EFFECTS OF D- AND L-SERINE, D- AND L-METHIONINE, GLYCINE AND PANTOIC ACID ON MONKEY KIDNEY EPITHELIAL TISSUE CELL CULTURES

By

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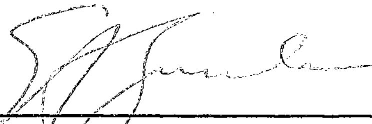

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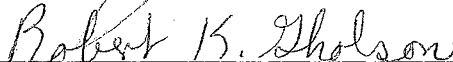

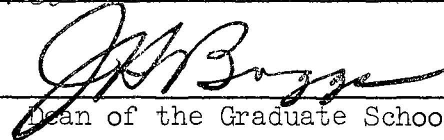

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PREFACE

It has conclusively been demonstrated that bacterial growth and division are inhibited by D-amino acids (Grula, 1960). In a chemically defined medium several D-isomers of amino acids, most notably serine, methionine, phenylalanine, threonine, tryptophan, and histidine, either alone or in combination with glucose, caused inhibition of division and growth in a species of <u>Erwinia</u> (Grula, 1960). Further studies have shown that inhibition of cell-division caused by D-serine is pH-dependent and reversable by several types of compounds (Grula and Grula, 1962a). Of the organic compounds tested, D- or L-<u>alpha</u>-alanine, pantoic acid, and pantoyl lactone are the most active in reversing division inhibition.

The primary objective of this study was to determine if compounds found to be effective in the bacterial cell division system could influence growth and morphology of monkey kidney cells <u>in vitro</u>. The approach was to determine if morphology changes were caused by addition of various concentrations of the different compounds. Compounds studied were D- and L-serine, D- and L-methionine, glycine, pantoyl lactone, and pantoic acid. Effective concentrations of each compound were determined. Experiments were conducted to study the effect on the cells of various lengths of administration of the inhibitors and the effect of the inhibitors on cultures of different ages. Photomicrographs of

morphological changes were made of cells grown in the presence of single compounds and combinations of various compounds. Levels of growth inhibition were determined by cell counts taken at the end of experiments.

Isolation and assay of the cell components DNA, RNA, and protein from cells under normal and inhibitory conditions were conducted. Isotope studies using carbon-14 labeled thymidine and uracil were undertaken to determine if a possible metabolic block existed at some stage in nucleic acid synthesis.

ACKNOWLEDGMENTS

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CHAPTER I

INTRODUCTION

A. Amino acid metabolism

The metabolism of mammalian and avian tissue cultures has been studied extensively in efforts to provide optimum growth conditions *in vitro* and to develop chemically defined media for use in more exacting metabolic studies. Current reviews of the evolution of semi-chemically defined media are those of Eagle (1955a), Swim (1959), Greenstein and Winitz (1961), Levintow and Eagle (1961a), and Paul (1960).

In general, thirteen L-amino acids (arginine, cystine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine), eight B-vitamins (biotin, choline, folic acid, nicotinamide, panthothenic acid, pyridoxal, thiamine, and riboflavin), several inorganic ions (Na^+, Mg^{++}, Ca^{++}, Cl^-, $H_2PO_4^-$ and HCO_3^-), glucose, and serum or plasma of various concentrations provide suitable growth conditions for a wide variety of tissue culture lines (Eagle, 1955a, 1956). The remaining amino acids are either not essential or are, in the case of glycine and serine, synthesized in quantities sufficient to support growth (Eagle, 1955a).

The nutritional requirements for monkey kidney cells in primary culture have been determined (Eagle, Freeman, and Levy, 1958). They were found to have the same requirements as listed above with an additional need for 0.1 mM glycine. However, other workers have found that monkey kidney cortex cells synthesized a slight excess of both serine and glycine (Pasieka, Morton, and Morgan, 1958).

Substitution for such ill-defined nutrients as serum, plasma, and embryo extract has been the main goal in finding optimum concentrations of amino acids for cell growth *in vitro*. The standard criterion for optimum concentration of an amino acid has been good growth after several subculture transfers in the test medium (Healy, Fisher, and Parker, 1954). Good growth is defined as the conditions under which there is no degeneracy of cells, an increased viability upon subculturing, or the ability to initiate growth from a primary explant of tissue (Sanford et al. 1958; Morgan, Morton, and Parker, 1950; Fischer, 1953).

Omission of individual amino acids from a basal medium was used to determine their usefulness to chicken embryonic heart and leg muscle explants (Fischer, 1953; Morgan et al. 1950). Any amino acids termed essential by these criteria were then tested in a fairly broad range of concentrations. Comments on the effects of these varied media were generally limited to the standards for good growth mentioned above. Eagle (1955b,c) has been one of the few to show that morphologic changes occur when a culture has been grown in amino acid deficient media. His studies were not complete, however, because only HeLa cells and strain L cells which had been grown in suboptimal concentrations of lysine, isoleucine, or methionine were reported. In

addition, he has made only general statements concerning growth inhibition due to high concentrations of amino acids, with no mention given of any morphologic or metabolic changes under these circumstances.

Originally, media contained DL-amino acids. Then, as purified L-amino acids became available, the DL-mixtures were replaced. Morgan et al. (1950) found no difference in growth promoting abilities between L-amino acid media and media containing racemic mixtures. Healy et al. (1955) felt it wise, for no certain reason, to replace the racemic and diastereoisomeric forms previously used.

With increasing availability of D-amino acids, several attempts have been made to test their usefulness as amino acid sources. McCoy, Maxwell, and Neuman (1956) found that D-isomers of asparagine, valine, tryptophan, phenylalanine, and methionine did not support growth of Walker carcinoma 256 when substituted at equal concentration for the L-isomers. A strain of rabbit fibroblasts would not utilize D-isomers of histidine, methionine, phenylalanine, serine, or tryptophan at twice the normal concentration of the L-isomers. There appeared to be no inhibition of growth when the normal concentration of one of the L-amino acids and a five times concentrated D-form were added together (Haff and Swim, 1957). This also held true with a permanent strain of altered uterine fibroblasts (Swim and Parker, 1958).

With strain L cells, when the natural L-amino acids were incorporated into the medium at one-half the levels previously used for the DL-mixture, there was a distinct improvement in the appearance of the cultures (Healy et al. 1955). Morgan and Morton (1957) felt that the inhibitory effects of M494, a medium containing eleven DL-mixtures, might be due to the D-isomers because M496, which contained only L-

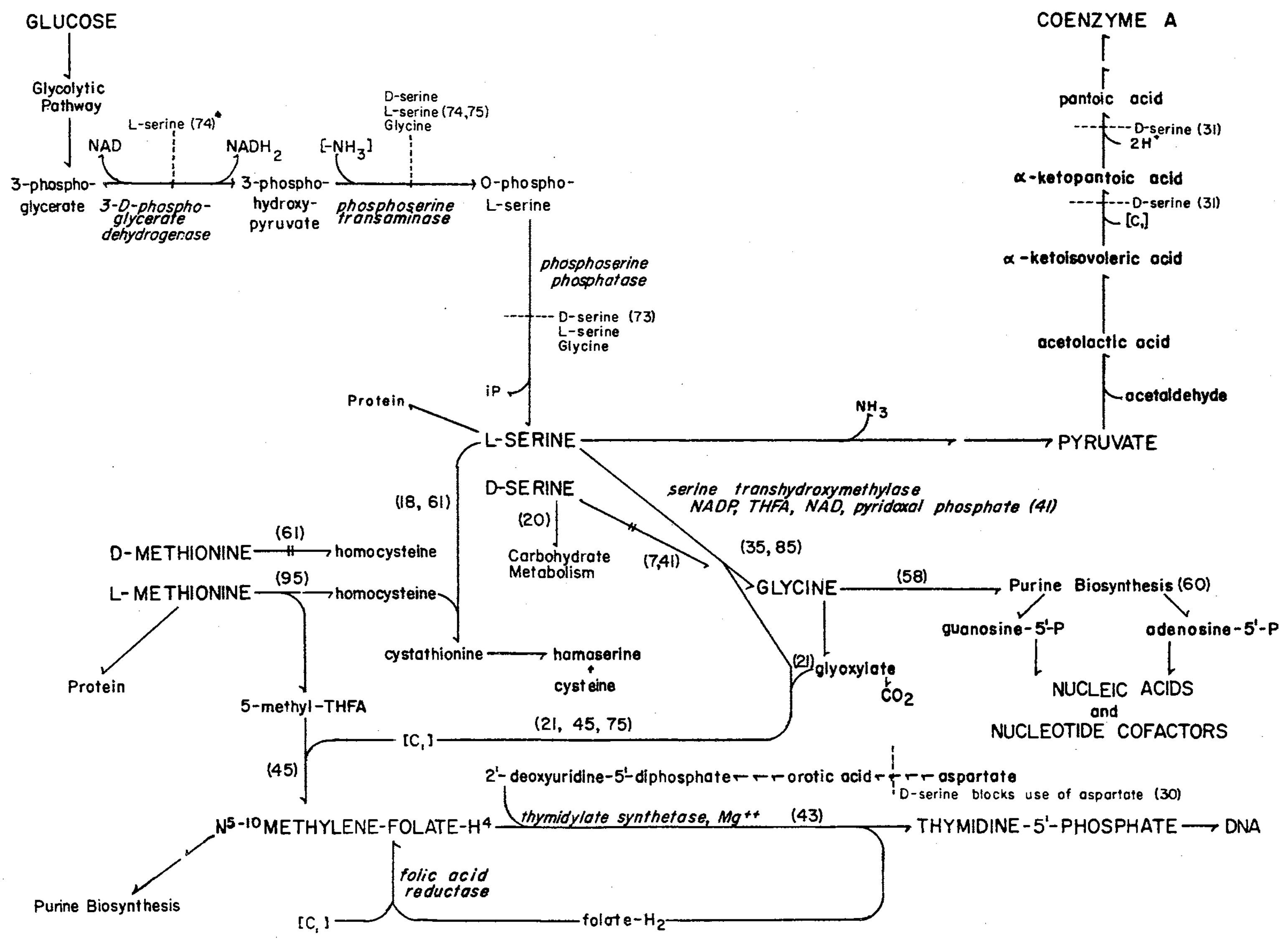
isomers at the same concentration allowed more growth.

Neither HeLa cells nor strain L cells would utilize D-isomers of any of the essential amino acids in place of the L-amino acids (Eagle, 1955 b,c). In several publications Eagle presented the opinion that D-isomers were not utilized by tissue cultures; neither were tissue cultures inhibited by high concentrations of the D-isomer when the L-amino acid was also present at optimum concentrations (1955 a,b,c; 1959). Thus, despite some evidence to the contrary, it has been generally accepted that D-amino acids have no effect on tissue cell growth *in vitro*.

Of the six compounds used in this study, three are methyl donors for one carbon (C_1) metabolism, i. e. L-serine, L-methionine, and glycine. They may also be readily incorporated into protein (Sinclair and Leslie, 1959; Rabinowitz, Olsen, and Greenberg, 1959; Manner, Broda, and Kellner, 1957), and have other metabolic functions as will be discussed later. The two "unnatural" amino acids, D-serine and D-methionine are related to the above three due to their possible effects on the metabolism of the "natural" amino acids. The metabolic pathway shown in Figure 1 illustrates these interrelationships.

Tissue cultures incubated in a medium containing glucose-U-^{14}C resulted in labeling of glycine, serine, alanine, and pyruvate (Herzenberg and Roosa, 1960; Fischer, 1953). In a definitive study of the enzymology of serine biosynthesis in KB cells Pizer (1964) reported that a pathway from 3-phosphoglycerate was the active pathway for serine biosynthesis. End product regulation was observed in that L-serine, when in sufficient concentrations, acted as a phosphoryl group acceptor in place of water, thereby reducing the

Figure 1. Metabolic Interrelationships of the Compounds Used in This Study. *Numbers refer to references listed in the bibliography.



GLUCOSE
Glycolytic Pathway
L-serine (74)*
NAD
NADH2
[-NH3]
D-serine
L-serine (74,75)
Glycine
3-phospho-glycerate
3-D-phospho-glycerate dehydrogenase
3-phospho-hydroxy-pyruvate
phosphoserine transaminase
O-phospho-L-serine
phosphoserine phosphatase
D-serine (73)
L-serine
Glycine
iP
Protein
L-SERINE
NH3
PYRUVATE
COENZYME A
pantoic acid
D-serine (31)
2H*
α-ketopantoic acid
D-serine (31)
[C1]
α-ketoisovaleric acid
acetolactic acid
acetaldehyde
D-SERINE
(20)
Carbohydrate Metabolism
(7,41)
serine transhydroxymethylase
NADP, THFA, NAD, pyridoxal phosphate (41)
(35, 85)
GLYCINE
(58)
Purine Biosynthesis (60)
guanosine-5'-P
adenosine-5'-P
NUCLEIC ACIDS
and
NUCLEOTIDE COFACTORS
(18, 61)
(61)
D-METHIONINE
homocysteine
(95)
L-METHIONINE
homocysteine
Protein
cystathionine
homoserine
+
cysteine
(21)
glyoxylate
CO2
5-methyl-THFA
[C1]
(21, 45, 75)
(45)
2'-deoxyuridine-5'-diphosphate
orotic acid
aspartate
D-serine blocks use of aspartate (30)
N5-10 METHYLENE-FOLATE-H4
thymidylate synthetase, Mg++
(43)
THYMIDINE-5'-PHOSPHATE
DNA
Purine Biosynthesis
folic acid reductase
[C1]
folate-H2

phosphatase-catalyzed hydrolysis of phosphoserine and the extent of serine accumulation. Cells grown in 1 mM L-serine had decreased levels of 3-phosphoglycerate dehydrogenase and phosphoserine phosphatase activities. The enzyme activities returned to normal when the cells were grown in a medium lacking L-serine. Similar data were obtained using Escherichia coli grown in the presence of L-serine or glycine (Pizer and Potochny, 1964). Activity of phosphoserine phosphatase in purified E. coli extracts was inhibited by 5 mM concentrations of L-serine (100%), D-serine (94%), and glycine (54%) (Pizer, 1963).

As first shown by Shemin (1946), the prinicple source of glycine in the animal body is the amino acid, serine. The conversion of serine to glycine without rupture of the carbon--nitrogen bond was proved by the use of doubly labeled serine, $HOCH_2CH^{15}NH_2{}^{13}COOH$; the COOH--$^{13}C/^{15}N$ ratio was the same in the glycine formed as in the serine administered.

This metabolic conversion of serine to glycine plus a C_1 unit is reversible and requires tetrahydrofolic acid (THFA), pyridoxal phosphate, nicotinamide adenine dinucleotide (NAD), and nicotinamide adenine dinucleotide phosphate (NADP) for the reaction (Hunnekens, Hatfi, and Kay, 1957). The reaction, catalyzed by the enzyme serine transhydroxymethylase sometimes known as serine aldolase, is specific for L-serine, while the D-isomer is almost devoid of activity.

The requirement for glycine, but not for L-serine, which appeared when amethopterin, an inhibitor of folic acid activity, is added to cell cultures indicates that normally the direction of the biosynthetic process is conversion of L-serine to glycine (Hakala and Taylor,

1959). Universality of the conversion of L-serine to glycine is indicated by the numerous types of tissue cells grown _in vitro_ which exhibit a requirement for glycine but not for serine when folic acid activity is blocked by amethopterin (Lieberman and Ove, 1960; Ham, 1962; McCarty, 1962). Eagle (1959) indicated the possibility that a partial block in conversion of serine to glycine caused a requirement for glycine by monkey kidney cells.

In rat liver slices, L-serine-3-^{14}C is metabolized giving the following distribution; 10-15% remains free or combined; 40% of the label goes to CO_2; 2% to formate, 3% each to pyruvate, and glucose and 40% unaccounted for (Elwyn et al. 1957). D-Serine-3-^{14}C is transformed to carbohydrate intermediates via glucose and is metabolized at a much slower rate. The increased conversion of L-serine to CO_2 was prevented by feeding glycine before killing the rats. When incubated with L-serine-3-^{14}C, the cells took up 37% of the added isotope. Incubation with D-serine-3-^{14}C gave 11% uptake.

Haff and Swim (1957) found that D-serine would not replace L-serine for growth of rabbit fibroblasts. Also, although D-serine was concentrated in the amino acid pool, it was not metabolized to glycine by Ehrlich's ascites cells (Christensen et al. 1952).

Methionine, an amino acid essential for growth of whole animals as well as for _in vitro_ cell cultures (Eagle, 1959), is metabolically related to serine in at least two ways. Homocysteine, the end-product of methionine demethylation, reacts with serine to form cystathionine which in turn is split into two molecules, cysteine and homoserine. This pathway is present an a human cell line and in

chicken heart explants (Eagle, Piez, and Oyama, 1961; Morgan and Morton, 1955). With the human cell cultures, a population minimum was evident (Eagle et al. 1961). With a 6 to 12 x 10^4 cells/ml, the population would survive with methionine and glucose without serine and homocysteine. With homocysteine and serine alone, minimum population per ml of culture was 100. With methionine, cysteine, and serine present even a single cell in 10 ml of medium would grow. The D-isomer of methionine could not substitute even partially for L-methionine (Morgan and Morton, 1955). Occasionally D-methionine suppressed the activity of L-methionine (Morton and Morgan, 1958).

The reversability of the reaction between homocysteine and methionine has been shown using THFA and homocysteine in stoicheometric amounts (Willmanns, Rucker, and Jaenicke, 1960). Using a preparation from sheep liver it was found that, in the presence of homocysteine, Mg^{++}, adenosine triphosphate, NAD, and THFA, radioactivity from DL-serine-3-^{14}C was incorporated into the methyl group of methionine (Nakao and Greenberg, 1958). This reaction is inhibited by amethopterin (Doctor, Patton, and Awapara, 1957). Kit (1957) reported incorporation of labeled carbon from methionine-$^{14}CH_3$, serine-3-^{14}C, and glycine-2-^{14}C into thymidine of Ehrlich's ascites cells.

The compound, pantoic acid, an intergral part of Coenzyme A, is effective in reversing growth inhibition and division inhibition caused by D-serine in cultures of an Erwinia species (Grula and Grula, 1962a). In the Erwinia species, synthesis of pantoic acid proceeds from pyruvate via acetolactate, alpha-ketoisovalerate, and alpha-ketopantoate (Grula and Grula, 1962b). D-Serine blocks this pathway

at the hydroxymethylation of alpha-ketoisovaleric acid and at the reduction of ketopantoic acid to pantoic acid. The greater inhibition occurs at the hydroxymethylation step. Only one-third the concentration of pantoic acid which was required to give optimal reversal of growth inhibition and division inhibition sufficed to restore pantothenate synthesis in the presence of beta-alanine. This indicated that pantoic acid was active in two roles: synthesis of pantothenic acid and triggering of cell division.

Beta-Alanine, another moiety of Coenzyme A is synthesized from aspartic acid (Grula and Grula, 1963). D-Serine blocks alpha-decarboxylation of aspartic acid, the initial step in this pathway. D-Serine also inhibits use of aspartic acid as the sole source of carbon, nitrogen, and energy by an *Erwinia* species.

