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Observations Related To The Mechanisms Of Tetracycline- Induced Lipid Accumulation In A Strain Of Mouse Fibroblasts Cultivated In Vitro

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OBSERVATIONS RELATED TO THE MECHANISMS OF
TETRACYCLINE-INDUCED LIPID ACCUMULATION
IN A STRAIN OF MOUSE FIBROBLASTS
CULTIVATED IN VITRO

A Dissertation
Presented to
the Faculty of the Graduate School
University of the Pacific

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

by
Dale William James

June 1971

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OBSERVATIONS RELATED TO THE MECHANISMS OF
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Abstract of Dissertation

Intravenous administration of tetracycline has been known to result in fatty degeneration of the liver. This study was undertaken in order to elucidate some of the actions of tetracycline on mammalian cells in culture and to attempt to relate these findings to the clinical condition of fatty degeneration of the liver mentioned above.

Low Line cells were exposed to various concentrations of tetracycline hydrochloride dissolved in the tissue culture medium. Under the conditions of this study no cytotoxic effects as evidenced by morphological changes were observed when the cells were exposed to concentrations of less than 10 mcg/ml. In fact this concentration resulted in a stimulation in growth. Concentrations greater than 50 mcg/ml depressed growth rate. Concentrations of 10, 50, and 100 mcg/ml caused an increase in the amount of glucose utilized by the cells and a corresponding increase in the amount of lactic acid produced. These concentrations also depressed succinic dehydrogenase activity, RNA content, and DNA content of the cells. Fifty mcg/ml stimulated acid phosphatase activity. No effect was observed on cytochrome oxidase activity and no alkaline phosphatase activity was observed in the cells studied. Concentrations of 50 and 100 mcg/ml resulted in an increase in the lipid content of the cells and a decrease in the rate of both total cellular protein and serum protein synthesis.

It is suggested that lipid accumulation occurring after tetracycline administration is possible the result of a decrease in B-lipoprotein synthesis and enzyme inhibition. These factors could result from the high blood levels of tetracycline seen after large and rapid intravenous injections of the drug into normal patients or those suffering from kidney impairment.

INTRODUCTION

CHAPTER I

INTRODUCTION

Early methodology associated with tissue culture developed quite naturally from work in the area of embryology. However, prior to its acceptance as a separate and unique prospective several significant advances occurred which should be noted. The first successful attempt at maintaining explanted tissue was by Roux (1885). In 1898 Ljunggren demonstrated that human skin could survive in vitro for many days if stored in ascitic fluid. Jolly (1903) maintained amphibian leucocytes in hanging drops for up to one month. By using this method he was able to report the first observations of cell survival and cell division in vitro.

The first work with sufficient sophistication to be called pure tissue culture research must be attributed to Ross G. Harrison (1907). He was able to successfully explant pieces of frog embryo medullary tissue into clotted frog lymph which resulted in the outward growth of small fibers from the original tissue.

Although extremely important, the techniques of Harrison were still limited and it remained for others to refine them. Two notable investigators were Burrows (1912) and Carrel (1914). The contributions of these two men are

too voluminous to review at this time. We should, however, mention that scientists are particularly indebted to Carrel for the techniques which allow the continuous cultivation of rapidly growing and dividing cells over long periods of time.

Presently, the amount and quality of work done in this field continues to grow although the majority of pharmacological studies are still being conducted utilizing animals or cell-free extracts. Tissue culture, however, offers the advantages of large quantities of homogeneous cell populations free from the interference of other cells, organs, and nervous control, as well as easy manipulation of experimental conditions.

Nevertheless, great care must be exercised in the selection of possible studies, for the following limitations must also be considered if the above advantages are to be used to obtain valid results. Most cells utilized when employing tissue culture techniques are quite different than those in vivo. Their chromosome number may have been altered so that most are polyploid, and they tend to dedifferentiate, hence, losing many of their specialized functions. The previously stated advantage of the absence of interfering influences from other systems of the whole animal can also be considered a possible disadvantage. Drugs work within the configuration of the systemic

environment, thus ultimate decisions about their action must be made accordingly. The in vitro and in vivo environments are also different. Although some cell strains can be cultured in a chemically defined medium (Evans et al., 1956), most require the addition of between 5 and 25% serum which is quite different from the amount present under in vivo conditions. Another factor which may affect the dynamic state of the cells is the lack of continuous removal of wastes and constant renewal of nutrients.

After consideration of these issues, an attempt was made to devise a study for determining some of the basic pharmacological actions of the antibiotic tetracycline. These findings were then related to fatty degeneration of the liver, a toxic response which has been attributed to this drug.

It appeared appropriate to study these toxic responses in conjunction with the pharmacological action, for the increased availability of potent drugs has resulted in a concurrent increase in the number of drug-induced pathological conditions.

Almost immediately after the discovery of the tetracyclines, Lepper et al., (1951) and Skorov and Sutherland (1951) reported many serious and sometimes fatal side effects resulting from administration of this

drug. The reports of ill effects continued to appear and at first seemed to suggest a correlation between tetracycline-induced fatty liver degeneration and intravenous administration of the drug during pregnancy (Davis and Kaufman, 1966; Allen and Brown, 1966; Dowling and Lepper, 1964; Whalley et al., 1964). However, after more careful examination of the literature it would appear that pregnancy is not the independent variable, since hepatotoxicity resulting from intravenous administration of tetracycline in non-pregnant women is also well documented (Lepper et al., 1951; Bateman et al., 1952; Wruble et al., 1965; Davis and Kaufman, 1966). Nor does it appear to be confined to females. Recently, Robinson and Rywlin (1970) have reported a male patient exhibiting symptoms of tetracycline-induced fatty liver.

It has been demonstrated that liver damage does not appear to be associated with the low blood levels which are obtained after oral administration of tetracycline (Cohn and Zaslow, 1955; Orenbreich, 1965). Therefore, it would appear that an adequate hypothesis should seek to establish a relationship between high blood levels and liver damage. In pursuing this association, another factor which might provide a connection for the above citations of clinical observations must be considered. The tetracyclines are excreted primarily by the kidneys (Goodman and Gillman, 1965),

therefore a decrease in renal function would result in increased blood levels. There is, then, a possible correlation between large intravenous doses of the tetracyclines in patients with poor renal function, which subsequently may result in high blood levels and liver damage.

In contrast to the abundance of reports citing clinical effects of tetracycline toxicity there is a paucity of studies dealing with in vitro attempts to study some of the metabolic actions of this drug. The most significant to date are as follows. Lepine et al., (1950) observed the presence of numerous vacuoles and fatty degeneration in epithelial and fibroblast cells derived from chick embryo when exposed to a concentration of 100 mcg/ml of chlortetracycline. DuBuy and Showacre (1961), utilizing the fluorescent property of the tetracyclines, demonstrated that the drug became localized in the mitochondria of monkey kidney cells after a dosage of 20 mcg/ml. Journey and Goldstein (1963) made ultrastructural observations of HeLa cells exposed to various concentrations of oxy-tetracycline and reported distortion of mitochondria in cells exposed to a concentration of 100 mcg/ml. Finally, Zuckerman et al, (1967) observed accumulation of lipid droplets in primary human embryo liver cells in culture when exposed to a concentration of 1000 mcg/ml of tetra-

cycline. The present study will attempt to add to this small body of literature dealing with metabolic actions of the drug in vitro.

It would appear that tissue culture techniques would be particularly useful for the study of lipid metabolism for as Mackenzie et al., (1964) have observed:

When analysis has shown that the amount of lipid in an organ or tissue is increased, staining reactions can only suggest, but cannot prove, that the increase has occurred primarily in specific cell types in the heterogeneous cell population. ...it would appear, therefore, that homogeneous populations of cultured cells offer distinct advantages in the investigation and evaluation of the intracellular (genetic) and extracellular (environmental) factors that regulate the lipid content of cells.

The appearance of lipid-staining material in cultured cells has been associated with aging of cultures, crowding, cessation of growth, toxic substances and degeneration (Bailey et al., 1959; King et al., 1959). Healthy cells do not normally exhibit distinct droplets of lipid and although lipid material can be extracted from them, it is not stained by the Sudan dyes. Originally a controversy existed as to whether the droplets arising in unhealthy cells were merely invisible lipids made visible by some of the previously stated conditions or an accumulation of lipid subsequent to the damage. It has now been documented that both factors are involved (Dixon, 1958). The invisible state of cellular lipids can be explained in terms of colloid chemistry since

lipid material is not soluble in the aqueous environment of the cell. It is held in a finely dispersed state by emulsifying agents containing a polar group on one end of the molecule. The phospholipids belong to this class of compounds. The lamellar micelle is an arrangement of these molecules oriented in parallel plates with non-polar ends internal, thus separated from the aqueous phase. Their polar ends are external and in contact with water¹. These micelles have the capacity to incorporate water-insoluble lipids in between the two layers and in this way form a stable emulsion. This can be termed micellar fat which is distinct from the observable lipid droplets or globular fat. Dixon (1958) has suggested that an equilibrium exists between these two forms. If there is an adequate amount of phospholipid and protein present to act as emulsifying agents the equilibrium favors the micellar fat. However, if conditions result in a decrease in emulsifying agents or an increase in lipid beyond the capacity of the available emulsifying agents, or a combination of both, the equilibrium will shift toward the globular fat.

