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Changes In Surface Properties Of Cultured Animal Cells Throughout The Growth Cycle

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CHANGES IN SURFACE PROPERTIES OF CULTURED ANIMAL CELLS THROUGHOUT THE GROWTH CYCLE

by

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ABSTRACT

To better characterize the numerous functions undertaken by the outer region of the mammalian cell, analyses of the cell and cell envelope throughout the life cycle was attempted. LM mouse fibroblast cells (clone 929) propagated in protein-free, serum-free, medium 199 peptone, were separated into three distinct cell fractions on a Ficoll-medium 199 gradient using low speed centrifugation. The cell populations banded characteristically at three different percentages of Ficoll, 17.2%, 18.4% and 20.4%.

The cell envelope was harvested from the LM cell by swelling, hardening and separating the envelope from the nucleus containing cytoplasmic mass with Tris-magnesium chloride-Gluteraldehyde solution, either by homogenization or by brisk mixing on a vortex mixer. Purification of the envelope fraction was accomplished by means of repeated sucrose gradient centrifugation.

From the kinetics of labelled thymidine incorporation into DNA it was confirmed that fraction #1 was primarily cells in G1, early interphase. The other two fractions were mixtures of cells in the other phases of the cell life

cycle, fraction #2 (S and G2) and fraction #3 (G2 and M).

Chemical analyses of the three cell fractions and their envelope portions were undertaken. Protein content of the cells and envelope doubled from fraction #1, G1, through fraction #2 (S and G2), to fraction #3 (G2 and M). Lipid (total and cholesterol) content of both cells and the envelope fraction quantitatively increased from fraction #1, G1, to fraction #3, G2 and M, but the ratio of lipid to protein per cell fell slightly during this time. Carbohydrate per cell remained quantitatively fairly constant. In both the cell and envelope however, when the ratio of carbohydrate to protein per cell was calculated a considerable drop was noted. (Fraction #1 : fraction #3 = 3:1).

Enzymatic studies using the fluorogenic substrate Fluorescein Diacetate (FDA), indicated a membrane associated acetic-ester hydrolase (3.1.1.6, acetylesterase), of the LM cell, split the nonfluorescent substrate in a two step procedure - first giving the intermediary product fluorescein monoacetate, (FMA), then finally the fluorescent product, fluorescein (F). The fluorescein accumulated inside the cell at a rate proportional to enzyme activity and could be monitored with a fluorometer. A four minute oscillatory mechanism of linked substrate influx and product efflux was noted. The esterase inhibitor, Paraoxon, specifically inhibited this enzymatic breakdown

and oscillatory mechanism. Fraction #2 (S and G2) was the most active cell population when the breakdown rate of the substrate was calculated per microgram of cell protein, or per cell. Fraction #1, (G1) was the most active cell population when the breakdown rate of the substrate was calculated on the basis of surface area or volume of the cells.

Immunological tests utilizing preimmune serum and anti LM serum prepared in rabbits indicated that fraction #1, G1 cells, was most affected both enzymatically and in subsequent growth tests by the addition of serum. The fluorogenic substrate method coupled with the addition of various dilutions of immune serum give similar values for antibody titer of the serum when compared to ring and agglutination tests. Chemical, enzymatic and immunological tests of LM mouse fibroblast cells during different phases of the cell's life cycle would indicate that the membrane is a dynamic cell region adapting itself continually throughout the cycle to the changing requirements and metabolic activities of the cell interior.

I. INTRODUCTION

A. MOTIVATION

Many investigations of the cell's near invisible skin, the plasma membrane, have been attempted using the electron microscope or various histochemical stains. Such tests undoubtedly change the membrane from the dynamic state seen in life to a shadow picture, perhaps a false shadow picture, of its former self. Additions of minute quantities of various substances, such as common salt, to the milieu surrounding the cell cause marked disturbances of the membrane and can result in the cell's inner contents becoming vacuolated and agitated. When no visible effect is noted in the cell due to the presence of these alien substances in the immediate environment, enzymatic monitoring of the cells shows significant fluctuations from the normal. This indicates the extreme sensitivity of the plasma membrane, and subsequently the whole cell, to any change in its immediate surroundings. The functions of membranes include the following: phagocytosis, pinocytosis, cell adhesion and mobility, the carrying of surface antigen and the limiting organ of growth, general and selective

diffusion of small molecules and ions, active transport, and control of influx and efflux of ions and substrate products between cell compartments (Parsons, 1967).

Recognizing the complexity of the living mammalian cell and its cornerstone role within the whole living animal, investigations of the plasma membrane, the surrounding membrane region, as well as the cell itself, were undertaken.

B. THE PROBLEM

The normal life cycle of the mammalian cell is highly complex. Conflicting data, conflicting theories for the simplest of cellular functions confront the investigator on viewing the literature. With this in mind the questions asked were simple in the hope of obtaining basic knowledge for use in future investigations.

Does the membrane vary during the cell's life cycle? If it does, and all previous investigations tend to support this assumption, in what manner does this variation express itself? Chemically? Physically?

During the life cycle is there a time at which the cell is more resistant to exterior factors, for example, virus attack? If so, in what form does this resistance express itself?

Having asked the questions the means to answer them could then be devised.

C. MEANS OF ATTACK

It is important to know the chemical composition of the cell throughout the life cycle, to note any variations which might consistently occur. Analysis of the cell using established chemical methods consequently was undertaken. Such analysis presupposes a method of dividing cells into their various phases of the cycle. It was of prime importance not to destroy the balances existing within the cell by use of metabolic blocks. Consequently, density gradient studies were undertaken exploiting density variation throughout the life cycle as a swift means of cell separation. As yet clear division into the various life phases has not been obtained; nevertheless, the fractions obtained have limited considerably the size variation normally noted in a logarithmically growing cell population. The findings of Killander and Zetterberg (1965), that within the L cell, (clone 929), population there was a very small variation in cell mass of those cells entering (DNA) synthesis phase, S, from the initial growth period G1, seems to indicate that the lightest cell fraction obtained may all be within this one particular phase, G1. Confirmation of the place of the various cell groups was undertaken using radioactive tracers.

Enzymatic studies utilizing a fluorogenic substrate, monitored on a fluorometer, allowed for precise measurement of cellular activity. Analysis of the cells and substrate

at specific time intervals showed an unsuspected cyclic cellular metabolic pattern for this substrate.

Extension of the fluorometric method to include various sera and serum components within the extracellular milieu showed variations of response during the cell's life cycle. Such variations may be indicative of phase dependent cellular mechanisms which cause corresponding fluctuations of cellular resistance to external stress.

The questions asked in this investigation were basic to an understanding of the cell's growth cycle and the functioning of the membrane during the cycle. Although the results obtained from this study supported many of the current suppositions as to the composition and functioning of the membrane, several of the findings raise new questions as yet unanswered.

II. HISTORICAL REVIEW

A. THE LM MOUSE FIBROBLAST CELL

1. The History of Cell Culture

"The cells are not very deep, but resemble many boxes, made by numbers of partitions dividing one long continued pore."

With these words Robert Hooke (1665), introduced into scientific literature, the cell. He was viewing a thin cork slice under his microscope and chose this term to describe the little sacs he observed in such abundance. However, not until the nineteenth century did the cell itself become an object for extensive research. It was Dutrochet (1824), who recognized that the cell was the basic life unit, but Schleiden (1837), and Schwann (1838), enunciated the theory in clear terms to the scientific world and consequently have overshadowed Dutrochet's original work. By the beginning of the twentieth century an extensive body of literature existed describing the role of the cell in such diverse fields as embryology, pathology and physiology.

It is but a short step from "describing the role of the cell" to attempting to mimic the tissue and organ by growing the cell under defined conditions. Claude Bernard, in 1878, had described the principles necessary for organ culture survival outside the whole organism and Wilhelm Roux (1885), performed the first successful transplant in vitro with the chick medullary plate, sustaining it in a physiological solution.

Harrison, in 1907, removed asceptically small pieces of frog neural lymph which grew actively for only a few weeks. He was able to suggest various refinements of his technique which Carrel and Burrows, in 1911, utilized. They found that embryo extract had a growth promoting action on chick heart cells on coagulated plasma bathed in embryo extract. The technique is still used today though with more complicated sustaining medium.

Developing these sustaining media was a more lengthy task. M. and W. Lewis, in 1911, had introduced the first liquid medium using some controlled components but the addition of sea water, aqueous embryo extracts and serum were still necessary. A. Carrel for thirty years led the search to define the different substances favoring cell growth necessary for a completely defined laboratory medium. When he died it was still missing. Not until Morgan and his associates (1950), with their introduction of medium 199 was a basic synthetic medium available.

The manipulation of large cell populations was made possible with roller tube cultures introduced by Gey in 1933. Moscona (1952), developed the technique of tissue trypsinization and made possible the cultivation of cell populations on a grand scale. By the 1950's therefore the methods had been devised to produce large quantities of sterile cells growing under defined chemical conditions. At the same time within the laboratory of W. R. Earle the defined cell line had been developed.

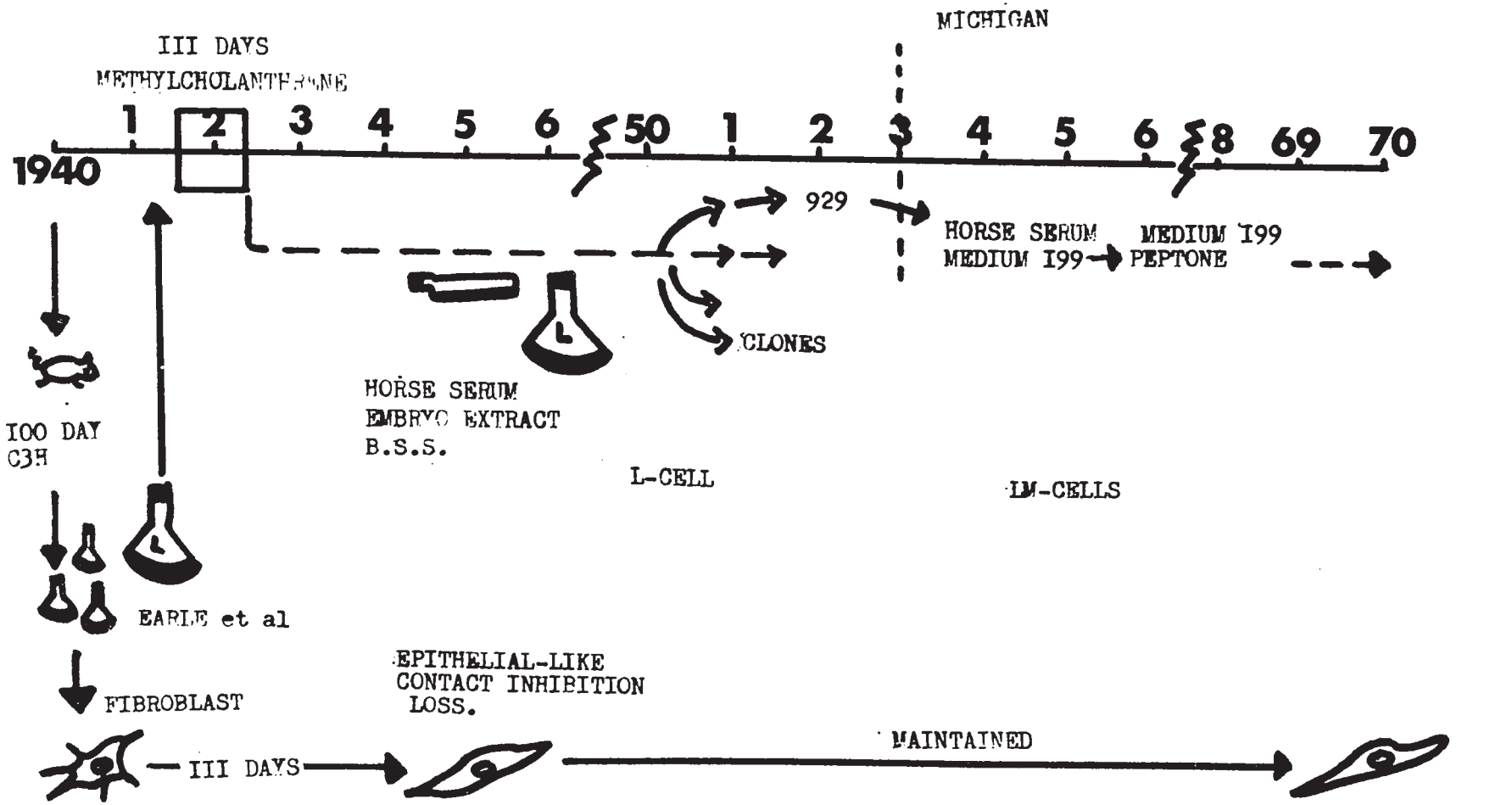
2. Origin and Development of the LM Mouse Fibroblast Cell Line

Quoting W. R. Earle's own words "the strain of fibroblast-like cells used was originally taken from a 100 day old male mouse of the C3H strain Andervont sub-strain. On October 18, 1940, the mouse was decapitated. Without including muscle tissue and the regional lymph node, the connective and fatty tissue pad along the side and just in front of one of the hind legs was removed and cut into six strips." After a year of cultivation and subdividing, one line, the L-line, was exposed for 111 days to the carcinogenic agent methylcholanthrene and then allowed to continue growing in normal medium thereafter. The first effect the carcinogen had on the fibroblast-like cells was to cause a decline in the rate of cell proliferation.

Earle concluded that the action was progressive and gradual and would have ultimately been lethal. After 40 days of exposure the cells showed a diminution in the length of the terminal cell process and the processes lateral edges became increasingly amoeboid. This change extended progressively from the tips of the cells towards the middle. Numerous short lateral processes were sent out and in tissue culture, cell ribbons and strands similar to epithelial sheets were seen. The progression of changes were gradual and appeared to affect the whole culture rather than an isolated cell or a small clump of cells. Stabilization at different levels of morphological alteration resulted after removal from the carcinogen.

Figure 1 gives a schematic picture of the subsequent growth history of the LM mouse cell. Sanford et al. (1948), utilizing a micropipette as the propagating vessel isolated a single mouse fibroblast cell and from it grew clone 929, the original source of our cell line. Merchant in Michigan in 1953, obtained a seed culture from this line, designated it LM clone 929, and began the gradual weaning of the cell from undefined growth substances such as horse serum, human ascitic fluid and embryo extract. By 1956 the cell had become adapted to medium 199 plus 0.5% Bacto Peptone, a completely defined chemical medium, in which it is sustained today. It is daughter cells grown in this defined medium by D. J. Merchant which were used in this study.

Figure 1 Cell Line History



B. THE CELL SURFACE

1. Physical and Chemical Properties of the Surface

a. The Extraneous Coat

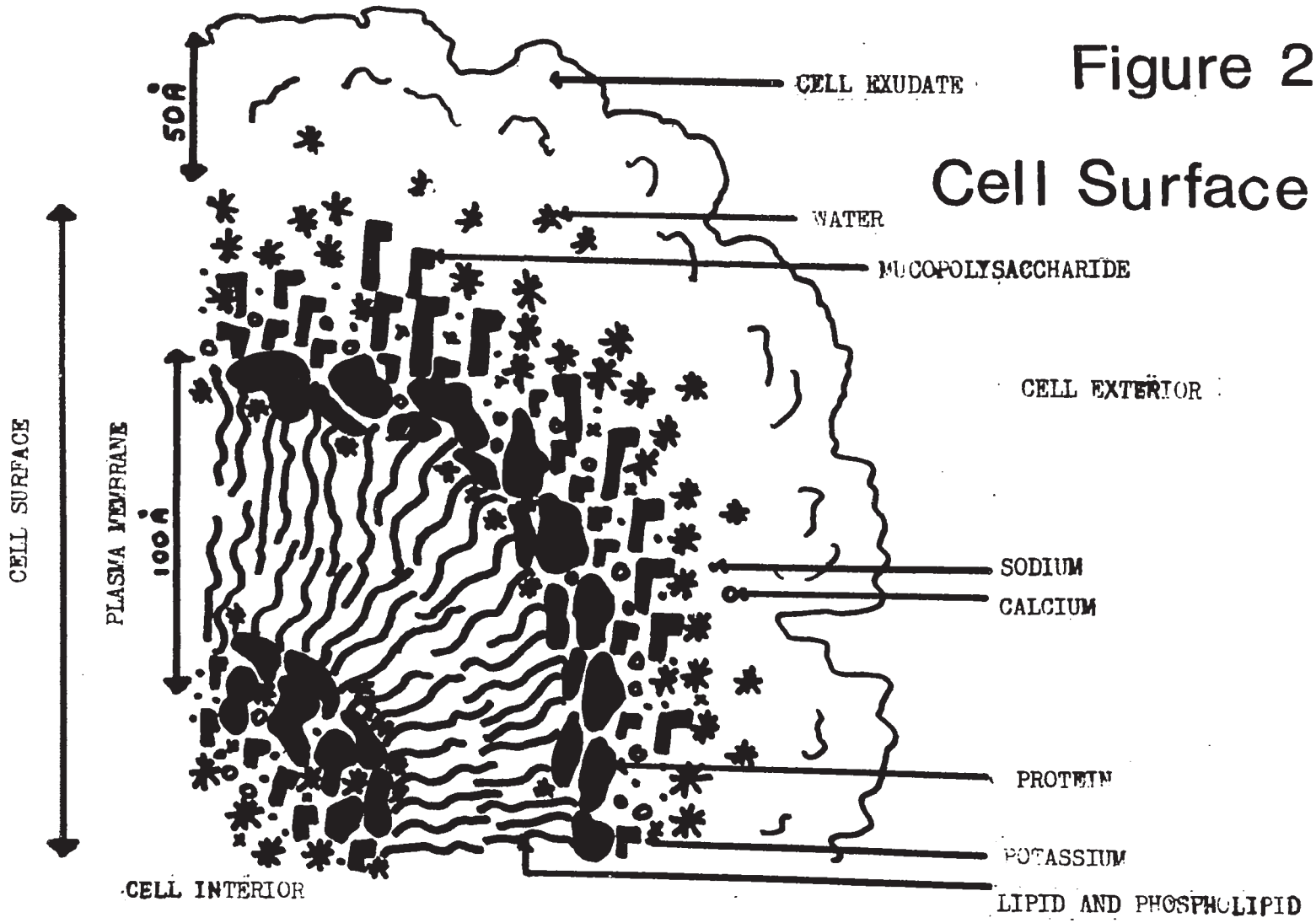
Chambers in 1940, studying the fertilized echinoderm egg found a hyaline layer outside the plasma membrane and suggested the universal extraneous coat. He postulated such a coat might cement cells together and be of importance in tissue organization and in cellular permeability phenomena. Hirst (1948), suggested the coat and viral receptors were primarily mucopolysaccharide. Figure 2 is a diagrammatic representation of the mammalian cell surface. The positioning of the various components follows the model of Davson and Danielli, (1935).

Klenk and Uhlenbruck, (1958), gave the first concrete evidence of carbohydrate in the extraneous layer on mammalian cells, when they isolated mucoproteins containing sialic acid from the red cell stroma. The extraneous coat has been described by Revel and Ito, (1967), as a fuzz layer and according to Lewis, (1958), it is invisible, elastic, viscous, water insoluble, and mucous or mucous-like. Cationic substances such as colloidal ferritin will combine with this outer layer indicating to Revel and Ito that it contains negatively charged colloid.

The study of the carbohydrate components of this extraneous cellular coat has been aided by investigations

Figure 2

Cell Surface



of other naturally occuring mucoproteins in salivary glands and urine. Blix, (1937), identified sialic acid in mucoprotein when he crystallized a carbohydrate from the submaxillary gland mucin and found a hexosamine, an acidic group and two acetyl groups. Odin in 1952 investigated the mucoproteins known to actively inhibit influenza virus hemagglutination. In all he found sialic acid.

Gottschalk, (1960, 1963), defined sialic acid as the generic name for all N-&-O-substituted neuraminic acids after having determined its structure. The linkage of sialic acid to bovine salivary gland mucoprotein was found to be an α-ketosidic linkage between the keto group of neuraminic acid and an hexosamine (D-galactosamine mainly); this in turn was joined by a glycosidic ester linkage to an acidic amino acid residue of the protein component.

Gottschalk found that neuraminidase cleaved the α-ketosidic linkage between the sialic acid and hexosamine, and caused a decrease in the protein viscosity suggesting that the sialic acid content of mucoproteins may be responsible for the normally viscous nature of mucoproteins. Weiss, (1963b), treated various cultured cells with neuraminidase and found that there was a significant increase in their detachment from glass, concluding that sialic acid is an important structural element in the cell's surface.

Burnet, (1951), isolated what he called a "Receptor-destroying enzyme" (RDE) from the bacteria Vibrio cholerae and Clostridium welchi, and showed that influenza virus also possessed it. This RDE, in actual fact the enzyme neuraminidase, would release sialic acid from the stroma. The conclusion was reached that the viral receptors of the red cell surface consisted of sialic acid as the prosthretic group of the stromal mucoprotein and that the negative charge of the cell could be ascribed to it.

Cook, Heard and Seaman, (1960), examined the tryptic degradation products of intact human erythrocytes using paper chromatography and showed that the decrease in the cells electrophoretic motility was accompanied by the production of sialomucopeptide with a terminal sialic acid.

Immunological work on the ABO and MN systems of human blood groups by Makela et al., (1960), Morton, (1962), Yokoyama and Trams, (1962), Ohkuma and Ikemoto, (1965), and Prager and Fletcher, (1966), has shown that removal of the sialic acid constituents of the erythrocyte membrane has a profound effect on certain immunological phenomena and they have concluded that these constituents confer antigenic properties on the membrane.

Histochemical studies of Gasic and his associates, (Gasic and Gasic, 1962a and 1962b; Gasic and Berwick, 1963; Defendi and Gasic, 1963) employed the Hale stain to

identify carbohydrate containing mucins. This Hale stain positive coating was either partially destroyed or removed completely by the action of trypsin, pepsin, pancreatin, and neuraminidase, whereas crude papain, crystalline papain, and hyaluronidase had no effect. These enzyme treated cells, however, were able to regenerate their cell coats within a few hours.

Rambourg and Leblond, (1967), have examined a variety of rat tissues and have demonstrated that the majority of cells examined are coated by an extraneous carbohydrate rich fuzz layer.

The possible function of this extraneous coat is a matter of discussion since the cell is viable without the coat. Whether it would remain so if the coat could be removed permanently is unknown.

Glick and Githens, (1965), using leukaemic cells showed that membrane-bound sialic acid mediates both the inward and outward diffusion of K^+ ions and that enzymatic removal of the extraneous coat inhibited the flow in both directions for potassium, although sodium, lysine and glucose were unaffected.

As well as this, the role of the extraneous coat in adhesion, both to other cells and to a foreign substrate, such as glass, is well documented, (Weiss, 1965; Gasic and Gasic, 1962; Kemp, Jones, Cunningham and James, 1967), Berwick and Coman, (1962). Gesner and Ginsburg, (1964),

have suggested surface sugars allow the lymphocytes to move normally through the body by acting as sites recognized by complementary structures on the surface of endothelial cells in the post capillary venules of the lymphoid tissues. Whatever the function, or functions, of the extraneous coat it would appear to be a universal layer on the cell surface.

b. The Plasma Membrane

Nägeli and Cramer, (1855), are credited with the first mention of the plasma membrane. They observed that small wounds of some plant cells were self healing, due to what they termed a denser, viscous, superficial material around the protoplasm - "The Plasma-membran." Further experiments with hypotonic or hypertonic salt and sucrose solutions led them to conclude that this membrane was responsible for the osmotic properties of the cell. In 1877, Pfeffer characterized it as osmotically active and semipermeable and Overton, (1899), and Kite, (1913), attempted to confirm this definition with their permeability studies. Overton noted that fat soluble substances passed through the red cell membrane readily while fat insoluble substances did not. He proposed the lipoid theory which visualized the plasma membrane of the living cell as one continuous lipoid layer. However, certain lipoid-insoluble substances such as sugars and amino acids

by entering the cell readily did not obey his permeability restrictions and required him to explain this apparent anomaly with the "adenoid" (or secretive) mechanism which transported these substances in an undisclosed manner across the membrane. Nathanson, (1904), suggested instead, aqueous channels dotted the cell's surface.

In 1925, Gorter and Grendel, using lipid extracted with acetone from erythrocytes, calculated that there was just enough lipid in the cell to form a bimolecular covering layer. These estimates are now considered to be too low by nearly 50 per cent. Technical difficulties of acetone extraction account for this discrepancy, however, the idea they put forth has been a direct stimulus to most of the later work.

Danielli and Davson, 1935, following the ideas of Gorter and Grendel, proposed that biological membranes were similar to a sandwich--two molecules thick of lipid in the center, the meat of the sandwich, covered on both sides by absorbed protein. The model proposes a continuous bi-molecular lipid layer of closely packed phospholipid molecules with rigid and with fully extended hydrocarbon chains. The polar ends of the lipid molecule are directed outward while the nonpolar turn inward. Wilmer, (1961), pointed out a layer of absorbed protein is almost inevi-table considering the lipids known to be present in the

membrane, and the known specific interactions of proteins with these lipids.

Fatty acid analysis of the lipid fractions of a number of mammalian tissues shows that the lipids present do possess species specificity, differing from animal to animal but showing between different tissues, within a given species, many similarities (Veerkamp et al., 1962). A wide variation in lipid composition of membranes is probably of importance in determining function and properties of the various membranes.

In animal membranes, cholesterol forms nearly half of the neutral lipid, and phospholipid just over half of the total lipid. Weinstein and his associates (1969), studying the LM mouse fibroblast cell, concluded that the lipid composition of the surface membrane is specific and differs from that of other membrane systems of the cell, and that specific fatty acid patterns may be useful in the classification of surface membranes.

Within the membrane the neutral lipids found usually included the unesterified fatty acids, the mono-, di- and tri esters of glycerol, the glycerides, cholesterol and its esters. It is however, the complex lipids such as glycolipids which vary enormously in both type and amount from species to species (Hakomori and Jeanloz, 1961; VanDeenen and DeGier, 1964).

Jorpes, (1932), showed, to his own and to many others satisfaction, that red blood cell membrane does contain a protein which was not either hemoglobin, globulin or fibrin, and that it comprises 80% of the total membrane dry mass.

Other attempts to extract protein from erythrocytes have been reported by Dandliker et al., (1950), Moscovitch et al., (1950), and Moscovitch and Calvin, (1952). They lysed erythrocytes in distilled water, washed them at pH 9.0 and obtained rod-like proteinaceous material (5 to 11 μ by 0.3 to 1.3 μ) which they called "reticulin", "stromin", or as the ether extract "elenin". Ponder, (1961), has concluded that present knowledge of physiochemical data on these protein fractions from erythrocyte ghosts tells us virtually nothing about their state in the intact cell.

Data available on other proteins, in the form of enzymes, located in the membrane are more abundant, though again attention is focussed almost exclusively on human erythrocyte stroma (Hokin and Hokin, 1961; Micheli and Graber, 1961; Novikoff et al., 1962; Férnandez-Moran et al., 1964; Green, 1964).

The nature of the sidechains of proteins determines the form any association with lipids will take. Since in many proteins some 35-50% of these sidechains are nonpolar (Tristam, 1953; Waugh, 1954), they will, in an aqueous environment, in the absence of strong interaction with

surrounding molecules, turn into the interior of the molecule to form a globular state (Langmuir, 1939; Cheesman and Davies, 1954; Klotz, 1958). Kalaidjiew and Segal, (1967), consider globular proteins to be hollow cylindrical structures and cite high resolution electron micrographs which they have taken as evidence for these "Faltentrommels". Other workers (Pauling and Corey, 1951; Nemethy and Scheraga, 1962), consider the protein of the membrane to consist of partially unfolded and uncoiled fabric proteins in an extended form resembling a pleated sheet. They visualize these sheets with extensible segments which are folded and coiled to various degrees into "knots" or globules which can react to external or internal forces, becoming unfolded or knotted as the stress is increased or diminished. Nevertheless, whatever the form, most investigators today accept the presence of both protein and lipid in the plasma membrane.

Although Davson and Danielli's model is still accepted by most workers, the subunit model advocated by Green and his associates (1967), explains many problems not adequately covered by the bimolecular leaflet model. A subunit structure system reduces the genetic information necessary to specify its structure and makes repair to the membrane simpler. In this membrane model a mosaic of units and consequently a mosaic of function is possible over the cell's surface. The subunit would be a

supramolecular complex of different chemical components, with a basic set of common binding sites, allowing the attachment of various chemical moieties to give a variety of function. Changeux et al., (1967), has theorized that the subunit may be capable of transforming between several alternative configurations to perform a different specific function in each configuration.

Cook, (1968), found that small amounts of sugar bound to the protein of the plasma membrane is usually about 1-5% of the dry weight. The presence of nucleic acids, especially ribonucleic acid, as component parts of the membrane has not as yet been settled.

The role of water, and the form of water, on the biological membrane is as yet the great unknown of membrane studies. It is thought to be in a highly structured ice-like form, (Pauling and Marsh, 1952; Bovey, 1961; Kavanau, 1965), but as yet experimental proof of the assumption has not been forthcoming.

The outer limits of the cell would seem to be composed therefore of the carbohydrate rich extraneous coat and the plasma membrane which is predominately lipid and protein. The form and structure of the components is unknown.

2. Immunological Loci Map of the Mouse

The arrangement on the cell surface of the components which carry the specificities dictated by inherited isoantigens of a species is unknown. It is assumed that these components are in some defined interrelated pattern but little attempt has been made to place them into the topographical details of the cell surface.

Based mainly on the results of tissue grafting experiments it is estimated that there are at least 14 gene loci in the mouse which regulate the formation of cellular isoantigens, (Snell, 1958). Serological analysis of cellular antigens has been limited almost exclusively to the histocompatibility-2 or H2 system (Gorer, 1937), although other systems (H1, H3, H4, H5, H6) have been mentioned, but only in broad outline. The H2 locus has at least 18 alleles and 25 or more specificities which combined together give the serological effect of the H2 (Snell et al., 1964; Amos, 1962; Amos et al., 1963). Recent studies (Kandutson and Stimpfling, 1966) claim that the H2 locus is a constituent of the cell membrane. However, the chemical nature, the function and production of this constituent are unresolved, (Snell, 1968).

Haughton and Davies, (1962), studying mouse tumor cell ghosts and the L cell, found that they retained all the agglutinogens and precipitating antigens that they had been able to detect in the intact cells. They concluded

that the H2 histocompatibility antigens of the mouse cell are very firmly bound to the structural material of the cell surface.

Considering the chromosomes of the LM mouse fibroblast which control the production of such antigens, the work of Hsu, (1959), Hsu and Merchant, (1961), and Giles et al., (1966), is very important. In 1959, Hsu studying the parent line of the LM mouse fibroblast cell, clone 929, found that the cells had a median number of 67 chromosomes with approximately 24% biarmed. The strain had an unique marker "T" and associated marker "D". By 1961, both markers were still noted but two new markers, E and F were also present in the "D" region. The median number of chromosomes had dropped from 67 to 64 and the biarmed chromosomes per cell had risen to 35%. In 1966, the range of total chromosome number had shrunk drastically but the overall increase in the mode was one chromosome to give a total of 65. The biarmed chromosome per cell had risen to 49%. From this Giles et al., (1966), concluded that the LM cell strain had undergone a gradual evolution after it was transferred to a serum-free medium, and that the population tended towards more uniformity in chemical nature, production and function, than the original cell population from which the line arises.

C. THE CELL LIFE CYCLE

1. Cytological Events

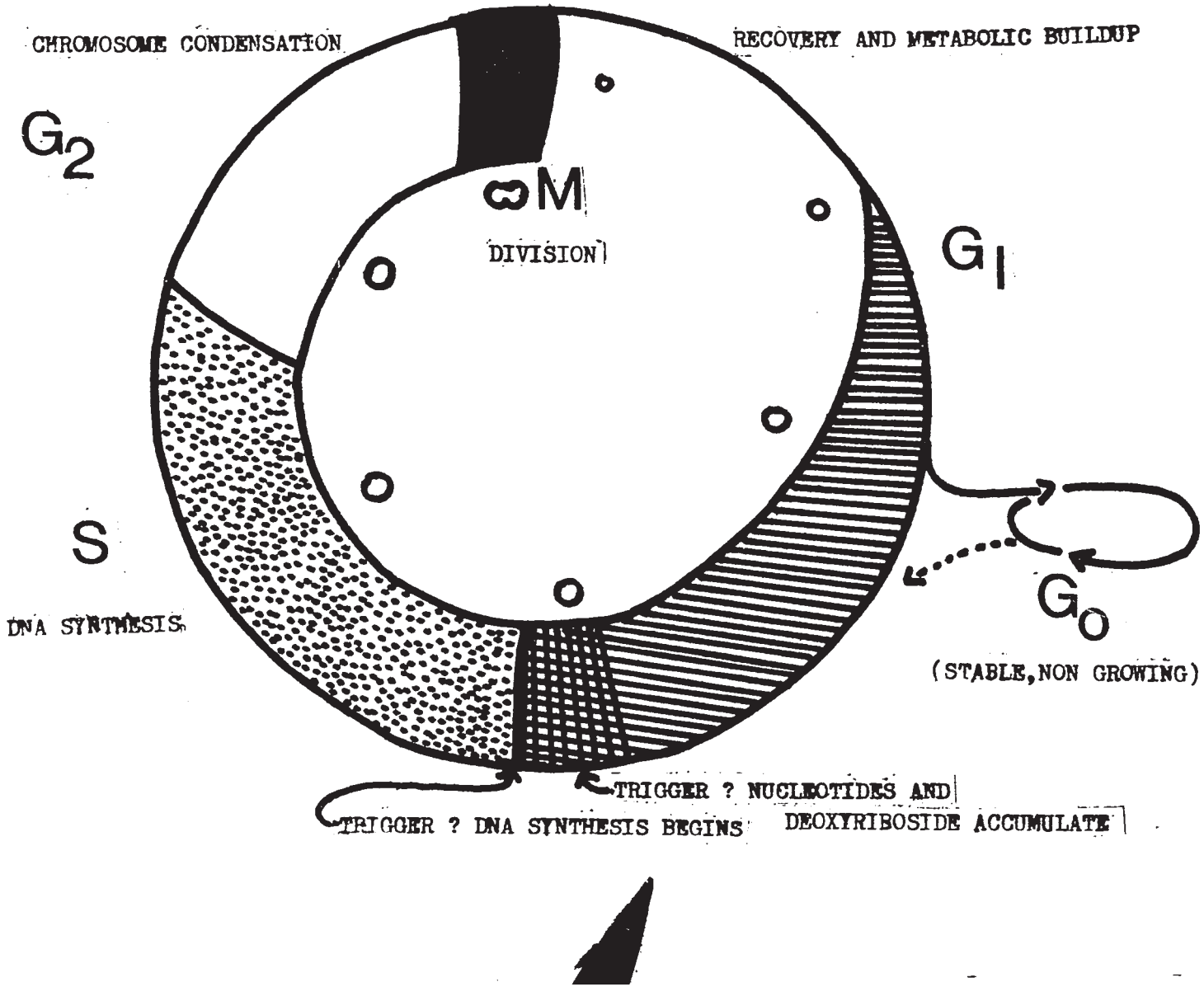
Within the cell population as it passes from lag phase, through logarithmic growth phase, to the stationary growth phase, the individual cells within the population proceed through their life cycle, (Appendix). The variation in time within the mitotic cycle of a cell has been found by Siskin and Kinosita, (1961), to be the rule rather than the exception within a population. Even daughter cells with an almost identical environment, followed through a single division do not have a similar mitotic cycle time. Figure 3 gives a diagrammatic representation of what is thought to be the stages followed by a cell from newly formed young cell (G1), during its preparation for division (S and G2 periods), to its final division time, M, (Howard and Pelc, 1953).

Cytologically, as Scherbaum, (1963), and Firket, (1965), have commented, the intermitotic period (G1, S and G2) has no spectacular morphological modifications visible. It is the very short mitotic period, M, which visually shows the cellular activity, (Wilson, 1925; Ris, 1955; Howard and Dewey, 1959; Gross, 1960; Brachet and Mirsky, 1961; and Levine, 1963).

During the interphase, the L cells appeared stellate, rounded or spindle shaped, with a prominent eccentric

Figure 3

Cell Cycle



nucleus. This nucleus contained many large pleomorphic nucleoli and a loose chromatin network. The cytoplasm next to the nucleus was slightly vaculated (Gey et al., 1949; Ludford, 1951; Gey, 1955), and a granular layer surrounded this in turn. The periphery of the cell was nongranular in appearance. Mitochrondria were located exclusively in the granular zone (Kahn et al., 1962). The nucleoli are pyroninophilic during interphase, indicating the presence of ribonucleoproteins, while the chromatin network stains dark green, indicating deoxyribonucleoprotein.

In Prophase, the first stage of mitosis, the chromatin condenses and the nucleolus begins to disintegrate, disappearing by late prophase. The forming chromosomes are embedded in a pyroninophilic matrix which remains through metaphase. At this time the nuclear membrane disappears and the chromosomes, in metaphase, spread from the nuclear area and attach themselves to the spindle. A to and fro motion is noticed until all the chromosomes are attached. Now they freeze in groups on the equatorial plate. Peripheral cytoplasmic "bubbling" is often observed at the same time. Anaphase, when the chromosomes draw apart in a precise formal dance, is perhaps the most fascinating moment in cell division. Telophase, which follows, finds the cytoplasm constricting in the region of the former equatorial plate and the nuclear membrane and nucleolus reappearing. The

final division of the cell into two daughter cells may take place immediately or hours later. Occasionally no such division takes place and giant multi-nuclear cells can be observed in the LM mouse fibroblast cultures.

2. Biochemical and Enzymatic Events

The most important cellular changes during the cycle are metabolic rather than morphological. In all cells studied the cycle is divided into four distinct periods based on the behaviour of the DNA at that time, (Woodard et al., 1961; Lajtha, 1957; Stanners and Till, 1960).

The G1 period, which is the most variable in length, is probably responsible for the variations in the total duration of the whole cycle since other periods appear more constant (Stanner and Till, 1960; Sisken and Kinosita, 1961). The new, small cell enters this phase which might be viewed as its childhood and spends the first hours in recovery after division. Previous to mitosis a choice had been made by the cell to either divide or differentiate. We therefore have a mixed population at the start of G1--those which will go immediately into preparation for division with every enzyme and metabolite produced or collected at this end, or else, those cells which will concentrate on metabolic build up alone. The latter cells are in a fairly stable state and may at any time go into the division cycle triggered by an unknown mechanism.

The cell preparing for division begins to produce protein (as shown by an increase in overall mass), and water and ions are absorbed, (Terasima and Yasukawa, 1966). The synthesis of RNA and protein would appear to be necessary for an orderly flow of cells from G1 to S (Baserga et al., 1965). For most of the G1 phase the cells appear to be stable metabolically, and nongrowing tissue, (GO), has been considered to be physiologically at this stage in the cell cycle.

Towards the end of the G1 phase some unknown mechanism causes the accumulation of deoxyribosides and nucleotides (Schneider and Brownell, 1959; Foster and Stern, 1959), and there is a marked decrease in potassium ion content of the cell and an increase of sodium (Jung and Rothstein, 1967). Killander and Zetterberg, (1965), have found with LM mouse fibroblast cells that there is a very small variation in the mass of these cells entering S from G1.

At the beginning of S, the period of DNA synthesis, activity begins very near the nucleolus (Firket, 1958; Harris, 1961). A parallel production of histones and DNA would seem to be necessary (Bloch and Godman, 1955; Mueller et al., 1962).

Amino acid lack in G1 will not prevent the entrance of the cell into S but it will gradually curtail DNA production once started. However, after DNA synthesis has

begun amino acid deprivation at that time does not affect the completion of synthesis (Baserga, 1968). Why DNA synthesis in mammalian cells is so tied to protein synthesis is still unknown.

During DNA synthesis an increase in thymidine kinase, thymidylic kinase, DNA polymerase, deoxycytidylate deaminase, and thymidylate synthetase (Baserga, 1968), takes place. Alkaline phosphatase activity decreases.

As the cell enters G2 the cell is preparing for division. The duration of time a cell is in this phase is fairly constant for a given cell type. In plants, yeasts, protozoa and other organisms (Woodard, Rasch and Swift, 1961; Williamson and Scope, 1960; Prescott, 1960; Woodard, Geller and Swift, 1961), G2 is characterized here by an increase in RNA synthesis, especially in the nucleus. Whether such an increase takes place in vertebrate cells is unknown. However, studies using biochemical inhibitors indicate that both protein and RNA synthesis is necessary during G2 if mitosis is to be completed (Tobey et al., 1966; Pfeiffer and Tolmach, 1967). Swann, (1954), postulates that there is some energy reservoir filling at a constant rate and that a certain energy level must be reached before mitosis could proceed. Numerous chemical substances such as cyanide (Pomerat and Willmer, 1939), fluoride or iodoacetate (Hughes, 1950), which affect the cell's energy system, do not affect the mitotic process once begun,

giving support to Swann's hypothesis.

At the end of G2, dry mass, due mainly to protein, reaches a maximum. About thirty minutes to an hour before mitosis a loss of water occurs (Sandritter, Schiemer, Kraus and Dorrien, 1960). The cell as it comes to mitosis, therefore, is in a very special metabolic state having a high chemical energy reservoir, protein mass at a maximum, double the number of deoxyribonucleoprotein strands in its chromosomes, and a nuclear accumulation of RNA.

During mitosis proper, the M phase, the chromosomes divide, two new nuclei are formed and the cell divides into two separate daughter cells both of which will then proceed through the cell cycle independent one of the other. It has been noted by Terasima and Tolmach, (1963), that the cells at mitosis are less sticky. As well as this, cell electrophoretic mobility studies have all shown an enhancement of the net negative charge associated with the mitotic state (Brent and Forrester, 1967; Mayhew and O'Grady, 1965). The accumulation of sialic acid residues has been postulated as the causal factor but the electrophoretic mobility increase is probably due to something more than just a surface concentration change of charge-bearing macromolecules.

3. Cell Synchrony

Two general approaches have been used to obtain synchrously growing populations of mammalian cells. The first approach involves the blocking and accumulation of the cells in a particular stage of the cell cycle and the subsequent release of the block. Such a population is termed a "Synchronized" culture. The blocking agent can be low temperature (Newton and Wildy, 1959); temperature cycling in which the culture is subjected to a cold-warm cycle equal to one generation time (Padilla and Cameron, 1964); temperature pulsing in which the culture is rapidly exposed to fairly wide temperature shifts (Zeuthen, 1964); nutritional growth methods in which the cell culture is grown on a basal medium then shifted up to a rich medium (Katsuta and Takaoka, 1962); gasometric methods in which single or multiple cycles of nitrogen gas bubbling accumulates cells at a specific stage of the cycle (Neff and Neff, 1964); or using an inhibitor or natural metabolite to block the cells (Zeros, 1962; Till et al., 1963; Firket, 1964; Sinclair, 1965; Galavazi et al., 1966). Removal of the block releases the cells and allows them to proceed through the cell cycle in a synchronized wave.

The second approach involves the selection of a group of cells in or close to one particular stage in the cell cycle without altering their metabolic state. Such cultures are called "Synchronous cultures". Mechanical separation

utilizing a particular cell characteristic is the basis of division here. Filtration, trapping large cells and allowing small cells to pass was used by Anderson and Pettijohn, (1960). Other methods include: density gradient separation, utilizing density variations of the cell population (Mitchison and Vincent, 1965; Sinclair and Bishop, 1965; Noble and Cutts, 1967; Boone, Harell and Bond, 1968); and substrate attachment variations. Terasima and Tolmach, (1963), took advantage of the fact that in monolayer cultures mitotic cells round up and detach themselves. By continually collecting cells shaken from monolayer cultures they were able to get a population of cells just entering mitosis. This second approach to cell population is most promising. The technique imposes minimal strain on the cell's metabolic system and is rapid in execution.

III. MATERIALS AND METHODS

A. CELL STUDIES

1. Cell Medium Preparation

Medium 199-peptone has been used for the propagation of the L cell since 1956 (Morgan, Morton and Parker, 1950; Merchant, Kuchler and Munyon, 1960). Four separate sterile solutions were mixed together asceptically and after a sterility check were completed by the addition of an antibiotic mix (Medzon and Merchant, 1970) (Appendix).

2. Culture of the Cells

Two methods of culture, suspension and monolayer, were used to obtain cell populations. In the suspension cultures adequate air space of approximately 3/5 the flask volume was left above the propagating medium, (Medzon and Merchant, 1970).

The growth curve observed for the LM mouse fibroblast cells was similar to that seen with any nonselected population of almost any single cell organism (Appendix). Merchant and his associates have well documented the growth characteristics of this cell line, (Merchant, Kuchler and

Munyon, 1960; Hsu and Merchant, 1961; Merchant and Hellman, 1962; Eidam and Merchant, 1965a, 1965b, 1965c). The LM cells used in this study were obtained from Dr. Merchant.

3. Viability Tests

There are many procedures used to differentiate between viable and non-viable cells. The ability of viable cells to exclude dye is one criterion used. Various dyes such as erythrosin B (Phillips and Terryberry, 1957) have been used in this manner. With other substances the metabolite used indicates metabolic cell activity. Fluorescein Diacetate (FDA) when used by Rotman and Papermaster, (1966), has such a function.

a. Erythrosin B-dye Exclusion Test (Fisher, 1953)

Erythrosin B (certified stain of National Division, Allied Chemical Corporation) was dissolved in Hanks' buffer to give a 1×10^{-5} M solution. The solution was filtered to remove any undissolved dye crystals. To 0.5 ml of a cell suspension (1×10^5 - 2×10^5 cells/ml) in a 12 x 75 mm tube was added 0.1 ml of the dye solution. A total count and a count of unstained cell was done microscopically at 100X magnification. Assuming that those cells which are unstained are viable, the results obtained were expressed as percentage viable cells.

b. Fluorescein Diacetate Fluorescent Metabolism Test (Rotman and Papermaster, 1966)

Fluorescein Diacetate, FDA, received from Mann Research Laboratories, N.Y., was initially dissolved in acetone (100 mg powder FDA/20 ml acetone), and placed in a screwcapped universal in the freezer at -20°C. To 10 ml of medium 199-peptone 50 λ of this concentrated FDA was added using a micropipette. To 0.5 ml of a suspension of LM mouse fibroblast cells in medium 199-peptone, in a 12 x 75 mm test tube, was added 0.5 ml of this FDA suspension. Living cells, after approximately 5 minutes in this mixture, exhibit a white-yellow glow when viewed microscopically under ultra violet light using a large Zeiss florescence microscope, a HB200 mercury vapor lamp, a number 2 exciter filter, an open condenser with a number 50 barrier filter. The florescence was caused by the cellular accumulation of fluorescein, the metabolic breakdown product of fluorescein diacetate. Non-viable cells having no such metabolic activity do not use the FDA and the florescent compound, fluorescein, did not accumulate in them. Comparison of the two viability tests were made counting twelve separate populations, one hundred fields each (Appendix).

c. Double Dye Technique

This is an extension of the two viability tests mentioned above. To 0.5 ml of a suspension of LM mouse

fibroblast cells in medium 199-peptone in a 12 x 75 mm test tube was added 3 drops (15λ) of a 1 x 10^{-5} M solution of Erythrosin B. The tube was gently shaken then 10 λ of the fluorescein diacetate concentrate (FDA) was also added to the cell suspension. After 5 minutes the cells were viewed, one field at a time, alternately under low power phase contrast and then under ultra violet light, using a large Zeiss florescence microscope, a HB200 mercury vapor lamp, a number 2 exciter filter, and an open condenser with a number 50 barrier filter. Comparison counts of viable and non-viable cells were made, (Figure 4).

d. Mitotic Cell Count (Schindler et al., 1967)

In order to obtain a mitotic count for any cell population used in these tests, the cells were first fixed by mixing an aliquot of the cell suspension culture with an equal volume of ethanol-acetic acid - water (5:2:3 v/v). The cells were subsequently centrifuged and pelleted in an International PR2-centrifuge at 2400 rpm (1200 g) and re-suspended in a small volume (0.5 ml) of 0.025% crystal violet in 1% acetic acid. In this suspension, using a haemocytometer, the percentage of cells in mitosis was determined. Only those cells in which the chromosomes were distinctly visible, through metaphase and into anaphase, were counted, (Figure 5).

FIGURE 4

DOUBLE DYE TECHNIQUE

a. Cell population viewed under high dry (magnification 1000 X) phase contrast. Non-viable cell dyed red with Erythrosin B. (arrow).

b. Same cell population viewed under ultra violet light (magnification 1000 X). Viable cells fluorescent. Non viable cells non fluorescent. (arrow).

Figure 4

a.

b.

FIGURE 5

MITOTIC STAINING OF LM MOUSE FIBROBLAST CELLS

a,b. Examples of cells in mitosis (arrow) stained according to the method of Schindler et al. (1967). (Magnification 1,200 X) viewed under high dry phase contrast.

Figure 5

a.

b.

4. Membrane Effects with Various Buffers, Chemical Solutions, Inhibitors and Enzymes

The method used to study the effects of various agents on the membrane of the cell was simple. The cells were suspended, at 37°C, either in a solution containing the agent or else the agent was added to the bathing medium surrounding the cells. Cells were viewed microscopically, using phase microscopy, continually for the first ten minutes. After this time the cells were allowed to incubate and at various times aliquots were removed and the cells inspected microscopically. All preparations were examined using a Zeiss microscope fitted for florescence and phase microscopy. This microscope was equipped with a Nikon 35 mm camera and photomicrographs were taken using Kodak plus X panchromatic film (ASA 125). Phase contrast photomicrographs were taken with an exposure time of 10 seconds. Florescent photomicrographs were taken with an exposure time of 2 minutes.

a. Buffers and Chemical Solutions

All chemicals mentioned were supplied either by Shawinigan Chemical Company, Montreal, Quebec, or by Fischer Scientific Company, Toronto, Ontario unless otherwise noted. All chemicals were reagent grade.

Buffered and chemical solutions tested were:

Dulbecco Phosphate Buffered Saline (Appendix),

Physiological Saline, (0.8%),

Sucrose Solution, (0.25 M),

Magnesium Chloride, ($MgCl_2$), 1 x 10^{-2} M to 1 x 10^{-7} M in medium 199,

Calcium Chloride ($CaCl_2$), 1 M to 1 x 10^{-7} M in medium 199,

Potassium Chloride (KCl), 1 M to 1 x 10^{-5} M in medium 199,

Nonidet, a nonionic detergent supplied by Shell Company of Canada, .0001% to .000001% in distilled water,

EDTA (Ethylenediaminetetraacetate), selective chelation concentration 5 x 10^{-4} M, and total chelation concentration 3.8 x 10^{-1} M in medium 199,

Sodium Fluoride, 3 x 10^{-4} M in medium 199, (Kirschner, 1964),

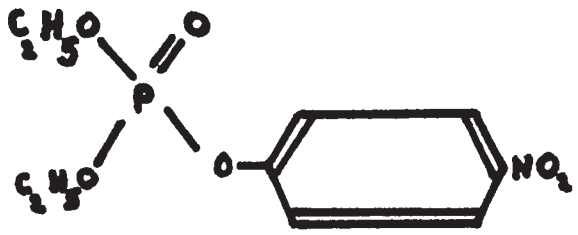
Vitamins A, C, and E (Appendix), in medium 199,

Ficoll (15%, 20% and 40%) in medium 199 (Appendix).

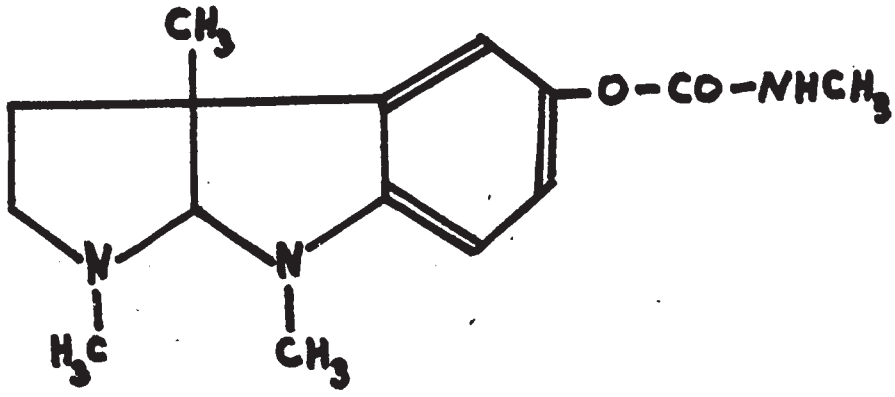
b. Inhibitors and Enzymes

Paraoxon, (Diethyl p-nitrophenyl phosphate) is an organophosphate esterase inhibitor widely used in the classification of esterases. A solution 1 x 10^{-3} M in medium 199-peptone was freshly made up and the cells suspended in it. This inhibitor was obtained from Nutritional

PARAOXON (DIETHYL p-NITROPHENYL PHOSPHATE)



ESERINE SULPHATE (PHYSOSTIGMINE)



Biochemicals, Cleveland, Ohio, U.S.A.

Eserine sulphate (Physostigmine) is a naturally occuring anticholinesterase obtained from the calibar bean. It is relatively specific for cholinesterase. A solution 1×10^{-3} M in medium 199-peptone was freshly made up and the cells suspended in it. This inhibitor was obtained from Nutritional Biochemicals, Cleveland, Ohio.

Lipase and Collagenase, obtained from Nutritional Biochemicals, Cleveland, Ohio, U.S.A., were made up in medium 199-peptone at a concentration of 0.05% and 0.1% respectively.

Neuraminidase, in Vibrio cholera filtrate, obtained from Nutritional Biochemicals, Cleveland, Ohio, U.S.A., was made up in medium 199-peptone at a concentration of both 300 units/ml and 50 units/ml. The cells were incubated with these solutions at 37°C in a constantly stirred water bath. Four tests were undertaken with aliquots of cells from the incubating cell population. Three of the tests involved viewing microscopically, under high dry phase contrast of a Zeiss microscope, a drop of cell suspension plus a drop of either Erythrosin B (1×10^{-5} M), Fluorescein Diacetate (1×10^{-4} M) or Fluorescein (1×10^{-4} M). In the fourth test the number of cells which adhered to a glass surface as compared to a control sample was ascertained.

5. Cell Envelope Preparation

A number of methods, described in the literature, were used to prepare cell envelopes (Warren and Glick, 1968; Emmelot and Bos, 1965; Wallach, 1967). The yields obtained in all cases were low and the final method of choice was developed during the investigation.

a. Developed Method

Materials

Tris-$MgCl_2$-Gluteraldehyde Solution: Tris (hydroxymethyl) Aminomethane primary standard, magnesium chloride (crystals), and Gluteraldehyde solution 50% w/w, biological grade, were obtained from Fischer Scientific Company, Fairlawn, New Jersey, U.S.A.

A solution was made up in 0.02 M Tris buffer of magnesium chloride (1×10^{-5} M) and 0.004 M gluteraldehyde. The pH was adjusted to 8.1 and the solution was kept in the cold at 4°C.

Sucrose Solutions: The sucrose solutions were made up according to the table in the Handbook of Chemistry and Physics (44th Edition, 1963, page 2272).

Method

5×10^5 to 5×10^6 LM mouse fibroblast cells were pelleted by centrifugation in a screwtopped 50 ml, conical centrifuge tube at 2400 rpm (1200 g) for 10 minutes in an

International centrifuge, model PR2. The supernatant was removed and the cells were washed in fresh 199-peptone and repelleted. From this point on two different methods of handling the cells were developed. Both methods gave high clean yields of cell envelopes.

In the first method the pellet was resuspended in approximately 1 ml of cold Tris-$MgCl_2$ buffer and the cells were "buzzed" on the vortex mixer. The cold Tris-$MgCl_2$-gluteraldehyde solution (10 ml-20 ml depending on the original cell number) was then added to the cells and they were allowed to swell and burst. After 5 minutes the cells were "buzzed" again and under the phase contrast microscopy it was noted that all cells had "popped" leaving the cell envelope free. Separation of these envelopes from the rest of the cell debris is indicated at the conclusion of the second method of cell envelope preparation.

