTS

It has been my privilege to work under the supervision of Dr. D. R. McCalla. To him, I extend my gratitude for patient assistance given in all aspects of study.

I also wish to thank my wife for her help and suggestions in preparing this manuscript.

I am grateful to the Department of Chemistry, for Departmental Scholarships and Summer Research Scholarships given to me for the academic years 1961-62 and 1962-63.

TABLE OF CONTENTS

I

INTRODUCTION

In 1955 de Duve and co-workers at the University of Louvain proposed the name lysosome for a newly-discovered rat liver cytoplasmic particle possessing only hydrolytic enzymes. When subsequent work in a number of laboratories identified lysosome-like particles in numerous mammalian tissues, de Duve postulated that lysosomes are a constituent of all cell types and are not a special class of particles restricted to hepatic tissue.

Plant tissues might also be expected to contain lysosomes since it is known that much of the polymeric material of leaves is degraded and returned to the stem before the leaves are lost in the fall. Another type of autolysis takes place in the endosperm of germinating seeds. However, when the present work was started, no lysosome-like granules had been demonstrated in plants. Therefore, tissue fractionation studies were initiated with the hope of finding lysosomes in pea roots, and thus extend the lysosomal concept.

II

LITERATURE SURVEY

1. Structure of the Living Cell

Since the work described in this thesis deals mainly with fractionation of ruptured cells and attempts to establish the particulate nature of acid phosphatase, it is pertinent to review briefly current knowledge of the structure of intact cells and of the biochemical function of the subcellular components. As there probably exists no "typical" cell, it is necessary to describe a generalized cell. Perhaps no cell will have all of the components discussed, but all living cells will have most of them.

The contents of animal cells are believed to be contained within a plasma membrane; plant cells have in addition, a rigid cell wall, of which cellulose is the most important structural component. The existence of the plasma membrane has never been unequivocally demonstrated, but is inferred from the requirement for a barrier to free diffusion and from permeability and electron microscopy studies of mammalian red blood cell ghosts. Ponder's (1961) critical analysis of the plasma membrane theory points out its severe limitations, but in lieu of any experimentally tested alternative

hypotheses, a plasma membrane with properties similar to those of red cell membranes must be assumed. The lipid and protein of the red cell ghost, probably exists as lipoprotein, the lipid being weakly or strongly bound, depending on its nature. From the ratio of lipid to protein, Parpart and Ballentine (1952) have calculated that each protein molecule (molecular weight 70,000) would have thirty lipid molecules associated with it. Although numerous workers have reported the separation of protein fractions from red cell ghosts, the protein composition is still undetermined, as sufficient physicochemical and immunological tests have not been applied to warrant these fractions being considered as homogeneous. The plasma membrane is reasonably elastic, but its toughness varies with cell type. For example, liver or lymphoid cell membranes are easily disintegrated by grinding, whereas, the membranes of chicken tumor cells can resist lengthy maceration with fine sand (Swanson, 1961). The membrane selectively absorbs solutes from the external environment and can be regarded as a living portion of the cell since it is repaired by the cytoplasm when punctured with a microneedle.

The most conspicuous particle in the cytoplasm is the nucleus. The interphase nucleus is generally a round body separated from the cytoplasm by a nuclear membrane,

which unlike the plasma membrane, is not capable of self-repair (Chambers and Fell, 1931). The semi-permeable nuclear membrane is in direct contact with the cytoplasm and is connected with the external fluids by a number of canals. The nucleus is responsible for overall cell organization and function during both interphase and division, for if it is removed from a mononucleate cell, the cell fails to divide and eventually dies, but in binucleates the removal of one nucleus usually does not destroy all cellular functions. Most of the cell's genetic information is carried by the nucleus. Such information has been found in chromosomes during division and in chromatin during interphase; in both cases the chemical substance which bears the genetic code is deoxy-ribosenucleic acid (DNA). The detailed chemical structure of any particular DNA has not been elucidated, but some recent data reported by independent investigators substantiate the helical structure proposed by Crick and Watson (1954) and the triplet code suggested by Gamow (1954). It is known that this information is transcribed into a ribosenucleic acid, called messenger-RNA (m-RNA), which migrates from the nucleus to other subcellular particles known as ribosomes. The m-RNA appears to attach itself to the ribosomes where it is used one or more times as a template for protein synthesis.

Mitochondria or chondriosomes lie free in the cytoplasm. As described by Lewis and Lewis (1914-15) they are "extremely variable bodies, which are continually moving and changing shape in the cytoplasm," two or more fusing to form a single mitochondrion, dividing into two, and increasing or decreasing in size without fusion or division. These astonishingly plastic organelles rapidly change shape and size in response to many chemical and physical stimuli. In addition to these active movements, they are also moved about in the cell by protoplasmic streaming, diffusion and Brownian forces. While the size and shape of a mitochondrion is related to the cell type, all have the same general morphology. They range from 4 to 5 microns in diameter or length down to submicroscopic particles. Most often they are pictured as rod-shaped bodies with a smooth outer membrane and an infolded inner membrane forming cristae which provide a large surface area for their many enzymic reactions. These membranes are also liproprotein in nature. From almost all the information collected on the biochemical activities of mitochondria, the supreme fact to emerge is that mitochondria are the chief if not the exclusive sites of oxidative phosphorylation (Novikoff, 1961a). Isolated mitochondria are reported to possess high transaminase activity (Gianetto and de Duve, 1955) and are the sole loci of glutamic

dehydrogenase (Hogeboom and Schneider, 1953); these results would link mitochondria with amino acid formation. Mitochondria are indirectly linked to protein synthesis since they provide a source of adenosine triphosphate (Bates et al., 1958); Burnett and Kennedy (1954) established that the enzyme involved in phosphorylation of proteins is localized in mitochondria. Penn (1959, 1960) has found mitochondria to be the site of catabolism of exogenous serum albumin, and Kennedy (1957) found that mitochondria can catalyze the synthesis of phosphatides. "The diversity and complexity of mitochondrial functions, revealed by the experiments of the past decade, cannot help but be a source of amazement, even to those who have been closely associated with this field since its inception" (Schneider, 1959).

Extending throughout the entire cytoplasm is a system of fine canals or vesicles made up of a lipoprotein double membrane, which in some cases appears to be an extension of the nuclear membrane. This structure is known as the endoplasmic reticulum. Much of the endoplasmic reticulum has ribosomes attached to it when isolated from disrupted cells, the nucleoprotein with pieces of the endoplasmic reticulum attached, are referred to as the microsomes. As noted above, ribosomes have been demonstrated to be the primary site of protein synthesis. In addition, certain enzymes such as glucose-6-phosphatase, which are not

involved directly in protein synthesis, are attached to the microsomal surface. No relationship between the endoplasmic reticulum structure and the function of such enzymes has been established.

In the majority of cell types studied, there exists a cytoplasmic area capable of reducing silver nitrate or osmium tetroxide, which was given the name Golgi body or Golgi apparatus. Since the discovery of these bodies in 1898, experiments have been conducted to show that they are involved in some direct way in secretory activity. Although improvements in isolation techniques have allowed Golgi material to be separated from the rest of the cell, the growing concensus of opinion is that these bodies are actually only a part of the complex matrix of cytoplasmic membranes which divide the cell into specialized areas or compartments (Dalton, 1961).

In the cytoplasm of cells, especially plant cells, there can be seen a clear region surrounded by a membrane called the tonoplast. These spherical bodies, referred to as vacuoles, have received comparatively little study, but it is known that in plant cells, water soluble pigments are concentrated in these bodies, while in animals, vacuoles are involved in the movement of materials in and out of the cell. Recently de Duve (1958) has proposed a physiological scheme relating vacuoles and a new cytoplasmic particle, the

lysosome, to phagocytosis and pinocytosis. Lysosomes will be the subject of more detailed discussion in later sections.

Within the cells of plants are found a group of related, self-perpetuating organelles, known as plastids. Some examples of these organelles are: (1) leucoplasts are storage bodies for starch; (2) elaioplasts produce oil; (3) chromoplasts contain the pigments such as carotene and xanthophyll. The most important plastid, however, is the chloroplast. This body contains the photosynthetic apparatus necessary for the production of hexose sugars upon which all higher forms of life depend. Chloroplasts appear to develope from smaller particles called proplastids, or alternatively by a process of division (Granick, 1961).

2. The Biochemical Properties of Lysosomes

In 1951 researchers at the University of Louvain reported that nonspecific acid phosphatase exhibits latent activity which can be 'activated' by a variety of physical and chemical treatments (Berthet and de Duve, 1951; Berthet et al., 1951). By modifying the classical differential centrifugation scheme of Claude (1946a, b) they obtained a fraction sedimenting between the mitochondrial and microsomal fractions possessing most of the acid phosphatase (de Duve et al., 1953; Appelmans et al., 1955). Further

studies on this fraction showed the existence of discrete particles containing all the acid phosphatase and further that activation results from rupture of the particle membrane, which releases the enzyme in a fully active, soluble state (Appelmans et al., 1955; Appelmans and de Duve, 1955). Four other hydrolytic enzymes with an acid pH optimum were found to be activated in a manner parallel to acid phosphatase; these particles were given the name lysosomes (Gianetto and de Duve, 1955; de Duve et al., 1955).