Although the increase in lipids observed in tetracycline-induced fatty liver have been shown to be mainly composed of triglycerides (Lewis et al., 1967), the

1 See Dixon (1958) or Alexander and Johnson (1951) for further explanation.

mechanism(s) responsible for this accumulation remain unknown. There are several possible mechanisms which could affect the above mentioned equilibrium between micellar and globular fat and result in visible lipid droplets:

1. Increase in fatty acid synthesis
2. Decrease in oxidation of fatty acids
3. Increase in mobilization of peripheral adipose depots
4. Decrease in secretion of triglycerides from the cells
5. Increase in formation of triglycerides from fatty acids
6. Decrease in phospholipid synthesis

The aim of this project was to study many of the effects of tetracycline on growth and metabolism of cultured cells and to attempt to correlate these observations with some or all of the suggestions for the possible causes of tetracycline-induced fatty liver listed above. In this way, an attempt was made to define the mechanism of tetracycline-induced fatty liver and also demonstrate the feasibility of using tissue culture as a tool for studying certain pharmacological actions of drugs.

MATERIALS AND METHODS

CHAPTER II

MATERIALS AND METHODS

Origin and Nature of the Cell Strains Used

Low Line cells, classified as NCTC clone #2555, were utilized for these studies. They, along with the High Line (NCTC clone #2742), were isolated from a 97 day-old parent culture of normal subcutaneous adipose connective tissue obtained from a C3H/He mouse (Likely et al., 1952). Before cloning, these cells were non-malignant, however after the 12th and 19th transplants the High and Low Line produces tumors in normal C3H/He mice. After $1\frac{1}{2}$ years, the High Line cells produced 63% sarcomas and the Low Line, none. Three years later, the numbers had increased to 97% and 1%, respectively (Sanford et al., 1954).

In one phase of this investigation the NCTC #2071 cell strain was employed. This strain was derived from Strain #929 established by Evans et al., (1956), but differs from it in that it is cultured in chemically defined medium. The original Strain L was established in 1940 by Earle and was one of the first cell strains to be grown in continuous culture. The parent Strain L was derived from normal subcutaneous areolar and adipose tissue of a 100 day-old male C3H/An mouse.

Cultivation of the Cells

Stock cultures of the cells were cultivated in Pyrex glass T-60 Flasks (Earle et al., 1951) and maintained at 37°C in a Sherer environmental chamber. Experimental studies were conducted using either T-15 or T-60 flasks. Cytochemical observations were made of cells grown on glass cover slips in Leighton tubes. Silicone stoppers were employed for all flasks in as much as they contain a minimum of toxic substances which could dissolve in the medium.

All glassware was rinsed with tap water and placed in a detergent solution for at least 30 minutes. It was then removed, rinsed with tap water and placed in chromic acid for several hours. Next it was rinsed with tap water 15 times and then with triple distilled water 5 times. Drying was accomplished by means of a drying oven at 70°C with subsequent wrapping for sterilization. Cover slips for use in Leighton tubes were subjected to 30 minutes ultrasonic cleaning, rinsed in tap water, dried at 70°C and placed in the Leighton tubes before sterilization. All sterilization was carried out in an American Sterilizer autoclave at 121°C for 20 minutes and dried under vacuum for 15 minutes.

The Low Line cells were cultured in a synthetic medium, NCTC #109¹ (Evans et al., 1956) supplemented with

1 Grand Island Biological Company, Berkeley, California

10% new-born calf serum². This medium was originally developed for long-term cultivation of Strain L cells, but it has also proven to be effective in the cultivation of other cell strains when supplemented with serum. After the addition of the serum, the medium was sterilized by filtration through a sterile Millipore filter (4-8 pounds pressure with compressed air containing 5% CO₂) with an average pore size of 0.22 micron into sterile 500 ml bottles for storage. The sterile medium was stored at 4°C until ready for use at which time it was warmed to room temperature and refiltered through a sterile 0.22 micron Millipore filter.

NCTC #2071 cells were maintained in a serum-free chemically defined medium designated NCTC #135 (Evans et al., 1964). This medium was obtained as a 2X concentrate³. Prior to its use the concentrate was diluted with tripple distilled water and sterilized by filtration as described above.

All cell transfer procedures were carried out under a Lab Con Co Tissue Culture Hood, which had been wiped clean with 70% ethanol and sterilized with ultraviolet irradiation for 20 minutes prior to use.

2 Hyland Laboratories, Inc., Los Angeles, California

3 Grand Island Biological Company, Berkely, California

Replicate Culture Techniques

The replicate techniques used during this study were similar to those used by Pace and Aftonomos (1957). The cells were removed from the stock T-60 flasks by scraping with a nichrome wire and gently shaken to disperse the individual cells. The cell suspension was then drawn into the replicator and the cell density adjusted to the desired level by dilution with medium. A cell density of 1 to 1.5×10^5 cells per ml was normally used. Two ml aliquots of the suspension were used for the T-15 flasks, 12 ml for the T-60 flasks, and $1\frac{1}{2}$ ml for the Leighton tubes. The flasks were then gassed with a mixture of 95% air and 5% CO₂, stoppered and placed in the incubator.

Forty-eight hours after replication, the medium was removed by suction and fresh medium and gas mixture added. This was repeated at 48 hour intervals throughout the duration of the experiment.

Measurement of Growth

For this study, cell numbers were obtained by means of a Coulter Counter Model B. The cells were prepared for counting by suspending in a counting solution composed of Darvan⁴, and EDTA in balanced salt solution (BSS). The cells

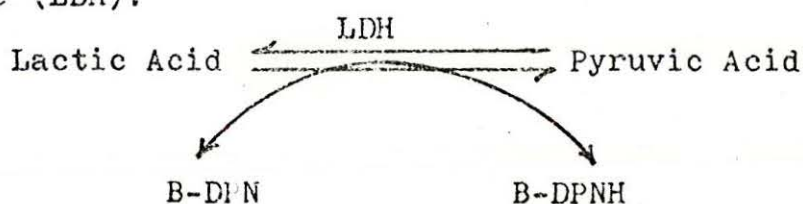
⁴ R. T. Vanderbilt, New York, New York.

from one T-15 flask were diluted to 25 ml with counting solution. This suspension was then drawn through the 100 micron aperture of the counter. As each cell passed through the aperture it displaced electrolyte, momentarily causing a reduction in current. This produced a voltage pulse which was amplified, scaled, and counted. Coincidence losses were corrected by a coincidence correction of plus 3%. In all cases counts were made of two and sometimes three flasks.

Chemical Analysis of the Medium

Glucose concentration of the medium was determined 24 hours after the addition of fresh medium to the flasks by the method of Hyvarinen and Nikkili (1962). This method also determines galactose and mannose; however, since these sugars are not present in the medium they are of little consequence. The results obtained were highly reproducible and checked to within 0 to 4 mg % of the enzymatic method of glucose analysis (Keston, 1956).

Lactic acid was ascertained by the method of Scholz et al., (1959) and involved measurement of the amount of DPN which was converted to DPNH in the presence of excess lactic dehydrogenase (LDH):



For this purpose a Coleman Hitachi 101 Spectrophotometer at 340 mμ was used.