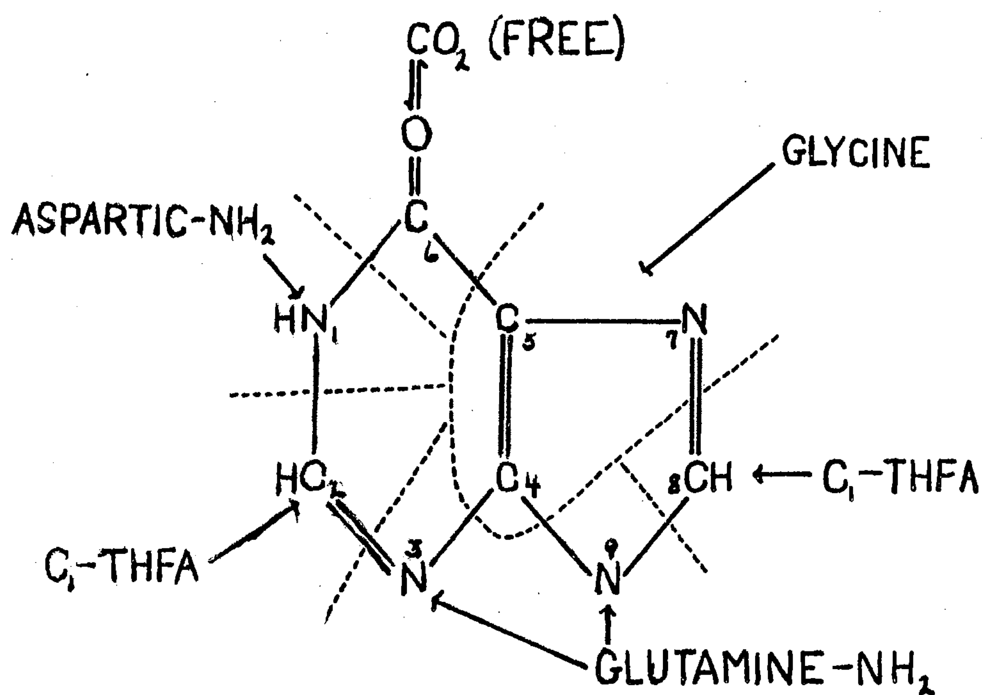
Amino acids are present within tissue culture cells in free amino acid pools (Christensen and Riggs, 1952; Piez and Eagle, 1958; Kuchler and Grauer, 1962). In general, the non-essential amino acids are more highly concentrated than essential amino acids when every amino acid is present in the medium. In L-cells, 90% of the amino acids contained in the pool were aspartic acid, threonine, serine, glutamic acid, proline, glycine, and alanine (Kuchler and Grauer, 1962). Per cents of the total pool for specific amino acids were: glycine (28%), serine (11%), alanine (12%), and methionine (1%). The internal level for any amino acid is characteristically determined by the concentration of that amino acid in the surrounding medium. In L cell cultures, when glycine was 1 mM exogenous concentration, it was present at 7-8 mM endogenous concentration and represented 28% of

the total pool. An increase of glycine concentration to 2 mM exogenous concentration caused the per cent of intracellular glycine to increase to 48.

The extent of concentration of any single amino acid is dependent upon the other amino acids within the medium. Glycine concentration by Ehrlich's ascites cells was decreased when alanine was also present in the incubation medium (Christensen and Riggs, 1952). Several D-amino acids, including D-serine, were found also to be concentrated but not as actively as the L-isomers (Christensen et al. 1952).

The C_1 units derived from serine conversion to glycine, from methionine conversion to homocysteine and, possibly, from glycine conversion to glyoxylate and the glycine molecule itself are utilized extensively for purine ring synthesis (Moat and Friedman, 1960). One carbon units are also active in methylation of deoxyuridine monophosphate to form thymidine monophosphate (Jaenicke, 1964) and methylation of alpha-ketopantoic acid to form pantoic acid (Grula and Grula, 1962b). Using glucose-6-^{14}C, McCarty (1962) obtained labeling in the methyl group of serine but glycine was unlabeled. Purines were labeled in the 2 and 8 positions and thymine in the methyl position. Glycine-1-^{14}C was shown to be a precursor of and was utilized equally for biosynthesis of both adenine and guanine in strain L cells and Ehrlich's ascites cells (McFall and Magasanik, 1960). Adenine completely suppressed this incorporation. These data conform to current knowledge of the source of atoms of the purine ring (Figure 2) as reported by Moat and Friedman (1960).

Figure 2.



Source of Atoms of the Purine Ring.

The *beta*-carbon of serine as well as the *alpha*-carbon of glycine was incorporated into the methyl group of thymine in the adult rat (Elwyn and Sprinson, 1950). In addition, formate-^{14}C served as a precursor of the methyl group of thymine both *in vivo* and *in vitro*. In bacterial systems, a serine auxotroph of *E. coli* was shown to utilize exogenously provided L-serine for synthesis of essentially 100% of both the glycine for purine synthesis and the C_1 fragments for thymine and purine synthesis (Pizer and Potochny, 1964).

B. *Cytology of tissue cells*

The nucleic acids of a tissue cell are compartmentalized in special locations within the cell nucleus. Within the nucleus, RNA is localized almost exclusively in the nucleoli, which are organelles within the nucleus. The majority of cellular RNA is contained within the cytoplasm either as part of the polysomes attached to the endoplasmic reticulum (Porter, 1961) or as soluble RNA (Paul, 1960). Within the nucleus, protein is associated with both DNA of the chromosomes and RNA of the nucleoli. Both acidic and basic proteins are present as molecules having either structural or enzymatic activity (Busch and Starbuck, 1964).

During the cell growth cycle there are four definite intervals of biosynthetic activity in relationship to formation of DNA, RNA, and protein (Gelfant, 1962; Schmidt, 1963; Prescott, 1964). There is a long postmitotic gap; (G_1) of 10 to 20 hr which corresponds to the interphase of a normal cell cycle. This is followed by a short 6 to 8 hr period (S) of DNA synthesis on the cell chromosomes. This period occurs during the preprophase stage of division. A second

premitotic gap (G_2) occurs for 1 to 2 hr just prior to a short period of mitosis (M). RNA and protein synthesis are carried out during all phases of division except the actual series involving mitosis. Just prior to mitosis, the nucleolus dissolves concomitantly with cessation of RNA synthesis (Busch et al. 1963).

In a recent review, Graham and Rake (1963) discussed the current knowledge concerning RNA synthesis and turnover in mammalian cells propagated *in vitro*. From this review several points may be made concerning RNA synthesis. (1) The kinetics of incorporation of labeled precursors into various fractions of RNA (nuclear, nucleolar, and cytoplasmic) have shown that the most rapidly labeled RNA of the cell is contained in the nucleus and that cytoplasmic RNA becomes labeled only after a relatively long delay. (2) There is a rapid turnover of nuclear RNA; label disappears from the nucleus and appears in the cytoplasm. (3) The synthesis of nuclear RNA is largely (70%) independent of RNA synthesis in the nucleolus but cytoplasmic RNA is dependent (70%) on nucleolar RNA synthesis. (4) Isotope exchange from nucleus to cytoplasm is theorized to occur either by transport of intact RNA molecules or by the transport of labeled degradation products of nuclear RNA.

The nucleoli of a tissue cell are, according to Sirlin (1962), proteinaceous intranuclear bodies whose extreme plasticity allows their morphologic appearance to vary according to the status of the cell. They are devoid of internal membranous structure and have no organized physical barrier separating nucleolar contents from nuclear contents (Moses, 1964). This implies an unusual degree of organization of the nucleolar material itself in order for it to be visible at all.

The nucleoli are the most dense of cell organelles, with a concentration of up to 90% protein which is predominantly acidic (Sirlin, 1962). Ribonucleoprotein is the most typical component in nucleoli.

Nucleoli are formed immediately after separation of the chromosomes during the telephase of mitosis. Control of nucleoli formation is by nucleoli organizers, or chromosomal loci, which are the same for all cells of an organism (Sirlin, 1962).

Nucleoli are characteristically prominent in cells actively synthesizing proteins, i. e. during multiplication, growth, or secretion.

The nucleoli are considered to be in some way essential to mitosis since damage to them by microbeam X-ray blocks mitosis (Moses, 1964).

C. Cellular RNA, DNA, and protein content

According to Mirsky and Osawa (1961), the following statements can be made concerning RNA and DNA content of normal cells: (1) DNA per cell nucleus is not only constant in the different somatic tissues of an organism but is also essentially invariant despite drastic changes in the physiological state of the organism, (2) the amount of RNA varies depending on the organ or cell line under study and the physiological conditions present.

Becker (1961) reported DNA and protein content of primary and established cell types which were 7-10 days of age. There was considerable difference between primary amnion cells, which had 17.6 x 10^{-12} g DNA/ cell and 250 x 10^{-12}g protein/ cell, and the established strains, which had 31-36 x 10^{-12}g DNA/cell and 271-396 x 10^{-12} g protein/ cell. McConkey and Hopkins (1964) found that HeLa cells

contained 25-30 x 10^{-12} g RNA/ cell and 20 x 10^{-12} g DNA/ cell. Salzman (1959) found that age of culture affected macromolecular content of HeLa cells. At 26 hr there was 53.7 x 10^{-12}g DNA/ cell, 85.8 x 10^{-12}g RNA/ cell and 0.790 x 10^{-6} absorption units protein/ cell. At 167 hr, or approximately 7 days, there was 19.8 x 10^{-12}g DNA/ cell, 23.1 x 10^{-12}g RNA/ cell and 0.179 x 10^{-6} absorption units protein/ cell. In a protein-free medium, at 7 days, strain L-M cells contained 21.5 x 10^{-12}g RNA/ cell; 19.8 x 10^{-12} g DNA/ cell and 607 x 10^{-12} g protein per cell (Eidam and Merchant, 1965).

In summary, at 7 days after subculture RNA and DNA are nearly equal in quantity per cell. This holds true for two cell lines, one of which is normal (L-M) and one neoplastic (HeLa) in origin. The amount of protein per cell is variable.

D. Morphologic changes known to occur in response to added compounds

Addition of certain compounds to tissue cultures or treatment with certain physical agents has been found to cause changes in cellular morphology and macromolecular constituents. Exposure of HeLa cells to X-rays caused the formation of giant cells seven times larger than control cells (Puck and Marcus, 1956). These cells, representing the 10% surviving cells, had huge nuclei, distinct nucleoli, and vast volumes of cytoplasm. Their overall size was such that individual cells were visible without microscopic aids.

Goldstein, Slotnick, and Journey (1960) studied the effects of actinomycin D on HeLa cells *in vitro*. Cells exposed to actinomycin D frequently contained large cytoplasmic vacuoles. Cells stained with haematoxylin showed decreased cytoplasmic basophilia. There was

greater loss of staining intensity with longer periods of exposure. These observations were taken to indicate that cytoplasmic RNA and protein, as well as nucleolar RNA and protein, were either decreased in amount or were altered by the antibiotic. Later it was found that concentrations of actinomycin D which could diminish RNA synthesis by 99% permitted substantial rates of protein and DNA synthesis for prolonged periods (Reich et al. 1962). The mode of action of actinomycin D appears to be interruption of RNA synthesis, particularly of the ribosomal RNA which is messenger RNA and DNA directed (Girard, Penman, and Darnell, 1964).

Thioacetamide, a carcinogenic compound, caused formation of large, dense nucleoli by liver cells treated in vivo (Kleinfield and von Haam, 1959). The cytoplasm of these cells had dispersed basophilic granules which gave it smooth staining characteristics. Smooth staining characteristics were correlated with disruption of the endoplasmic reticulum. A decrease in ribosomal RNA was found which resulted in net decrease of RNA even though there was formation of larger nucleoli. In vitro treatment of HLM cells and strain L showed similar changes (Danes, 1961). When growth limiting concentrations of thioacetamide were used in cultures of strain L cells, cell nuclei became pycnotic and fat vacuoles accumulated along with other signs of injury. HLM cells which had been treated with thioacetamide gradually increased in size with equal increase occurring in both nucleus and cytoplasm. Basophilia of the nuclei and nucleoli (which were prominent) was unchanged. There was a slight decrease in stain intensity of cytoplasmic contents. Nuclei were large (greater than 8 μ); there was increased cytoplasmic vacuolization.

Purines and pyrimidines cause morphologic changes when added to primary explants of chick heart muscle (Hughes, 1951; Necco, 1958). Adenosine (7.4 mM in Tyrode salt solution) caused fragmentation of cell nucleoli within 10-15 min. Thymidine, cytosine, and uracil (10 mM in Hanks' balanced salt solution) caused structural changes in the nucleoli without affecting the mitotic process. Use of balanced salt solutions as incubation media may have aided the effects observed because Swift (1959) reported that the number of nucleoli per cell was related to the medium in which the cells were incubated. Incubation in balanced salt solutions caused an increase in the number of nucleoli per cell.

Sanford, Dupree, and Covalesky (1963) characterize folic acid deficiency morphology in strain L cells as an increase in cell size, enlargement of nuclei, spinning out of long thread-like filaments and cessation of cell division. Glycine, L-serine, L-methionine, and deoxycytidine were present in a medium lacking folic acid, vitamin B_{12}, and all the other nucleic acid derivatives.

Rueckert and Mueller (1960) report that in amethopterin treated Novikoff hepatoma cells *in vitro* there was a cessation of division but the cells continued to increase in size over a period of 72 hr or longer. RNA synthesis ceased after 16 hr but protein continued to accumulate slowly. The addition of thymidine halted degeneration and caused a burst of cell division.

Yoneda and Krasnoschecoff (1958) reported cellular morphology changes in primary explants of chick heart muscle when 30 mM L-methionine was present. D-Methionine was not reported as being used. The morphologic changes observed were retraction of cell processes,

vacuolation and fragmentation of the cytoplasm, rounding and shortening of the mitochondria, production of irregular contouring of the nucleus and nucleolus, decrease of cytoplasmic basophilia, and pycnosis of the cell.

In a work published after this research problem was initiated, Best et al. (1963) reported that relatively high concentrations of D-serine could cause large cell formation with increased nuclear size and was more toxic to cell growth than L-serine. The increase in nuclear size was attributed to a stoppage of nuclear division without inhibiting DNA synthesis.

In general, these research workers attribute morphological changes to changes in the amounts of RNA, DNA, and protein present in the cells. Thus, large nuclei are considered to be a criterion of increased DNA content and, subsequently, cell division inhibition. Decreased cytoplasmic basophilia is indicative of decreased nucleic acids and acidic proteins within the cytoplasm. A smoothly stained cytoplasm in a criterion of dispersed basophilic granules. No chemical data were resented to establish a firm basis for the various morphologic changes observed.

CHAPTER II

MATERIALS AND METHODS

A. Tissue culture cell line

Monkey kidney epithelial cell tissue cultures were used. The first starter culture was obtained from a cell line carried in continuous culture 2 years by Dr. Paul Barto, Oklahoma State University, Stillwater, Oklahoma. It was lost after a few weeks. The second starter culture was obtained by Dr. Barto from Fort Sam Houston Tissue Culture Laboratory and was maintained in continuous culture for the duration of the study.

B. Growth medium

The growth medium was concentrated Basal Medium Eagle diluted 1 in 100 v/v with Hanks' balanced salt solution and supplemented with 10% horse serum (Eagle, 1959; Hanks and Wallace, 1948). The detailed composition of this medium may be seen in Table I. All medium components were obtained from Microbiological Associates, Inc., Bethesda, Maryland, packaged aseptically.

TABLE I

DETAILED COMPONENTS AND PREPARATION OF REGULAR ANTIBIOTIC STOCK MEDIUM

A. Basal Medium Eagle (Eagle, 1955a)

1. L-Amino Acid Mixture--concentrations are in terms of mMoles and mg/ liter of medium before addition of horse serum. Stored at 4 C.

Amino Acid	mMolar	mg/ liter
Arginine	.12	21
Cystine	.05	12
Histidine	.05	8
Isoleucine	.2	26
Leucine	.2	26
Lysine	.2	26
Methionine	.05	8
Phenylalanine	.1	16
Threonine	.2	24
Tryptophan	.025	4
Tyrosine	.1	18
Valine	.2	24

2. Vitamin Mixture--concentrations in mg/ liter of medium before addition of horse serum. Stored frozen.

Biotin	1.0	Pyridoxal	1.0
Choline	1.0	Thiamine	1.0
Folic Acid	1.0	Riboflavin	1.0
Nicotinamide	1.0	i-Inositol·2 H_2O	1.8
Pantothenic Acid	1.0		

3. L-Glutamine--292 mg/ liter or 2.0 mMolar. Stored frozen.

(These three components are prepared separately as 100x concentrated solutions and stored as indicated until used.)

B. Hanks' Balanced Salt Solution (Hanks and Wallace, 1948). Concentrations are in terms of g/ liter of medium before addition of horse serum. Stored at 4 C.

NaCl	8.00	$Na_2HPO_4 \cdot 2H_2O$	0.06
KCl	0.40	NaH_2PO_4	0.06
$CaCl_2$	0.14	Glucose	1.0
$MgSO_4 \cdot 7H_2O$	0.10	Phenol Red	0.02
$MgCl \cdot 6H_2O$	0.10	$NaHCO_3$	0.35

TABLE I (continued)

(Prepared as a 10x concentrated solution and stored as indicated. The The sodium bicarbonate is prepared and stored separately as a 4.4% solution.)

C. Antibiotics--components in concentrations per ml of medium after addition of horse serum.

Aqueous potassium penicillin G* 100 units
Aqueous dihydrostreptomycin* 100 µg

(Prepared as 110x concentrated stocks and stored frozen.)

D. Horse Serum--100 ml/ liter of medium. Stored frozen.

For the preparation of 110 ml of medium add the following components to 82.5 ml of sterile glass distilled water in the order given.

10	ml	10x Hanks' balanced salt solution
1	ml	100x Basal Medium Eagle amino acid solution
1.8	ml	4.4% Sodium bicarbonate solution
1	ml	100x Basal Medium Eagle vitamin solution
0.6	ml	200 mM Sodium glutamine solution
1	ml	each of 110x penicillin and streptomycin solutions
10	ml	Horse serum

Final pH of the medium 7.2. Stored at 4 C. Sterilization of individual component solutions is by filtration through sintered glass filters.

*E. R. Squibb and Sons, New York
All other medium components were obtained from Microbiological Associates, Bethesda, Maryland.

C. Compounds tested

Stock amino acid solutions were made up in 0.085% NaCl, (0.85% NaCl, but not 0.085% NaCl, was found to be toxic in itself) adjusted to pH 7 with HCl or NaOH, autoclaved at 10 lbs/ 10 min, and refrigerated until used.

Pantoyl lactone was prepared by dissolving 2 g pantoyl lactone in 18 ml of glass distilled water. The pH was carefully adjusted (usually unnecessary) to pH 6.8 with 0.1 N NaOH at the time of preparation. The solution was again checked after a period of time for a decrease in pH indicating hydrolysis to pantoic acid.

Pantoic acid was made by dissolving 1 g pantoyl lactone in 13 ml 0.55 N NaOH (CO_2 free). The solution was boiled in a water bath for exactly 11 min, cooled, and adjusted to pH 7.0. The volume was increased to 20 ml with glass distilled water.

Sterilization of both solutions was accomplished by filtering through ultrafine sintered glass filters.

D. Culturing procedure

Cultures were maintained as monolayers in milk dilution bottles (milk dilution cultures). Suspension of the cells in fresh medium was by trypsinization or by loosening from the glass surface by scraping off with a rubber policeman. With either method the final step involved pipeting of the suspension to break cell clumps. Trypsinization was used in preparation for taking cell counts. Routine cell transfers were made using the second technique. The trypsin solution used was made of lyophilized crystalline trypsin

(Worthington Biochemical Corporation, Freehold, New Jersey) 30 µg/ ml of Puck's saline A (NaCl, 8.00; KCl, 0.40; glucose, 1.00; and $NaHCO_3$, 0.35 g/ liter of solution)(Marcus, Cieciura, and Puck, 1956). Numbers of cells in suspension were determined from replicate counts of diluted samples using a Coulter Counter Model B (Coulter Electronics, Inc., Chicago, Illinois). Stock cultures were transferred weekly and the medium was completely replaced every three to four days. Experimental cultures were grown in either milk dilution cultures or in 20 x 125 mm screwcapped test tubes (test tube cultures) which contained an 11 x 22 mm glass coverslip. Culture bottles and tubes were kept on stationary trays in a 37 C constant temperature room. Atmosphere within a culture tube or bottle was room air which entered the container prior to air-tight sealing.

E. Staining procedure

Cultures grown on coverslips were fixed for 30 min in a buffered formalin solution of the following composition: 10% formalin in a buffered saline solution (NaCl, 9.00; KCl, 0.42; $CaCl_2$, 0.24; and $NaHCO_3$, 0.20 g/ liter of solution). After fixation, the coverslips were treated with 70% isopropanol for 5 min and immediately transferred to Ehrlich's acid haematoxylin stain (Guyer and Bean, 1953) for 5 min.

The following is the method of preparation of Ehrlich's acid haematoxylin stain. Two g of haematoxylin are dissolved in 10 ml glacial acetic acid with 25 ml of absolute ethanol. To this solution is added 100 ml of glycerin and 75 ml absolute ethanol. Ten g potassium alum is dissolved in 100 ml H_2O by the aid of heat. The warm alum solution is combined with the haematoxylin solution slowly

with stirring. The solution must be exposed to light and air at least 3 weeks to allow oxidation of the haematoxylin to haematein. A deep-red color is indicative of completion of the oxidation process. Ehrlich's acid haematoxylin stain is an excellent nuclear stain and with proper care will keep several years.