At least ten lysosomal enzymes are now known. Acid phosphatase, acid deoxyribonuclease, acid ribonuclease, β-glucuronidase (de Duve et al., 1955), cathepsins A, B and probably cathepsin C (Finkenstaedt, 1957), arylsulfatases A and B (Viala and Gianetto, 1955; Roy, 1958), β-galactosidase, β-N-acetylglucosaminidase, α-mannosidase (Sellinger et al., 1960) have been shown to be lysosomal in rat liver; and a phosphoprotein phosphatase has been shown to be lysosomal in mouse liver (Paigen and Griffiths, 1959). It is likely individual lysosomes contain all the acid hydrolases recognized as lysosomal, but in variable proportions (de Duve, 1961). However, Shibko et al. (1963), investigated the presence of lysosomal enzymes in tissues of various species representative of the principal phylla of the animal kingdom, and found that certain liver lysosomal enzymes were absent from muscle

lysosomes. Their data suggest that the enzyme complement of lysosomes is more tissue type dependent, than species dependent.

Lysosomes isolated from rat liver in 0.25M sucrose have an average density of 1.15 and vary in diameter from 0.25 microns to 0.8 microns, the mean diameter being 0.4 microns when calculated from sedimentation data (de Duve, 1958; Appelmans et al., 1955). Novikoff et al. (1956) obtained a size range in good agreement with these values obtained from electron micrographs.

Lecithinase, trypsin, chymotrypsin and pancreatin are all capable of disrupting the lysosomal membrane, indicating that this membrane consists of lipoprotein (Beaufay and de Duve, 1959). Fell et al. (1962) reported that vitamin A as the lipid-soluble free acid or alcohol can also release lysosomal enzymes. In addition, the acid hematin staining procedure specific for phospholipid (Deane, 1958) gives a positive reaction throughout the granules in kidney and liver (Oliver et al., 1954). Lysosomes isolated in sucrose solutions have this membrane intact, so that the enzymes are inaccessible to their substrates (Berthet and de Duve, 1951; Berthet et al., 1951). In all probability this same condition exists in normal intact cells.

Besides the enzymic methods previously mentioned,

many other treatments result in activation of the enzymes. Such treatments include blending (i.e. waring blendor treatment); incubating at 37°; freezing and thawing; and exposure to variations in pH, hypotonic media, isotonic sodium chloride, detergents or carbon tetrachloride (de Duve, 1958). Weissmann and Dingle (1961) have found that ultra violet radiation releases lysosomal enzymes while hydrocortisone increases the resistance of lysosomes to ultra violet radiation. de Duve et al., (1961) reported that, in vivo and in vitro, hydrocortisone lowers the sensitivity to rupture by pH and heat.

3. Cytological Identification of Lysosomes

The first electron micrographs of a lysosomal fraction (Novikoff et al., 1956) showed the presence of single outer-membraned particles, some possessing internal cavities, but all containing many electron-opague grains resembling ferritin molecules. These granules resembled the dense bodies seen along the bile canaliculi of liver. Although iron is found in all particulate fractions from hepatic tissue, Beaufay et al. (1959) have shown it to be most abundant in the lysosomal fraction. By using density-gradient centrifugation, de Duve et al. (1960) separated lysosomes from another group of single membrane particles containing the enzymes uricase, D-amino acid oxidase and

catalase. Electron microscopic examination of fractions obtained in this way reveal ferritin grains in lysosomes but none in the uricase particles (Novikoff, 1961b) suggesting that the latter may be the microbodies described by Rouiller and Bernhard (1956).

The original histochemical procedure for acid phosphatase (Gomari, 1941, 1952) has undergone a variety of modifications, greatly increasing the confidence that can be placed in this technique. However, caution should be used in assuming that any staining procedure for enzymes is reliable in the absence of suitable control experiments and biochemical assay (Holt, 1959). Diengdoh (1963) has shown, that acid phosphatase granules in mouse skin correspond to lysosomes, as their apparent rupture (seen by diffusion of the enzyme) in response to hypotonicity, to acid conditions, to heat, to freezing and thawing, and to non-ionic detergents (Triton X-100) parallels that of isolated lysosomes from rat liver.

The pericanalicular dense bodies seen in hepatic tissue have also been shown to be lysosomes. With osmium-fixed sections, the size, shape and distribution of these dense bodies is similar to granules containing acid phosphatase (Essner and Novikoff, 1961). More conclusive studies by Holt and Hicks (1961a, b) show that (1) acid phosphatase granules are ruptured by Triton X-100 and

lecithinase; (2) they are located mainly in the peri-canalicular region of the cell; and (3) they are morphologically similar to the dense bodies.

Essner and Novikoff (1961) also reported that the increase in the number of microbodies in hepatic tissue from bilirubin-infused rats parallels the increase in the number of acid phosphatase granules, suggesting that microbodies are also lysosomes. However, Holt and Hicks (1961b) could not find acid phosphatase in microbodies from normal rats. Although further study is needed to relate these different observations, it is possible that all single-membraned bodies are different functional states of the same entity (Claude, 1960).

Droplets seen in proximal convoluted kidney tubules have also received detailed study. Holt (1959) and Novikoff (1961b) demonstrated that these droplets contain acid phosphatase, and thus, can be considered to be lysosomes.

In a brief communication, Koenig (1963a) reported that lysosomes in rat kidney are autofluorescent. Should this prove to be a general characteristic, it will greatly facilitate the identification of lysosomes and lysosomal constituents.

4. Lysosomes in Other Tissues

It is desirable to review some of the published

reports which indicate that lysosomes are a characteristic organelle of many cell types. de Duve (1958) has summarized the biochemical data from his laboratory indicating the presence of lysosome-like particles in mammalian brain, spleen and thyroid. Similar data has been found for mammary glands (Greenbaum et al., 1960) and pancreas (Van Lancker and Morrill, 1958; Van Lancker and Holtzer, 1959). Cytological studies show acid phosphatase granules in mammalian cerebral capillary endothelium, adrenaline secreting cells of the adrenal medulla (Novikoff, 1961b), prostatic epithelium (Brandes et al., 1962) and trigeminal ganglion cells (Tewari and Bourne, 1963).

Biochemical studies show lysosomes to be present in the Müllerian duct of the chick embryo (Brachet et al., 1958), the giant amoeba Chaos chaos (Holter, 1954, 1956; Holter and Lowy, 1959), and in Amoeba proteus (Quertier and Brachet, 1959). Acid phophatase granules are seen in the food digesting phagocytes of planarians (Rosenbaum and Rolon, 1960) and in Tetrahymena pyriformis (Klamer and Fennell, 1963).

Few investigations have been made with plants. In a recent abstract Harrington and Altschul (1963) have reported that acid phosphatase from roots of germinating onion seeds sedimented in a particulate form and could be released by treatment with Triton X-100. Novikoff (1961b)

also identified acid phosphatase granules in onion root tips cytochemically, and the work of Avers and King (1960) suggests acid phosphatase granules are present in grass root tips.

It can be seen that lysosome-like particles are found in many organisms and tissue types. However, lysosomes can still not be called a universal organelle of living cells, as there have been cases reported where cytoplasmic granules with acid phosphatase activity could not be found. Novikoff (1961b) reported that such granules could not be seen in skeletal or cardiac muscles. However, such results could be attributed to cytological manipulations in tissue preparation. Tappel et al. (1962) and Zalkin et al. (1962) reported that the specific activity of lysosomal enzymes is very low in normal mouse muscle, but increases greatly as genetic and vitamin E induced muscular dystrophy developes. Koszalka et al. (1961) suggested that the invasion of macrophages into the muscle tissue is the major source of the lysosomal enzyme, cathepsin. Acid phosphatase could not be detected in a variety of other muscle tissues using conventional techniques (Shibko et al., 1963). Schmidt et al. (1963) found that acid phosphatase in bakers' yeast is not contained in granules but is probably contained in compartments near the cell surface. Mitchell and Moyle (1956) also reported

that acid phosphatase is associated with the cell surface in Staphylococcus aureus.

5. Physiological Function of Lysosomes

The physiopathological role of lysosomes in cell metabolism, has not been conclusively demonstrated, even though a large volume of supporting literature has accumulated in the past decade. This subject has been reviewed by de Duve (1958) and Novikoff (1961b). The following will present only the principal current speculations.

Any hypotheses on the biochemical function of lysosomes has to account for the following observations: (1) lysosomes appear to contain only acid hydrolases; (2) the lysosomal membrane restricts enzyme diffusion; (3) the substrates are inaccessible to the enzymes except under certain conditions. There is reason to believe that enzymes which catalyze hydrolysis in vitro will normally catalyze the same reaction in vivo. If this is true one could expect lysosomes to be involved in intracellular digestion, and physiological and pathological autolysis (de Duve, 1958).

Lysosomes are probably involved in pinocytosis and phagocytosis. In pinocytosis, "a certain area of the surface membrane of the cell encloses a droplet of the surrounding medium, separates from the surface and migrates

into the cell" (Holter, 1961). In phagocytosis, particles are also ingested. Studies of pinocytosis in cells of the proximal convoluted tubules of kidney have provided most of the information linking lysosomes with pinocytosis. The material to be ingested by the cell enters into microcytotic vacuoles by the canaliculi extending from adjacent microvilli (Novikoff, 1959, 1960b, 1961b; Novikoff and Essner, 1960; Burgos, 1960). Straus (1957, 1958, 1959) injected horseradish peroxidase intravenously, and studied the location of the enzyme in the cells after various lengths of time. Vacuoles with peroxidase activity were visible from 5 to 30 minutes after injection, but after 2 hours there was no activity in the lumen, brush border, or vacuoles. Instead, the peroxidase activity was localized in droplets containing acid phosphatase and possessing a single membrane. It would seem, that the microcytotic vacuoles fuse together forming larger vacuoles, which migrate to that portion of the cell rich in Golgi bodies, where they acquire sufficient acid phosphatase to give a staining reaction. Farquhar and Palade (1960) have carried out an excellent cytochemical study of pinocytosis in rat glomerular epithelium cells, from which they concluded that the microcytotic vacuoles fuse to form larger vacuoles.