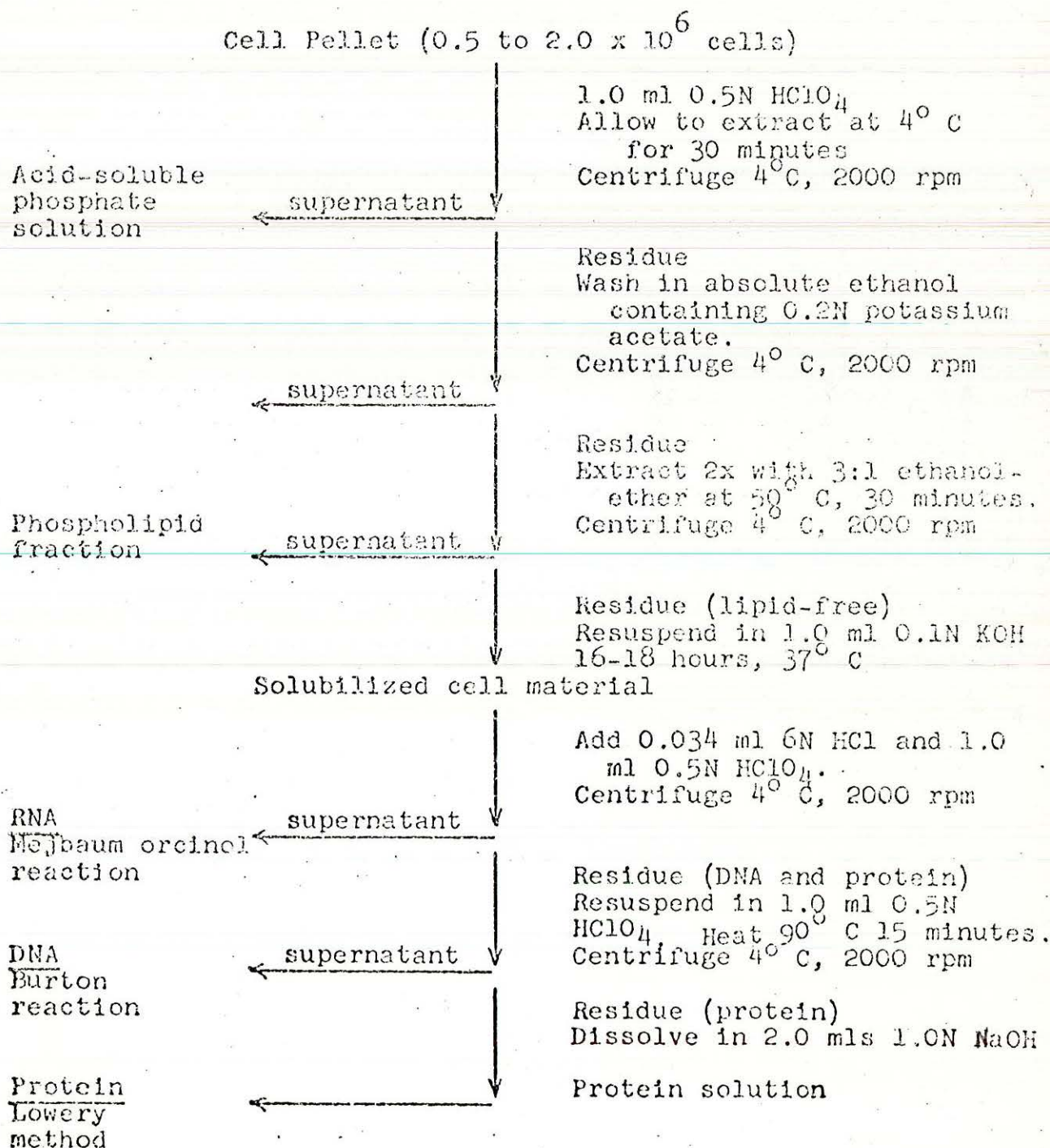
Chemical Analysis of the Cells

Test for total protein, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) were made 24 hours after the addition of fresh medium. At this time the culture medium was replaced with warm calcium and magnesium-free balanced salt solution (CMF-BSS) containing 0.25% trypsin. After the cells became detached the suspension was centrifuged at 2000 RPM for 15 minutes at 4°C in a Sorvall refrigerated centrifuge (Model RC-2). The cell pellet was then extracted according to the method described by Merchant et al., (1964). The total protein content of the cells was ascertained by means of the Folin-Ciocalteu phenol reagent (Lowry et al., 1951). This method is based on the color reaction of the aromatic amino acids tyrosine and tryptophan with the Folin-Ciocalteu reagent. DNA content was obtained by means of the method described by Burton (1956) which is based on the reaction of diphenylamine with deoxyribose. RNA was estimated by the orcinol reaction (Mejbaum, 1939), which measures the pentose content. The above procedures are summarized in Table I.

The colorimetric determinations of acid and alkaline phosphatase activity required a greater quantity of cells to get within the sensitivity of the procedures employed. For

TABLE I

FLOW SHEET FOR THE SEPARATION OF RNA, DNA, AND PROTEIN
IN MAMMALIAN CELLS CULTURED IN VITRO



this reason T-60 flasks were used for culturing. Twenty-four hours after the addition of fresh medium the cells were treated with 0.25% trypsin in CMF-BSS which caused them to detach from the floor of the flask. The cell suspension was then centrifuged at 2000 RPM for 15 minutes. The cell pellet was washed 3 times with BSS and recentrifuged after each washing. The washed pellet was then extracted with 2 ml of an extraction medium prepared according to Shonk and Boxer (1964). The cell pellet was suspended in this solution and then homogenized for 2 minutes in a motor driven, previously chilled 10 ml Potter-Elvehjem homogenizer. The homogenate was then divided into 3 portions: 1 for alkaline phosphatase, 1 for acid phosphatase, and 1 for protein determination. The phosphatase activity was expressed as "phosphatase activity per mg of total protein". The phosphatases were determined by the method of Bessie et al., (1946) which depends on the enzymatic hydrolysis of p-nitrophenylphosphate. Appropriate aliquots of the cell homogenate were incubated with buffered substrate for 30 minutes at 37°C. At the end of the incubation period the reaction was terminated by the addition of alkali and the p-nitrophenyl measured colorimetrically at 410 mμ. Tests for acid phosphatase activity were made in 0.05 M citrate buffer at pH 4.8 and those for alkaline phosphatase activity in 0.05 M glycine buffer at pH 10.5.

Cytochemical Procedures

At the designated time the cover slips were removed from Leighton tubes and the cells stained for neutral fats, succinic dehydrogenase, and cytochrome oxidase.

The presence of neutral fats was indicated by the pink to bright red droplets which became obvious after fixing in formalin and staining with Oil Red-O.

Succinic dehydrogenase activity was ascertained using the method of Seligman and Rutenburg (1951). The cover slips on which the cells were attached were incubated in a mixture of equal parts of 0.1 M phosphate buffer (pH 7.6), 0.2 M sodium succinate, and ditetrazolium chloride (1.0 mg/ml). After a 2 hour incubation period the cover slips were rinsed in BSS and mounted in glycerogel. Succinic dehydrogenase causes the pale ditetrazolium to be reduced to either a blue, water-insoluble pigment (diformazan), or a reddish-purple, water-insoluble pigment (monoformazan) depending upon the enzyme activity.

For determination of cytochrome oxidase activity the cover slips were incubated in a solution of p-aminodiphenylamine plus tetrahydro quinoline in tris buffer (pH 7.4) for 15 minutes or longer. These were then fixed in 10% cobaltous acetate in 10% formalin for 1 hour, washed in water, and mounted in glycerogel. Cytochrome oxidase activity was evidenced as a blue color forming at the site of enzyme

activity (Burstone, 1961).

Lipid content, succinic dehydrogenase activity, and cytochrome oxidase activity were determined 4 hours after exposure of the cells to various concentrations of tetracycline. The recovery from the effects of tetracycline was also studied by treating the cells as described above, removing the treated medium, rinsing the flasks 3 times with BSS and adding fresh medium. Cytochemical observations were then made on the cells 24 hours after the addition of the fresh medium.

The effect of adenosine triphosphate (ATP) on lipid content, succinic dehydrogenase activity, and cytochrome oxidase activity was also ascertained. ATP was added to the control and tetracycline medium and the cells were incubated for 4 hours. The cover slips were removed and the tests performed as described earlier.

Representative samples were photographed using a 35-mm Zeiss Ikon camera attachment on a Standard Zeiss GFL microscope. High speed Ektachrome film (Daylight) and the appropriate color conversion filters were used. Black and white photomicrographs were made using Addox KB-14 film processed in Neofin developer.