After staining, the culture slips were destained in 0.1% HCl for 2 sec. A 2 sec rinse in distilled water made alkaline by addition of 1 g $NaHCO_3$/ 100 ml solution intensified the blue color of the stain. Excess bicarbonate water was removed with two rinses in distilled water. The culture slips were upgraded at 4 sec intervals through 70, 95, and 100% isopropanol; isopropanol-xylene (1/1 v/v mixture), and 100% xylene before mounting in Permount (Fisher Scientific Company, USA). Preparations stained in this manner are stable in color for two to three years if protected from direct light. Within a stained cell, normally, the nucleolus is stained intensely blue, the nucleus medium blue, and the cytoplasm light blue.

Nuclei average diameter was determined by selecting at random 100 nuclei from each of several coverslips for measurement.

F. Photomicrographic techniques

Photomicrographs of haematoxylin stained cell preparations were made using an A. O. Spencer Microstar with a Kodak 35 mm camera attachment (American Optical Company, Buffalo, New York). Light source was from built-in substage illumination. Kodak Plus-X Black-and-White Panchromatic film was used (Eastman Kodak Company, Rochester 4, New York). All photographs were taken and developed at equal magnification.

G. Total cell counts and determination of cell sizes

Cell preparations for terminal counts were made by using 30 µg/ml trypsin in Puck's saline A (10 ml total volume for each milk dilution culture) to free the cells from the glass. The cell suspension was pipetted back and forth into the bottle to break up cell clumps. The resulting suspension was checked microscopically for absence, or near absence, of clumps. The suspension was diluted with more suspending medium so that the total cell count from 0.5 ml of counting suspension was 3-10 x 10^3. Between these concentrations the counts are considered to be statistically accurate with no need to correct for the coincidence of two cells entering the counting orifice simultaneously. Four replicate counts were made at each lower threshold setting selected. When counts were taken from a single sample over a long period of time, it was necessary to pipet the counting suspension occasionally to keep cells from settling.

Cell sizes were estimated by recording cell counts at 4 unit increases in the lower threshold setting. Basic instrument settings were determined by preliminary calibration with ragweed pollen suspended in saline. Ragweed pollen particle size closely approximates the size of monkey kidney epithelial cells and has an advantage of size stability in solution.

Mean cell threshold is that lower threshold setting at which one-half the total cell number is automatically rejected as causing too small an electrical current variation (pulse) to be recorded by the instrument. The mean cell threshold setting is considered representative of the average size of particles present in suspension.

H. Analysis for ribonucleic acid, deoxyribonucleic acid, and protein

The nucleic acids and protein were precipitated and extracted using a trichloroacetic acid (TCA) procedure involving treatment of the centrifuged cell mass with cold 10% TCA for 30 min followed by centrifugation to collect the precipitate. The precipitate, containing nucleic acids, protein, and other macromolecules, was then hydrolyzed at 100 C for 15 min in 3 ml 5% TCA and centrifuged. The supernatant liquid, containing the hydrolyzed nucleic acids, was collected and the precipitate was again hydrolyzed with 5% TCA. After centrifugation the supernatant liquids were pooled and the precipitate was dissolved in 0.1 N NaOH and used in protein analysis.

Ribonucleic acid content of the hot TCA extract was analyzed by the procedure of Morse and Carter (1949) using D-ribose (50 µg/ml) as the standard. Deoxyribonucleic acid content of the same extract was analyzed according to Burton's procedure (Burton, 1956). The standard was 2-deoxy-D-ribose (135 µg/ ml). Both standards were obtained from California Corporation for Biochemical Research.

Protein analysis was according to the procedure given by Sutherland et al. (1949) with 0.1 mg/ml bovine plasma albumin as the standard.

I. Isotope labeling of cultures and counting procedures

Replicate milk dilution cultures inoculated with approximately 2.0×10^4 cells/ ml were incubated 48 hr to establish growth. Cultures with the most uniform growth and conformity were then selected for an isotope tracer experiment. This group of uniform cultures was divided

into two sets. Set number 1 was prelabeled with either thymidine-2-^{14}C (0.1 μC/ ml or 0.8 μg/ ml medium) or uracil-2-^{14}C (0.1 μC/ ml or 1.3 μg/ ml medium). Set number 2 received no isotope at that time. The cultures in both sets were allowed to continue growth for 48 hr, at which time they were rinsed and incubated with 1x Hanks' balanced salt solution twice for 7 min each time. Fresh medium in 20 ml volumes was added to all cultures. Along with the medium was added D- or L-serine, 9.5 mM; D- or L-methionine, 19.2 mM; pantoic acid, 51.5 mM or glycine, 10.6 mM final concentration. At this time radioactive thymidine or uracil, in the concentrations given above, was added to the unlabeled cell cultures (set number 2). To bring all media to the same volume, 0.085% NaCl was added to both the culture sets. Samples were removed from set number 1 to determine the amount, if any, of residual radioactivity present. Samples were removed from set number 2 to determine the initial radioactivity present. Replacement of media, addition of test compounds and isotopes and removal of samples at zero time was carried out while the culture bottles were turned so that the cell monolayers were not in contact with the media. After the zero hr samples were removed, the cultures were turned into contact with the media and incubated for 5 days without further change of media. Samples were taken at 24 hr intervals with omission of the fourth day to conserve volume. Free floating cells were either not present or were collected by centrifugation of the samples.

Aliquots of samples were plated at infinite thickness on ringed aluminum planchets and counts were made in an open window, Picker

automatic gas flow isotope counter (Picker Nuclear Instruments, New Haven, Connecticut).

CHAPTER III

RESULTS

A. Changes in morphology in response to addition of different compounds

1. Normal morphology of monkey kidney epithelial cells

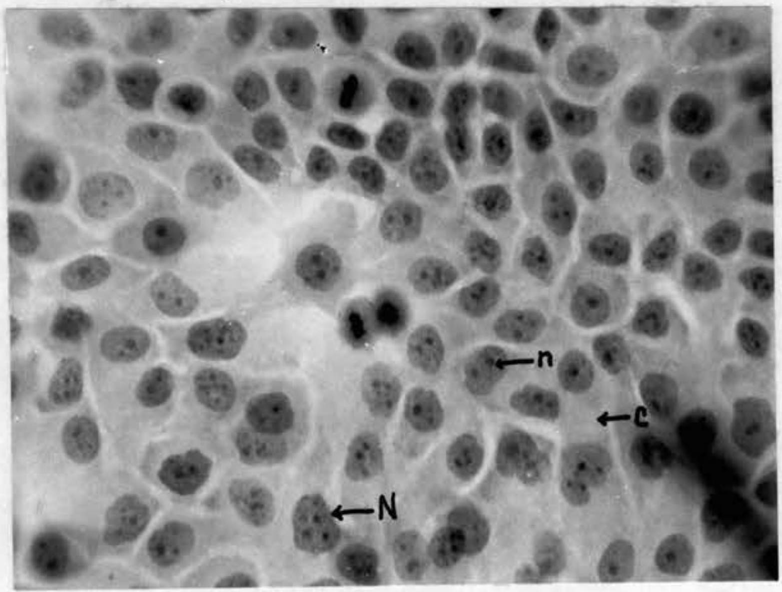
The morphology of tissue culture cells is not a stable, uniform characteristic. It must be described in terms of the most predominant features which appear in the majority of the cells present in a culture. Thus, when the normal morphology of monkey kidney epithelial cells is described as being flat, shield-like cells with single round, acentrically located nuclei, it must be understood that there will be a certain per cent of cells present which vary from the normal in some manner.

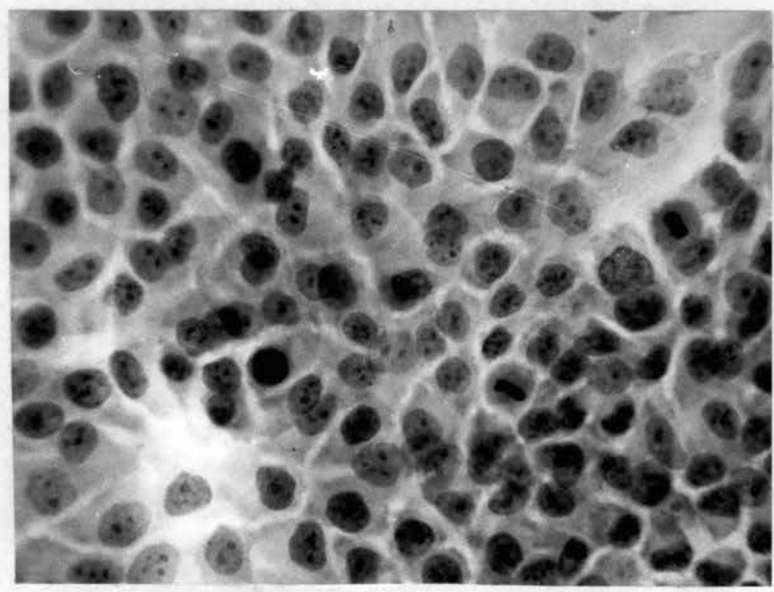
Plate I.1 is an illustration of a normal culture, seven days old, grown from an inoculum of 20,000 cells/ml. The photograph was taken of an area midway between the edge of the clone and the center. It must be pointed out that the cytoplasm (C) of properly stained cells may not show clear delineation between cells. For this reason, many of the cells appear to be dinucleated. This is an unavoidable artifact introduced by the staining and photographic methods employed. There are, however, some instances of dinucleation occurring in any normal culture.

In seven day cell cultures, the cytoplasmic to nuclear ratio (based on visual comparisons of nuclear and cytoplasmic areas as shown in

Plate I.1. 7 day culture of normal monkey kidney epithelial cells. 2×10^4 cells/ml inoculum. 1720x. Nucleus, N; Nucleolus, n; and Cytoplasm, C.

Plate I.2. Same as above. Different area within the clone.


n
C
N



photomicrographs of stained preparations) was approximatly 1 to 1. This ratio changed or was not so clearly evident in other areas of a normal clone. Plate I.2 is an illustration from an area near the center of the same clone. The cells here had begun to form a multicellular layer which obscured the individual cell morphology. At the edge of the clone, where there was more area per individual cell, the cyto-plasmic: nuclear ratio approached 2: 1. For these reasons, all illustrative photographs were made of the cells from the same general area, i. e. midway between the edge and the center of their respective clones. In some instances, the whole clone may be shown.

The nuclei (N) of cells stained with medium density and were located slightly acentrically in a light staining, smooth, nonvac-uolated cytoplasm (Plate I.1). In very young, rapidly growing cultures, there appeared 1-2% of the population with two to three nuclei per cell. After five to seven days, the frequency of these multinucleated cells decreased to less than 1% of the total population.

Typical control cell nuclei were round to oblong in shape with occasional irregular forms. The nuclear boundaries were distinct and fairly entire. Within each nucleus appeared darkly stained forms, the nucleoli (n). Their number varied from cell to cell with the normal limits being from one to five per cell. In instances of cells with only one or two nucleoli, the nucleoli appeared large, round, and densely stained. As the number of nucleoli per cell increased the size of individual nucleoli decreased.

At seven days a normal culture was still actively multiplying as indicated by the three metaphase mitotic figures present. Continuing

cell division in a culture was somewhat limited by the initial cell inoculum because cultures with a low initial inoculum tended to continue active cell multiplication longer than cultures with a high initial inoculum.

Older cultures of monkey kidney epithelial cells showed a changed morphology. The cells had shrunken, vacuolated cytoplasms with small threadlike projections of cytoplasmic material along the cell boundary (Plate II). The nuclei lost their smooth staining characteristic also. The nucleoli were no longer clearly visible with increased instances of fragmentation. These changes in morphology were typical for cultures older than ten days and appeared in normal cultures regardless of the cultural conditions or total cell population. The increased vacuolization within the cytoplasm is possibly due to accumulation of fat (King, Socolow, and Bensch, 1959).

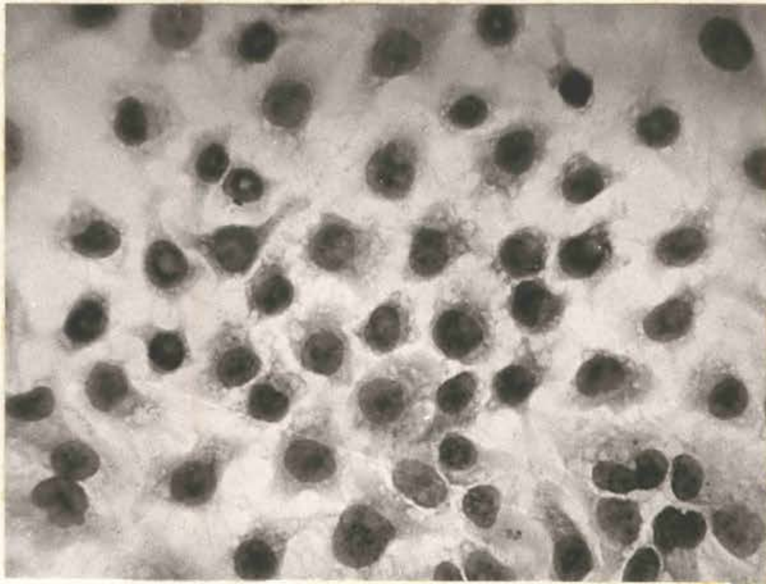
2. Changes in morphology in response to addition of single compounds

a. Cultural conditions allowing maximum expression of effect

Reproduction of the observed morphology changes and their related biochemical alterations proved to be dependent upon three cultural conditions: the culture had to be of a certain age range, there was a pH requirement, and there was a maximum population requirement. Even in a normal culture, these three requirements were interrelated.

Normal animal cell cultures exhibit growth kinetics similar to those of bacterial cultures except that the different phases of growth are in terms of days rather than hours. According to Paul (1960), the lag

Plate II. 12 day culture of normal monkey kidney epithelial cells. 2×10^4 cells/ml inoculum. 1720x.



phase of growth is from one to two days depending on the physiological condition and age of the inoculum, the number of viable cells in the inoculum, the plating efficiency of the strain, and the pH of the growth medium. The logarithmic phase of growth occurs during the third to fifth day of culture. Depending on the number of cells present at the end of the lag phase and the pH of the medium, the log phase may be expanded to include the seventh day of growth after inoculation. An inoculum using cells in log phase growth produced a rapidly growing new culture within 48 hours. An old inoculum affected the number of viable cells added to a new culture, decreased plating efficiency of the inoculum, and lengthened lag phase. A large initial inoculum actually acted to shorten the log phase of active division.

The pH for optimum outgrowth of monkey kidney epithelial cells appeared to be between 7.0 and 7.3. A more acidic medium (6.8 and below) slowed the outgrowth abruptly with death of the culture occurring in 3 to 4 days. A more alkaline medium (7.8 and above) completely halted outgrowth with cell death occurring 2 to 3 days later. These data correlate with data of a similar nature for HeLa and Chang liver cells (Mackenzie, Mackenzie, and Beck, 1961).

The exact age of a culture necessary to give reproducible results was of a relative nature because slightly older cultures which had taken two or three days to enter the logarithmic growth phase produced the same changes as a younger culture which had had a short one-day lag phase. The real requirement was for cultures which were in the logarithmic growth phase. That this occurred was expected because the logarithmic growth stage is the one most sensitive to metabolic imbalances (Paul, 1960).

That there could be an optimum pH for appearance of effects was indicated by Grula and Grula (1962a). With Erwinia there was a lesser extent of inhibition of division by D-serine if the initial pH of the medium was not 6.5-7.2. This correlated with the finding that both D-serine and pantoic acid gave maximum effects in culture media at pH 6.9-7.2.

The unexpected requirement for maximum effect by the compounds tested in this study was that of inoculum size. In preliminary experiments little attention was given the variation of inoculum numbers from one experiment to another. There was a tendency to use large inocula because of the increased plating efficiency and because most literature reported use of 1×10^5 to 1×10^6 cells/ml inoculum (Eagle, 1955b,c; Eagle, Freeman, and Levy, 1957). Later, it was discovered, with a large initial inoculum, there was difficulty in determining whether or not the desired effects were present or were just disguised by the increased number of cells. On the other hand, with a sparce initial inoculum, the control bottle also contained many abnormal forms and irregular sizes. A possible explanation for this phenomenon came from Eagle's laboratory (Eagle and Piez, 1962). Using several cell types, it was found that with inocula of 5,000 cells/ml or less there were additional metabolic requirements for survival of the cultures. This was particularly noticeable with regard to serine and glycine, which are both normally non-essential. As cell inoculum increased in number, there was shortening of generation time and increased survival rate. At 60,000 cells/ml inoculum, cultures grew normally.

Analysis of the growth medium before and after growth of the cultures showed a 10 to 15 fold increase in serine and glycine con-

centration after growth of the cells. Eagle and Piez felt that this "conditioning" of the medium to an optimum exogenous concentration caused a serine starvation condition in low population cultures due to the cells' inability to synthesize serine and glycine rapidly enough to meet all metabolic demands. When serine was added to the medium at a concentration equal to that synthesized by the cells in a non-serine medium, growth was normal.

Using Eagle's data as reference, an experiment in which the initial inoculum of monkey kidney epithelial cells varied from 5,000-50,000 cells/ml was carried out. Results showed that controls with less than 10,000 cells/ml contained aberrant forms similar to cultures containing L- and D-serine (Plate III). This could indicate a serine or glycine imbalance as theorized by Eagle and Piez. With 40,000-50,000 cells/ml, the morphologies normally observed in D- and L-serine containing cultures were no longer apparent (Plate IV). Use of 20,000 to 30,000 cells/ml gave reproducible results with maximum morphologic effects. This cell inoculum size was therefore utilized for all succeeding experiments.

b. Morphologic changes in response to D- or L-serine

Cultures containing D- or L-serine at 38, 19, 9.5, or 6.3 mM final concentrations were observed for periods of time varying from one to seven days. The lowest serine concentration giving obvious morphological changes was 9.5 mM. Higher concentrations gave more pronounced morphological changes, increased toxicity and, in some instances, additional effects. Morphology changes began to appear 48 hr after addition of either isomer and continued to increase in

Plate III.1. 7 day culture of normal monkey kidney epithelial cells. 5×10^3 cells/ml inoculum. 1720x.

Plate III.2. 7 day culture of monkey kidney epithelial cells grown with 9.5 mM L-serine 5 days. 5×10^3 cells/ml inoculum. 1720x.

Plate III.3. 7 day culture of monkey kidney epithelial cells grown with 9.5 mM D-serine 5 days. 5×10^3 cells/ml inoculum. 1720x.