There have been numerous speculations as to how acid phosphatase 'enters' the vacuole. The possibility of fusion

of an acid hydrolase granule with a vacuole is indicated in a brief communication by Brewer and Heath (1963), who reported that vacuoles in liver cells formed after injection of sucrose, arise from lysosomes. Trump and Janigan (1962) had also reported that vacuoles were formed by enlargement of lysosomes, and observed the aggregation of acid phosphatase droplets prior to enlargement. Lysosomes could originate in turn from Golgi bodies. Lodja (1960) found acid phosphatase-rich bodies to be concentrated in the Golgi zones of basophiles of rat pituitary, and Novikoff (1959) noted that in parenchymal cells of liver, experimental procedures that change the distribution of Golgi bodies change the lysosome distribution correspondingly. Ogawa et al. (1960, 1961) and Koenig (1963b) reported that lysosomes are stained with neutral red, as are Golgi bodies and vacuoles. Essner and Novikoff (1962) concluded from an electron microscopy study of hepatoma tissue that lysosomes originate from the Golgi apparatus.

Phagocytosis has been reported to be similar to pinocytosis in mechanism and in relation to lysosomes. The process has been studied in phagocytes (Essner, 1960) in polymorphonuclear leucocytes (Cohn and Hirsch, 1960a, b; Hirsch and Cohn, 1960), in Kupffer cells (Novikoff and Essner, 1960; Essner and Novikoff, 1961), and in parenchymal cells of liver (Hampton, 1958).

It should be remembered, that relatively few cell types are capable of pinocytosis or phagocytosis (Holtzer and Holtzer, 1960), and so the temptation to unite Golgi apparatus, lysosomes, and vacuoles in an oversimplified picture should be avoided. These bodies all appear to be involved in intracellular digestion or storage of nutrients, but it is conceivable that this may not be the main function or even an important function in many cells.

Physiological autolysis is known to occur in many developemental processes. Examples are involution of the thymus at puberty; involution of the uterus after parturition; and in the metamorphosis of insects and other zoölogical groups (de Duve, 1958). Physiological autolysis probably occurs in all tissues, as is evidenced by the observed turnover of their main constituents. The mechanism of this process has received very little study, most workers being concerned with synthesis in the cell. However, lysosomes have been implicated in studies by Weber (1957a, b) and de Duve (1963a, b) on catheptic activity in the tails of Xenopus larvae. It was found that lysosomal enzymes decrease during the growth phase of the tail, and increase during its subsequent resorption. Weber and Salzmann (1963) have shown that one of these enzymes, acid phosphatase, is localized in lysosome-like granules. Brachet et al. (1958) have demonstrated a marked increase in lysosomal enzyme

activities, and in the proportion of these activities that is unsedimentable, in regressing Müllerian ducts of the chick embryo. A similar situation was found for regressing Rana tadpole tail (Novikoff, 1960a).

de Duve (1958) has suggested that rupture of the lysosomal membrane might initiate necrosis or cell death. He found that the intensity of necrotic phenomena was proportional to the lysosomal enzymes in the free or unsedimentable form. Cytological studies (Trump et al., 1962), however, indicate that considerable mitochondrial degeneration appears before any autolysis around lysosomes can be seen. For the present, it is perhaps best to consider lysosomes as scavengers which clear tissues of dead cells.

6. Centrifugation Technique

Centrifugation is the most versatile technique used for fractionation of subcellular components. Since it has been used extensively in this work, a brief discussion of the main principles will be presented. No attempt has been made to provide a complete survey of the literature, but attention is called to reviews by Svedberg and Pedersen (1940), Pickels (1943, 1952), Anderson (1956), de Duve and co-workers (1954, 1959) and Allfrey (1961).

Theory

Fractionation by differential centrifugation is

based on the fact that particles of different sizes or densities sediment at different speeds in a field of centrifugal force. The rate of sedimentation of a spherical particle is given by Stokes' law:

$$\frac{dx}{dt} = \frac{2 r^2 (d_p - d_m)}{9 \eta} g$$

where x is the distance from the axis of rotation (in cm.), t the time (in sec.), r the radius of the particle (in cm.), d_p its density (in g./cu. cm.), d_m the density of the suspension medium (in g./cu. cm.), η the viscosity of the medium (in poises), and g the gravitational acceleration (in cm./sec.^{2}).

In a centrifuge the sedimentation is increased by the centripetal acceleration produced which may be expressed in multiples of the gravitational acceleration, g. The centripetal acceleration is given by

$$\omega^2 x$$

in cm./sec.^{2}, and where ω is the angular velocity (in rad./sec.), and x the distance from the center of rotation (in cm.). These expressions can be combined and integrated to give the equation:

$$2.3 \log_{10} \frac{R_{max}}{R_{min}} = \frac{2r^2(d_p - d_m)}{9\eta} \int_0^t \omega^2 dt$$

where R_{MAX} is the maximal distance between the bottom of the liquid and the axis of rotation, R_{min} is the minimal distance between the miniscus and the axis of rotation.

When the time for acceleration and deceleration is only a small fraction of total time of centrifugation, the integral $\int_0^t \omega^2 dt$ is closely approximated by $\omega^2 t$. Experimental conditions can then be described by stating the time (t) during which the centrifuge is run and the centripetal acceleration at the plateau speed (i.e. g_{max}).

However, in modern high speed machines capable of providing very strong centrifugal forces, centrifugations may last only a few minutes with the time to accelerate and decelerate making up a significant fraction of the total. Under these circumstances the simple statement of g_{MAX} and t does not allow reproducibility from one instrument to another. de Duve and Berthet (1953) have suggested that the conditions of sedimentation be given by the sedimentation constant (S) of the lightest spherical particles which are completely sedimented:

$$S = \frac{2r^2(d_p - dm)}{9\eta} = \frac{2.3 \log_{10} \frac{R_{MAX}}{R_{min}}}{\int_0^t \omega^2 \, dt}$$

An approximate value of the integral $\int_0^t \omega^2 \, dt$ can be obtained experimentally. In this equation **S** is given in seconds or in Svedberg units (10^{-13} sec.) multiplied by 10^{13}. The value of **S** may be expressed in the conventional way as a function of g average (the centripetal acceleration in the center of the tube). From the expression:

$$g_{av.} = \frac{1}{981} \omega^2 R_{av.}$$

it can be shown that:

$$S = 3.9 \times 10^{-5} \frac{R_{av} \log_{10} \frac{R_{MAX}}{R_{min}}}{\int_0^T g_{av} \, dT}$$

where **T** is the time in minutes. $\int_0^T g_{av} \, dT$ is expressed as the composite unit, g-min., or alternatively, as the

equivalent of x minutes at y times g. The preceding formulae are strictly only valid if sedimentation occurs parallel to the direction of the field. However, Pickels (1943) has shown that a tube inclined at a fixed angle may be treated for most practical purposes as a horizontal tube.

Practical

A Potter-Elevehjem homogenizer with a smooth-walled glass tube and plastic pestle is the best device for grinding most tissue. Simple rubbing in a mortar disrupts only a fraction of the cells, while a mechanical chopper (Waring Blendor) or an all glass type homogenizer (releases powered glass) causes excessive damage to the particulate components of cells. Damage to these particles is also minimized by appropriate grinding of the tissue. The tissue is first gently ground in a minimum volume of fluid (1-1.5 ml. of grinding fluid to 1 g. of tissue) and then centrifuged at a speed just sufficient to sediment the nuclei and unbroken cells. The particles released are then drawn off with the supernatant so that they are not damaged by subsequent grinding. The precipitate is rehomogenized in another portion of fluid and centrifuged as before; a further homogenization generally yields near quantitative release of the subcellular components.

"Physiological" salt solutions have been found to cause damage to the subcellular particles, so isotonic sucrose solutions are now used as grinding media. Sucrose solutions tend to cause agglutination of particulate material; this effect is minimal at approximately neutral pH, and increases markedly below pH 6. With certain tissues the addition of base may be necessary during grinding. Decreasing the centrifuging time also minimizes agglutination, and Versene (ethylenediamine tetraacetic acid) is frequently added to grinding media, as it appears to decrease aggregation of cytoplasmic material by complexing with calcium ions.

In simple differential centrifugation the homogenate is centrifuged so as to sediment one particle type completely. The pellet is removed and the supernatant again centrifuged to obtain the smaller or less dense particles, the process being continued until only 'soluble' material remains. Soluble material is generally taken to be that which will not sediment with the microsomes or ribosomes. Pellets obtained in this manner are contaminated by particles from other fractions. Examination of Stokes' law shows that a small dense particle could sediment with the larger granules, thus some of the smaller particles will have sedimented with the larger ones. This type of contamination is decreased by 'washing' the pellet with more grind-

ing medium, and then recentrifuging.