Measurement of Protein Synthesis

The rate at which carbon-14 labeled L-leucine⁵ was

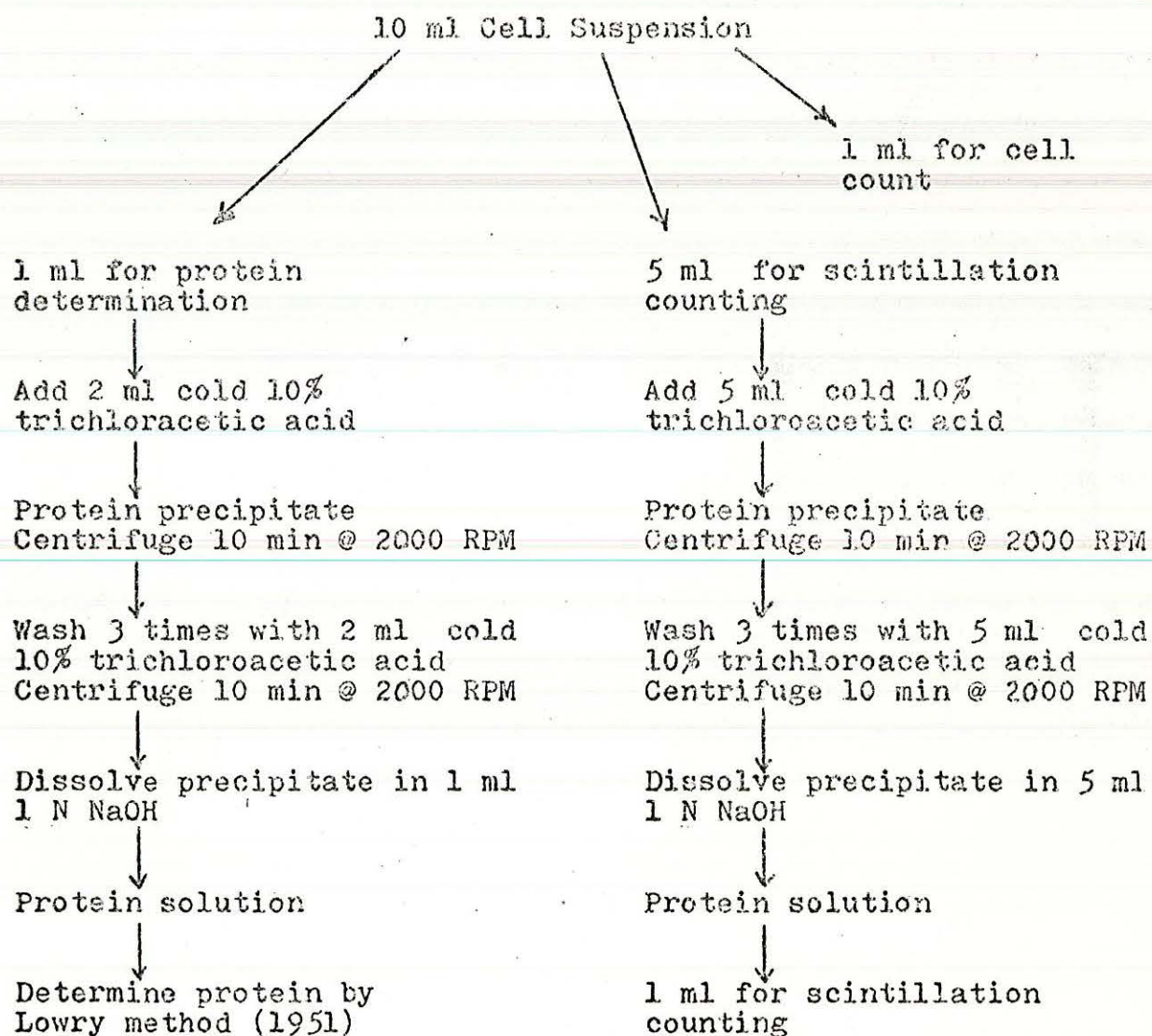
incorporated into cellular protein was used as an indication of the rate of protein synthesis. Leucine is required in the medium of cultured mammalian cells (Levintow and Eagle, 1961) and is thus actively incorporated into cellular protein.

For the purpose of this investigation the tetracycline was added 24 hours prior to the addition of the labeled amino acid or, in a separate experiment, at the same time as the radioactive tracer. In all instances the labeled medium, both control and that containing the tetracycline, contained 0.01 uCi/ml of carbon-14 leucine. This level of radiation provided sufficient incorporation of labeled amino acid to allow scintillation counting of 1% accuracy within a reasonable amount of time. The cultures were then incubated at 37°C for 2 hours. After the incubation period the medium was removed and the flasks rinsed gently three times with BSS in order to remove as much of the radioactive medium as possible. Ten ml of a balanced salt solution containing Darvan and EDTA was added to each flask to detach the cells. The treatment of the resulting cell suspension is summarized in Table II. One ml of the suspension was analyzed for total cellular protein by the method of Lowry (1951). One ml was used to ascertain the number of cells per ml and 5 ml were analyzed for the incorporation of radioactivity

5 L-Leucine-UL-C-14 was obtained from International Chemical and Nuclear Corporation, City of Industry, California. The specific activity was 240 mc/mM.

TABLE II

PROCEDURE FOLLOWED IN TREATING THE CELL SUSPENSION
AFTER INCUBATION WITH RADIOACTIVE LEUCINE



into cellular protein. Analysis of the trichloroacetic acid used to wash the protein precipitate demonstrated that by the third wash the radiation level had returned to that of background, indicating that the precipitate was washed free of residual carbon-14 leucine.

The amount of carbon-14 leucine which had been incorporated into cellular protein was determined by standard liquid scintillation methods. The scintillation cocktail used for this study was first used by Newman (1968). The formulation is as follows:

Solvent	Toluene ⁶
Primary Fluor	PP0 ⁷ (8 gm/l)
Secondary Fluor	POPOP ⁷ (50 mg/l)

One ml of the protein solution was solubilized in the cocktail by first mixing it with $2\frac{1}{2}$ ml of Bio-Solv⁶ and then adding 10 ml of cocktail. The samples were then shaken vigorously to clear the solution and counted at the 1% error level in a Beckman CPM-100 liquid scintillation counter.

6. Scintillation Grade, Beckman Instrument Company, Fullerton, California

7. Scintillation Grade, Packard Instrument Company, La Grange, Illinois

Isolation of Serum Proteins

For the purpose of this study serum proteins will be defined as those proteins synthesized by the cells and released into the medium. Replicate cultures of Low Line cells were established in T-60 flasks at a cell density of approximately 1.75×10^5 cells per ml. Four days after replication, 12 ml of fresh culture medium containing tetracycline at 0 and 100 mcg/ml was added to the flasks which were then allowed to incubate at 37°C for 12 hours. Eight hours after the addition of the drug, a quantity of carbon- 14 labeled leucine was added to each flask to give a final concentration of 0.5 uCi/ml. Four hours later the medium was removed and centrifuged at 2000 RPM for 15 minutes at 0°C to remove any cell debris which might be present. The medium was then fractionated by ultracentrifugation according to a modification of the procedure as specified by Radding and Steinberg (1960). Ten ml samples of each were removed for analysis. The density of these samples was adjusted to 1.063 by the addition of a solution of NaCl-KBr ($d=1.35$)⁸. The samples were then placed in cellulose nitrate centrifuge tubes, capped and filled with a solution of $d=1.063$ ⁹. They were then centrifuged at 41,000 RPM for 22 hours in a No. 65 centrifuge head in a Beckman

8 Solution of $d=1.35$ was prepared by dissolving 153 gm NaCl and 354 gm KBr in 1 liter of water.

9 Solution of $d=1.063$ was prepared by mixing 10 ml of a solution of $d=1.006$ (9 gm NaCl dissolved in 1 liter) and 2 ml of solution $d=1.35$.

Ultracentrifuge Model L2-65B at 12°C. After the centrifugation all material down to 2.1 cm below the bottom of the tube cap was removed and placed in 15 ml Pyrex glass centrifuge tubes. The bottom 2 cm sample was also removed and placed in centrifuge tubes. The serum proteins in both fractions were precipitated by the addition of ethanol and trichloroacetic acid to a final concentration of 70% v/v and 5% w/v respectively. The mixtures were then shaken and allowed to stand at 0°C for 24 hours. After this time the samples were centrifuged at 2000 RPM for 10 minutes at 0°C. The precipitate was then washed with 70% ethanol containing 5% trichloroacetic acid until the radiation level of the wash solutions approximated the normal background level. The precipitates were then washed twice in hot (50°C) acetone and ethanol (1:1). After the lipids were removed the remaining protein precipitate was solubilized in 5 ml 1 N NaOH and 1 ml samples were prepared for liquid scintillation counting as described earlier. One ml samples were also removed for determination of total protein. Robinson and Harris (1961) reported the results of two experiments which were carried out to determine whether carbon-14 leucine that was not incorporated into protein would be removed from the protein precipitates. In both instances they found virtually no incorporation. In order to verify this, a 12 ml sample of medium containing 0.5 uCi/ml of carbon-14 leucine was used as a control. Serum proteins isolated from this sample contained no detectable radiation.

Lipid Extraction

For the purpose of this investigation, replicate cultures were established in T-60 flasks. The cells were incubated for 4 hours in a culture medium containing either 0 or 100 mcg/ml of tetracycline. Both the control and the tetracycline-containing medium also contained carbon-14 labeled sodium pyruvate¹⁰ at a concentration of 0.025 uCi/ml. At the end of the designated incubation period, the medium was removed and the flask gently rinsed 2 times with BSS. Ten ml of BSS containing Darvan and EDTA were added to each flask. One ml of the resulting cell suspension was removed for determination of cell count after which the cell densities were adjusted by dilution in order to obtain the same number of cells in each flask. Eight ml of cell suspension was then removed and centrifuged for 10 minutes at 2000 RPM. The cell pellet was extracted for 15 minutes with two 5 ml portions of hot (50°C) chloroform and methanol (2:1). This mixture of a polar and non-polar organic solvent removes not only the free lipids but also those bound to protein (Scott, 1969). The total lipid extract (10 ml) was washed with 2 ml of 0.05 N NaCl according to the procedure of

¹⁰ Sodium pyruvate-2-C¹⁴ was obtained from New England Nuclear, Boston, Mass. The specific activity was 4.04 mc/mM.