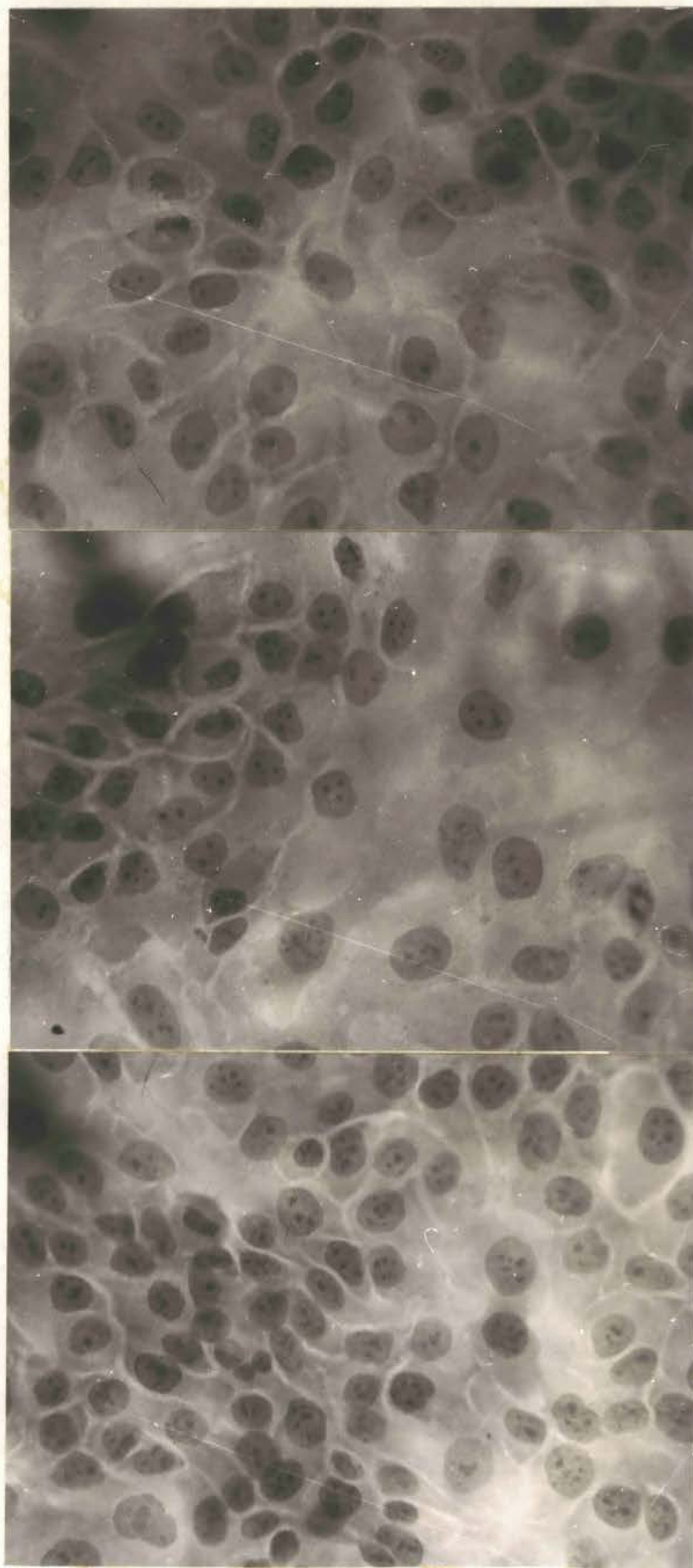
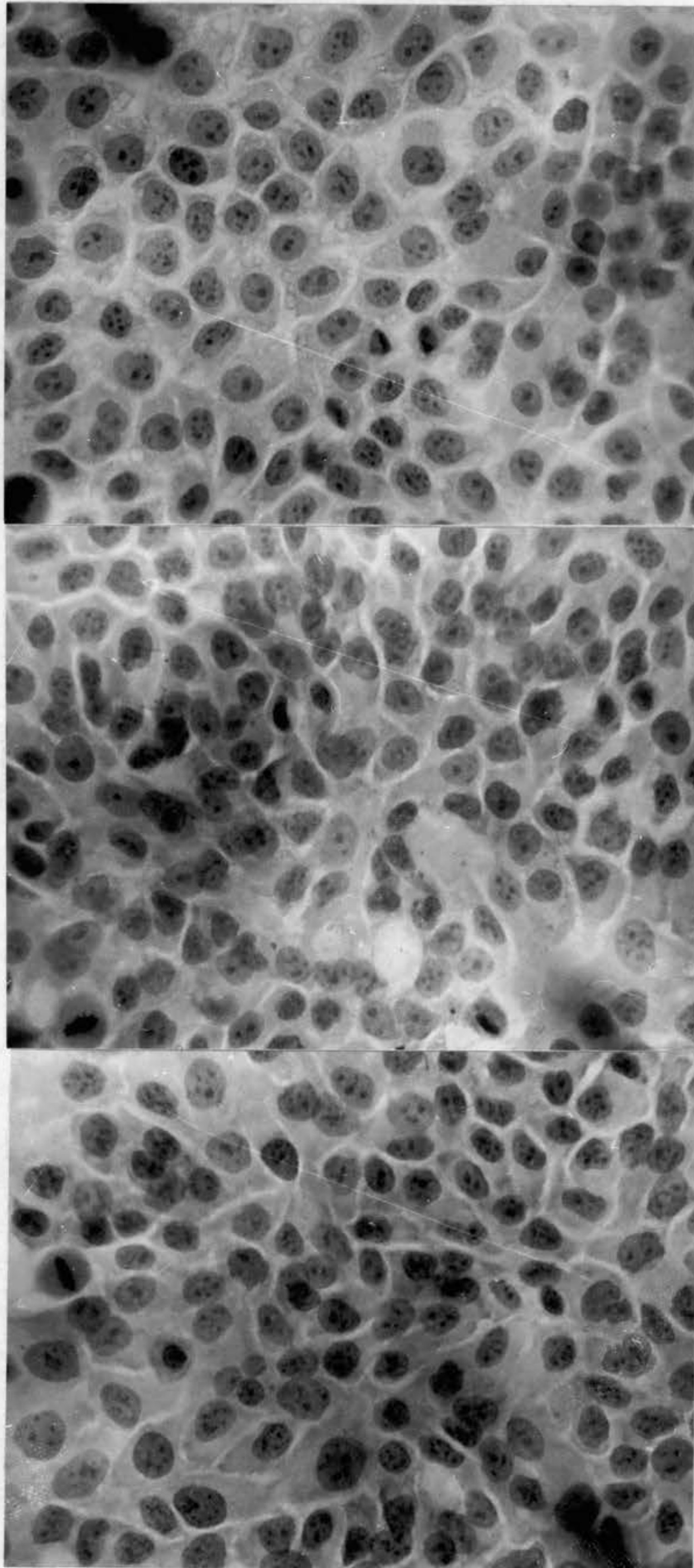


Plate IV.1. 7 day culture of normal monkey kidney epithelial cells. 5 x 10^4 cells/ml inoculum. 1720x.

Plate IV.2. 7 day culture of monkey kidney epithelial cells grown with 9.5 mM L-serine 5 days. 5 x 10^4 cells/ml inoculum. 1720x.

Plate IV.3. 7 day culture of monkey kidney epithelial cells grown with 9.5 mM D-serine 5 days. 5 x 10^4 cells/ml inoculum. 1720x.



effect with length of exposure. Maximum effect appeared at 5 to 7 days.

Plate V.1 shows an example of morphology changes occurring in a 12 day culture grown in the presence of L-serine (9.5 mM) for six days. With respect to control, the cell cytoplasm stained lightly, was smooth and contained a moderate number of vacuoles. Rather than becoming crenated, the cytoplasmic volume increased. The cytoplasmic: nuclear ratio approached 3: 1. The nuclei maintained the smooth stain characteristics of a younger culture and showed increased size over control. The nucleoli fragmented to some extent but still stained with good intensity.

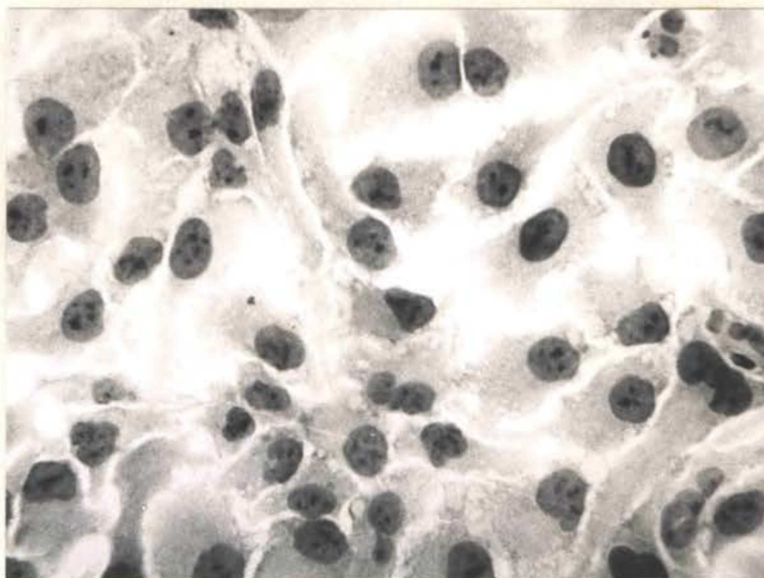
Higher concentrations of L-serine introduced a new morphological change unique to this compound. At 38 mM L-serine, cell cultures became highly vacuolated. In many instances this vacuolation was so extensive as to completely fill the cell with large round vacuoles (Plate VI).

Addition of D-serine (9.5 mM) caused the formation of very large, lightly stained cells (Plate V.2). Gross observation of stained coverslips gave very clear identification of D-serine cultures due to this change in stain intensity. Microscopically, the D-serine cells were very large with a 3: 1 cytoplasmic: nuclear ratio. The cytoplasm appeared thin due to light stain retention and a moderate number of vacuoles present. The nuclei increased greatly in size over the nuclei in both control and L-serine grown cells. The nucleoli, although varibly stained, were always fragmented. Morphologic changes due to D-serine were enhanced when the medium was kept at pH 6.9-7.2.

Morphologic changes caused by L- or D-serine were distinguished on the

Plate V.1. 12 day culture of monkey kidney epithelial cells grown with 9.5 mM L-serine 6 days. 2×10^4 cells/ml inoculum. 1720x.

Plate V.2. 12 day culture of monkey kidney epithelial cells grown with 9.5 mM D-serine 6 days. 2×10^4 cells/ml inoculum. 1720x.



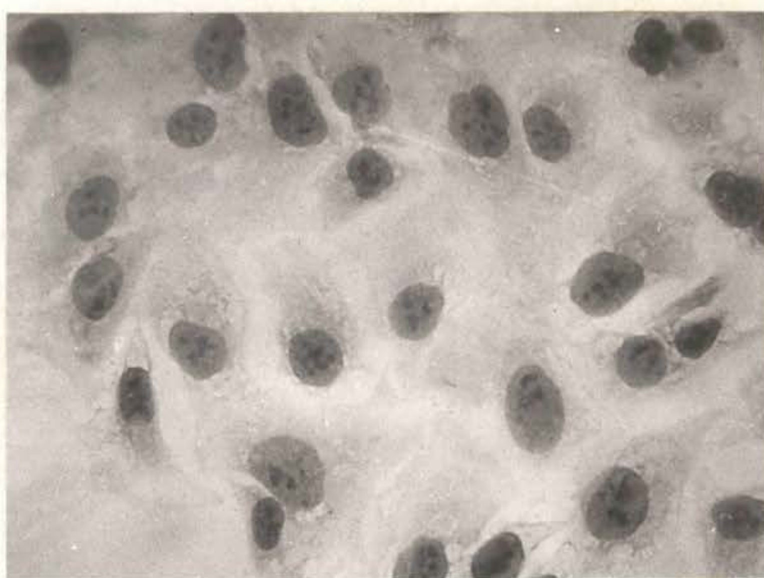
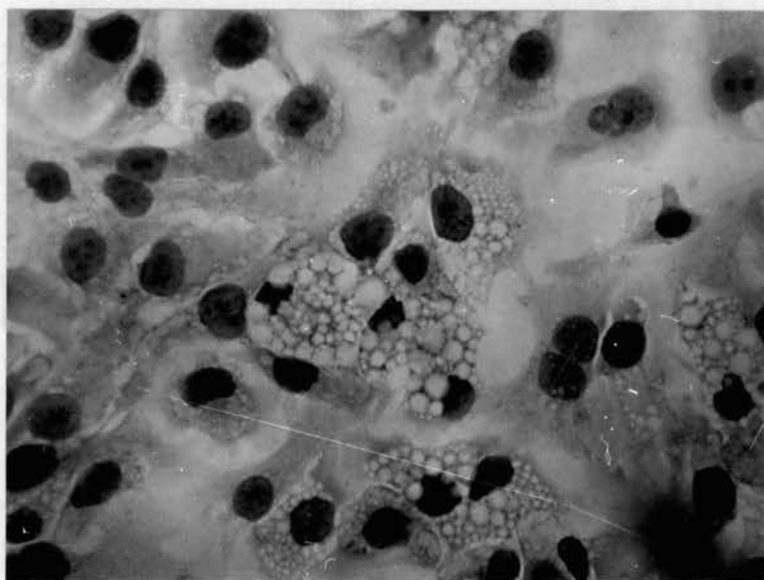


Plate VI. 12 day culture of monkey kidney epithelial cells grown with 38 mM L-serine 6 days. 2×10^4 cells/ml inoculum. 1720x.



basis of greater nucleolar fragmentation and the decreased intensity of staining in cultures containing D-serine. The increase in cell size was not always as extreme as the examples given. However, D-serine grown cell nuclei were at all times at least an average of 2 µ larger in diameter than the nuclei of L-serine grown cells.

From studies begun after this research project was underway, Best et al. (1963) reported similar results from experiments using L-cells.

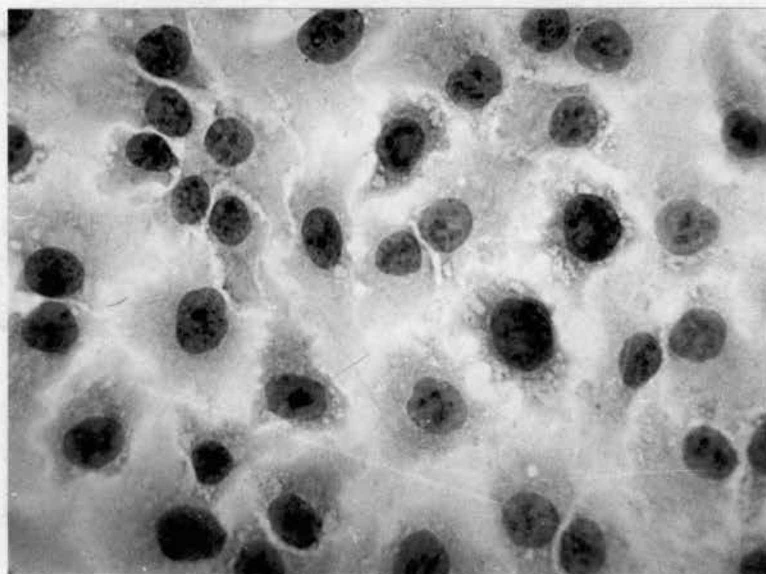
The concentration they found effective, 5 mg/ml medium, was far in excess of concentrations found effective during this study.

In succeeding experiments, the final concentration of either serine isomer was 9.5 mM.

c. Morphologic changes in response to addition of glycine

Contrary to Eagle et al. (1958) neither cell line of monkey kidney epithelial cells utilized in this study required glycine for growth. On the possibility that excess amounts of D-serine were interrupting normal glycine metabolism, studies were begun involving the use of excess glycine. Glycine was added to cell cultures at 42.6, 21.3, or 10.6 mM final concentration to test for the ability to cause morphology changes similar to those of D-serine. When glycine (10.6 mM) was added during the logarithmic growth phase and kept in the medium for 5 to 7 days, the morphology shown in Plate VII was obtained. The changes which occurred are similar to those occurring in D-serine grown cells except that the cells are smaller and have less cytoplasm. The nuclear and cytoplasmic contents stained more intensely than those of D-serine grown cells. The cytoplasm of glycine grown cells

Plate VII. 12 day culture of monkey kidney epithelial cells grown with 10.6 mM glycine 6 days. 2×10^4 cells/ml inoculum. 1720x.



did not resemble that of control cells because it was larger and did not have the same stringy texture (Plate II). On a concentration basis, glycine was not as effective as D- or L-serine in producing large cells, nor was it as toxic to growth.

For succeeding experiments the glycine concentration was maintained at 10.6 µmoles/ml medium.

d. Morphologic changes in response to pantoyl lactone

Pantoyl lactone at 58, 29, or 14 mM final concentration was unstable in tissue culture media. A short time after addition of pantoyl lactone, the pH of the medium dropped from 7.6 to 5.5-6.0 due to hydrolysis of the lactone by some agent, possibly a lactonase, present in the horse serum of the medium.

Because pantoyl lactone is a key reversing agent in the bacterial division inhibition system (Grula and Grula, 1962a), a study was made of methods of counteracting this pH change so that the effects of the lactone could be studied in the tissue culture system. Heating the serum at 60 C for 2 hr was partially effective but only if the serum was utilized immediately. Growth of the cultures using horse serum from which the gamma globulin fraction had been removed gave evidence that the agent was not in that serum fraction.

The most logical remedy for the pH shift, i. e. addition of stronger buffer or of a neutralizing agent, was unsatisfactory because an ionic imbalance was introduced which also caused morphologic abnormalities. An experiment in which 4.4% $NaHCO_3$, 0.1 N NaOH, or 0.1 N KOH was used to adjust the pH of media made equally acidic by either pantoyl lactone or 0.1 N HCl gave clear indications that the reagent

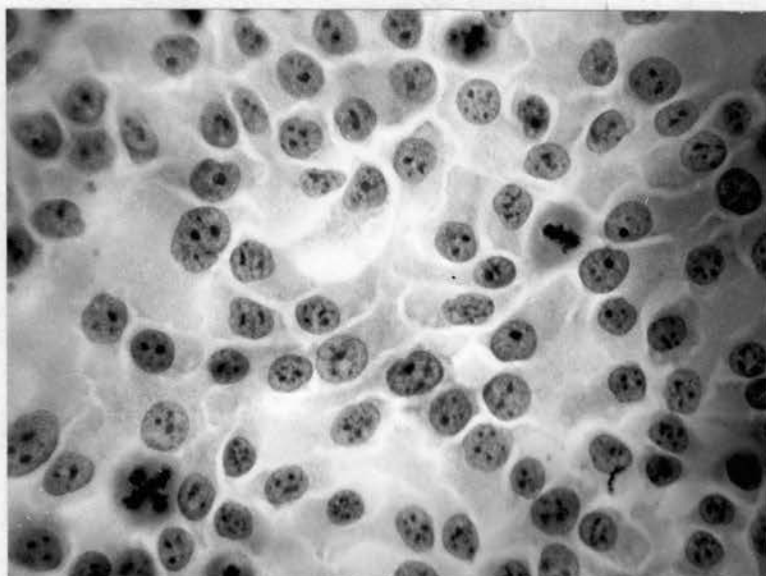
used to counteract acid production affected the system considerably. Pantoyl lactone cultures buffered with $NaHCO_3$ produced larger than normal cells having very uniformly stained cytoplasm, slightly granular nuclei and very dark, distinct nucleoli (Plate VIII.1). Addition of NaOH gave cells with the same size nuclei but less cytoplasm which did not stain smoothly. The nuclei had very pronounced membrane boundaries and a granular texture. The nucleoli appeared to be fragmented and difficult to distinguish from the granular nuclear material (Plate VIII.2). Neutralization using KOH produced cells with extremely light cytoplasm and elongated, amorphous nuclei (Plate VIII.3).

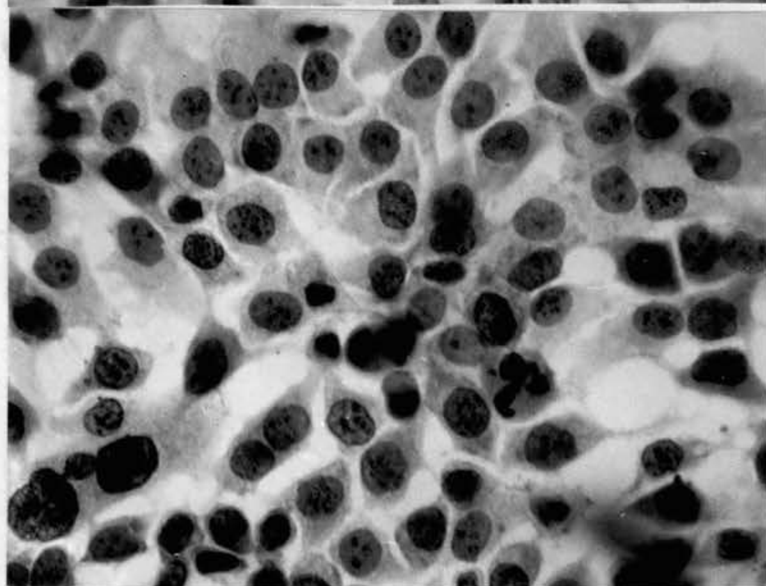
Cultures in which 4.4% $NaHCO_3$ was utilized to buffer acidity produced by 0.1 N HCl showed many morphology changes similar to the ones present in pantoyl lactone cultures which had been buffered with bicarbonate (Plate IX). Sorokin (1962) states that bicarbonate concentrations above 0.01 M have inhibitory effects on cell division. This concentration is below that (0.028 M) utilized to buffer acid produced from the pantoyl lactone present in these cultures. There was no appreciable difference between cultures in which HCl acidity had been neutralized with KOH or NaOH and the control cultures. This gives no explanation for the variation in morphology between the three types of pantoyl lactone containing cultures. It is known that excess amounts of sodium chloride have extensive effects on HeLa cell metabolism with decreased growth and decreased DNA synthesis being the most noticeable (Stubblefield and Mueller, 1960). High salt medium caused chromosome clumping in mitotic cells also. The effect of excess potassium ion was possibly due to its relationship with sodium balance.

Plate VIII. 1. 9 day culture of monkey kidney epithelial cells grown with 58 mM pantoyl lactone 2 days. Acidity buffered with 4.4% $NaHCO_3$. 1720x.

Plate VIII. 2. 9 day culture of monkey kidney epithelial cells grown with 58 mM pantoyl lactone 2 days. Acidity neutralized with 0.1 N NaOH. 1720x.