With density gradient centrifugation, the particles are fractionated according to density only. A density gradient is prepared by appropriate mixing of two liquids or solutions, one of which may contain the material to be fractionated. In this manner the material is distributed throughout the tube. An alternative is to place a thin layer of the homogenate over the gradient. The tubes are then spun in a centrifuge until equilibration has been reached and the particles have moved to a point in the tube where their density is matched by that of the surrounding fluid.

III

EXPERIMENTAL

1. Tissue Sources

Adult hooded male rats were obtained from the Department of Psychology at McMaster University. The rats were fed a standard diet and attained an average weight of 290 g.

Spergon DDT treated peas (Pisum sativum L. var. Alaska) were purchased from Dominion Seed House, Georgetown, Ontario. The peas were soaked for 6-8 hrs. in just enough distilled water to cover the seed, and then placed between pieces of moist filter paper in a shallow pan which was covered with aluminum foil. They were germinated at 20-23°. Thiram treated onion seeds (Allium cepa L. var. Yellow Globe Danvers), purchased from Dominion Seed House, were placed on moist filter paper in a covered shallow pan, and germinated at 25°.

2. Chemicals

Reagent grade β-glycerophosphate, glucose-6-phosphate and type III cytochrome C were purchased from Sigma Chemical Company. Sodium cacodylate was obtained from Matheson Coleman and Bell, while an "Extra Pure" sample of

1-amino-2-naphthol-4-sulfonic acid was obtained from British Drug Houses. All other chemicals used were reagent grade.

3. Evaluation of the Integral $\int_0^t \omega^2 dt$

Mention has already been made of the advantage of reporting differential centrifugation conditions (especially when using an ultracentrifuge) in the manner suggested by de Duve and Berthet (1953), in terms of the smallest particle which is completely sedimented. For this purpose, it is necessary to obtain a value for the integral $\int_0^t \omega^2 dt$. An approximate value for the integral was obtained experimentally for a Beckman Model L Preparative Ultracentrifuge, using the manufacturer's #50 rotor.

The integral $\int_0^t \omega^2 dt$ was considered to consist of the sum of three parts which could be evaluated independently. Thus, it was evaluated for acceleration, plateau run, and deceleration.

Ten $^5/_8$" x $2\,^1/_2$" cellulose centrifuge tubes were filled with ice cold distilled water, capped, and then placed in the #50 rotor. The ultracentrifuge was adjusted so that the rotor temperature would be about 0° for prolonged runs (longer than 1 hr.). The "speed selector" was set at 5,000 revolutions per minute (R.P.M.) and the speed attained recorded every 15 seconds until maximum speed was

reached; the procedure was repeated for every 5,000 R.P.M. increment on the speed selector. Similar data were collected for deceleration with the automatic brake "on".

At each point, the value of ω^2 was calculated from the relationship

$$\omega^2 = \frac{4\pi^2 (R.P.M.)^2}{3600}$$

and the values obtained plotted as a function of time for each speed setting. The area under the curve then gives the value of $\int_0^t \omega^2 dt$ for acceleration or deceleration at a particular speed setting (see table 1). The value of the integral $\int_0^t \omega^2 dt$ during the plateau portion of the run is given by $\omega^2 t$ and is readily calculated. The values of $\int_0^t \omega^2 dt$ during acceleration and deceleration were plotted as a function of the speed setting and the time required to accelerate or decelerate was also plotted as a function of speed setting. S_{min} was calculated using the equation presented on page 23.

4. Homogenization

Rat Liver

Rats were fasted for 12 hrs. to eliminate liver glycogen, after which they were killed by decapitation, bled, and the liver quickly removed and chilled in ice

Table 1: Experimentally determined values of $\int_0^t \omega^2 dt$ and time to accelerate or decelerate.

Speed Setting	Acceleration		Deceleration	
(R.P.M.)	$\int_0^t \omega^2 dt$ ($rad^2/sec \times 10^6$)	time (sec)	$\int_0^t \omega^2 dt$ ($rad^2/sec \times 10^6$)	time (sec)
10,000	25	45	30	45
15,000	68	60	77	81
20,000	135	75	142	95
25,000	250	90	237	110
30,000	422	120	365	123
35,000	750	150	512	137
40,000	1,270	195	700	151
45,000	2,040	240	1,000	165

cold 0.25M sucrose solution. The liver was cut into several pieces, washed in 0.25M sucrose, blotted to remove excess sucrose and weighed. It was then gently ground in a minimum volume (1-1.5 ml. of medium per 1 g. of wet tissue) of 0.25M sucrose containing 0.001M ethylenediamine tetraacetic acid, and adjusted to pH 7.0. The grinding was done in a Potter-Elevehjem type homogenizer consisting of a smooth glass-walled tube and a teflon pestle. The suspen-

sion obtained was centrifuged at 700 g for 10 min. The supernatant was removed with a hypodermic syringe fitted with a #18 canula bent slightly at the tip. The pellet was resuspended in the plastic centrifuge tube using a glass tube blown out into a close fitting bulb, which was operated as a microhomogenizer, the glass tube being rotated by an electric motor. The 0.25M sucrose solution was added dropwise at first and then as the lumps were dispersed it was added in progressively larger portions. The resuspended pellet was rehomogenized, centrifuged at 600 g for 10 min., and the supernatant removed as before. The pellet was resuspended again, homogenized, centrifuged for 10 min. at 500 g, and the supernatant removed. The pellet obtained was now almost free of intact cells, and consisted mainly of intact nuclei and cell debris. The combined supernatants were used for further fractionation. All steps in the above procedure were performed at 0°.

Plant Embryos

About 6 g. of pea embryos were collected from seed germinated for 3 to 4 days. The embryos were placed in ice cold 0.25M sucrose grinding medium, and ground with a Potter-Elevehjem homogenizer fitted with a tapered teflon pestle. A "nuclear-debris fraction" was then prepared under the same conditions used for rat liver. In this case

the final pellet was also contaminated with cell wall material.

Onion embryos were ground in an identical manner.

5. Fractionation of Homogenates

The combined supernatants from the homogenization were diluted to form a 1:10 (1 g. wet tissue in 10 ml. of suspension) extract in 0.25M sucrose. The extract was then transferred to 10 ml. cellulose or polypropylene centrifuge tubes which were capped and placed in a Spinco #50 rotor. These were centrifuged at 0° in the preparative ultracentrifuge for 33,000 g-min. (S_{min} 17,300 S). The supernatant was removed with a hypodermic syringe fitted with a 3 inch #19 needle. The precipitate was resuspended with the glass pestle described in the previous section, and then recentrifuged under the same conditions. This washing was repeated once more. The pellet so obtained was resuspended to make a 1:10 or 1:5 extract, and was labelled mitochondrial fraction. The supernatant was centrifuged for 250,000 g-min. (S_{min} 2,300 S) and washed twice, as above. The resuspended pellet was called the lysosomal fraction. The supernatant was centrifuged for 3,000,000 g-min. (S_{min} 190 S), the pellet resuspended, and labelled the microsomal fraction. The final supernatant was considered to contain only 'soluble' material.

This fractionation procedure was used for both rat liver and pea embryos.

In some experiments a crude "lysosomal preparation" was made by centrifuging the combined supernatants from homogenization, for 250,000 g-min. (S_{min} 2,300 S), washing the pellet and centrifuging again. The pellet was resuspended to make a 1:10 or 1:5 extract.

6. Enzyme Assays

Cytochrome Oxidase

Cytochrome oxidase was assayed by the spectrophotometric method of Cooperstein and Lazarow (1951). The initial homogenate and the mitochondrial fraction were diluted with 0.005M phosphate buffer (pH 7.4) to form 1:20 extracts. The microsomal and lysosomal fractions were diluted to give 1:10 extracts (particles from 1 g. wet tissue in 10 ml. of suspension), while the final supernatant was not diluted. These dilutions were often sufficient for the assay described below, but sometimes further dilutions were required. To insure the release of enzyme contained in sac-like membranes, the fractions were blender treated for 20 seconds in a Servall "Omni-Mixer" at 16,000 R.P.M. and then kept at 0° until used.

Three ml. of cytochrome C preparation (1.7×10^{-5}M cytochrome C in 0.03M phosphate buffer) were pipetted into

a 1 cm. glass cuvette, and the absorbancy measured at 550 mμ against a 0.03M phosphate buffer (pH 7.4) blank. The cytochrome C was reduced by adding 2 drops of freshly prepared 0.05M sodium dithionite ($Na_2S_2O_4$) from a #23 needle, and the absorbancy was again recorded. A change of about 0.25 absorbancy units was found to indicate that approximately 95% of the cytochrome C had been reduced; this could also be checked by taking the ratio of the absorbancy at 550 mμ to that at 565 mμ, a ratio of about 10 gave satisfactory results. Complete reduction was avoided as then the cytochrome C could not be reoxidized. The reaction was started by adding 0.05 to 0.1 ml. of the diluted extract to the reduced cytochrome C and also to the phosphate buffer blank. The absorbancy was recorded every 15 to 45 seconds until about 10 points had been collected, then the cytochrome C was completely reoxidized by adding a few grains of potassium ferricyanide. The absorbancy was recorded. The assay was done in a constant temperature room at 25°, as the reaction rate is quite sensitive to temperature.