Folch et al. (1957). The combined extracts were mixed and allowed to separate into two phases. After removing and discarding the upper phase, the interface was carefully rinsed three times with 1 ml portions of "pure phase upper"¹¹. After rinsing the interface, the lower phase and washed interface were made into one phase by the addition of methanol. This washing procedure removes essentially all the non-lipid contaminants from the extract with a concomitant loss of relatively few lipids. In order to verify this, 0.5 ml of the labeled medium was added to 10 mls of chloroform and methanol (2:1) and subjected to the washing procedure as described above. When prepared for liquid scintillation counting, this sample averaged no more than 4 counts per minute above the background level. After the extracts had been washed, they were evaporated to dryness under nitrogen and redissolved in 1 ml of petroleum ether. One-half ml samples were then added to 10 mls of scintillation cocktail and the uptake of carbon-14 pyruvate into total cellular lipid was determined.

¹¹ Pure phase upper was previously prepared by mixing chloroform, methanol and an aqueous solution of 0.58% NaCl in a separatory funnel in the proportions 8:4:3 by volume. When the mixture is allowed to stand, a biphasic system is obtained. The upper phase, termed pure phase upper, was saved.

Time-Lapse Cinematography

Cells utilized for time-lapse studies were grown in Carrel flasks. These flasks have a sufficiently thin bottom to allow good photomicrography and also allow the medium to be changed while minimizing the danger of contamination.

A flask containing the cells to be photographed was secured on the stage of an inverted Model M Nikon microscope. The medium could then be changed and the flasks gassed as required.

Motion pictures were made using a Bolex 16 mm camera mounted on a Sage Model 500 Cinematographic apparatus. The temperature of the flasks was maintained at approximately 37°C using a Sage Air Curtain Incubator. Kodak Plus-X Reversal film (7276) exposed at 1 frame per 45 seconds was used for these studies. Control cultures and those cultures exposed to tetracycline were photographed for approximately 48 hours.

Nature of the Chemical Agent

The antibiotics have become so widely and successfully used in the practice of medicine that they are now considered to be essential. Among the antibiotics, the tetracycline group has achieved an enviable reputation for dependable medication. In fact it has been estimated that the world production of the tetracyclines exceeds 1,000 tons per year

(Schindler, 1965).

Most of the tetracyclines are obtained from *Streptomyces* species by fermentation procedures. Of the available forms, four are most frequently used. Chlortetracycline was isolated from *Streptomyces aureofaciens* by Duggar (1948). This was followed by the isolation of oxytetracycline from *Streptomyces rimosus* by Finlay (1950). Tetracycline was obtained by a process of catalytic hydrogenolysis of chlortetracycline developed by Conover (1955). Demethylchlortetracycline was isolated by McCormick (1957) from a mutant strain of *Streptomyces aureofaciens*.

The tetracycline antibiotics are known as broad-spectrum antibiotics because they are effective against a variety of gram-positive and gram-negative microorganisms, many of which are also susceptible to other antibiotics. They are also effective against rickettsia and the large viruses of lymphogranuloma, psittocosis, and viral pneumonia. In addition they are active against the intestinal forms of *Entamoeba histolytica* (Sellers and Marine, 1965).

Their mode of action has been reviewed by Gale (1963) and Jackson (1964) who suggest that they act primarily by inhibition of protein synthesis, specifically the transfer reaction (to be reviewed in more detail in the discussion).

The tetracyclines, not unlike many other organic compounds (Udenfriend, 1962), are capable of exhibiting

fluorescence when excited by ultraviolet irradiation (Saltzman, 1950). Bottiger (1955), the first to report a practical use of this phenomenon, studied the distribution of chlortetracycline in the various tissues and organs of white mice. The retention of the tetracyclines by tumor tissue was reported by Rall et al., in 1957. The properties of retention by tumor tissue and fluorescence were thought to be an ideal method of cancer detection. However, it was later shown that tumor retention and fluorescence were not only quite species specific but also dependant on such factors as type of tumor, temperature, pH, and dose¹³.

The chemical structures of these compounds are shown in Figure 1. All are amphoteric crystalline compounds which have a common octahydronaphthacene ring structure (White, 1966).

For these investigations tetracycline hydrochloride¹⁴, the most widely used of the tetracycline group, was employed. Hereafter it will be referred to as tetracycline.

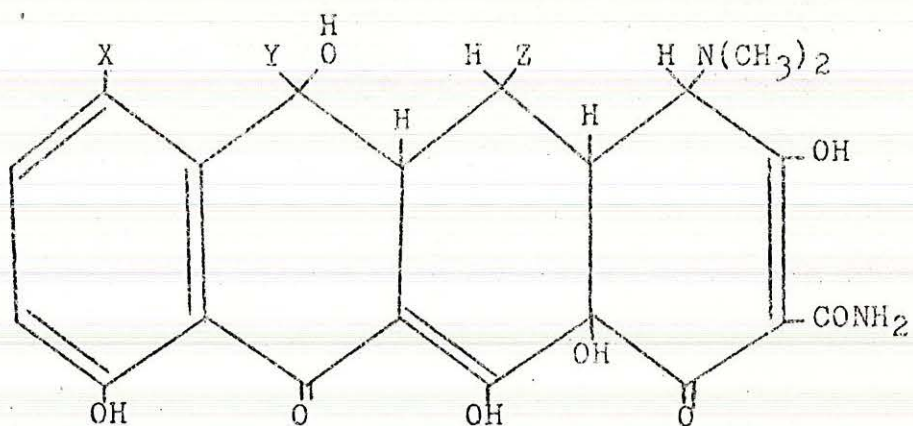
Just prior to use, tetracycline was dissolved in the medium in the highest concentration required. This solution was then sterilized as described and dilutions were made with

¹³ See James, 1969 for further review.

¹⁴ Provided by Charles Pfizer and Company, Inc., Brooklyn, New York.

Figure 1.

Chemical structures of four commonly used tetracycline homologues.



Tetracycline: $X, Z = H; Y = CH_3$

Chlortetracycline: $X = Cl; Y = CH_3; Z = H$

Oxytetracycline: $X = H; Y = CH_3; Z = OH$

Demethylchlortetracycline: $X = Cl; Y, Z = H$

sterile medium to obtain the desired lower concentrations.

A concentration of 100 mcg/ml resulted in no change of the pH of the culture medium.

RESULTS

CHAPTER III

RESULTSCytotoxicity of Tetracycline

The initial phase of this investigation was concerned with determining the cytotoxic level of tetracycline in Low Line cells. These preliminary observations were based on gross morphological changes only. Low Line cells were cultured in T-15 flasks and exposed to 0, 1, 5, 10, 50, 100, and 250 mcg/ml of tetracycline. Those cells exposed to 0, 1, 5, and 10 mcg/ml evidenced no changes in morphology. Seventy-two hours after the addition of the drug the cells exposed to 50 mcg/ml appeared more granular and contained several vacuoles which were later determined to be composed of neutral lipids. Seven days after the initial treatment these cells were very granular and many were beginning to round up. In medium containing 100 mcg/ml of tetracycline, increased vacuolization and the appearance of numerous granules occurred within 48 hours. A large percentage of the cells were becoming spherical in shape and by 72 hours, many of the cells were detached from the flask. A concentration of 250 mcg/ml resulted in toxic effects within 24 hours with no viable cells remaining after 36 hours exposure. Photographs of cells exposed to the above concentrations of tetracycline for 72 hours are shown in Figure 2.

Figure 2

Typical examples of Low Line cells exposed to various concentrations of tetracycline for 72 hours.

A. Control

B. 1 mcg/ml

C. 5 mcg/ml

D. 10 mcg/ml

E. 50 mcg/ml

F. 100 mcg/ml

G. 250 mcg/ml (24 hours)

A.



B.



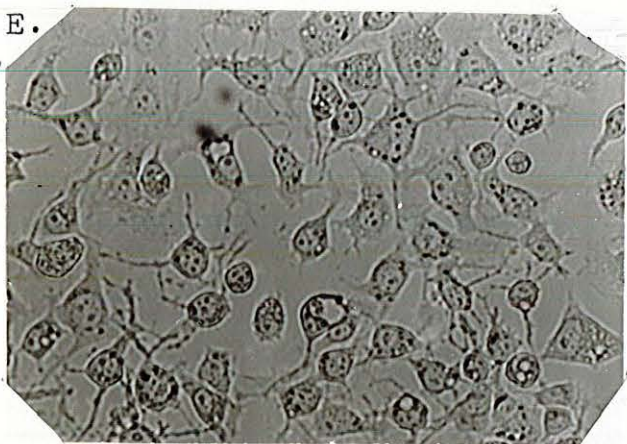
C.