Plate VIII. 3. 9 day culture of monkey kidney epithelial cells grown with 58 mM pantoyl lactone 2 days. Acidity neutralized with 0.1 N KOH. 1720x.





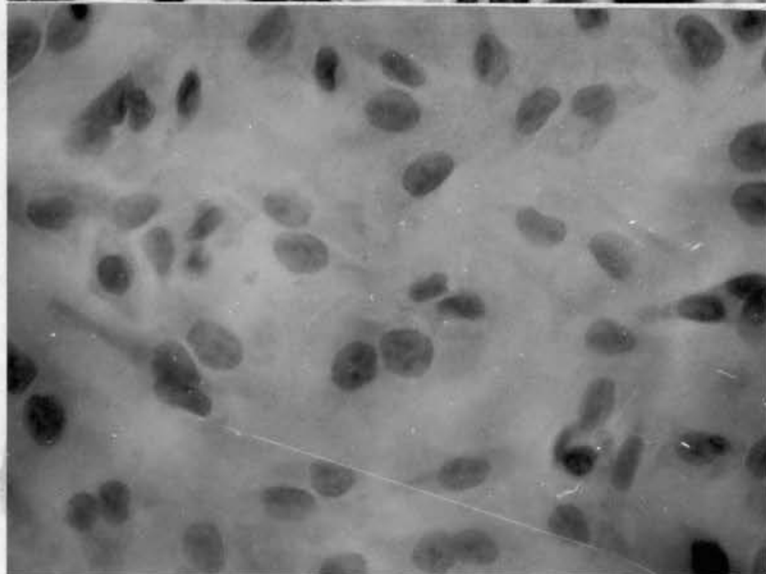
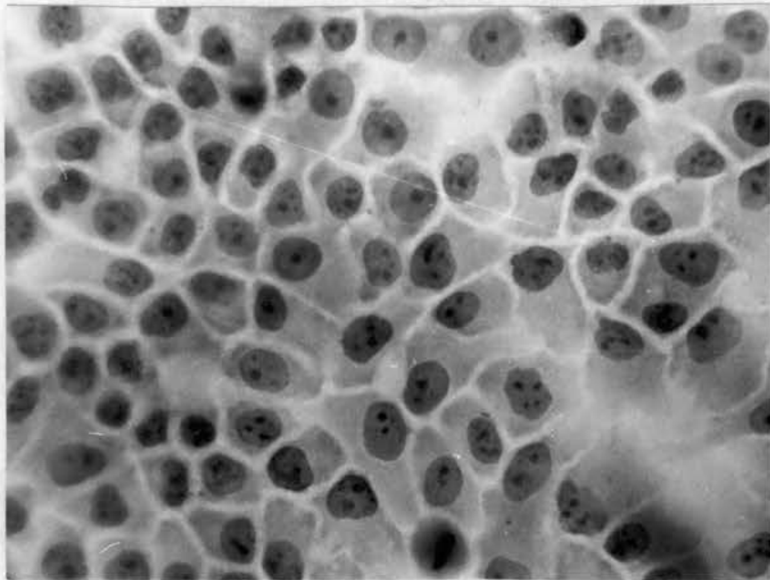


Plate IX. 9 day culture of monkey kidney epithelial cells HCl acidity buffered with 4.4% $NaHCO_3$. 1720x.



e. Morphologic changes in response to pantoic acid

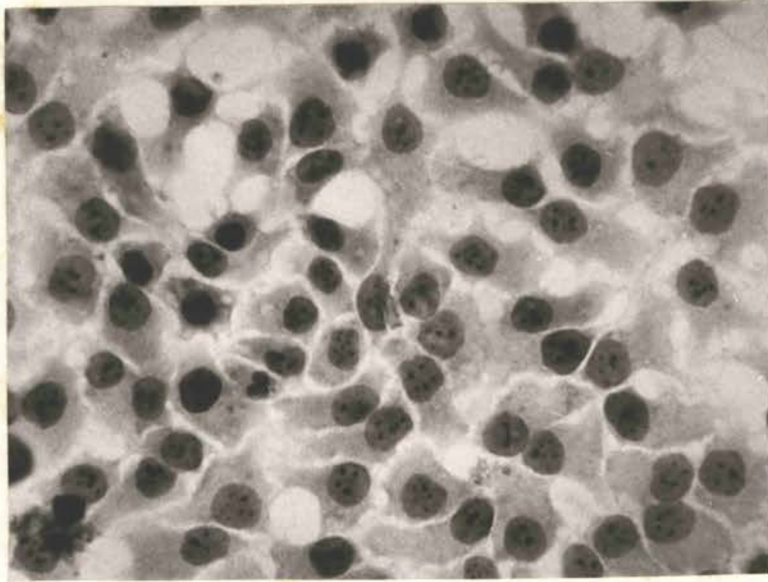
When it was found that use of pantoyl lactone in a serum containing medium was impractical from a technical standpoint, attention was shifted to the use of pantoic acid, even though this compound was less effective as a division-inhibition reversing agent in the D-serine bacterial system (Grula and Grula, 1962a). Use of 51.5 mM pantoic acid final concentration (Plate X.1) in the medium caused the formation of cells similar in morphology to the pantoyl lactone grown cells (Plate VIII.1) with the exception that the nuclei of the pantoic acid grown cells appeared smooth in contrast to the granular nuclei of the pantoyl lactone grown cells. Decreasing concentrations of pantoic acid were decreasingly effective on morphology and growth of cells.

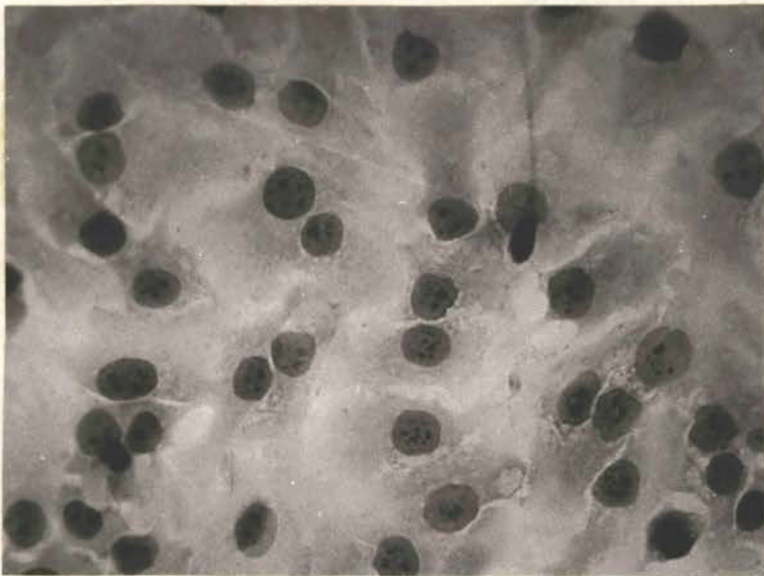
When the medium was maintained at a pH of 7.3 to 7.6, cells grown with pantoic acid (Plate X.1) did not increase significantly in size over that of the control (Plate II). The nuclei, however, were fuller, more uniform in shape and contained densely stained nucleoli. When the medium was maintained at pH 6.9 to 7.2, there was a significant increase in cell size (Plate X.2). The cytoplasmic: nuclear ratio changed from 1.5: 1 to 3: 1. Nuclei of the test cells increased in size over the nuclei of the control cells (Plate II). Compared to control cells, pantoic acid grown cells had dense, discrete nucleoli, moderately stained nuclear material and smooth staining, nonvacuolated cytoplasmic contents.

Pantoic acid grown cells were distinguished from D- and L-serine and glycine grown cells on the basis of two characterisitics: (a) the nucleoli were uniformly more discrete and more densely staining than any of the

Plate X.1. 11 day culture of monkey kidney epithelial cells grown with 51.5 mM pantoic acid 6 days. pH of the medium 7.3-7.6.

Plate X.2. 12 day culture of monkey kidney epithelial cells grown with 51.5 mM pantoic acid 6 days. pH of the medium 6.9-7.3.





others and (b) the cytoplasm of each cell was thinly spread over a great area and stained so lightly that the boundaries were indistinct. The discrete and densely stained nucleoli were different from the nucleoli found in either D-serine or glycine cells and L-serine cells did not have the same cytoplasmic changes.

f. Morphologic changes in response to D- or L-methionine

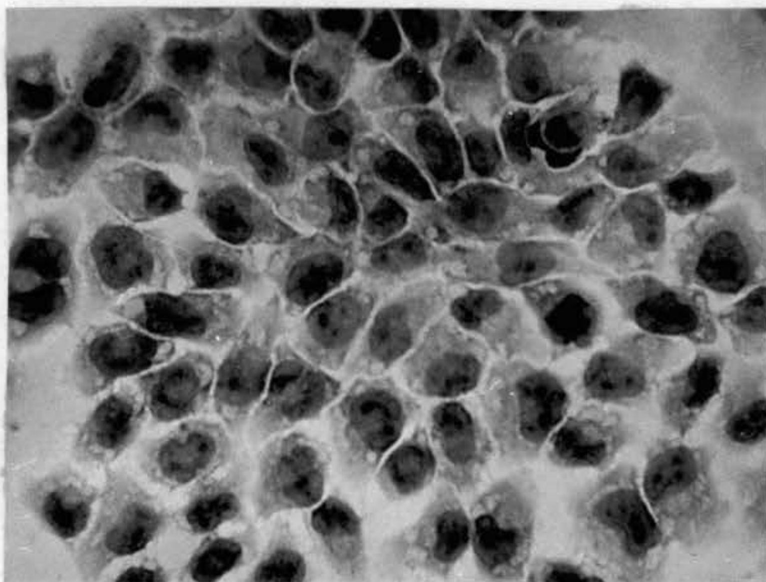
Cultures containing D- or L-methionine at 19.2, 9.6, or 4.8 mM final conqentrations were observed for periods of time varying from one to seven days along with effects of methionine addition on cultures of varying age. Only the 19.2 mM concentrations caused significant morphological changes (Plate XI.1 and XI.2). Morphology changes appeared 72 hours after addition of either isomer and increased in intensity with length of exposure. Addition of the compounds during log phase was essential with maximum effect appearing in 5 to 7 days.

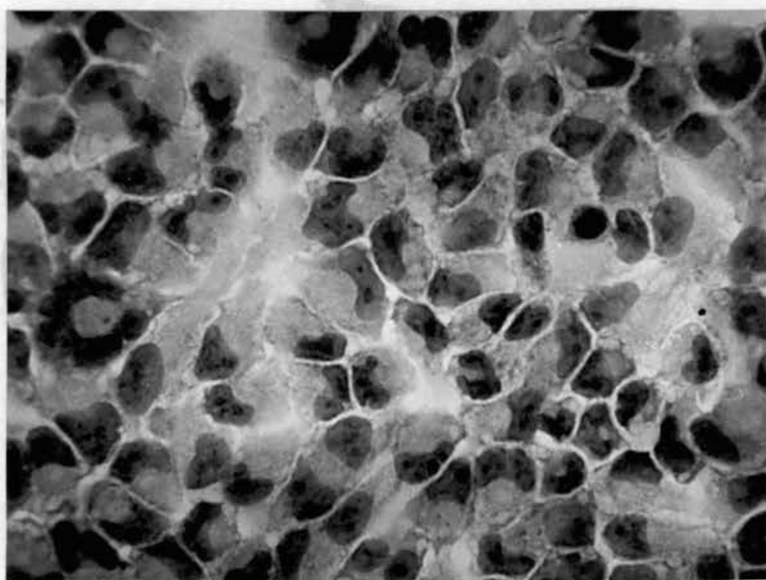
The morphologic changes which appeared in response to addition of L- or D-methionine were entirely different from those of the previously discussed compounds. Cells from these cultures were equal to or smaller than the control cells. The cytoplasmic: nuclear ratio was a maximum of 1: 1. The cytoplasm contained large, irregularly shaped vacuoles and was stringy in appearance. The nuclei stained darkly, were irregular in shape, and were displaced to the edge of the cell with the effect more pronounced in the D-methionine cultures than it was in the L-methionine cells. The nucleoli were large and indistinct.

The only point of differentiation between D-methionine and L-methionine grown cells was the greater extent of nuclear deformation in D-methionine grown cells. In succeeding experiments methionine was

Plate XI.1. 12 day culture of monkey kidney epithelial cells grown with 19.2 mM L-methionine 6 days. 2×10^4 cells/ml inoculum. 1720x.

Plate XI.2. 12 day culture of monkey kidney epithelial cells grown with 19.2 mM D-methionine 6 days. 2×10^4 cells/ml inoculum. 1720x.





kept at 19.2 mM final concentration. It should be noted that the true concentration of L-methionine was 19.25 mM when consideration of the amount of L-methionine normally present in the regular stock medium was made.

L-Methionine was more toxic than D-methionine but neither showed very great toxicity (in terms of total growth) for monkey kidney epithelial cells.

A summary of the morphological changes observed for each compound tested is given in Table II. Included are averages of measurements of nuclei of the larger than normal cells. Nuclei from cells grown in the presence of D- or L-methionine were too irregular in shape to be accurately measured.

B. Changes in morphology in response to addition of combinations of compounds

1. Effects caused by addition of pantoic acid in combination with D- or L-serine, D- or L-methionine, or glycine

Attempts were made to reverse the morphologic and growth effects of the amino acids by adding pantoic acid (51.5 μ moles/ml medium) simultaneously with any of the amino acids. Pantoic acid in combination with any of the other large cell producing compounds, D-serine, L-serine, or glycine, rather than decreasing cell size, produced even larger cells (Plate XII). Although there was no increase in nuclear volume, there was a particularly obvious increase in the cytoplasmic volume of each cell. There was little vacuole formation and, in each test combination, the cytoplasm stained evenly with medium to light intensity. The second significant morphological change observed was

TABLE II

SUMMARY OF CHANGES IN MORPHOLOGY

Compound Added mM	Cyto-plasmic: Nuclear Ratio	Avg. Nuclear Size μ	Morphology of the Nucleus	Morphology of the Nucleolus	Morphology of the Cytoplasm
None (7 day cells)	1:1	7.3	Round with distinct boundaries. Smooth, medium staining.	One to 5 per cell. Usually round. Variable size. Densely staining.	Lightly staining, smooth, non-vacuolated.
None (12 day cells)	1:1	7.3	Round with fairly distinct boundaries, Dark, non-uniformly staining.	Large, dark, hardly distinguishable from nuclear matrix.	Stringy, shrunken, and vacuolated.
L-Serine, 9.5 (12 day cells)	3.1	8.8	Round to oblong with distinct boundaries. Smooth, medium staining.	Irregular in shape, size and number. Variable staining.	Light staining, smooth, occasionally vacuolated.
L-Serine, 38	---	---	As above.	As above.	Abundant cytoplasmic vacuoles.
D-Serine, 9.5 (12 day cells)	3:1	9.2	Round with distinct boundaries. Large, lightly staining.	Fragmented. Lightly staining.	Very lightly staining, smooth, small vacuoles.

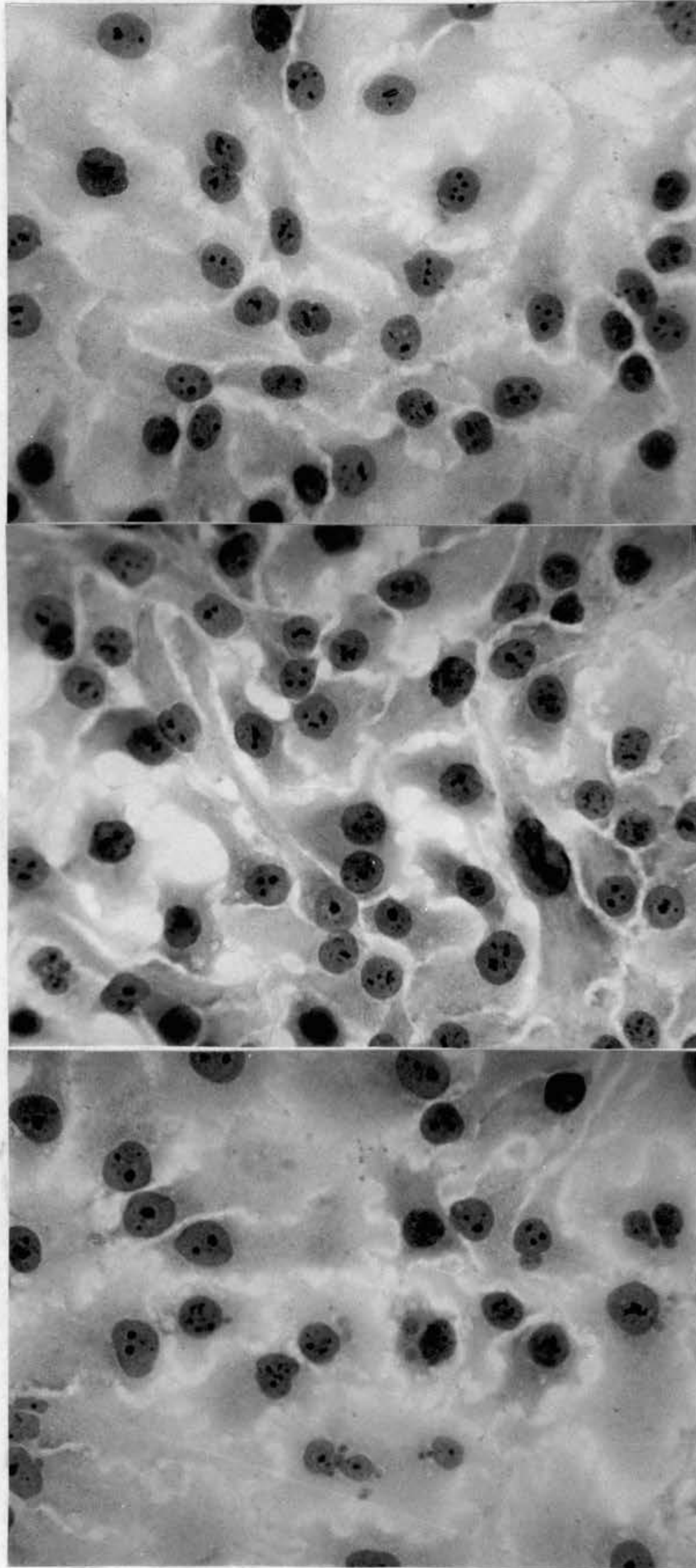
TABLE II (CONTINUED)

Compound Added mM	Cyto-plasmic: Nuclear Ratio	Avg. Nuclear Size μ	Morphology of the Nucleus	Morphology of the Nucleolus	Morphology of the Cytoplasm
Glycine, 10.6 (12 day cells)	2:1	8.4	Round with distinct boundaries. Large, light, smooth staining.	Fragmented, medium staining.	Lightly staining, moderate amount of vacuoles.
Pantoyl Lactone, 58 (9 day cells)	2:1	6.6	Round with very distinct boundaries. Medium staining.	Dark, distinct, fairly discrete forms.	Smooth, medium staining.
Pantoic Acid 51.5 (12 day cells)	3:1	8.0	Round with distinct boundaries. Medium staining.	May or may not be fragmented. Darkly staining, very distinct.	Medium to lightly staining, some vacuoles.
L-Methionine 19.2 (12 day cells)	1:1	---	Irregularly shaped and stained. Boundaries indistinct. Nuclear mass usually located near edge of cell.	Large darkly stained. Indistinct from nuclear mass.	Darkly stained, large frequent vacuoles.
D-Methionine 19.2 (12 day cells)	1:1	---	Very irregularly shaped and stained. Nuclear mass always pushed against cell boundary. Nuclear boundaries fairly distinct	Large, darkly stained. Slightly distinct from nuclear mass.	Rough, darkly staining, occasional vacuoles.

Plate XII.1. 10 day culture of monkey kidney epithelial cells grown 5 days with L-serine (9.5 mM) and pantoic acid (51.5mM). 2×10^4 cells/ml inoculum. 1720x.

Plate XII.2. 10 day culture of monkey kidney epithelial cells grown 5 days with D-serine (9.5 mM) and pantoic acid (51.5 mM). 2×10^4 cells/ml inoculum. 1720x.

Plate XII.3. 10 day culture of monkey kidney epithelial cells grown 5 days with glycine (10.6 mM) and pantoic acid (51.5 mM). 2×10^4 cells/ml inoculum. 1720x.



in the appearance of discrete, dense, heavily stained nucleoli even where previously they had been extensively fragmented.