In this second method the cell pellet was resuspended in cold (4°C) Tris-$MgCl_2$-gluteraldehyde solution (10-20 ml depending on the original cell number) and placed in a Dounce homogenizer (50 ml) in an ice bath for 10 minutes to allow the cells to swell, then the cells were gently homogenized using 15 strokes of a tight fitting pestle. The homogenate after this treatment should contain free nuclei, cell envelopes and debris, the number of strokes being adjusted--either decreasing or increasing the number--

depending on the state of the homogenate when viewed by phase-contrast microscopy.

After homogenizing or cell popping, the cell mixture was layered on top of 45% sucrose in a cellulose nitrate tube (30 ml). The tube was centrifuged at 1200 rpm (550 g) for 30 minutes to obtain the envelope fraction. About mid-way down the tube a turbid band containing predominately cell envelopes appeared. The nuclei and broken cells either pelleted or ran very near the bottom of the tube. Above the envelope fraction light debris and vesicles appeared. The envelope fraction was removed by puncturing the cellulose nitrate tube with a syringe needle and drawing off the layer.

This layer was mixed with an equal volume of 45% sucrose and layered on a continuous 35% to 65% sucrose gradient. The tubes were centrifuged at 2000 rpm (1000 g) for 30 minutes. The envelope fraction appeared at about the 45% sucrose region. This layer was removed and found to be almost pure cell envelopes. If it was not pure the fraction was rerun on another continuous sucrose gradient and recollected. This fraction, if not used immediately, was stored in the 45% sucrose in sterile universals for up to a month without any visible deterioration (Warren, Glick and Nass, 1966). If used immediately it was washed in 0.02 Tris-$MgCl_2$ (1×10^{-5} M) buffer after pelleting out of the sucrose by centrifugation.

Centrifugation

In all the following methods pelleting of the cells prior to utilization was necessary. The International centrifuge PR2, swing bucket, #269, or #284 rotor with cups of three sizes was used (Size 356, 320, 384). Using a nomogram the gravity force was calculated. Hereafter only this force will be given. Unless otherwise noted the centrifugation time was 10 minutes.

6. Cold Shock Synchrony of Cells

Following the method of Newton and Wildy, (1959), the LM mouse fibroblast cells were incubated immediately after a one hour chill at 4°C. From hour 17 for each half hour of incubation until hour 24, aliquots of the two populations, test and control, were taken and a cell count, viability and mitotic count recorded. Each of the two cell populations were then divided into two, one portion being allowed to continue normal incubation while the other was separated on a continuous Ficoll gradient. The cells thus divided were placed in small Erlenmeyer flasks (100 ml) with fresh medium 199-peptone and incubated at 37°C. Growth counts and visual monitoring of the various populations continued for the next 3 days.

7. Ficoll Gradient Method for Separating Cells

a. Ficoll Gradient Preparation

Ficoll was obtained from Pharmacia, Uppsala, Sweden. It is a large inert molecule; its molecular weight obtained by light scattering was approximately 400,000 and there was a chloride content of less than 1%.

Sterile medium-199-peptone without methyl cellulose but containing penicillin (100 IU/ml) and streptomycin (100 ug/ml), was used as the suspending fluid. To prepare the Ficoll-199 peptone 50 to 75 ml of sterile medium 199-peptone was placed into sterile (250 ml) beakers covered with foil wrap. The desired percentage, by weight of Ficoll, for a final total of 100 ml, was swiftly weighed in weighing boats. This was the only step in the operation that was not done under completely sterile conditions. However, very little loss, due to contamination, ever resulted. The Ficoll was added to the sterile 199 (plus penicillin and streptomycin) and a sterile magnetic stirrer bar was dropped into the beaker. The foil wrap was tightly pinched around the lip of the beaker and a covering of parafilm placed over and taped down around the beaker. The beaker was then placed on a magnetic stirrer in a refrigerator and the Ficoll, resting on top of the medium 199, was gradually pulled into the liquid. After 12 hours all of the Ficoll was dissolved in the medium 199. The liquid was poured

aseptically, after flaming the beaker lip, into a sterile 100 ml volumetric flask and the level of liquid made up to the 100 ml line with fresh medium 199-peptone containing antibiotics. This liquid was then poured into a sterile medical flat and stored at 4°C until needed. All glassware and instruments used in preparing the gradient were sterilized if possible. In the case of the continuous gradient machine (Buchler Instruments, Fort Lee, N.J., U.S.A.), 500 ml of sterile distilled water was run through prior to using with the Ficoll-199-peptone in order to minimize any possible contamination of the cells subsequently by diluting out any bacteria which might be in the machine and polyethelyne lines. This allowed for adjustments of the blade stirrer, flow, and cleansing of the apparatus. After the sterile distilled water had passed through the machine, the stopcock joining the two chambers was closed.

The two Ficoll solutions were added to their respective chambers, the exact amount varied depending on the size of the cellulose nitrate tubes to be filled. For example if three gradient tubes containing a final volume of 30 ml each (plus cushion) was used, 90 ml total of both concentrations of Ficoll solutions was necessary (45 ml of the weaker, 45 ml of the stronger). To provide a cushion of the higher percentage solution (2 ml extra for each tube) an extra 6 ml was added to the chamber containing the higher percentage solution. This extra solution not

only provided a cushion in each tube but also filled the delivery lines prior to generating the gradient. After the cushion had been delivered to each of the three cellulose nitrate tubes, the blade stirrer was started and the stopcock between the two Ficoll solutions opened. The most commonly used gradient spread was between 22% Ficoll and 15% Ficoll although other limits were tried.

b. Method of Cell Separation

When a continuous gradient was made up using the small cellulose nitrate tubes (15 ml), a total of 5 ml of 5×10^5 cells/ml was the maximum count. When the large cellulose nitrate tubes (30 ml) were used a total of 10 ml of 5×10^6 cells/ml was the maximum count used. An equal volume of 24% Ficoll in 199 was mixed with the cell suspension and the resulting mixture layered on top of the continuous gradient in the cellulose nitrate tubes. The tubes were centrifuged in a swing bucket rotor (#269) on the International PR2 centrifuge, 1000 rpm (350 g) for 1 hour.

A continuous spread of cells from just below the miniscus of the inoculum to almost three quarters of the way down the tube was obtained. Three more compact band regions were usually noted. With either a syringe, puncturing just below the band, or with a sterile Pasteur pipette, the cells of the band and the region immediately

above it were removed and placed in sterile screwcapped centrifuge tubes. An equal volume of fresh medium 199 was added and the cells washed. These tubes were centrifuged at 2400 rpm (1200 g) for 10 minutes and the supernatant subsequently removed. The cell pellet was resuspended in a known amount of fresh medium 199-peptone and a cell count taken using the Coulter counter.

c. Radioactive Verification of Cell Synchrony

Source and Concentration of Label

Thymidine methyl - H_3 was supplied by New England Nuclear, Boston, Mass., U.S.A. The original thymidine solution contained 5 x 10^{-1} mci/ml at a specific activity of 6.7 c/mmole. It was diluted 1/1000 and 1 ml gave a count rate of 300,000 cpm.

Method of Labelling

Prior to the following experiments the most advantageous labelling time was ascertained for this type of cell population and this particular radioactive label (Appendix). The tritiated thymidine was incubated either 30 minutes or 60 minutes with the LM mouse fibroblast cells at 37°C. The labelling took place either before or after Ficoll gradient separation of the cells.

If the labelling took place prior to the Ficoll gradient separation (prelabelling) the incubation time

was 30 minutes and the cells were pelleted by centrifugation at 1000 g for 10 minutes, resuspended in 10 mls of 10% Ficoll in 199 and separated on the Ficoll gradient. A total of 20 x 10^5 cells were used in all tests. After washing with fresh 199 the cells were resuspended to a count of 2 x 10^5 cells/ml, as indicated by a Coulter counter, in fresh medium 199.

If the labelling took place after separation on a Ficoll gradient (post labelling), the three bands of washed cell populations were brought to a count of 4 x 10^5 cells/ml in medium 199, as indicated on a Coulter counter, model A. An equal amount of medium 199 containing the thymidine label was added (2 x 10^5 cells/ml final count 300,000 cpm final label) and the cells were incubated at 37°C for 60 minutes.

Method for Extraction

In the case of the prelabelled cell populations from each of the three populations two aliquots of 0.5 ml (1 x 10^5 cells) were poured into two millipore filters apparati (0.2 u filter) and the cells trapped on the filters. One ml of cold TCA, (5% plus 100 micrograms

thymidine/ml), was used to wash the cells. The filters were removed, dried on an absorbent towel under a warm lamp and placed in a scintillation vial containing 10 ml of the fluor.

In the case of the post-labelled cell populations after the incubation time, two aliquots of 0.5 ml (1×10^5 cells) were also separated on a millipore filter apparatus (0.2 u filter) and cold TCA (5%) plus thymidine (100 micrograms/ml) was added to stop the reaction, as well as wash the cells. The cells remaining in each incubation flask also were stopped by the addition of an equal amount of 5% TCA and thymidine to the flask. The filters were removed from the millipore apparatus, dried and placed in the scintillating vials containing the fluor.

Counting Cocktails

For both the prelabelled populations and the post labelled populations, whole cells, the fluor in the scintillating vials was PPO-POPOP (Nuclear Chicago):

Toluene	1000 ml
PPO (2,5,Diphenyloxazole)	4 g
POPOP	0.1 g
(1,4,bis [2-(4-methyl-5-phenyloxazolyl] benzene)	

Control vials of the fluor alone, unlabelled mother population trapped on the filter, the radioactive tracer, and standard vials giving low and high machine quenching rates were run with each test.

Methods for Solubilizing for Counting

For all cells chemical analysis and the identification of the site of the radioactive label was undertaken. The DNA isolated by the method in Section IIIB, page 48 of this study was suspended in Trichloracetic acid. The sample was placed in a scintillation vial and evaporated to dryness in a water bath (Hanson and Bush, 1967). A white flaky powder remained in the vial and was readily dissolved in 100 λ of sterile distilled water. 100λ of 0.6N NCS (Nuclear Chicago, Des Plaines, Illinois) in toluene completes the solubilization of the DNA after 3 hours. To this vial 10 ml of the fluor PPO in toluene was added:

Toluene	1000 ml
PPO	6 g

Hanson and Bush cautioned that in some cases a fine precipitate was noted; however, in these particular samples none appeared. The vials were wiped clean with lens paper and placed in the scintillation trays. Control vials of fluor, NCS and PPO, and toluene, and the radioactive tracer were run with each test.

Calculation

Counting of all samples was done using a Philips liquid scintillation analyser. A total of 12 separate, 10 minute, gross counts was done for each vial in order

to obtain significant results. These gross counts were averaged, corrected for background and system counts to give counts per minute for 1 x 10^5 cells.

B. CHEMICAL ANALYSIS

Chemical analyses were undertaken both for whole cells and for membrane envelopes obtained from these same populations. Using logarithmically growing cell populations protein, deoxyribonucleic acid, ribonucleic acid, carbohydrate and lipid (both total and cholesterol) analyses were done on both whole cells and membrane envelopes of the mother populations as well as the Ficoll gradient cell fractions. Cell counts and ghost counts were performed so that the analytical value could be expressed as a function of these parameters. Detailed protocol, for each method, may be found in the Appendix.

1. Protein Determination

Two methods were used to determine the protein content of the LM mouse fibroblast cell and envelope fraction of the same cell population:

a) The Dry Weight Method - Modified Schmidt Tannhauser as described by Merchant, Kahn and Murphy, 1964.

b) The Folin - Ciocalteau Phenol Method described by Lowry, 1951.

2. DNA Determination

The sample of DNA tested, as a fraction suspended in 6% TCA, was obtained from the procedure used to ascertain total protein, Folin-ciocalteau phenol method.

Two variations of the diphenylamine method were used to determine the DNA content of the LM mouse fibroblast cells. The two methods differed in whether or not acetaldehyde was used and by a slight difference in the reagent concentration.

a. Method 1 - (Burton 1956, modification of Dische 1930, as Quoted by Merchant, Kahn and Murphy 1964)

In this method a stock solution was prepared containing 1.5 gm of crystalline diphenylamine dissolved in 100 ml of glacial acetic acid plus 1.5 ml of concentrated sulphuric acid. Just before the test 0.1 ml of an acetaldehyde solution (16 mg acetaldehyde in 1 ml of glass distilled water), was added to 20 ml of the stock diphenylamine solution.

b. Method 2 - (Dische, 1930)

In this method 1.0 gm of crystalline diphenylamine was dissolved in 100 ml of glacial acetic acid containing 2.74 ml of concentrated sulfuric acid. No acetaldehyde was added in this test.

3. RNA Determination

The orcinol test of Drury, (1948), with the modifications of Miller et al., (1951), was done on the RNA samples obtained as a by product during the Folin-Ciocalteau Phenol Test for protein.

4. Lipid Analysis

Two methods of lipid extraction from the LM mouse fibroblast cells were used. These methods were that used by Weinstein et al., (1969), and the classic Schmidt-Tannhauser method. After extraction, the purification, gravimetric determination of total lipid and the cholesterol determination methods were the same for both extracted samples.

a. Extraction

i. Method of Weinstein, Marsh, Warren and Glick (1969)

In this method four separate lipid extractions were made of the cells with chloroform-methanol (1:1), one extraction at 0°C, two at room temperature and the final one at 45°C. All the extracts were combined in a universal vial and retained for purification.

ii. Schmidt-Tannhauser Method (Merchant, Kahn and Murphy, 1964)

In this method two extractions, with chloroform-methanol (2:1), at room temperature were combined to give the unpurified extract.

b. Lipid Purification

Extracts from both extraction methods were handled in the same manner. Water soluble cell constituents were removed using the purification method of Sperry, (1955).

c. Lipid Distillation and Gravimetric Total Lipid Determination

After purification the remaining extract was distilled, to separate the lipid from the solvent, using the method of Sperry, 1955. A pale yellow greasy deposit remained in the bottom of the flask. After 16 hours drying in a desiccator the total lipid content of the sample was determined using a gravimetic scale.

d. Cholesterol Determination

The method of Quaife, Geyer and Bollinger, (1959), was used to determine the cholesterol content of both the whole cell and of the cell membrane fraction.

5. Carbohydrate Determination

Two classic methods of carbohydrate determination were used to ascertain the amount present in LM mouse fibroblast cells and in the envelope fraction of these cells. The anthrone method (Seibert and Affronti, 1963), which is relatively unspecific and which is harsh enough to break glycoprotein bonds in the membrane, was compared to the slightly less destructive thymol-sulphuric acid procedure (Shetlar and Masters, 1957).

The cell pellet or membrane fraction, after two saline washes, was solubilized in 5 ml of 0.1% Nonidet (37°C) in distilled water prior to the tests. The exact chemical nature of Nonidet was unknown due to the reluctance of the producer to divulge it, however, a control sample of Nonidet was included in each test and gave optical density readings only slightly higher than the blank sample.

C. ENZYMATIC STUDIES

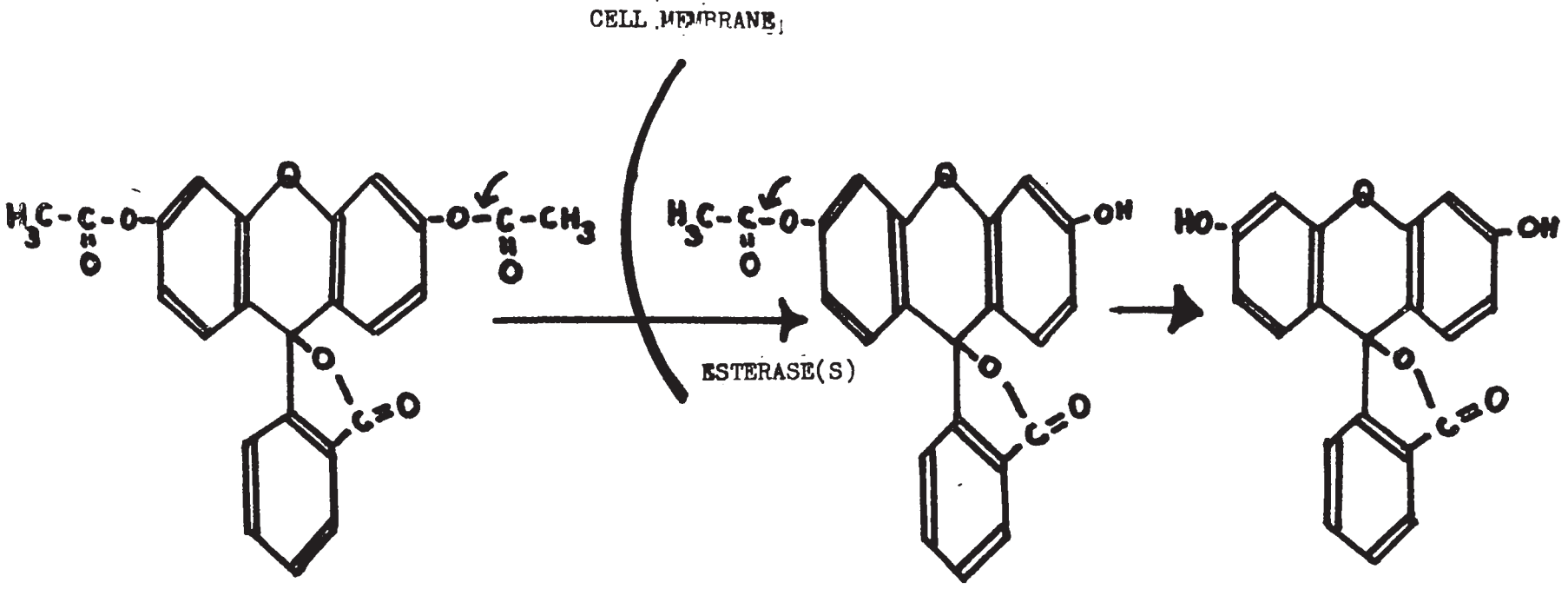
1. The System Used

a. The Substrate

Fluorescein diacetate, (FDA), the substrate used for these studies was obtained from Mann Research Laboratories, New York, U.S.A. It is a non-fluorescent acetylesterase substrate initially used with mouse fibroblast cells by

Figure 6

Fluorogenic Substrate Breakdown



Rotman and Papermaster, (1966). They found that within the cultured animal cells it was broken down to allow the intracellular release of the fluorescent product fluorescein, F (Figure 6). The FDA molecule is attacked by an esterase(s) (3.1.1.6), thought to be membrane bound, and an acetate moiety is released to give the intermediary product, fluorescein monoacetate (FMA). Continued action by the esterase(s) releases the other acetate moiety to give the fluorescent compound, fluorescein, (F). The rate at which the fluorescent compound, fluorescein, appears within the cell is a measure of the rate of breakdown of the substrate, fluorescein diacetate (FDA). Only living cells will utilize the substrate. Therefore, under ultra violet light the living cell fluoresces. The product fluorescein, is not able to pass easily through the membrane. The compound therefore accumulates in the cell.

Heat and light will cause the gradual breakdown of the substrate. A control sample was carried in all experiments to calculate the corrected rate of substrate breakdown by the cells.

b. The Fluorometric Method

A Turner Fluorometer, model 111, from G. K. Turner Associates, Palo Alto, California, was used to monitor the rate of breakdown of the substrate fluorescein diacetate (FDA).

The fluorometer is basically an optical bridge which shows the differences between light emitted by the sample and that from a calibrated rear light path. A single photomultiplier surrounded by a mechanical light interrupter sees light alternately from the sample and from a rear light path. The quantity of light required in the rear path to balance that from the sample is indicated by the fluorescence dial. If a compound is fluorescent any wavelength of light absorbed will cause it to fluoresce. The emitted light spectrum is normally quite broad and its shape and location quite independent of the light used to excite it. The only variation is one of intensity, that is, where less light is absorbed (less energy) less fluorescent light will be emitted. The correct choice of filters minimizes the fluorescence of blanks and other compounds present while retaining high sensitivity for the fluorescent compound being measured. The filters used were 47B and 2A giving excitation up to 436 nm in the primary filter slot, and 2A-12 giving emission over 510 nm in the secondary filter slot. For this substrate over 80% of the fluorescent light emitted was thus measured, this is considered to be a very high sensitivity for the Turner Fluorometer. Sensitivity of this machine allowed measurement of 1×10^{-8} Moles of fluorescein within the sample for each fluorometric unit.

The Turner Fluorometer was attached to a Servo Recorder, model EVW 20A (Heath Company, Benton Harbor,

Michigan). Such an attachment allowed for continual and accurate monitoring of the rate of production of fluorescence from the sample.

c. The Basic Test Method

Half an hour before the beginning of the test the Turner Fluorometer, the spectronic 20, and the Heath recorder were turned on. The fresh medium 199 (4.25 ml) was placed in the test tubes (Spectronic 20 tubes, Bauch and Lomb) held in a rack in the constantly stirred 37°C water-bath. The test tubes were always arranged in a certain order, at least two for each factor being tested. The ultra violet light was turned on in the fluorometer after checking that the correct filters were in place before the primary and secondary light aperatures. All tests were conducted with the sensitivity at 3X. The machine was checked to confirm that the fluorescence dial and the recorder readings coincided. The fluorometer was zero'd using the control tube of medium 199. The cell population, 250 λ of a cell suspension, OD 0.2 at 680 nm measured on the spectromic 20 (see Appendix for standard curve cell count vs. Optical Density), was added to the medium 199 in the test tube, (4.25 ml) at 37°C. Preincubation of the cells proceeded for 15 minutes prior to the test, to allow for equilibration.

At zero time 500 λ of the substrate of a predeterminated strength, was added to the test tube giving a total

of 5.0 ml in each tube (4.25 ml medium 199 + 0.25 ml cells + 0.50 ml substrate). At the same time a stopwatch was begun. The liquid was mixed using a Vortex mixer and then placed in the fluorometer. The recorder, on chart, with the roller moving at a known rate of speed (2 inches/minute) recorded the fluorescence of tube #1. While this reading was taking place, alloting a 30 second interval between substrate addition to the first and second tube, the substrate was being placed in the second tube and mixed. At 45 seconds from the start of the test, the first and second tubes were exchanged, the first tube being put into the rack in the constant temperature water bath in a designated position, and the second tube being placed in the fluorometer where its reading was recorded. This rhythm was maintained until a maximum of 12 tubes were being tested at any one time. By judicious handling of the tubes it was possible to read two tubes at 15 second intervals. Thus all tubes were reread at numerous times during a ten minute test run.

Constant Experimental Factors

The method used for the fluorometric cell studies requires rigid adherence to a number of conditions in order that experimental results may be meaningfully compared. In all cases the following requirements were met.

a. Cell Number. A standard curve showing cell number as measured on a Coulter counter, model A, serial 923,

Coulter Electronics, Chicago, Illinois, compared to the optical density of the cells suspended in medium 199 measured on the Spectronic 20, Bauch and Lomb, Rochester, New York, was made (Appendix). This curve was the result of four separate experiments using four completely independent cell populations. In all fluorometric experiments 250 λ of a cell suspension optical density 0.2 at 680 nm was used (1.1×10^5 mid log-phase LM mouse fibroblast cells/ml suspension). In a normal logarithmically growing LM cell population this number of cells was found to take about 10 minutes to metabolize enough fluorescein diacetate (FDA), at the standard concentrations used, to measure from zero to one hundred on the fluorescence dial of the Turner Fluorometer. Such a time interval allowed for a number of enzyme determinations to be compared at one time. The same mother population of cells was used in comparing the effect of various factors or substances on the cell's substrate breakdown rate.

b. Substrate concentration and preparation. A concentrated solution of fluorescein diacetate (FDA) was prepared using 100 mgm of the pure compound in 20 ml of acetone. This was kept at all times in the freezer compartment of a refrigerator (-20°C). From this concentrate the test substrate was made immediately prior to the fluorometric test. The suspending fluid for the test substrate was medium 199-peptone (Appendix). Various concentrations

of substrate were tested ranging from 2.4×10^{-6} M to 48.0×10^{-6} M of the compound. The concentration found to give the most accurate reproducible results and also allow ample time for manipulation of numerous test samples, was 12.0×10^{-6} M of fluorescein diacetate. This was made by adding 50 λ of the acetone concentrate to 10 mls of medium 199-peptone in a brown bottle.

c. Temperature, Light and Timing of Experiments. The temperature of the cell-substrate interaction was kept at 37°C. A constant temperature water bath kept the extracellular medium at this temperature.

It was found that the temperature within the Turner Fluorometer maintained itself at 37°C, after an initial warm up time of half an hour, consequently the enzymatic breakdown of the substrate was not exposed to wide temperature variations during fluorometric measurement. Addition of 500 λ of the cold test substrate caused an initial temperature drop of 0.2°C but the suspension returned to 37°C after less than a minute of incubation in the constant temperature water bath.

At all times the fluorometric test was carried out in a darkened room with the window blinds down and no fluorescent lights on overhead. A remote incandescent desk lamp was kept on to allow the experimenter to distinguish the sample tubes. The temperature controlled water bath containing the tubes was covered with a dark velvet cloth

in such a manner as to allow it to be easily moved to take out the tubes. The substrate brown bottle was covered in foil to prevent light deterioration and was kept on ice.

Addition of the substrate, capping with parafilm, mixing by Vortex mixer, and placing of the test tube in the Turner fluorometer for the initial recording of the fluorescence observed, was timed with a stopwatch. Manipulation time from the substrate addition to the reading was 5 seconds. All tests were accurately timed so that comparison of the enzymatic breakdown rate would be relevant.

Controls

The cells alone in 199 medium, as well as the substrate breakdown control tube were always included. Results presented were corrected taking both these possible sources of breakdown rate variation into consideration. In all experiments appropriate controls for all other variant factors were included.

d. Chromatographic Analysis

The thin layer chromatography of the substrate, fluorescein diacetate (FDA); the product, fluorescein (F), and any of the various chemicals tested alone, and mixed with FDA; was done using the method modified from Rotman and Papermaster, (1966). Silica gel G glass fiber paper (Gelman ITLC) was wrapped in foil and activated by heating

at (90°C) in a hot air oven for 20 minutes, spotted with the test solution (10λ) and dried. The running solvent for the chromatogram was 2% chloroform in methanol. The separation was carried out for half an hour in a Gelman TLC chamber. After air drying for 5 minutes the fluorescent compounds were visualized by spraying with 1 M alcoholic potassium hydroxide. The chromatogram was viewed under long wave UV using a chromato-vue (San Gabriel, California, U.S.A.) cabinet. In this system FDA has an rf. of 0.9, and fluorescein remains at the origin. In later tests analysing cell and supernatant contents at various times, the intermediary breakdown product of FDA, fluorescein monoacetate (FMA) (rf. approximately 0.6) was observed on the chromatograms). In the initial chromatograms analysing the possible interaction between various chemicals and the substrate, this intermediary product rarely, if ever, appeared.

2. Exit - Entry Experiments

a. The Method (Figure 7)

This method is designed to combine fluorometric and chromatographic techniques in order to analyse quantitatively the metabolic breakdown of the substrate, fluorescein diacetate, by the cell. The cellular reaction with the substrate was stopped at a predetermined time by separating the cells from the extracellular bathing medium by

FIGURE 7

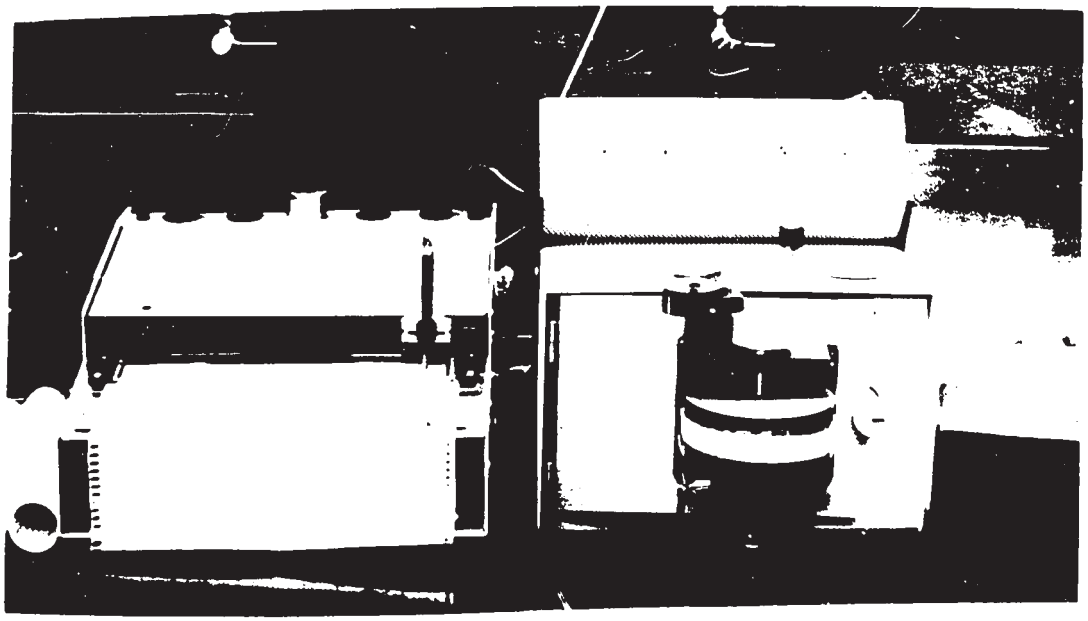
MACHINERY AND METHOD USED IN

EXIT-ENTRY EXPERIMENTS

a. Turner Fluorometer, model 111, showing chromatogram strip taped in place on the uncovered drum. Servo Recorder is attached to record fluorometric readings on attached recording paper.

b. Typical plot obtained from chromatogram strip (at left). Positioning of Gelman Planimeter to measure area under the curve for the compound Fluorescein.

Figure 7



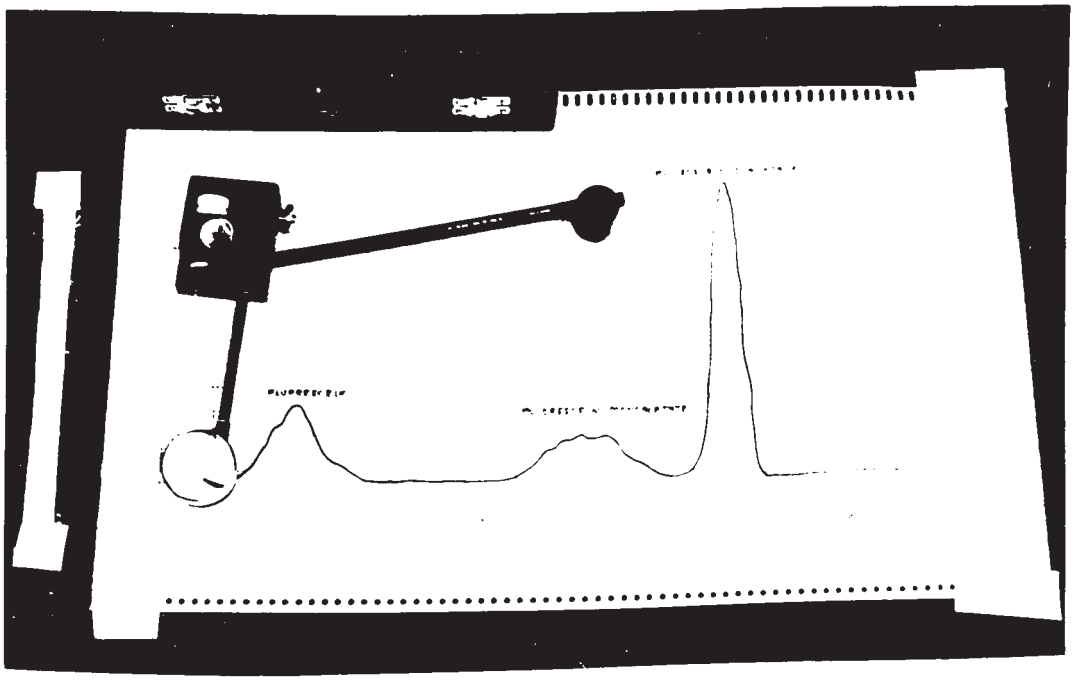
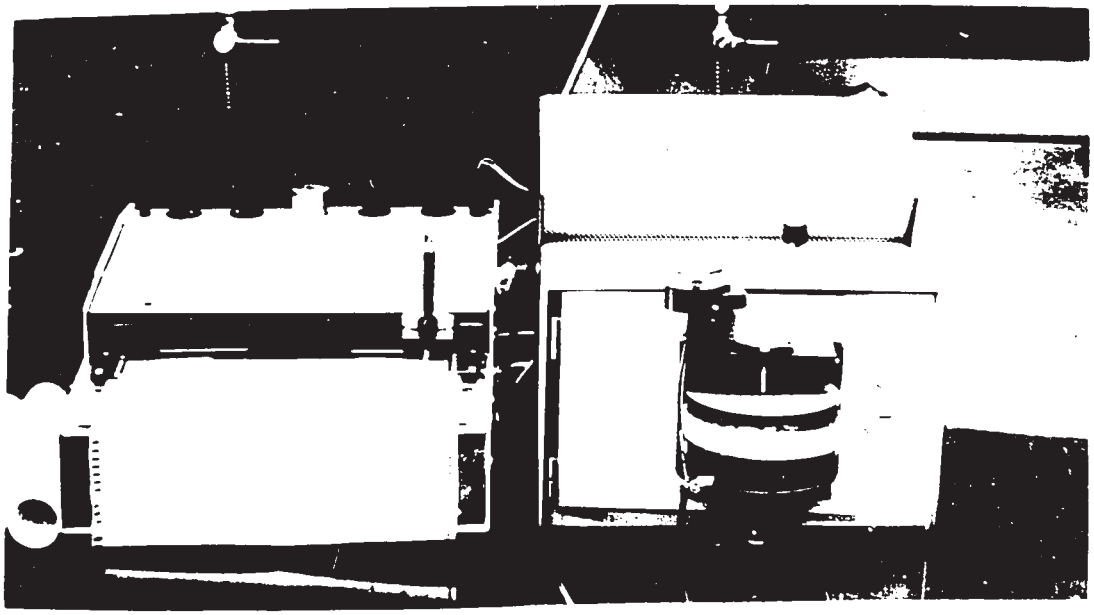
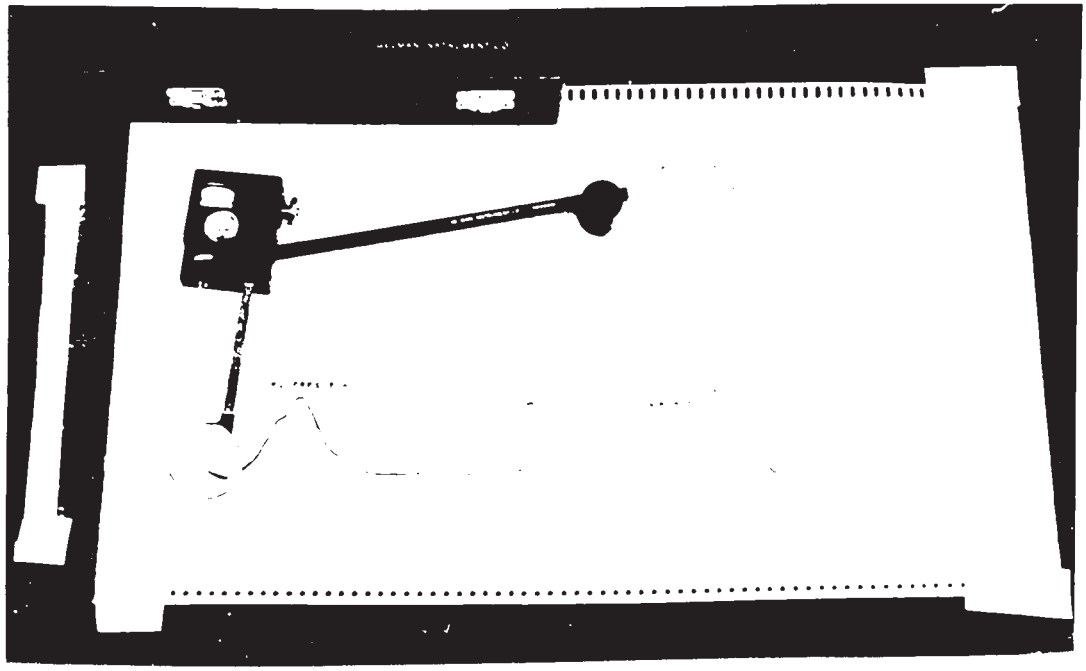


Figure 7





FLOW SHEET 1

CELLS (O.D. 680 nm, 0.2 , in 199)

250 λ

1. Medium L99 plus FACTOR IF APPLICABLE , 4.25 ml

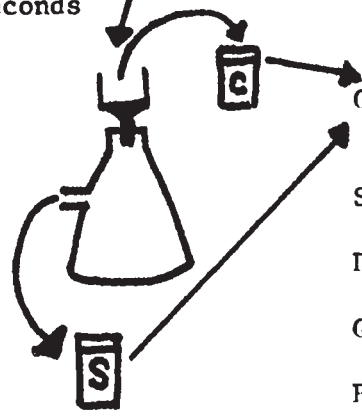
2. PREINCUBATION ZERO TIME

15 minutes — 37°

3. ZERO TEST TIME ← 500λ of FDA (12.0×10^{-6} M in 199)

X — 30 seconds — FLUOROMETRIC READING

X — 5 seconds



CHROMATOGRAM

STRIPS

DRUM FLUOROMETRY

GRAPH READING

PLANIMETER READING

CALCULATIONS

filtration. The cells adhering to the filter were washed with 5 ml of fresh medium 199, then immediately solubilized in 1 ml of methanol in a screwcapped bijoux bottle and placed in the dark in the refrigerator. The suspending fluid was also immediately removed from the flask, placed in a screwcapped universal in the dark in the refrigerator. These fluids were subsequently analysed chromatographically. Using drum-chromatography quantitative results were obtained for the compounds present in both the cells and supernatant. All the requirements for a normal fluorometric test were met except that instead of allowing each tube to go ten minutes, tubes were removed at a predetermined time and filtered to separate the cells and the supernatant. To insure that no unknown rythmical handling by the investigator was responsible for the results obtained the regime of timing the test samples for each population was randomized. As Flow Sheet 1 shows, preincubation of 15 minutes in the medium at 37°C before addition of the substrate was followed for each sample.

After a test sample of cells and substrate had been allowed to interact for the predetermined time minus thirty seconds, a fluorometric reading was taken. Five seconds before the test time had elapsed the vacuum was turned on and the sample was slowly poured over the filter of the millipore apparatus (0.45 u). The vacuum was turned off after no more liquid was present on the filter. The

supernatant fluid was poured off through the vacuum spout and placed in a screwcapped universal. The vacuum was re-applied and a chaser of 5 ml fresh 199 medium poured over the filter to rinse off any adhering supernatant. When the filter was once more dry the vacuum was turned off, and the filter was removed with forceps, placed in a bijoux containing 1 ml of methanol. The filter was submerged in the liquid to immediately solubilize the cells, then the bijoux cap was replaced and the bottle placed in a blackened container surrounded by ice. The universal containing the supernatant fluid was also placed here. Manipulation time from the pouring of the liquid till the placing of the bijoux bottle in the iced and blackened container, with practice, was kept to 30 seconds.

After all cell samples and supernatant samples had been collected, they were tested chromatographically using the method modified from Rotman and Papermaster, (1966). This method is given on page 58. 5 λ of cell extract in methanol and 10 λ of extracellular media were spotted for chromatography.

The method used to analyse quantitatively the compounds present in the cells and supernatant required the use of a drum attachment for the fluorometer (Figure 7). The chromatograms were cut into one inch strips, wrapped in a transparent, non-fluorescent plastic wrap, and taped with masking tape to the drum so that the complete width

of the strip would pass the slit opening which is exposed to the light beam from the fluorometer. The strip was set so that the start of the paper was prior to the origin spot. The metal drum covering was placed in position, and the motor gears were cranked down to mesh the teeth. The motor, plugged into the sample outlet, turned the drum at a constant rate, so that the speed (4 inches/minute) was half that of the recorder. The zero setting of the recorder was set at 20 units to allow for any adsorption dips which might appear in the tracing. The drum motor and the recorder were turned on at precisely the same moment and a tracing of fluorescent peaks at the appropriate positions for fluorescein (F), fluorescein monoacetate (FMA), and fluorescein diacetate (FDA) was drawn (Figure 7). With a Gelman Planimeter the area under the curves was calculated (Stahl and Jork, 1968; Mefferd et al., 1968). The base line was drawn using the product of the tracings of control curves always done on chromatograms, in every test, for the medium 199, methanol, solubilized cells not tested fluorometrically, and the paper. This product of these control curves normally hovered around the zero line (20 fluorometric units). Standard curves were constructed for both fluorescein and fluorescein diacetate and the amount of each compound in the test samples was calculated with reference to them. For each compound graphs were then drawn, each timed test giving one point, and the metabolic activity of

the cells analysed. Calculations were done to total the compounds present--both cellular and extracellular. The method of calculation is given in the Appendix.

D. IMMUNOLOGICAL STUDIES

1. Preparation of the Immune Rabbit Sera

Five female rabbits randomly bred were obtained from a local supplier. They were housed in wire mesh cages and were fed rabbit chow and watered regularly. Prior to injection of the rabbits with LM cells 25 ml of blood was obtained from each rabbit. The sera obtained from this blood was designated "preimmune rabbit serum". The immunization program took place over four weeks. A suspension of living cells (1.27×10^7 cells/ml) in saline, 0.25 ml, were injected subcutaneously on day 0, 2, 4, 7, 9, 11, 15 and 17. First bleeding was taken on day 19, two days after last injection.

Serum was produced by first incubating the blood sample for approximately half an hour at 37°C. The clot was rimmed from the sides of the tube with a sterile inoculation loop, and the sterile screwcapped tube placed in the refrigerator overnight. The serum was poured off, care being taken not to include any blood cells, passed through a sterile millipore filter (pore size, 0.20 microns) to ensure sterility and placed into sterile universal

bottles. Sera were stored in the deep freeze (-100°C) for later use.

Presence or absence of humoral antibodies in these sera was determined by means of a ring test (Oudin, 1948). A few drops of the serum to be tested was placed in Durham tubes, tiny fermentation tubes, and on top of this a fine sonicate, (15 minutes, 30 second bursts), of LM cells in distilled water was layered. The tubes were left undisturbed at room temperature for an hour after which time the appearance of a fine visible precipitate at the interface between the two layers confirmed the presence of antibody (Figure 8).

The slide agglutination test was also used to confirm the presence or absence of humoral antibodies in these sera. With a glass pencil a ring was drawn in the center of a clean dry glass slide. Within this ring a drop of LM cell suspension in medium 199 was placed (5.5×10^3 cells, 5 λ of a suspension OD. 0.2 at 680 nM). To this a drop of (5 λ) serum being tested was added. The slide was rocked gently back and forth to insure good mixing. Visual clumping of the cells, checked under low power white light microscopy, indicated the presence of antibodies to LM cells in the serum being tested. If the cells did not clump together but stayed separated, the same as a control drop of cells in saline or with preimmune serum, then the serum was considered negative (Figure 8).

FIGURE 8

IMMUNOLOGICAL TESTS CARRIED OUT ON IMMUNE AND PREIMMUNE RABBIT SERA

a. Ring Test. Preimmune serum test, "0", no ring at junction of serum and sonicated LM mouse cell suspension. Negative ring test.

Anti-LM rabbit serum test, "1", shows a strong positive ring test reaction. A visible precipitate appears at the junction of the serum and cell sonicate.

Anti-LM rabbit serum test, "2", shows a weak positive ring test reaction. A slight precipitate appears at the junction of the serum and cell sonicate.

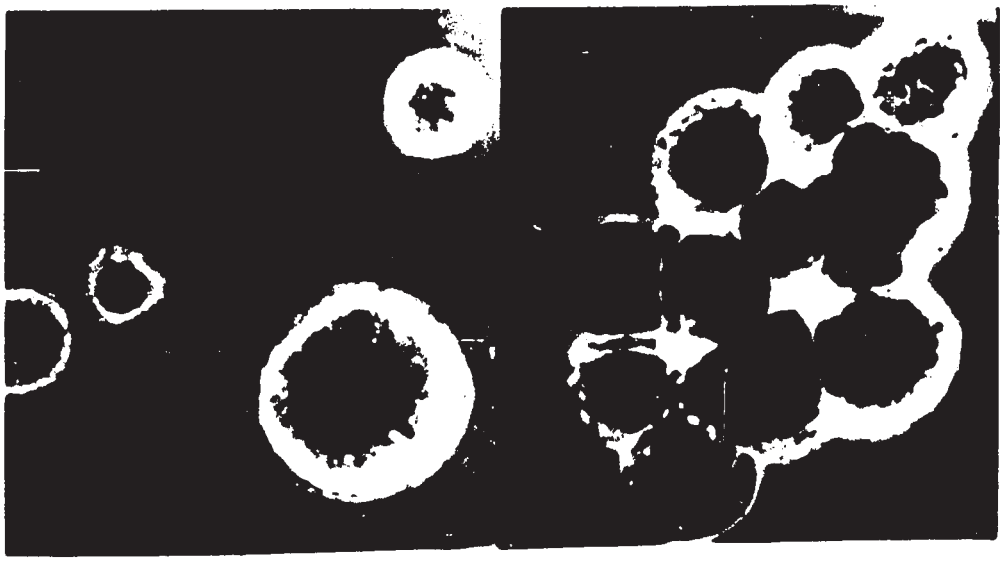
b. Negative agglutination test - Cells remain separated though preimmune serum present (1000 X).

c. Positive agglutination test - Cells clump together in the presence of anti-LM serum (1000 X).

Figure 8

a.

b.



c.

Figure 8

a.

b.

c.

2. Animal Source of Serum

Sera from calves, horses, guinea pigs and rabbits were also tested both cytologically (.01% in medium 199) and fluorometrically (.10% → .0001% in medium 199). Normal serum containing complement (C^1), normal inactivated serum (56°C for 30 minutes) were used. The calf serum and horse serum were obtained from Grand Island Biological Company, New York, U.S.A. Guinea pig and rabbit sera were obtained from our own animals, those without any history of previous inoculations or bleedings. The anti LM rabbit serum was purified, (Campbell et al., 1964), using first ammonium sulphate precipitation (3 times), then dialysis against cold borate-buffered saline for 3 days to remove salts. The final solution was slightly opalescent. This method is sufficient to separate gamma globulin in fairly pure form.

IV. RESULTS

A. EFFECTS OF BUFFERS, CHEMICAL SOLUTIONS, INHIBITORS AND ENZYMES ON CELL CYTOLOGY

In order to distinguish between visual effects caused by substances used to hold or prepare a cell population for investigation, and the effects caused by an experiment itself, it was necessary to test these various substances by incubation with the cell population. The cytological effects observed on the membrane and cell contents of the LM mouse fibroblast cell are discussed in Table Ia, b, c, d, and shown in Figures 9 and 10.

In all subsequent experiments the normal incubating medium 199-peptone was used as the holding fluid for the cells.

More extensive tests were conducted with the enzyme neuraminidase (Table II). The enzyme preparation used (cholera filtrate), did not contain only neuraminidase but was contaminated with proteases. The results obtained from these experiments cannot therefore be attributed to

TABLE I

CYTOLOGICAL EFFECT OF BUFFERS AND COMPOUNDS

ON THE LM MOUSE FIBROBLAST CELL

A. BUFFERS

Substance	Concentration	Cytological Effect on LM Mouse Fibroblast Cell*				Type of Membrane and Cell Effect
		5 mins	15 mins	30 mins	60 mins	
Medium 199	Normal pH 7.2	-	-	-	-	Normal cell in every way
A. Buffers						
Dulbecco	Normal pH 7.2	-	-	-	-	More vaculation than normal cells, membrane normal.
Physiological saline 22 milliosmoles/liter	0.8% pH 7.2	+	+	++	++	Cell swollen, nucleus defined and swollen, cytoplasm vaculated, membrane taut.
Sucrose Solution 260 milliosmoles/liter	0.25 M pH 7.2	+	-	-	-	More vaculation than normal cells. Membrane normal.

* -, no effect on cell
+, slight damage to cell
++, moderate damage to cell.

TABLE I (Continued)

Substance	Concentration	Cytological Effect on LM Mouse Fibroblast Cell*				Type of Membrane and Cell Effect
		5 mins	15 mins	30 mins	60 mins	
Tris	0.2 M pH 7.2	++	+++	++++	++++	Membrane ruffled breaks easily, nuclei swollen, cytoplasm normal

B. IONS

Substance in Medium 199	Concentration Molar	Cytological Effect on LM Mouse Fibroblast Cell*				Type of Membrane and Cell Effect
		5 mins	15 mins	30 mins	60 mins	
Magnesium chloride (Normal level .200 gms liter)	10^{-2}	+++	+++	+++	+++	Blebbing of membrane in higher concentrations. Many microvilli present, nucleus more defined. Cytoplasm appears normal
	10^{-3}	++	++	++	+++	
	10^{-4}	+	+	++	++	
	10^{-5}	-	+	+	+	
	10^{-6}	-	-	-	-	

* - No effect
+ Slight change in cell
++ Moderate damage to cell
+++ Extensive damage to cell
++++ Cell destroyed

TABLE I (Continued)

Substance in Medium 199	Concentration Molar	Cytological Effect on LM Mouse Fibroblast Cell*				Type of Membrane and Cell Effect
		5 mins	15 mins	30 mins	60 mins	
Calcium chloride (Normal level .140 gm/liter)	1	+++	+++	+++	+++	Cells shrink and clump at high concentrations. Vaculation at lower concentrations, some microvilli, nucleus defined and non refractile
	10^{-1}	+++	+++	+++	+++	
	10^{-2}	++	+++	+++	+++	
	10^{-3}	+	++	++	+++	
	10^{-4}	-	+	++	++	
	10^{-5}	-	-	+	+	
	10^{-6}	-	-	-	-	
Chemical solutions potassium chloride (normal level 0.400 gms./ liter	10^{-1}	++	++	+++	+++	Cells become elongated; blebbing of membranes of some cells. Extensive microvilli formation.
	10^{-2}	+	++	++	++	
	10^{-3}	-	+	++	++	
	10^{-4}	-	-	+	+	
	10^{-5}	-	-	-	-	
Sodium fluoride	3×10^{-4}	+	+	+	+	Wavey membrane, nucleus and cytoplasm normal

* - No effect
+ Slight change in cell
++ Moderate damage to cell
+++ Extensive damage to cell

TABLE I (Continued)

Substance in Medium 199	Concentration Molar	Cytological Effect on LM Mouse Fibroblast Cell*				Type of Membrane and Cell Effect
		5 mins	15 mins	30 mins	60 mins	
EDTA	Selective chelation 5×10^{-4}	+	++	++	++	Extensive microvilli production. Nucleus slightly swollen.
	Total chelation 3.7×10^{-2}	++++	++++	++++	++++	

C. SUGARS AND VITAMINS

Glucose in	10X normal 199 conc.	+	+	+	+	Cell membranes blebbed; microvilli present. Cytoplasm agitated. Nuclei appear normal.
	100X normal 199 conc.	++	+++	++++	++++	
Ficoll	15%	-	-	-	-	In 40% Ficoll cell membrane has pollen grain appearance gradually blebs and bursts. In 20% after 60 minutes slight wavey membrane. Some vaculation.
	20%	-	-	-	+	
	40%	+	++	+++	++++	

* - No effect
\+ Slight damage to cell
++ Moderate damage to cell
+++ Extensive damage to cell
++++ Cell destroyed

TABLE I (Continued)

Substance in Medium 199	Concentration	Cytological Effect on LM Mouse Fibroblast Cell*				Type of Membrane and Cell Effect
		5 mins	15 mins	30 mins	60 mins	
Vitamin A	10X normal 199 conc.	+	+	+	+	Cell membrane looks like rigid parchment. Cell appears set or frozen into place
	100X normal 199 conc.	+	+	+	+	
Vitamin C	10X normal	-	-	-	-	Microvilli extensive at higher concentrations of vitamin C.
	100X normal 199 conc.	+	+	+	+	
Vitamin E	1×10^{-4} M	++	+++	+++	+++	Membrane appears as mosaic. Some cells shrunken. All cells granular.
	1×10^{-5} M	++	++	++	++	

* - No damage
\+ Slight damage
++ Medium damage
+++ Extensive damage
++++ Cell destroyed

TABLE I (Continued)

D. INHIBITORS AND ENZYMES

Substance in Medium 199	Concentration	Cytological Effect on LM Mouse Fibroblast Cells*				Type of Membrane and Cell Effect
		5 mins	15 mins	30 mins	60 mins	
Inhibitors and Enzymes paraoxon	1×10^{-3} M	-	-	-	-	Cell looks normal. Around 15 minutes appearance of mitochondrial clumps which disappeared by 40 minutes. Other-wise cell normal throughout
Eserine	1×10^{-3} M	-	+	+++	++++	Membrane first blebs, then ballooned, by 60 minutes most cells have crumbled membranes
Lipase	0.05%	-	-	++	+++	Cells appear normal for first 20 minutes then clump, membranes appear full of holes, cytoplasm swells, nucleus normal.

* - No Damage
\+ Slight Damage
++ Medium damage
+++ Extensive damage
++++ Cell destroyed

TABLE I (Continued)

Substance in Medium 199	Concentration	Cytological Effect on LM Mouse Fibroblast Cells*				Type of Membrane and Cell Effect
		5 mins	15 mins	30 mins	60 mins	
Collaganase	0.1%	+	+++	+++	++++	Microvilli extensive, cells gradually break up.
Neuramini-dase	50 units/ml	-	-	+	+	At 50 units/ml after 30 minutes cells became slightly swollen, membrane appears normal. At 300 units/ml membrane blebs swollen after 5 minutes.
	300 units/ml	+	+	+	++	
Solutions Nonidet	1×10^{-4} %	++++	++++	++++	++++	Even at highest dilution Nonidet still can damage membrane, nuclei swell, cytoplasm becomes vaculated and agitated.
	1×10^{-5} %	++	+++	+++	++++	
	1×10^{-6} %	+	++	++	+++	

* - No Damage
\+ Slight damage
++ Medium damage
+++ Extensive damage
++++ Cell destroyed

TABLE II

EFFECT OF NEURAMINIDASE INCUBATION ON CELLULAR PROPERTIES

MINS INCUBATION	NORMAL CONTROL POPULATION			NEURAMINIDASE TREATED (50 units/ml)		
	% Viable	Fluorescein Exclusion	Stickiness	% Viable	Fluorescein Exclusion	Stickiness
0	96%	+	100%	95%	+	100%
5	95%	+	100%	94%	+	100%
10	96%	+	100%	94%	+	100%
15	94%	+	100%	96%	+	100%
20	95%	+	100%	96%	+	100%
25	96%	+	100%	94%	+	100%
30	97%	+	100%	95%	+	100%
35	96%	+	100%	95%	+	100%
40	95%	+	100%	96%	88% + 12% -	100%
45	94%	+	100%	96%	86% + 14% -	90%
50	95%	+	100%	95%	75% + 25% -	75%
55	95%	+	100%	95%	60% + 40% -	44%

TABLE II (Continued)

MINS INCUBATION	NORMAL CONTROL POPULATION			NEURAMINIDASE TREATED (50 units/ml)		
	% Viable	Fluorescein Exclusion	Stickiness	% Viable	Fluorescein Exclusion	Stickiness
60	96%	+	100%	95%	37% + 63% -	35%
65	94%	+	100%	94%	10% + 90% -	20%
70	95%	+	100%	96%	-	-
80	95%	+	100%	95%	-	-
90	95%	+	100%	96%	-	-

this enzyme alone but to a mixture in which neuraminidase was the predominant material present.

Neuraminidase, at the lowest concentration, 50 units/ml, after 50 minutes eliminated the cell's ability to attach itself to a glass surface. Otherwise only a very slight swelling of the cell was noted. The cell's stickiness was tested by placing a drop of the test cell suspension on a clean, dry, coverslip allowing it to stand for 20 minutes. Then while holding the coverslip on its edge gently allowing a stream of fresh medium 199 from a Pasteur pipette to pass over the coverslip washing off any cells which had not as yet adhered to the glass. A few moments were allowed for draining. The coverslip was then viewed microscopically, and twenty fields were counted to ascertain the number of cells which adhered to the glass.