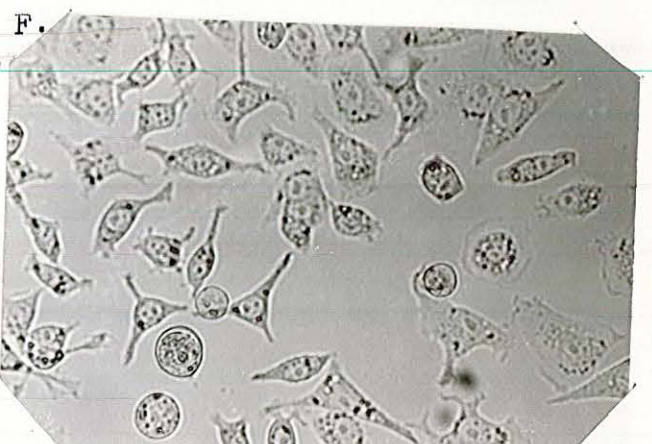
D.



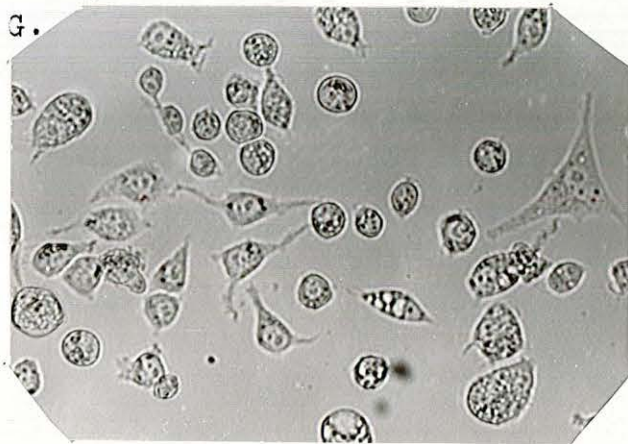
E.



F.



G.



Time-lapse photographs were taken in order to study the development of the morphological changes observed above. These photographs were made of the cells in the control cultures and those exposed to 100 mcg/ml of tetracycline. The most significant observation was the development of numerous vacuoles as a result of an increase in pinocytotic activity. Photomicrographs taken from widely spaced frames of the film are shown in Figure 3.

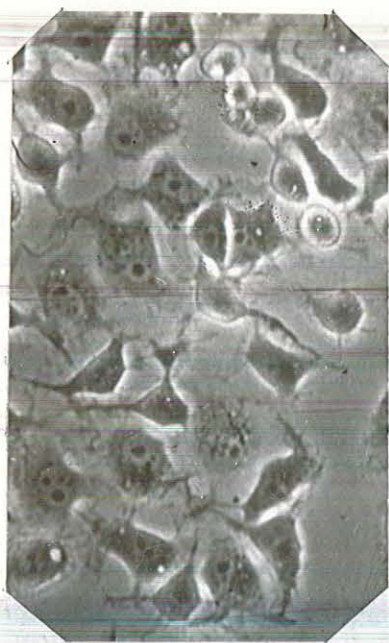
Growth

Low Line cells in T-15 flasks were exposed to tetracycline concentrations of 0, 10, 50, and 100 mcg/ml for 9 days. Samples were counted every 2 days as described earlier. For the first 3 days cell proliferation in control and treated cultures remained approximately the same. However, by the 5th day those cells exposed to a concentration of 100 mcg/ml demonstrated a marked decrease in growth rate and after day 7 an actual decline in cell number indicating possible sloughing off of injured or dead cells. Ten and 50 mcg/ml actually seemed to stimulate growth through day 7 after which those cells exposed to 50 mcg/ml also began to detach. The results of these studies indicate that a concentration of 10 mcg/ml had no deleterious affect on growth rate and actually stimulated it slightly. The results of 20 consecutive runs are compiled in Figure 4.

Figure 3

Photomicrographs taken from four widely spaced frames of a 16 mm film used in a time-lapse study of the development of the cytotoxic effects in Low Line cells exposed to a concentration of 100 mcg/ml of tetracycline.

1.



2.



3.



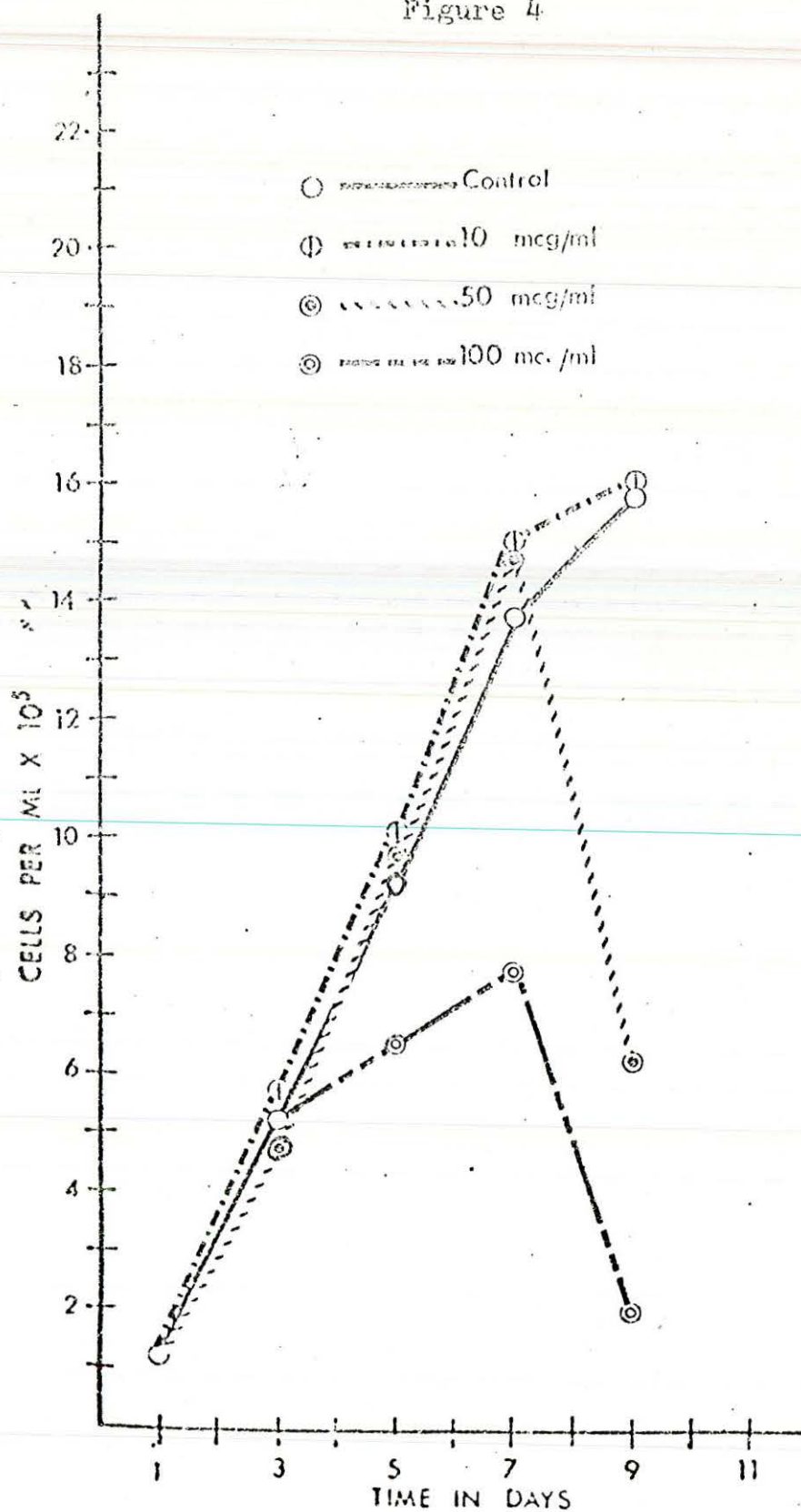
4.



Figure 4

Average of two experiments demonstrating the effect of various concentrations of tetracycline on growth of Low Line Cells. (See Appendix I.).

Figure 4



Glucose Utilization and Lactic Acid Production

The results of the effects of various concentrations of tetracycline on glucose utilization and lactic acid production by Low Line cells are compiled in Figures 5 and 6, respectively. It is apparent from these results that all concentrations of tetracycline employed caused an increase in the amount of glucose utilized per cell and a corresponding increase in the amount of lactic acid produced. Three days following the initial treatment, the cultures exposed to 100 mcg/ml showed a sharp increase in both glucose utilization and lactic acid production. On the 5th day a noticeable decrease in both occurred, followed by a steady increase. Identical results were produced by a concentration of 50 mcg/ml, however, to a lesser degree. Exposure to 10 mcg/ml evidenced a slight increase in glucose utilization and then a steady decline similar to that observed in the control cultures, yet remaining slightly higher throughout the study. The lactic acid production of those cells exposed to 10 mcg/ml followed a pattern similar to that of glucose utilization, although the amount of lactic acid produced seemed to be higher than the amount of glucose utilized.