A combination of pantoic acid with either D- or L-methionine resulted in complete loss of the methionine induced morphology (Plate XIII). Where normally the cytoplasm was crenated and the nuclei were irregular in shape and had indistinct boundaries, the cells became larger, had fuller nuclei, and had more cytoplasmic contents which stained smoothly. As with the other compounds, the nucleoli became dense and were stained heavily.

With decrease in concentration of pantoic acid to 25 mM, morphology changes associated with that compound also decreased.

There was some indication that 12.5 mM pantoic acid in combination with 9.6 mM D- or L-methionine would allow the production of near normal cells in comparison to control.

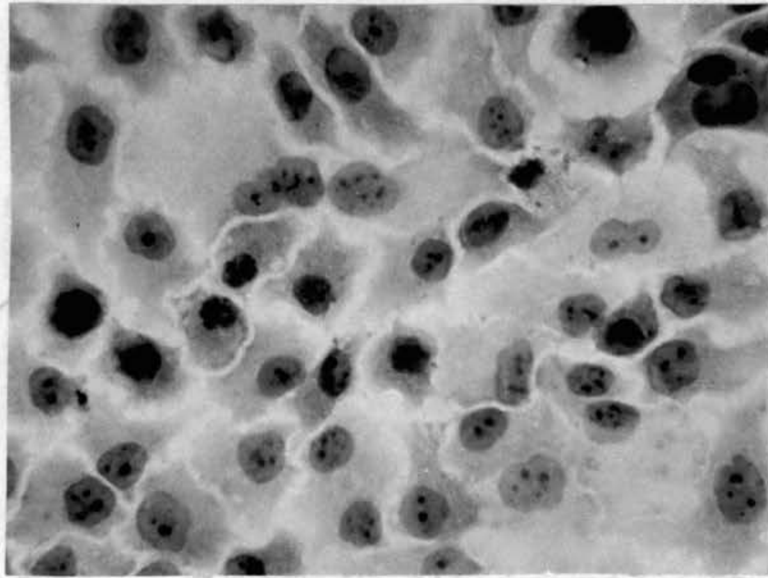
Previous experiments involving combination of pantoic acid with the various amino acids had not shown any morphologic changes other than fuller, more uniform nuclei and slightly larger cytoplasmic content. The extremely obvious change in nucleolar density and shape was observed only in one experiment.

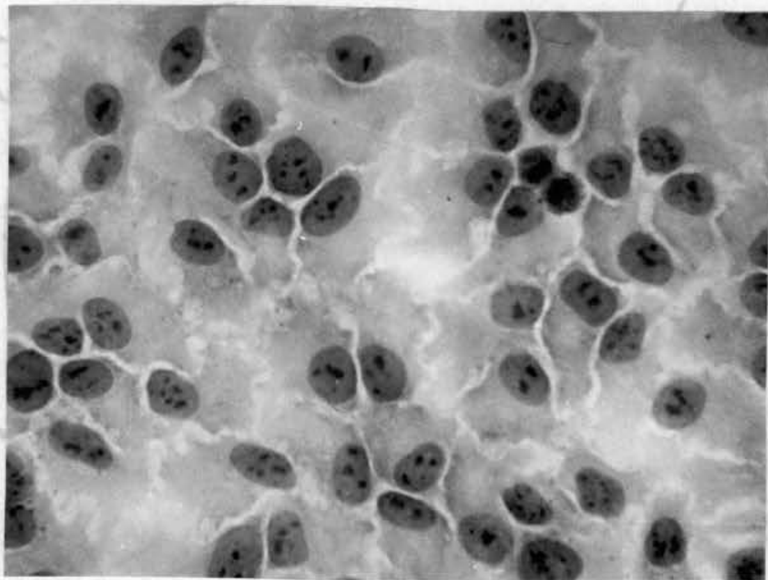
2. Effects caused by addition of beta-alanine to cultures containing L- or D-methionine, L- or D-serine, glycine, or pantoic acid

Beta-Alanine, whose synthesis by an *Erwinia* species is inhibited in the presence of D-serine (Grula and Grula, 1963), was added to monkey kidney cell cultures both individually and in combination with L- or D-methionine, L- or D-serine, glycine, or pantoic acid. Final concentrations per ml medium employed were 32.4, 16.2, and 8.2. There

Plate XIII. 1. 10 day culture of monkey kidney epithelial cells grown 5 days with L-methionine (19.2 mM) and pantoic acid (51.5 mM). 2×10^4 cells/ml inoculum. 1720x.

Plate XIII. 2. 10 day culture of monkey kidney epithelial cells grown 5 days with D-methionine (19.2 mM) and pantoic acid (51.5 mM). 2×10^4 cells/ml inoculum. 1720x.





was little or no added toxicity and no further variation in cellular morphology observed under any of the test conditions.

3. Effects caused by addition of glycine to D-serine, L-serine, D-methionine, or L-methionine containing cultures

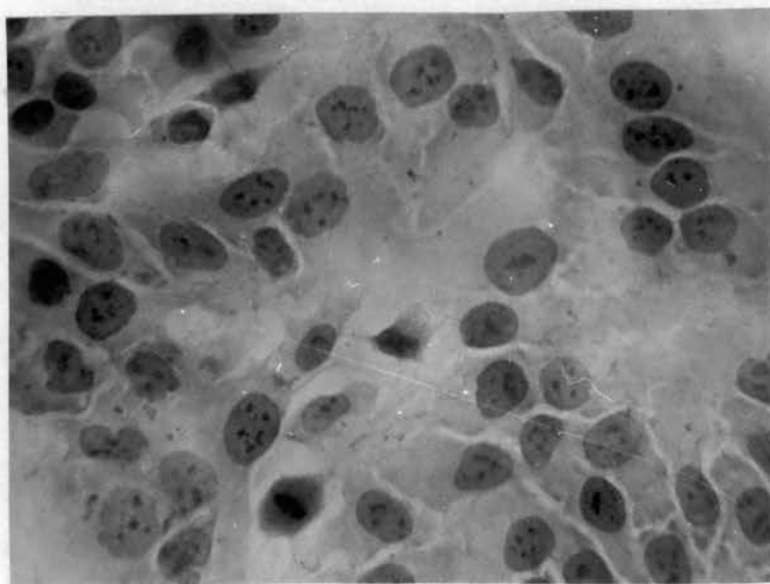
Because glycine, serine, and methionine are interrelated with respect to one carbon metabolism, experiments were set up in which glycine was added to cultures containing D-serine, L-serine, D-methionine, or L-methionine to check possible additive or reversal effects.

Cells grown in the presence of both glycine and L- or D-serine were slightly larger than those grown with D- or L-serine alone. There was no great increase in cytoplasmic contents as there had been when cells were grown with L- or D-serine and pantoic acid. The nucleoli, even though they retained more stain, remained fragmented when D-serine also was present (Plate XIV.1). In the presence of L-serine there appeared to be increased nucleolar fragmentation (Plate XIV.2). With L- or D-methionine grown cells, glycine caused formation of larger cells having more cytoplasm and increased numbers of nucleoli (Plate XV.1 and XV.2). The shape of the nuclei remained irregular. Although these cells were larger, they did not appear similar to controls.

These photographs cannot be compared for sharpness or density of stain with other photographs presented because the film development was not the same, causing a variation in the intensity of the photographic image.

Plate XIV.1. 10 day culture of monkey kidney epithelial cells grown 5 days with D-serine (9.5 mM) and glycine (10.6 mM). 2×10^4 cells/ml inoculum.

Plate XIV.2. 10 day culture of monkey kidney epithelial cells grown 5 days with L-serine (9.5 mM) and glycine (10.6 mM). 2×10^4 cells/ml inoculum.



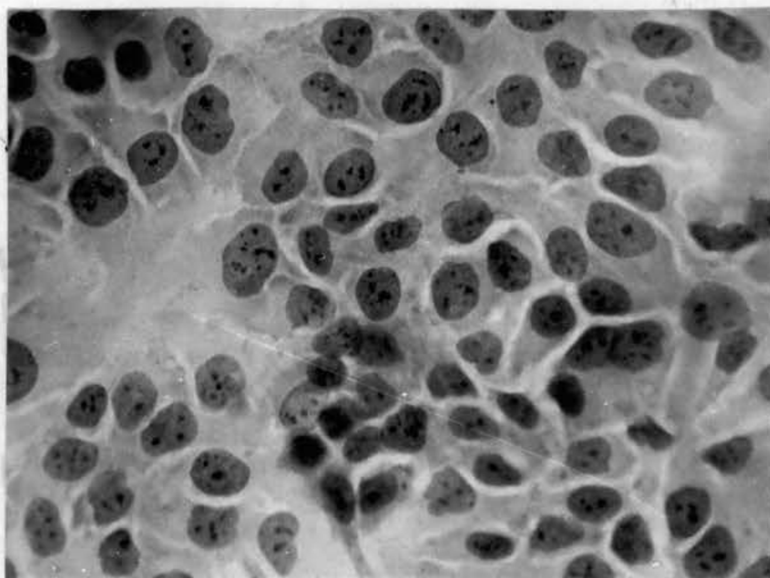
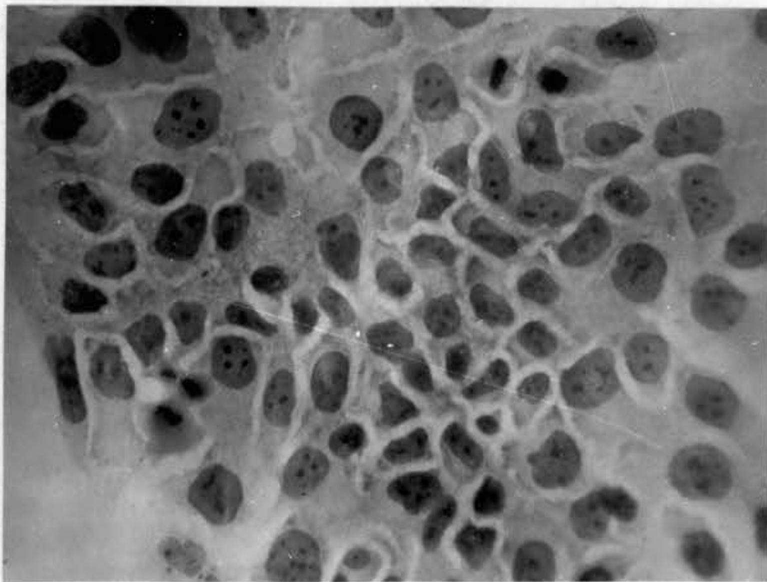
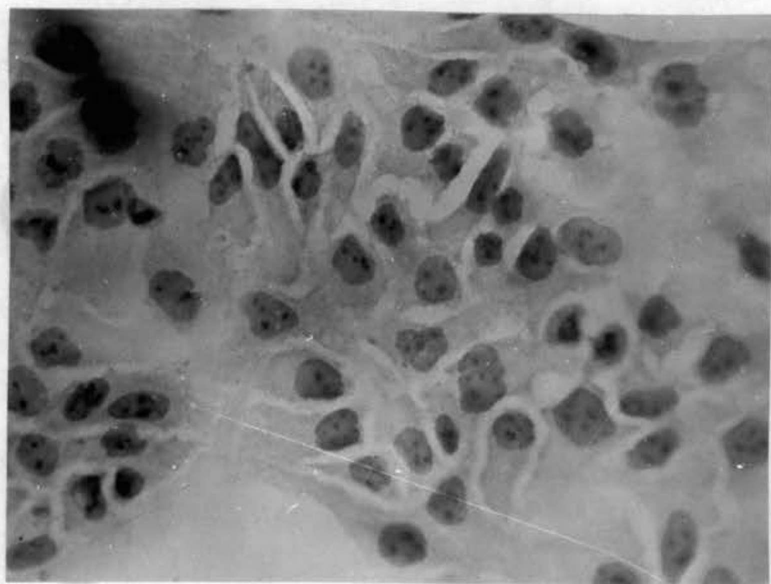


Plate XV.1. 10 day culture of monkey kidney epithelial cells grown 5 days with L-methionine (19.2 mM) and glycine (10.6 mM). 2 x 10^4 cells/ml inoculum.

Plate XV.2. 10 day culture of monkey kidney epithelial cells grown 5 days with D-methionine (19.2 mM) and glycine (0.6 mM). 2 x 10^4 cells/ml inoculum.





4. Summary of results obtained from morphology studies

From observations of amounts of growth remaining on stained coverslip preparations and from the morphological observations of changes resulting in the presence of abnormal concentrations of single compounds at least three conclusions could be made: (1) there were different levels of toxicity for the individual compounds, (2) there were changes in overall cell size together with internal structural changes and (3) there were changes in dye retention of various cell structures. Combination of pairs of compounds resulted in visible decreases in total growth. These decreases were much greater than any seen in those cultures containing single compounds.

C. Determination of toxicity levels and cell sizes by direct cell counts

Toxicity levels, as indicated by making terminal cell counts of cultures grown with L- or D-serine, L- or D-methionine, pantoic acid or glycine, were determined. Data from two experiments are given in Table III. It was found that D-serine, L-serine, and pantoic acid decreased final cell numbers considerably more than the other compounds. Glycine was intermediate in toxicity between these three compounds and L-methionine. D-Methionine was the least toxic of any of the compounds at the concentration employed. D-Serine was more toxic than L-serine, a non-essential amino acid. L-Methionine, an essential amino acid, proved to be more toxic than the D-isomer. Pantoic acid, because of the relatively high concentration used, could be considered the least toxic on a molar concentration basis.

Final cell numbers may be affected by decreased division rate or

TABLE III

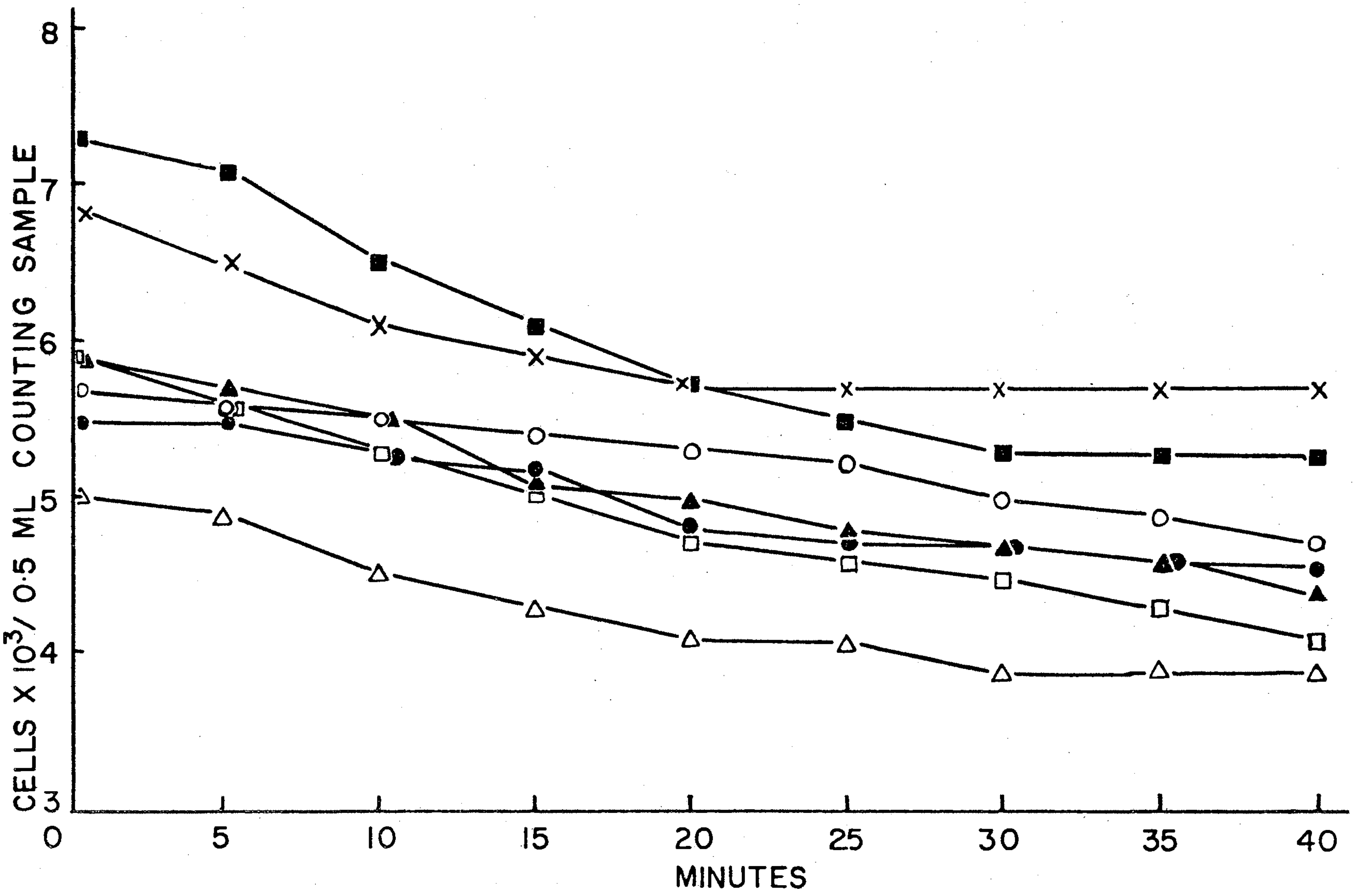
PER CENT DECREASE IN GROWTH CAUSED BY ADDITION OF D- OR L-METHIONINE, D- OR L-SERINE, PANTOIC ACID OR GLYCINE TO CULTURES OF MONKEY KIDNEY EPITHELIAL CELLS

Compound Added mM	Total Cells/ Culture x 10^6	Average Number Cells/ Culture x 10^6	Per Cent of Control
None	2.36	2.48	—
(All cultures	2.24		
12 days old)	2.70		
	2.60		
	2.44		
	2.44		
D-Serine,	1.36	1.38	55
9.5	1.40		
L-Serine,	1.54	1.54	62
9.5	1.52		
Pantoic Acid,	1.68	1.78	72
51.5	1.88		
Glycine,	1.98	1.98	79
10.6			
L-Methionine,	2.16	2.18	88
19.2	2.20		
D-Methionine,	2.28	2.31	93
19.2	2.34		

increased death rate. In a monolayer culture of moderate cell population, dead or dying cells tend to loosen from the glass surface and float free in the medium. Daily observation of experimental cultures indicated that at least part of the decrease in final cell numbers was due to increased cellular death. Because of the increase in cell size with addition of some of the compounds, it might be suggested that a part of the decreased final cell number was due to decreased cell division without appreciable inhibition of macromolecular synthesis (See Table IV for RNA, DNA, and protein analysis).

Efforts to determine cell sizes yielded invalid data due to instability of the cells while suspended in the counting fluid. It was discovered that both 0.85% NaCl and Hanks' balanced salt solution were slightly hypertonic to the cells. As a result, the cells decreased in size even while counts were being made. An experiment was conducted in which counts were made at constant lower threshold levels. These levels were arbitrarily set at values ten units apart so that they would cover the entire range of cell sizes. Figure 3 shows the change in cell counts at a constant threshold setting slightly below the peak of control cell size. The decrease in cell counts at a set threshold level may be affected not only by the number of cells which have decreased in size but also by the relationship of that particular threshold setting to the peak of cell pulse height or mean cell threshold. A threshold setting at or near the mean cell threshold will show only moderate change in cell count while a setting very much above or very much below will show greater change. Thus the data given in Figure 3 cannot be used to quantitatively determine relative amounts of shrinkage from one type cell culture

Figure 3. Decrease in Total Counts at a Constant Threshold Level When Cells Were Suspended in Hanks' Balanced Salt Solution. Lower Threshold Setting 35. Control x—x; L-Methionine, 19.2 mM ■—■; D-Methionine, 19.2 mM □—□; L-Serine, 9.5 mM ●—●; D-Serine, 9.5 mM ○—○; Pantoic Acid, 51.5 mM △—△; Glycine, 10.6 mM ▲—▲.


CELLS X 10^3/ 0·5 ML COUNTING SAMPLE
8
7
6
5
4
3
0
5
10
15
20
25
30
35
40
MINUTES

to another.