When a neuraminidase concentration of 300 units per ml was used, however, the membrane became blebbed and all the cells were markedly swollen after 5 minutes. Table II shows the effect on cells of incubation in medium 199-peptone containing 50 units/ml of neuraminidase. When viability tests were conducted using both erythrosin B and fluorescein diacetate no increase in the presence of non-viable cells was noted over the whole observation period of 90 minutes. By 15 minutes, however, a slight swollen

condition was evident in most of the cells. Blebbing of the membranes of a few cells began by 45 minutes and by 90 minutes about 15% of the total viable cells counted exhibited this abnormal condition. Fluorescein itself is normally excluded by the cellular membrane and the cells incubated in the 199 medium with the neuraminidase also excluded it until around 40 minutes (Table II) after the start of incubation. At this time a very faint glow appeared in about 12% of the cells indicating that the fluorescein had passed the membrane barrier. By 70 minutes after the start of incubation all the cells allowed its entry and a few "hot spots" of fluorescein accumulation were evident in most cells. The stickiness of the cells to the glass substrate remained normal for the first 45 minutes then the percentage adhering to the glass fell quickly so that by 70 minutes no cellular sticking was observed. The correlation of loss of cell stickiness and loss of cellular ability to exclude fluorescein is noted but not understood. When the cells were removed from incubation with neuraminidase and placed in fresh medium 199 after 3 hours, 50% of the cells when compared to a control population, were able to adhere to the glass once more. After 12 hours both the viable count and the ability to adhere to the glass substrate had returned to normal.

FIGURE 9

CELLULAR EFFECTS OF VARIOUS COMPOUNDS AND BUFFERS ON THE LM CELLS

All cells are shown at a magnification of 2500X and after 60 minutes of incubation, viewed under high dry and phase contrast.

a. Control LM mouse fibroblast cell incubated in medium 199.

b. Cells incubated in Dulbecco phosphate buffer. Note increased vaculation and the partial definition of the nucleus.

c. Cell incubated in 0.25 M sucrose solution. Note increased vaculation and granulation. The cell is slightly swollen.

d. Cell incubated in 0.8% saline (NaCl). Note swollen appearance of cell and the nonrefractile nature of the nucleus. Brittle membrane protrusions are abundant.

Figure 9

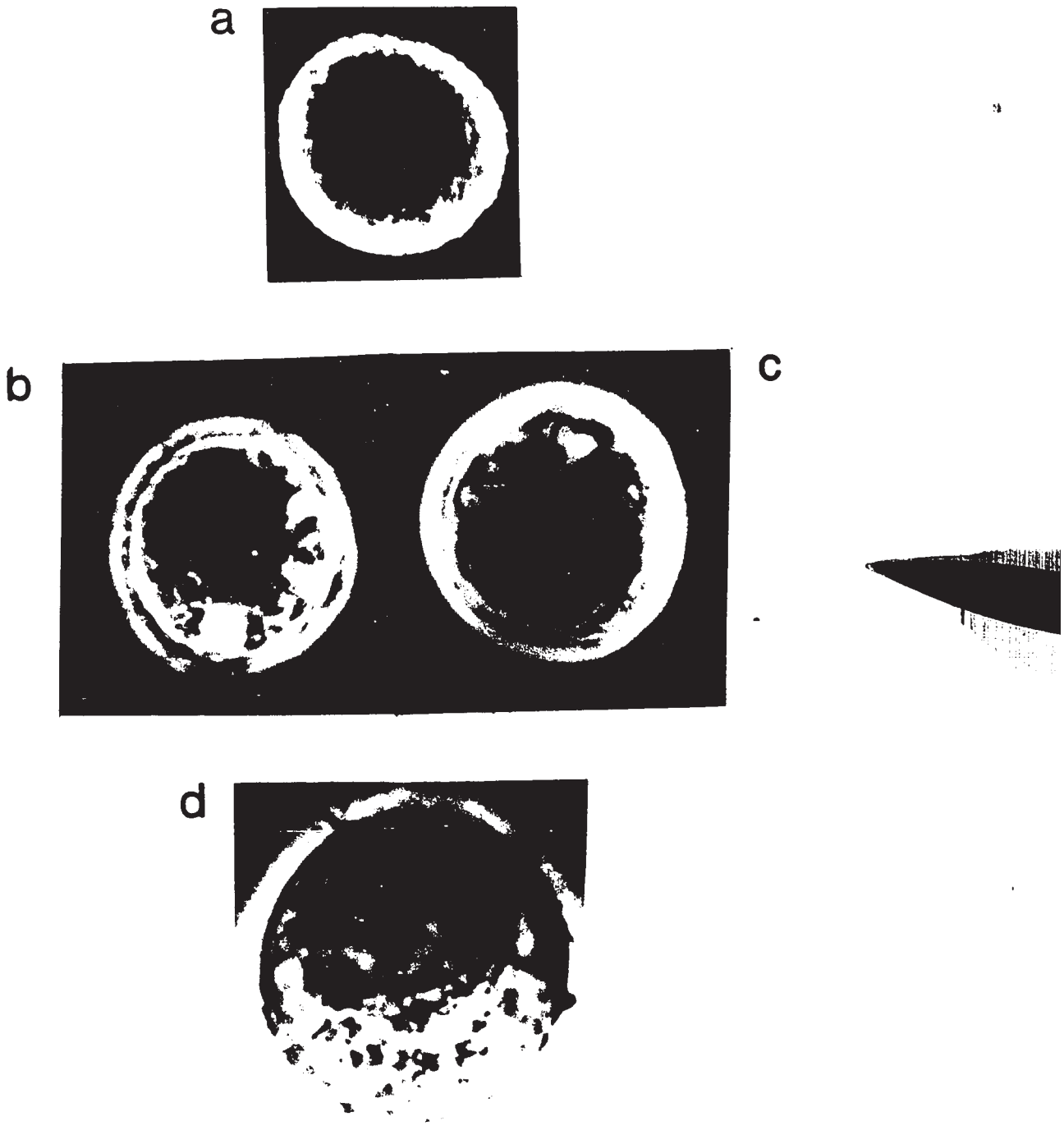


Figure 9

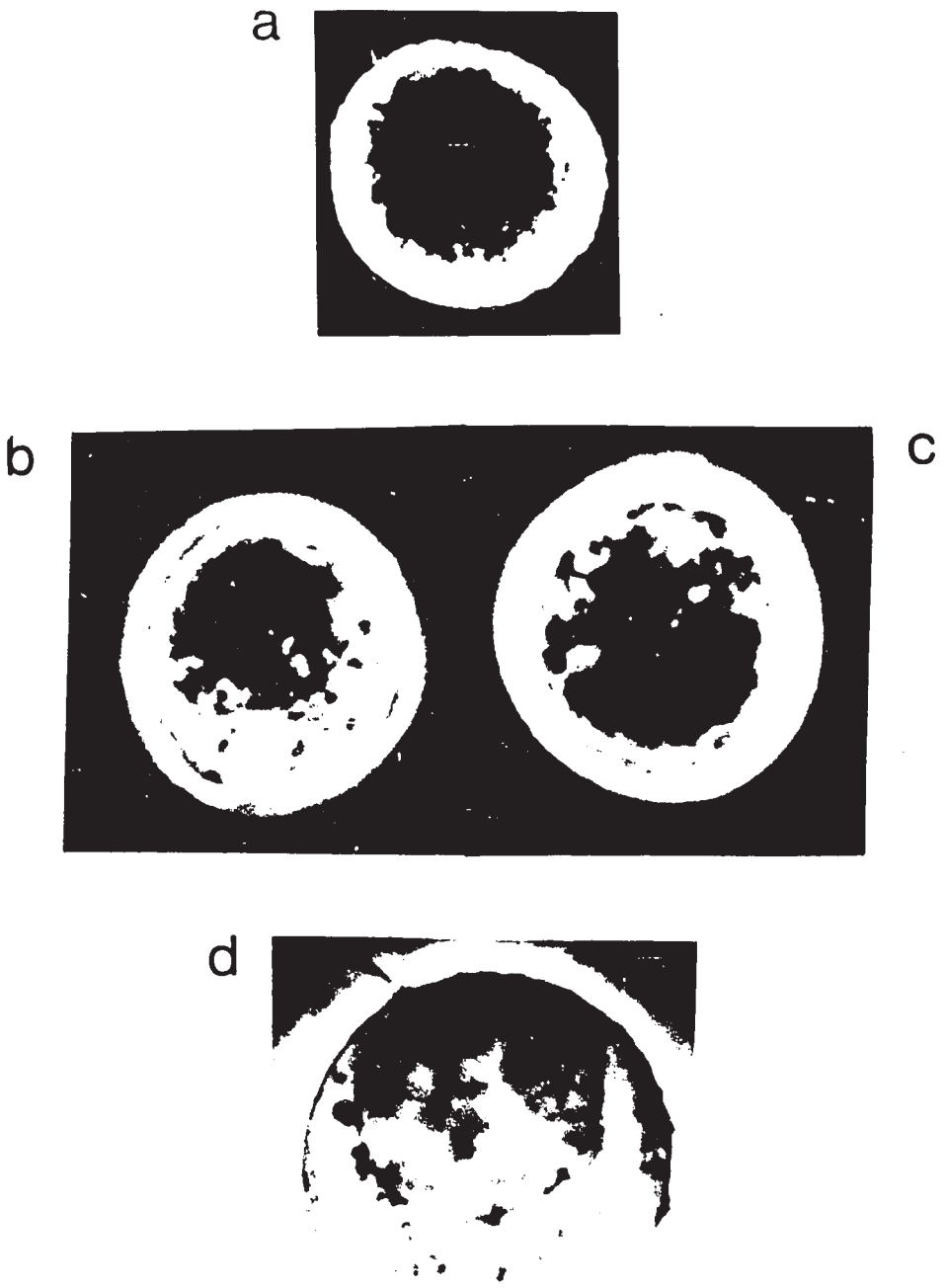


FIGURE 10

OBSERVED ABNORMAL CELL CYTOLOGY OF LM CELLS

All cells are shown at a magnification of 2500X.

a. Elongated misshapened cell. The nucleus is non-refractile and clumps of organelles can be seen in the cytoplasm.

b. Prominent non-refractile nuclei are shown in these cells.

c. A slightly swollen cell with numerous rigid microvilli protruding from the membrane is shown here.

d. Cells with blebbed membranes--that is slight ballooning in various regions of the membrane, are pictured here.

Figure 10

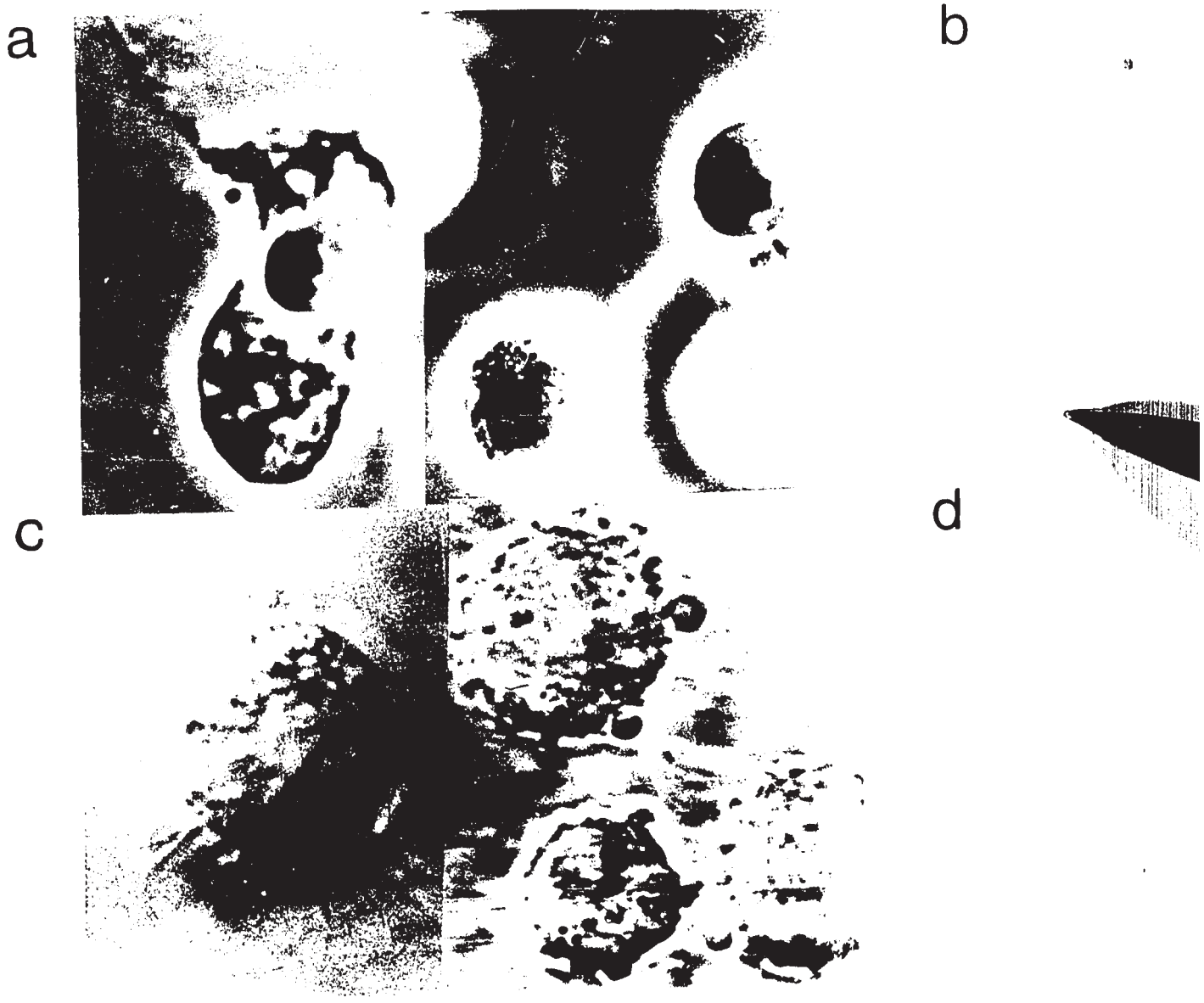
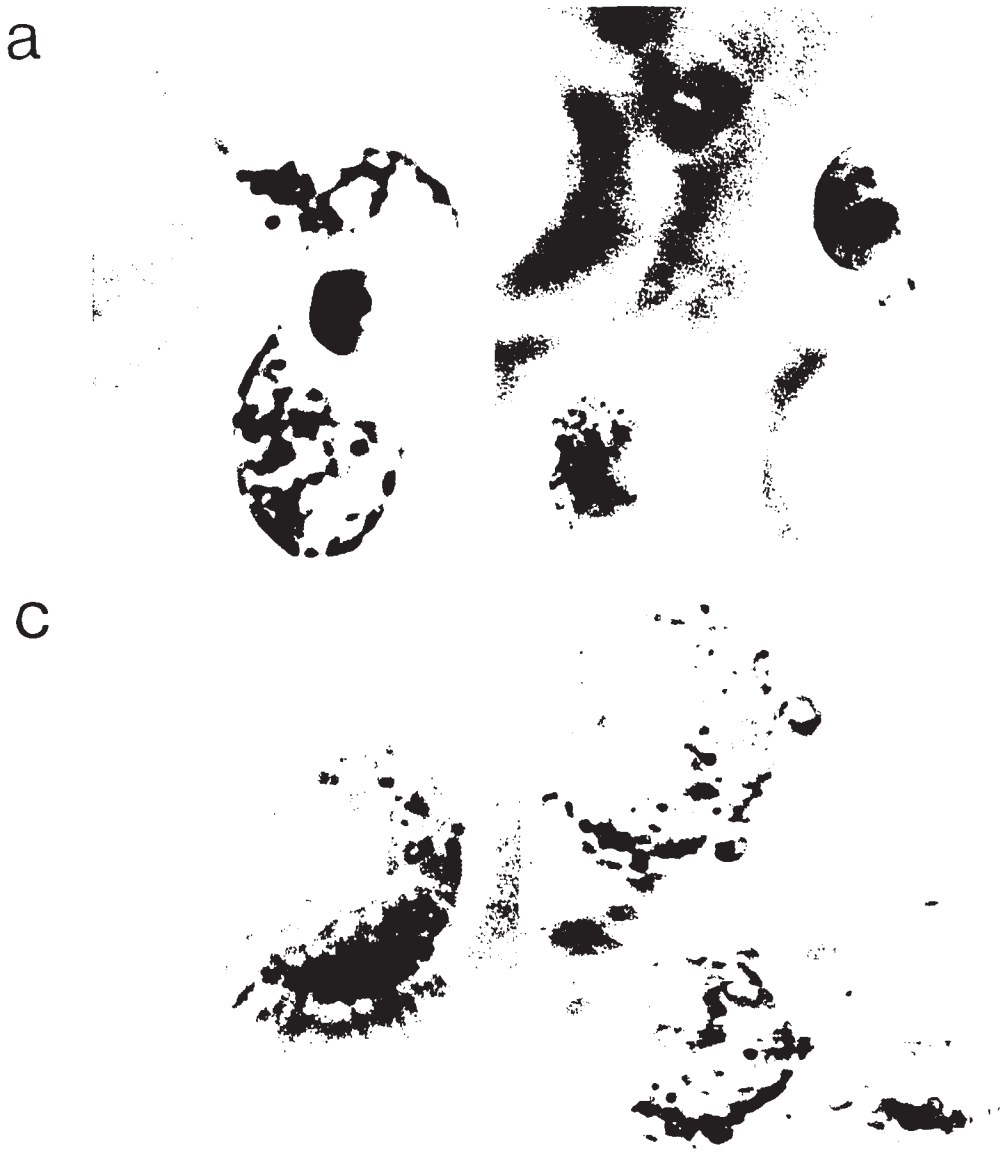


Figure 10



It was found that Nonidet, a nonionic detergent, even in dilute solutions, had the ability to damage and eventually destroy the cell. The cell membrane appeared to gradually dissolve, presumably destroyed by the detergent action. Within the cell, nucleus and cytoplasm are also affected suggesting that the detergent may flow into the cell as well, inflicting irreparable damage on all the metabolic systems.

Because of the observed cytological effects noted with these various materials and solutions, in later experiments analysing the chemical, enzymatic, and immunological properties of the LM mouse fibroblast cells, strict attention was paid to the holding and experimental milieus. The normal growth medium 199 peptone (Appendix) was used at all times to insure that the effects observed were due solely to the factor under consideration.

B. EFFECTIVENESS OF PROCEDURES USED TO SEPARATE CELL FRACTIONS AND PREPARE MEMBRANES

One of the most likely sources of cell membrane variation throughout the cell's life cycle is in the chemical composition of the membrane. Consequently experiments were undertaken to analyse both the whole cell and the cell membrane at various stages in the cell cycle. Such analysis presupposes a method to obtain large

quantities of cells in the same phase of the cycle. To obtain such populations two basic approaches were tried: cold shock and mechanical separation. The final method of choice was Ficoll gradient cell population separation.

1. Ficoll Gradient Cell Population Separation

Upon completion of the centrifugation of a logarithmically growing cell population on a continuous Ficoll gradient (15% - 22%), a spread of cells from just below the miniscus, at approximately 16% Ficoll, to about 20% Ficoll was noted. Figure 11 shows the three more opaque band regions at 17.2% (rf. 1.3583), 18.4% (rf. 1.3600) and 20.4% (rf. 1.3630). Fraction 1, from the upper band always contained only small single cells (diameter approximately 8 u). Fraction 2 was a mixture of medium sized cells (12.6 u in diameter) and some doublets, those cells in the last stages of mitosis or having just finished but not yet divided. Fraction 3 was composed of large cells (16.5 u to 20 u in diameter) and most of the doublets in the population. Cell counts of all fractions and the mother population showed that in each case the percentage of the total cells in the population stayed fairly constant. In fraction 1, approximately 14% of the total cell population was obtained. In fraction 2, 52%, and fraction 3, 34% of the total cell population was found. Knowledge of the cell cycle and the volume changes throughout it led to the conclusion that

FIGURE 11

FICOLL GRADIENT OF NORMAL LOGARITHMIC CELL POPULATION

Cells magnified 1200X. Three bands and the types of cells found in these bands are shown.

Fraction #1 - 17.2% Ficoll single small cells (8 u diameter).

Fraction #2 - 18.4% Ficoll, mixture of medium single (12.6 u diameter) and double cells.

Fraction #3 - 20.4% Ficoll, mixture of large single cells (16.5 u to 20 u diameter) and double cells.

Figure 11

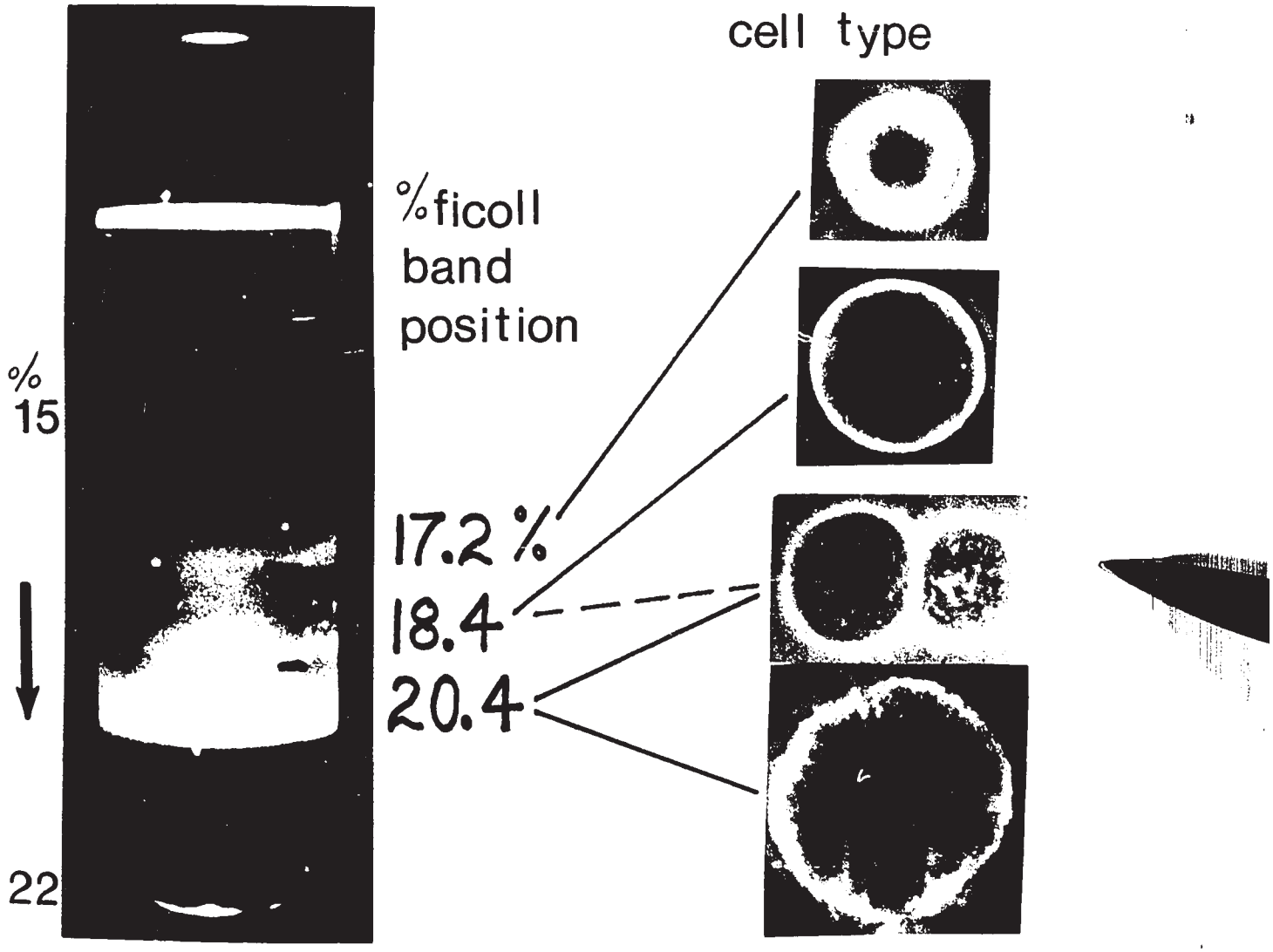
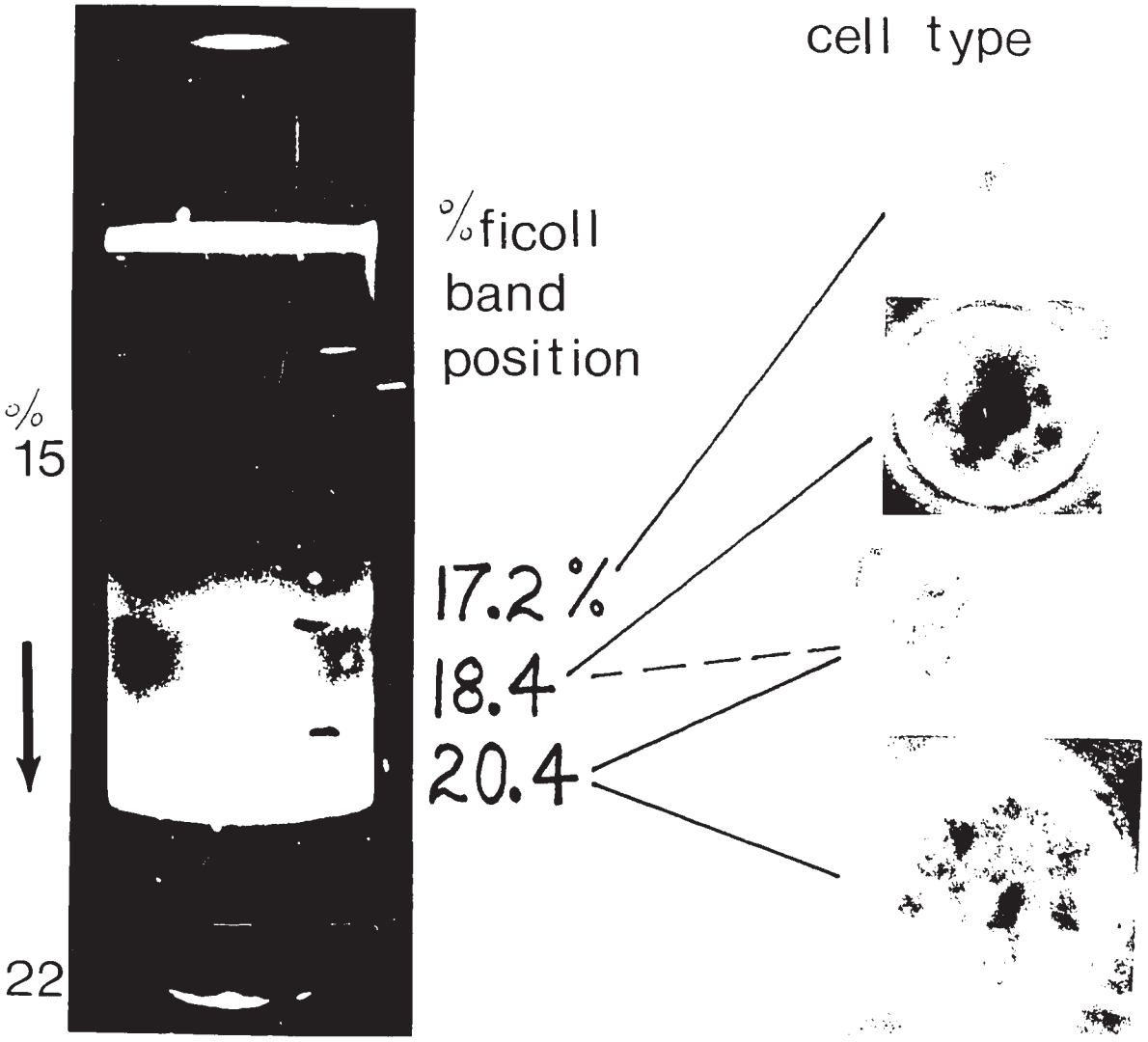


Figure 11



fraction 1 was mainly composed of cells in G1, fraction 2 of those cells in S and G2 with some in late M (Telophase), while fraction 3 contained mainly G2 and M cell with some in S.

Cells from all three fractions were tested for viability using both the double Dye Test (page 31), to test initial viability, and also growth in suspension and monolayer culture. Table XIV, page 110 shows variation in growth rate (doubling time) for the three cell fractions.

2. Radioactive Verification of Cell Synchrony Using the Ficoll Gradient Cell Separation Method

It was postulated that fractions 2 and 3 only from the Ficoll separated cell population would contain cells in S--that is cells actively synthesizing DNA. If such was the case then only these fractions would be expected to incorporate the DNA precursor, H3 thymidine, after a short pulse. To verify this assumption radioactive incorporation of thymidine was undertaken.

Post labelling and pre-labelling of cell populations separated on continuous Ficoll gradients gave the results (Specific Radioactivity) noted in Table III A and B (for the raw data see Appendix). As expected only fractions 2 and 3 incorporated the tritiated thymidine, the DNA fraction of these cells showing 90% of the label. Fraction 1 did not have cells synthesizing DNA as shown by its failure to

TABLE III

SPECIFIC RADIOACTIVITY OF CELL FRACTIONS SEPARATED ON A FICOLL GRADIENT. THE TOTAL CELL POPULATION.

Population	Cell Number	Corrected Total Count Per Minute	(1) % of Total Population	(2) % of Total Radioactive Count	Ratio (2)/(1)	DNA Content μg*
Mother						
Postlabelled	20.0×10^5	82,605	100%	100%	1.0	0.138
Prelabelled	20.0×10^5	62,338	100%	100%	1.0	0.145
Fraction #1						
Postlabelled	2.6×10^5	3	13%	<.01%	<.01	0.086
Prelabelled	2.3×10^5	1	11.5%	<.01%	<.01	0.091
Fraction #2						
Postlabelled	10.0×10^5	53,040	50%	63%	1.26	0.121
Prelabelled	9.9×10^5	40,500	49.5%	65%	1.29	0.124
Fraction #3						
Postlabelled	6.7×10^5	28,500	33 1/3%	34.5%	1.03	0.147
Prelabelled	7.0×10^5	22,325	35%	35%	1.00	0.146

*for 1×10^4 LM mouse fibroblast cells.

incorporate the thymidine. The conclusion, up to this point based on visual evidence, that fraction 1 contains cells solely in G1 is therefore reinforced. It should be noted that Tobey and Ley, (1970), working with Chinese Hamster cells found that viable cells in G1 arrest would not incorporate thymidine. After 80 hours of this arrest, if resuspended or diluted with fresh medium the cells again proceeded through the normal cell cycle and also once again incorporated thymidine at the appropriate phase (S). For this experiment, Table IIIB, the specific activities per cell of fractions 2 and 3 show that fraction 3 was higher. The more actively DNA producing cells of the S phase, larger in volume, are probably in this band. However, Table IIIA shows that the total incorporation is higher in fraction 2.

It can be noted, in conclusion, that although a clean division of all the cell population into its various phases was not obtained, fraction 1 would appear to be almost a pure population of cells in G1. The long period of time observed necessary for this population to double also would suggest that it is in G1 of the cell life cycle (Table XIV, page 110).

3. Cold Shock Synchrony

Although this method was not used extensively in this study, attempts were initially made to obtain large cell populations in this manner.

The methods of Padilla and Cameron, (1964), and Zeuthen, (1964), were investigated exposing LM mouse fibroblast cells to single and to cycles of cold exposure of various lengths (12 hours and 24 hours). The results obtained were inconclusive and consequently not pursued.

However, following the method of Newton and Wildy, (1959), in which cells were treated to a cold shock for one hour at 4 °C then returned to 37°C for continued incubation, the results noted in Table IV and Figure 12 were obtained. Comparison of the control untreated population and the cold shocked population for both cell count and mitotic percentage were noted every half hour beginning 17 hours after the shock and monitored until 23 hours. At 18 hours a marked increase (40.3% as opposed to 12.6% for the control population) in the number of cells in mitosis was observed in the shocked population. The percentage remained high gradually falling towards that of the control population. After Ficoll separation (Figure 11, page 71) all the cell counts for the three separated cell populations fractions show an increase, sometimes substantial, in the cold shocked population. The smaller new cells of G1 show a significantly higher growth increase than the other two fractions.

Watanabe and Okada, (1967), and Sisken, (1963), have suggested that the G1 phase, which is most affected by temperature changes, when released from such a block show

TABLE IV

GROWTH COMPARISON OF CELL POPULATIONS GIVEN ONE HOUR COLD SHOCK

(AFTER SEPARATION ON A FICOLL GRADIENT)

Population	0 Day		1 Day		2 Day		3 Day	
	Count	Cell type	Count	cell type	Count	Cell type	Count	Cell type
Mother Control	2.0	mixed	4.0	mixed	9.0	mixed	22.4 11.2 X original count	mixed
Band 1 Control	1.0	small singles	1.6	medium cells some doubles	2.4	mixed	4.2 4.2 X original count	mixed
Band 2 Control	1.0	medium & doublets	2.1	mixed	4.2	mixed	12.4 12.4 X original count	mixed
Band 3 Control	1.0	large cells doublets	3.0	medium small singles	6.2	mixed	18.5 18.5 X original count	mixed
Test Mother	2.0	small & medium	4.6	mixed	10.8	mixed	28.5 14.25 X original count	mixed
Band 1 Test	1.0	small & medium sized	2.4	mixed	5.7	mixed	15.6 15.6 X original count	mixed

TABLE IV (Continued)

Population	0 Day		1 Day		2 Day		3 Day	
	Count	Cell type	Count	Cell type	Count	Cell type	Count	Cell type
Band 2 Test	1.0	large & doublets	2.5	mixed	6.3	mixed	16.6 16.6 X original count	mixed
Band 3 Test	1.0	doublets mainly	4.0	small mainly	6.5	mixed	20.1 20.1 X original count	mixed

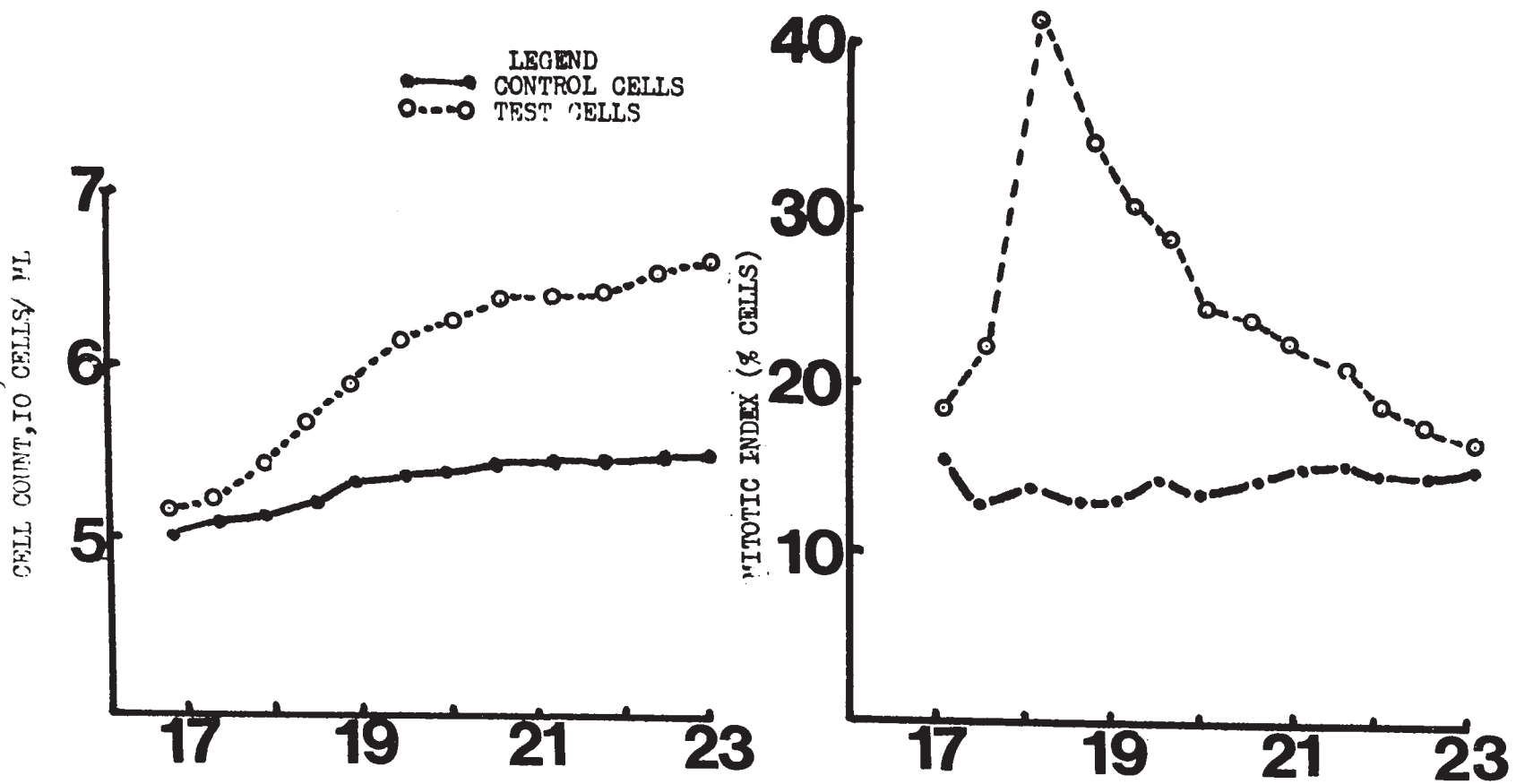
FIGURE 12

EFFECT OF ONE HOUR COLD SHOCK ON THE CELL COUNT AND MITOTIC PERCENTAGE OF LM CELLS

a. Variation in Cell Count. The comparison between the normal control population cell count and that of a cold shocked population tested 17 hours to 23 hours after the hour's cold shock is shown.

b. Variation in Mitotic Percentage of LM cells using the method of Schindler et al., (1967), to compare a normal control population and a cold shocked cell population 17 hours to 23 hours after the hour's cold shock.

Figure 12


LEGEND
CONTROL CELLS
TEST CELLS
CELL COUNT, 10^5 CELLS/ ML
7
6
5
17
19
21
23
TIME AFTER COLD SHOCK IN HOURS
MITOTIC INDEX (% CELLS)
40
30
20
10
17
19
21
23
TIME AFTER COLD SHOCK IN HOURS

a burst of enzyme production resulting in an increased cellular growth not normally observed. The results observed here would tend to support such a conclusion.

Although Galavazi et al., (1966), and others have found Newton and Wildy's, (1959), method not reproducible in this particular study such was not the case. It was concluded from this set of experiments that a one hour cold shock of a logarithmically growing cell population was a feasible way to obtain a large number of cells in one phase of the cell cycle. Judicious harvesting on a Ficoll gradient of cells obtained from such a method could produce cell populations containing varying percentages of cells in mitosis (Figure 13, page 75).

4. Cell Membrane Preparation

Figure 14, page 75, shows various stages observed with the LM cells when the developed method was used to produce a membrane fraction. In all experiments the cell membranes popped off with ease giving a yield of between 95 - 98%. Although the membrane fractions have not been viewed under the electron microscope they are strikingly similar to those pictured by Warren, Glick and Nass, (1966), even having the rolled scroll appearance they commented on.

FIGURE 13

FICOLL GRADIENT SEPARATION OF VARIOUS CELL POPULATIONS COLD SHOCKED INTO SYNCHRONY

using the method of Newton and Wildy (1959)

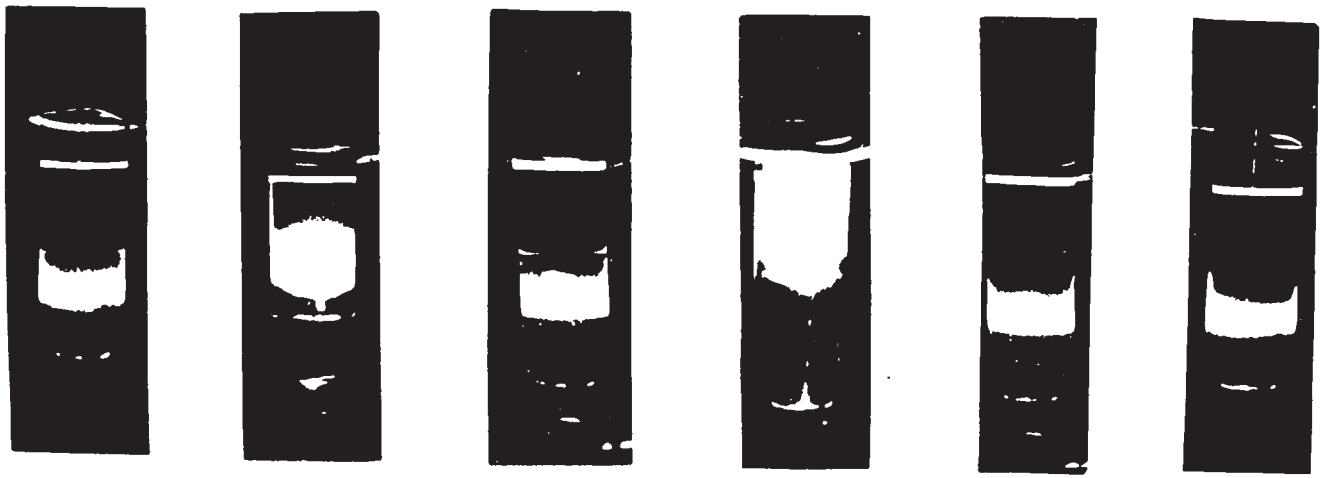
The percentage noted below each tube indicates the mitotic percentage obtained when the cell populations were characterized using the method of Schindler et al., (1967), (page 32 of this study).

Note the variation in bands' position and concentrations for the different cell populations.

Figure 13

in mitosis

Figure 13



in mitosis

FIGURE 14

CELL POPPING AND MEMBRANE PRODUCTION

USING THE DEVELOPED METHOD

Cell (magnification 1000X) gradually swells finally bursting the cell membrane and allowing the cytoplasm and nucleus to flow out. The scroll-like membrane fraction is shown.

Figure 14

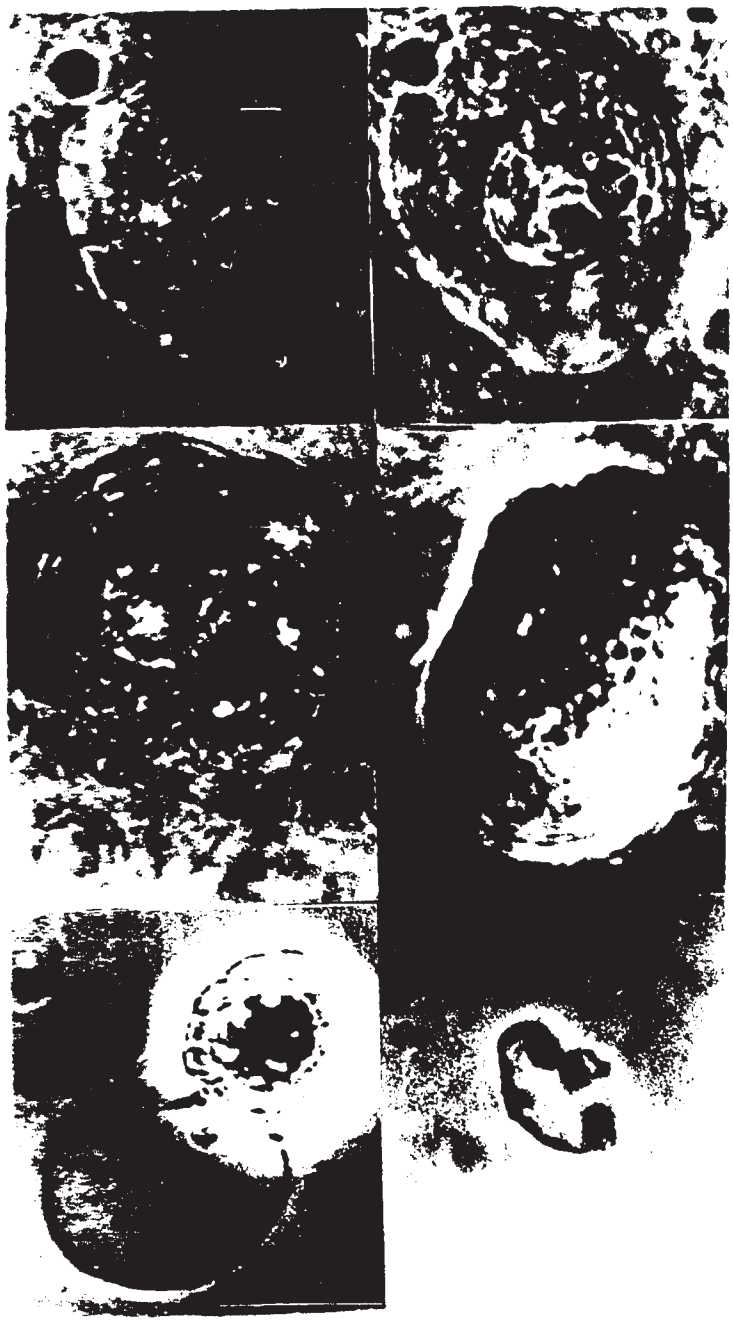
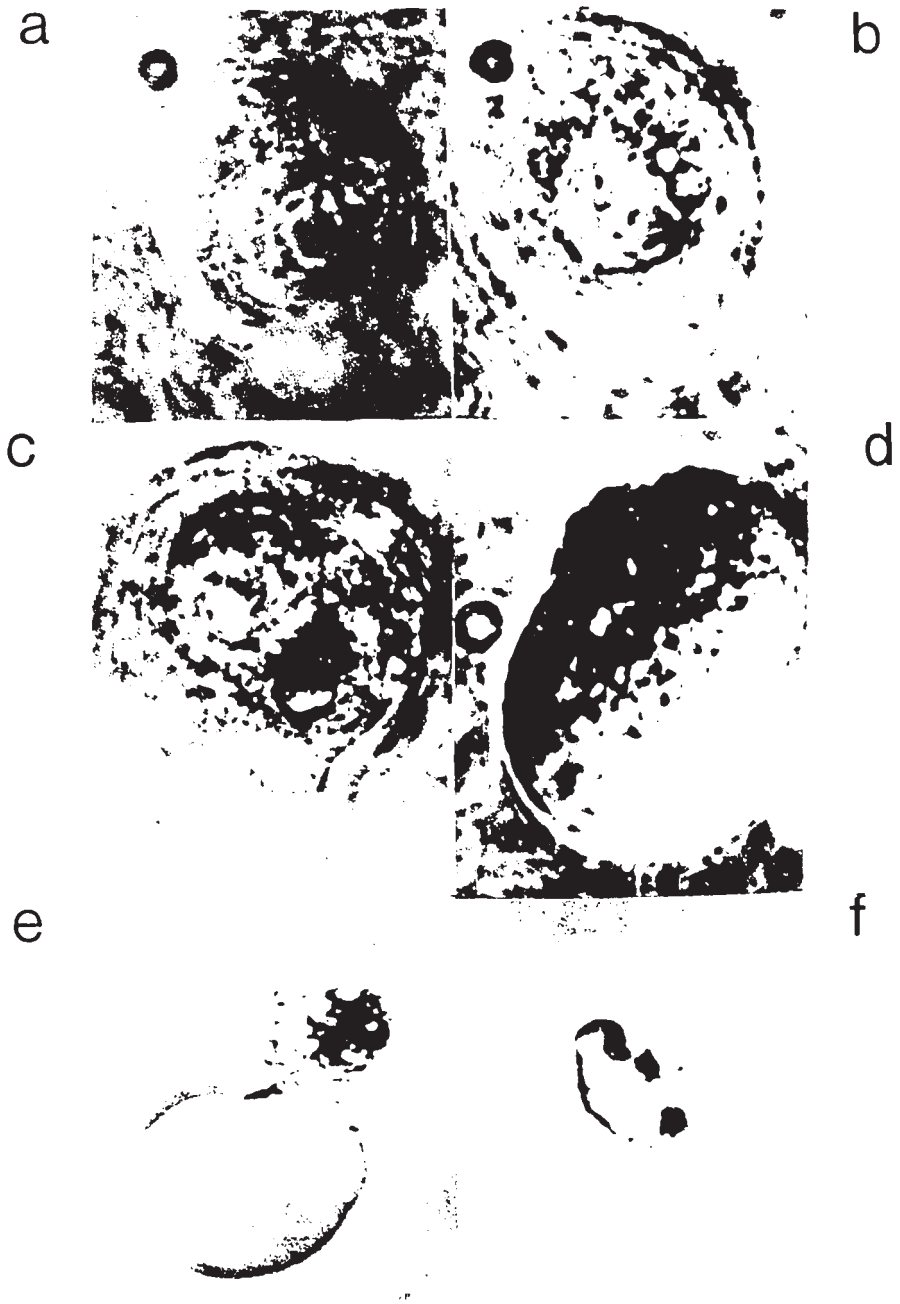


Figure 14



5. Fluorescein Mercuric Acetate Incubation of Cells

When a logarithmic cell population was incubated with fluorescein mercuric acetate (2.2×10^{-3} M) it was noted that only 30% of the cells took up the compound which was expected to react with free sulphydryl groups and to fix, or harden, the surface membrane. Ficoll separated populations of cells previously incubated in fluorescein mercuric acetate showed that only the medium sized cells allowed its uptake (S, G2 and early M). Newly formed cells, G1, and cells of late mitosis (telophase) excluded the compound, indicating different membrane permeability characteristics at this time.

C. CHEMICAL ANALYSIS OF LOGARITHMICALLY GROWING LM CELLS

Cells were separated into fractions on a Ficoll gradient. Envelope fractions were prepared using the developed method. The following chemical analyses for both whole cells and membrane fractions were made:

Protein,

DNA,

RNA,

Carbohydrate,

Lipid (Total and Cholesterol).

Four separate logarithmically growing populations were analysed; the variation of results obtained was minimal

TABLE V

*CHEMICAL ANALYSIS OF LOGARITHMICALLY GROWING LM MOUSE FIBROBLAST CELLS

ORIGIN	PROTEIN (a)		CARBOHYDRATE (a)		DNA (a)		RNA (a)		LIPID (a)			
									TOTAL		CHOLESTEROL	
	Cell	Membrane	Cell	Membrane	Cell	Membrane	Cell	Membrane	Cell	Membrane	Cell	Membrane
									Warren and Glick			
	Dry Weight 35.9	1.7 (4.8%)	Thymol 5.8	Thymol 0.61 (10%)	1.49	(b)	3.46	(b)	71.4	8.4 (12%)	8.2 (11.5%)	1.58 (18.9%)
									S-T			
	Folin-Phenol 35.7	1.7 (4.8%)	Anthrone 7.6	Anthrone 0.76 (10%)					80.6	9.2 (11.0%)	9.4 (11.6%)	1.8 (19.6%)
Fraction 1 G1 predominately	Dry Weight 26.7	1.2 (4.5%)	Thymol 71.	Thymol 0.58 (8.2%)	1.23	(b)	3.00	(b)	Warren and Glick 64.1	6.8 (10.7%)	7.38 (11.5%)	1.26 (18.5%)
	Folin-Phenol 26.7	1.2 (4.5%)	Anthrone 8.3	Anthrone 0.63 (7.7%)					S - T 68.0	7.2 (10.6%)	7.83 (11.5%)	1.33 (18.5%)
Fraction 2 G2? S	Dry Weight 33.0	2.1 (6.3%)	Thymol 6.1	Thymol 0.58 (9.5%)	1.50	(b)	3.64	(b)	Warren and Glick 67.5	9.3 (13.8%)	7.6 (11.6%)	1.94 (20.9%)
	Folin-Phenol 34.0	2.1 (6.3%)	Anthrone 7.4	Anthrone 0.71 (9.6%)					S - T 69.4	9.7 (13.9%)	7.88 (11.4%)	2.05 (21.1%)
Fraction 3 S G 2 M	Dry Weight 54.6	4.9 (9.0%)	Thymol 7.8	Thymol 0.75 (9.7%)	1.96	(b)	3.68	(b)	Warren and Glick 98.6	23.3 (23.9%)	11.3 (11.5%)	5.9 (25.1%)
	Folin - Phenol 54.7	4.9 (9.0%)	Anthrone 8.5	Anthrone 0.86 (10.0%)					S - T 100.1	24.7 (24.7%)	11.6 (11.5%)	6.4 (25.9%)

(a) $\gamma \times 10^{-6}$/cell

(b) undetectable

* Variation in per cell content less than 0.5%.

The standard deviation was calculated and the coefficient of variation obtained was less than 0.5% for the four experiments. Table V reports the average results obtained from these four separate experiments.

Compared to the values obtained by other workers (Healy et al., 1956; Siminovitch et al., 1957; Salzman, 1959; and Hill, 1959), the mother populations show similar results for both protein and DNA content. Total lipid and cholesterol content for both the mother and membrane fractions agree well with those reported by Weinstein et al., (1969).

Analysis of the Ficoll separated fractions showed the expected doubling of both protein and lipid percentages within the whole cell and the membrane between the small cells of fraction 1 (G1) and the large cells of fraction 3 (G2 and M). Although the actual amount of carbohydrate within the total cell remains fairly constant, the ratio of carbohydrate to protein falls continually as the cells move from G1 to M. In fraction 3 the ratio is one half that of fraction 1. The decline of carbohydrate in the cell envelope is even more evident, fraction 3 having only one third the amount found in fraction 1. Table VI gives the ratio variation noted in the whole cells and the cell membranes in the various fractions.

TABLE VI

RATIO OF WEIGHT OF LIPID AND CARBOHYDRATE TO PROTEIN IN BOTH THE WHOLE CELL AND IN ENVELOPE PORTIONS FOR VARIOUS CELL FRACTIONS OBTAINED BY FICOLL GRADIENT SEPARATION

ORIGIN	% of Constituent of Whole Cell/% of Protein			% of Constituent of Cell Envelope/% of Protein		
	Carbohydrate	Total Lipid	Cholesterol	Carbohydrate	Total Lipid	Cholesterol
NORMAL	0.21	0.22	0.03	0.44	0.49	0.093
FRACTION 1	0.31	0.25	0.03	0.53	0.57	0.11
FRACTION 2	0.21	0.20	0.02	0.34	0.44	0.092
FRACTION 3	0.16	0.19	0.02	0.18	0.48	0.12

No attempt was made to characterize the carbohydrates present in this investigation though in retrospect such an analysis would have been worthwhile.

The conclusions reached from the chemical analyses of total cell and cell envelope are:

1. As the cell proceeds through the life cycle from G1, through S and G2 to M, quantitatively the total cell and envelope fraction protein and lipid content increases to twice its original value. The total cell and membrane carbohydrate content, however, remain fairly constant.

2. The ratio of lipid to protein in both the whole cell and in the envelope fraction remains fairly constant throughout the cell's life cycle but the ratio of carbohydrate to protein falls to one half in the total cell and to one third the value in the cell envelope as the cell proceeds from G1 to M.

D. ENZYMATIC STUDIES

As Shibko and Tappel, (1964), note esterases are associated with the lysosomal membrane and other membraneous constituents of the cell, and are probably responsible for the hydrolysis of some of the naturally occuring esters such as cholesteral ester and vitamin A ester. To them the prime function of the cellular esterases is to directly control

membrane permeability processes through this hydrolysis. Rotman and Papermaster, (1966), note that the fluorogenic substrate fluorescein diacetate, (FDA), is particularly useful in quantitative measurements of enzymatic activity at the cellular level because its hydrolysis results in the intracellular accumulation of fluorescein. They showed that such hydrolysis and intracellular retention of fluorescein was dependent on the integrity of the cell membrane. In this present investigation experiments studying the effects of esterase inhibitors, paraoxon and eserine sulphate (page 35) showed that fluorogenic enzyme assay is a valid means of measuring cell membrane activity.