Acid and Alkaline Phosphatase Activity

Acid phosphatase activity, expressed as Sigma units

Figure 5

Average of three experiments showing the effects of various concentrations of tetracycline on glucose utilization by Low Line cells. (See Appendix I.).

Figure 5

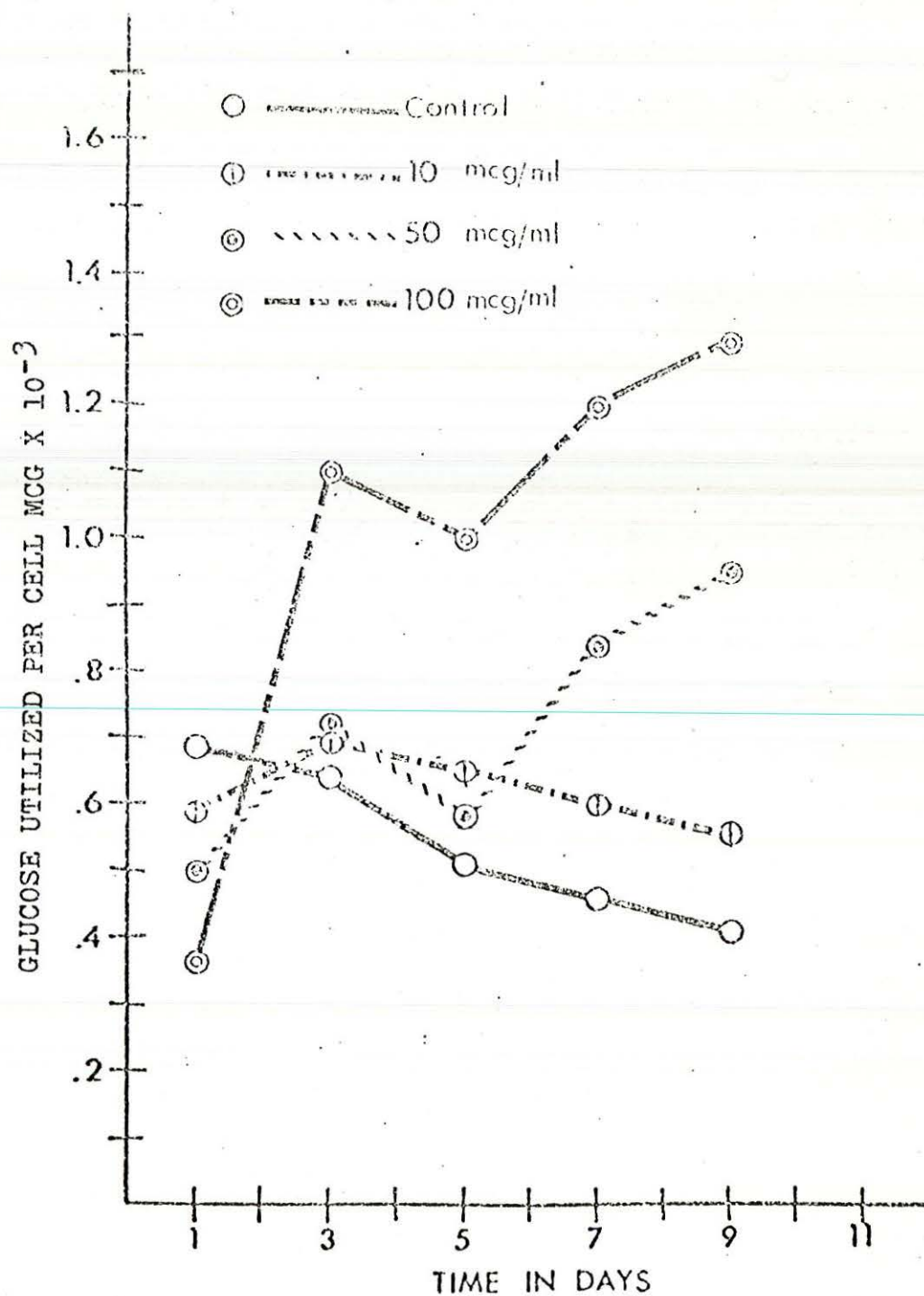
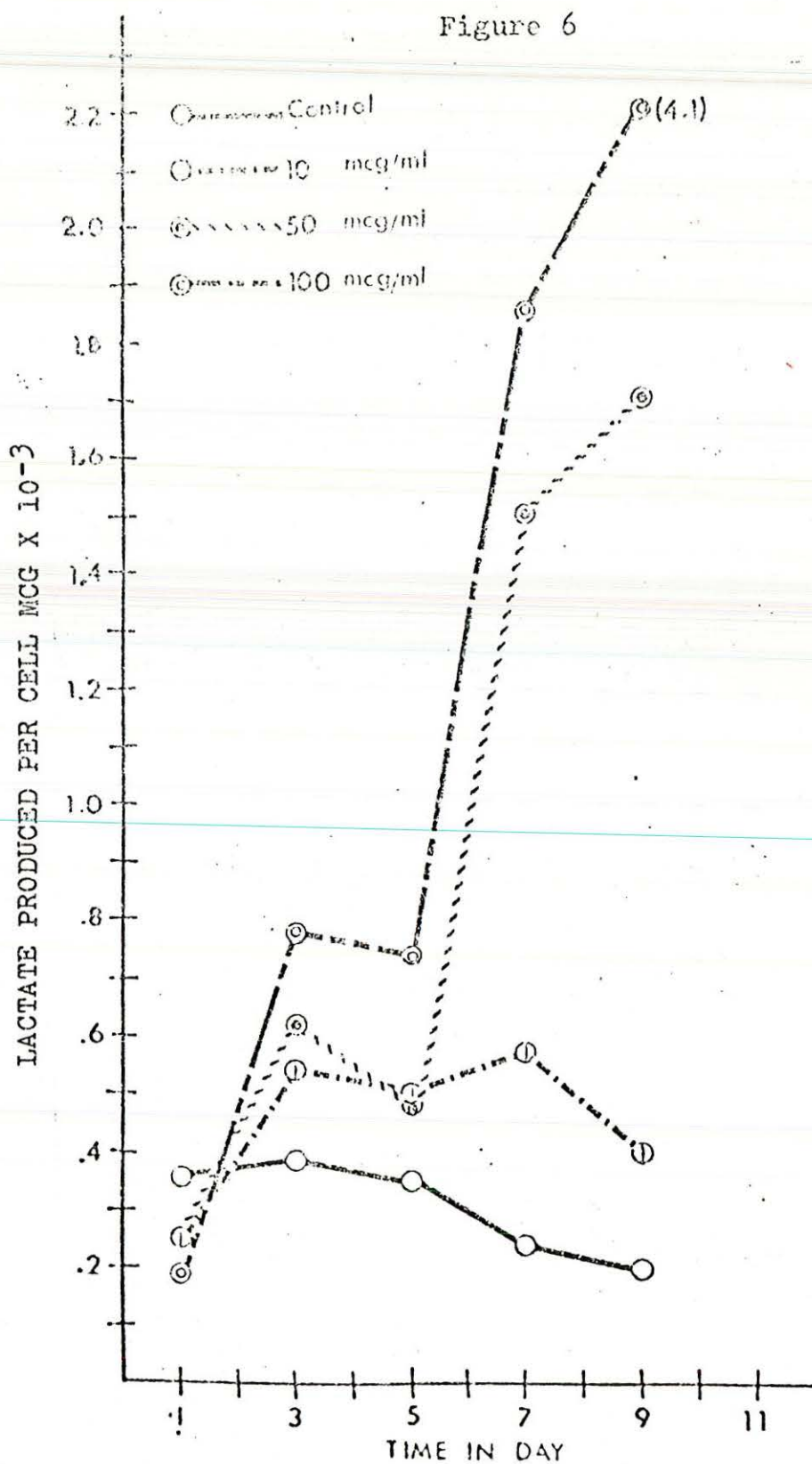


Figure 6

Average of three experiments showing the effects of various concentrations of tetracycline on lactic acid production by Low Line cells. (See Appendix II.).

Figure 6



per mcg of protein, increased sharply in both the control and tetracycline treated cultures until the 3rd day. At this time the activity in the cells of the control cultures increased less sharply and by the 5th day began to decrease gradually and continued to do so throughout the duration of the experiment. The activities in the cells of the cultures exposed to 10 and 50 mcg/ml are approximately similar until the 4th day at which time the activities in cells exposed to 50 mcg/ml began a sharp increase. The activity of those cells exposed to 10 mcg/ml closely parallels that of the control cultures and it is questionable whether the slight decreases and increases above and below the control cultures after the 5th day are notable. These results are presented in Figure 7.

Repeated attempts were made to ascertain the presence of alkaline phosphatase by both cytochemical and colorimetric analyses on the culture medium, cell homogenates, and the cells themselves. At no time was any alkaline phosphatase activity observed. The significance of these findings will be considered in the discussion.