D. Ribonucleic acid, deoxyribonucleic acid, and protein content of normal and treated cultures

Changes in nuclear size and density; in nucleolar number, size, and conformity; and in cytoplasmic volume and density as seen in the stained cell preparations could indicate changes in the DNA, RNA, and protein content of cultures after addition of the test compounds. Consideration of the mode of action of haematoxylin upon fixed cell preparations further suggested there might be changes in nucleic acid and protein content of the cells. Oxidized haematoxylin, haematein, acts as a basic dye when combined with a mordant such as the potassium alum present in Ehrlich's acid haematoxylin stain. This complex dye-mordant compound stains the nucleic acids of the nucleus and the cytoplasm and other acidic compounds within the cell (Gurr, 1960). Under controlled conditions, haematoxylin can act as a differential stain and a quantitative indicator of the amount of acidic compounds present in cells. It was tentatively concluded that the variations in staining of cell organelles seen while making microscopic observations indicated changes in the macromolecular content of the cells.

These observations led to studies of the cellular content of RNA, DNA, and protein. Table IV shows data obtained from these analyses. The total RNA, DNA, and protein content of cultures within each treatment is given. All data represent the average of values obtained from two or more experiments. Included for purposes of comparison are the data shown in Table III concerning changes in total cell number. Using these figures, relative decreases or increases

TABLE IV

RIBONUCLEIC ACID, DEOXYRIBONUCLEIC ACID, AND PROTEIN CONTENT OF 7 DAY AND 11 DAY CULTURES AND TREATED CULTURES

Compound Added mM	RNA			DNA			PROTEIN			GROWTH
	Total µg	$g \times 10^{-11}$* /Cell	% Change from Control	Total µg	$g \times 10^{-11}$* /Cell	% Change from Control	Total µg	$g \times 10^{-11}$* /Cell	% Change from Control	% of Control
None, 7 day culture	444	4.44	0	112	1.12	0	7800	78	0	100
L-Serine, 9.5	290	4.7	6	76	1.22	9	5500	89	14	62
D-Serine, 9.5	200	3.64	-18	72	1.31	17	5000	91	17	55
None, 11 day culture	630	6.30	0	156	1.56	0	8200	82	0	100
L-Methionine, 19.2	480	5.45	-13.5	152	1.73	11	7400	84	2	88
D-Methionine, 19.2	460	4.95	-21.5	141	1.52	-2.5	7100	76.5	-6.5	93
Glycine, 10.6	470	5.80	-8	133	1.64	5	7100	87.5	7	81
Pantoic Acid, 51.5	425	5.90	-6.5	127	1.76	13	6900	96	17	72

*Computed from comparative amounts of cells present. This is an attempt to compute a specific amount of substance per mass of cells.

in either DNA, RNA, or protein can be more clearly defined. The column "g x 10^{-11}/ cell" was determined by using an arbitrary number of cells for control and assigning a number to each experimental condition equivalent to the per cent change in growth. Thus it can be shown that for a given unit number of cells present there is a unit amount of RNA, DNA, or protein present. The selection of g x 10^{-11} as the unit amount per cell was based on data published by Becker (1961) and Hill et al. (1959).

The data produced by this type calculation cannot be taken literally since the actual number of cells present at the end of each experiment was not known. Thus the "g x 10^{-11}/ cell" for the 7 day and 11 day controls cannot be compared. However, the per cent change from control calculated from these data is significant. From a comparison of per cent change from control it can be seen that growth in the presence of L-serine increased the cell content of RNA, DNA, and protein with the greatest increase occurring in the protein fraction. These data are in agreement with the increased nuclear size and the change in cytoplasmic: nuclear ratio observed. Although D-serine caused a 17% increase in DNA and protein content per cell over control cells, there was a very obvious decrease in RNA content (18% less than control). Increased DNA and protein correlated with increased nuclear and cytoplasmic volume. Decreased RNA content was not unexpected because of the observed fragmentation of nucleoli, nuclear sites of RNA synthesis (Busch et al. 1963), and the less densely stained cytoplasm, which contains most of the cellular RNA in ribosomes attached along the endoplasmic reticulum (Porter, 1961).

In the second group of experimental cultures a similar pattern of

changes occurred. All types of cultures showed decreased RNA/cell in comparison to control. D-Methionine grown cells were the only cultures which showed decrease in all three macromolecular constituents. These data concerning D-methionine grown cells are in agreement with observed morphologic changes of crenated nuclei and small volumes of cytoplasm in these cultures.

Increased DNA content of L-methionine grown cells without obvious increase in nuclear size may be explained by the increased density of stain of the cell nuclei, indicating a higher DNA concentration per unit area. Measurement of nuclei from either L- or D-methionine grown cells was not made because of the irregular shapes of nuclei and ill-defined nuclear boundaries present in these cells.

The per cent decrease of RNA in D-methionine grown cells was nearly that observed in D-serine grown cells when both were compared to control. However, in D-serine grown cells, the overall increase in DNA and protein concomitant with RNA decrease gave these cells a 35 percentile difference between DNA and protein content and RNA content. In D-methionine cells, there was a 15-19 percentile difference between DNA and protein content and RNA content. Thus, in relationship to the other macromolecular constituents of cells grown with D-methionine, there was a smaller change in RNA content than that of D-serine grown cells.

Loss of RNA in glycine grown cells may have been from the partially fragmented nucleoli. On the contrary, in pantoic acid cells, the nucleoli were very dense and much more uniform in size and shape than those of the control which should have indicated greater RNA content. Busch et al. (1963) point out that in many instances cells grown in the

presence of carcinogenic agents such as thioacetamide or on a non-protein medium show remarkable increase in size of nucleoli without a parallel increase in total RNA due to a decrease in cytoplasmic RNA.

In Table V internal ratios are given comparing the amounts of macromolecular constituents to each other with the per cent change between ratios of experimental cultures and ratios of the controls.

The ratio of 3.9 for RNA/DNA for 7 day cultures and 4.0 for 11 day cultures shows an apparent small amount of DNA present for the amount of RNA in comparison to data presented by other workers (Becker, 1961; McConkey and Hopkins, 1964; Salzman, 1959; Eidam and Merchant, 1964). It must be reemphasized that RNA, DNA, and protein content of one type cell line will not necessarily be equivalent to that of another type cell line (Mirsky and Osawa, 1961; Becker, 1961; Leslie, 1955).

Salzman (1959) reported a decrease in both RNA and protein during stationary growth phase. A comparison of controls of both ages showed that while the RNA/DNA ratio remained similar, there were significant decreases in the protein/RNA and protein/DNA ratios for the 11 day cultures. This is indicative of a decreased protein content. If RNA also decreased, the RNA/DNA ratio should have varied but it did not. Using L-M cells, Eidam and Merchant (1964) present data from RNA and DNA content studies which indicate a parallel linear decrease of both constituents starting shortly before 7 days after subculture. There may be a similar parallel decrease occurring in this system which would result in the same RNA/DNA ratio although decreases in RNA and DNA had occurred.

All experimental cultures had a positive per cent change in the

TABLE V

RATIOS OF RIBONUCLEIC ACID, DEOXYRIBONUCLEIC ACID, AND PROTEIN CONTENT OF 7 AND 11 DAY NORMAL CULTURES AND TREATED CULTURES

Compound Added mM	Protein / RNA	% Change	Protein / DNA	% Change	RNA / DNA	% Change
None, 7 day culture	17.6	0	69.6	0	3.9	0
L-Serine, 9.5	19.0	8	73.0	5	3.8	-2
D-Serine, 9.5	25	42	69.3	.05	2.8	-28
None, 11 day culture	13	0	52.5	0	4.0	0
L-Methionine, 19.2	15.4	18.5	48.5	-7	3.2	-20
D-Methionine, 19.2	15.4	20	50.3	-4	3.3	-18
Glycine, 10.6	15.1	16	53.3	2	3.5	-12.5
Pantoic Acid, 51.5	16.2	24	54.5	4	3.3	-17.5

protein/RNA ratio indicating that all cultures had a decrease of RNA in respect to any protein changes (either increase or decrease) which occurred. The same held true in the relationship of RNA/DNA because, regardless of increase or decrease of DNA/culture, there was less RNA present which gave negative per cent change in comparison to control.

Total cell counts taken at the conclusion of one of the seven day experiments in which L- or D-serine was added to the cultures allowed the determination of RNA, DNA, and protein on a per cell basis for cells grown with these compounds. These data, given in Table VI, verify the previous data concerning these two amino acids and give support for the method used in calculating the amount of RNA, DNA, or protein/cell for the remaining cultures. Cell counts for the eleven day cells and the other experimental cultures were not made.

In Table VII, the extent of change of DNA/cell and change of nuclear size are not in agreement if increased DNA is considered to be the sole cause of increased nuclear size. If it is not, two factors may be affecting the greater increase in nuclear size: (1) some of the increased protein content is not totally cytoplasmic but is partially nuclear in origin or (2) there may be osmotic effects introduced by excess exogenous L- or D-serine or glycine. There are large amounts of histone, protamine, and other types of protein present in nuclei whose concentrations may be affected by metabolic imbalances (Mirsky and Osawa, 1961). An increase in any of the basic proteins would not have increased stainability; however, an increase in acidic proteins would result in increased stainability.

Christensen and Riggs (1952) report that high exogenous concentrations

TABLE VI

RNA, DNA, AND PROTEIN CONTENT PER CELL OF L-SERINE, D-SERINE, AND CONTROL CULTURES

	Control	L-Serine, 9.5 mM	% Change from Control	D-Serine, 9.5 mM	% Change from Control
Total Cells	2.3×10^{7}	1.5×10^{7}	−35	1.25×10^{7}	−45.5
RNA / Cell, g	19.3×10^{-12}	19.3×10^{-12}	0	16.0×10^{-12}	− 18
DNA / Cell, g	4.87×10^{-12}	5.07×10^{-12}	4	5.75×10^{-12}	18
Protein / Cell, g	340×10^{-12}	366×10^{-12}	7	400×10^{-12}	18
Nuclear Size, Average	7.3μ	8.8μ	20	9.2μ	26

TABLE VII

COMPARISON BETWEEN INCREASED NUCLEUS SIZE AND INCREASED DNA CONTENT

Compound Added	% Change from Control Nucleus Size	% Change from Control DNA Content (Table IV)
L-Serine, 9.5 mM	20	9
D-Serine, 9.5 mM	26	17
Glycine, 10.6 mM	15	5
Pantoic Acid, 51.5 mM	10	13

of such amino acids as glycine and L-serine caused 6-16% swelling of ascites cells in suspension. In later studies D-amino acids were added to this list (Christensen et al. 1952). The swelling, caused by excessive concentration of exogenous amino acids into cellular amino acid pools, decreased as the cells were incubated one to two hours in physiological saline.

Suspensions of monkey kidney epithelial cells which had been grown in the presence of optimum concentrations of any of the test compounds did not show any shrinkage in excess of control. Suspending media were either Hanks' balanced salt solution or 0.85% NaCl.

In D-methionine grown cultures, smaller, crenated nuclei are apparently due to decreased DNA and protein content. L-Methionine containing cultures, although they showed 11% increase of DNA/cell over control, had very little or no increase in nuclear size compared to contemporary control cells. Also, in these L-methionine containing cultures, there was insignificant increase in protein content. Even though there was increased DNA content per cell, there was no increase in nuclear size in L-methionine grown cells. In contrast, L-serine grown cells with only 9% increase in DNA/cell content had a 20% increase in nucleus size. There was a concomitant 14% increase in protein content. Thus at least part of the increase in nuclear size of L-serine grown cells appears to be caused by an increased protein content. This appears to be the case also in D-serine and glycine grown cells. However, DNA appears to be the major cause of increased size of nuclei in pantoic acid grown cells since per cent increase in DNA/cell is approximately equivalent to per cent increase in nuclear

size.

Similar inconsistancies between nuclear diameter and increased DNA content have been reported for cells treated with nitrogen mustard (Levis and De Nadai, 1964).

E. Uptake and release of radioactive material from thymidine-2-^{14}C or uracil-2-^{14}C

Studies of the uptake and release of uracil-2-^{14}C or thymidine-2-^{14}C were undertaken to determine if the changes in RNA and DNA content were due either to increased or decreased leakage or increased or decreased synthesis of the macromolecules.

Cultures grown in the presence of uracil-2-^{14}C did not show any significant uptake therefore release of radioactivity or synthesis of RNA could not be studied in this manner. This correlates with reports by Harris (1952) and Healy, Fisher, and Parker (1955) that free nucleic acid bases either are not utilized or are toxic to strain L cells. Pileri and Ledoux (1957) found that, of the nucleic acid bases tested, uracil was the least utilized for synthesis of any of the nucleic acids (DNA and various types of RNA). Amount of uptake in relation to amount of isotope added to cultures of HeLa cells was reported to be in the vicinity of 0.13 to 0.2%.

There was release of radioactivity from cultures prelabeled with thymidine-2-^{14}C. The release was nearly linear and followed the same pattern for all cultures, including control. By applying the same mathematical principles that were used to determine unit amounts of RNA, DNA, and protein/ unit number of cells (Table IV), an estimation of counts/min of isotope released per unit of cells was made. The

results of these calculations are given in Table VIII. All experimental cultures, except D-methionine, showed a positive per cent change from control indicating there was increased leakage of radioisotope from the cells.

To analyze properly the data obtained from use of radioactive thymidine, the method used to calculate uptake of ^{14}C as given in Figure 4 must be considered. Amount of uptake of isotope was considered to be the difference between counts/min per ml of the zero hour sample and the counts/min per ml of the current sample, i. e. counts/min per ml of the 0 hr samples minus counts/min per ml of the 48 hr samples. This difference in counts covered not only the amount of radioisotope taken up by the cells but also the amount of isotope released by the cells since it measured only the change in isotope content of the medium from one sampling to another. A continued positive slope on the graph then indicated that uptake was in excess of release; the more acute the slope, the greater the amount of uptake over the amount released. A line horizontal to the abscissa results when uptake is in equilibrium with release. When release is greater than uptake, a negative slope to the graph of "counts per min per ml" appears.

In the control culture, uptake was slow for 24 hr after addition of thymidine; at that time uptake became rapid. At 72 hr uptake of isotope ceased to exceed release as indicated by the graph line becoming horizontal.

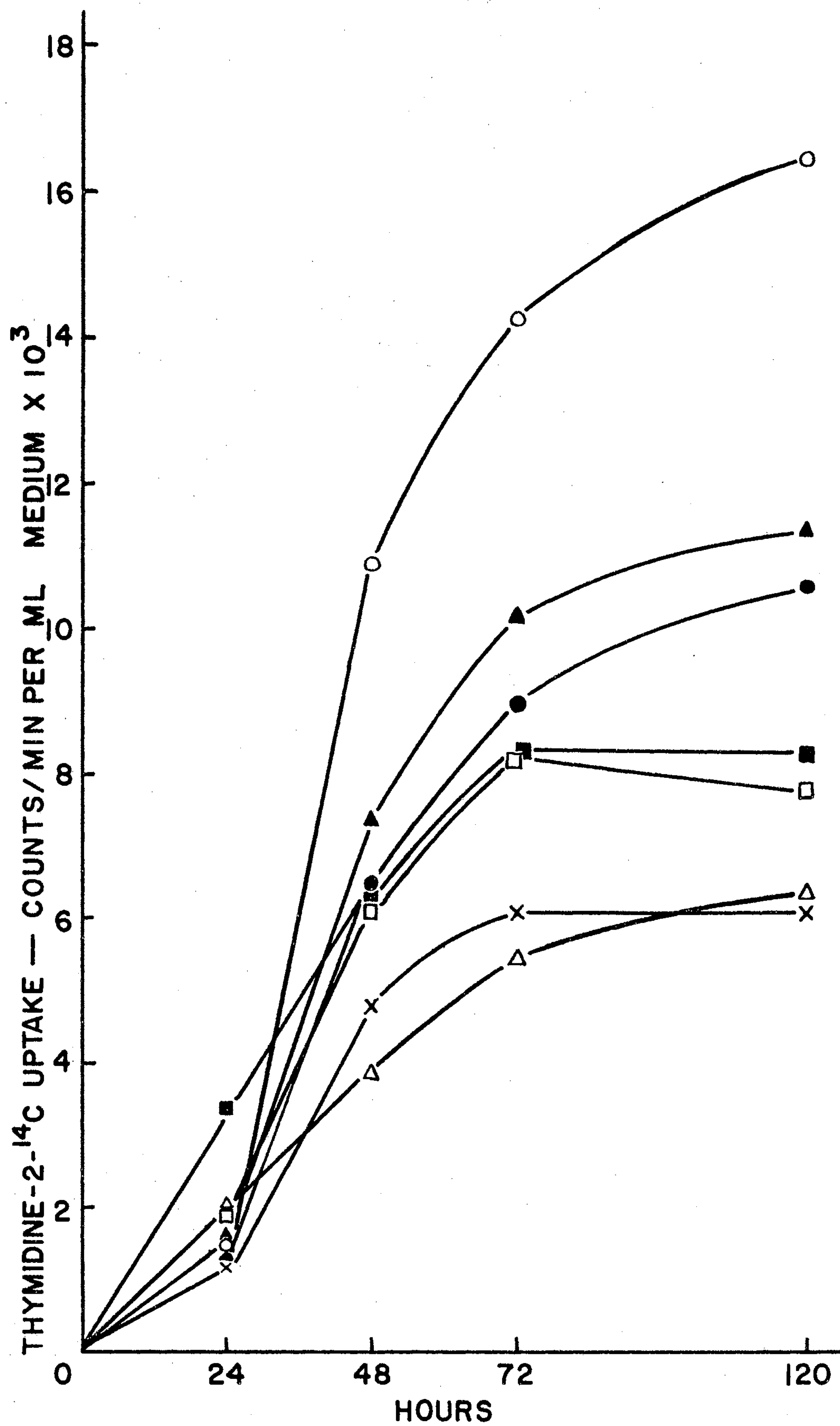
As in control, little uptake occurred in D-serine, L-serine, glycine, and pantoic acid grown cultures until 24 hr had passed. Despite decreased growth, cells grown with D-serine, glycine, and L-serine took

TABLE VIII

CHANGE IN RATE OF RELEASE OF RADIOACTIVITY FROM CULTURES PRELABLED WITH THYMIDINE-2-^{14}C

Culture	Final cpm/ml Medium	Growth, % of Control	Release, % Change from Control	DNA / Cell % Change from Control (Table IV)
Control	520	100	0	0
L-Serine, 9.5 mM	550	62	71	9
D-Serine, 9.5 mM	530	55	88	17
L-Methionine, 19.2 mM	600	88	31	11
D-Methionine, 19.2 mM	480	93	0	-2.5
Pantoic Acid, 51.5 mM	410	72	11	13
Glycine, 10.6 mM	540	79	31	5

Figure 4. Uptake of Thymidine-2-^{14}C by Control and Experimental Cultures. Control ×—× ; L-Methionine, 19.2 mM ■—■; D-Methionine, 19.2 mM □—□; L-Serine, 9.5 mM ●—●; D-Serine, 9.5 mM ○—○; Pantoic Acid, 51.5 mM △—△ ; Glycine, 10.6 mM ▲—▲.



THYMIDINE-2-^{14}C UPTAKE — COUNTS/MIN PER ML MEDIUM X 10^{3}
18
16
14
12
10
8
6
4
2
0
24
48
72
120
HOURS

up more radioactivity than did the control. Cells grown in the presence of pantoic acid had a lower but more consistant uptake than control.

D-Methionine and L-methionine grown cultures began to take up radioactivity from thymidine from time of addition. This indicates a more rapid effect of L- and D-methionine on the cell cultures. Rate of uptake was in the order of that of control and followed the same pattern of uptake as did the control, but was greater in amount.

Without further manipulation of data other than calculation of counts/min per ml it can be seen that all cultures containing compounds which caused cells to have increased DNA/cell, i. e. all compounds except D-methionine, showed continued uptake of thymidine-2-^{14}C throughout the incubation period (Figure 4). This is in contrast to the control culture which entered a plateau at 72 hr and showed no further change in radioactivity content of the medium. D-Methionine grown cells, which had decreased DNA/cell, also had negative uptake between 72 and 120 hr indicating that release of isotope was in excess of uptake. The greatest variation in amount of uptake for most cultures occurred after 72 hr which corresponds to appearance of morphologic changes.