1. Factors Affecting the Experimental System

a. Normal Fluorometric Run

The fluorometric rates obtained for a normal run with logarithmically growing suspension cells is shown in Figure 15a and in Appendix 4. When the velocity, V, the fluorometric rate, is plotted against the substrate concentration (S) the normal kinetic curve is obtained (Figure 15a). A Michaelis plot for this substrate and the acetylesterase of the normal LM mouse fibroblast cell is shown in Figure 15. From this the fluorometric rate, Km, value would appear to be 10×10^{-6} moles per liter, and the V_{max} 5.0 fluorometric units/minute. Preliminary experiments utilizing various substrate

FIGURE 15

FLUOROGENIC SUBSTRATE AND THE CELL

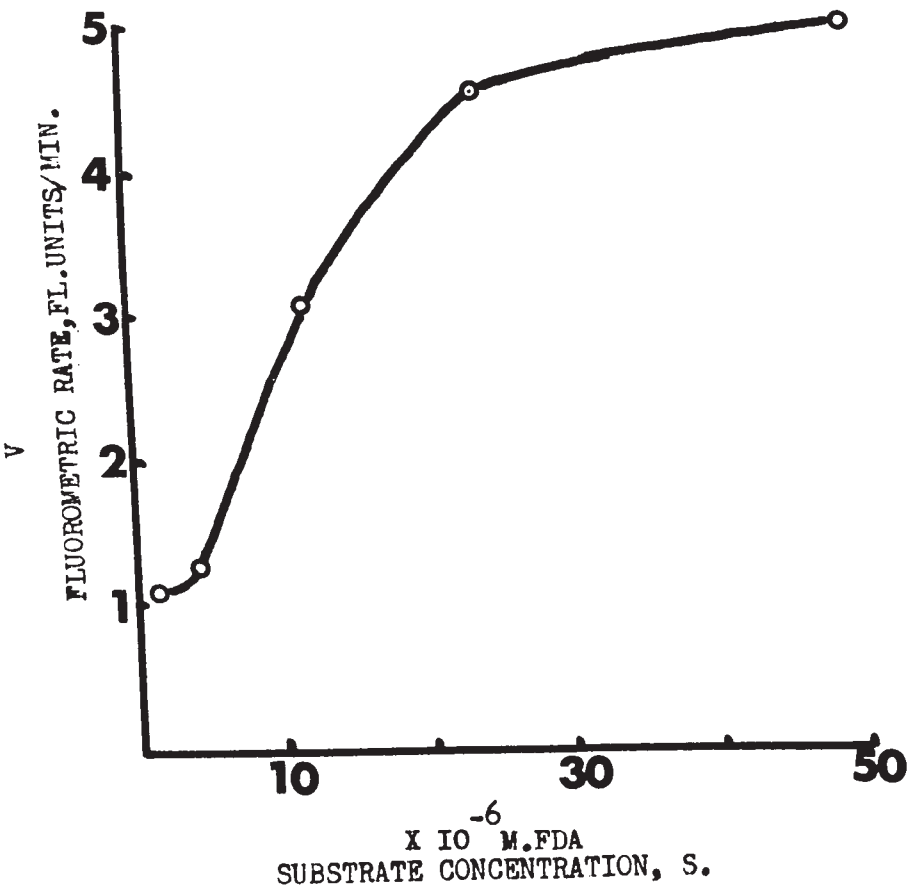
a. Normal Cell Population. The fluorometric rate (i.e., the rate at which the substrate FDA is broken down by the LM cell, measured by the accumulation of the product Fluorescein) observed when various concentrations of substrate are added to a system where all other factors are constant (cell number, temperature, extracellular medium and method of analysis).

b. Normal Cell Population. The fluorometric rate observed when the temperature is varied for three separate substrate cell interactions in a system where all other factors are constant (cell number, extracellular medium and method of analysis).

c. Lineweaver-Burk Plot for a normal LM mouse fibroblast cell population metabolizing Fluorescein Diacetate (various concentrations) at 37°C.

Figure 15 Fluorogenic Substrate &The Cell

a. NORMAL CELL POPULATION



b. TEMPERATURE EFFECT ON FDA- ACETYLESTERASE SYSTEM OF THE LM MOUSE FIBROBLAST CELL. 37 C and 27 C

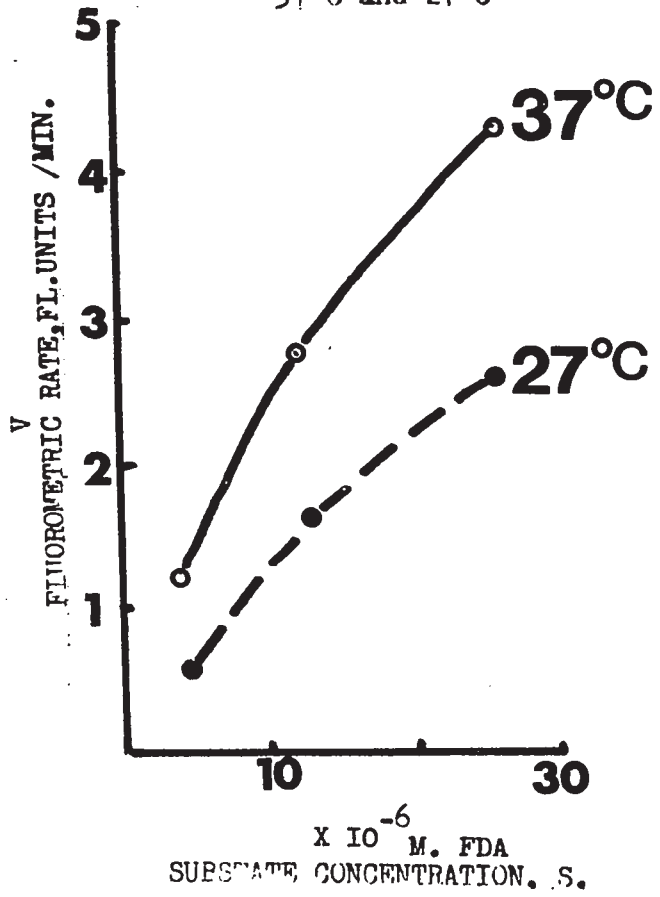
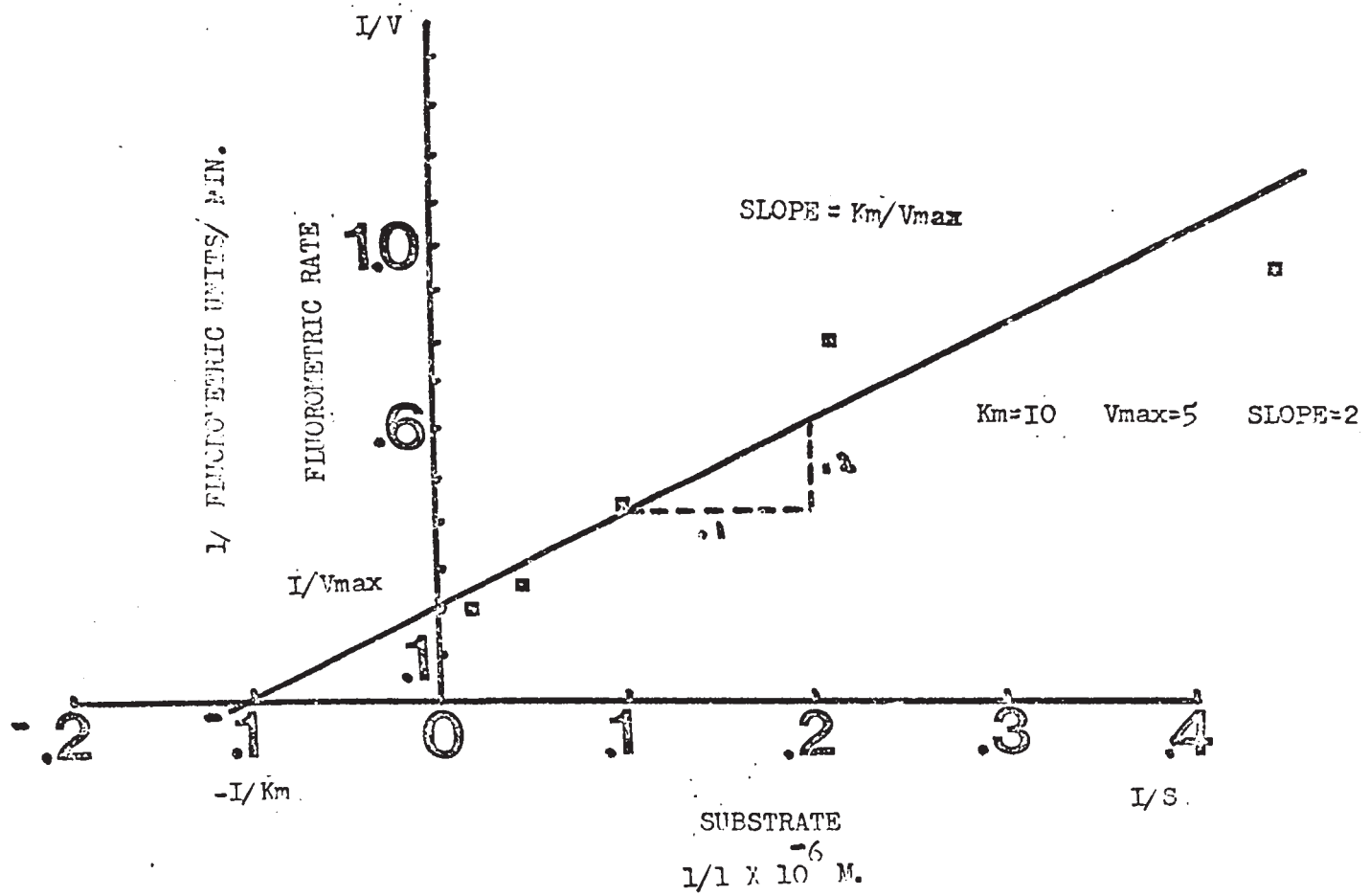


Figure 15

C LINEWEAVER-BURK PLOT, RECIPROCAL FORM OF THE RATE EQUATION FOR FDA-ACETYLESTERASE SYSTEM OF THE LM MOUSE FIBROBLAST.



concentrations (Appendix), and cell concentrations resulted in the standardizing of the final substrate concentration within the system (5 ml) 6.0×10^{-6} M of FDA and the final cell concentration to 2.75×10^{5} cells.

b. Effects of Temperature on the Metabolic System

The fluorometric substrate breakdown rate obtained for a normal cell population - FDA interaction allowed to run at two different temperatures (27°C and 37°C) with three different substrate concentrations is given in Figure 15 (Appendix 4). The Q_{10} for each concentration of substrate was calculated and the average result was 1.70 (27° - 37°C), which compares well with that of Rotman and Papermaster, (1966), of 1.71 (20° - 30°C).

c. Effect of pH Variation on the Metabolic Breakdown Rate of FDA by whole Cell Populations in Various Stages of the Growth Curve (Figure 16)

The hydrogen ion concentration exerts a strong influence on the protein components of the cell by affecting not only the degree of dissociation and thereby the structural arrangement but also its charge. The tension of oil to water interfaces, such as those proposed to be an integral part of the cell membrane structure, also are known to be affected markedly by pH shifts (Danielli, 1937). For a cell, therefore, the bulk pH surrounding it is of extreme importance in determining its ability to tolerate

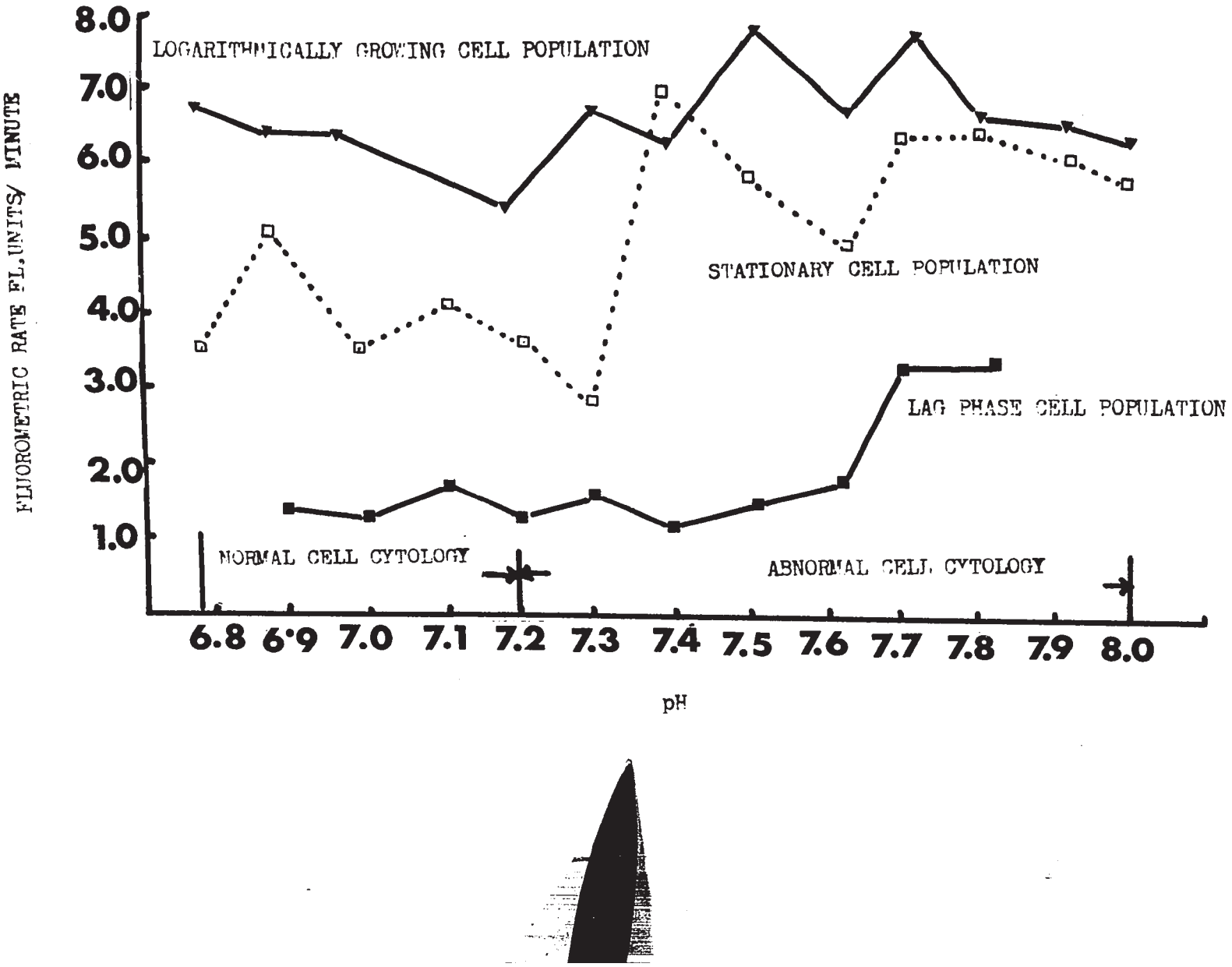
FIGURE 16

EFFECT OF pH VARIATION ON THE FDA-ACETYLESTERASE SYSTEM OF THREE DIFFERENT CELL POPULATIONS

A cell population at various stages of the growth curve (Appendix) was tested flurometrically with FDA. The pH of the medium 199 was varied by increments of 0.1 from pH 6.8 to pH 8.0. Population differences as well as pH effect differences are noted.

Logarithmically growing cells give higher flurometric breakdown rate readings than either stationary cell cultures or cells in the Lag phase, indicating a more active acetylesterase system at this time. Stationary cells are most affected by the pH variation of the medium with regards to this enzymatic interaction. All three cell populations are cytologically adversely affected by alkaline medium.

Figure 16


8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
FLUOROMETRIC RATE FL.UNITS/ MINUTE
LOGARITHMICALLY GROWING CELL POPULATION
STATIONARY CELL POPULATION
LAG PHASE CELL POPULATION
NORMAL CELL CYTOLOGY
ABNORMAL CELL CYTOLOGY
6.8 6.9 7.0 7.1 7.2 7.3 7.4 7.5 7.6 7.7 7.8 7.9 8.0
pH

the environmental conditions and continue life.

Rotman and Papermaster, (1966), when studying the FDA - acetylesterase system found that the cells would metabolize the substrate over a wide range (pH 6.7 - pH 8.0). Although in this study this possible range for substrate metabolism was confirmed (Figure 16), the adverse cytological effects noted with the cells incubated in medium 199-peptone above pH 7.2 did not recommend the higher, more alkaline, pHs. It was found, when whole cell populations were compared, that logarithmically growing cell preparations are more active metabolically than either the lag or stationary phase cell populations. However, all three populations showed membrane disturbance in the form of crinulation or wavey membranes above pH 7.2. On the acidic side of neutral, pH 7.0, the cells were tested with the FDA - acetylesterase system only as low as pH 6.8. At this pH both the metabolic activity and the cellular cytology remained normal.

The total effect noted for any population is the sum of the combined effects of the individuals within the population. Since the percentages of cells at different phases of the cell cycle varies during the growth curve the weight of influence they exert on the final results observed for the FDA - acetylesterase system will fluctuate in accordance with this percentage. It might well be that at different phases of the cell life cycle different ionizable

groups are present at the cell surface. The pH effects noted on the FDA - acetylesterase system may reflect this difference.

d. Effects of Pretreatment of Cells with Esterase Inhibitors on the FDA - Acetylesterase System of the LM Mouse Fibroblast Cells

Paraoxon, (Table VIIA) would appear to specifically inhibit the esterase responsible for the FDA substrate breakdown. Cells incubated 15 minutes prior to a fluorometric test in medium containing as little as 1×10^{-5} M paraoxon show an inhibition. Almost complete inhibition is noted at 1×10^{-3} M. The cells are not visually disturbed, the membranes remaining taut and normal in appearance throughout the incubation period. After 10 minutes clustering of granules at the membrane is the only abnormality noted. After 20-30 minutes these clusters disappear and the cells appear normal. If the cells are removed from the inhibiting medium and resuspended in fresh medium 199, after 2 hours they will once more metabolize FDA normally.

Eserine sulphate, (Table VIIA), on the other hand, although it does inhibit the system to some degree would appear to do it by destroying the membrane and possibly some related energy system. After 15 minutes incubation in eserine sulphate the cells begin to have wavey membranes though the cellular contents remain normal in appearance.

TABLE VII

EFFECT OF INHIBITORS AND IONS ON FDA-ACETYLESTERASE SYSTEM

OF THE LM MOUSE FIBROBLAST

Factor Molar Concentration	Corrected Fluorometric Rate	Ratio of Rates	Cytology and Conclusion
A. Esterase Inhibitors	Normal 3.10		
Paraoxon			Paraoxon, though not disturbing the membrane, inhibits the esterase necessary for the substrate breakdown
10^{-2}	0.0	*NA	
10^{-3}	0.28	0.009	
10^{-4}	1.0	0.322	
10^{-5}	1.5	0.50	
10^{-6}	2.9	0.94	
10^{-7}	3.1	1.0	
Eserine			Eserine disturbs the membrane. Possible complex inhibition on the specific substrate system.
10^{-2}	1.04	0.34	
10^{-3}	1.62	0.52	
10^{-4}	2.2	0.71	
10^{-5}	2.7	0.87	
10^{-6}	3.0	0.97	
10^{-7}	3.0	0.97	
B. Respiratory Inhibitors	Normal 2.95		
Antimycin A			Inhibits co-enzyme Q;no cellular effect noted
10^{-3}	1.6	0.54	
10^{-4}	2.2	0.75	
10^{-5}	2.9	0.99	
10^{-6}	3.0	1.1	
10^{-7}	3.0	1.1	

TABLE VII (Continued)

Factor Molar Concentration	Corrected Fluorometric Rate	Ratio of Rates	Cytology and Conclusion
Oligomycin			
10^{-1}	0.0	NA	Cells swollen.
10^{-2}	0.0	NA	Inhibits mem-
10^{-3}	0.0	NA	brane bound
10^{-4}	1.0	0.34	ATPase. Upsets energy system
Potassium Cyanide			
10^{-1}	0.0	NA	Membranes dis-
10^{-2}	0.0	NA	torted; granu-
10^{-3}	0.0	NA	lar cytoplasm.
10^{-4}	1.0	0.34	
10^{-5}	1.5	0.51	
2,4 Dinitrophenol**			
10^{-1}	0.0	NA	Cells appear nor-
10^{-2}	0.0	NA	mal. Strong
10^{-3}	0.0	NA	energy inhibi-
10^{-4}	1.0	0.34	tor of
10^{-5}	2.5	0.85	phosphorylation.

*NA - No Activity

** - Pre incubation and removal of this inhibitor necessary before fluorometric test, due to interaction with substrate FDA.

TABLE VII (Continued)

Factor Molar Concentration	Corrected Fluorometric Rate	Ratio of Rates	Conclusion
NaCl, Sodium Chloride			
10^{-1}	3.1	1.0	Cell membrane was disturbed above 1×10^{-3} M. No affect on FDA-enzyme system.
10^{-2}	3.0	1.0	
10^{-3}	3.0	1.0	
10^{-4}	3.0	1.0	
10^{-5}	3.1	1.0	
10^{-6}	3.0	1.0	
Sodium Acetate			
10^{-1}	3.0	1.0	No competitive inhibition noted. Cells appear normal.
10^{-2}	3.1	1.0	
10^{-3}	3.1	1.0	
10^{-4}	3.1	1.0	
$MgCl_2$, Magnesium Chloride			
10^{-1}	3.0	1.0	Above 1×10^{-4} M membrane disturbed, blebbing, but FDA-enzyme system unaffected.
10^{-2}	3.0	1.0	
10^{-3}	3.0	1.0	
10^{-4}	3.0	1.0	
10^{-5}	3.0	1.0	

TABLE VII (Continued)

Factor Molar Concentration	Corrected Fluorometric Rate		Ratio of Rates		Conclusion
C. Ion Excess	Normal 3.0				
$CaCl_2$ Calcium Chloride	0 Time	15 mins	0 Time	15 mins	Cells incubated in medium with an excess of calcium chloride within 15 mins have adjusted their metabolic systems to the excess. Cells added to such a medium however will initially have high metabolic activity.
IM	12.0	3.5	4.0	1.2	
10^{-1}	12.0	3.2	4.0	1.1	
10^{-2}	12.0	3.0	4.0	1.0	
10^{-3}	7.5	3.1	2.5	1.0	
10^{-4}	7.0	3.1	2.4	1.0	
10^{-5}	6.5	3.0	2.2	1.0	
10^{-6}	4.0	3.0	1.3	1.0	
10^{-7}	3.1	3.0	1.0	1.0	
KCl, Potassium Chloride					
10^{-1}	3.0		1.0		Cells elongate and produce many microville but their metabolic activity with regards to FDA seems unaffected by the excess of Potassium chloride.
10^{-2}	3.1		1.0		
10^{-3}	3.0		1.0		
10^{-4}	3.1		1.0		
10^{-5}	3.1		1.0		
10^{-6}	3.0		1.0		

Continued incubation in 1 x 10^{-3} M eserine sulphate causes destruction of 90% of the cells after one hour. There is always a residual group of cells, approximately 10%, which were unaffected by the eserine incubation. These cells might possibly be in one specific phase of the cell's life cycle. Mayhew and O'Grady, (1965), commenting on the findings of Dustin, (1947), and Walker and Helleiner, (1963), proposed that surface charge changes during different stages of the cell's mitotic cycle might account for the variations in sensitivity to cytotoxic drugs. This would appear to be another such case.

e. Effects of Pretreatment of Cells with Respiratory Inhibitors on the Acetylesterase System

The respiratory inhibitors tested, (Table VIIB), all had a marked effect on the breakdown rate of FDA by LM mouse fibroblast cells. Both Oligomycin, which blocks ATP-energized mitochondrial reactions (Mahler and Cordes, 1966), and potassium cyanide, which inhibits the oxidation of the cytochromes, had extremely adverse effects on the cells, distorting the membranes, causing granulation of the cytoplasm and swelling of the cell. Consequently, the exact point at which these inhibitors interfered with the system being studied is not able to be stated. Antimycin A, an antibiotic which inhibits the energy requiring step cytochrome B - cytochrome C in oxidative phosphorylation,

and 2,4,dinitrophenol, an uncoupler of oxidative phosphorylation, do not visually disturb the cell. When cells are removed from the presence of these two inhibitors they regain their normal FDA metabolizing ability within an hour. From this experiment it would appear that energy is necessary for the penetration or breakdown of fluorescein diacetate by the LM mouse fibroblast cell.

f. Effect of Additional Ions, to Medium 199-Peptone, on the Acetylesterase Activity of the Cells

Of the five chemicals tested, (Table VIIC), only one had any effect on the FDA - acetylesterase system of the LM mouse fibroblast cell. Cells preincubated 15 minutes in calcium chloride did not show any excessive increase in metabolic breakdown rate of the substrate, but, the cells from the same original population when added to the medium containing calcium chloride excess at the same time as the substrate addition, showed marked enhancement of the system. Manery, (1966), in his review of the myriad effects of calcium on membranes postulates that calcium induces conformational changes in the lipoprotein structure of the cell membrane. Such a change might result in a transitory permeability increase to some substances, in this case the substrate, FDA. The cells all appeared normal and glowed very brightly, no background fluorescence was noted, the cells were not, therefore, leaking due to breaks in the membrane.

g. Effect of Enzyme Pretreatment of Cells on the FDA - LM Mouse Fibroblast Cell Metabolic Breakdown Rate

Cells were incubated in neuraminidase, at two concentrations (300 units/ml for 60 minutes and 50 units/ml for 60 minutes), and tested fluorometrically immediately upon removal from the presence of the enzyme and also 30 minutes later after a rest period in fresh medium 199 for the cells (Table VIII). The stronger concentration of enzyme, 300 units/ml, destroyed a number of cell membranes, caused blebbing and marked swelling of the cells. Tested fluorometrically this cell population showed some inhibition at both zero and at 30 minutes. The cells incubated in the weaker concentration of neuraminidase, 50 units/ml, though they still were unable to stick to glass (page 67), did manage to recover their enzymatic activity after 30 minutes rest in fresh medium.

Collagenase, probably by removing collagen from the cell surface, gave a slight enhancement of the fluorometric rate. Lipase as well allowed increased substrate breakdown rate due possibly to increased permeability brought about by the stripping of the cell's surface of easily digestible lipids.

TABLE VIII

EFFECT OF ENZYMES, MEMBRANE ACTING COMPOUNDS AND THE PRODUCT OF THE FDA-ACETYLESTERASE SYSTEM ON THE LM MOUSE FIBROBLAST CELL

Factor	Corrected Fluorometric Rate	Ratio of Rates	Cytology and Conclusion
Enzyme preincubation Neuraminidase	Normal 3.0		300 units/ml of neuraminidase was too concentrated for the cells; 50% of cells were damaged. 50 units/ml of neuraminidase did not visibly affect the cells (some slight swelling). Only a slight inhibition was noted in the FDA esterase system. The cells quickly recovered their enzymatic ability.
300 units/ml	1.5	0.5	
0 time	1.5	0.5	
30 mins	2.3	0.8	
50 units/ml			
0 time	2.5	0.8	
30 mins	3.0	1.0	
Collagenase (0.1%)	4.3	1.4	Removal of collagen may increase permeability to FDA.
Lipase (0.05%)	3.9	1.3	

TABLE VIII (Continued)

Factor	Corrected Fluorometric Rate	Ratio of Rates	Cytology and Conclusion
Membrane Acting Substances	Normal Rate 2.9		
Vitamin A			
1×10^{-2}M	3.0	1.0	Membrane disturbed, but FDA-esterase system unaffected.
1×10^{-3}M	2.8	1.0	
1×10^{-4}M	2.9	1.0	
1×10^{-5}M	3.0	1.0	
1×10^{-6}M	3.0	1.0	
Vitamin C			
1×10^{-3}M	3.5	1.2	Cell membranes taut. FDA-esterase system enhanced.
1×10^{-4}M	3.0	1.0	
1×10^{-5}M	3.0	1.0	
Vitamin E			
2×10^{-4}M	2.3	0.8	Cell membrane like cracked mosaic. Slight FDA inhibition.
2×10^{-5}M	3.0	1.0	
Methylcellulose	3.0	1.0	Methylcellulose had no effect on the system.
Product Excess			
Initial run			
Control normal	3.2	1.0	Fluorescein inhibits the FDA-esterase system but the same cells show a priming effect if removed from this
Fluorescein 1.0×10^{-7}M	1.8	0.6	

TABLE VIII (Continued)

Factor	Corrected Fluorometric Rate	Ratio of Rates	Cytology and Conclusion
Fresh Medium			
Control Normal	2.5	1.0	medium and allowed to metabolize the substrate in medium 199 without F.
Fluorescein 1.0×10^{-7}M	5.2	2.1	

h. Effect of Various Membrane Active Compounds on the Acetylesterase Activity of LM Mouse Fibroblast Cells

Vitamins A, C and E, did not markedly affect the breakdown rate of the FDA - LM mouse fibroblast system (Table VIII). Vitamin A and E did disturb the membrane but vitamin C appeared to improve the cell. The membrane was taut and the cells appeared metabolically active. Methyl cellulose, normally in the suspension culture medium to prevent cell clumping did not affect the fluorometric rate of the cells at all.

i. Effect of Product Concentration in the Extracellular Medium on the Metabolic Breakdown Rate of Six Cell Populations

Preliminary experiments showed that fluorescein, though nonpenetrating to the cell, in a concentration of 1×10^{-7} M had an inhibitory effect on the LM cell - acetylesterase system. The effect of fluorescein on six populations of cells cold shocked into synchrony and having different mitotic percentages was studied (Newton and Wildy method, page 39). The cells ranged from one having no visible cells in mitosis, (after a count of 500 cells) to one having 48% in mitosis (page 32) (Schindler et al., 1967). The added concentration of the normal end product fluorescein (F.) varied in the medium 199 from nil to 1×10^{-7} M.

TABLE IX

FLUOROMETRIC RATE VARIATION CAUSED BY FLUOROSCEIN IN THE BATHING MEDIUM

	Fluorometric Rate Observed in Cell Populations					
	% Mitosis					
Product Concentration	0%	5-10%	15%	25%	34%	48%
6×10^{-6} M Substrate (25)	Cells S & G2	mix	mix but more doublets	50% small, early G1, 25% late G1	large cells S & G2 M	large, G2, M
Medium 199 (control)	1.0	4.0	4.2	10.0	5.5	3.5
1×10^{-9} M F1	0.97*	4.1	4.2	10.0	5.4*	3.3*
2.5×10^{-9} M F1	0.91	4.0*	4.3	10.0	5.0	3.1
5.0×10^{-9} M F1	0.90	3.6	4.2*	9.8*	4.7	3.0
7.5×10^{-9} M F1	0.85	3.3	4.0	8.5	4.6	2.7
1×10^{-8} M F1	0.80	2.5	3.7	8.3	3.7	2.8
1×10^{-7} M F1	0.86	2.0	3.2	7.9	3.9	2.6
12.0×10^{-6} M Substrate (50)						
Medium 199 (control	2.0	6.0	7.0	15.0	8.0	5.0
1×10^{-9} M F1	1.9*	6.1	7.1	15.1	8.1	5.0
2.5×10^{-9} M F1	1.5	5.9*	7.1	15.0	8.1	5.1
5.0×10^{-9} M F1	1.4	5.7	7.0*	15.1*	8.0*	5.0*
7.5×10^{-9} M F1	1.3	4.5	6.8	14.8	7.4	4.8
1×10^{-8} M F1	1.2	4.0	5.9	14.0	6.9	4.3
1×10^{-7} M F1	1.0	4.0	5.0	13.6	6.3	4.3

TABLE IX (Continued)

Product Concentration	Fluorometric Rate Observed in Cell Populations					
	% Mitosis					
	0%	5-10%	15%	25%	34%	48%
24.0×10^{-6} M Substrate (100)						
Medium 199 (control)	2.8	10.0	11.2	25.0	12.5	9.7
1×10^{-9} M Fl	2.6*	10.1	11.1	25.2	12.5	9.7
2.5×10^{-9} M Fl	2.2	9.8*	11.1*	25.1	12.5	9.7
5.0×10^{-9} M Fl	2.2	8.9	11.0	25.0*	12.6*	9.8*
7.5×10^{-9} M Fl	2.0	7.7	8.9	23.6	12.3	9.6
1×10^{-8} M Fl	1.7	7.5	8.6	21.7	11.4	8.4
1×10^{-7} M Fl	1.5	6.0	7.5	20.6	10.8	7.7

Normal Logarithmic Population

6.0×10^{-6} M - 2.0
12.0×10^{-6} M - 3.0
24.0×10^{-6} M - 5.0

* Point at which fluorometric rate approaches normal cell rate of control cells.

Three concentrations of the substrate, FDA, were tested. Table IX shows the effect both of the product concentration on the fluorometric rate of FDA breakdown and the variation noted between populations with different mitotic counts. The results obtained from this experiment show that cells in G1 (note 25% mitosis population) metabolize the substrate at a higher rate than those in S, G2 and M, and would seem to be more resistant to the inhibition effect of the product. Cells in other phases of the cell cycle would appear to have varying degrees of resistance. The concentration of the substrate within the extracellular medium 199 also plays a role in the metabolism of this substrate. When it is in low concentration, the product is more effective as an inhibitor than when the substrate is at a higher concentration. It would appear that certain phases of the cell cycle are greatly influenced in their ability to take up and metabolize the substrate by a delicate balance of product to substrate which must be maintained. The variation in membrane state throughout the cell cycle is evident from these results.

2. Exit-Entry Experiments

Initially this method (page 59) was devised to analyze the intracellular metabolic breakdown of the substrate fluorescein diacetate (FDA) by the LM mouse fibroblast cell, and to ascertain if it was a membrane dependent

phenomenon. To this end both whole cells and broken cell preparations were analysed.

a. Normal Cell Population

For every test conducted using the exit-entry method a control normal whole cell population was also run. Within the whole system, cells and extracellular fluid, a gradual decrease of the substrate fluorescein diacetate (FDA) and a gradual increase of the product fluorescein (F) was noted. The extracellular fluid alone showed a similar decrease of the substrate (Figure 17b) and increase of the product. At all times the cyclic results shown in Figure 18 was obtained when the cellular content of FDA, FMA, and F, were analysed from a chromatogram such as that shown in Figure 19.

The concentration of the substrate, FDA, within the cell, rose and fell rhythmically with a period of approximately 3 to 4 minutes. At the same time an opposite wave was noted for the end product, F. FMA, the intermediate metabolite, always peaked just before the end product F. The swift shift of concentration highs and lows within the cell as opposed to the slower extracellular loss of FDA and accumulation of F, (Figures 17b and 18), suggests a dynamic exchange of compounds at the cell membrane. The accumulation of F within the cells and its slow release from the cells allowed the use of fluorescence microscopy to confirm the fluorometric data by visualization and photography (Figure 4).

TABLE X

CALCULATION OF COMPOUND CONTENT OF CELLS AND EXTRACELLULAR FLUID FROM PLANIMETER READINGS OBTAINED FROM GRAPHS OF EXIT-ENTRY CHROMATOGRAPHIC PAPER STRIPS

A. CELLULAR CONTENT

Time in Minutes	FLUORESCEIN			FLUORESCEIN DIACETATE			FLUORESCEIN MONOACETATE			TOTAL
	Planimeter Reading Units	Factor* $1.6181X10^{-14}$ X Planimeter =moles/cell	A Cells in System X moles/cell	Planimeter Reading Units	Factor* $1.9418X10^{-14}$ X Planimeter moles/cell	B Cells in System X moles/cell	Planimeter Reading Units	Factor* $1.6181X10^{-14}$ X Planimeter =moles/cell	C Cells in System X moles/cell	A + B + C (Fl + FDA + FMA) in cells
1.	3.5	5.66×10^{-14}	15.57×10^{-9}	47.2	91.65×10^{-14}	252.05×10^{-9}	6.3	10.19×10^{-14}	28.0×10^{-9}	$.2956 \times 10^{-6}$
2	8.0	12.95	35.60	36.1	70.10	192.78	11.0	17.80	49.0	.2774
3	14.7	23.79	65.41	18.0	35.15	96.65	7.1	11.49	32.0	.1940
4	23.0	41.78	142.39	10.1	19.61	53.93	8.0	12.95	35.0	.2313
5	7.0	11.33	31.15	38.9	75.54	207.72	5.5	8.90	24.0	.2628
6	9.0	14.56	40.05	25.4	49.32	135.63	15.0	24.27	67.0	.2431
7	21.0	33.98	93.45	10.5	20.39	56.07	6.8	11.00	30.0	.1795
8	4.0	6.47	17.80	40.2	78.06	214.67	6.0	9.70	27.0	.2594
9	9.8	15.86	43.61	24.5	47.57	130.83	14.0	22.65	62.0	.2364
10	22.3	36.08	99.23	11.0	21.36	58.74	16.2	26.21	72.0	.2299
11	11.0	17.79	48.95	44.7	86.80	238.70	7.7	12.46	34.0	.3216
12	19.4	31.39	86.33	23.0	44.66	122.82	9.4	15.21	42.0	.2511

*For calculation of specific factor see Appendix.

TABLE X

B. EXTRACELLULAR FLUID CONTENT

Time in Minutes	FLUORESCEIN			FLUORESCEIN DIACETATE			FLUORESCEIN MONOACETATE	Total System Cells & Fluid
	Planimeter Reading Units	Factor* 2.23×10^{-7} X Planimeter	D $\times 10^{-6}$	Planimeter Reading	Factor* 2.67×10^{-7} X Planimeter	E $\times 10^{-6}$	Planimeter Reading F	(A + B + C) + (D + E + F) cell + fluid (Table Xa) = Total
1	0	0	0	22.4	59.80	5.980	0	6.248×10^{-6} M
2	0	0	0	23.1	61.68	6.168	0	6.396×10^{-6} M
3	0	0	0	21.0	56.07	5.607	0	5.769×10^{-6} M
4	.15	.335	.0335	20.8	55.54	5.554	0	5.783×10^{-6} M
5	.28	.624	.0624	20.0	53.40	5.340	0	5.641×10^{-6} M
6	.40	.892	.0892	19.9	53.13	5.313	0	5.578×10^{-6} M
7	.66	1.472	.1472	18.9	50.46	5.046	0	5.343×10^{-6} M
8	.78	1.739	.1739	19.2	51.26	5.126	0	5.533×10^{-6} M
9	.90	2.007	.2007	15.8	42.19	4.219	0	4.594×10^{-6} M
10	.97	2.163	.2163	17.4	46.46	4.646	0	5.020×10^{-6} M
11	1.24	2.765	.2765	16.0	42.72	4.272	0	4.836×10^{-6} M
12	1.27	3.278 $\times 10^{-7}$	.3278	17.9	47.79	4.779	0	5.316×10^{-6} M

Original Inoculum

6.0×10^{-6} M FDA

* For calculation of specific factor see Appendix.

FIGURE 17

NORMAL CELL EXIT-ENTRY ANALYSIS

a. Fluorometric Breakdown Rate of the Control Mother Population and the three cell fractions from Ficoll Gradient Separation.

The control mother population - the graph recording obtained on a Turner Fluorometer when a tube containing cells and substrate are read, to assess the rate at which the substrate is broken down, normally is a straight line. With time the readings recorded go from zero (no visible Fluorescein in the system) to 100 Fl units, showing 1 x 10^{-7} moles in the fluorescent product in the whole system - cells and extracellular fluid (page 52). Each point on this graph indicates the reading obtained for the Exit-Entry tube, after that length of time metabolizing the substrate, just before the cells and extracellular fluid were separated (page 59). This graph shows that the LM cells were metabolizing the substrate in a normal manner prior to separation of the two component parts of the system. The graph recordings for the three fractions indicate the fluorometric breakdown rates of the three populations analyzed in Section C2.g page 94 of this study.

b. Plot of data from Table Xb showing the gradual decrease in substrate concentration and gradual increase in product concentration in the extracellular fluid of a normal Exit-Entry analysis.

Figure 17

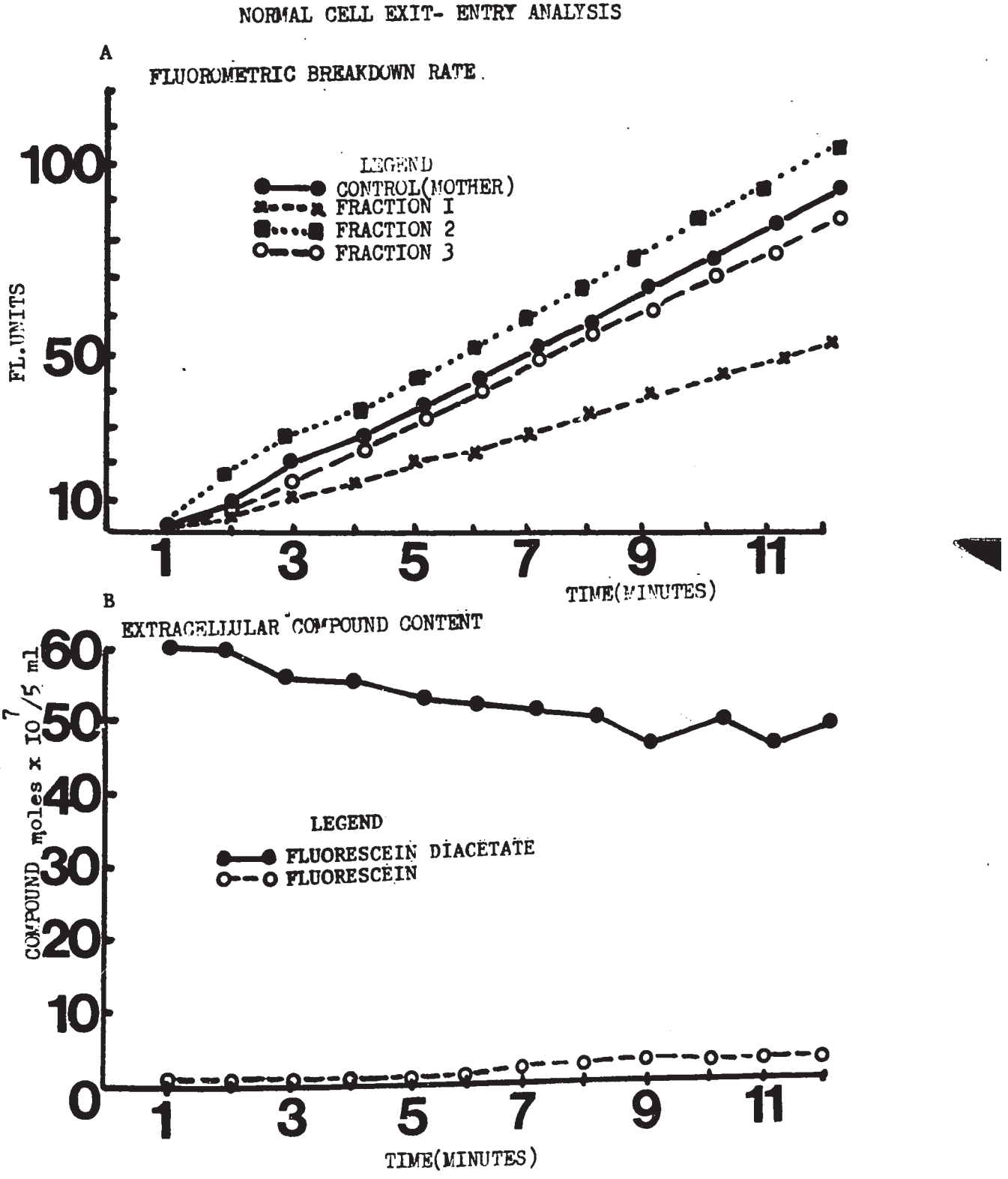

NORMAL CELL EXIT- ENTRY ANALYSIS
A
FLUOROMETRIC BREAKDOWN RATE.
LEGEND
CONTROL(MOTHER)
FRACTION I
FRACTION 2
FRACTION 3
FL.UNITS
100
50
10
1
3
5
7
9
11
TIME(MINUTES)
B
EXTRACELLULAR COMPOUND CONTENT
COMPOUND moles x IO⁷/5 ml
60
50
40
30
20
10
0
LEGEND
FLUORESCEIN DIACETATE
FLUORESCEIN
1
3
5
7
9
11
TIME(MINUTES)

FIGURE 18

NORMAL CELL EXIT-ENTRY ANALYSIS -

CELL CONTENT

Plot of data Table Xa, showing the cyclic nature of the compound content of the LM cell. Each minute the cells were analysed for the quantity of the three compounds present. From these results one point was obtained for each compound. The variations observed, with time, of the three compounds results in the three curves drawn here.

Figure 18

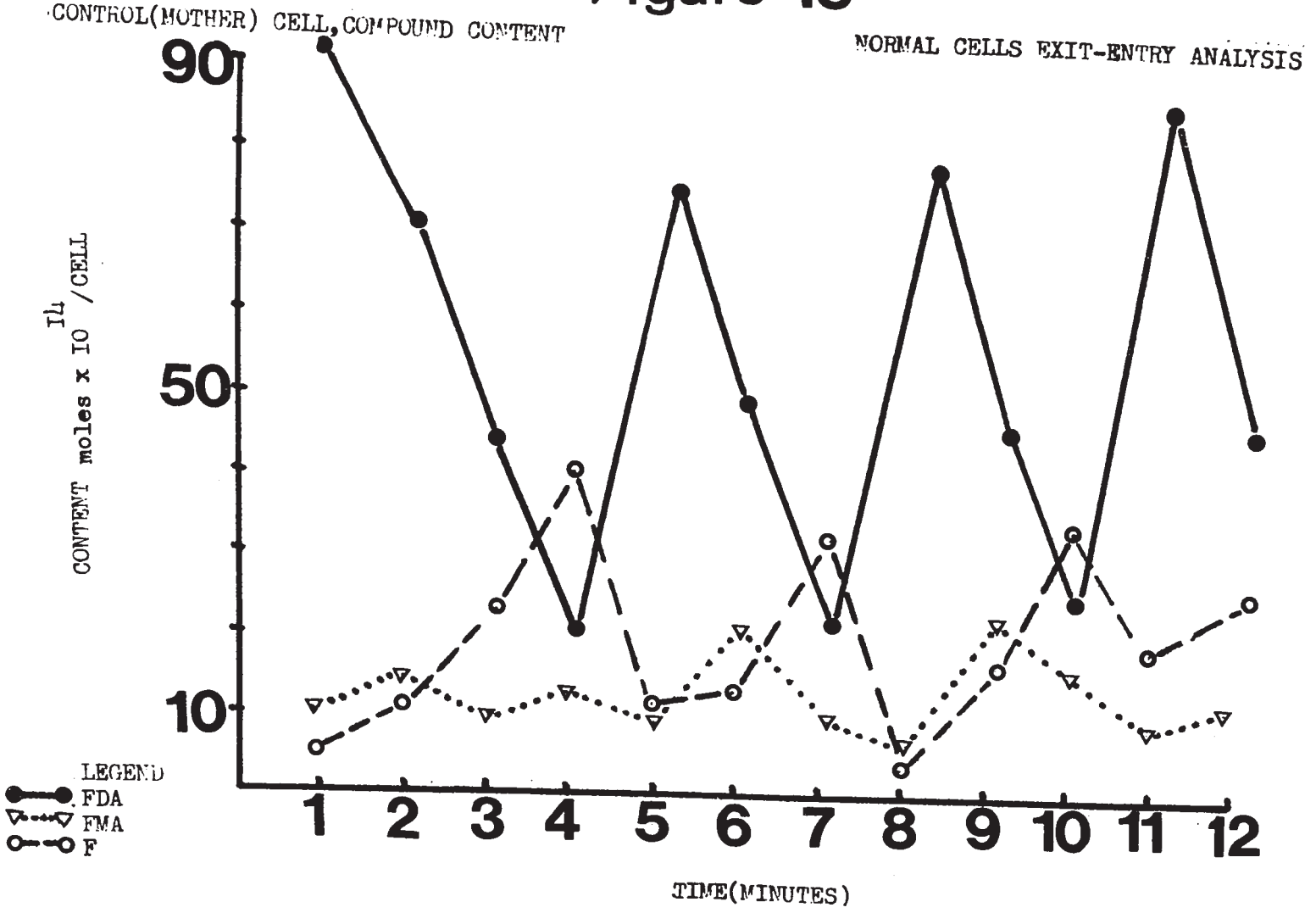

CONTROL(MOTHER) CELL, COMPOUND CONTENT
NORMAL CELLS EXIT-ENTRY ANALYSIS
CONTENT moles x IO -14 /CELL
90
50
10
LEGEND
FDA
FMA
F
1 2 3 4 5 6 7 8 9 10 11 12
TIME(MINUTES)

FIGURE 19

Exit-Entry chromatogram taken under ultra-violet light showing the variation in position and intensity of FDA, FMA and F in the methanol cell extract, 1 minute to 12 minutes. 5 ul samples were spotted on the activated silica gel paper after solubilization of the cells. The chromatograms were run according to the method of Rotman and Papermaster, (1966) (see page 61). Fluorescein stays at the origin, Fluorescein Monoacetate has an rf value between 0.6-0.8, whereas fluorescein diacetate has an rf. of 0.9.

Figure 19

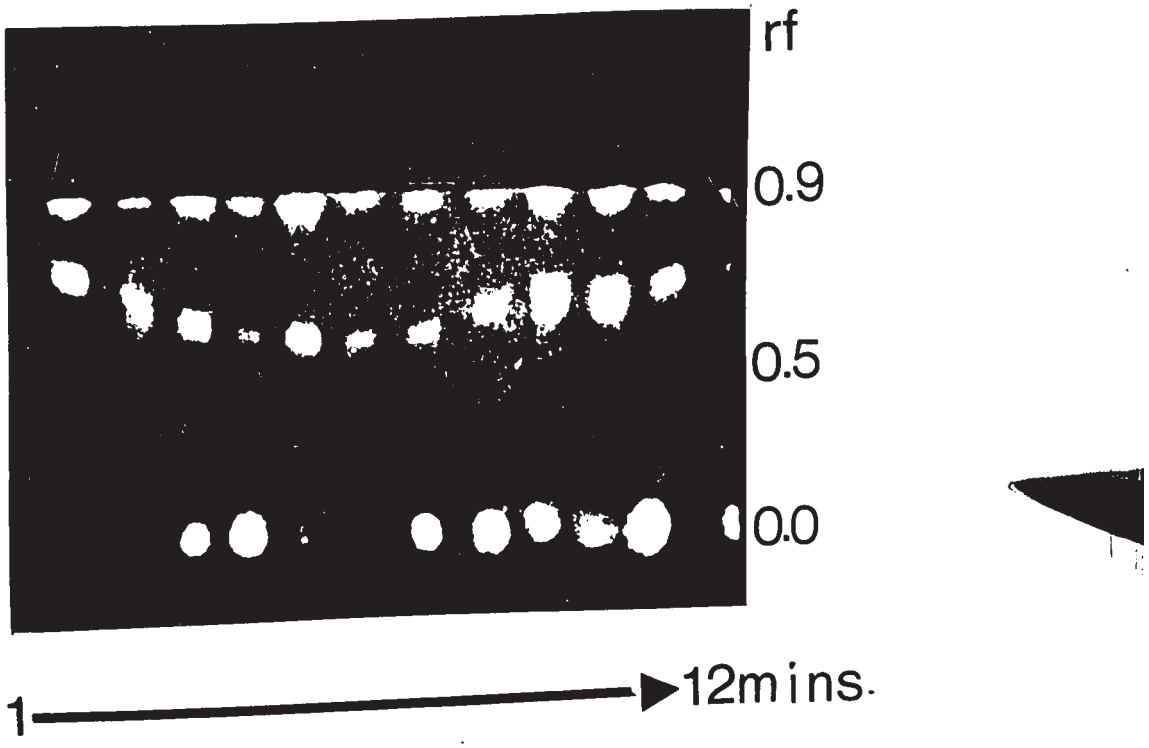


Figure 19

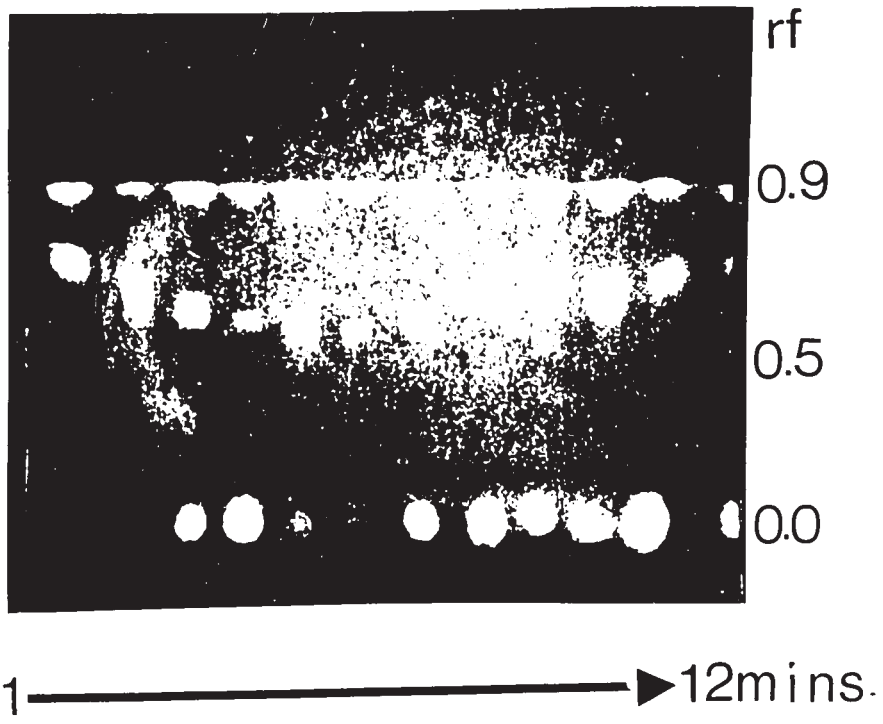


Table XA gives the readings obtained (cellular compound content) for one such normal experiment. It is from this data that Figure 18 was drawn. Table XA shows that for each minute a separate test was made. The amount of each compound was calculated for each minute giving one point on the appropriate compound curve. Table Xb shows the total amounts of the three compounds within the whole system - cells and extracellular medium. Sixteen experiments were done with normal cells, tested either at 30 seconds or minute intervals. The results obtained were virtually identical.

Although periodicities have been recognized in whole organisms as circadian rhythms for many years (Halberg, 1969), virtually nothing has been done at the cellular level. Periodicity of enzyme production during the generation cycle in yeast and bacteria have been cited by many workers, (Sylven et al., 1959; Gorman et al., 1964; Masters et al., 1964; and Kuempel et al., 1965), but except for the work of Chance and his associates the search for possible metabolic cellular oscillations has not been pressed (Chance and Schoener, 1964; Gosh and Chance, 1964; Chance et al., 1965).

Rashevsky, (1968), has shown mathematically that within a single cell, producing or consuming only one substance, periodicities may theoretically occur. Kraeplin, (1969), found leakage rhythms about 5 minutes in length for

yeast cells and concluded that such oscillating properties were indicative of a complex intra and intercellular regulating system. A cycle of approximately 3 minutes was found by Frenkel, (1966), when observing the reduced pyridine nucleotide levels in heart muscle extracts supporting once again the possibility of the universal occurrence of such metabolic cycles.

b. Broken Cell Analysis by the Exit-Entry Method

Two broken cell preparations were analysed to ascertain if the observed, cyclic nature of the metabolic substrate breakdown was dependent upon a functioning membrane. A cell homogenate (40 strokes in a Dounce homogenizer), and a cell sonicate (6 minutes of 30 second bursts on a MSE instrument), were tested (page 59). The results obtained are shown in Figure 20a and 20b, and would seem to indicate that a functioning membrane is necessary for the observed cycle. However, it was concluded that the Exit-Entry method is not an accurate enough method to give definitive results. The swift decrease of FDA within both broken cell systems is unexplained when the slow accumulation of the product and intermediary product is noted. The large quantity of test material (5 ml) from which the small aliquot (10λ) was taken for testing on the chromatogram, plus the lack of homogenity of the test material (chunks of cellular debris were evident) may account for this result.