The specific activity of the cytochrome oxidase preparations was expressed as an arbitrary unit for a 1:10 extract:

$$\frac{\Delta \log (\text{ferrocytochrome C})}{\Delta t}$$

where t is in minutes. The values were calculated from:

$$\frac{\Delta \log (\text{ferrocytochrome C})}{\Delta t} =$$

$$\frac{\log (A_{t_1} - A_\infty) - \log (A_{t_2} - A_\infty)}{t_2 - t_1} \times \frac{\text{final dilution}}{10}$$

where A_t is the absorbancy at time t and A_∞ is the absorbancy of completely oxidized cytochrome C. The first term of this expression is equal to the slope of the line obtained by plotting log $(A_t - A_\infty)$ against time.

Each analysis of cytochrome C was done twice and the average taken, unless these values differed markedly, in which case a third determination was made. Reactions which are too slow or too rapid do not yield satisfactory results and so dilutions were made in such a way that the linear portion was readily apparent in a plot of log $(A_t - A_\infty)$ versus time.

Acid Phosphatase

Acid phosphatase was determined by the method of Berthet and de Duve (1951). Equivalent quantities of sodium acetate, sodium borate, and sodium cacodylate were dissolved in distilled water and adjusted to pH 5.0 with 0.1N hydrochloric acid to make a 0.15M cacodylate buffer. Equal volumes of 0.15M cacodylate buffer, 0.1M β-glycero-

phosphate in 0.25M sucrose, and the 1:10 enzyme preparation were mixed and incubated for 10 min. at 37°. The total volume of the incubation mixture varied from 1 to 6 ml. Trichloroacetic acid (8%) was added to stop the enzyme reaction, the final concentration being greater than 2%. In some experiments, a blank was run to determine inorganic phosphate added as contaminant. For this purpose, the same volume of enzyme preparation was placed in a boiling water bath for 5 min., the same volumes of substrate and buffer added after cooling, and the mixture incubated for 10 min. at 37°.

The above procedure was used to assay for free enzyme. Total enzyme was determined in the same way, except the enzyme preparation was first treated to rupture any particles present. Freezing and thawing six or more times, blending for 2-3 min., or assaying in the presence of 0.1% Triton X-100, was sufficient to release bound enzyme to determine total activity. The bound enzyme was taken as the difference between total enzyme and free enzyme.

Determination of Inorganic Phosphate

The method of Fiske and Subbarow (1925) was used to determine inorganic phosphate. The incubation mixture was centrifuged to precipitate coagulated protein, and then

the supernatant was transferred to a 100 ml. volumetric flask. To this was added 10 ml. of "Molybdate Reagent", 4 ml. of "1, 2, 4-Aminonaphthol sulfonic acid Reagent", and distilled water to make up 100 ml. The color was allowed to develope for 15 min. and the absorbancy was determined with an Evans Electroselenium colorimeter using the red filter (OR-1). The blank and also a standard phosphate solution were treated in the same way and their absorbancies determined.

Glucose-6-Phosphatase

Glucose-6-phosphatase was determined in a manner described by Gianetto and de Duve (1955), and differed only from the acid phosphatase procedure in the reagents used. The 1:10 enzyme preparations were assayed immediately after fractionation, as the enzyme activity was found to decrease with time even in frozen preparations. The buffer adjusted to pH 6.5 was either 0.05M acetate or 0.05M cacodylate (the latter takes longer to prepare, but is a better buffer in this range). Disodium salt of glucose-6-phosphate was dissolved in 0.25M sucrose (adjusted to pH 6.5) to make a 0.04M solution. A blank was run as described for acid phosphatase, and inorganic phosphate was determined as on page 36.

IV

RESULTS

Since the results of this investigation are dependent upon the assay for acid phosphatase, experiments were done to establish the reliability of the assay. The effect of varying the enzyme concentration on the reaction

Table 2: Variation of Reaction Rate With Enzyme Concentration. The appropriate amount of 0.15M cacodylate buffer (pH 5.0) was added to the volumes of 1:10 enzyme preparation indicated. The β-glycerophosphate concentration was kept constant at 0.033M. The reaction mixture incubated at 37° for 10 min.

Enzyme Added (ml)	Phosphate Produced (mg $PO_4^{\equiv}$ /g wet tissue)
2.0	0.11
1.5	0.13
1.0	0.13

rate in the range which was routinely employed in the acid phosphatase assay is shown in table 2. These results indicate that the enzyme was 'saturated' with substrate under the conditions used.

Table 3: Effect of Varying Blending Time on Acid Phosphatase Activity. The enzyme preparation was blended at 16,000 R.P.M.

Time Blended (sec)	*Activity
15	0.10
30	0.09
60	0.08
90	0.10

*mg $PO_4^{\equiv}$ /g wet tissue/10 min

Figure 1 shows that the reaction rate was constant over a 2 hr. period, and therefore, the reaction has apparent zero-order kinetics, a situation which could be expected if the enzyme was saturated with substrate. Under these conditions the reaction rate constant or the amount of product produced in unit time, can be used to

determine the amount of enzyme present.

Treatment of isolated fractions with a blender was used to rupture lysosomal particles before total enzyme assays were performed. The data shown in table 3 establish that no loss in activity occurs even when treated in such

Table 4: Data for a Beer's Law Plot for the Colorimetric Determination of Phosphate.

Concentration $PO_4^{\equiv}$ (mg/100 ml)	Absorbancy
0.450	3.00
0.225	1.50
0.113	0.75
0.057	0.38
0.029	0.19
0.015	0.10

a way that considerable foaming occurs. Again, reference to figure 1 shows that no loss in activity occurred even when the enzyme was blended for 4 minutes.

The reliability of the phosphate analysis was also checked. Temperature was found to have no effect on the

final color produced, but did have a very slight effect on the rate of color developement. Generally the absorbancy increased rapidly for the first five minutes and then increased slowly for the next ten minutes; for this reason, better results were obtained when the color was allowed to develope for 15 minutes, and a standard was run simultaneously. Table 4 shows that the absorbancy was proportional to the phosphate concentration over a sixty-four fold range. In phosphate determinations appropriate dilutions were made so that the concentration was within this range.

The enzymes cytochrome oxidase, acid phosphatase, and glucose-6-phosphatase have been used by de Duve as markers for mitochondria, lysosomes, and microsomes respectively. These enzymes are now believed to be associated only with these particles, and so were used as markers in this work. The data of table 5 show that the various groups of subcellular particles were largely separated from one another but were still contaminated with the other fractions. If all the lysosomes had been broken a more even distribution of enzyme in all particulate fractions would be expected due to nonspecific adsorption, and most of the acid phosphatase would appear in the supernatant. The fact that each enzyme sedimented in the fraction expected also indicates that intact particles were separated, and that the calculations used

Table 5: Centrifugation Fractionation of Hepatic Tissue.

Fraction	*Cytochrome Oxidase	Acid Phosphatase	Glucose-6-Phosphatase
Homogenate	378	2.28	7.40
Mitochondrial	127 (67)	0.64 (17)	1.22 (7)
Lysosomal	59.3 (31)	1.55 (42)	2.57 (15)
Microsomal	5.3 (3)	0.53 (14)	10.00 (60)
Supernatant	0.0 (0)	1.01 (27)	2.94 (18)
Recovery	191.6	3.73	16.73

*Cytochrome oxidase is expressed as the activity of a 1:10 particle resuspension in arbitrary units as defined on page 34. Acid phosphatase and glucose-6-phosphatase activities are expressed as mg $PO_4^{\equiv}$/g wet tissue/10 min. The figures in parentheses give percentage distribution.

as a basis for the centrifugation procedure are essentially correct.

The same homogenization and fractionation procedure was extended to the pea embryos. The figures given in table 6 are representative of the enzyme distributions obtained in fractions from this plant material. In all, three complete fractionations of this type were done, the distribution of glucose-6-phosphatase was measured once, that of cytochrome oxidase twice, and that of acid phospha-

Table 6: Centrifugation Fractionation of Pea Embryos. The units are the same as given in table 5.

Fraction	Cytochrome Oxidase	Acid Phosphatase	Glucose-6-Phosphatase
Nuclear + Debris	0.262 (34)	0.27 (28)	
Mitochondrial	0.129 (17)	0.00 (0)	0.028 (5)
Lysosomal	0.263 (35)	0.00 (0)	0.031 (6)
Microsomal	0.108 (14)	0.08 (8)	0.034 (6)
Supernatant	0.000 (0)	0.63 (64)	0.440 (83)

tase three times. In addition eight other partial fractionations were performed using variations in homogenization technique. In these experiments an average of 5% of the acid phosphatase sedimented in the "lysosomal" fraction. These results, like the figures in table 6 indicate that a negligible fraction of the acid phosphatase sedimented in the "lysosomal" fraction.