When data are recalculated on the basis of established amounts of growth usually present within test cultures, it appears that all cultures had increased uptake of isotope over control uptake, including pantoic acid grown cells (Table IX). By this method of calculation, uptake of thymidine-2-^{14}C approximated DNA synthesis in the pantoic acid grown culture. At the same time, however, there appears to be excessive uptake of isotope in all other cultures,

TABLE IX

INCREASE IN UPTAKE OF THYMIDINE-2-^{14}C BY EXPERIMENTAL CULTURES AS COMPARED TO UPTAKE BY CONTROL CULTURES

Culture	Final Net ^{14}C Uptake cpm/ml	Growth, % of Control	Uptake, % Change from Control	DNA/Cell, % Change from Control (Table IV)
Control	6000	100	0	0
L-Serine, 9.5 mM	10,600	62	77	9
D-Serine, 9.5 mM	16,500	55	175	17
L-Methionine, 19.2 mM	8300	88	38	11
D-Methionine, 19.2 mM	7700	93	28	-2.5
Pantoic Acid, 51.5 mM	6400	72	7	13
Glycine, 10.6 mM	11,400	79	90	5

particularly in D-serine grown cultures. This finding is in agreement with the observed increased DNA content of the experimental cultures. However, the amount of uptake is much greater than can be accounted for if thymidine was used in normal fashion for synthesis of DNA at a rate equivalent to that of the control.

CHAPTER IV

DISCUSSION

The compounds studied in this investigation were seen to cause changes in total cell growth; cellular morphology; cellular content of RNA, DNA, and protein; and in rate and amount of thymidine-2-^{14}C uptake and release of and by monkey kidney epithelial cells *in vitro*.

Low population levels were essential for the production of morphologic variations (and their seemingly related macromolecular changes). Apparently this placed the cells at a critical metabolic balance in which rate of synthesis of compounds such as serine and glycine was equivalent to the rate of utilization for conditioning of the medium and for cell metabolism. Thus, metabolic imbalances induced at this point would be more effective than those induced later when growth had slowed and cultural metabolism had reached a balance.

The time lapse of 48 to 72 hr before appearance of morphologic changes indicates the compounds acted either indirectly, by incorporation into macromolecules in substitution for the natural compound or by metabolic starvation through depletion of a compound already supplied in the medium, or directly, by low level inhibition of synthesis of other metabolically important compounds. Metabolic starvation through depletion of compounds supplied in the medium can be immediately ruled out because media were replenished completely on every second or third

day of the experiment.

Total cell growth, in terms of total numbers, was decreased by all six compounds. These decreases were caused, in part, by increased rates of cell death as indicated by greater numbers of floating cells. However, these losses were not enough to totally account for the decreased growth that occurred in D-serine, L-serine, glycine,or pantoic acid containing cultures. This suggests inhibition of cell division or growth by these compounds.

The variation of extent of growth inhibition (in terms of cell numbers) by equal concentrations of different compounds (e. g. D-serine, glycine, and L-serine) or the excessive amounts of one compound required to produce effects equivalent to those caused by much lower concentrations of another (e. g. pantoic acid and glycine) may possibly be related to the number of sites of inhibition each compound had in the system, to the effectiveness of the compounds as inhibitors, or to the relative metabolic importance of the inhibited reaction(s).

Because cultures containing L-serine, glycine, or D-serine showed morphologic changes (large cells with large nuclei and fragmented nucleoli); RNA, DNA, and protein content changes (losses of RNA concomitant with large increases in DNA and protein) and thymidine uptake patterns (continued uptake thoughout the experiment) which were similar to each other, these three compounds may well have similar inhibitory functions within the system studied.

Cultures grown with pantoic acid differed from those described above because the nucleoli of the cells in these cultures were distinct and densely stained in contrast to the fragmented nucleoli of L-serine, glycine, and D-serine grown cells. Thus pantoic acid appears to have a different mode of action from these three compounds.

Addition of L- or D-methionine to monkey kidney epithelial cell cultures caused morphologic changes (normal or smaller than normal cells with crenated nuclei) which were unlike changes seen in cultures containing any of the other compounds. Further dissimilarities with respect to relative changes in RNA, DNA, and protein content (slightly increased or decreased protein/cell) and patterns of thymidine uptake (no uptake in excess of release after 72 hr) indicate L- and D-methionine affected the cells in a manner unlike any of the other compounds.

Thus, on the basis of morphologic and macromolecular changes and results from a thymidine uptake experiment, the six experimental compounds can be divided into three groups: (1) L-serine, glycine, and D-serine, (2) pantoic acid, and (3) L- and D-methionine.

The biological significance of each of the compounds used in this study has been discussed in Chapter I. As was shown, imbalance of any one compound could have caused far reaching effects in any of several metabolic sequences. Within the first group, as delineated above, L-serine, glycine, and D-serine, in that order, caused increasingly extensive modes of inhibition in both bacterial and animal systems.

As indicated by increased DNA/cell, these compounds all inhibited cell division in monkey kidney epithelial cells.

In addition, L-serine may have disrupted metabolism of other non-essential amino acids, i. e. alanine, aspartic acid, glutamic acid, and glycine, at either the biosynthetic or the degradation level. By limiting either aspartic acid or glycine availability, L-serine could have directly affected pyrimidine and purine biosynthesis.

Glycine could have been acting in either or both the following ways: (1) inhibiting L-serine synthesis at the steps involving 3-

phosphoglycerate dehydrogenase or phosphoserine phosphatase or (2) because of its high level of endogenous and exogenous concentrations, lowering the concentrations of other amino acids present in the amino acid pool to levels insufficient for proper cell metabolism. By limiting L-serine synthesis, glycine would have removed a major source of C_1 units for both purine and pyrimidine synthesis. On the other hand, if the conversion of serine to glycine is a reversible reaction, synthesis of L-serine from glycine and a C_1 unit would only further drain the cellular supply of C_1 units. The second possibility, that of lowering concentrations of other non-essential amino acids, seems less probable since protein synthesis continues in the presence of glycine.

D-Serine, the compound which caused the most extensive effects on monkey kidney epithelial cells also has the greatest number of inhibitory roles in bacterial and animal metabolic systems. As discussed in the introduction, D-serine can inhibit enzymes involved in serine biosynthesis by E. coli and is inactive as a substrate for the enzyme serine hydroxymethylase from sheep liver. In a species of Erwinia, D-serine blocks both the use of aspartate as a carbon and nitrogen source and the alpha-decarboxylation of aspartate in the formation of beta-alanine. In addition to these inhibitions, D-serine also inhibits cell division in this organism.

D-Serine inhibition of L-serine synthesis could have caused a decrease in supply of L-serine and glycine for protein synthesis; cut off a major source of C_1 units for purine, pyrimidine, and pantoic acid synthesis, and removed the supply of glycine vital to purine synthesis.

On the other hand, if D-serine were simply a competitor for the

active site of, or in some other manner inhibited conversion of L-serine to glycine and a one carbon unit by the enzyme serine hydroxymethylase, the net result would be limitation of supply of glycine and C_1 units for purine and pyrimidine synthesis as well as for protein synthesis and other metabolic functions. Presence of folic acid in the medium would not alleviate the need since folic acid is active only as a C_1 carrier and does not in itself supply one carbon units.

Blocking synthesis of glycine was only one action of D-serine in this system because addition of glycine to D-serine containing cultures resulted only in darkening of the nucleoli, an indication of increased RNA content. Reversal of other morphologic changes attributed to presence of D-serine within the medium did not occur. Nor was there any increase in total cell numbers. Addition of glycine would have relieved the requirements for purine synthesis and added a possible means of supplying C_1 units.

D-Serine inhibition of aspartic acid utilization by the cells could have directly affected synthesis of purines, pyrimidines, and beta-alanine. Because addition of beta-alanine to D-serine containing cultures did not cause any reversal of observed D-serine morphologic changes and because pantothenic acid itself is supplied in the medium, inhibition of beta-alanine synthesis, if it occurs in this system, is not a significant factor in D-serine inhibition.

In an *Erwinia* species D-serine inhibits pantoic acid synthesis. Both D- and L-serine inhibit pantothenate synthesis from pantoic acid and beta-alanine. Pantothenate, however, is supplied in the culture medium for the growth of monkey kidney epithelial cells because tissue culture cells have been shown to be dependent on exogenously supplied

pantothenate (Sanford et al. 1958) but do not require exogenous sources of Coenzyme A. Thus, unless D- or L-serine affect synthesis of Coenzyme A from pantothenate, ATP, and cysteine, they would have no effect on this particular pathway.

In the same bacterial system, pantoic acid has been shown to be active as a reversal agent for cell division inhibition by D-serine. At a lower concentration, pantoic acid stimulates pantothenate and Coenzyme A synthesis in D-serine inhibited cells. No morphologic changes were observed. Because pantoic acid caused morphologic and macromolecular changes in tissue culture cells grown *in vitro* and could not reverse D-serine inhibition in these same cells this indicates that a different mechanism of action exists for one or both of these compounds in mammalian cells grown *in vitro* as compared to their activities in cultures of the *Erwinia* species. Inability of cells grown *in vitro* to synthesize pantothenate, as indicated by their dependence on the added compounds, (Sanford et al. 1958), would negate that mode of pantoic acid activity. However, stimulation of Coenzyme A synthesis may occur. In this case, increased levels of Coenzyme A could affect any of the several Coenzyme A mediated reactions, many of which are CO_2 yielding and which are related to synthesis of adenosine triphosphate.

The limited knowledge of the mammalian metabolic role of pantoic acid other than as a moiety of, first, pantothenic acid and, subsequently, of Coenzyme A allows no further speculation concerning the mode of action of pantoic acid.

In the last group of compounds (L- and D-methionine), similarities in morphologic alterations indicate similarities in function. However,

the differences observed in RNA, DNA, and protein content indicate diverse functions also. L-Methionine, an essential amino acid which must be supplied exogenously, is active as a methyl group donor, is utilized intact in protein synthesis, and can be a source of sulfur for synthesis of cysteine. Presence of excess L-methionine is known to affect the type of nucleic acid bases present in _E. coli_ RNA by causing an increased amount of methylated bases to be present in the RNA (Mandel and Borek, 1961). Excess L-methionine may act as repressor or inhibitor for metabolic reactions within this mammalian cell system. Excess of either methionine isomer would not interrupt cysteine synthesis, if these cells are capable of synthesizing that compound, because it also is supplied in the medium. D-Methionine may be functioning as a repressor or inhibitor in a manner similar to L-methionine and also may be interrupting reactions involving L-methionine, e. g. protein synthesis and transmethylation.

Apparent reversal of D- and L-methionine morphology changes by the addition of pantoic acid is questionable on the basis that the results may only reflect superimposition of pantoic acid effects over those of D- and L-methionine. Lower levels of pantoic acid which are not capable of causing morphologic changes themselves are not capable of giving good reversal of the methionine effect when methionine is present in high concentrations. However, the increase in nuclear size and cytoplasmic area is a good indication that addition of pantoic acid overcame or reversed at least the effects of L- and D-methionine on cellular DNA and protein content.

Levels of DNA were elevated in all the experimental cell cultures , except the D-methionine grown cells. This increase, because DNA is

located solely within the nucleus, was expressed morphologically as increased nuclear size or density. Data obtained from thymidine-2-^{14}C uptake studies indicated that all experimental conditions causing greater DNA content also had greater and continuing thymidine uptake over that of the control. For L-methionine and pantoic acid containing cultures, per cent increase in thymidine uptake was in reasonable agreement with per cent increase of DNA/cell. However, D-serine, L-serine, and glycine containing cultures took up greater quantities of thymidine than could be accounted for in terms of amount of DNA found to be synthesized when thymidine was not added.

Thymidine uptake is considered to be a criterion of DNA synthesis within tissue culture cells. According to Kit, Dubbs, and Frearson (1965), the ability of cells in culture to incorporate thymidine-^{3}H into DNA depends upon a number of factors, including the thymidine kinase activity of the cultures and the percentage of cells passing through the "S" period of the mitotic cycle. Incorporation increases from zero to 48 hours but at 72 hours after subculture, incorporation of thymidine into cellular DNA decreases to a very low point. If however, thymidine is added to 48 hour cultures (the method employed in this investigation), the decrease in enzyme activity normally observed at 72 hours is postponed for up to 48 hours. Thus, under experimental conditions involving the presence of D-serine, L-serine, or glycine in conjunction with thymidine, if thymidine uptake and retention represents amount and rate of DNA synthesis, there is greater DNA synthesis than when thymidine is not present.

Thymidine uptake also depends on the number of cells in the "S" or DNA synthesizing period of cell growth. Thus it appears that D-serine

and, to a lesser extent, glycine and L-serine, inhibit cell division and that added thymidine allows uncontrolled DNA synthesis. A theory has been proposed and supporting evidence published which shows that deoxythymidine monophosphate is the major regulatory compound in DNA synthesis (Maley and Maley, 1964). Increased conversion of thymidine to deoxythymidine monophosphate in cultures containing D-serine, glycine, and L-serine could have been the mechanism triggering excessive DNA synthesis.

Thymidine added to pantoic acid grown cultures did not affect rates of DNA synthesis. Although there was continuing uptake of thymidine, in pantoic acid plus thymidine grown cultures, in excess of uptake in the control, the increase approximated the excess amount of DNA synthesized when only pantoic acid was present in the medium. This indicates a mechanism which is completely independent of the presence of thymidine, unlike the effect thymidine had in combination with the other compounds tested as shown in Table IX.

Instability of the DNA present in the test cultures was demonstrated by the raised amounts of isotope released by the cultures. Lysis could have been a contributing factor but not the sole cause of the increase since the amount of loss was not related to the decrease in cell number of the experimental culture (Table VIII).

There were lowered levels of RNA in all cultures except L-serine grown cells. Less RNA could have resulted from either lack of synthesis or accelerated loss.

As proposed previously, the compounds used in this study could interrupt synthesis of nucleic acid bases. Inhibition of RNA formation could have been caused by insufficient levels of the precursor bases. However, DNA synthesis, also dependent upon nucleic acid bases, was

shown to be unaffected and even enhanced under the experimental conditions employed. Thus the synthesis of purine and pyrimidine bases is not the main reason for the observed decrease in cell content of RNA.

The biosynthetic life of a cell has been divided into gap_1 (G_1), synthesis (S), gap_2 (G_2), and mitosis (M) phases. RNA is synthesized during the G_1, S, and G_2 phases while DNA is synthesized only during the S phase (Zetterberg and Killander, 1965). Thus there is no normal period when DNA synthesis proceeds in the absence of RNA synthesis. This eliminates the possibility of the test compounds having inhibited cell division at a stage in which DNA, but not RNA is being synthesized.

According to Prescott and Bender (1962) RNA synthesis ceases just prior to nucleolar disappearance and before nuclear membrane disintegration. At the time of nuclear membrane breakdown the nucleolus disappears and a large amount of nuclear RNA is released to the cytoplasm. The compounds used in this study might have disrupted cell timing mechanisms to such an extent that DNA synthesis overlapped the period of interrupted RNA synthesis. Prolonged arrest at this point does not seem feasible because after a short period of interrupted RNA synthesis, protein synthesis decreases considerably (Prescott and Bender, 1962). The reverse of this phenomenon occurred in response to the presence of all experimental compounds except D-methionine. The lack of extensive division or growth inhibition in D-methionine grown cells eliminates the above mechanism as one occurring in these cells.

Protein synthesis is dependent on the presence of functional polysomes (Ingram, 1965). Polysomes are formations of nucleoprotein particles (Ribosomal-RNA complexed with protein) connected by a messenger-RNA (m-RNA) molecule. Magnesium ions are essential for integrity of

the structure. For protein to have been synthesized in the excessive amounts observed, polyribosomes and, inherently, m-RNA and ribosomal-RNA (r-RNA) must have been present and functional. It follows then that RNA must have been synthesized in amounts sufficient to allow excessive protein synthesis. An alternate situation would be cellular production of a small amount of an abnormally formed RNA which functions at excessive or uncontrolled rates or for an unusually long time.

For the amount of RNA/cell to have been severely depressed at the time of assay some mechanism(s) must have been previously triggered by which the cellular RNA was either degraded or lost from the cells.

The swelling of D-serine, glycine, and pantoic acid grown cells and the increased loss of isotope (Table VIII) from these cells points to membrane barrier breakdown due possibly to increased internal pressures. Swelling and increased loss of isotope was also observed within cultures containing L-serine yet these cultures had an increased content of RNA. Cells did not increase in size in response to L- or D-methionine although cultures grown in media containing these compounds had a largely decreased RNA content. From this it can be concluded that RNA loss involved more complex causes than a simple physical breakdown of membrane barriers due to swelling. Other causes for membrane breakdown could be synthesis and incorporation of abnormal proteins, activation of degradative enzymes, or lack of synthesis of proper membrane precursors other than protein molecules.

The plasma membrane, which limits the cell and provides a barrier or dividing wall between the cell contents and the surrounding medium, is not the only membrane of a cell. There are also nuclear membranes and the endoplasmic reticulum within the cytoplasm. Sjöstrand (1964)

defines alpha-cytomembranes as special parts of the endoplasmic reticulum having ribosomes attached. Disruption of this membrane obviously could have affected the RNA content of cells. Evidence supporting possibility of disruption of this membrane and its associated ribosomes in at least D-serine and pantoic acid grown cultures lies in the low hematoxylin retention by the cytoplasmic contents of these cells.

Although membrane breakdown might have affected activation of lytic enzymes such as RNases and proteases, the excessive synthesis of RNA might have affected release of RNases as a mechanism to control the cellular RNA content. Non-selective activation of RNases might have destroyed not only part of the functional RNAs (messenger and soluble) but also the informational RNA of the nucleus from which de novo synthesis of most cytoplasmic RNA is thought to originate (Graham and Rake, 1963). Presence of RNases in nuclei has been shown (Villalobos, Steele, and Busch, 1965). Instability of the DNA being synthesized could also lower the ability of the cells to produce new RNA. Thus a dicotomous situation would develop in which the cells, now unable to synthesis new RNA to replace the RNA being degraded, would loose RNA to levels below that of the control cells due to the action of the released RNases. The fragmentation or other alterations in the form of cell nucleoli may then have been either the cause or the result of RNA loss.

So far as the types of RNA which might have been lost from the cells is concerned, it is quite obvious that partial amounts of any of the three major cytoplasmic RNAs might have accounted for this net loss with a minimum of impairment of cell functions. However, when one considers the relative amounts of the various types of cellular RNA; cytoplasmic RNA (mitochondria, 16%; ribosomes, 52.4-66%; and soluble, 10-16%)

constitutes the majority of the cell RNA while nuclear RNA is about 11% of whole cell RNA and m-RNA, with its high turnover rate, is the smallest fraction (Hogeboom and Schneider, 1955; Graham and Rake, 1963); it becomes obvious that the most loss with the least amount of cell impairment would necessarily have to be in the ribosomal fraction.

Although experimental results obtained from cultures of cells grown with D- or L-serine, D- or L-methionine, glycine, or pantoic acid were in many places similar, the numerous dissimilarities observed leads to a conclusion that each of these compounds affected cellular growth, metabolism, and division in a different way. For instance, L- and D-serine, glycine, and pantoic acid caused formation of cells with larger nuclei and higher DNA content. On the other hand, the ability of thymidine to stimulate an even greater excess of DNA in L-serine, D-serine, and glycine but not in pantoic acid containing cultures shows that there were at least two mechanisms operating to cause DNA increase. Similarly, the variation in morphologic expression of RNA loss in D-serine grown cells as opposed to D-methionine or pantoic acid grown cells points to differences in the means by which RNA was lost from these cells.

The overlapping effects observed point to similarities in modes of action of the various compounds. Concomitantly, the appearance of varied responses indicates multifunctional action of the test compounds. Thus no singular theory on mode of action can be derived which fits the conditions indicated by the results obtained.

CHAPTER V

SUMMARY

Cultures of monkey kidney epithelial cells grown in the presence of D- or L-serine, D- or L-methionine, glycine, or pantoic acid were found to have altered morphologic and staining qualities. Analysis for RNA, DNA, and protein content of these cultures gave results which could be related to the observed morphologic and stain retention changes. Generally speaking, there was increased DNA and protein content and decreased RNA content of the experimental cells. Studies with thymidine-2-^{14}C showed that the compounds and possibly even the thymidine itself affected the rate of synthesis and the amount of DNA synthesized. As indicated by increased DNA/cell and by greater decrease in cell numbers than could be accounted for by cell loss, D-serine, L-serine, glycine, and, possibly, pantoic acid inhibited cell division.

The possible involvement of one carbon metabolism at the micromolecular level was discussed. Mechanisms which could have lead to the observed effects in cellular growth, metabolism of macromolecules, and division were examined. It was noted that there was both unity and diversity of results obtained from cultures treated with the different compounds. This indicates that each compound had multifunctional activity with some functions being shared by other compounds and some functions being unique for that particular compound.

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VITA

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