FIGURE 20

EXIT-ENTRY ANALYSIS OF CELL HOMOGENATE AND SONICATE

a. Plot of data obtained from an Exit-Entry analysis of a broken cell preparation - homogenate, 40 strokes in a Dounce homogenizer. Note the lack of any cyclic phenomenon.

b. Plot of data obtained from an Exit-Entry analysis of a cell sonicate (6 minutes of 30 second bursts). Note the lack of any cyclic phenomenon.

Figure 20

EXIT-ENTRY ANALYSIS OF CELL HOMOGENATE AND SONICATE

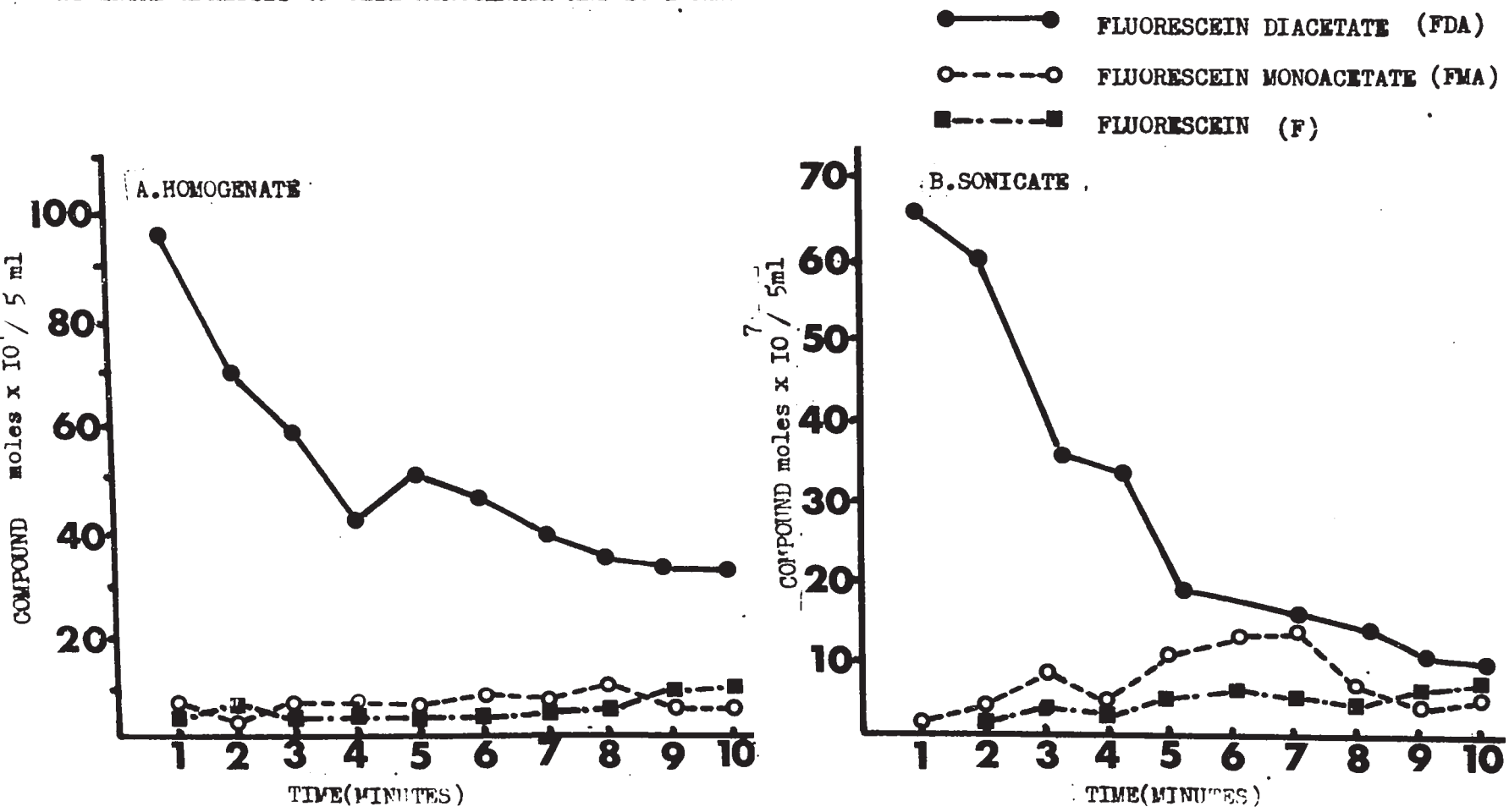


FIGURE 21

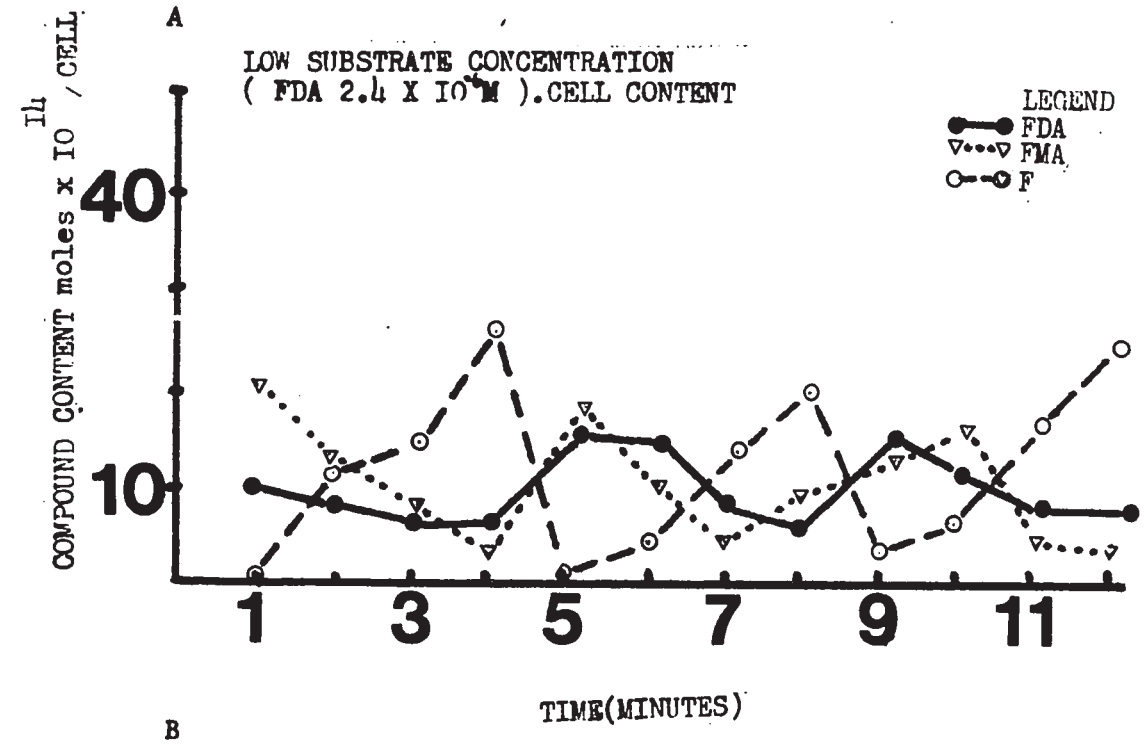
INVESTIGATIONS OF VARIATIONS OF

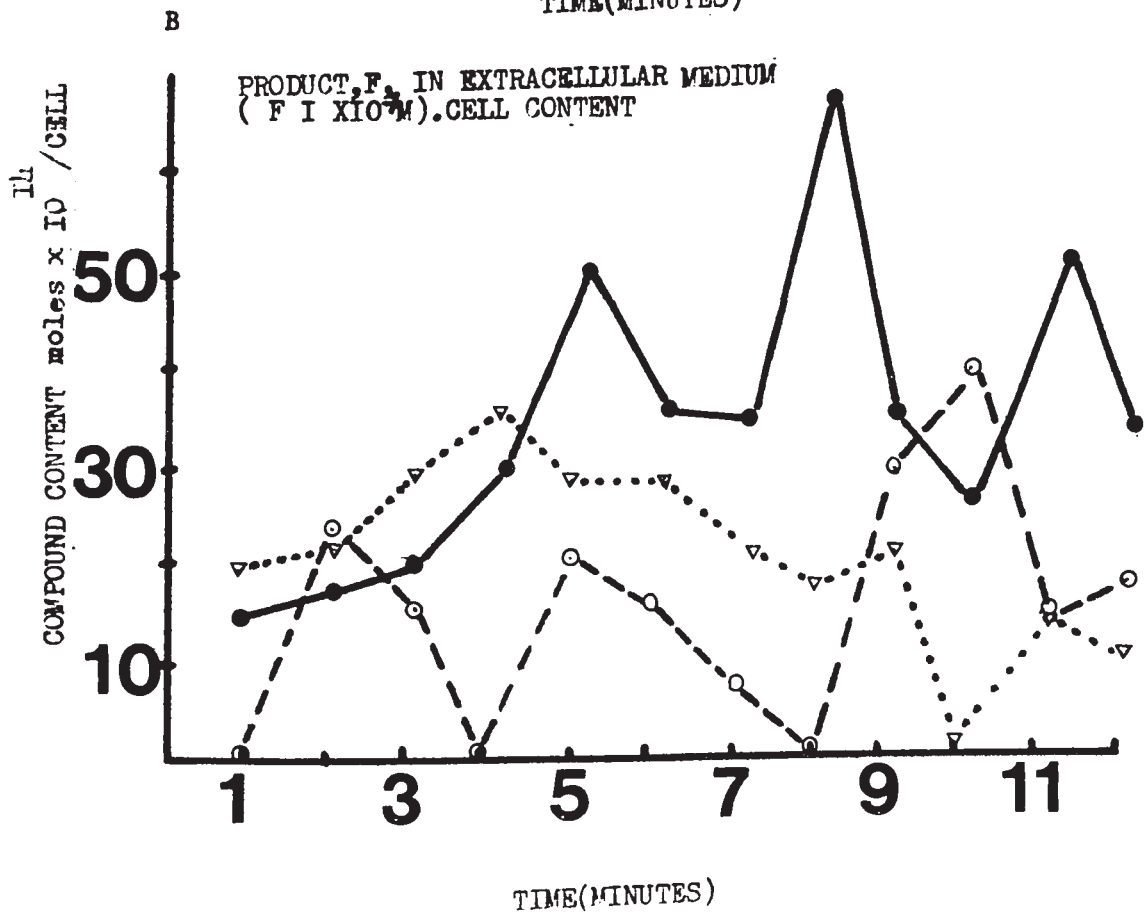
BASIC EXIT-ENTRY METHOD

a. Plot of data obtained from cells when substrate concentration was reduced. Lower limit of metabolic cyclic phenomenon indicated for cells.

b. Plot of data obtained from cells equilibrated in Medium 199 containing 1×10^{-7} M Fluorescein prior to the Exit-Entry analysis. Note the 8 minute lag before initiation of normal cyclic FDA metabolism.

Figure 21


A
LOW SUBSTRATE CONCENTRATION
(FDA 2.4 X IO⁻⁵M).CELL CONTENT
LEGEND
FDA
FMA
F
COMPOUND CONTENT moles x IO⁻¹⁵ /CELL
40
10
1
3
5
7
9
11
TIME(MINUTES)


B
PRODUCT,F, IN EXTRACELLULAR MEDIUM
(F I XIO⁻⁵M).CELL CONTENT
COMPOUND CONTENT moles x IO⁻¹⁵ /CELL
50
30
10
1
3
5
7
9
11
TIME(MINUTES)

Nevertheless, a cyclic fluctuation was not noted, suggesting the possible need of a functioning membrane.

c. Effect of Low Substrate Concentration on the Oscillatory Phenomenon noted for the Substrate FDA when Metabolized by the LM Cell

Frenkel, (1966), noted that theoretical analysis of systems capable of generating oscillations indicates that these cyclic phenomena can occur over clearly defined regions of substrate concentration. The lower limit of substrate FDA concentration was sought and found to be 2.4×10^{-6} M. Figure 21a shows the cellular variation of FDA, FMA and F obtained at this concentration. Increasing the substrate concentration above 6.0×10^{-6} M resulted in manipulation problems which made suspect the results obtained, consequently they are not reported here.

d. Effect of the Addition of Fluorescein (1×10^{-7} M) to the Extracellular Medium prior to the Exit-Entry Analysis

When FDA was added to the extracellular medium in which cells had been allowed to equilibrate with 1×10^{-7} M fluorescein for 15 minutes, it was found that the normal cycle still appeared but only after an 8 minute adjustment period (Figure 21b). The FDA entered slowly and was metabolized at a slower rate. The concentration of the intermediate metabolite, FMA, rose to nearly twice that normally

measured, (Figure 18, page 83), during the first four minutes but gradually fell to the normal level. Comparison of the periodic fluctuations of a normal population showed that the fluorescein forces the cycle out of phase. This appears to be accomplished by slowing down the uptake of FDA and with a back pressure preventing the accumulation of fluorescein within the cell thereby allowing the intermediate product FMA to accumulate. A form of negative feedback as well as an apparent permeability interference is involved.

e. Effect of Esterase Inhibitors, Paraoxon and Eserine Sulphate on the Oscillatory Metabolic Phenomenon Noted for the Substrate FDA with LM Cells

Preincubation of LM cells with either paraoxon (1×10^{-3} M) or eserine sulphate (1×10^{-3} M) in the medium 199 prior to the Exit-Entry Test (page 59) gave the results shown in Figure 22a and b when the chromatogram of cellular compound content was analysed (Figure 19, page 88). Paraoxon would appear to be specifically inhibiting the esterase (S) responsible for the breakdown of FDA, whereas eserine sulphate disrupts the membrane function and only incidentally causes partial inhibition of the substrate's metabolism.

FIGURE 22

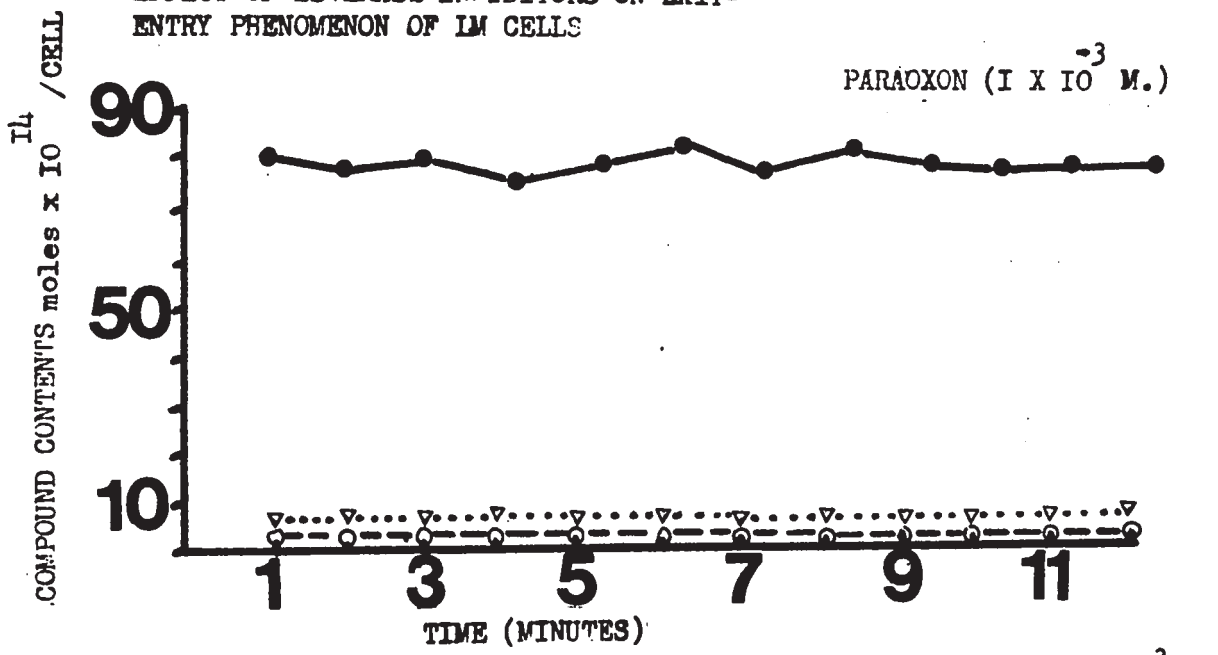
EFFECT OF ESTERASE INHIBITORS ON CYCLIC PHENOMENON OF FDA-ACETYLESTERASE METABOLISM IN THE LM CELL

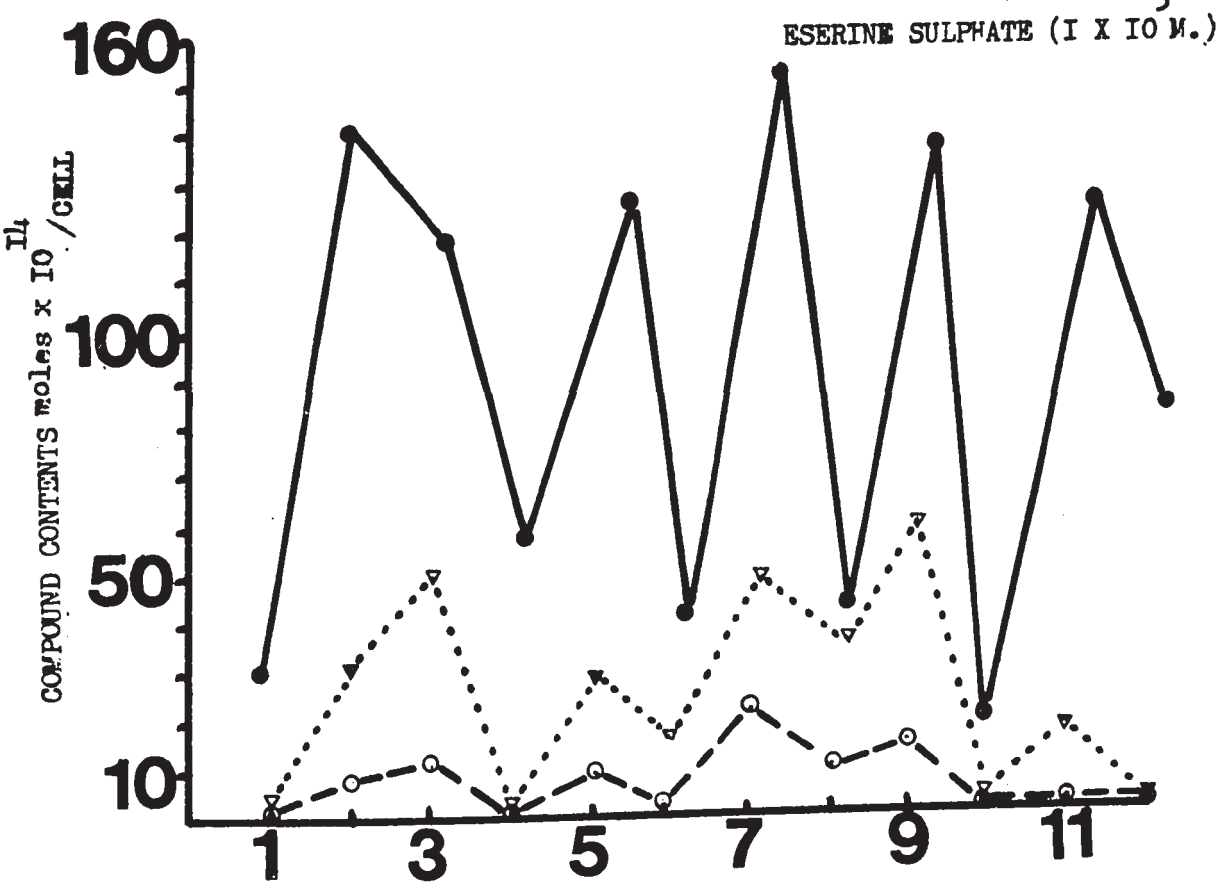
a. Plot of data obtained from cells equilibrated in medium 199 containing 1 x 10^{-3} M paraoxon prior to the Exit-Entry Analysis. Note the cessation of FDA breakdown by the cells. The substrate however, does enter the cell.

b. Plot of data obtained from cells equilibrated in medium 199 containing 1 x 10^{-3} M eserine sulphate (page 35) prior to the Exit-Entry Analysis. Note the destruction of the normal metabolic cycle and the appearance of a pulse-like substrate breakdown.

Figure 22

EFFECT OF ESTERASE INHIBITORS ON EXIT-ENTRY PHENOMENON OF LM CELLS





LEGEND
FDA
FMA
F

f. Possible Mechanism of FDA Metabolism by the LM Mouse Fibroblast Cell

In normal cells the initial high level of FDA, only 30 seconds after the addition of the substrate, indicates that the metabolite does not have any difficulty getting through the cell membrane. However, the quantity of substrate which enters the cell normally remains within a restricted range, suggesting a finite space for the substrate within the cell. Microscopic observations show a few small intracytoplasmic "hot spots" which may be fluorescing lysosomes or other organelles. Once the finite space is filled the membrane becomes impermeable to more substrate and the cell's enzymatic systems, in the form of acetylesterase(s) attacks the metabolite, breaking it down in two steps to fluorescein (Figure 6, page 52). Upon completion of the breakdown of most of the substrate present, the membrane once again allows substrate entry and the swift shift of cell's content from high concentration of the product, F, and low concentration of the substrate, FDA, to the opposite condition with a space of 30 seconds takes place (Figure 18, page 88). Such results support the suggestion of Katchalsky and Spangler, (1968), that the membrane is the gate keeper of the cell and that although an enzyme process itself may be constant the product concentration affects the permeability of the

membrane giving discernible periodic membrane processes.

The initial entry of the substrate cannot be by diffusion as pretreatment of the cells with respiratory inhibitor prevents the entry of the substrate FDA (page 52), suggesting active transport as the possible mechanism of entry. The carrier molecule does not transport fluorescein itself into the cell, although it does transport it out (page 87). Incubation with neuraminidase removes the source of this membrane exclusion (page 67), allowing both entry and exit of the product. The mechanism of transport does not adhere to the classic models (Pardee, 1968; Park et al., 1968), although it may be basically similar to the "pump-leak" model suggested for sodium and potassium.

Although the enzyme(s) activity varies with the age of the cell population (Figure 16), the enzyme would appear to be noninducible. This result is at variance with those results noted for cells incubated in medium containing the product, fluorescein, during a fluorometric run then removed from its presence and another fluorometric test conducted (Table VIII). In this case (page 85) a priming effect was noted. Such a priming effect may be solely characteristic of the product of the FDA - acetylesterase system of the LM mouse fibroblast cell, both this ability and the feedback inhibition effect being linked.

g. Cell Cycle Variations of Enzymatic Cell Activity Analysed Using the Exit-Entry Method

Analysis of the breakdown of FDA by the LM mouse fibroblast cell during different phases of the cell cycle was necessary to ascertain if there was a variation either in the method of breakdown, the rate of breakdown or in the oscillatory nature of the breakdown.

When analysed separately, (Figure 23a, b, c), the three cell fractions, separated on the Ficoll gradient, still showed the oscillation noted with the mixed logarithmic control populations (Figure 18, page 88). However, variations from it and between one another are noted. All three fractions are slower to begin the oscillation taking five or six minutes before the first wave as compared to 3 or 4 minutes for the normal population. This could be accounted for by the fact that these three populations were in 199-Ficoll solution for one hour and although the Ficoll has not been shown to damage the cell membrane it may have a dulling effect on the membrane processes.

Fraction 1 cells which are primarily small (diameter 8u) and in G1, would appear to metabolize the substrate slower as indicated both by the fluorometric rate (Figure 17, page 88), and by the shallowness of the peaks and troughs for the three compounds (Figure 23a). However, considering both the volume and surface area of these small cells compared to the larger cells in the other two

FIGURE 23

EXIT-ENTRY ANALYSIS OF FICOLL GRADIENT SEPARATED LM CELLS

a. Fraction #1 - cells predominantly in G1, cellular compound content. Cyclic fluctuation of compounds evident. Compare to mixed logarithmic culture, Figure 18, page

b. Fraction #2 - mixture of cells, predominantly in S with some in G2 and M. Cyclic fluctuation of compounds evident.

c. Fraction #3 - mixture of cells, predominantly in G2 and M with some late S. Cyclic fluctuation of compounds evident.

Figure 23

EXIT-ENTRY EXPERIMENT OF FICOLL GRADIENT
SEPARATED LM MOUSE FIBROBLAST CELL FRACTIONS

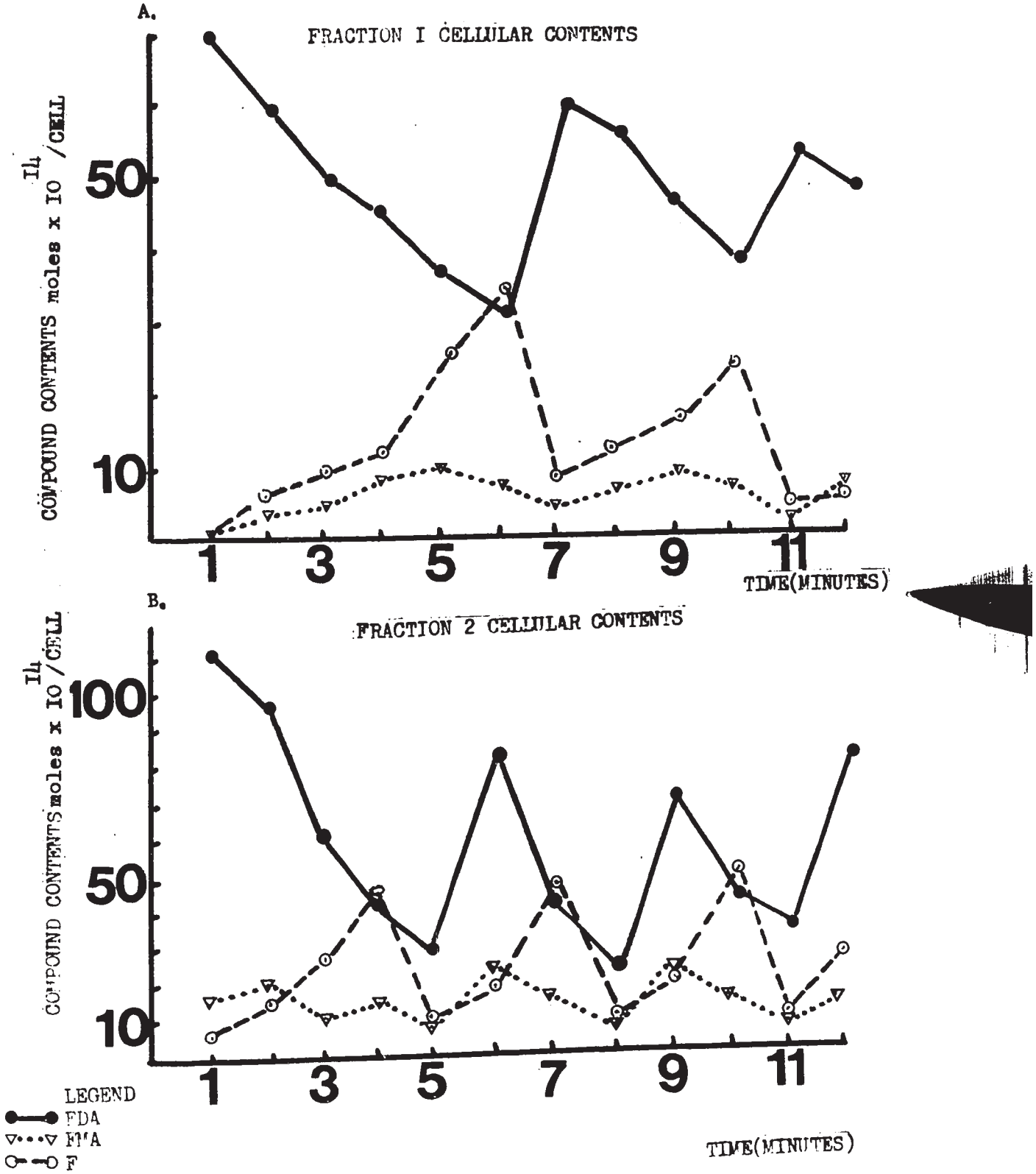
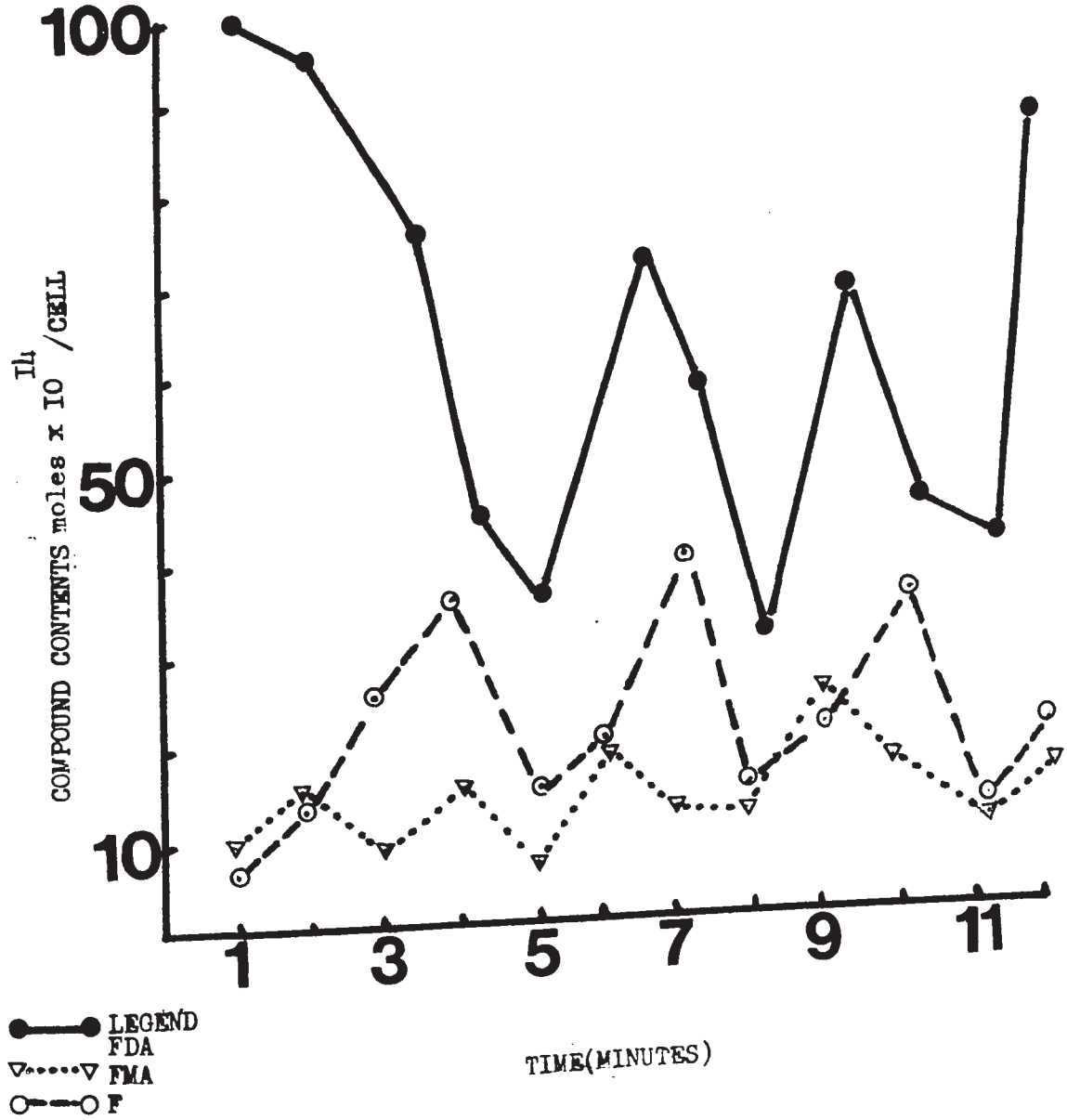


Figure 23

EXIT-ENTRY EXPERIMENT OF FICOLL GRADIENT
SEPARATED LM MOUSE FIBROBLAST CELL FRACTIONS

C. FRACTION 3 CELLULAR CONTENTS



fractions, per unit volume and per unit surface area (see Table XI, page 96), they not only take up 2 or 3 times as much substrate but are more active metabolically. When the specific ratios of various cell fractions characteristic to the observed fluorometric rate of the fraction are calculated, fraction 2, per microgram of protein, is more active fluorometrically than fraction 1 (Table XI, page 96). An increased percentage of enzymes per total cell protein, in this fraction, as compared to fraction 1, may account for this. The larger cells in fraction 3 (diameter 12-16 u) would actually be the most sluggish metabolically despite the fact that on a per cell basis they take up more substrate than either of the other two fractions. Rotman and Papermaster, (1966), observing single cells of approximately 20 u in diameter (mouse lymphoma ML - 3882 B2 clone), found that kinetically both the substrate accumulation and the product rate of exit varied extensively from cell to cell. The cells they viewed might have been in different phases of the cell cycle, since they made no attempt to characterize the cells they observed in this manner. Single cell analysis during a complete life cycle might yield some interesting results with regard to FDA metabolism variations. However, as Kahn et al., (1962), points out, differences in population density, mitotic phase, and propagation methods in themselves produce heterogeneity in the metabolic and synthetic activity of individual cells.

TABLE XI

SPECIFIC RATIOS OF FLUOROGENIC SUBSTRATE BREAKDOWN ACTIVITY FOR CELL FRACTIONS SEPARATED ON A FICOLL GRADIENT WHEN VARIOUS PHYSICAL CHARACTERISTICS OF THE CELL ARE CONSIDERED

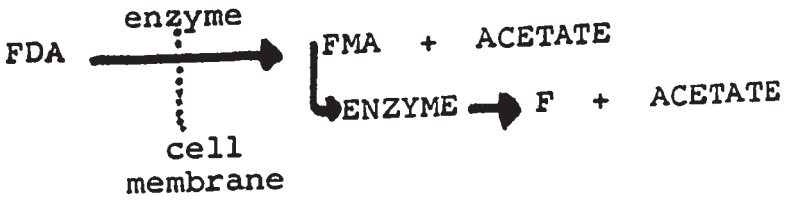
Gradient Fraction	X/Cell		X/unit cell surface area		X/unit cell volume		X/Ug Protein	
		RATIO		RATIO		RATIO		RATIO
#1	0.89	1.0	2.45	1.0	2.45	1.0	0.33	1.0
#2	1.40	1.6	1.54	0.63	0.96	.39	0.42	1.3
#3	1.27	1.4	0.88	0.36	0.39	.16	0.23	0.70
Mother	1.09	1.2	-	-	-	-	0.30	0.90

X = Fluorometric units/min

Since the biochemical approach necessitates the use of large cell populations which tend to mask the intrinsic variations between cells, at the present time, only general statements as to cellular activity at different phases of the cell cycle can be made. The cyclic breakdown of the substrate fluorescein diacetate exists throughout the cell cycle with the smaller cells in G1 more metabolically active than the larger cells in S, G2 and M.

From these enzymatic experiments the following conclusions concerning the metabolism of the molecule fluorescein diacetate (FDA) by the LM mouse fibroblast cell can be made:

1. The substrate FDA is broken down by the LM mouse fibroblast cell (Figure 6, page 52).



2. This breakdown takes place throughout the cell cycle.

3. The breakdown process is accompanied by an opposing oscillatory accumulation of substrate and product within the LM mouse fibroblast cell.

4. Paraoxon (E 600), an esterase inhibitor, inhibits the breakdown: FDA ✳→ FMA

5. The enzyme(s) considered responsible for the substrate breakdown is an acetylesterase(s) (3.1.1.6). The enzyme(s) is probably membrane bound, lysosomal as well as plasmallema.

6. An unknown source of energy (ATP?) is required for the entry of the substrate.

7. An intact functioning membrane is probably necessary for the oscillatory phenomenon of substrate hydrolysis.

8. Removal of sialic acid residues by enzymatic action only slightly inhibits the substrate's breakdown by the cell.

9. There is an inhibition by the product fluorescein. Smaller, younger cells in G1 are most resistant to this inhibition whereas cells in other phases of the life cycle have a variable resistance. This resistance would appear to be a function of the membrane. Sodium acetate does not inhibit the reaction.

10. Small young cells, from logarithmically growing cell populations, metabolize the substrate at a higher rate than cells in other phases of the cell cycle.

11. Logarithmically growing cell populations are more active in substrate breakdown than those in either the lag phase or stationary phase cell population.

Although stationary cell populations have a greater percentage of small cells (33% as opposed to 12.7% for the logarithmically growing cell population) these are not all in G1. Non-essential protein and carbohydrate stores have probably been used up by these cells to overcome adverse growing conditions within the culture.

E. IMMUNOLOGICAL STUDIES

The fluorometric enzyme monitoring system was used with immunological specific immune serum to determine if the method was adaptable to immunological testing of the cell. To ascertain if either the acetylesterase system or cell cytology would be adversely affected by nonimmune serum or by serum components, preliminary tests were conducted investigating various parameters of the method. Table XII shows these parameters.

1. Animal Source of Serum

Figure 24a and b shows the results obtained with serum from various animal sources, both fresh and inactivated, 30 minutes at 56°C. Rabbit serum was judged to be the ideal animal source of LM cell antibodies, (Figure 24a and b).

In data not presented here, utilizing Immune Gamma Globulin (IGG) it was found that fresh complement potentiates the system and that unexplained noncomplement effects were possible.

TABLE XII

ANALYSIS OF THE FLUOROMETRIC SYSTEM:

ADDITION OF VARIOUS SERA & SERUM COMPONENTS

Factor Investigated	Concentration and Characteristics of Added Substances
1. Animal source of serum	Calf serum) Horse serum) - Grand Island Biological Company Suppliers
	Guinea pig serum) Rabbit serum) - Dept. of Bacteriology animals.
	Anti LM rabbit serum - Two sources, unpurified and purified according to Campbell et al., 1964 (page 89).
	Concentrations varied from 0.10% to .0001% in Medium 199
	Active and Inactivated (30 mins at 56°C) both tested
2. Albumin	Concentrations varied from .03 g/100 ml to 3.608 g/100 ml of medium 199. That is 1/100th, to the normal amount found in serum. Bovine serum Albumin was supplied by Calbiochem, Los Angeles, Calif., U.S.A.
3. Merthiolate	0.01% to 0.1% in medium 199. This is a normal serum preservative.

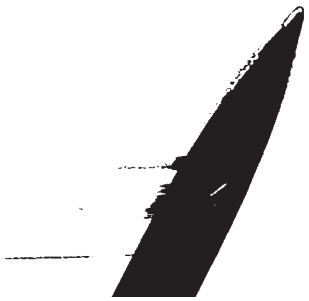


TABLE XII (Continued)

Factor Investigated	Concentration and Characteristics of Added Substances
4. Comparison of Fluorometric test with added anti LM serum and Ring test and Agglutination tests with same serum dilutions	Inactivated Anti LM rabbit serum dilutions 1:50 to 1:5,000
5. Complement	Effect of Addition; active & inactivated to system with anti LM serum (inactivated) & preimmune rabbit serum

FIGURE 24

CYTOLOGICAL AND METABOLIC EFFECTS OF VARIOUS SERA ON THE LM MOUSE FIBROBLAST CELL

Cytological testing was conducted as noted on page 33
Fluorometric testing was conducted as noted on page 52

a. Sera from various animal sources. Guinea pig, calf and horse sera had adverse effects on both factors tested, whether serum was fresh or inactivated. Rabbit serum inactivated did not affect either factor tested.

b. Rabbit Sera Effects. Rabbit serum with an unrelated antibody (anti-horse) had adverse effects on both factors tested whether fresh or inactivated serum was used. Specific anti-LM rabbit serum shows potentiation effect of the fluorometric system and disturbs the cell cytology (blebbing membranes).

Figure 24

a. EFFECT OF VARIOUS SERA ON THE L M MOUSE FIBROBLAST CELLS, CYTOLOGICAL AND METABOLIC.

OBSERVATIONS	STATE	NORMAL CELLS	NORMAL PREIMMUNE RABBIT SERUM	GUINEA PIG SERUM	CALF SERUM	HORSE SERUM
FLUOROMETRIC RATE, fl units per minute.	FRESH	NORMAL	4X	I	2X	I plus X
FLUOROMETRIC RATE, fl units per minute.	INACTIVE (30 min. at 56 C)	NORMAL	NORMAL	Iplus X	2X	I plus X
CYTOLOGY OF CELLS	FRESH	NORMAL				
CYTOLOGY OF CELLS	INACTIVE (30 mins at 56 C)	NORMAL	NORMAL			

LEGEND : THE MORE SHADED A SQUARE, THE MORE EXAGGERATED THE CELLULAR EFFECT. CLEAR SQUARES INDICATE NORMAL CELL APPEARANCE OR FUNCTION. THE NUMBERS INDICATE DEGREE OF FLUOROMETRIC RATE INCREASE. "I" INDICATES INSTANTANEOUS FLOODING OF THE SYSTEM WITH FLUORESCENCE.

Figure 24

b. EFFECT OF VARIOUS SERA ON THE L M MOUSE FIBROBLAST CELL

CYTOLOGICAL AND METABOLIC.

OBSERVATIONS	STATE	RABBIT ANTI HORSE SERUM ANTIBODY	RABBIT ANTI LM CELL Ab. UNPURIFIED	RABBIT ANTI LM CELL Ab. PURIFIED
FLUOROMETRIC RATE, fl units per minute	FRESH	I	I	I plus X
	INACTIVE (30 mins at 56 C)	3X	4X	I plus X
CYTOLOGY OF CELLS	FRESH			
	INACTIVE (30 mins at 56 C)			

LEGEND AND CELL CONTROL AS OF FIGURE 23 a.

The effect on the fluorometric system of rabbit serum containing antibodies to either horse or the LM mouse fibroblast cells also was studied. Potentiation of the metabolic breakdown rate was noted for all the sera, whether fresh or inactivated (Figure 24). Comparing the preimmune rabbit serum with the anti LM serum, since both sera came from the same rabbit, it was concluded that the cell specific antibodies were responsible for the increase in substrate breakdown rate, by the LM cells noted. Merthiolate, a serum preservative, was found to enhance the substrate breakdown rate and consequently was not used.

2. Addition of Albumin to Medium 199 and Its Effect on the Fluorometric Rate and Cytology of the LM Mouse Fibroblast Cell

Within serum the most abundant protein present is albumin, while humoral immunoglobulins are in minimal quantities. The normal effect of such a macromolecule on the cell was therefore of importance. Dilutions of purified bovine albumin (BSA) were made in 199 medium ranging from that amount normally found in serum to .01% (Figure 25). Fluorometrically all the dilutions enhanced the FDA breakdown rate by the mouse cell, but the degree of enhancement varied. The enhancement plateau indicates perhaps a dilution dependent conformational change of the complex macromolecule.

100 A

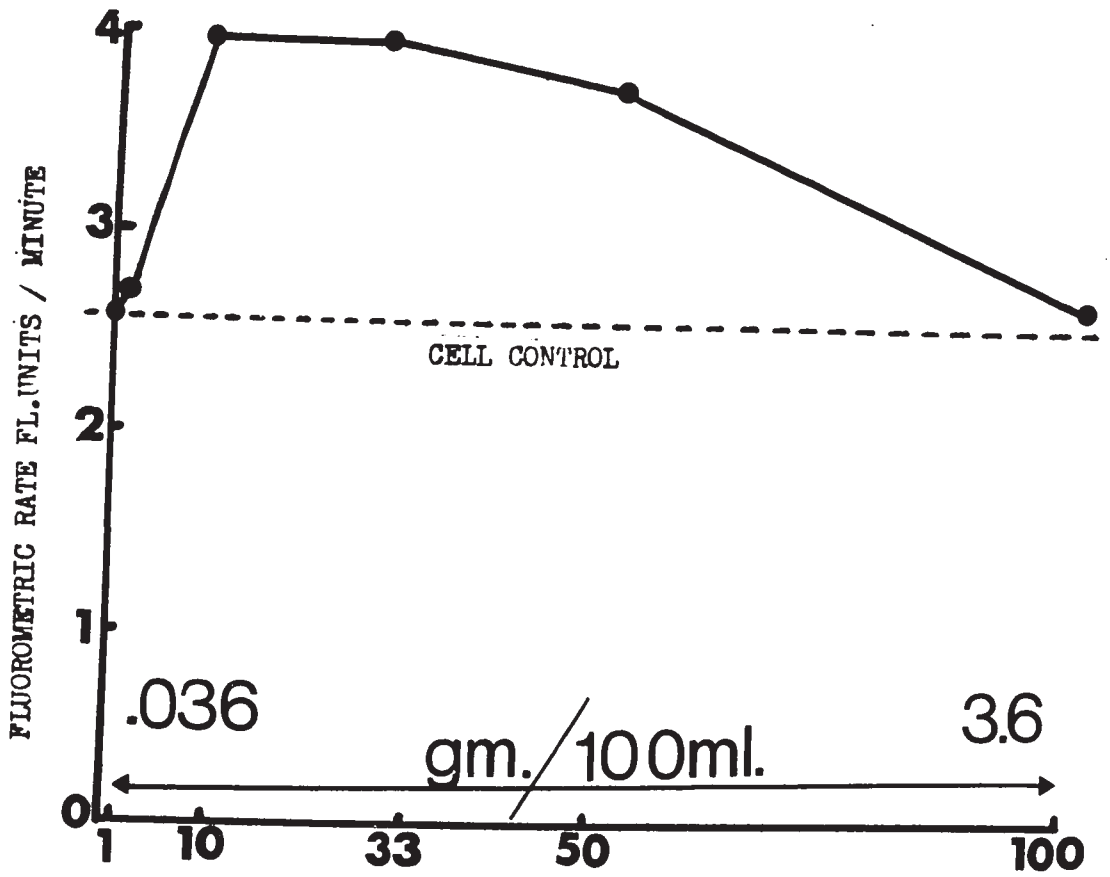
FIGURE 25

EFFECT OF ALBUMIN ON THE FDA-ACETYLESTERASE SYSTEM OF THE LM MOUSE FIBROBLAST CELL

Various concentrations of Bovine Serum Albumin in medium 199, ranging from 1/100 for the amount normally present in serum to the normal level, were tested.

Figure 25

EFFECT OF ALBUMIN ON THE FDA-ACETYLESTERASE SYSTEM OF THE LM MOUSE FIBROBLAST CELL



% ALBUMIN NORMALLY PRESENT IN RABBIT SERUM

Such results underline the necessity both to accurately control the serum dilutions tested on the LM mouse fibroblast cells and to interpret any results obtained in this enhancement plateau region with caution. Cultured cells often are grown in medium containing serum. The effect of such constant exposure to albumin is unknown although growth enhancement phenomena are reported (Sanford et al., 1955).

3. Dilution Effects of Anti LM Rabbit Serum Upon the Observed Fluorometric Breakdown Rate of FDA by the LM Mouse Fibroblast Cell

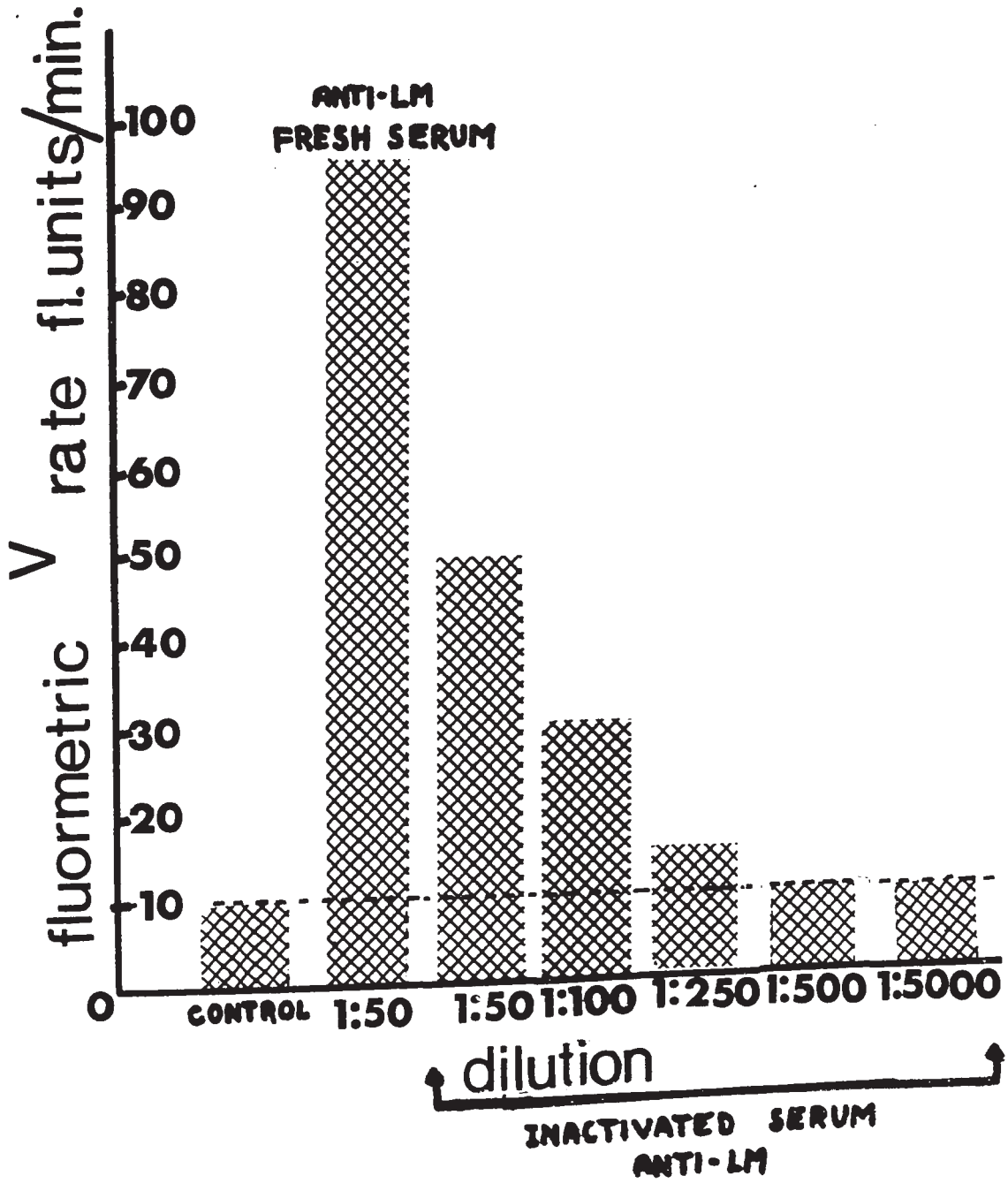
When anti LM serum is added to the extracellular medium 199 of a fluorometric test in various dilutions the effect on the fluorometric breakdown rate observed are interesting. An antibody titre was measured in LM cells agglutinating units (5.5×10^3 cells in 5 λ of saline plus 5 λ of serum). The bargraph in Figure 26 shows the results of the anti LM rabbit serum dilution on this breakdown rate by the LM mouse fibroblast cells. Fresh anti LM rabbit serum causes almost instantaneous flooding of the system with fluorescence, as was previously noted (Figure 24). When diluted the inactivated anti LM rabbit serum exerts an enhancing effect on the system to somewhere between the serum medium 1:250 and 1:500 dilutions. The serum was also tested using slide agglutination (page 65). Here cell clumping was observed

FIGURE 26

SERUM DILUTION EFFECT ON LM MOUSE FIBROBLAST CELLS

Fresh and inactivated anti-LM rabbit sera were tested using the fluorometric method (page 52). Dilutions of inactivated sera from 1:50 to 1:5000 were used.

Figure 26


fluormetric V rate fl.units/min.
100
90
80
70
60
50
40
30
20
10
0
ANTI-LM
FRESH SERUM
CONTROL
1:50
1:50
1:100
1:250
1:500
1:5000
dilution
INACTIVATED SERUM
ANTI-LM

down to the serum 1:256 dilution. Such close correlation between the two sets of results would lead to the conclusion that the fluorometric enhancing mechanism or factor and the clumping factor (the antibody-antigen complex) at the very least are interdependent and at the most the same thing.

From this series of experiments it was concluded:

1. The animal source of anti LM serum is important. Numerous unknown species factors have a deleterious effect on the LM mouse fibroblast cell membrane and metabolism. Rabbit is the best source of serum, in this case, showing the least cytological and metabolic cell effects.

2. Some of the unknown species factors, mentioned above, are heat labile. However, this property varies from species to species.

3. Albumin, a complex macromolecule in serum, itself can have membrane effects. This property is dilution dependent and probably indicates conformational changes of the molecule as it interacts with the cell.

4. Serum preservatives such as merthiolate disturb the metabolic equilibrium of the LM cell and should not be used.

5. Fresh complement (rabbit serum) contains nonspecific esterases which destroy the usefulness of the FDA-LM cell metabolic breakdown system method. These esterases are heat labile.

6. Inactivated anti-LM rabbit serum (IAR) enhances the observed fluorometric breakdown rate of FDA by the LM mouse fibroblast cell, whereas inactivated preimmune serum from the same rabbit does not. Humoral antibodies would appear to be implicated.

7. Titration of inactivated anti-LM rabbit serum using the FDA-LM cell metabolic breakdown method gives elevated rates up to the point of antibody activity noted using the agglutination test.

4. LM Cells in Various Stages of their Cell Cycle and the Effect of Serum (Inactivated Preimmune and Inactivated anti LM) on the Fluorometric Rate of Breakdown of FDA, their Subsequent Growth Patterns and their Protein and DNA Content as compared to a Normal Ficoll Gradient Divided Population

The basic question, "Does immune serum affect cells in different parts of the cell cycle in a different way?" underlies this experiment. Fluorometry, growth patterns, and protein and DNA content were investigated comparing normal cells to preimmune serum treated populations and to anti LM

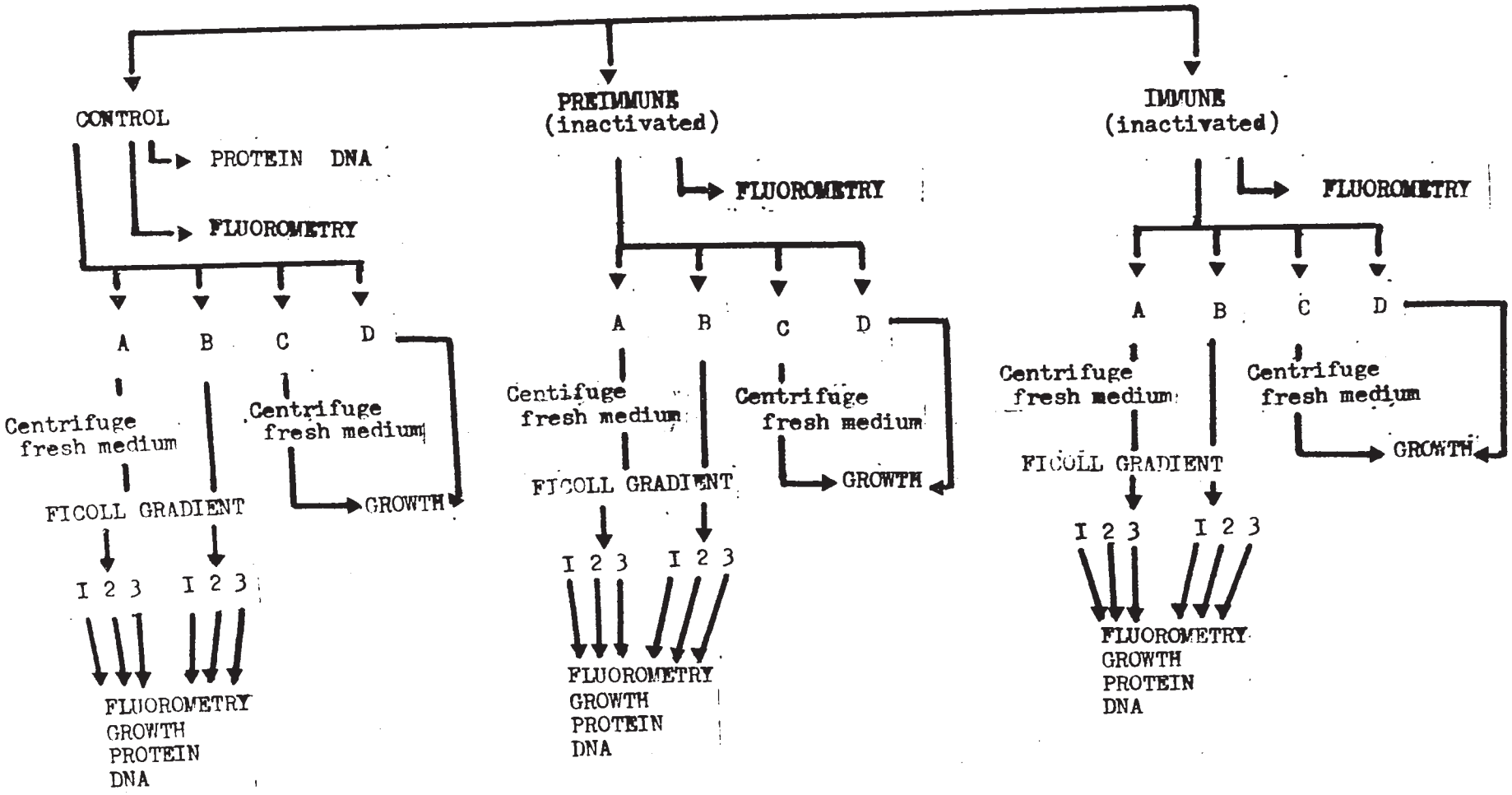
FIGURE 27

PROTOCOL FOLLOWED FOR LM CELLS

AND SERA TEST

Investigation of metabolic, chemical and growth action of preimmune and anti-LM serum was undertaken using a single cell population. Subsequent separation of cell fractions on Ficoll gradients allowed for monitoring of variations of effect within the cell cycle.