Figure 1 shows the results of an experiment designed to determine whether the small amount of acid phosphatase found in the "lysosomal" fraction is really contained in lysosomes. The usual 1:10 extract was made and divided into two portions, one of which was blender

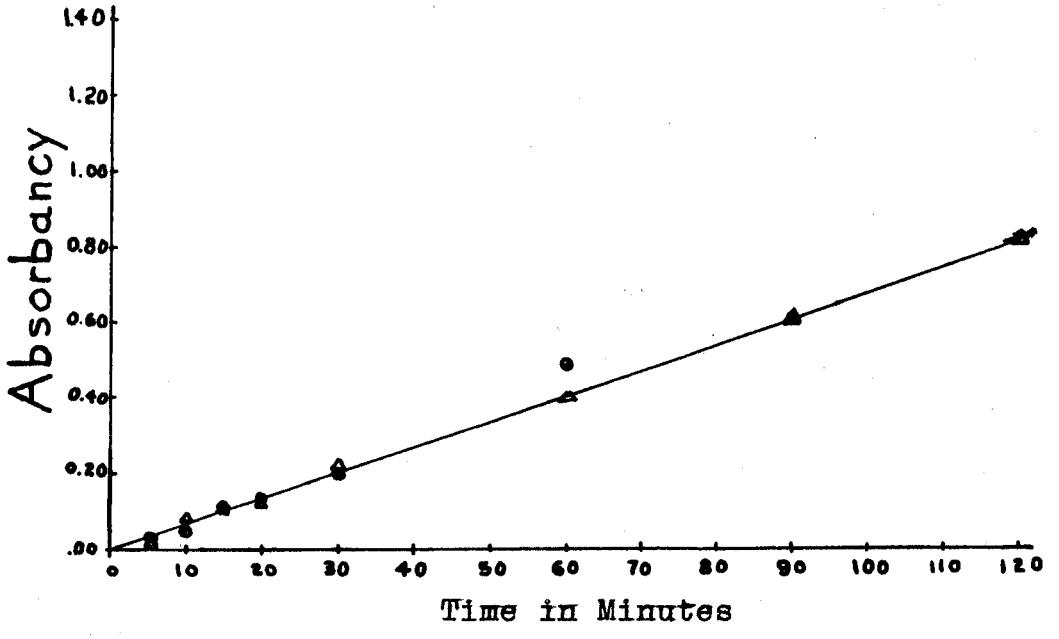

Figure 1: Effect of incubating at 0° a "lysosomal" preparation from pea embryos in the assay medium for acid phosphatase. The values given by ▲ are for a sample blended for 4 min. at 16,000 R.P.M.; the values given by O are not blender treated.

treated for 4 min. Both were then incubated under the usual conditions of assay for acid phosphatase; samples of the mixture were taken periodically and analyzed for inorganic phosphate. The blender-treated portion was used as a control to determine any change in reaction rate with time. Now, if intact lysosomes had been present

it would be expected that incubating at 37° for prolonged times, would result in progressive rupture of particles (if they were at all similar to lysosomes isolated from rat liver). Acid phosphatase would be released causing an increase in reaction rate which would be seen as an increase in the slope of the line in figure 1. Within experimental error, both curves were coincident over their entire length. This establishes that lysosomes were not present, as no enzyme release occurred on blending or on prolonged incubation at 37°.

The results of the above experiment are substantiated by an experiment designed to detect any osmotic rupture of lysosomal particles. In this experiment samples of a "lysosomal" preparation were incubated at 0° for 2 hrs. in various concentrations of sucrose. The results of two experiments are shown in figure 2. Although the points fluctuate appreciably the curve probably should be a straight line. In any case there is no characteristic minimum at an isotonic sucrose concentration, therefore, it is clear that no rupture of lysosomal particles occurred under hypo or hypertonic conditions and lysosomes could not have been present.

Since acid conditions can cause rupture of rat liver lysosomes, the pH of the pea embryo homogenate was measured with a glass electrode. It was found that

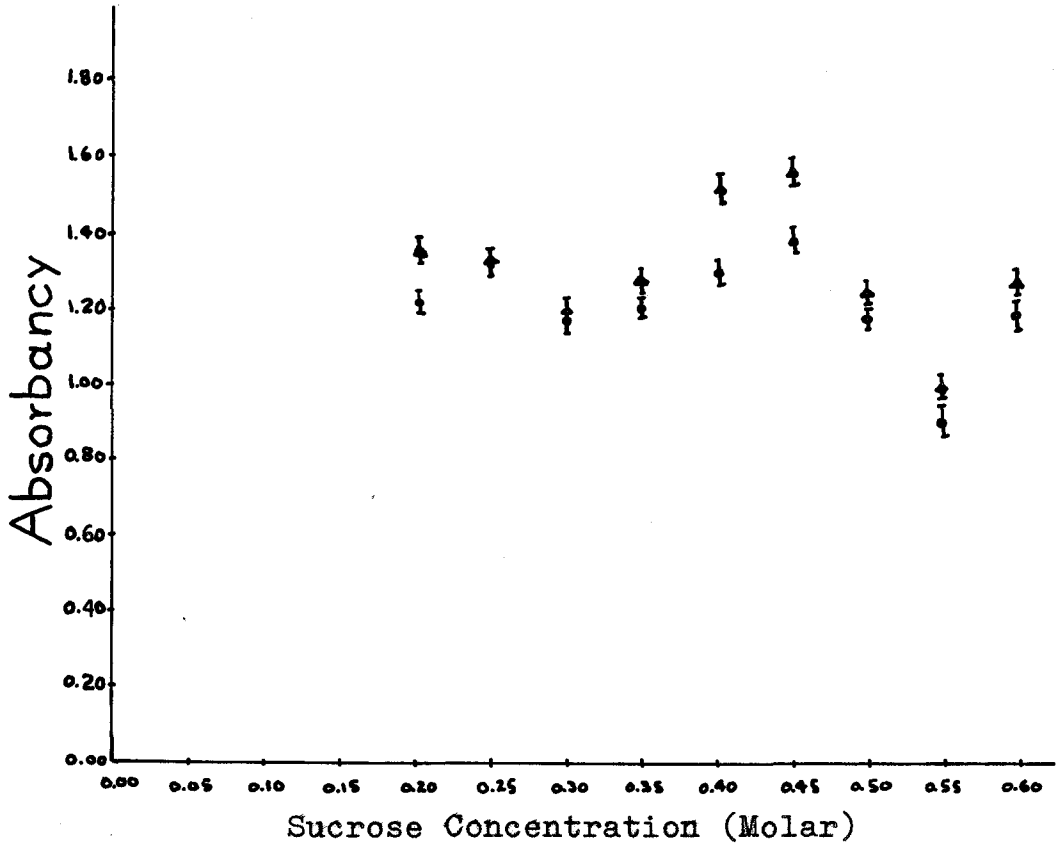

Figure 2: Effect of incubating a "lysosomal" preparation from pea embryos for 2 hr. in various concentrations of sucrose at 0°. The results of two experiments are shown.

grinding in 0.25M sucrose pH 7.0 resulted in a suspension with pH 6.1. Under these conditions the hydrogen ion concentration is neither high enough to cause much agglutination of particulate material, nor is it likely to damage lysosomes.

Many other experiments were performed in an attempt to obtain acid phosphatase in a particulate form. Grinding at 0° in a mortar with or without fine or coarse sand, blending at various speeds and for various times, grinding in an all glass homogenizer, and combinations of these were investigated and found to give results similar to those obtained when the tissue was ground in a tapered teflon homogenizer. Peas were used which were germinated for various times and the same results were obtained. Various parts of the embryo were homogenized, and "lysosomal" preparations made which were examined for lysosomes by measuring acid phosphatase activity before and after treatment with a blender or Triton X-100. In no case was there a significant increase in activity which could be attributed to rupture of particles and release of acid hydrolases.

Preliminary experiments were then conducted to detect lysosomes in onion embryos in the manner reported in abstract by Harrington and Altschul (1963). Homogenizing with the tapered teflon pestle gave lysosomal fractions containing 51% of the total acid phosphatase of which at least 15% appeared to be contained in particles since it was detectable only after blender treatment.

V

Discussion

Many problems are encountered in enzyme assays, these difficulties becoming particularily acute with differential centrifugation fractions where it is necessary to estimate enzyme quantitatively in a complex environment. An ideal blank can only rarely be found for such assays. The blank which was finally adopted for acid phosphatase assay (viz. boiled enzyme preparation incubated with substrate) was felt to be the most reliable. Other problems involve enzyme lability, structural barriers which limit access of substrate to enzyme, presence of inhibitors or activators, lack of specificity of the substrate, and the presence of competing systems. In the following, some of these problems will be discussed in relation to the assays used.

If cytochrome C was completely reduced with sodium dithionite ($Na_2S_2O_4$) as reported by Cooperstein and Lazarow (1951), it could not be reoxidized by cytochrome oxidase, air, or potassium ferricyanide. Hence the substrate was reduced only to the extent of 94-96%. The amount of sodium dithionite necessary to bring about 94-96%

reduction was determined experimentally. Although controlled tests were not run, there appeared to be no interference from cytochrome C peroxidase or turbidity. The mitochondria are permeable to cytochrome C. However, difficulties can occur in assaying the nuclear fraction or initial homogenate which may contain unbroken cells, as the substrate may not penetrate the plasma membrane. This possibility can be lessened by blender treating all fractions. The poor recovery of cytochrome oxidase in the rat liver fractionations is not surprising since enzyme in the nuclear-debris fraction was not assayed. It is extremely difficult to prepare this fraction free of mitochondria.

Few difficulties are encountered using the assay conditions for acid phosphatase given by Berthet and de Duve (1951). The β-glycerophosphate substrate must be as free as possible from the α-isomer, as the latter is also acted upon by glucose-6-phosphatase; for the same reason another commonly used substrate, phenyl phosphate, is of little value in this type of experiment. The β-glycerophosphate used was stated by the manufacturer to contain a maximum of 0.1% of the α-isomer. One of the main difficulties lies in the fact that the intact lysosomal membrane does not allow access of the substrate to the enzyme. Thus, if total activity is desired, the

material must be subjected to a treatment which will rupture the membrane. Based on de Duve's results, blending for 2-4 minutes was taken to be sufficient to rupture all lysosomes and still not inactivate the enzyme. Measurement of free enzyme activity in the presence of lysosomes is more difficult as the membrane is sensitive to extremes of pH, heat, and ruptures upon prolonged exposure even to isotonic sucrose. In this respect, it was assumed that lysosomes in plant material are similar to those in rat liver, and hence the assay conditions described by de Duve would be satisfactory. This assumption appeared to be valid in the preliminary experiments with onion embryos, as 15% of the activity was found only after blender treatment of the lysosomal fraction. The high recovery of acid phosphatase in the fractionation of rat liver was due to a failure to realize that the substrate was contaminated with inorganic phosphate, and hence an inappropriate blank was used in these experiments. The values which were measured represent inorganic phosphate released enzymically plus the amount contained as a contaminant in the substrate. If the activity of the four fractions is summed, four times the amount of contaminant appears in the total as is measured in the initial homogenate.