Figure 27


CELLS
CONTROL
PROTEIN DNA
FLUOROMETRY
A B C D
Centrifuge fresh medium
Centrifuge fresh medium
GROWTH
FICOLL GRADIENT
I 2 3 I 2 3
FLUOROMETRY
GROWTH
PROTEIN
DNA
PREIMMUNE (inactivated)
FLUOROMETRY
A B C D
Centifuge fresh medium
Centrifuge fresh medium
FICOLL GRADIENT
GROWTH
I 2 3 I 2 3
FLUOROMETRY
GROWTH
PROTEIN
DNA
IMMUNE (inactivated)
FLUOROMETRY
A B C D
Centrifuge fresh medium
Centrifuge fresh medium
GROWTH
FICOLL GRADIENT
I 2 3 I 2 3
FLUOROMETRY
GROWTH
PROTEIN
DNA

serum treated populations. Figure 27 gives the outline of this experiment. The aliquots of cells used for these analyses (fluorometry and chemical analyses), were taken 85 minutes after the initial addition of the normal logarithmically growing cells to the various media. Initially, a cell population was divided into three equal portions and placed into centrifuge bottles. The cells were pelleted (1000 g for 10 minutes) and the liquid evacuated from two of the bottles. The exact amount of medium 199 removed was measured using a graduated cylinder. The one population, from which the medium was not removed, was gently rocked to resuspend the cells once more in the medium 199. This centrifuge bottle was placed in the water bath at 37°C. To the other two bottles was added two different solutions of medium 199 equal to the amount removed. In one the medium contained .05% of inactivated preimmune rabbit serum, and in the other medium there was .05% of inactivated anti LM rabbit serum. Both sera came from the same rabbit. These two centrifuge bottles, after gently rocking to resuspend the cells, were also placed in the water bath at 37°C, and a 15 minute preincubation period was given to all three bottles. Gentle mixing by shaking of the bottles was done a number of times during the preincubation period. Just before the 15 minute preincubation time was up cell inoculums from the bottles were added to spectronic colorometric tubes in the water bath, also containing medium such as was in the

centrifuge bottles and at the same concentration. Each of the 3 centrifuge bottles had their cells divided into four parts. One portion A, after centrifugation to remove cells from the bathing medium and after resuspension in fresh medium 199, was to be run on a Ficoll gradient (page 40). The second portion B, was to be run on a Ficoll gradient immediately without removing the bathing medium. The third portion, C, was to be placed in fresh medium 199 for incubation (starting concentration 5.0×10^5 cells/ml) after centrifugation to remove cells from the bathing medium. The last portion, D, was adjusted to the cell count desired as a starting point for all the incubating populations (5.0×10^5 cells/ml) with medium containing the inactivated sera, where applicable, placed in a sterile, stoppered Erlenmeyer flask and put in the shaker culture incubator. The first portion, A, for all three populations, was spun down on the International centrifuge, 1000 g for 10 minutes, to pellet the cells. Afterwards the cells were suspended in fresh medium 199 and layered over Ficoll gradients. Four large continuous gradient tubes, 15% to 22% Ficoll, were needed for each of the three populations. The second portion, B, from the 3 populations was layered over the Ficoll gradients, four large continuous gradient tubes being necessary for each population, and spun down immediately on a second International centrifuge. The third portion, C, from the three populations was spun down in three small desk

centrifuges (UJI, Christ Co., Germany) each having been divided equally among 6 sterile centrifuge tubes. The liquid was evacuated from the cells and the cells resuspended, collected and brought to the desired cell count for all the incubating populations (5.0×10^5 cells/ml) and placed in a sterile, stoppered, Erlenmeyer flask and put in the shaker culture incubator.

During the time that these various cell populations were being spun down the fluorometric test, (page 52), was being conducted testing the effect of preimmune inactivated rabbit serum and anti LM inactivated rabbit serum on the FDA metabolic breakdown rate of the LM mouse fibroblast cells.

Upon completion of the Ficoll gradient runs similar bands of the same population from the four gradient tubes were combined. An aliquot of cells was taken from each band, of each population, washed in medium 199, centrifuged down to pellet the cells, resuspended to an OD. of 0.2 at 680 nM in fresh medium 199 and tested fluorometrically. Half of the cells from each population band were taken, washed with medium 199 to remove any adhering Ficoll, pelleted down by centrifugation and using the methods described on page 40 were used to ascertain the protein and DNA content of the cells. The other half of the cells from the Ficoll gradient bands, if they came from portion A which had been removed from contact with serum, were adjusted to a cell count of 5.0×10^5 cells/ml with fresh medium 199, placed in a

stoppered sterile flask, then incubated in the shaker culture incubator. If, however, the cells came from portion B, those cells in constant contact with the serum, they were adjusted to the required cell count with medium containing the appropriate serum.

All 24 cell populations had equal amounts of medium and cells. Of the 24 flasks, there were 6 each for the normal divided cell population, cells treated with pre-immune inactivated serum and cells treated with immune anti LM serum, as well as 2 for the undivided populations of each of the three experimental populations. The growth patterns and cytology were monitored for three days after the initial test.

In Table XIIIa the effect of the sera on the cell's fluorometric rate of FDA breakdown is shown. In the control population it will be noted that fractions 2 and 3, containing the larger cells in S, G2 and M, show a higher gross fluorometric rate than the cells in fraction 1. This small cell fraction, however, although according to cell count is equal that of fractions 2 and 3, have nevertheless a smaller cell surface area and volume: (Table XI, page 96).

FRACTION	1	2	3
AREA	1 :	2.5 :	4.25
VOLUME	1 :	4.0 :	9.0

TABLE XIII

COMPARISON OF METABOLIC ACTIVITY AND CHEMICAL CONTENT OF FICOLL SEPARATED CELLS AFTER TREATMENT WITH PREIMMUNE SERUM OF ANTI-LM SERUM

A. COMPARISON OF FLUOROMETRIC RATES

Population	Control	Preimmune	Immune	Ratio Preimmune/Immune
Initial	3.00	+ 3.70	+ 3.67	1.01
Gradient				
Fraction #1		+		
Serum Present	-	3.20	3.00	1.06
No Serum	2.45	2.80	3.45	0.81
Fraction #2				
Serum Present	-	4.50	3.56	1.26
No Serum	3.85	3.75	4.00	0.93
Fraction #3				
Serum Present	-	4.25	3.85	1.10
No Serum	3.50	4.00	4.17	0.95

TABLE XIII (Continued)

B. COMPARISON OF PROTEIN AND DNA CONTENT OF LM CELL POPULATIONS

Population	Control		Preimmune		Immune Anti LM	
	*Protein	*DNA	*Protein	*DNA	*Protein	*DNA
Initial	33.7 X	1.43 X	33.7 X	1.43 X	33.7 X	1.43 X
Fraction #1 Serum Present	24.3	0.84	24.8	0.90	25.4	1.1
Fraction #2 Serum Present	28.1	1.21	27.1	1.28	26.0	1.4
Fraction #3 Serum Present	50.2	1.47	51.7	1.45	47.9	1.49

* γ/cell x 10^{-6}

These smaller cells in fraction 1 are therefore actually the most metabolically active cell fraction. When cells incubated in preimmune sera are viewed it is noted that in all cases the fluorometric rate has been enhanced by the presence of serum. Those cells which have remained continually in the serum medium have an elevated rate above that of the cells washed clean of the serum before separation on the Ficoll gradient. Sanford et al., (1955), found that serum stimulated the growth of L cells. Increased cellular activity as noted here doubtless would be the first step in such an action. When the fluorometric rate of the cells incubated in immune sera medium is studied though, the opposite effect is noted. Those cells which have remained in the presence of the immune serum have a reduced rate, indicating cellular distress whereas those cells no longer exposed to the immune serum have a rate elevated above that for cells similarly handled but with preimmune serum (Table XIIIa). A priming effect of the cell's metabolic system would seem to be the initial effect of exposure to cellular anti sera. All three separated cell fractions show the same reaction fluorometrically. The small newly formed G1 cells would appear to be most affected by serum incubation especially by immune serum if it is promptly removed. Shodell and Rubin, (1970), observed a similar serum stimulation in G1 of chick embryo cells and suggest that the complex mixture of lipids,

hormones, enzymes and other proteins and peptides present in serum can be expected to stimulate a variety of cellular activities in this phase, a phase which normally is concerned with the production of various substances of later use in DNA synthesis and also mitosis (Taylor, 1965; Mazia, 1961; Donnelly and Sisken, 1967; and Robbins et al., 1968).

Analysis of the continuing growth effect of preimmune and immune sera on the various populations and cell fractions is shown in Table XIV. Actual cell count is not given. Instead two ratios which better characterize the results are given. The first, a count ratio:

$$C_{(\text{count ratio})} = \frac{\text{count of population on day indicated}}{\text{original count of culture}}$$

The second is a growth ratio:

$$G_{(\text{growth ratio})} = \frac{\text{cell fraction count ratio C on day indicated}}{\text{count ratio of control cell fraction on the same day}}$$

If both populations at day zero start out at the same count then

$$\text{Growth Ratio} = \frac{\text{count of test cells}}{\text{count of control}}$$

These two ratios give a clearer population comparison than simple population counts. It should be stated that all cell populations in this experiment began with the same cell

FIGURE 28

EFFECT OF PREIMMUNE RABBIT SERUM (INACTIVATED) AND ANTI-LM RABBIT SERUM (INACTIVATED) ON THE GROWTH OF THE LM MOUSE FIBROBLAST CELL

A. Control Populations (mixed phases). The priming effect of a short incubation with anti-LM rabbit serum in contrast to the retarding influence of the same serum in constant association with the mixed cell population is shown.

B, C, D. Comparison of sera effects on the three cell fractions is shown. Fraction #1 shows the most pronounced priming effect after short term anti-LM rabbit serum incubation.

Figure 28

EFFECT OF PREIMMUNE RABBIT SERA (INACTIVATED) AND ANTI-LM IMMUNE RABBIT SERA (INACTIVATED) ON THE GROWTH OF THE LM MOUSE FIBROBLAST CELL

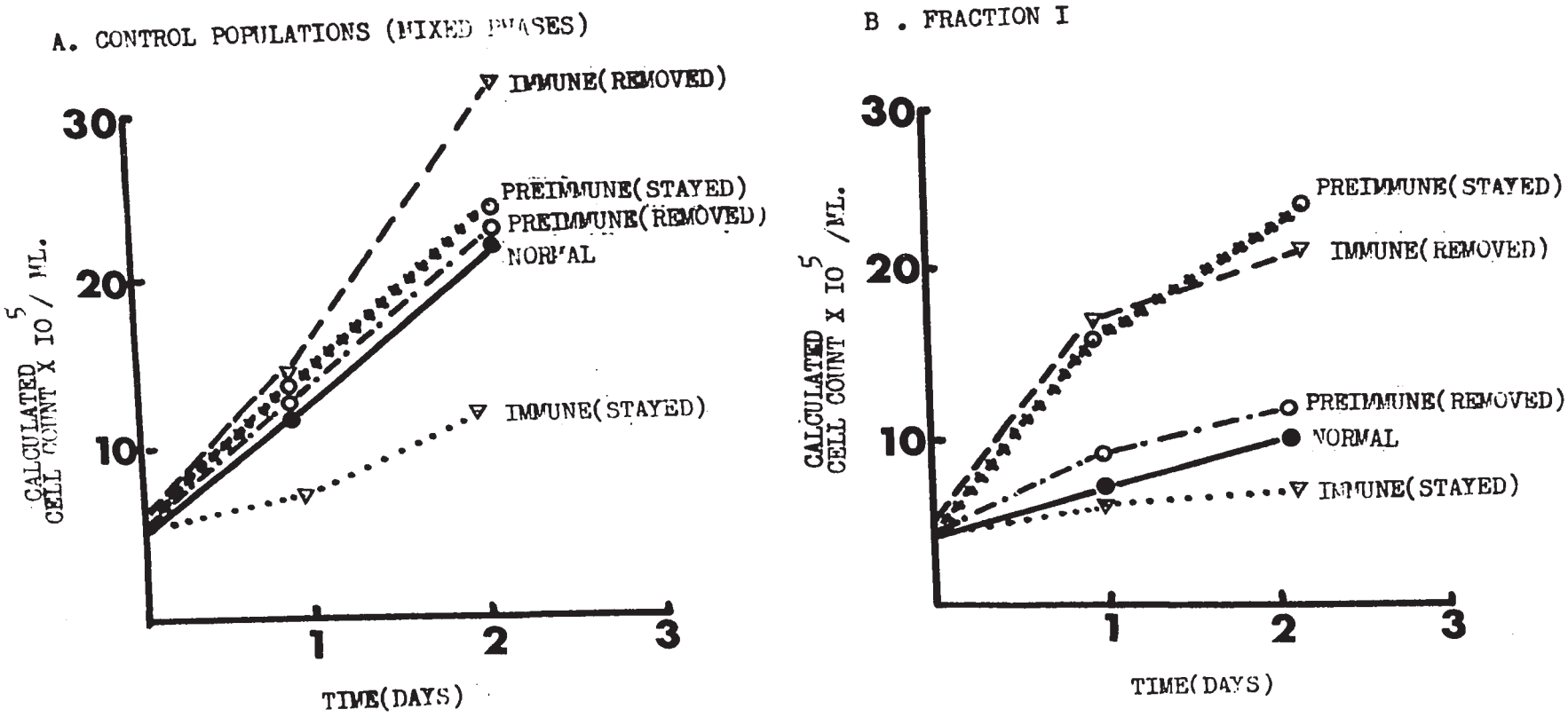
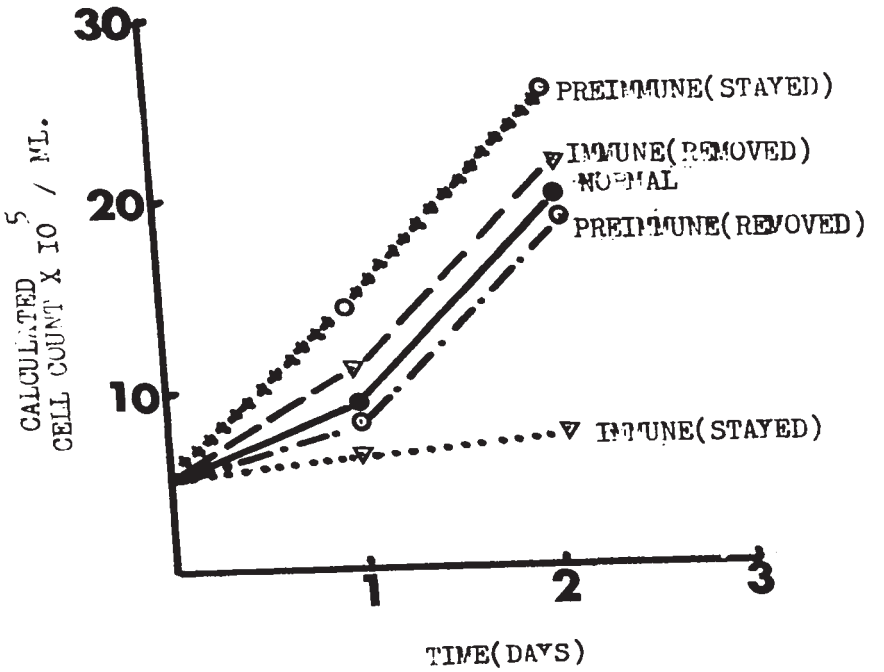


Figure 28

EFFECT OF PREIMMUNE RABBIT SERA (INACTIVATED) AND ANTI-LM IMMUNE RABBIT SERA (INACTIVATED) ON THE GROWTH OF THE LM MOUSE FIBROBLAST CELL

C. FRACTION 2



D. FRACTION 3

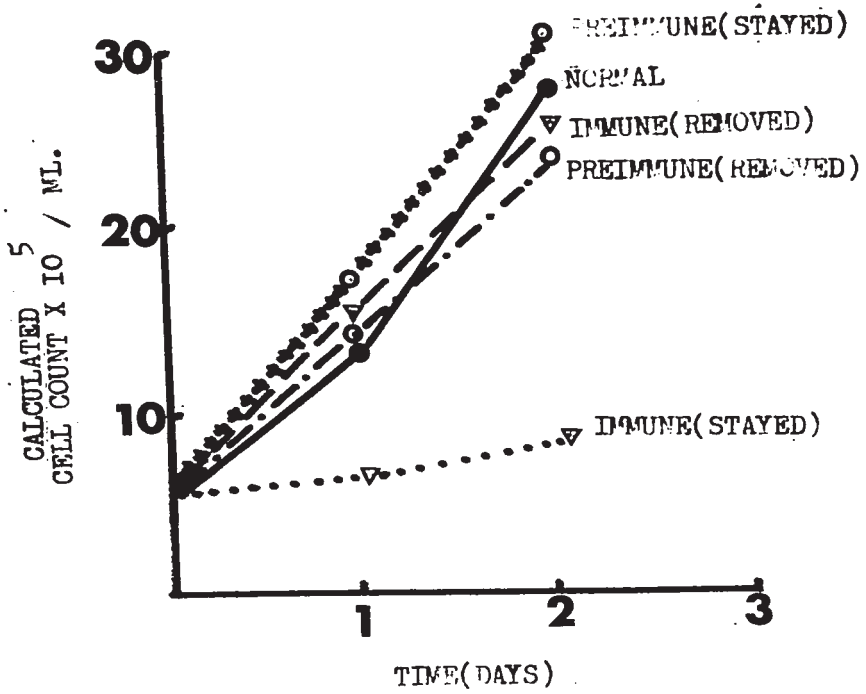
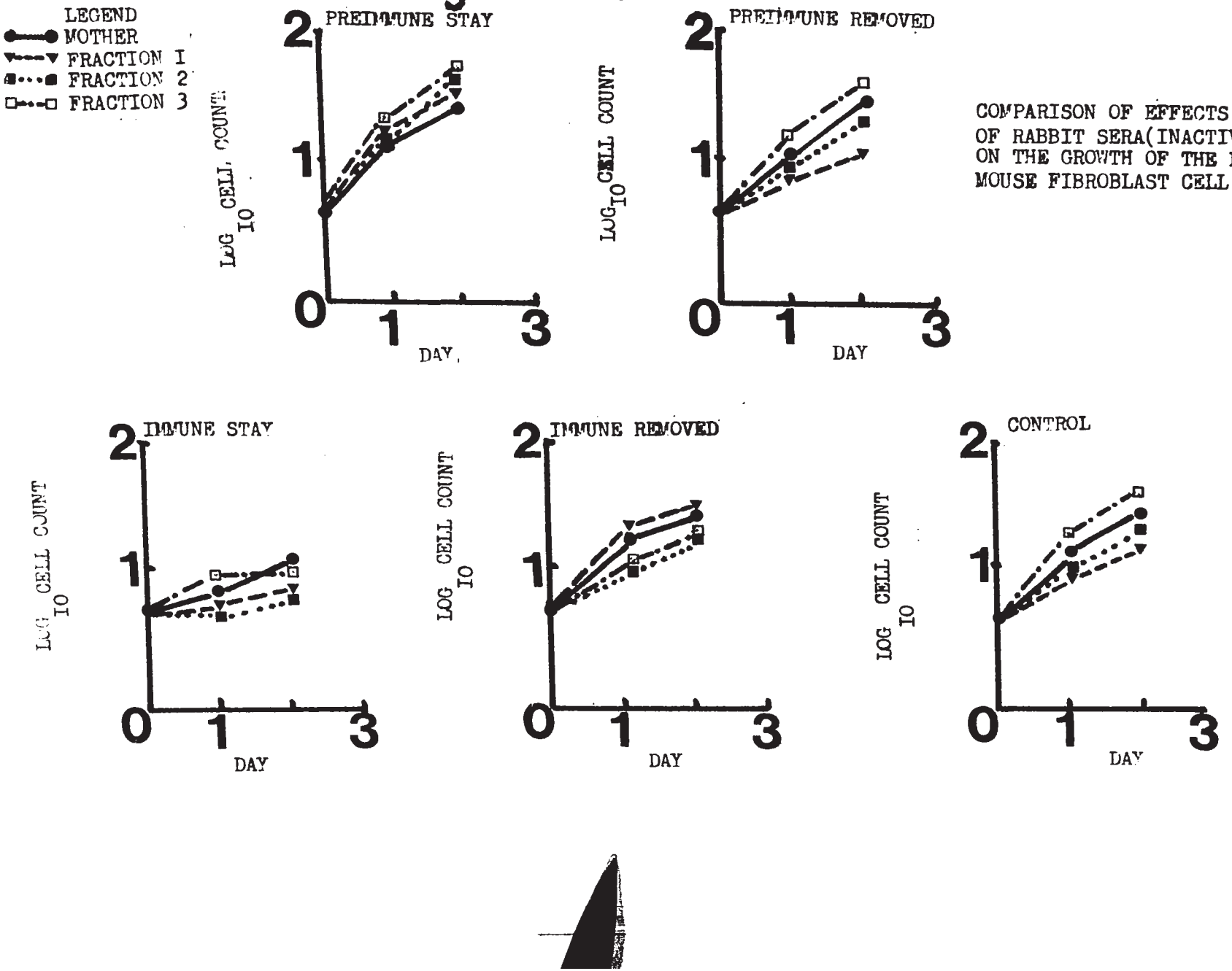


FIGURE 29

COMPARISON OF EFFECTS OF RABBIT SERA (INACTIVATED) ON THE GROWTH OF THE LM MOUSE FIBROBLAST CELL

Grouping of the three cell fractions according to the sera tested and whether growth experiments were conducted in its presence or not. The marked priming effect of short term incubation in anti-LM serum on fraction #1 is evident.

Figure 29



COMPARISON OF EFFECTS OF RABBIT SERA(INACTIVATED) ON THE GROWTH OF THE LM MOUSE FIBROBLAST CELL

count per ml (5×10^5 cells/ml) and were handled in exactly the same manner throughout the investigation.

Figures 28 and 29 show the actual cell count variation between the various cell fractions and the control population, according to the method of handling and type of serum tested.

When the effect of inactivated preimmune serum and of inactivated immune (anti LM) serum on the whole population are compared to the control population (Tables XIV and XV) it is noticed that preimmune serum,if remaining,does give a slight growth enhancement. This enhancement is less than would have been expected considering the original stimulation the presence of such serum had on the fluorometric breakdown rate of FDA, an indication of a more active metabolic rate. The slight enhancement is probably explained by the low concentration of serum in the propagating medium (0.05%). Such a low level was maintained to allow comparison with the immune serum populations which would have been destroyed by higher concentrations of that serum in their propagating medium. Other investigators who have reported marked growth stimulation used serum concentrations varying from 10 to 20%. In those total populations which had the brief exposure to inactivated immune anti LM serum, and were subsequently grown in medium free of it, the priming effect of even a small dose (0.05%) for a short time (25 minutes) gave elevated counts when compared to

TABLE XIV

ANALYSIS OF THE GROWTH EFFECT OF PREIMMUNE AND IMMUNE SERA ON VARIOUS MIXED CELL POPULATIONS AND CELL FRACTIONS (FICOLL GRADIENT SEPARATED) AFTER SERUM TREATMENT.

POPULATION	DAY	CONTROL NORMAL			PREIMMUNE SERUM			IMMUNE ANTI-LM SERUM		
		Count Ratio	Growth Ratio	Appearance	Count Ratio	Growth Ratio	Appearance	Count Ratio	Growth Ratio	Appearance
Original C	0	1.0	1.0	mix	1.0	1.0	mix	1.0	1.0	mix
Control	1	2.1	1.0	mix	2.2	1.05	mix	2.9	1.33	mix
No Serum	2	4.3	1.0	mix	4.5	1.04	mix	5.5	1.30	mix
Serum	0	1.0	1.0	mix	1.0	1.0	mix	1.0	1.0	mix
(Control D	1.	2.2	1.0	mix	2.5	1.14	mix	1.58	0.71	mix
Method)	2	4.4	1.0	mix	4.78	1.09	mix	2.7	0.60	small, giants unhealthy
Fraction No. 1	0	1.0	1.0	small singles	1.0	1.0	small singles	1.0	1.0	small, singles
No Serum	1	1.75	1.0	med. singles doubles	1.84	1.05	clumps medium singles	3.2	1.83	many small cells, no clumps
Doubling time 43.6 hours	2	2.2	1.0	mix	2.3	1.04	mix	4.1	1.86	mix
	0	1.0	1.0	small singles	1.0	1.0	small singles	1.0	1.0	small singles
Serum	1	1.80	1.0	med. singles doubles	3.0	1.71	cells medium no clumps	1.4	0.80	very small cells, some giants
	2	2.1	1.0	mix	5.1	2.32	mix	1.75	0.79	not healthy giants, small cells
Fraction No. 2	0	1.0	1.0	medium	1.0	1.0	medium	1.0	1.0	medium cells
No Serum	1	2.0	1.0	mix	1.96	0.98	mix	2.2	1.1	no clumps, mix
Doubling time 24 hrs.	2	4.1	1.0	mix	4.0	0.98	mix	4.6	1.12	mix
	0	1.0	1.0	medium	1.0	1.0	medium	1.0	1.0	medium cell
	1	2.0	1.0	mix	2.8	1.4	clumps giants	1.24	0.62	membrane disturbed, small
	2	4.0	1.0	mix	5.5	1.34	mix	1.42	0.35	giants, few healthy
Fraction No. 3	0	1.0	1.0	large doubles	1.0	1.0	large doubles	1.0	1.0	large doubles
No Serum	1	2.7	1.0	singles medium	2.8	1.03	mix	3.0	1.11	medium singles
Doubling time	2	5.5	1.0	mix	5.2	0.95	mix	5.4	0.98	mix
18 hrs.	0	1.0	1.0	large, doubles	1.0	1.0	large doubles	1.0	1.0	large cells, doubles
Serum	1	2.8	1.0	singles medium	3.2	1.19	mix	1.66	0.62	small vacuolated cells
	2	5.4	1.0	mix	6.0	1.09	mix	1.9	0.35	giant cells, unhealthy

TABLE XV

GROWTH RATIO COMPARISONS BETWEEN VARIOUS CELL POPULATIONS

A. GROWTH RATIO COMPARISON FOR VARIOUS CELL FRACTIONS TO THE CONTROL POPULATION

Fraction	No Serum	Preimmune		Immune	
		Stayed	Removed	Stayed	Removed
Control Mother	1.0	1.0	1.0	1.0	1.0
#1	0.5	1.0	0.5	0.6	0.8
#2	1.0	1.2	0.9	0.5	0.8
#3	1.2	1.3	1.2	0.7	1.0

B. GROWTH RATIO COMPARISON FOR VARIOUS CELL FRACTIONS COMPARED TO A NORMAL CONTROL POPULATION, INCUBATED WITHOUT SERUM

Control Mother	1.0	1.1	1.0	0.6	1.3
#1	0.5	1.2	0.5	0.4	1.0
#2	1.0	1.3	0.9	0.3	1.1
#3	1.2	1.5	1.2	0.5	1.2

both the control and preimmune serum total populations (Table XVb). If, however, the inactivated anti LM serum remained in the propagating medium a completely opposite effect was noted. The cell population gradually died showing a highly disturbed cell population with many giant cells. Fraction 1, which contains the small cells predominately in G1, was the cell group most affected by serum incubation (Table XV, page 110). Those exposed to inactivated preimmune serum, grown after removal from it, had only a very slight enhanced growth rate if any. If the cells were grown in medium containing even the small amount of preimmune serum, however, they were stimulated and increased in number over twice as fast as the control fraction 1 population. A similar effect was noted with those cells from fraction 1 which had the brief encounter with the inactivated anti LM serum, they too were stimulated and increased in number twice as fast as the control (Table XIV, page 110). If the inactivated anti LM serum remained, however, the deterioration and death of the culture resulted. Fractions 2 and 3 were not as stimulated in their growth rates as fraction 1. Fraction 2 showed only a slight enhancement and fraction 3 virtually none. Both these fractions, however, deteriorated and died if the inactivated anti LM serum remained in the propagating medium. In fact the deleterious effect was more pronounced and earlier than that noted in fraction 1. From this it would appear that

cells in G1 might have a membrane slightly more resistant to external stresses than cells in other phases of the cell cycle, or else might be less reactive to antibodies due to fewer antigenic sites on the cell surface.

From this experiment the following conclusions were reached:

For a Total Cell Population

1. The addition of inactivated preimmune rabbit serum to the environment of the logarithmically growing LM cell population causes an initial metabolic enhancement and a slight initial increase in cellular protein and DNA content. Removal of the serum after a brief interlude causes a return to normal level.

2. Continued incubation of a total cell population with inactivated preimmune serum (0.05%) causes a slight growth enhancement. Those cells having the brief serum exposure show less growth enhancement.

3. The addition of inactivated immune (anti LM) rabbit serum to the environment of a logarithmically growing LM cell population causes an initial metabolic enhancement and an increase in cellular protein and DNA content. Brief serum exposure subsequently resulted in an observed growth enhancement.

4. Continued incubation of a total cell population with inactivated immune (anti LM) serum (0.05%) causes deterioration and population death. Increased membrane damage and the production of giant cells is noted. Those cells having a brief anti LM serum exposure show growth enhancement in excess of that noted with the total cell population continually incubated with inactivated preimmune serum.

For the Three Cell Fractions

1. Fraction 1, the smallest cells predominately in G1, is most affected by the addition of serum--preimmune or immune--to the propagating medium. Continuous incubation in the presence of inactivated preimmune rabbit serum causes an increase in cell numbers twice that observed for the control fraction 1. Removal of the serum after a brief exposure, however, results in virtually no effect on the growth rate of this fraction, as compared to the control fraction, being observed. The effect of inactivated immune anti LM rabbit serum is exactly the opposite--removal of the serum causes enhancement whereas continued presence of the serum results in death of the culture. Fraction 1 cells survived longer than fractions 2 and 3 suggesting that these small cells are more resistant to environmental stress.

A doubling time of 43.6 hours noted for the control fraction suggests also the extreme youth of this particular fraction.

2. Fraction 2, containing medium sized cells and some double cells, is affected in a similar manner as that noted for fraction 1 except to a much reduced extent. Continued incubation of this fraction with the inactivated anti LM serum destroys cells from this fraction quicker than those of fraction 1. The observed doubling time of 24 hours would suggest that the majority of these cells were in S or G2.

3. Fraction 3, containing large cells and doublets mainly G2 and M, is least affected by the sera. However, here too continued incubation in the presence of inactivated anti LM-cell serum does destroy the cells at a faster rate than that observed for fraction 1. The observed doubling time of 18 hours for this population would also indicate that the majority of these cells were in G2 and M.

V. DISCUSSION

To the cell, its membrane is the first line of defence, the last barrier, and the selector which divides the controlled, known, interior from the hostile, unknown, exterior environment. Where does the membrane begin within the cell and where does it end in the external environment? As both Ponder, (1961), and Weiss, (1967), have noted, delineating the exact limits of the cell membrane or surface has become almost a matter of personal choice. It is extremely difficult to know where to draw the line when defining the membrane. In this study, therefore, no such definition was attempted. Instead, in the chemical analysis, reliance was placed on the equivalence of similar cell envelope fractions prepared in an identical manner, as the basis for comparison. Enzymatic and immunological studies with living metabolizing cells do not require an exact delineation of the membrane; nevertheless, speculations as to the surface activity of the cell membrane have been attempted in the light of results obtained utilizing known membrane acting agents.

The advantages of using a known cell system, such as that presented by the LM mouse fibroblast cell, are numerous. The cultivation of enormous cell populations is fairly easy; only strict adherence to sterile technique, to prevent bacterial contamination, and numerous tests to insure that the cells are free of mycoplasma and murine leukemia viruses being a necessary addition. Extensive knowledge of the cell's previous history is also available forming a good background for speculation on cell membrane activity. As well as this, the specialized characteristics of this cell line allows it to be grown both in suspension cultures as individual globular cells or alternately in sheets on monolayer cultures, making it a most attractive cell to study. The medium in which the cells are grown, is protein free. The peptone used contains nitrogen in a readily utilizable form--as amino acids and peptones, with only a negligible quantity of proteoses and more complex nitrogenous constituents. There are disadvantages in this cell line which makes exact characterization difficult. There is a tendency to form both giant cells and multi-nucleate cells. The conditions which trigger the production of such cells is unknown. As well as this, the lack of a mean chromosome number in the cells--only a modal number is possible--makes genetic studies difficult. Despite these drawbacks, however, the LM mouse fibroblast cell is a most useful tool in the definition not only of cellular membrane

activity but also of the ultimate aim of cell study, understanding of the complex mechanisms of cellular formation, development and behaviour.

It is realized that the cells used in this study are a selected population; those cells which have been able to overcome the disadvantage of an unnatural environment and to continue to grow. Consequently, we would expect that the membranes would have more uniformity both in chemical nature, production and function, than the original mouse cell population harvested by Earle in 1940. Investigation of the membrane composition and functions of the LM cell, which might vary throughout the single cell life cycle, were limited to cells taken from the logarithmically growing mixed cell population. This is the time of maximum cell proliferation when population growth conditions are optimum.

The problems inherent in gradient centrifugation sometimes are overlooked by investigators. The trauma experienced by cells (and cell membranes) during gradient centrifugation procedures can be extensive. In order to minimize this stress on the LM cells a large, inert, uncharged molecule of high molecular weight, Ficoll, was dissolved in medium 199, thereby preventing both osmotic damage and metabolic starvation of the cells during the centrifugation procedure. It was also noted that other workers in comparing Ficoll to various gradient materials found it to be relatively mild, indeed innocuous, with cells and their

membranes, (Boone, Harrell and Bond, 1968; Wallach, 1967; Bernfield and Fell, 1967). A slight sluggishness was noted in the subsequent enzymatic activity of such separated cells, (compare Figure 18, page 88 and Figure 23, page 95). However, sucrose normally used in gradients for cell separation does cause cytological aberrations in the cell (Table I, page 67 and Figure 9c, page 69). As well as this, bovine albumin gradients add to these traumatic stress on the cells, unknown immunological effects, (Figure 24, page 100); (Ryser, 1968).

The value of Ficoll-medium 199 gradient as a tool to separate and analyse various cell populations should be stressed. Figure 13b, page 75 shows the results obtained from six cell populations containing various cell types and mitotic indices.

Most of the methods used to obtain synchronized cell populations, demand the blocking of the cells in one phase, accumulation, then release of the block. Such action disrupts the normally functioning regulatory system of the cell and as a consequence unbalanced growth results (Lambert and Studzinski, 1969). It is known that the regulation of gene activity underlies cell differentiation, but the possibility that a new set of regulatory mechanisms for a cell can be the result of a single initiatory event is only now being considered (Britten and Davidson, 1969). The complete cellular reaction to such blocking agents as nitrogen gas, thymidine and colchicine for example, is unknown.

Wilson (1963), in his studies on the disruption of the mitotic cycle comes to the conclusion that mitotic poisons are the "sledge hammer" approach to cell study, and that the warning given by Mitchison (1957), that results obtained from synchronized systems, so produced, do not necessarily apply to normal unsynchronized growth, is valid. Living systems, such as the cell, will continually adjust their activities in response to their internal state. This internal state, in turn, is controlled by the regulatory mechanisms which integrate all metabolic processes. The two methods used in this study to obtain cells in phase--mechanical separation or a short term temperature shock--give the least stress to the cell (Scherbaum, 1963). For this reason the experimental results obtained are probably closer to those which would be observed if the cell population could be separated without any external stress, than those obtained using metabolic blocks.

The cell populations obtained by mechanical separation were not completely separated into the different cell phases--G1, S, G2 and M. Only fraction 1, the smallest cells in volume would appear to be fairly homogeneous population in G1 (Table III, page 72; Table XIV, page 110). The other two fractions were mixtures, with cells in S predominating in fraction 2, and G2 and M cells predominating in fraction 3.

The method of Newton and Wildy, (1959), which required an hour's cold shock to the cell population, undoubtedly

disturbed the cells regulatory mechanism. Such altered physical surroundings undoubtedly would upset the sensitive cell regulatory mechanisms. However, it was argued that this short period of protoplasmic immobilization was undoubtedly less of a strain on the cell than a blocking agent would have been (Sisken et al., 1965; Wiemken et al., 1970).

Physiological Studies

As Webb, (1966), has noted, living cells are complex, highly organized factories in which a fine balance of chemical and functional activities take place with accurate co-ordination according to strict patterns. These reactions are mediated and directed by enzyme systems which provide the energy necessary for all the various functions of the cell, maintenance, growth and multiplication. Webb, (1966), has listed numerous requirements which must be met to ensure experimental validity. In this study a definite number of cells, grown under similar conditions of nutrition and environment, in the same phase of the population growth cycle, and handled in precisely the same manner, were used as the basic cell population being tested.

Enzymatic studies of L cells have been attempted previously, but interest has centered mainly on those enzymes concerned with DNA synthesis (Littlefield et al., 1963; and Gold and Helleiner, 1964). The esterase content of mouse cells has been analysed (Templeton, 1963; Hunter

and Strachan, 1961), and recently the specific activity of esterases during the population growth curve has been followed (Ruddle and Rappola, 1970). However, extended observation of one particular enzymatic reaction, not knowingly concerned with DNA synthesis, throughout the growth cycle, has not been attempted. When the substrate to be used with an active membrane system was considered, certain prime requirements were noted. A substrate as near to a normal cell metabolite as possible was needed, one which would enter and be broken down by the cell in a normal kinetic manner. It must be nontoxic to the cell, allowing the continuation of normal growth after its removal, and it must also be able to be measured accurately and with ease. Fluorescein diacetate, FDA, was just such a compound. Not only did it fill the above prerequisites but the final product of the metabolic breakdown, unlike the substrate, was a molecule which fluoresced and which would accumulate in the cell allowing quantitative monitoring by fluorometry and visual monitoring by fluorescent microscopy (Rotman and Papermaster, 1966; Meczon and Brady, 1969). The distinct chromatographic separation and fluorescent visualization of the substrate, fluorescein diacetate, the intermediary product, fluorescein monoacetate, and the product, fluorescein, also allowed quantitative analysis of the complete cell-substrate system, both intracellular and extracellular components (Srere, 1967).

It was concluded that an acetic-ester hydrolase (3.1.1.6, acetylesterase) most likely was the enzyme or enzyme group involved in the metabolic breakdown. The ubiquitous nature of these enzymes in the living cell is well known (Myers, 1961). Although esterases as a group are specific in their action, being capable only of hydrolyzing ester linkages, they have among themselves shown a very low degree of substrate specificity (Fishman and Green, 1961; Hunter and Strachan, 1961). Consequently, owing to this low absolute specificity these enzymes generally cannot be identified by their action on a single substrate or by the effect of a single inhibitor or activator (Hofstee, 1961). Shibko and Tappel, (1964), have suggested that esterases may be directly concerned with the processes controlling the permeability of membranes. Burnstone, (1962), suggests also that these enzymes may be active in phagocytosis and related phenomena. Such possibilities, as these, are supported by the fact that the normal physiological substrates for most simple esterases are still unknown (Myers, 1961; Deduve, Wattiaux and Baudhuin, 1962).

The utilization of the known esterase inhibitor, paraoxon (Table ID, page 67; Table VII, page 82, Figure 22, page 92), confirmed that the metabolic breakdown of fluorescein diacetate was accomplished by an esterase. The lack of visible cytological distress of the cells when exposed to this inhibitor, and the subsequent regaining of the cell's

ability to metabolize the substrate after removal from the presence of this inhibitor, would suggest that only this metabolic function was affected by paraoxon incubation. The cholinesterase inhibitor, eserine sulphate, (Table ID, page 67; Table VIIA, page 82; Figure 22, page 92), although it does cause some inhibition of the substrate breakdown, produces such marked cytological distress in the cell that wholesale destruction of many metabolic processes, rather than the specific inhibition of fluorescein diacetate, would seem to be the cause. Bowman, Rand and West, (1968), have pointed out that eserine sulphate does inhibit active transport of sodium across frog skin, muscle and membrane. However, no evidence of similar action has been reported in mammalian tissues.

When various respiratory inhibitors were tested (Table VII, page 82) it became evident that energy was required for the enzymatic interaction to take place. Energy was required either for transport of the substrate, for the enzyme reaction, or for both processes. Antimycin A, oligomycin, 2,4, dinitrophenol and potassium cyanide, all inhibitors which block various steps of cellular respiration, caused inhibition of the FDA-acetylesterase system of the LM mouse fibroblast cell.

Enzyme digestion of the LM cells was attempted in order to ascertain if specific membrane molecules either were involved in, or restricted, the FDA-acetylesterase system.

Table I, page 67, and Table VIII, page 85, show the action noted for lipase and collagenase both cytologically and enzymatically. Both these enzymes allowed a slight enhancement of the fluorometric rate normally noted for the LM mouse fibroblast cell. Collagenase probably strips the cellular product, collagen, from the outer cell region and allows easier access of the substrate to the membrane. Lipase hydrolyses lipid membrane components, perhaps producing holes through which the substrate molecule passes easily. Neuraminidase, (Table II, page 67 and Table VIII, page 85), which attacks the sialic acid moieties of the mucopolysaccharide in the cell surface region, shows a slight inhibition of the FDA-acetylesterase system. The action of this enzyme is most interesting. Incubation of the cells with a dilute concentration of the enzyme (50 units/ml) gradually causes the cells to lose their ability to stick to glass (Table II, page 67). Weiss, (1963), also found this result when he treated various cell types with enzymes. I noted (Table II, page 67) that the ability of the LM cells to exclude fluorescein and to stick to the glass substrate diminished at the same time. It is possible that the molecules removed by the neuraminidase are in some way also involved in permeability control or function of the cell membrane.

Observing the effect of known membrane acting substances (Table I, page 67, Table VII, page 82), gave varied

results both cytologically and enzymatically with the LM mouse fibroblast cells. The large complex molecule, methyl cellulose, used as a dispersant in the suspension cell cultures of LM cells, did not disturb either system. However, vitamins A, C and E, which could, in concentrations in excess of that normally observed in medium 199, disturb the cell cytologically, had slight, if any, effect on the enzymatic system studied. Nonidet, P40, a nonionic detergent was observed to inhibit the FDA-acetylesterase system, (Medzon and Brady, unpublished results), and it was concluded that its action on the lipid of the membrane might result in the plugging of membrane pores.

The product, fluorescein, (Table VII, page 82), held a special place when its effect was studied on the LM mouse fibroblast acetylesterase system. Cytologically, it was found that the LM cell could exclude the product, F, indefinitely if the membrane integrity was not disturbed. The cell would grow and divide in medium with this molecule in a concentration of up to 1×10^{-5} M without any variation noted in the cell population when compared to a control population. At strengths greater than this (up to 1×10^{-2} M) a toxic effect was noted suggesting that the inclusion of the fluorescein molecule might disturb the fine nutritional balance in the medium and by some unknown action disturb the membrane function of the cell. Naturally, continued incubation with this molecule at even 1×10^{-5} M

over many months might have resulted in a membrane change, however, such an incubation and study was not attempted here.

When a mixed logarithmically growing LM mouse fibroblast population was incubated 15 minutes prior to the fluorometric test in medium 199 with a concentration of 1×10^{-7} M fluorescein, the subsequent substrate breakdown rate compared to a normal population showed marked inhibition. However, if these same cells were separated from this medium, placed in fresh medium 199 and another fluorometric run with the substrate FDA alone, then a breakdown rate twice as fast as the original control rate was noted. The mechanism of fluorescein action is unknown, however membrane permeability changes may account for the observed increased hydrolysis of the substrate.

Examination of the inhibition effect of fluorescein on the FDA-acetylesterase system of cell populations cold shocked into synchrony, and showing various percentages of cells in mitosis, gave even more interesting results (Table IX, page 86, Figure 13, page 75, Figure 17, page 88). The normal control, mixed phase cell population in logarithmic growth phase has a mitotic index of between 12-15%. When the populations cold shocked into synchrony were observed, it was noted that those populations with mitotic indices below 15% were more sensitive to fluorescein concentration in the medium, at all three substrate concentrations, than

those of 15% or over. Although the population showing no visible cells in mitosis were composed of large cells assessed to be mainly S and G2 (figure 13b, page 75), the cells of the 5-10% mitosis population appeared to be a normal mixed population. The cell population showing 15% mitosis varied from the normal control in the increase in number of doublets (M) observed. The substrate breakdown rate for this particular population was greater than that of the control (not cold shocked) population. Whether this increased metabolic activity is the result of the cold shock synchrony process or of the presence of an excess of doublets in the population is unknown. The cell population showing 25% of the cells in mitosis, had nearly 50% of the remaining cells in the G1 phase of the growth cycle. The FDA-acetylesterase breakdown rate was considerably higher (5X) than that of the normal mixed control population. Both the 34% mitotic cell population and the 48% mitotic cell population showed an elevated fluorometric rate as well. Ruddle and Rappola, (1970), found that the specific activity of esterase for PK cells decreased from a high when the cells were in lag phase, to a low at the logarithmic growth phase, again becoming high as the population entered the stationary phase. Tobey and Ley, (1970), have stated that Chinese hamster cells in the stationary phase are essentially in G1. Extrapolating back, and recognizing that different cell lines are involved, these two observations may explain

the high esterase activity observed with a cell population of LM cells containing nearly 50% of the cells in G1. The fact that all, but the 0% mitotic, cell populations showed FDA-acetylesterase breakdown rates in excess (2X and up) of the normal control population suggests that the process of cold shock synchrony has disturbed the normal cell metabolism. Perhaps this shows a metabolic compensation by the cells, after returning to the 37°C incubation temperature, for the unfavourable conditions experienced for one hour when gradually cooled to 4°C.

The interesting results noted with the fluorescein mercuric acetate, (FMA), when I attempted to obtain the cell envelope fraction, using the method of Warren and Glick, (1968), (Section III A 5, page 36), points out the complex nature of cell membrane activity. Fluorescein mercuric acetate is thought to react with free sulphydryl groups to fix and harden the surface membrane. The "hot spots" observed within some cells suggests that it does not remain just on the surface but also attacks sulphydryls within (on the lysosome membrane? Chromatin?).

Exclusion of the FMA by cells in G1 (fraction 1), and the doublets (late M), would suggest an altered cell membrane permeability and sulphydryl content at this time. Sakai and Dan, (1959), found that the TCA soluble SH fraction of sea urchin's eggs fell almost to zero during mitosis, and after division once more reached a peak,

giving a wave-like fluctuation throughout the mitotic cycle. This supported Chatton, Lwoff and Rapkine, (1931), who first postulated the SH fluctuation and suggested its importance in cell division. Klein and Robbins, (1970), assaying the soluble sulphydryl content of Hela cells during the life cycle in order to assess the glutathione levels, found a consistent 10% drop in the glutathione levels between mitosis and early G1. All of these investigators would support the claim that the membranes of cells at this time (late M to early G1) are altered.

Chemical Analysis of Whole Cell and Envelope Fraction

Biochemical studies in the form of chemical analysis for protein, DNA, lipid and carbohydrate have long been the vogue in the study of living populations, whether it be composed of bacteria, plant or animal cells (Healy et al., 1956; Hill et al., 1959; Salzman, 1959; Swaffield and Foley, 1960). However, Kahn et al., (1962), have cited a valid criticism when they note that "the cell biologist has turned to quantitative methods as more critical and perhaps more revealing indices of cell metabolism and function (than cytological methods). However, technical limitations have required that such studies be performed on cell populations with little regard to the viability between individual cells observed within such populations."

Within the literature some excellent studies have been done on the content of various chemical components within the L-cell and/or the membrane fraction of the L-cell. However, all studies were confined to mixed populations of cells, (Weinstein et al., 1969; Mackenzie et al., 1966; Swaffield and Foley, 1960; Eidam and Merchant, 1965), or else as Till et al., (1963), studied only the variation of DNA or RNA content. Table V, page 76, and Table VI, page 77 are the results of experiments to analyse mixed control cell populations, and Ficoll separated cell populations, both whole cell and envelope fractions, for protein, lipid (total and cholesterol), carbohydrate, DNA and RNA. Table XI, page 96, gives specific enzymatic activity ratios when unit of surface area, unit volume and gamma of protein are equated.

Table V, page 76, and Table VI, page 77, give the results obtained for protein, DNA, RNA, carbohydrate and lipid (total and cholesterol) in whole cells and in the cell envelope fraction. The values obtained from the analysis of the mother logarithmic control cell populations agree very well with those of other investigators who have analysed total LM cell populations (Healy et al., 1956; Siminovitch et al., 1957; Salzman, 1959; Hill et al., 1959). Weinstein et al., (1969), analysed the L cell, both whole cell and envelope fraction, for lipid content (total and cholesterol). The results I obtained agree fairly well

considering that the two populations have been propagated differently for many years (Anderson et al., 1969).

It is when a consideration is made of the results obtained from the three Ficoll separated cell populations that some interesting facts are noted, (Table V, page 76, Table VI, page 77).

The whole cells of fraction 1, G1, had about half the amount of protein and DNA compared to the large cells of fraction 3 (G2 and M mainly), (Healy et al., 1956; Hill et al., 1959; Siminovitch et al., 1957; Till et al., 1963). This is not unreasonable considering that the larger cells are about to divide into two cells, (Figure 3, page 22). It was noted also that the percentage of protein and total lipid in the cell envelope, as compared to that in the whole cell, doubled between fraction 1 and 3. Only the carbohydrate, both total cell and envelope portion, stayed fairly constant in quantity. Table VI, page 77, which shows the specific content of the various cell components per gamma of protein indicates the relative amounts of these components both in the whole cells and envelope during the cell's life cycle. Total lipid and cholesterol follow a similar synthesis rate as that of protein (Geyer, 1966; Warren and Glick, 1968). However, the synthesis rate for carbohydrate is different. In the whole young cell of G1

there is twice as much carbohydrate per gamma of cell protein than in the older whole cells of fraction 3. The drop of carbohydrate ratio in the cell envelope portion between fraction 1 and 3 is even more pronounced, there being nearly three times as much in G1 cells as in the G2 and M cells. These older cells in a phase of their life which requires a high rate of energy utilization, may be metabolizing both the stored cell glycogen and the freshly acquired carbohydrate from the medium so quickly that only a constant residual carbohydrate pool remained to be analysed in this investigation. The reduced ratio of carbohydrate to protein in the envelope is not easy to explain. Moscona, (1961), has postulated that carbohydrates are the possible basis for cellular adhesion. If such is the case the rounding up of cells at mitosis when carbohydrate ratio is at a minimum may be explained. However as Weiss, (1967), has observed the factors influencing cell adhesion, contact and separation indicate a most complex cellular function. Mayhew and O'Grady, (1965), have found that the electrophoretic mobility of parasynchronous suspension cultures is significantly higher during the mitotic peak phase and suggest that an increased density of ionized carboxyl groups of sialic acid moieties at the cellular electro kinetic surface is responsible. A structural rearrangement of sialic acids within the peripheral zone as distinct from de novo synthesis is proposed,

(Weiss, 1969). Brent and Forrester, (1967), concluded that the increase in electrophoretic mobility is not just due to increase in surface concentration of charge-bearing macromolecules as cells round up in mitosis, but offer no suggestions as to what may be the causal factor. Lippman, (1965), noting that the absence of polysaccharides on the cell surface is associated with unblocked cell division cycles, and that quantitative and qualitative differences in the mucopolysaccharide coat of the cell do occur in the course of a cell's lifetime, suggests that the absence or reduction of carbohydrate at this time, (M), may remove the block to cellular proliferation. This proliferation she imagines is then checked ("reblocked") by fresh production of mucopolysaccharides by the new daughter cell. Gerner et al., (1970), studying the biosynthesis of surface membrane during the cell cycle of the KB cell found that just after division, when cells were in G1, there was a marked increase (3X) of the specific activity per micrograms protein of C^{14}-D-glucosamine indicating a marked increase of carbohydrate synthesis in the cell at this time. Both these findings would agree with my results.

The inability to find any DNA or RNA in the envelope fraction tested, although up to 10 ml of 1×10^{10} cells/ml were harvested and the Hitachi spectrometer had a reproducible sensitivity of 5 ug of RNA/ml of sample, suggests that the envelope fraction was fairly clean of inner cellular

material, (Figure 14, page 75). Although RNA has been demonstrated using microelectrophoretic techniques in numerous cells, (Weiss, 1968), it would not seem the case in the L cell. However, since no attempt was made to study the envelope fraction with the electron microscope, and conscious of Curtis', (1967), criticisms of those workers who assume on the basis of a method alone that they have a pure membrane fraction, I will not suggest that the analyses of my envelope fraction represent that of a pure cell membrane.

Immunological Studies

As Weiss, (1963a) has noted, the immunochemical approach affords one of the most elegant means of cell surface study. He has, however, pointed out that some important surface components, such as water, do not have the necessary immunological properties to enable these methods to be of use in their case. Despite this obvious drawback using immunochemical methods, it is a fact that almost any biological macromolecule can cause the formation of antibodies. Purified polysaccharides and nucleic acids usually fail to induce antibody formation unless complexed with proteins. The anti LM serum produced for these experiments, contain antibodies produced in response to whole living LM cells, and as such possibly contain a mixture of antibodies to all components of the cell, whether membraneous, cytoplasmic or nuclear. With these limitations in mind only

general statements as to the significance of the results obtained from my experiments can be made.

Since the enzymatic system with the fluorogenic substrate was one of the tools used to study the effect of immune anti LM serum on the LM cell, preliminary tests studying various aspects of normal cell-serum interaction was necessary. From these studies various interesting facts immerged. It was found that the animal source of the serum was important (Figure 24, page 99), and that inactivation of the serum was necessary (30 minutes at 56°C). The fresh sera, from all sources, possibly contained non-specific esterases which broke down the fluorogenic substrate FDA within the extracellular medium causing a flooding of the system with the fluorescent product, fluorescein. Continued adverse cytological and enzymatic enhancement action noted with guinea pig, horse and calf serum, suggests that other factors not heat labile after 30 minutes at 56°C, were still present and acting on the LM cells. The sensitivity of the FDA method is useful in determining such effects.

Since albumin is the most abundant protein present in serum, while immunoglobulins are in minimal amounts, tests to ascertain the effect of this molecule at various dilutions was attempted. Figure 25, page 100, shows the unexpected results obtained. When albumin was present in the normal amount found in rabbit serum little enhancement of the FDA-acetylesterase system was noted. However, dilution of the

albumin (down to 1/100th the amount normally present in serum) caused a marked enhancement plateau to result. No background glow was noted when the cells and extracellular medium were observed using fluorescent microscopy. There was no cytological damage or distress recorded for any of the cells bathed in the medium containing various amounts of albumin. Albumin is an extremely complex flexible molecule and it is possible that a dilution dependent molecular conformational change of the molecule causes the enhancement plateau of the acetylesterase-FDA system studied.

As a result of these preliminary cell serum experiments, anti LM serum was produced in rabbits, inactivated before use with the FDA-acetylesterase system and accurately diluted, with the results obtained from the albumin plateau enhancement region being viewed with caution. The ring test and slide agglutination test were used to confirm the presence of humoral antibodies to LM cells (Figure 8, page 65). Another subline of the L-cells, LT cells, were obtained from Dr. I. Walker, Cancer Research, and the anti LM serum was cross agglutinated with these cells, 1 hour at room temperature. After pelleting of the cells, the serum was retested with the LM cells. The ring test and agglutination tests remained positive. When the serum was agglutinated with the LM cells first, the cells pelleted out, and the serum then tested with both fresh LT cells and LM cells, neither test was positive. This result suggests that the agglutinating

antibodies in the rabbit serum were specific for the LM mouse fibroblast cells (Kite and Merchant, 1961).

When the inactivated anti-LM rabbit serum was tested, in various dilutions, with the FDA acetylesterase system the results obtained are as noted in Figure 26, page 101 . Concurrent with this test slide agglutination tests were attempted with the serum, in similar dilutions. The last point at which the fluorometric rate increase of the substrate breakdown was recorded which showed a slightly elevated rate above the normal (1:250 dilution), coincided with the last dilution of serum (1:256) where positive cell agglutination was noted. Such close correlation between the two sets of results would lead to the conclusion that the fluorometric enhancement mechanism or factor, and the clumping factor (antigen-antibody complex) at the very least are interdependent and at the most, the same thing.

Table XI, page 96 , Table XIII, page 107, Figure 28, page 109 , Figure 29, page 109 , Table XIV, page 110 , Table XV, page 110 , indicate the results obtained from an experiment designed to answer the basic question, "Does immune serum affect cells in different parts of the cell cycle in a different way?" Figure 27, page 103, shows the protocol for this experiment. From these results it will be seen that for the total population the addition of inactivated pre-immune rabbit serum to the environment of a mixed phase logarithmically growing cell population causes an initial

metabolic enhancement (Table XIIIa, page 107) and a slight initial increase in cellular protein and DNA (Table XIII, page 107). Continued incubation of the cell population with the inactivated preimmune serum does cause a slight growth enhancement (Table XIV, page 110), but if the cells are removed from the serum medium and allowed to grow in medium 199 alone, growth levels return to the normal level after a brief interlude (Table XV, page 110).

The addition of inactivated immune anti LM serum to the environment of a mixed phase logarithmically growing cell population causes an initial metabolic enhancement (Table XIII, page 107) and an increase in cellular protein and DNA content (Table XIII, page 107). Removal of this serum after a brief exposure causes a continued growth enhancement (Table XV, page 110), whereas leaving the serum in the medium results in cellular deterioration and final population death. Increased membrane damage and the production of giant cells was noted. The cells which had a brief exposure to the medium containing inactivated anti-LM serum showed growth enhancement in excess of that noted with the total cell population continually incubated with preimmune serum (Table XV, page 110, Figure 28, page 109, Figure 29, page 109).

When the different cell fractions separated on the Ficoll gradient are considered it becomes evident that relatively speaking the young cells in G1 are most affected

by the addition of serum, preimmune or immune, to the propagating medium. For fraction 1, G1, continuous incubation in the presence of inactivated preimmune rabbit serum causes an increase in cell number twice that observed for the control fraction 1 (Table XV, page 110). Removal of the serum after a brief exposure, however, results in virtually no effect on the growth rate of this fraction, as compared to the control fraction. For the other two fractions a very slight growth enhancement is noted if the serum is allowed to stay in the medium (Table XV, page 110). For G1 cells the effect of inactivated anti-LM serum incubation is exactly the opposite to that observed with the preimmune serum. If the serum stays, death to the culture finally results. If it is removed, however, growth enhancement is noticed (Figure 28, page 109 , Figure 29, page 109). A similar depression of growth is noticed for fractions 2 and 3 if the anti LM serum is allowed to stay, however, growth enhancement is not noted if the serum is removed (Table XV, page 110). Since the only difference between the two rabbit sera tested is that the immune serum contains specific anti LM antibodies, the variations in results noted may be attributed to these antibodies. Table XIII, page 107 , shows the ratio comparison between rates of the FDA acetylesterase breakdown by the LM mouse fibroblast cells preimmune serum treated cells, to immune serum treated cells. If the immune serum is allowed to remain in contact with the cells

a depression of enzymatic function is already noted even after only 85 minutes of incubation. Removal of the offending anti LM serum (after a total of 25 minutes incubation) instead stimulates the enzymatic breakdown rate of the fluorogenic substrate. As Weiss, (1965), has noted, release of endogenous enzymes may be triggered off by physiological means. Although he was speaking of active antisera and in our case the antiserum was inactivated 30 minutes at 56°C, the mechanism whereby the antiserum produces a permeability change which in someway activates lysosomes, may function here as well.

There is an observed difference in the effect inactivated immune anti LM serum has on the LM cells in different phases of the cell cycle. As Möller and Möller, (1964), noted, a variation exists in the concentration of surface receptors to antibodies between new young cells and older cells. Davis and Silverman, (1968), found that the intensity of the specific ferritin labelling for the H_2 iso-antigens varied considerably from cell type to cell type of the CR strain mice with lymphocytes showing specific labelling on all the cell surface in comparison to spotty labelling in leukocytes and platelets. More relevant to our case they also noted that erythrocytes varied from uniform labelling over the whole cell circumference to those with little or no labelling. They offered no suggestion as to why this should be so but it is possible that they were observing an age-

concentration H_2 iso-antigen difference in these cells. When the L cell itself is considered Nathenson, (1968), found after membrane harvesting that all the H_2 iso-antigens were essentially on the cell envelope. He released a water soluble glycoprotein form of the H_2 iso-antigen from the cell membrane by papain digestion. The fact that any chemical analysis showed more carbohydrate in the cell membrane in the G1 phase might indicate the presence of more H_2 iso-antigens at this time and explain the more pronounced cellular response to inactivated antisera incubation in G1.

Drysdale et al., (1967), using the fluorescent antibody technique showed (Figure 1) fluorescent stained LM cells. There is a definite difference between degree of staining noted on the small cells (possibly young) and the larger cells, although they do not comment on this phenomenon.

Continued incubation of cells in medium containing inactivated anti-LM serum causes cellular death. A variation was noted between cell fractions however. The older and larger cells of fractions 2 and 3 may just be committed to finish the particular cycle they are in and the unhospitable medium may then cause their death. The young cells in G1, after initial stimulation by the antiserum, may maintain their commitment to growth and are able to resist longer the inhospitable medium.

The Oscillatory Phenomenon of the FDA-acetylesterase System of the LM Cells

It is perhaps the unexpected oscillatory nature of substrate (FDA)-acetylesterase system of the LM cell membrane which is of most interest here. The fortuitous nature of the substrate, having a readily visualized two step breakdown procedure, made the recognition of the oscillation possible. Figure 18, page 88, Figure 20, page 90, Figure 22, page 92, Figure 23, page 95, and Table X, page 88, give the relevant graphs and calculations for this membrane phenomenon.

Periodicities, mostly in the form of circadian rhythms, are not a new finding in nature (Halberg, 1969), however, at the cellular level very little of a concrete nature is known (Goodwin, 1967). Periodicity of enzyme production during the generation cycle has been shown for yeasts and bacteria (Sylven et al., 1959; Gorman et al., 1964; Masters et al., 1964; Kuempel et al., 1965) but the mammalian cell has not been studied even along this particular line to any great extent. Chance, (1964, 1965), has shown the presence of a sinusoidal cycle in the DPNH level of the yeast Saccharomyces carlsbergensis, and Frenkel, (1966), a similar 4 minute oscillation in cell free extracts of beef heart, but oscillatory phenomenon in living metabolizing mammalian cells has not been shown. I have found a periodicity, in suspensions of LM mouse fibroblast cells (Figure 18, page 88) which is dependent on the dynamic functioning of the cell

membrane (Figure 20, page 90). Fluorometric and chromatographic (Figure 13, page 75), studies using known amounts of substrate and known numbers of logarithmically growing LM cells (Figure 17, page 88, Table X, page 88,) showed a gradual increase in the end product, F, and a gradual decrease in the substrate FDA within the whole system. When the intracellular concentrations of FDA, FMA and F, (Figure 6, page 52) were plotted at minute intervals over a period of 12 minutes a cyclic phenomenon was immediately noted (Figure 18, page 88). The substrate, FDA, concentration within the cell rose and fell rhythmically with a period of approximately 3 to 4 minutes at the same time an opposite wave was noted for the product F. FMA, the intermediate metabolite, always peaked just before the end product F. The swift shift of concentration high and lows within the cell as opposed to the slower extracellular loss of FDA and accumulation of F (Figure 18, page 88), suggests a dynamic exchange of compounds at the cell membrane. It should be noted that the accumulation of F within the cells and its slow release from the cells allows the use of fluorescence microscopy to confirm the fluorometric data by visualization and photography (Figure 4, page 33).

The necessity of a membrane bounded system to produce this cycle is shown by Figure 21,(page 90). Although there was still a gradual increase in the amount of fluorescein, and decrease of fluorescein diacetate no cycle was seen for

either the homogenate or sonicate tests. The higher than normal accumulation of FMA within these systems might indicate that the enzyme is normally membrane bound and is therefore unable to fully cleave the FDA molecule under broken cell conditions. These experiments indicate the need for a living active membrane to analyse this cell function.

Purified fluorescein was added to the bathing medium to observe the effect on the rate of substrate metabolism and on the intracellular cycle (Figure 21, page 90). When FDA was added to the bathing medium in which cells had been allowed to equilibrate with F for 15 minutes, it was found that the normal cycle still appeared but only after an 8 minute adjustment period. The FDA entered slowly and was metabolized at a slower rate. The concentration of the intermediate metabolite, FMA rose to nearly twice that obtained normally (Figure 18, page 88) during the first 4 minutes but gradually fell to the normal level subsequently. The fluorescein appears to force the cycle out of phase by slowing down the FDA uptake and causing an accumulation of FMA, thus a form of negative feedback as well as interference with permeability is involved.

The addition of esterase inhibitors, paraoxon and eserine sulphate, to the preincubation medium caused the effects noted in Figure 22, page 92. Paraoxon inhibits almost completely the initial cleavage of the acetate from the FDA (Figure 6, page 52). It would appear to be inhibiting

the enzyme(s) responsible for the breakdown FDA - F. Eserine sulphate, however, does not seem to be specific for this particular interaction. It would appear to destroy the complex membrane permeability control mechanism and only incidently upset the FDA metabolism at the same time. Membrane leakiness would appear to allow the escape of the FMA (intermediary product) from the cell before it can be broken down to F. It should be noted that to assure myself that an oscillatory FDA-acetylesterase system did exist within the LM cell, the tests were randomly done and even the timing of the switch on the constant temperature water bath heater was monitored to assure me that it was not an unknown mechanical or human oscillation which was responsible for the results obtained. Sixteen separate tests with sixteen logarithmically growing LM mouse fibroblast cell populations gave virtually identical results and convinced me that the oscillatory membrane phenomenon with this system does exist.

Considering then the possible mechanism of FDA metabolism by the LM mouse fibroblast cell, it was noted that the substrate entrance, initially at least, would appear to be by simple diffusion. The quantity of substrate which enters the cell normally remains within a restricted range, suggesting a finite space for the substrate within the cell.

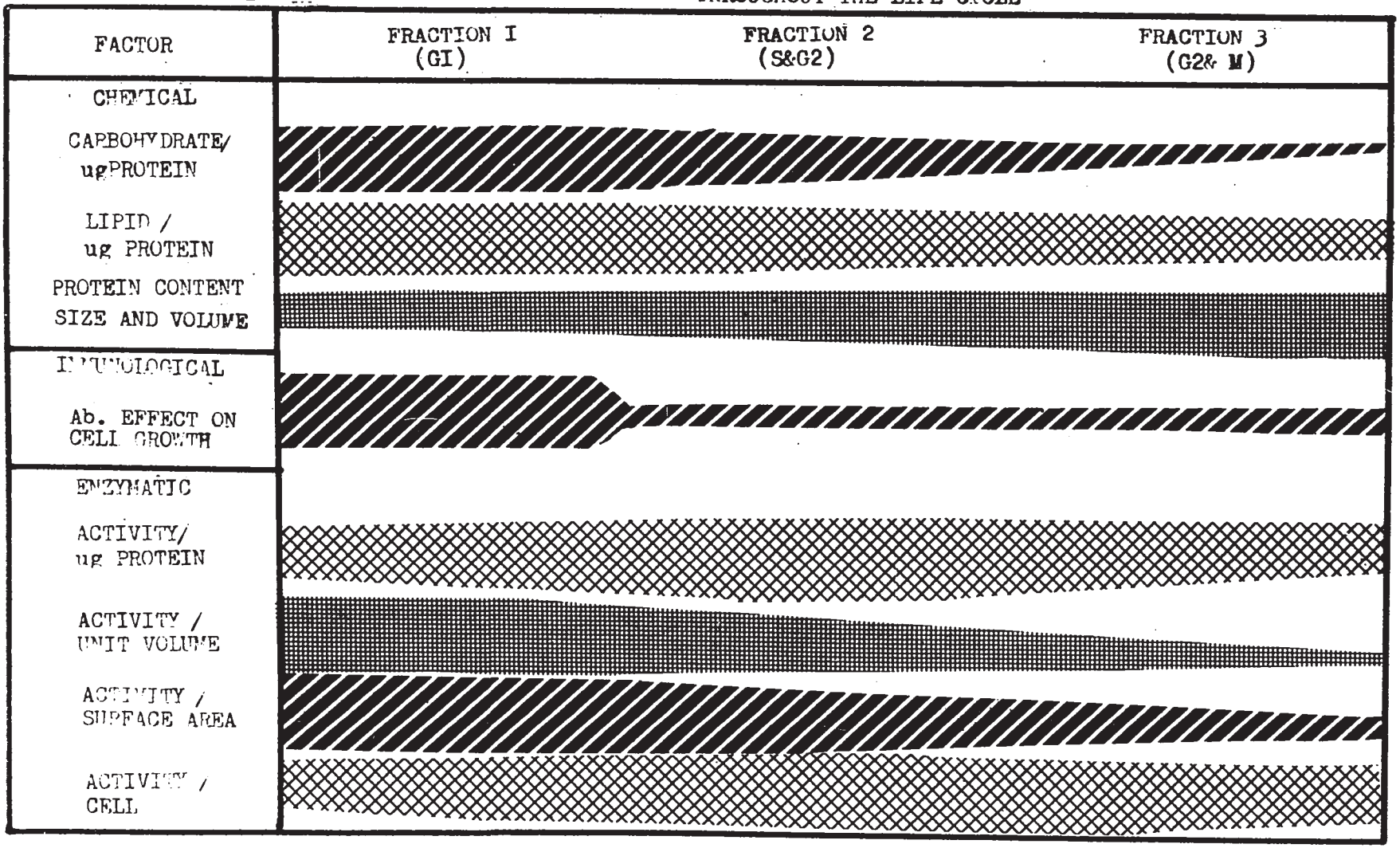
Intracytoplasmic "hot spots" often observed in these experiments may be fluorescing lysosomes or other organelles and would support such an assumption of finite substrate space. In the normal metabolizing cell, once this finite space is filled, the membrane closes to more substrate and the cell's enzymatic systems, in the form of acetylesterase(s), attacks the metabolite, breaking it down in two steps to fluorescein. Upon completion of the breakdown of most of the substrate present, the membrane once again opens and the swift shift of cell's content from high concentration of the product (F) and low concentration of the substrate (FDA) to the opposite condition within a space of 30 seconds takes place. Such results support the suggestion of Katchalsky and Spangler, (1968), that the membrane is the gatekeeper of the cell and that although an enzyme process itself may be constant the product concentration affects the permeability of the membrane giving discernible periodic membrane processes. The inability of the cell to metabolize the substrate if energy was denied it (Table VIII, page 85), would indicate that the enterance of the substrate or the breakdown process itself requires energy.

The speed with which the LM mouse fibroblast cell begins FDA breakdown and the observed cycle would seem to indicate that the enzyme is non-inducible. The presence of the cycle in all phases of the cell life cycle (Figure 23, page 95) would seem to indicate that the oscillatory system

exists independently of any growth activity of the cell. Burton, (1970), has proposed that all cells maintain an oscillatory regulating mechanism which functions continually and which is unaffected by any variations of the cell's metabolic life, including growth and division. Table XI, page 96, which shows the specific ratios of various cell fractions characteristic to the observed fluorometric rate of the fraction, does indicate that fraction 2 (S and G2), per microgram of protein, is more active fluorometrically than fraction 1 (G1) and 3 (G2 and M). An increased percentage of enzyme protein per total cell protein in this fraction 2 as compared to fraction 1 and 3, may account for this. The nonspecific nature of esterases should not be forgotten in this regard since fraction 2 may contain more esterases capable of attacking the FDA molecule should it appear. The priming effect noted with fluorescein incubation (Table VIII, page 85) may be a characteristic solely of the product of the FDA-acetylesterase system. Both this priming and the feedback inhibition noted for fluorescein may be linked.

Figure 30

VARIATIONS IN LV MOUSE FIBROBLAST CELL THROUGHOUT THE LIFE CYCLE



147A

Conclusion

When all the various aspects of this investigation are considered (Table VI, page 77, Table XI, page 96, Table XV, page 110 , Figure 20, page 147) it becomes apparent that there is a marked difference, both in the whole cell and in the cell membrane, between young newly formed cells in G1 and the older cells in the other phases of the cell cycle. Cells in G1, although the smallest in mass and volume, have per microgram of cell protein much more carbohydrate, (twice the amount in total cell and three times the amount in the membrane), and slightly more lipid than the older cells. They have greater enzymatic activity per unit of surface area and per unit volume, though not per cell or per microgram of cell protein. G1 is the cell phase most affected by the addition of sera, (both pre-immune and immune) in subsequent growth action as well as initial enzymatic reaction. Permeability of the cells in G1 seem to share, with those in the last stages of M, a resistance to sulphydryl acting agents such as fluorescein mercuric acetate, suggesting again an altered membrane at this time.

The basic questions asked initially in this investigation can be tentatively answered.

Does the membrane vary during the cell's life cycle? Yes, chemically, enzymatically and immunologically the membrane changes as the cell progresses from G1 through

the cell cycle to M. The changes are gradual and would appear to be consistent cellular changes for the LM mouse fibroblast cells.

During the life cycle is there a time at which the cell is more resistant to exterior factors? It would appear that resistance to exterior factors depends on the factor. Young cells in G1 appear to have more resistance to chemical stress--such as fluorescein mercuric acetate and fluorescein, but are the prime target for immunological stress such as specific cell antibodies.

It was evident throughout this investigation that the cell membrane is of paramount importance in all phases of cellular metabolism and the extent of its workings have so far just begun to be investigated.

LIST OF APPENDICES

APPENDIX 1

a. MEDIUM 199 (Grand Island Biological Company, Grand Island, N.Y., U.S.A.)

Component	Gms/Liter
Sodium Chloride (NaCl)	8.000
Potassium Chloride (KCl)	0.400
Magnesium Sulphate ($MgSO_4 \cdot 7H_2O$)	0.200
Disodium Phosphate ($Na_2HPO_4 \cdot 7H_2O$)	0.090
Potassium Dibasic Phosphate (KH_2PO_4)	0.060
Dextrose (anhydrous)	1.000
Phenol Red	0.020
Calcium Chloride ($CaCl_2$) anhydrous	0.140
Sodium Acid Carbonate ($NaHCO_3$)	0.350
	Mgs/Liter
L-arginine	70
L-histidine	20
L-lysine monohydrochloride	70
DL-tryptophan	20
DL-phenylalanine	50
DL-methionine	30
DL-serine	50
DL-threonine	60
DL-isoleucine	40
DL-leucine	120
DL-valine	50
DL-glutamic acid monohydrate	150
DL-aspartic acid	60
DL-alpha-alanine	50
L-proline	40

APPENDIX 1 (Continued)

Component	Mgs/Liter
L-hydroxy proline	10
Glycine (aminoacetic acid)	150
L-glutamine	100
Sodium acetate trihydrate	50
L-cystine	20
L-tyrosine	40
L-cysteine hydrochloride	0.1
Adenine	10
Guanine	0.3
Xanthine	0.3
Hypoxanthine	0.3
Uracil	0.3
Thymine (5-methyl uracil)	0.3
Disodium Alpha Tocopherol Phosphate	0.01
Thiamine	0.01
Pyridoxine Hydrochloride	0.025
Riboflavin	0.01
Pyridoxal Hydrochloride	0.025
Niacin	0.025
Niacinamide	0.025
Calcium pantothenate	0.01
i-inositol	0.05
Ascorbic Acid	0.05
Folic acid	0.01
p-Aminobenzoic acid	0.05
Ferric Nitrate	0.1
Biotin	0.01
Menadione	0.01
Glutathione	0.05
Vitamin A	0.1
Calciferol	0.1
Cholesterol	0.2
Tween 80*	5.0

APPENDIX 1 (Continued)

Component	Mgs/Liter
Adenosinetriphosphate (sodium salt)	1.0
Adenylic acid	0.2
Desoxyribose	0.5
D-ribose	0.5
Choline Chloride	0.5

APPENDIX 1 (Continued)

a. Medium 199 - peptone Preparation

This completely defined chemical medium has been used for LM mouse fibroblast cells since 1956. (Morgan, Morton and Parker, 1950). It was made from four separate sterile solutions mixed together asceptically and checked for sterility overnight in an incubator at 37°C. A fifth sterile solution of penicillin and streptomycin was added to the medium just before using. The five separate solutions are:

i) Medium 199 (10X)

ii) Glutamine solution

iii) Bicarbonate solution

iv) Peptone water - with or without methyl cellulose depending on the method of cell propagation.

v) Penicillin and streptomycin solution.

i) Medium 199 (10X)

Powdered Medium 199 (Hank's salts) was obtained from Gibco, Grand Island Biological Company, Grand Island, New York, U.S.A. For the chemical composition of this powder see the Appendix. A liter of medium 199 (10X) was prepared as follows.

In a large clean flask 111.0 gms of the 199 powder was placed. To this one liter of glass distilled water was added. The contents were mixed on a magnetic stirrer until

APPENDIX 1 (Continued)

all the powder was dissolved. This liquid was sterilized by filtration through a previously sterilized membrane filter (0.2μ filter). The liquid was measured asceptically (30 ml) into sterile screwcapped tubes using a repeating syringe apparatus. The tubes were kept in a refrigerator at 4°C until used for the complete H-199-peptone medium.

ii) D.L. Glutamine Solution

This powder was obtained from Nutritional Biochemical Co., Cleveland, Ohio, U.S.A. To give a 100X solution 10 gm of powder was dissolved in 100 ml of glass distilled water. The liquid was filtered through a sterile Seitz filter into a flask with a sterile delivery bell attachment. The liquid was measured, (3 ml) into sterile screwcapped tubes. The tubes were kept in the freezer at -20°C until adding to the complete H-199 peptone medium.

iii) Sodium Bicarbonate Solution

This sodium bicarbonate powder, reagent grade, was supplied by the Shawinigan Chemical Company, McCarter Chemical Company, Montreal, Quebec (Distributors). A 5.25% solution of the bicarbonate was made in glass distilled water, placed into small screwcapped bottles and autoclaved 20 minutes at 120°C. The solution was kept at room temperature until used.

APPENDIX 1 (Continued)

iv) Peptone Water (0.5%)

The peptone water, 0.5%, is the basis for the H-199 medium constituting 90% of the final propagating medium bulk. Bacto-peptone was supplied by Difco, Detroit, Michigan. The medium can be made either with or without methyl cellulose. The former basic medium was used for suspension cultures (Bryant, 1961); the latter for monolayer cultures.

Peptone	22.22 gm	Peptone Water	Peptone Water, Methyl Cellulose
Glass distilled water	4000 ml		
Methyl Cellulose	5.33 gm		

The water was gradually added to the peptone powder and mixed on a magnetic stirrer until all the powder was dissolved. If methyl cellulose, Dow Chemical of Canada, Sarnia, Ontario, was added, the peptone powder and the methyl cellulose powder were first dry mixed to facilitate the dissolving of the methyl cellulose.

Medical flats, 400 ml, were filled with 270 ml of the peptone water and autoclaved at 120°C for 30 minutes. The mixing of the medium takes place subsequently in these bottles. The peptone water was kept at room temperature until used.

APPENDIX 1 (Continued)

v) Preparing the Cell Medium 199-peptone Water

To the medical flat containing 270 ml of 0.5% sterile peptone water was added:

Medium 199 (10X)	30 ml
Sodium bicarbonate solution	3 ml
D-L Glutamine solution	3 ml

This was incubated at 37°C overnight to check the mixture's sterility, then a sterile solution of penicillin and streptomycin was added.

b) Penicillin and Streptomycin Solution

Penicillin G sodium and D-hydrostreptomycin sulphate obtained from Nutritional Biochemical Corporation, Cleveland, Ohio, were used to make this solution.

Penicillin G Sodium	0.6765	g	) 100X
Dihydrostreptomycin sulphate	1.418	g	) Solution
Water	400	ml	)

The antibiotics were dissolved in the water and filtered through a sterile membrane filter into a sterile flask with a dispensing bell attachment. 3 ml was placed into each sterile screwcapped tube, and subsequently frozen. When added to the 300 ml of prepared medium 199-peptone the final concentration is 100 International units of Penicillin/ml

APPENDIX 1 (Continued)

and 100 ug of Streptomycin/ml. The medium was now ready for use either for suspension or monolayer cultures.

b. Dulbecco Phosphate Buffered Saline

Three separate solutions A, B and C were made up, mixed and adjusted to a pH of 7.2. Osometer readings of the buffer were taken and compared to those for medium 199-peptone.

Solution A

Sodium chloride (NaCl)	8.0 g/liter	Dissolved in 980 ml Distilled Water
Potassium chloride (KCl)	0.20 g/liter	
Hydrated sodium phosphate dibasic ($Na_2HPO_4 \cdot 7H_2O$)	2.16 g/liter	
Potassium acid phosphate (KH_2PO_4)	0.2 g/liter (final concentration)	

Solution B

Calcium chloride ($CaCl_2$)	0.10 g/liter (final concentration)	Dissolved in 10 ml of distilled water

Solution C

Hydrated magnesium chloride ($MgCl_2 \cdot 6H_2O$)	0.10 g/liter (final concentration)	Dissolved in 10 ml of distilled water

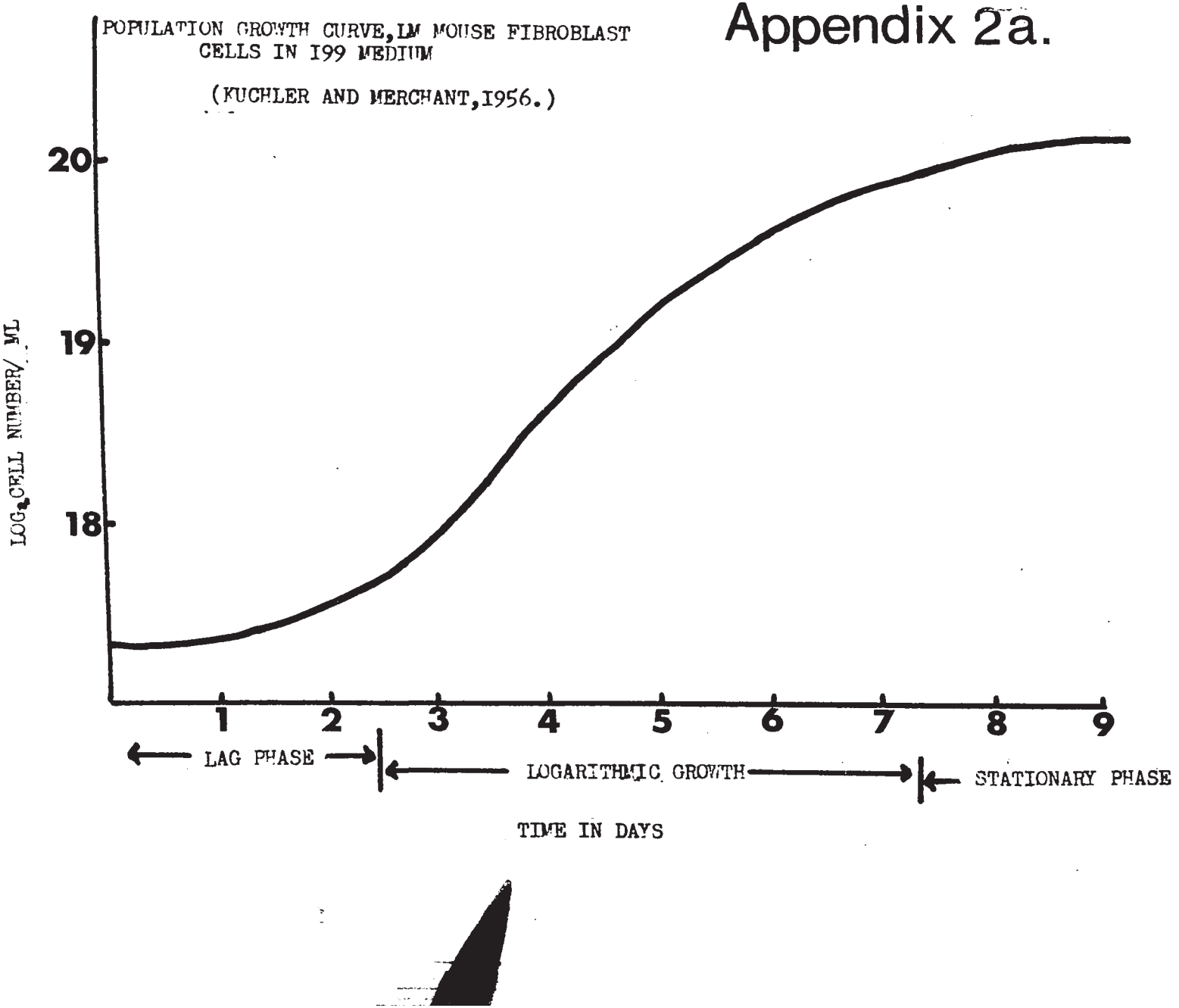
Together the three solutions give 1000 ml (1 liter) of Dulbecco phosphate buffer.

APPENDIX 1 (Continued)

c. Vitamins

Vitamin A and E, concentrate, (1×10^{-1} M) in chloroform and Vitamin C in water were freshly made up. Both Vitamin A and C, in medium 199-peptone, were made up to give 10X and 100X the normal concentration in the medium. Vitamin E was made up in medium 199 to give solutions containing 1×10^{-4} M and 1×10^{-5} M.

Appendix 2a.



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APPENDIX 2b

DOUBLING TIME CHART

POPULATION DOUBLING TIME RATIOS "R"

Growth Interval									
Days	Hours	20 hours	25 hours	30 hours	35 hours	40 hours	45 hours	50 hours	55 hours
½	12	1.516	1.394	1.319	1.269	1.231	1.203	1.181	1.163
1	24	2.297	1.945	1.741	1.608	1.516	1.447	1.394	1.319
1½	36	3.481	2.713	2.297	2.043	1.866	1.741	1.647	1.574
2	48	5.276	3.784	2.587	2.297	2.094	1.945	1.831	1.741
2½	60	7.998	5.276	3.281	2.828	2.520	2.297	2.129	2.000
3	72	12.13	7.358	4.161	3.481	3.031	2.713	2.477	2.300
3½	84	18.38	10.27	5.276	4.296	3.646	3.646	3.203	2.882

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Department of Bacteriology & Immunology
University of Western Ontario

APPENDIX 2b

Calculation of Population Doubling Time

1. Using population doubling time chart accompanying.

   At zero time the initial cell count "A" is taken. The second count "B", can be taken at any time, in 12 hour intervals after that. We will assume it is at 24 hours. Find the ratio B/A = R

   In the column (24 hours growth interval) find the number nearest (but slightly less than) "R" in the population doubling time column (Y). Find the difference between Y and the next highest doubling time ratio (X). "R" will be found somewhere between X and Y.

   $X - Y = Z$ The difference between the two ratios. The doubling time interval is a standard 5 hours.

   Next find the difference between Y and R

   $Y - R = Y'$

   The actual time in excess of X for the experimental doubling time is therefore

   $\frac{Y'}{Z} \times 5 = s =$ Time in excess of X for the experimental population

APPENDIX 2b (Continued)

The doubling time for the population is

X + s = Experimental doubling time.

2. Example. (using accompanying doubling time chart)

Count of cell population at zero time = A = 5.0×10^5 cells/ml

Count of cell population 24 hours later = B = 7.5×10^5 cells/ml

Doubling time ratio "R"

$$\frac{7.5}{5.0} = R = 1.5$$

From growth interval column, 24 hours, we see 1.5 ratio is between 45 hours and 40 hours.

At 45 hours the ratio is 1.447 = Y

At 40 hours the ratio is 1.516 = X

The difference X - Y = 1.516 - 1.447 = .069 = z

The difference between R and Y is 1.5000 - 1.447 = .053 = Y'

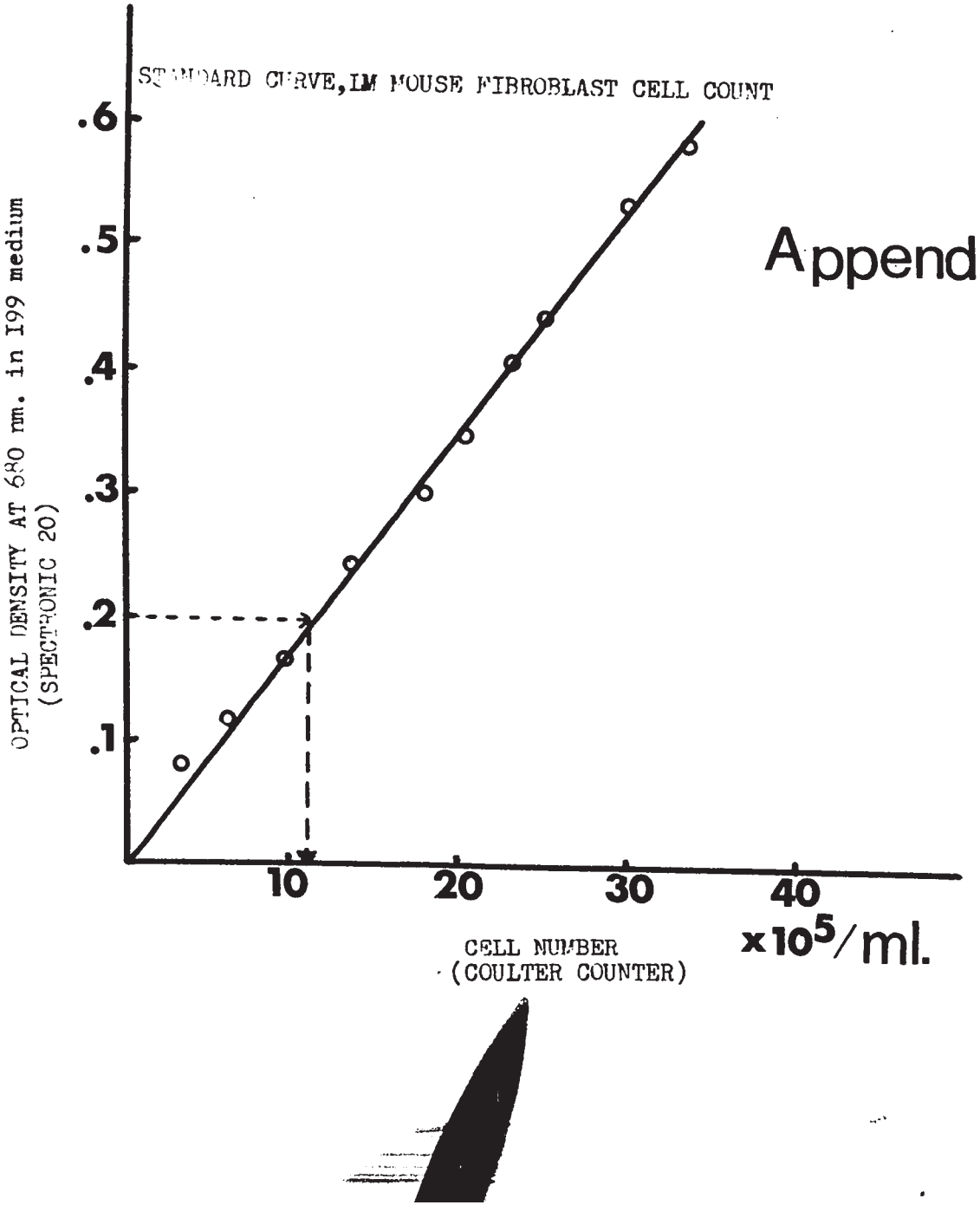
now actual time in excess of X (40 hours) is

$$\frac{.053}{.069} \times 5 \text{ interval hours} = 3.8 \text{ hours} = s$$

Therefore the actual doubling time for this population is

X + s = 40 + 3.8 = <u>43.8 hours</u>

The doubling time for the experimental population.


STANDARD CURVE, LM MOUSE FIBROBLAST CELL COUNT
OPTICAL DENSITY AT 680 nm. in I99 medium
(SPECTRONIC 20)
.6
.5
.4
.3
.2
.1
10
20
30
40
x10^5/ml.
CELL NUMBER
(COULTER COUNTER)

Appendix 2c

APPENDIX 2d

COMPARISON OF ERYTHROSIN B AND FLUORESCEIN DIACETATE

VIABILITY TESTS

Population	Erythrosin B-Dye Exclusion Method				Fluorescein Diacetate Method			
	Total cell count	Viable cells	Non Viable	% Viability	Total Cell count	Viable cells	Non Viable	% Viability
1	2821	2641	180	93%	2671	2433	238	91%
2	2720	2491	229	91%	2554	2378	176	93%
3	1886	1723	163	91%	2004	1913	91	95%
4	1735	1579	156	91%	1935	1784	151	92%
5	2114	1998	116	94%	2067	1901	166	92%
6	2026	1884	142	93%	2131	1925	206	90%
7	1524	1271	253	80%	1971	1624	347	82%
8	2283	2094	189	92%	2004	1931	73	95%
9	2055	1947	108	94%	2260	2135	125	94%
10	2346	2168	178	92%	2232	2105	127	94%
11	2455	2321	134	94%	2136	2097	39	97%
12	2670	2554	116	95%	2537	2449	88	96%

APPENDIX 2e

POPULATION AND INDIVIDUAL LM CELL DATA - OTHER INVESTIGATORS

1. Kahn, R.H., Conklin, J.L., & Dewey, M.M. (1962). Suspension cells range in size from 12 to 18μ in diameter. Differences in population density, mitotic phase, produce heterogeneity in the metabolic and synthetic activity of individual cells.

2. Sinclair, R. & Bishop, D.H. (1965). Suspension cells range in size from 10 to 20μ in diameter. Low speed centrifugation (100 g) gives best separation of cells.

3. Killander, D., & Zetterberg, A. (1965). If cells spend a long time in G1 will spend a shorter time in S and G2. Generation time fairly constant.

4. Merchant, D.J. & Eidam, C.R. (1965). As cell population goes into stationary phase percentage of small cells increase. Addition of glucose to exhausted medium will stimulate growth.

APPENDIX 2e (Continued)

5. Rothblat, G.H. (1969). If serum added, as concentration goes up the L cell suppresses cellular sterol synthesis and there is an enhanced release of synthesized sterol. If free cholesterol added the cells cease to produce cholesterol from added acetate.

6. Wiebel, F., & Baserga, R., (1969). Addition of serum causes an immediate effect on the function of the cell membrane, there is a shift in the amino acid pool and immediate protein synthesis.

7. Daniels, W.F., (1968). L cells need controlled CO_2, air, nitrogen, temperature, and pH. The pH and oxidation reduction potential most important (100 millivolts optimum).

8. Warren, L. & Glick, M. (1968). In growing cells and non growing cells approximately the same amounts of surface membrane and particulate material are synthesized. In growing cells the material is incorporated with net increase in substance. There is relatively little turnover. In non growing cells the newly synthesized material is incorporated and a corresponding amount eliminated

APPENDIX 2e (Continued)

there is a rapid turnover and no net increase of substance.

9. Weinstein, D.B., Marsh, J.B., Glick, M.S., & Warren, L., (1967). Membrances of L cells (grown in Eagle's medium plus serum) contained approximately 4.7% of the protein and 13.8% of the lipid of the total cell. Neutral lipids made up 40% of the total membrane lipid with cholesterol 20% and triglycerides 13%.

10. Geyer, R.P. (1966). L cells in medium with serum have high amounts of linoleic acid. L cells in medium without serum have low amounts of linoleic acid.

11. Mackenzie, C.G., Mackenzie, J.B., Reiss, O.K. (1966). When L cells transferred to a serum free medium there was an increase in the synthesis of fatty acids from glucose even though medium contained acetate. Striking increase in synthesis of cholesterol noted. Serum lipids suppress both synthesis of fatty acids and cholesterol.

12. Pirt, J., & Thackery, E.J. (1964). Tonicity optimum 300 - 363 milliosomoles/liter for L cell. Tolerance varies with sublines and pH of culture. Glycolysis increases with increasing pH.

13. Heard, D.H., Seaman, G.V.F., & Simon-Reuss, I., (1961). Also Ban-Or, S., Eisenberg, S., & Doljanski, F. (1960). Electrophoretic motilities of young cells, adult cells and neoplastic cells vary, but they are only a manifestation of normal growth process, not malignancy.

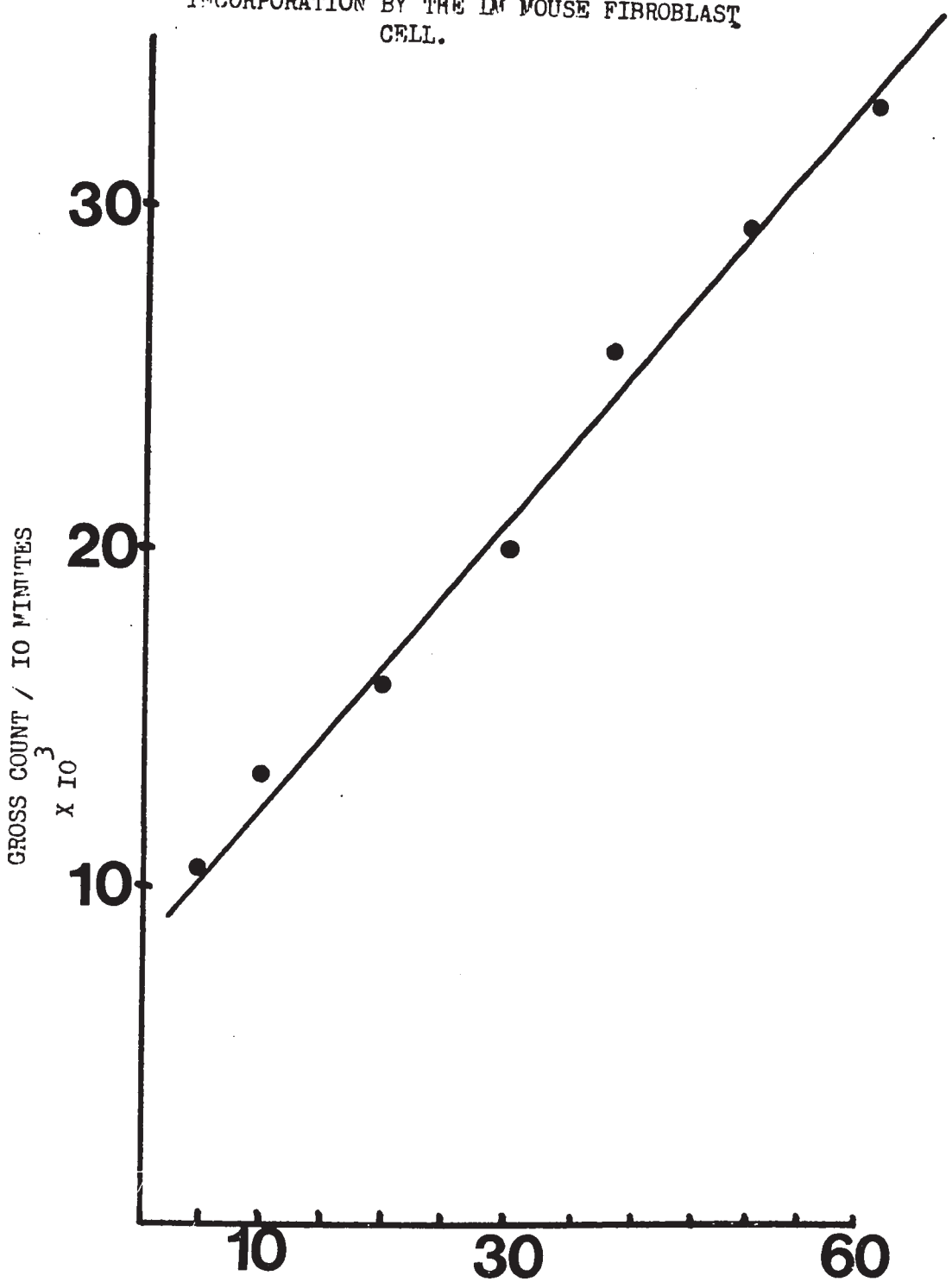
14. Filkuka, J., Svejda, J., & Aubrechtova, V., (1969). There is no parallelism between number of chromosomes and modal DNA content established at interphase. There is a correlation if measurement s are performed at metaphase. L-cells have a modal number of 54 chromosomes.

15. Rixon, R.H. & Whitefield, J.F. (1962). 0.24 M NaCl and 0.12 M KCl give structures in nuclei of L cells similar to those observed in nuclei in early prophase. Changes of osmoregulatory system may be part of mechanism initiating prophase.

xd

Appendix 3

STANDARD CURVE FOR RADIOACTIVE THYMIDINE INCORPORATION BY THE IM MOUSE FIBROBLAST CELL.



APPENDIX 3b

RAW DATA SHOWING RADIOACTIVE TRACER INCORPORATION

BY VARIOUS CELL FRACTIONS

	POST LABELLING		PRELABELLING	
WHAT	1	2	1	2
1. Standards				
a. Background	210 gross count	201 gross count	190 gross count	198 gross count
b. Efficiency of the Machine	71%	71%	71%	71%
c. Efficiency of the Label	29%	30%	30%	30%
d. PPO - Toluene	202 gross count	213 gross count	209 gross count	-
e. NCS - PPO Toluene	324	312	299	-
f. Filter plus unlabelled Mother	189	200	212	209 gross count
2. Mother				
a. Total counts per minute (corrected)	3,793	5,352	2,261	2,231

APPENDIX 3b (Continued)

WHAT	POST LABELLING 1	POST LABELLING 2	PRELABELLING 1	PRELABELLING 2
2. (cont'd)				
b. Counts corrected in DNA (cpm)	3,598 94% (DNA)	4,711 89% (DNA)	2,044 90% (DNA)	-
c. Counts corrected in rest of cell	219 5% (rest of cell)	241 4% (rest of cell)	91 4% (rest of cell)	
3. Fraction 1				
a. Total counts per minuted corrected	1.37	.06	.18	.22
b. Counts corrected in DNA	.1	.09	.26	-
c. Counts corrected in rest of cell	.76	.94	.80	-
4. Fraction 2				
a. Total counts corrected	3,076	2,398	1,763	1,899
b. Counts in DNA corrected	2,737 89% DNA	1,939 82% DNA	1,609 91% DNA	-

APPENDIX 3b (Continued)

	POST LABELLING		PRELABELLING	
WHAT	1	2	1	2
4. (Cont'd)				
c. Counts in rest of cell	154.5 5% (rest of cell)	87.2 4% (rest of cell)	70.0 4% (rest of cell)	-
5. Fraction 3				
a. Total counts corrected	3,558	4,640	2,364	2,129
b. Count in DNA corrected	3,145 89% CNA	3,810 82% DNA	2,179 92% DNA	-
c. Counts in rest of cell	235 6% (rest of cell)	170.2 4% (rest of cell)	75.8 3% (rest of cell)	-

xdiv

APPENDIX 4

FLUOROMETRIC CALCULATIONS

a. Calculation of the standard method of fluorometric rate for a cell population.

a = initial tube reading in fluorometric units recorded by the Heath Recorder from the Turner Fluorometer reading.

b = final reading of tube in fluorometric units recorded by the Heath Recorder from the Turner Fluorometer reading.

X = time in minutes from start of test when "a", initial reading was made as indicated by a stopwatch.

Y = time of final reading "b" according to a stopwatch.

Uncorrected Fluorometric rate in fluorometric units/minute $= \frac{b-a}{y-x} = V_u$

Corrected Fluorometric rate in fluorometric units/minute $= V_{u_{test}} - V_{\substack{FDA \\ control}} = V_{c_{test}}$

Example - initial reading 5.0 fl. units (a)

time of reading 0 minutes (X)

final reading 75.0 fl. units (b)

time of final reading 10 minutes (Y)

$\frac{75.0 - 5.0}{10 - 0} = \begin{matrix} 7.000 \\ \text{fl units/} \\ \text{min} \end{matrix} =$ Uncorrected Fluorometric rate V_u

APPENDIX 4 (Continued)

If breakdown rate of FDA in this system gave a rate of 0.2000 fl. units/min then

the Corrected Fluorometric rate in Fluorometric units/minute = 7.000 - 0.2000 = 6.8000

b. Lm cells and various concentrations of the fluorogenic substrate FDA. Rate variations

Total Substrate Concentration	(Fl. units/minute) Corrected Fluorometric Rate
2.4×10^{-6} M FDA	1.06
4.8×10^{-6} M FDA	1.17
12.0×10^{-6} M FDA	3.00
24.0×10^{-6} M FDA	4.44
48.0×10^{-6} M FDA	4.90

c. Lm cells and temperature variations effect on the substrate LM cell acetylesterase interaction

Total Substrate Concentration	Corrected Fluorometric Rate (Fl. units/min)		
	$27^{o}C$	$37^{o}C$	Q_{10}
4.8×10^{-6} M FDA	0.69	1.17	1.70
12.0×10^{-6} M FDA	1.60	2.80	1.72
24.0×10^{-6} M FDA	2.50	4.20	1.70

d. Effect of pH variation of the extracellular medium on the FDA acetylesterase system of the LM cell. Three populations

	Corrected Fluorometric Rate (Fl. units/min)												
Population pH	6.8	6.9	7.0	7.1	7.2	7.3	7.4	7.5	7.6	7.7	7.8	7.9	8.0
Logarithmic Growing	6.8	6.4	6.4	-	5.5	6.9	6.5	7.8	6.5	7.8	6.6	6.6	6.4
Lag Phase	-	1.6	1.5	1.9	1.5	1.7	1.2	1.5	1.7	3.6	3.6	-	-
Stationary Phase	3.4	5.1	3.3	4.3	3.9	2.8	7.2	5.6	4.8	6.3	6.3	6.0	5.9

Total substrate concentration 24.0 X 10^{-6} M of FDA

APPENDIX 5

CALCULATION OF EXIT-ENTRY SYSTEM COMPOUND SUMMATION

1. Molarity of substrate

   a. Concentrate

   Molecular weight of fluorescein diacetate 416.369.

   100 mg of FDA placed in 20 mls of acetone is our concentrate.

   $$\frac{100 \text{ mg}}{X} = \frac{20}{1000} \text{ or 5 gms in 1000 ml}$$

   now 416.369 g/liter = 1 M*

   $$\frac{1}{416.369} \times 5.0 = .012 \text{ M solution is concentrate (in acetone)}$$

   b. Test substrate

   50λ of the concentrate (.012 M FDA in acetone) is placed in 10 ml of medium 199, and 500λ (0.5 ml) of this is placed in the final bathing medium to make a total of 5.0 ml.

   $$.0120 \text{ M} \times \underset{\text{(test substrate)}}{\frac{50}{10{,}000}} \text{ ml} \times \underset{\text{(final)}}{\frac{0.5}{5.0}} = 6.0 \times 10^{-6} \text{ M of FDA in the extracellular medium and cells}$$

   6.0×10^{-6} M of FDA is the total amount of substrate in the system.

   * M = Molar Concentration

APPENDIX 5 (Continued)

2. From a standard curve it was found that one (1.0) planimeter unit of FDA was equal to 5.34×10^{-9} M of FDA (5 λ spot) of FDA dissolved in 1 ml of methanol.

Planimeter Reading on Record Paper of FDA Peak (X) · $(5.34 \times 10^{-9}$ M) ÷ cell number = M of FDA/cell

The cell number was kept constant at 2.75×10^{5} cells <u>total</u> added.

$X \cdot (5.34 \times 10^{-9}$ M of FDA) ÷ (2.75×10^{5})
$= 1.9418 \times 10^{-14}$ (X) M of FDA/cell

1.9418×10^{-14} was the factor by which the planimeter reading of the FDA peak was multiplied to get M of FDA/cell.

For the summation of FDA in the total system (cells and extracellular fluid) the amount of FDA in the total <u>cell</u> population was

$X \cdot (5.34 \times 10^{-9}$ M of FDA) = Total FDA for cells in system

Planimter reading · Amount of FDA for each planimeter unit

3. From a standard curve it was found that one (1.0) planimeter unit of fluorescein was equal to 4.44×10^{-9} M of Fluorescein (5 λ spot) of known amount of F dissolved in 1.0 methanol.

xdix

APPENDIX 5 (Continued)

Planimeter Reading on Record Paper of Fluorescein Peak "Y" $\times$ $(4.44 \times 10^{-9}$ M) $\div \frac{(2.75 \times 10^{5})}{\text{cell number}}$ = M of F/cell

$= 1.6181 \times 10^{-14}$ Y M of F/cell

1.6181×10^{-14} was the factor by which the planimeter reading of the fluorescein peak was multiplied to get M of F/cell.

4. Since the intermediary product (fluorescein monoacetate (FMA) was unavailable to make a standard curve the factor used to calculate M of FMA/cell was the same as the fluorescein, i.e. 1.5181×10^{-14} X the planimeter reading obtained for the FMA peak.

5. Extracellular fluid.

Since 10 λ of a total 5 ml extracellular bathing medium was spotted

$\frac{5000}{10}$ = 500 was the correction factor to account for all the compound in the medium.

From standard curves it was found that a known amount of FDA dissolved in medium 199, one planimeter unit was

5.34×10^{-9} M of FDA

$5.34 \times 10^{-9} \times 500 = 2.67 \times 10^{-7}$ M of FDA

APPENDIX 5 (Continued)

For fluorescein, one (1.0) planimeter unit was

$$4.44 \times 10^{-9} \times 500 = 2.23 \times 10^{-7} \text{ M of Fl.}$$

These are the factors by which the planimeter readings obtained for the extracellular fluid are multiplied.

6. Summations of System

$$\frac{\text{(FDA in cells + FMA in cells + F in cells)}}{\text{Total Cells}} + \frac{\text{(FDA in medium + FMA in medium + F in medium)}}{\text{Total Medium}} = \text{Total substrate originally added to the system}$$

APPENDIX 6

CLASSIC CHEMICAL METHODS

a. Protein Analysis

Dry Weight Method - Modified Schmidt - Tannhauser Method (Merchant, Kahn and Murphy, 1964)

i) Preparation of Cells

The population of LM mouse fibroblast cells, being studied, after washing with 199 peptone was re-suspended in a measured amount of 0.16 M NaCl. A cell count was taken using a Coulter counter (Model A), and the number of cells used in the test ascertained. In the case of the cell membrane fraction (page 50) originally suspended in 45% sucrose in Tris, the pellet was washed with .02 M Tris-$MgCl_2$ (1×10^{-5} M) buffer resuspended after pelleting in a measured amount of 0.16 M NaCl and a ghost count made using a hemocytometer.

ii) Extraction of Protein

After two fresh saline washes (Mackenzie et al., 1966), the cells were suspended in 5 ml of 6% Trichloracetic acid. The mixture was heated at $90^{o}C$ for 15 mins. and centrifuged to pellet the precipitate. Two lipid extractions of the precipitate were done using 5 ml of chloroform - methanol (2:1). The first

APPENDIX 6 (Continued)

extraction was done 10 mins. at room temperature, and the second 10 mins at 45°C. After recentrifugation the extract (called Lipid I) was placed in a screw-capped universal and treated as in Section III 8.4. of this study. The precipitate was resuspended in a chloroform ether solution (4:1) and transferred to a constant weight aluminum dish. The chloroform and ether were evaporated off in a fume cupboard and the aluminum dish placed in a desicator and allowed to come to a constant weight. The dish was weighed and the dry weight (protein) of the known number of cells calculated.