The assay for glucose-6-phosphatase presented some

difficulty. Since all of the activity of glucose-6-phosphatase is lost in a preparation frozen 24 hrs., it is possible that much of the activity is lost in the 12 hrs. required for a complete fractionation. This necessitates assaying for the enzyme as soon as fractionation is complete. Assays for the enzyme in the initial homogenate and the supernatant can be complicated by a soluble inhibitor which is found in these fractions. While the lability of glucose-6-phosphatase was reported by de Duve and Berthet (1954) to frequently offset the increase in activity expected in washed microsomes, unusually high recoveries are often obtained. The activity of glucose-6-phosphatase measured in the initial homogenate of rat liver doubled if the sample was frozen and thawed ten times. The reasons for this increase in activity are not understood. As with the acid phosphatase assay, the importance of inorganic phosphate in the substrate was not immediately recognized. All of the above factors can easily account for the high recoveries of glucose-6-phosphatase.

Table 5 showing the distribution of enzymes in rat liver particle fractions is self-explanatory. Table 7 compares data collected by de Duve in similar experiments with those found in this study. The values obtained in this study are, in general, comparable to those obtained by

Table 7: Comparison of Hepatic Tissue Fractionation Results. The values listed in columns marked "1" were reported by de Duve and co-workers (1955). The statistical variations are deviations from the mean obtained from 19 fractionations. Columns marked "2" give values obtained in the present investigation. All columns give percentage distribution of the enzymes.

Fraction	Cytochrome Oxidase		Acid Phosphatase		Glucose-6-Phosphatase	
	1	2	1	2	1	2
Nuclear	10.1 ± 5.4		3.6 ± 1.3		6.8 ± 2.3	
Mitochondrial	57.7 ± 12.0	67.0	24.1 ± 1.6	17.3	2.7 ± 1.2	7.3
Lysosomal	17.4 ± 8.7	31.1	40.7 ± 6.7	41.6	7.0 ± 2.0	15.3
Microsomal	3.7 ± 3.7	2.7	20.1 ± 4.8	14.2	73.8 ± 9.7	59.7
Supernatant	0.0 ± 0.0	0.0	13.3 ± 2.9	27.1	2.7 ± 0.6	17.6
Recovery	88.9 ± 8.1	50.5	101.8 ± 5.1	164.0	92.8 ± 8.7	226.0

de Duve. Since the proportion of acid phosphatase in the supernatant is greater in the present study, it must be concluded that more damage was done to the lysosomes. This conclusion is supported by the greater amount of glucose-6-phosphatase appearing in the supernatant. It can also be seen that a more diffuse distribution of the two phosphatases was obtained in this study. However, as noted before, in this part of the work blanks were used which did not take into consideration inorganic phosphate present in the substrates, and such contamination could cause this diffuse distribution. In all probability the separation is better than it appears from the data of tables 5 and 7.

In an earlier section examples of lysosomes or lysosome-like particles found in a variety of animal tissues were cited. In most instances these bodies were similar in size to lysosomes in hepatic tissue, thus, as a working hypothesis it was assumed that lysosomes in plants would also have a similar size and density, and that the same centrifugation conditions could be used. This hypothesis was verified in the preliminary investigation of acid phosphatase distribution in onion embryos. It was found that most of the enzyme sediments in a fraction similar to that used to obtain lysosomes from rats. Moreover, a significant increase in activity occurs on treating

this fraction with a blender, suggesting that a successful isolation of lysosomes from onion embryos had been achieved. Critical comparison with the work of Harrington and Altschul (1963) is not possible as their report lacks sufficient information.

Table 6 shows the distribution of enzymes in fractions of pea homogenates. Since acid phosphatase is found almost entirely in the nuclear-debris (contains unruptured cells) and the supernatant fractions, it appears that the forces which are necessary to break the cell are such that the subcellular particles are also badly damaged. The glucose-6-phosphatase distribution also points to excessive damage of particulate material during grinding or from autolysis. The distribution of cytochrome oxidase could arise from redistribution of fragmented mitochondria, but could also mean that the centrifugal forces were not high enough to bring about the desired sedimentation.

The Louvain group has shown that when extracts containing lysosomes are incubated with the assay mixture for long periods, the measured acid phosphatase activity of the extract increases for the first 45 minutes and then remains constant. This increase in activity is attributed to rupture of the lysosomal membrane and release of soluble acid phosphatase. Since no such increase in acid phospha-

tase activity was obtained with the "lysosomal" fraction from pea embryos (figure 1), it must be concluded that lysosomes are not present in this fraction. de Duve considers that acid phosphatase which sediments in the particulate fractions and is not contained inside the lysosomes is due to nonspecific adsorption of enzyme protein by the particle membrane. It is probable that the 5% of the total acid phosphatase found in the "lysosomal" fraction is adsorbed enzyme, and does not arise from insufficient centrifugation of lysosomes. A similar conclusion is reached from the results of an experiment designed to detect osmotic rupture of any lysosomes which might be present in this fraction. The data presented in figure 2 have been interpreted to represent a straight line. The same type of experiment done by de Duve and co-workers shows that the amount of free enzyme increases rapidly in sucrose solutions of less than 0.25M, and increases slowly in sucrose solutions of higher concentration. The points shown in figure 2 cannot be joined by a smooth curve to give a graph similar to that of de Duve's. The scattering of these points may be attributed to variations in the pH of the solutions, as these were made by diluting different stock solutions. The pH of the diluted solutions used in this experiment was later measured and found to vary over the range 5 to 7.

The results of this study indicate that in pea embryos, acid phosphatase is not contained in lysosome particles. However, it is felt that this result was obtained due to excessive damage to the subcellular particles, as much higher shear forces were required to shread the tissue and rupture the cells, than were required for either rat liver or onion embryo tissue. Although there remain other ways of applying and controlling these forces, it is felt that a positive identification of lysosomes in pea embryos would be achieved more rapidly through application of cytochemical techniques.

REFERENCES

Allfrey, V. (1961). "The Cell" (J. Brachet and A. E. Mirsky, eds.). Vol. 1, pp. 193-290. Academic Press, New York.

Anderson, N. G. (1956). "Physical Techniques in Biological Research" (G. Oster, and A. W. Pollister, eds.). Vol. 3, pp. 299-352. Academic Press Inc., New York.

Appelmans, F., and de Duve, C. (1955). Biochem. J., 59, 426.

Appelmans, F., Wattiaux, R., and de Duve, C. (1955). Biochem. J., 59, 438.

Avers, C. J., and King, E. E. (1960). Am. J. Botany, 47, 220.

Bates, H. M., Craddock, V. M., and Simpson, M. V. (1958). J. Am. Chem. Soc., 80, 1000.

Beaufay, H., and de Duve, C. (1959). Biochem. J., 73, 604.

Beaufay, H., Bendall, D. S., Baudhuin, P., and de Duve, C. (1959). Biochem. J., 73, 623.

Berthet, J., and de Duve, C. (1951). Biochem. J., 50, 174.

Berthet, J. Berthet, L., Appelmans, F., and de Duve, C. (1951). Biochem. J., 50, 182.

Brachet, J., Decroly-Briers, M., and Hoyez, J. (1958). Bull. Soc. Chim. Biol., 40, 2039.

Brandes, D., Groth, D. P., and Gyorkey, F. (1962). Exptl. Cell Research, 28, 61.

Brewer, D. B., and Heath, D. (1963). Nature, 198, 1015.

Burgos, M. (1960). Anat. Record, 137, 171.

Burnett, G., and Kennedy, E. P., (1954). J. Biol. Chem., 211, 969.

Chambers, R., and Fell, H.B. (1931). Proc. Roy. Soc., B109, 380.

Claude, A. (1946a). J. Exptl. Med., 84, 51.

Claude, A. (1946b). J. Exptl. Med., 84, 61.

Claude, A. (1960). Nature, 187, 836.

Cohn, Z. A., and Hirsch, J. G. (1960a). J. Exptl. Med., 112, 983.

Cohn, Z. A., and Hirsch, J. G. (1960b). J. Exptl. Med., 112, 1015.

Cooperstein, S. J., and Lazarow, A. (1951). J. Biol. Chem., 189, 665.

Crick, F. H. C., and Watson, J. D. (1954). Proc. Roy. Soc., A223, 80.

Dalton, A. J. (1961). "The Cell" (J. Brachet and A. E. Mirsky, eds.), Vol. 2, pp. 603-618. Academic Press, New York.