The dried precipitate in the aluminum dish was dissolved in 5 ml of 0.1 NaOH and a Folin-Phenol determination done on the resulting liquid.

Folin - Ciocalteau Phenol method (Lowry, 1951)

Protein content determined by this method utilizes the color reaction of the aromatic amino acids tyrosine and tryptophane with the Folin-Ciocalteau phenol reagent. The method therefore assumes a constant tryptophane and tyrosine content in the protein. The modification developed by Lowry was used.

All chemicals used for test procedures and standard solutions were reagent grade. Bovine Serum Albumin (BSA) Calbiochem, Los Angeles, California, U.S.A. was used as

APPENDIX 6 (Continued)

the standard. Serial dilutions of this material were prepared ranging from 10 - 500 mg/ml. Duplicate tests were performed on each of these dilutions to verify the method and to determine at which concentrations the results were most reproducible. In each test the resulting optical density (OD) of the sample was compared to that of a known quantity of BSA. In this manner an estimate was obtained of protein present in the cell test sample.

Protein Extraction

A population of cells washed and pelleted as noted in Section III B. 1. (a) was handled in a similar manner to the final extraction of Lipid I (page 66). After this point the precipitate was suspended in 0.1 ml of 0.5 N potassium hydroxide (KOH) and incubated at 37°C for 16 hours. The mixture was neutralized with hydrochloric acid (HCl) using approximately 75λ of a 6.2 N solution. Cold 6% trichloracetic acid (0.5 ml) was added and the mixture incubated at 4°C for 30 minutes, then centrifuged at 1000 g for 10 minutes. The fluid was removed and placed in a screwcapped bijoux bottle to use in the orcinol test for RNA. The pellet was resuspended in 1 ml of 6% trichloracetic acid and incubated at 90°C for 15 minutes. After

div

APPENDIX 6 (Continued)

centrifugation the supernatant was removed and placed in a screwcapped bijoux bottle for the test for DNA. Once more the pellet was resuspended, this time in 5 ml of 0.1N NaOH (sodium hydroxide) and held at 37°C for 30 minutes during which time the precipitate dissolved.

Previously to this, 100 ml of a two percent aqueous sodium carbonate solution was prepared. 2 ml of a mixed solution, containing one volume of a 2% aqueous tartrate and one volume of 1% cupric sulphate in glass distilled water, was added to the sodium carbonate solution just prior to the protein test.

To 0.1 ml of the solubilized protein was added one ml (1 ml) of this carbonate - sulphate - tartrate mixture. It was allowed to stand 10 minutes at room temperature, then 100 ul of Folin-phenol reagent was added (commercial grade, titrated to 1 N). The reaction mixture was allowed to develop for 30 minutes at room temperature after which the optical density was read on the Hitachi spectrophotometer at a wavelength of 750 nm, (Hitachi-Perkin-Elmer 139, UV-VIS, Hitachi Ltd., Tokyo, Japan). Amounts of protein were determined from a BSA standard curve, done at the same time as the test material.

APPENDIX 6 (Continued)

b. DNA Determination

Method 1 (Burton, 1956 modification of Dische)

A stock solution was prepared containing 1.5 gm of crystalline dephenylamine dissolved in 100 ml of glacial acetic acid plus 1.5 ml of concentrated sulfuric acid. To 1 ml of glass distilled water 16 mg of acetaldehyde was added and well mixed. To 20 ml of the stock diphenylamine solution, just before using, was added 0.1 ml of the acetaldehyde solution.

To 1 ml of the test material, or to a standard DNA solution (commercial DNA), (Nutritional Biochemicals, Cleveland, Ohio, U.S.A.) was added two ml of the fresh diphenylamine reagent. The mixture was allowed to develop for 16 hours at 30°C after which time the optical density was read at 600 nm on the Hitachi spectrophotometer and the amount of DNA in the sample calculated by comparison to the standard DNA solutions readings.

Method 2 - (Dische, 1930)

1.0 gm of crystalline diphenylamine was dissolved in 100 ml of glacial acetic acid containing 2.74 ml of concentrated sulfuric acid. A blank control of 2.74 ml concentrated sulfuric acid in 100 ml of

APPENDIX 6 (Continued)

glacial acetic acid was also included in the test. The stock solution for the DNA standard contained 10 mg of the DNA dissolved in 50 ml glass distilled water containing a few drops of sodium hydroxide 1N to facilitate its dissolving.

To 0.6 ml of the unknown or standard 1.2 ml of the dephenylamine reagent was added and mixed. Tubes were placed at 37°C for 24 hours after capping. The optical density of the resulting solutions was measured at 600 nm on a Hitachi spectrophotometer.

c. RNA Determination (Drury, 1948; Miller et al., 1951)

Orcinol Test

The sample of RNA tested was obtained as a by-product in the procedure used to ascertain total protein, Folin ciocalteau phenol method.

Standard solutions of RNA were from yeast RNA (Mann Research Laboratories, New York, N.Y.). 1.6 ml of the test material or standard was added to 3 ml of the orcinol reagent. The stock reagent was made up by mixing 13.5 g of ferric ammonium sulphate with 20.0 g of orcinol and bringing the resulting mixture up to 500 ml, in a volumetric flask, with glass distilled water. The test reagent was prepared by taking 25 ml of this stock solution, 415 ml of concentrated hydrochloric

APPENDIX 6 (Continued)

acid and mixing the two. From this comes the 3 ml mixed with the test material. The reaction mixture was heated in boiling water for 20 minutes, cooled to room temperature and read on Hitachi spectrophotometer at a wave length of 760 nm.

d. Lipid Extraction Determination

i) Method of Weinstein, Marsh, Warren & Glick (1969).

A population of LM mouse fibroblast cells was pelleted out of the 199-peptone medium, the supernatant was removed, and the cells resuspended in a measured amount of 0.16 M NaCl. A cell count was taken using a Coulter counter. In the case of the cell envelope fraction, page 36 and 38, the washed pellet was resuspended in a measured amount of 0.16 N NaCl and a ghost count made using a hemocytometer.

After two fresh saline washes (Mackenzie et al., 1966) the pellet was suspended in 5 ml of methanol and extracted 15 minutes at 0°C stirring occasionally. After this time, 5 ml of chloroform was added, making an extraction solution 1:1, and the lipid extraction allowed to continue at 0°C for an additional 15 minutes, stirring occasionally. Four such extractions were

APPENDIX 6 (Continued)

carried out, one at 0°C, two at room temperature and the final one at 45°C.

All the extracted solutions were collected in a universal and retained for purification.

ii) Schmidt-Tannhauser Method (Merchant, Kahn, Murphy, 1964).

A population of LM mouse fibroblast cells or the envelope fraction was pelleted, washed and counted in a similar manner to that of Weinstein et al (1969) method (page 36). After the second saline wash the pellet was resuspended in 5 ml of 6% trichloracetic acid and held in a water bath at 90°C for 15 minutes. After centrifugation the cell pellet was resuspended in 10 ml of chloroform-methanol (2:1) and held at room temperature for 10 minutes. A second extracton was carried out at room temperature for 10 minutes and the clean extract placed in a universal.

Lipid Purification (Sperry, 1955)

Extracts from both the Weinstein et al method and the Schmidt-Tannhauser method were handled in the same way.

To rid the extracts of water soluble constituents of the cells purification was necessary. The screwcap

APPENDIX 6 (Continued)

from the universal were removed and glass distilled water was gently pipetted on top of the extract solutions. The universals were then placed into a large container, three quarters full of glass distilled water, care being taken not to spill the water suspended on top of the extract. Diffusion out of water soluble tissue components was allowed to proceed overnight at room temperature. In the morning the universals were removed from the large container of water and the water suspended above the extract was removed by suction using a Pasteur pipette. Care was taken not to remove any of the interface fluff between the two liquid layers.

Lipid Distillation and Gravimetric Total Lipid Determination (Sperry, 1955).

The extracted layer plus the interface fluff were placed in a 50 ml round bottomed flask which had been previously rinsed three times with ether, and dried to constant weight in a desiccator before use. The universal vial was rinsed twice with 5 ml of chloroform-methanol (2:1). These rinses were added to the 50 ml round bottomed flask. The flask was clamped into a water bath (35° - 40°C) and connected by means of a

APPENDIX 6 (Continued)

Claisen distilling head (Y-attachment and still head) and a close fitting elbow tube with a vacuum attachment to a distillation flask. Through the close fitting rubber stopper, immediately above the round bottomed flask, a capillary tube was led from a funnel, with stopcock attachment, to about two thirds of the way into the round bottomed flask. As distillation proceeded to dryness there was a possibility that residual water might cause frothing. Such frothing was controlled by the addition of small amounts (1-2 ml) of ethanol through the funnel capillary tube attachment.

After distillation the lipid remained as a pale yellow, greasy deposit in the bottom of the round bottomed flask. The flask was removed from the apparatus, the outside polished with lense paper to remove any residual water or grease from handling, and placed in a desiccator for 16 hours before the flasks were weighed on a gravimetric scale to determine total lipid content of the sample.

Cholesterol Determination - Quaife, Geyer and Balliger Method (1959)

i) Materials

A Ferric chloride reagent was prepared by dissolving 0.05% $FeCl_3 \cdot 6H_2O$ in a volumetric flask half full

APPENDIX 6 (Continued)

of glacial acetic acid. The final volume was made up to 100 ml.

Concentrated stock solutions of cholesterol were made in hexane using recrystallized cholesterol obtained from Nutritional Biochemicals, Cleveland, Ohio, U.S.A. The cholesterol, 0.0967 g, was dissolved in about 10 ml of hexane in a 25 ml volumetric flask and the volume made up to 25 ml with hexane. Cholesterol standards in hexane containing from .001 μ moles/ml to 1.0 μ mole/ml were made up using the concentrated stock solution (10.0 μ moles/ml) as the basis.

ii) Method

The pale yellow greasy deposit of fat in the bottom of the round bottomed flask was repeatedly washed with hexane (to a final total of 10 ml) to redissolve the lipid. If the test for cholesterol was not done immediately this liquid was stored at 4°C in a screw-capped universal container.

When the cholesterol test was begun, 1.0 ml of this lipid containing hexane was placed in the bottom of a test tube, lightly stoppered with a marble, and the sample taken to dryness in a 45°C water bath. To the test tube 3 ml of ferric chloride reagent was added, washing down the inside wall of the tube in the process.

APPENDIX 6 (Continued)

The tubes were warmed 15 seconds in a 70°C water bath to ensure that any residue was dissolved; they were then cooled to room temperature.

With the test tube tilted at a 45° angle, 2 ml of reagent grade concentrated sulphuric acid was added, so that a bottom layer formed. A footed glass rod was used to mix the layers, although vigorous mixing was avoided to prevent the introduction of air bubbles. After mixing, the tubes stood for exactly 30 minutes at room temperature. Then the optical densities for the standards and the samples were read using a Hitachi spectrophotometer set at a wave length of 560 nm. Contents of the samples were calculated using the standard curve so determined with the known cholesterol standards.

e. Carbohydrate Determination.

a) Sample Preparation of Cell and Envelope Fraction

The population of LM mouse fibroblast cells, being studied were pelleted, the supernatant was removed, the cells resuspended in a measured amount of 0.16 M NaCl. A cell count was taken using a Coulter counter. In the case of the cell envelope fraction, originally suspended in 45% sucrose in Tris, the pellet was washed

APPENDIX 6 (Continued)

with 0.02 M Tris-$MgCl_2$ (1 X 10^{-5} M), the liquid removed after recentrifugation, and the new pellet resuspended in a measured amount of 0.16 M NaCl. A ghost count was made using a hemocytometer. After a fresh saline wash the pellet was solubilized in 5 ml of 0.1% Nonidet in distilled water. This solubilized sample was used in both the anthrone test and the thymol test.

For all carbohydrate determinations glucose (Fischer Scientific, Toronto, Ontario) was used as the standard. Serial dilutions of this material were prepared ranging from 1 g/ml to 1 X 10^{-5} g/ml, in both distilled water and in 0.1% Nonidet. Duplicate tests were performed on each of these dilutions to verify that the testing procedures were accurate and comparable, and also to determine at which concentrations the results were most reproducible. When the cell carbohydrate samples were being tested a number of tubes, in water and in 0.1% Nonidet, were always included and the resulting optical densities of the samples compared to those of known quantities of carbohydrate. Water blanks and Nonidet blanks also were included to compensate for any contributions from these sources.

dxiv

APPENDIX 6 (Continued)

b) Anthrone Method (Seibert and Affronti, 1963)

The anthrone procedure consisted of pipetting 1 ml of the reagent (0.2% anthrone in a 5:2 solution of concentrated sulphuric acid and water) into tubes which had been precooled in an ice bath. On top of this was layered 250 ul of the sample. The mixture was shaken vigorously, heated at 90°C for one hour, cooled and the optical density read on the Hitachi spectrophotometer at a wavelength of 625 nm.

c) The Thymol-Sulphuric Acid Procedure (Shetlar and Masters, 1957)

Since our sample contained other biological material besides carbohydrate this method was included. 3.5 ml of 77% sulphuric acid was added to precooled tubes in an icebath, and chilled at 4°C for half an hour. At this time 0.5 ml of the sample was layered on top. Chilling continued for half an hour, then 0.05 ml of thymol reagent (10% w/v in absolute alcohol) was added using a micropipette. 0.45 ml of glass distilled water was added, the tubes stoppered with a marble and mixed well by gently shaking. Any precipitate that formed redissolved after shaking. The tubes were placed in an actively boiling water bath (100°C) for 20 minutes,

after which they were allowed to cool for 30 minutes. The optical density of the samples and standards were read at 500 nm on the Hitatchi spectrophotometer. A water blank and a Nonidet blank were always included in the test and the amount of sample carbohydrate calculated after adjustment for these.

BIBLIOGRAPHY

Amos, D. B., (1962). Isoantigens of mouse red cells. Ann. N. Y. Acad. Sci. 97: 69-82.

Amos, D. B., Zumpft, M., & Armstrong, P., (1963). HfA, H6A, two mouse isoantigens on red cells and tissues detected serologically. Transplantation 1: 270-283.

Anderson, P. A., & Pettijohn, D. E. (1960). Synchronization of division in Escherichia coli. Science 131: 1098.

Anderson, R. E., Cumming, R. B., Walton, M., & Snyder, F., (1969). Lipid metabolism in cells grown in tissue culture: O-Akyl, O-Alk-1-enyl, & acyl moieties of LM cells. Biochem. Biophys. Acta, 176: 491-501.

Baserga, R., (1968). Biochemistry of the cell cycle: A Review. Cell Tissue Kinetics 1: 167-191.

Bernard, C., (1878). Leçons sur les phénomènes de lavie communs aux animaux et aux végetaux. Baillier, Paris, France.

Bernfield, M. R., & Fell, P. E., (1967). Separation of proliferating and differentiating cell populations of cultured embryonic pancreatic epithelium. Proc. Nat. Acad. Sci. (Wash.) 58: 2227-2234.

Berwick, L., & Coman, D. B., (1962). Some chemical factors in cellular adhesion and stickiness. Cancer Res. 22: 982-986.

Blix, G., (1937). Uber die kohlenhydratengruppen des submaxillarismusins. Hoppe-Seyler Z. Physiol. Chem. 240: 43-54.

Bloch, D. P., & Godman, G. C., (1955). A microphotometric study of the synthesis of deoxyribonucleic acid and nuclear histone. J. Biophys. Biochem. Cytol. 1: 17-28.

Boone, C. W., Harrell, G. S., & Bond, H. E., (1968). The resolution of viable mixtures of mammalian cells into homogenous fractions by zonal centrifugation. J. Cell Biol. 36: 369-378.

Bovey, F. A., (1961). Nuclear magnetic resonance spectroscopy of proteins: the hydrogen bonding of water in Bovine serum albumin solutions. Nature 192: 324-326.

Bowman, W. C., Rand, M. J., & West, G. B., (1968). Textbook of Pharmacology. Blackwell Scientific Publications, Oxford & Edinburgh.

Bracket, J., & Mirsky, A. E. (Editors) (1961). The Cell. Volumes I and II, Academic Press, New York.

Brent, T. P., & Forrester, J. A., (1967). Changes in surface charge of Hela cells during the cell cycle. Nature 215: 92-93.

Britten, R. J., & Davidson, E. H., (1969). Gene regulation for higher cells: A theory. Science 165: 349-357.

Bryant, J. C., Evans, V. J., Schilling, E. L., & Earle, W. R., (1961). Effects of chemically defined culture medium NCTC 109 supplemented with methocel and silicone coating the flasks, on strain 2071 cells in suspension cultures. J. Natl. Cancer Inst. 26: 239-252.

Burnet, F. M., (1951). Mucoproteins in relation to virus action. Physiol. Rev. 31: 131-150.

Burnstone, M. S., (1962). Enzyme histochemistry and its application in the study of neoplasm. Academic Press, New York.

Burton, A. C., (1970). Perspectives in Biology. In Press.

Campbell, D. H., Garvey, J. S., Cremer, N. E., & Sussdorf, D. H., (1963). Methods in Immunology. W. A. Benjamin, Inc., New York & Amsterdam.

Carrel, A., & Burrows, M. T., (1911). Cultivation of Tissues in vitro and its technique. J. Exp. Med. 13: 387-398.

Chambers, R., (1940). The relation of the extraneous coats to the organization and permeability of cell membranes. Cold Spring Harbour Symp. Quant. Biol. 8: 144-153.

Chance, B., & Schoener, B., (1964). Control of oscillations of DPNH level in cell free extract of saccharomyces

carlsbergensis by 3'5' cyclic AMP. Biochem. Biophys. Res. Commun. 17: 416-423.

Chance, B., Williamson, J. R., Jamieson, D., & Schoerer, B., (1965). Properties and kinetics of reduced pyridine nucleotide fluorescence of the isolated in vivo rat heart. Biochem. Z. 341: 357-377.

Changeux, J. P., Thiery, J., Turg, Y., & Kittel, C., (1967). On cooperativity of biological membranes. Science 57: 335-341.

Chatton, E., Lwoff, A., & Rapkine, L., (1931). L'Apparition de groupements - SH avant la division chez les Foettingeriidae (Cilies) Compt. rend. Soc. biol. 106: 626-628.

Cheesman, D. F., & Davies, J. T., (1954). Physiochemical and Biological aspects of proteins at interfaces. Advan. Prot. Chem. 9: 439-501.

Cook, G. M. W., (1968). Glycoproteins in membranes. Biol. Rev. 43: 363-391.

Cook, G. M. W., Heard, D. H., & Seaman, G. V. F., (1960). A sialomucopeptide liberated by trypsin from the human erythrocyte. Nature 188: 1011-1012.

Curtis, A. S. G., (1967). The cell surface, its molecular role in morphogenesis. Academic Press, Logos Press, London, England.

Dandliker, W. B., Moscowitz, M., Zimm, B., & Calvin, M., (1950). The physical properties of Elinin, a

lipoprotein from human erythrocytes. J. Am. Chem. Soc. 72: 5587-5592.

Danielli, J. F., (1937). The relations between surface pH, ion concentration and interfacial tension. Proc. Roy. Soc. B. 122: 155-174.

Danielli, J. F., & Davson, H., (1935). A contribution to the theory of permeability of thin films. J. Cell. Comp. Physiol. 5: 495-508.

Daniels, W. F., (1968). Comments on technology of production - continuous cell lines. Nat. Cancer Inst. Monograph 29: 71-72.

Davis, W. C., & Silverman, L., (1968). Localization of mouse H-2 histocompatibility antigen with ferritin-labelled antibody. Transplantation 6: 535-543.

DeDuve, C., Wattiaux, R., & Baudhuin, P., (1962). Distribution of enzymes in animal tissues. Advances in enzymology 24: 291-358.

Defendi, V., & Gasic, G., (1963). Surface mucopolysaccharide of polyoma virus transformed cells. J. Cell. Comp. Physiol. 5: 495-508.

Dische, Z. Microchemie, 8: 4 (1930). Cited in methodology manual for investigators of mycobacterial and fungal antigens. Amer. Thor. Soc. New York. (1963).

Donnelly, G. M., & Sisken, J. F., (1967). RNA and protein synthesis required for entry of cells into mitosis and during the mitotic cycle. Exp. Cell Res. 46: 93-105.

Drury, H. F., (1948). Identification of pentoses in the presence of glucose. Arch. Biochem. 19: 455-466.

Drysdale, R. G., Merchant, D. J., Shreffler, D. C., & Parker, F. R., (1967). Distribution of H-2 specificities within the LM mouse cell line and derived lines. Proc. Soc. Exp. Biol. & Med. 124: 413-418.

Dustin, P., (1947). Some aspects of mitotic poisoning. Nature 159: 794-797.

Dutrochet, P., (1824). Recherches anatomique et physiologiques sur le structure intime des animaux et des végètaux, et sur leur motilité. Paris.

Earle, W. R., Schilling, E. L., Stark, I. H., Strauss, N. P., Grown, M. F., & Shelton, E., (1943). Production of malignancy in vitro. IV the mouse fibroblast cultures and changes seen in the living cells. J. Nat. Cancer Inst. 4: 165-212.

Eidam, C. R., & Merchant, D. J., (1965a, b, c). The plateau phase of growth of the LM strain mouse cell in a protein-free medium.

I. Patterns of protein and nucleic acid synthesis and turnover. Exp. Cell Research 37: 132-139.

II. Prolongation of the plateau phase by supplemental glucose. Exp. cell Research 37: 140-146.

III. The effect of added glucose on protein and nucleic acid synthesis and on carbohydrate utilization. Exp. Cell Research 37: 147-160.

Emmelot, P., & Bos, C. J., (1965). Differential effect of neuraminidase on the Mg^{++}- ATPase, Na^{+}- K^{+}- Mg^{++} ATPase, and 5' nucleotidase of isolated plasma membrane. Biochim. Biophys. Acta. 99: 578-580.

Fernandez-Moran, H., Oda, T., Blair, P. V., & Green, D. E., (1964). A macromolecular repeating unit of mitochondrial structure and function. J. Cell Biol. 22: 63-100.

Filkuka, J., Svejda, J., & Aubrechtova, V., (1969). DNA content and chromosome number of L cells. Neoplasma 16: 257-264.

Firket, H., (1958). Recherches sur la synthese des acid deoxyribonucleiques et la préparation à la mitose. Arch. Biol. (Liége) 69: 1-166.

Firket, H., (1964). Synchronisation de cultures de cellules HeLa par un excés de thymidine. Comp. rend de Soc. Biol. 158: (part 1): 1408-1410.

Firket, H., (1965). Cell division. In "Cells and Tissue in culture. Methods, Biology and Physiology". Wilmer E. N. (editor) Academic Press, New York.

Fischer, A., (1948). Nutrition of animal tissue cells. Scientia (Milano) 6: 170-175.

Fishman, W. H., & Green, S., (1961). B-glucuronidase, peptidases and esterases. Methods in Medical Research 9: 56-78.

Foster, T. S., & Stern, H., (1959). The accumulation of soluble deoxyribosidic compounds in relation to nuclear division in anthers of Lilium longifolium. J. Biophys. biochem Cytol. 5: 187-192.

Frenkel, R., (1966). Reduced diphosphopyridine nucleotide oscillations in cell free extracts from beef heart. Arch. Biochem. and Biophysics 115: 112-121.

Galavazi, G., Schenk, H., & Bootsma, D., (1966). Synchronization of mammalian cells in vitro by inhibition of DNA synthesis. Exp. Cell Res. 41: 428-451.

Gasic, G., & Berwick, L., (1963). Hale stain for sialic acid containing mucins. J. Cell Biol. 19: 223-228.

Gasic, G., & Gasic, T., (1962a). Removal of sialic acid from the cell coat in tumor cells and vascular endothelim, and its effect on metastasis. Proc. Nat. Acad. Sci. 48: 1172-1177.

Gasic, G., & Gasic, T., (1962b). Removal and regeneration of the cell coating in tumor cells. Nature 196: 170.

Gerner, E. W., Glick, M. C., Warren, L., (1970). Membranes of Animal cells. V Biosynthesis of the surface membrane during the cell cycle. J. Cell Physiol. 75: 275-280.

Gesner, B. M., & Ginsburg, V., (1964). Effect of glycosidases on the fate of transfused lymphocytes. Proc. Nat. Acad. Sci. 52: 750-755.

Gey, G. O., (1933). An improved technique for massive tissue culture. Amer. J. Cancer 17: 752-756.

Gey, G. O., (1954-1955). Some aspects of the constitution and behaviour of normal and malignant cells maintained in continuous culture. Harvey Lect. 50: 154-229.

Gey, G. O., Gey, M. K., Firor, W. M., & Self, W. O., (1949). Cultural and cytologic studies, autologous normal and malignant cells of specific in vitro origin: Conversion of normal into malignant cells. Acta Unio Intermat. contra cancrum 6: 706-712.

Geyer, R. P., (1966). Uptake and retention of fatty acids by tissue culture cells. Wistar Inst. Sympos. Monograph 6: 33-44.

Ghosh, A., & Chance, B., (1964). Oscillation of glycolytic intermediates in yeast cells. Biochem. Biophys. Res. Commun. 16: 174-180.

Giles, R. E., Merchant, D. J., & Masselink, E., (1966). Chromosomes of LM mouse cells and variants. J. Nat. Cancer Inst. 37: 663-676.

Glick, J. L., & Githens, S., III, (1965). Role of sialic acid in potassium transport of L1210 leukaemia cells. Nature 208: 88.

Gold, M., & Helleiner, G. W., (1964). Deoxyribonucleic acid polymerase in L cells. I. Properties of the enzyme and its activity in synchronized cell cultures.

Biochim. biophys. Acta. 80: 193-203.

Goodwin, B. C., (1967). Biological control processes and time. Ann. N. Y. Acad. Sci. 138: 748-758.

Gorer, P. A., (1937). The genetic and antigenic basis of tumor transplantation. J. Path. Bact. 44: 691-700.

Gorman, J., Tauro, P., Laberge, M., & Halvorson, H. O., (1964). Timing of enzyme synthesis during synchronous division in yeast. Biochem. Biophys. Res. Commun. 15: 43-49.

Gorter, E., & Grendel, F., (1925). On bimolecular layers of lipoids in the chromocytes of the blood. J. Exp. Med. 41: 439-443.

Gottschalk, A., (1960). Correlation between composition, structure, shape and function of a salivary mucoprotein. Nature 186: 949-951.

Gottschalk, A., (1963). The basic structure of glycoproteins and problems of their chemical and physiochemical analysis. Ann. N. Y. Acad. Sci. 106: 168-176.

Green, D. E., (1964). The mitochondrion. Sci. Am. 210: 63-74.

Green, D. E., Allman, D. W., Bachmann, E., Baum, H., Popaczyk, K., Korman, E. F., Lipton, S., MacLennan, D. H., McConnell, D. G., Perdue, J. F., Rieske, J. S., & Tzagoloff, A., (1967). Formation of membranes by repeating units. Arch. Biochem. Biophys. 119: 312-335.

Gross, P. R., editor, (1960). Introductory remarks. Second conference on the mechanism of cell division. Ann. N. Y. Acad. Sci. 90: 349-352.

Hakomori, S. I., & Jeanloz, R. W., (1961). Isolation and characterization of glycolipids from erythrocytes of human blood A (plus) and B (plus). J. Biol. Chem. 236: 2827-2834.

Halberg, F., (1969). Chronobiology. Ann. Rev. of Physiology 31: 675-725.

Hansen, D. L., & Bush, E. T., (1967). Improved solubilization procedures for liquid scintillation counting of biological materials. Nuclear-Chicago Publications. Des Plaines, Illinois.

Hanig, M., (1948). Electrokinetic changes in human erythrocytes during adsorption and elution of PR8 influenza virus. Proc. Soc. Exp. Biol. Med. 68: 385-392.

Harris, H., (1961). Formation of the nucleolus in the animal cell. Nature 190: 1077-1078.

Harrison, R. G., (1907). Observations on the living developing nerve fiber. Proc. Soc. Exp. Biol. 4: 140-143.

Haughton, G., & Davies, D. A. L., (1962). Tissue cell antigens. Antigens of mouse tumour cell ghosts. Brit. J. Exp. Path. 43: 488-495.

Healy, G. M., Siminovitch, L., Parker, R. C., Graham, A. F., (1956). Conservation of deoxyribonucleic acid phosphorus in animal cells propagated in vitro.

Biochemica et Biophysica Acta 20: 425-426

Hill, R. B., Jr., Bensch, K., Simbonis, S., & King, D. W., (1959). Variability of contents of deoxyribonucleic acid in L-strain fibroblasts. Nature 183: 1818-1819.

Hirst, G. K., (1948). The nature of the virus receptors of red cells. I. Evidence on the chemical nature of the virus receptors of red cells and of the existance of a closely analogous substance in normal serum. J. Exp. Med. 87: 301-313.

Hofstee, B. J. H., (1961). Fatty acid esterases of low eserine sensitivity and related enzymes. In "The Enzymes". Volume 4. (Boyer, Lardy & Myrback, editors). Academic Press. New York.

Hokin, L. E., & Hokin, M. R., (1961). Diglyceride kinase and phosphatidic acid phosphatase in erythrocyte membranes. Nature 189: 836-837.

Hooke, R., (1745). Micrographia restauranta. John Bawles Publ. London.

Howard, A., & Dewey, D. L., (1959). The cell nucleus. The Faraday Society, p. 155-162. Butterworth, London.

Howard, A., & Pelc, S. R., (1953). Synthesis of deoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. Heredity. Supp. 6: 261.

Hsu, T. C., (1959). Mammalian chromosomes in vitro. XI. Variability among progenies of a single cell. University of Texas Publication 5914: 129-134.

Hsu, T. C., & Merchant, D. J., (1961). Mammalian chromosomes *in vitro*. XIV. Genotypic replication in cell populations. J. Nat. Cancer Inst. 26: 1075-1083.

Hughes, A. F. W., (1950). The effect of inhibitory substances on cell division. Quart. J. Micr. Sci. 91: 251. Cited but not seen Firket, H., (1965). q.v.

Hunter, R. L., & Strachan, D. S., (1961). The esterases of mouse blood. Ann. N. Y. Acad. Sci. 94: 841-867.

Jorpes, E., (1932). The protein component of the erythrocyte membrane or stroma. Biochem. J. 26: 1488-1503.

Jung, C., & Rothstein, A., (1967). Cation metabolism in relation to cell size in synchronously grown tissue culture cells. J. Gen. Physiol. 50: 917-932.

Kahn, R. H., Conklin, J. L., & Dewey, M. M., (1962). Cytological and cytochemical characterization of cells grown *in vitro*. Nat. Cancer Inst. Monograph 7: 123-137.

Kandutsen, A. A., & Stimpfling, J. H., (1966). Properties of purified isoantigenic preparations from a mouse sarcoma. In "Immunopathology". IV International Symposium, New York.

Kalaidjiew, A., & Segal, J., (1967). Molecular structure and properties of cell membranes. Protides of the biological fluids. 15: 105-108.

Katchalsky, A., & Spangler, R., (1968). Dynamics of membrane processes. Quart. Rev. of Biophys. 1: 127-175.

Katsuta, K., & Takoaka, T., (1962). Synchronous cultivation of a substrain of L cells (mouse fibroblast) in a protein free medium. Jap. J. Exptl. Med. 32: 279-287.

Kavanau, J. L., (1965). Structure and function in biological membranes. Volumes I and II. Holden-Day Inc., London & Amsterdam.

Kemp, P. B., Jones, B. M., Cunningham, I., James, M. C. M., (1967). Quantitative investigations of the effect of puromycin on the aggregation of trypsin and versene dissociated chick fibroblast cells. J. Cell Sci. 2: 323-340.

Killander, D., & Zetterberg, A., (1965). Cell mass and initiation of DNA synthesis in fibroblasts (L cell clone 929). Exp. Cell Res. 40: 12-20.

Kite, G. L., (1913). The relative permeability of the surface and interior portions of the cytoplasm of animal and plant cells. Biol. Bull. 25: 1-7.

Kite, J. H., & Merchant, D. J., (1961). Studies of some antigens of the L strain mouse fibroblast. J. Nat. Cancer Inst. 26: 419-434.

Klein, P., & Robbins, E., (1970). An ultra sensitive assay for soluble sulphydryl and its application to the study of glutathione levels during the HeLa life cycle. J. Cell Biol. 46: 165-168.

Klenk, E., & Uhlenbruck, W. G., (1958). Uber ein neuraminsurehaltiges mucoproteid aus rindererythrocytenstroma.

Hoppe-Seyler Z. Physiol. Chem. 311: 227-233.

Klotz, I. M., (1958). Protein hydration and behaviour. Science 128: 815-822.

Kraepelin, G., (1969). Physiological rhythms in petite mutation. Antonie Van Leeuwenhoek 35: C13-14 Supplement: Yeast Symposium.

Kuempel, P. L., Masters, M., & Pardee, A. B., (1965). Bursts of enzyme synthesis in bacterial duplication cycle. Biochem. Biophys. Res. Commun. 18: 858-867.

Lajtha, L. G., (1957). Bone marrow cell metabolism. Physiol. Rev. 37: 50-65.

Lambert, W. C., & Studzinski, G. P., (1969). Thymidine as a synchronizing agent. II Partial recovery of HeLa cells from unbalanced growth. J. Cell Physiol. 73: 261-266.

Langmuir, I., (1939). Cited but not seen. Proc. Roy. Soc. A 170: 1-22, from Kavanau, J. L., (1965) q.v.

Levine, L. (editor), (1963). The cell in mitosis. Academic Press, New York.

Lewis, M. R., & Lewis, W. H., (1911). The cultivation of tissues from chick embryo in solutions of NaCl, $CaCl_2$, KCl, and $NaHCO_3$. Anat. Rec. 5: 277-293.

Lewis, W. H., (1958). Mucos coat of amebos. Anat. Rec. 130: 426 (Abstract).

Lippman, M., (1965). A proposed role for mucopolysaccharides in the initiation and control of cell division.

Trans. of N. Y. Acad. Sci. (2) 27: 342-360.

Littlefield, J. W., McGovern, A. P., & Margeson, K. B., (1963). Changes in the distribution of polymerase activity during DNA synthesis in mouse fibroblasts. Proc. Nat. Acad. Sci. (Wash.) 49: 102-107.

Lowry, O. H., Rosenough, N. S., Farr, A. A., & Randall, P. J., (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

Ludford, R. J., (1951). Pathological aspects of cytology. In "cytology and cell physiology", 2nd ed., Bourne, G. H. editor. Oxford Clarendon Press.

MacKenzie, C. G., MacKenzie, J. B., & Reiss, O. K., (1966). Regulation of cell lipid metabolism and accumulation. V. Quantitative and structural aspects of triglyceride accumulation caused by lipogenic substances. Wistar Inst. Symp. Monograph 6: 63-81.

Mahler, H. R., & Cordes, E. H., (1966). "Biological chemistry", Harper & Row Publishers, New York & London.

Mäkelä, O., Miettinen, T., & Pesola, R., (1960). Release of sialic acid and carbohydrate from human red cells by trypsin treatment. Vox Sang. 5: 492-496.

Manery, J. F., (1966). Effect of Ca ions on membranes. Fed. Proc. 25: 1804-1810.

Masters, M., Kuempel, P. L., & Pardee, A. B., (1964). Enzyme synthesis in synchronous cultures of bacteria. Biochem. Biophys. Res. Commun. 15: 38-42.

Mayhew, E. H., & O'Grady, E. A., (1965). Electrophoretic mobilities of tissue culture cells in expotential and parasynchronous growth. Nature 207: 86-87.

Mazia, D., (1961). Mitosis and the physiology of cell division in "The Cell", Brachet & Mirsky editors. Volume III, Academic Press, New York.

Medzon, E. L., & Brady, M. L., (1969). Direct measurement of acetylesterase in living protist cells. J. Bact. 97: 402-415.

Mefferd, R. B., Summers, R. M., & Clayton, J. D., (1968). Correlation analysis of seven procedures for determining area of chromatographic curves. J. Chromatog. 35: 469-474.

Merchant, D. J., & Hellman, K., (1962). Growth of LM strain mouse cells in chemically defined medium. Proc. Soc. Exptl. Biol. & Med. 110: 194-198.

Merchant, D. J., Kahn, R. H., & Murphy, W. H. Jr., (1960). Handbook of cell and organ culture. Burgess Publishing Company, Minneapolis.

Merchant, D. J., Kuchler, R. H., & Munyon, W. H., (1960). J. Biochem. Microbiol. Tech. Eng. 2: 253. Cited but not seen from Eidam, C. R., & Merchant, D. J., (1965). q.v.

Micheli, A., & Grabar, P., (1961). Etude immunochemique des henolysats de globules rouge humains. Ann. Inst. Pasteur 100: 569-580.

Miller, G. L., Golden, R. H., & Miller, E. E., (1951). Determination of Pentoses. Effect of varying properties of components of Beal's color reagent. Anal. Chem. 23: 903-905.

Mitchison, J. M., (1957). The growth of single cells. I. Schizosaccharomyces pombe. Expt. Cell Res. 13: 244-262.

Mitchison, J. M., & Vincent, W. S., (1965). Preparation of synchronous cell cultures by sedimentation. Nature 205: 987-989.

Moëller, G., & Moëller, E., (1964). Phenotypic expression of mouse Isoantigens. J. Cell & Comp. Physiol. 60: Suppl. 107-128.

Morgan, J. F., Morton, H. J., & Parker, R. C., (1950). Nutrition of animal cells in tissue culture. Initial studies on a synthetic medium. Proc. Soc. Exptl. Biol. Med. 73: 1-10.

Morton, J. A., (1962). Some observations on the action of blood group antibodies on red cells treated with proteolytic enzymes. Br. J. Haemat. 8: 134-148.

Moscona, A., (1952). Cell suspensions from organ rudiments of chick embryos. Expt. Cell Res. 3: 535-537.

Moscona, A., (1961). Effect of temperature on adhesion to glass and histogenetic cohesion of dissociated cells. Nature 190: 408-409.

Moscovitch, M., Calvin, M., (1952). On the components and structure of the human red cell membrane. Expt. Cell Res. 3: 33-46.

Moscovitch, M., Dandliker, W. B., Calvin, M., & Evans, R. S., (1950). Studies on the antigens of human red cells. J. Immunol. 65: 383-392.

Mueller, G. C., Kajuuara, K., Stubblefield, E., & Reuckert, R. R., (1962). Molecular events in the reproduction of animal cells. I The effect of puromycin on the duplication of DNA. Cancer Res. 22: 1084-1090.

Myers, D. K., (1961). Carboxyl ester cleavage. In "The Enzymes" Volume 4, Boyer, Lardy & Myrback, editors. Academic Press. New York.

Nägeli, C. W., & Cramer, C., (1855), cited but not seen from Smith, H. W., (1962) q.v.

Nathanson, A., (1904). Über die regulation der aufrahme anorganischer Salze durch die knollen von Dahlia. Jahrb. Wiss. Bot. 39: 607-644.

Nathenson, S. G., (1968). Discussion p. 116 of Biological Properties of the Mammalian Surface Membrane. Wistar Inst. Symp. Monograph 8: 116-117.

Neff, R. J., & Neff, R. H., (1964). In "Synchrony in Cell Division and Growth." Zerthain E., editor. Wiley (Interscience), New York.

Nemethy, G., & Scheraga, H., (1962). J. Phys. Chem. 66: Cited but not seen from Kavanau, J. L., (1965). q.v.

Newton, A. A., & Wildy, P., (1959). Parasynchronous division of HeLa cells. Exp. Cell Research 16: 624-635.

Noble, P. B., & Cutts, J. H., (1967). Separation of blood leukocytes by Ficoll gradient. Can. Vet. Journal 8: 110-111.

Novikoff, A. B., Essner, E., Goldfischer, S., & Haus, M., (1962). Interpretation of ultrastructure. Volume I, Academic Press, New York.

Odin, L., (1952). Carbohydrate residue of a urine muco-protein inhibiting influenza virus haemagglutination. Nature 170: 663-664.

Ohkuma, S., & Ikemoto, S., (1965). Release of a sialo-mucopeptide from human red cells and destruction of the I blood group receptors of their cells by crystalline trypsin treatment. Proc. Jap. Acad. 41: 482-487.

Okada, H., & Halvorson, H. O., (1964). Uptake of α-thio etyl d-glucopyranoside by Saccharomyces cerevisiae. Biochem. Biophys. Acta. 82: 538-546.

Oudin, J., (1948). L'analyse immunochemique qualitative méthode par diffusion des antigènes au sein de l'immunserum precipitant galose. Ann. Inst. Pasteur. 75: 30-51.

Overton, E., (1899). Über die allgeneinen osmotischen Eigenschaften der Zellen, ihre vermotlichen ursachen und ihre Bedeutung für physiologie. Vierteljahrschrift

Naturforsh Ges. Zurich 44: 88-135.

Padilla, G. M., & Cameron, H., (1964). Synchronization of cell division in Tetrahymena pyriformis by repetitive temperature cycle. J. Cell Comp. Physiol. 64: 303-307.

Pardee, A. B., (1968). Membrane transport proteins. Science 162: 632-637.

Park, C. R., Crofford, O. B., & Kono, T., (1968). Mediated (nonactive) transport of glucose in mammalian cells and its regulation. Biological Interfaces: Flows & Exchanges. Little, Brown & Co., Boston.

Parsons, D. F., (1967). Ultrastructural and molecular aspects of cell membranes. Can. Cancer Conference 7: 193-246.

Pauling, L., & Corey, R. B., (1951). Proc. Nat. Acad. Sci. 37: 729. Cited but not seen from Kavanau, J. L., (1965). q.v.

Pauling, L., & Marsh, R. E., (1952). Proc. Nat. Acad. Sci. 38: 112. Cited but not seen from Kavanau, J. L., (1963). q.v.

Pfeffer, W. F. P., (1877), cited but not seen from Smith, H. W., (1962). q.v.

Pfeffer, S. E., & Tolmach, L. J., (1967). Selecting synchronous populations of mammalian cells. Nature 213: 139-142.

Phillips, H. J., & Terryberry, J. E., (1957). Counting actively metabolizing tissue cultured cells. Exp.

Cell Res. 13: 341-347.

Pomerat, C. M., & Willmer, E. N., (1934). Studies on growth of tissues in vitro; carbohydrate metabolism and mitosis. J. Exper. Biol. 16: 232-249.

Ponder, E., (1961). In "The Cell", Brachet, J., & Mirsky, A. E. editors. Academic Press, New York.

Prager, M. D., & Fletcher, M. A., (1966). The effect of enzymatic release on sialic acid from erythrocytes on Rh agglutination. J. Immun. 97: 165-170.

Prescott, D. M., (1960). Relation between cell growth and cell division IV. The synthesis of DNA, RNA and protein from di 'ision to division in tetrahymena. Exp. Cell Res. 19: 228-235.

Quaife, M. L., Geyer, R. P., Bollinger, H. R., (1959). Rapid paper chromatographic micro assay of free and ester cholesterol of blood. Analyt. Chem. 31: 950-955.

Rambourg, A., & LeBlond, C. P., (1967). Electron microscope observations on the carbohydrate-rich cell coat present at the surface of cells in the rat. J. Cell Biol. 32: 27-53.

Rashevsky, N., (1968). A suggestion of a new approach to the theory of some biologi al periodicities. Bull. Math Biophys. 30: 751-760.

Revel, J. P., & Ito, S., (1967). In "The specificity of cell surfaces." Davis, B., & Warren, L., editors. Prentice-Hall, Englewood Cliffs, N.J., p. 211-234.

Ris, H., (1955). Cell Division. Saunders, Philadelphia.

Rixon, R. H., & Whitfield, J. F., (1962). The effect of high salt concentration on nuclear structure of L mouse cells. Exp. Cell Res. 26: 591-594.

Robbins, E., Jentzsch, G., Micali, A., (1968). The centriole cycle in synchronized HeLa cells. J. Cell Biol. 36: 329-339.

Rotman, B., & Papermaster, B. W., (1966). Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. Proc. Nat. Acad. Sci. USA 55: 134-141.

Rothblat, G. H., (1969). The effect of serum components on sterol biosynthesis in L cells. J. Cell Physiol. 74: 163-170.

Roux, W., (1885). Beitrage zur Entwicklungsmechanik des embryo. Z. Biol. 21: 411-524.

Ruddle, F. H., & Rappola, J., (1970). Changes in lactate dehydrogenase and esterase specific activities, isozymic patterns and cellular distribution during the growth cycle of PK cell in vitro. Exp. Cell Res. 59: 399-

Sakai, H., & Dan, K., (1959). Studies of sulphydryl groups during cell division of sea urchin egg. Exp. Cell Res. 16: 24-41.

Sandritter, W., Schiemer, H. G., Kraus, H., & Dorrien, U., (1960). Das washstum von einzelzellen in der gewebekulture. Interferenz mikroskopesche untersuchungen an Hela Zellen. J. biophys. biochem. Cytol. 7: 579-582.

Sanford, K. K., Earle, W. R., & Likely, G. D., (1948). The growth in vitro of single isolated tissue cells. J. Natl. Cancer Inst. 9: 229-246.

Sanford, K. K., Westfall, B. B., Fioramonti, M. C., McQuillcin, W. T., Bryant, J. C., Peppers, E. V., Evans, V. J., & Earle, W. R., (1955). The effect of serum fractions on the proliferation of strain L mouse cells in vitro. J. Natl. Cancer Inst. 16: 789-802.

Salzman, N. P., (1959). Systematic fluctuations in the cellular protein, RNA and DNA, during growth of mammalian cell cultures. Biochimica et Biophysca Acta. 31: 158-163.

Scherbaum, O. H., (1963). Chemical prerequisites for cell division. In "The cell in mitosis." Levine, L., editor. Academic Press, New York.

Schindler, R., Odertchenko, N., Ramseier, L., & Grieder, A., (1967). Causal relationship between completion of DNA synthesis and the onset of G2 period of the division cycle. Europ. J. Cancer 3: 349-354.

Schneider, W. C., & Brownell, L. W., (1957). Deoxyribonucleic compounds in regenerating liver. J. Nat. Cancer Inst. 18: 579-586.

Schwann, T., (1839). Mikroskopische untersuchungen über die Uberenstimmieng in der struktur und dem Wachstum der thiere und pflanzen. Berlin, 1938. Translation, H. Smith, Sydenham Society, London, 1847.

Seibert, F. B., & Affronti, L. F., (1963). An analytical chemical method . In "Methodology manual for the investigation of mycobacterial and fungal antigens." Amer. Thor. Soc. New York.

Scheiden, M. J., (1838). Müller archiv für anatomie and physiologie. Part II. Translation, H. S. Smith, Sydenham Society, London, 1847.

Shetlar, M. R., & Masters, Y. F., (1957). Use of thymol sulfuric acid reaction for determination of carbohydrates in biological materials. Anal. Chem. 29: 402-405.

Shibko, S., & Tappel, A. L., (1964). Distribution of esterases in rat liver. Arch. Biochem. & Biophys. 106: 259-266.

Shodell, M., Rubin, H., (1970). Studies on the nature of serum stimulation of proliferation in cell culture in vitro 6: 66-74.

Siminovitch, L., Graham, A. F., Lesley, S. M., Nevill, A., (1957). Propagation of L-strain mouse cells in

suspension. Exp. Cell Research 12: 299-308.

Sinclair, W. K., (1965). Hydroxyurea, differential lethal effects on cultured mammalian cells during the cell cycle. Science 150: 1729-1731.

Sinclair, R., & Bishop, D. H. L., (1965). Synchronous culture of strain L mouse cells. Nature 205: 1272-1273.

Sisken, J. E., (1963). Analysis of variations in inter-mitotic time. In "Cinemicrography in cell biology." Rose, G. C. editor. Academic Press. Cited but not seen from Watanabe, I., & Okada, S., (1967). q.v.

Sisken, J. E., Morasia, L., Kibby, S., (1965). Effects of temperature on the kinetics of the mitotic cycle of mammalian cells in culture. Exp. Cell Res. 39: 103-116.

Sisken, J. E., & Kinosita, R., (1961). Variations in the mitotic cycle in vitro. Exp. Cell Res. 22: 521-525.

Smith, H. W., (1962). The plasma membrane, with notes on the history of botany. Circulation 26: 987-1012.

Snell, G. D., (1958). Histocompatibility genes of the mouse. II Production and analysis of isogenic resistant lines. J. Nat. Cancer Inst. 21: 843-877.

Snell, G. D., (1968). The H-2 locus of the mouse. Folia biologica 14: 335-358.

Snell, G. D., & Bunker, H. P., (1964). Histocompatibility genes of the mouse. IV. The position of H-3 in the

fifth linkage group. Transplantation 2: 743-751.

Sperry, W. M., (1955). Methods of biochemical analysis 2: 83-111.

Srere, P. A., (1967). Enzyme concentration in tissues. Science 158: 936-937.

Stahl, E., & Jork, H., (1968). "Thin layer chromatography. XIX, Direct evaluation." Zeiss Information 68: 52-65.

Stanners, C. P., & Till, J. E., (1960). DNA synthesis in individual L strain mouse cells. Biochem. biophys. Acta. 37: 406-419.

Swaffield, M. N., & Foley, G. E., (1960). Changes in the cellular content of Ribonucleic acid, Deosyribonucleic acid and protein in cultured cells during logarithmic growth. Arch. Biochem. & Biophys. 86: 219-224.

Swann, M. M., (1954). The mechanism of cell division. A study of carbon monoxide on the sea urchin egg. Quart. J. micr. Sci. 94: 367. Cited but not seen from Firket, H., (1965). q.v.

Sylven, B., Tobias, C. A., Malmgnen, R., Ottoson, R., & Thorell, B., (1959). Cyclic variations in the peptidase catheptic activities of yeast cultures synchronized with respect to cell multiplication. Expt. Cell. Res. 16: 75-87.

Taylor, E. W., (1965). Control of DNA synthesis in mammalian cells in culture. Exp. Cell Res. 40: 316-332.

Templeton, M., (1963). Mouse erythrocyte esterases and an interaction with Heparin as shown by starch gel electrophoresis. J. Hist. & Cytol. 17: 95-101.

Terasima, T., & Tolmach, L. J., (1963). Growth and nucleic acid synthesis in synchronously dividing populations of HeLa cell. Exp. Cell Res. 38: 344-352.

Terasima, T., & Yasukawa, M., (1966). Synthesis of G1 protein proceeding DNA synthesis in cultured mammalian cells. Exp. Cell Res. 44: 669-672.

Till, J. E., Whitmore, G. F., & Gulyas, S., (1963). Deoxyribonucleic acid synthesis in individual L strain mouse cells. II Effect of thymidine starvation. Biochem. Biophys. Acta 72: 277-289.

Tobey, R. A., Anderson, L. C., & Petersen, D. T., (1966). RNA stability and protein synthesis in relation to the division of mammalian cells. Proc. Nat. Acad. Sci. 56: 1520-1531.

Tobey, R. A., & Ley, K. D., (1970). Regulation of Initiation of DNA synthesis in Chinese hamster cells. J. Cell Biol. 46: 151-157.

Tristam, G. R., (1953). In "The Proteins", Neurath, H., and Bailey, K., editors, Vol. I. Academic Press, New York.

Van Deenen, L. L., & De Geir, J., (1964). In "The Red Blood Cell. A comprehensive treatise." Bishop, D., and Surgenor, D. M. (editors) Academic Press, New York.

Veerkamp, J. H., Mulder, I., & Vandeenen, L. L., (1962). Comparison of the fatty acid composition of lipids from different animal tissues including some tumours. Biochem. Biophys. Acta. 57: 299-309.

Walker, I. G., & Helleiner, C. W., (1963). The sensitivity of cultured mammalian cells in different stages of the division cycle to nitrogen and sulfur mustards. Cancer Res. 23: 734-738.

Wallach, D. F., (1967). Isolation of plasma membranes of animal cells. In "The specificity of cell surfaces." Davis, B. D., & Warren, L., Editors Prentice Hall, Englewood Cliffs, New Jersey.

Warren, L., & Glick, M., (1966). Membranes of animal cells. I. Method of isolation of the surface membrane. J. Cell Physiol. 68: 269-288.

Warren, L., & Glick, M., (1968). Membranes of animal cells. II. The metabolism and turnover of surface membranes. J. Cell Biol. 37: 729-746.

Watanabe, I., & Okada, S., (1967). Effects of temperature on growth rate of cultured mammalian cells. J. Cell Biol. 32: 309-323.

Waugh, D. F., (1954). Protein-protein interactions. Advan. Protein Chemistry 9: 324-437.

Webb, J. L., (1966). Enzymes & Metabolic Inhibitors. Academic Press, New York, N.Y.

Weinstein, D. B., Marsh, J. B., Glick, M., & Warren, L., (1969). Membranes of animal cells. IV. Lipid of the L cell and its surface membrane. J. Biol. Chem. 244: 4103-4111.

Weiss, L., (1963a). The mammalian tissue cell surface. Biochem. Soc. Symp. 22: 32-50.

Weiss, L., (1963b). Studies on cellular adhesion in tissue culture. V. Some effects of enzymes on cell detachment. Exp. Cell Res. 30: 509-520.

Weiss, L., (1965). Studies on cellular adhesion in tissue culture. VII. Some effects of antisera on cell detachment. Exp. Cell Res. 37: 540-551.

Weiss, L., (1967). The cell periphery, metastasis and other contact phenomena. Frontiers of Biology Vol. 7, Neuberger, A., & Tatum, E. C., editors. North Holland Publishing Company, Amsterdam.

Weiss, L., (1968). Some comments on RNA as a component of the cell periphery. Wistar Inst. Symp. Monograph 8: 73-77.

Weiss, L., (1969). The cell periphery. Int. Rev. of cytology 26: 63-105.

Wiebel, F., & Baserga, R., (1969). Early alteration in amino acid pools of protein synthesis of diploid fibroblasts, stimulated to synthesis DNA by addition of serum. J. Cell Physio. 74: 191-202.

Wiemken, A., Matile, P., Moor, H., (1970). Vacuolar dynamics in synchronously budding yeast. Arch. Mickrobiol. 70: 89-103.

Williamson, D. M., & Scopes, A. W., (1960). The behaviour of nucleic acids in synchronously dividing culture of Saccharomyces cerevisiae. Exp. Cell Res. 20: 338-349.

Wilmer, E. N., (1961). Steroids and cell surfaces. Biol. Rev. 36: 368-398.

Wilson, E. B., (1925). The cell in development and heredity. 3rd edition. MacMillan, New York.

Wilson, G. B., (1963). Studies on disruption of the mitotic cycle. In "The cell in mitosis." Levine, L., editor. Academic Press, New York.

Woodard, J., Geller, B., & Swift, H., (1961). Nucleo-protein changes during the mitotic cycle in Parmecium aurelia. Expt. Cell Res. 33: 258-264.

Woodard, J., Rasch, E., & Swift, H., (1961). Nucleic acid + protein metabolism during the mitotic cycle in Vicia folia. J. biophys. biochem. Cytol. 9: 445-462.

Xeros, N., (1962). Deoxyriboside control and synchronization of mitosis. Nature 194: 682-683.

Yokoyama, M., & Trams, E. G., (1962). Effects of enzymes on blood group antigens. Nature 194: 1048-1049.

Zeuthen, E. (editor) (1964). Synchrony in cell division and growth. Interscience, New York.