Deane, H. W. (1958). "Frontiers of Cytology" (S. L. Palay, ed.), pp. 227-263, Yale Univ. Press, New Haven, Connecticut.

de Duve, C. (1958). "Subcellular Particles" (T. Hayashi, ed.), pp. 145-155. Ronald Press, New York.

de Duve, C. (1961). "Biological Aspects of Cancer Chemotherapy" (R. J. C. Harris, ed.). Academic Press, New York.

de Duve, C. (1963a). Scientific American, April.

de Duve, C. (1963b). Ciba Found. Symp. Lysosomes (Churchill, London), in press.

de Duve, C., and Berthet, J. (1953). Nature, 172, 1142.

de Duve, C., and Berthet, J. (1954). Inter. Review Cytol., 3, 225.

de Duve, C., Gianetto, R., Appelmans, F., and Wattiaux, R. (1953). Nature, 172, 1143.

de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., Appelmans, F. (1955). Biochem. J., 60, 604.

de Duve, C., Berthet, J., and Beaufay, H. (1959). Progress in Biophysics and Biophysical Chemistry, 9, 326.

de Duve, C., Beaufay, H., Jacques, P., Rahman-Li, Y., Sellinger, O.Z., Wattiaux, R., de Coninck, S. (1960). Biochim. Biophys. Acta, 40, 186.

de Duve, C., Wattiaux, R., and Wibo, M., (1961). Proc. 1st. Pharmacol. Meeting, Stockholm.

Diengdoh, J. V. (1963). Biochem. J., 87, 41P.

Essner, E. (1960). J. Biophys. Biochem. Cytol., 7, 329.

Essner, E., and Novikoff, A. B. (1961). J. Biophys. Biochem. Cytol., 9, 773.

Essner, E., and Novikoff, A. B. (1962). J. Cell Biol., 15, 289.

Farquhar, M. G., and Palade, G. E. (1960). J. Biophys. Biochem. Cytol., 7, 297.

Fell, H. B., Dingle, J. T., and Webb, M. (1962). Biochem. J., 83, 63.

Finkenstaedt, J. T. (1957). Proc. Soc. Exptl. Biol. Med., 95, 302.

Fiske, C. H., and Subbarow, Y. (1925). J. Biol. Chem., 66, 375.

Gamow, G. (1954). Nature, 173, 318.

Gianetto, R., and de Duve, C. (1955). Biochem. J., 59, 433.

Gomori, G. (1941). Arch. Path., 32, 189.

Gomori, G. (1952). "Microscopic Histochemistry: Principles and Practice", pp. 189-194. University of Chicago Press, Chicago.

Granick, S. (1961). "The Cell" (J. Brachet and A. E. Mirsky, eds.). Vol. 2, pp. 489-602. Academic Press, New York.

Greenbaum, A. L., Slater, T. F., and Wang, D. Y. (1960). Nature, 188, 318.

Hampton, J. C. (1958). Acta Anat. 32, 262.

Harrington, J. F., and Altschul, A. M. (1963). Federation Proc., 22, 475.

Hirsch, J. G., and Cohn, Z. A. (1960). J. Exptl. Med. 112, 1005.

Hogeboom, G. H., and Schneider, W. C. (1953). J. Biol. Chem., 204, 233.

Holt, S. J. (1959). Exptl. Cell Research Suppl. 7, 1.

Holt, S. J., and Hicks, R. M. (1961a). J. Biophys. Biochem. Cytol., 11, 31.

Holt, S. J., and Hicks, R. M. (1961b). J. Biophys. Biochem. Cytol., 11, 47.

Holter, H. (1954). Proc. Roy. Soc., B142, 140.

Holter, H. (1956). "Fine Structure of Cells", pp. 71-76. Noordhof, Groningen.

Holter, H. (1961). "Biological Aspects of Cancer Chemotherapy" (R. J. C. Harris, ed.). Academic Press, New York.

Holter, H., and Lowy, B. A. (1959). Compt. Rend. Trav. Lab. Carlsberg, 31, 105.

Holtzer, H., and Holtzer, S. (1960). Compt. Rend. Trav. Lab. Carlsberg, 31, 373.

Kennedy, E. P. (1957). Federation Proc., 16, 847.

Klamer, B., and Fennell, R. A. (1963). Exptl. Cell Research, 29, 166.

Koenig, H. (1963a). J. Histochem. Cytochem., 11, 556.

Koenig, H. (1963b). J. Histochem. Cytochem., 11, 120.

Koszalka, T. R., Mason, K. E., and Krol, G. (1961). J. Nutrition, 73, 78.

Lewis, M. R., and Lewis, W. H. (1914-15). Am. J. Anat., 17, 339.

Lodja, Z. (1960). Morfologie, 8, 148.

Mitchell, P., and Moyle, J. (1956). Discussions Faraday Soc., 21, 258.

Novikoff, A. B. (1959). Biol. Bull., 117, 385.

Novikoff, A. B. (1960a). "Developing Cell Systems and Their Control" (D. Rudnick, ed.), pp. 167-203. Ronald Press, New York.

Novikoff, A. B. (1960b). "Biology of Pyelonephritis" (E. Quinn and E. Kass, eds.), pp. 113-144. Little, Brown, Boston, Massachusetts.

Novikoff, A. B. (1961a). "The Cell" (J. Brachet and A. E. Mirsky, eds.), Vol. 2, pp. 339-343. Academic Press, New York.

Novikoff, A. B. (1961b). "The Cell" (J. Brachet and A. E. Mirsky, eds.), Vol. 2, pp. 423-488. Academic Press, New York.

Novikoff, A. B., and Essner, E. (1960). Am. J. Med., 29, 102.

Novikoff, A. B., Beaufay, H., and de Duve, C. (1956). J. Biophys. Biochem. Cytol., 2, Suppl., 179.

Ogawa, K., Mizuno, N., Hashimoto, K., Fujii, S., and Okamoto, M. (1960). Proc. Dept. Anat., Kyoto Univ., School Med., 4, 1.

Ogawa, K., Mizuno, N., Okamoto, M. (1961). J. Histochem. Cytochem., 9, 202.

Oliver, J., Moses, M. J., MacDowell, M. C., and Lee, Y. C. (1954). J. Exptl. Med., 99, 605.

Paigen, K., and Griffiths, S. K. (1959). J. Biol. Chem., 234, 299.

Parpart, A. K., and Ballentine, R. (1952). "Modern Trends in Physiology and Biochemistry" (E. S. G. Barron, ed.), p. 139. Academic Press, New York.

Penn, N. W. (1959). Federation Proc., 18, 301.

Penn, N. W. (1960). Biochim. Biophys. Acta, 37, 55.

Pickels, E. G. (1943). J. Gen. Physiol., 26, 341.

Pickels, E. G. (1952). "Methods in Medical Research" (A. C. Corcoran, ed.), Vol. 5, pp. 107-133. The Year Book Publishers, Chicago.

Ponder, E., (1961). "The Cell" (J. Brachet and A. E. Mirsky, eds.), Vol. 2, pp. 1-80. Academic Press, New York.

Quertier, J., and Brachet, J. (1959). Arch. biol. (Liege), 70, 153.

Rosenbaum, R., and Rolon, C. I. (1960). Biol. Bull., 118, 315.

Rouiller, C., and Bernhard, W. (1956). J. Biophys. Biochem. Cytol., 2, Suppl. 355.

Roy, A. B. (1958). Biochem. J., 68, 519.

Schmidt, G., Bartsch, G., Laumont, M. Herman, T., and Liss, M. (1963). Biochemistry, 2, 126.

Schneider, W. C. (1959). Adv. in Enzymol., 21, 1.

Sellinger, O. Z., Beaufay, H., Jacques, P., Doyen, A., and de Duve, C., (1960) Biochem. J., 74, 450.

Shibko, S., Caldwell, K. A., Sawant, P. L., and Tappel, A. L. (1963). J. Cellular Comparative Physiol., 61, 85.

Straus, W. (1957). J. Biophys. Biochem. Cytol., 3, 1037.

Straus, W. (1958). J. Biophys. Biochem. Cytol., 4, 541.

Straus, W. (1959). J. Biophys. Biochem. Cytol., 5, 193.

Svedberg, T., and Pedersen, K. O. (1940). "The Ultracentrifuge" The Clarendon Press, Oxford.

Swanson, C. P. (1961). "Cytology and Cytogenetics", p. 21. Prentice-Hall, New Jersey.

Tappel, A. L., Zalkin, H., Caldwell, K. A., Desai, I. D., and Shibko, S. (1962). Arch. Biochem. Biophys., 96, 340.

Tewari, H. B., and Bourne, G. H. (1963). Experientia, 19, 238.

Trump, B. F., and Janigan, D. T., (1962). Lab. Invest., 11, 395.

Trump, B. F., Goldblatt, P. J., and Stowell, R. E. (1962). Lab. Invest., 11, 986.

Van Lancker, J. L., and Holtzer, R. L. (1959). Am. J. Pathol. 35, 563.

Van Lancker, J. L., and Morrill, G. A. (1958). Federation Proc. 17, 463.

Viala, R., and Gianetto, R. (1955). Can. J. Biochem. Physiol., 33, 839.

Weber, R. (1957a). Experienta, 13, 153.

Weber, R. (1957b). Rev. Suisse Zool., 64, 326.

Weber, R., and Salzmann, R. (1963) Experienta, 19, 352.

Weissmann, G., and Dingle, J. (1961). J. Exptl. Cell. Res., 25, 207.

Zalkin, H., Tappel, A. L., Caldwell, K. A., Shibko, S., Desai, I. D., and Holliday, T. A. (1962). J. Biol. Chem., 237, 2678.