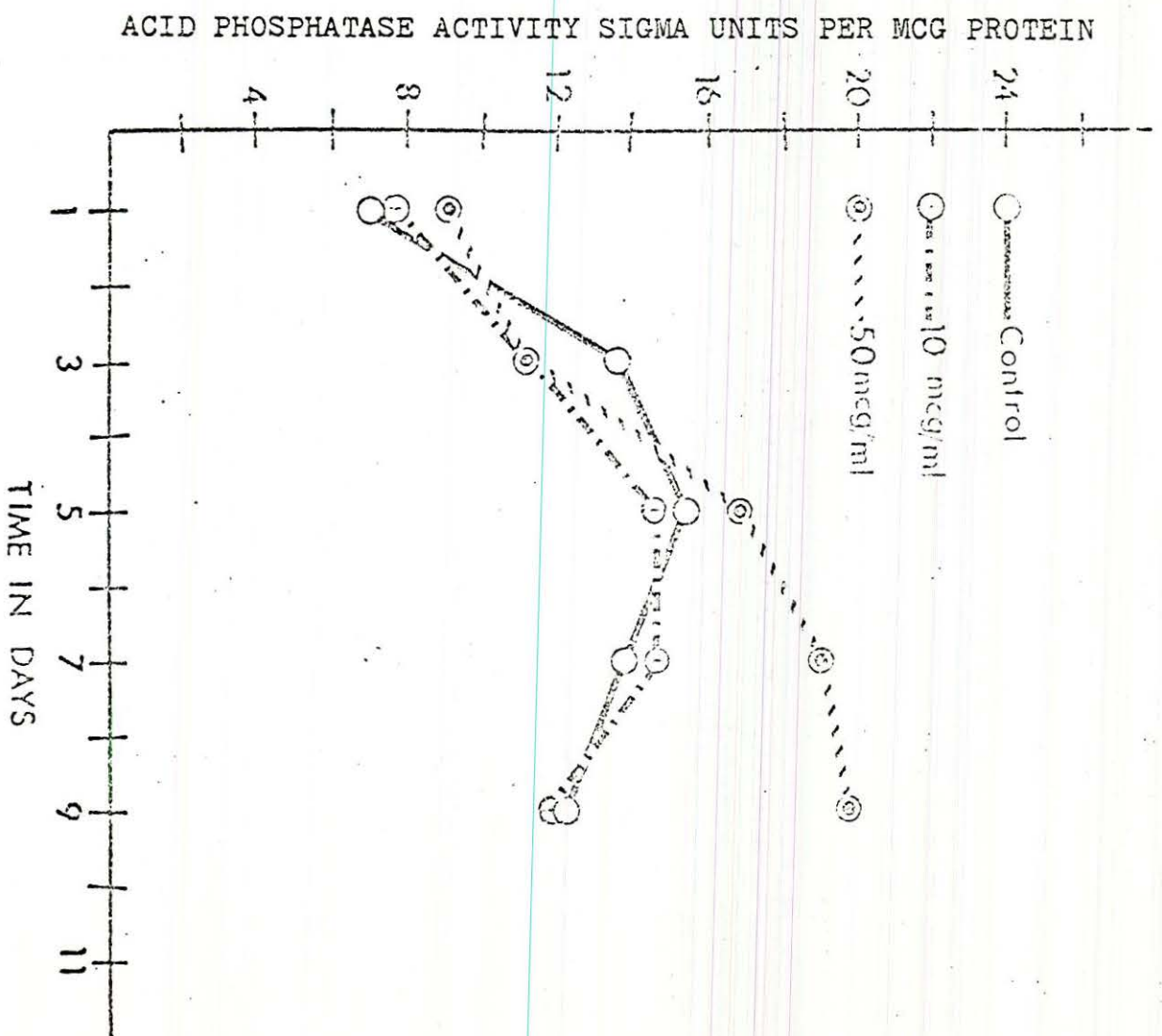
Deoxyribonucleic Acid (DNA)

The curves representing the amount of DNA per cell approximated the various growth curves presented earlier. The amount of DNA per cell in the control cultures increased

Figure 7

Average of three experiments showing the effect of various concentrations of tetracycline on the acid phosphatase activity of Low Line cells. (See Appendix II.).

Figure 7



sharply during the period corresponding to the logarithmic growth phase of the cells. It then reached a plateau and after several days, began to decline. A concentration of 10 mcg/ml of tetracycline resulted in a DNA content per cell which approximated that of the control cultures, although, there did seem to be a slight inhibition prior to the 4th or 5th days. Both 50 and 100 mcg/ml of tetracycline inhibited DNA production, with the 100 mcg/ml exhibiting marked inhibition after the 3rd day. The results demonstrating the effects of tetracycline on DNA content of the cells are seen in Figure 8.

Ribonucleic Acid (RNA)

The results obtained for RNA content per cell are quite different from those of the DNA per cell and they are presented in Figure 9. The amount of RNA per cell in the control cultures increased sharply during the logarithmic growth phase (days 1 through 3) then began to gradually decline until the termination of the experiment. Those cells exposed to 10 mcg/ml of tetracycline did not increase in RNA content as rapidly as the controls, however, they did remain elevated through day 7 before declining sharply to the level of the control cultures. The plotted curve of the RNA content of those cells exposed to 50 mcg/ml parallels very closely that of the control cells only at a reduced amount.

Figure 8

Average of three experiments showing the effects of various concentrations of tetracycline on the DNA content of Low Line cells. (See Appendix III.).

Figure 8

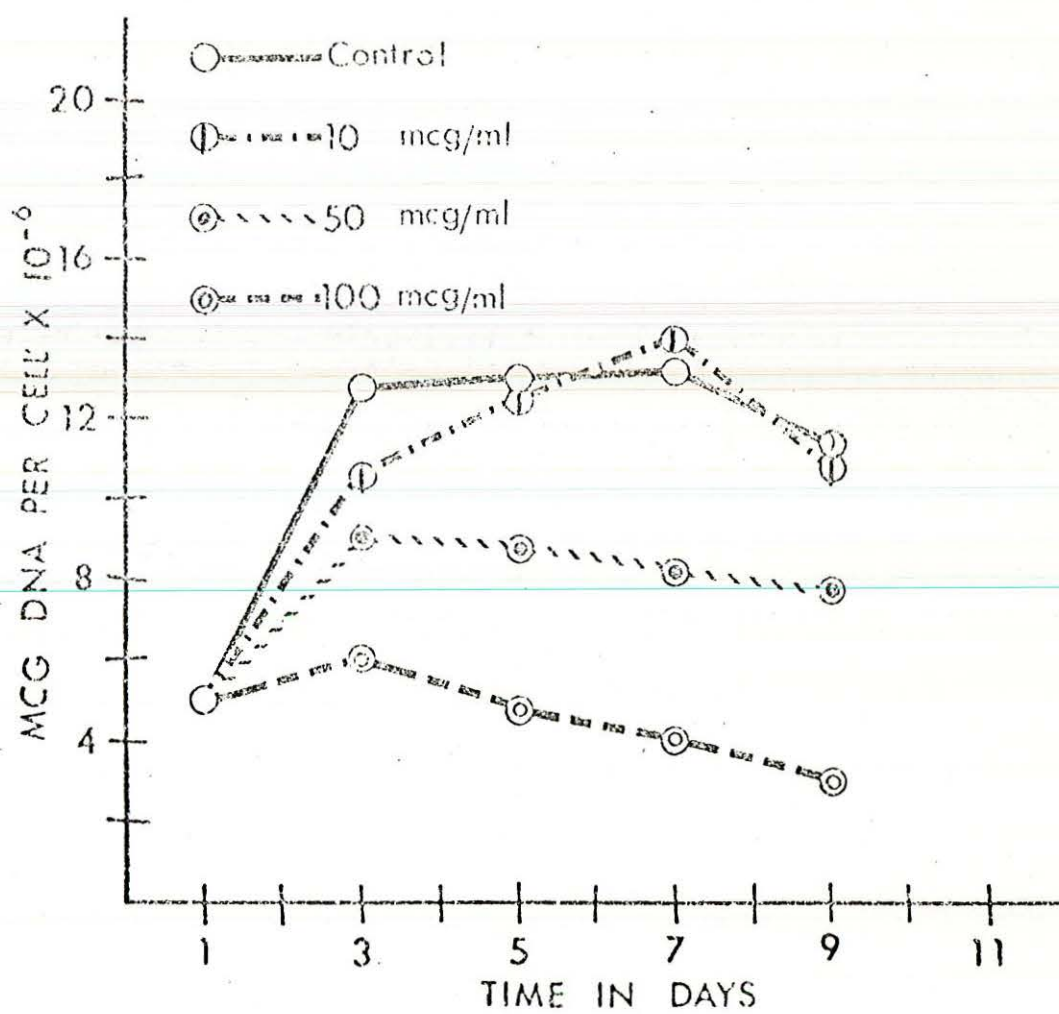
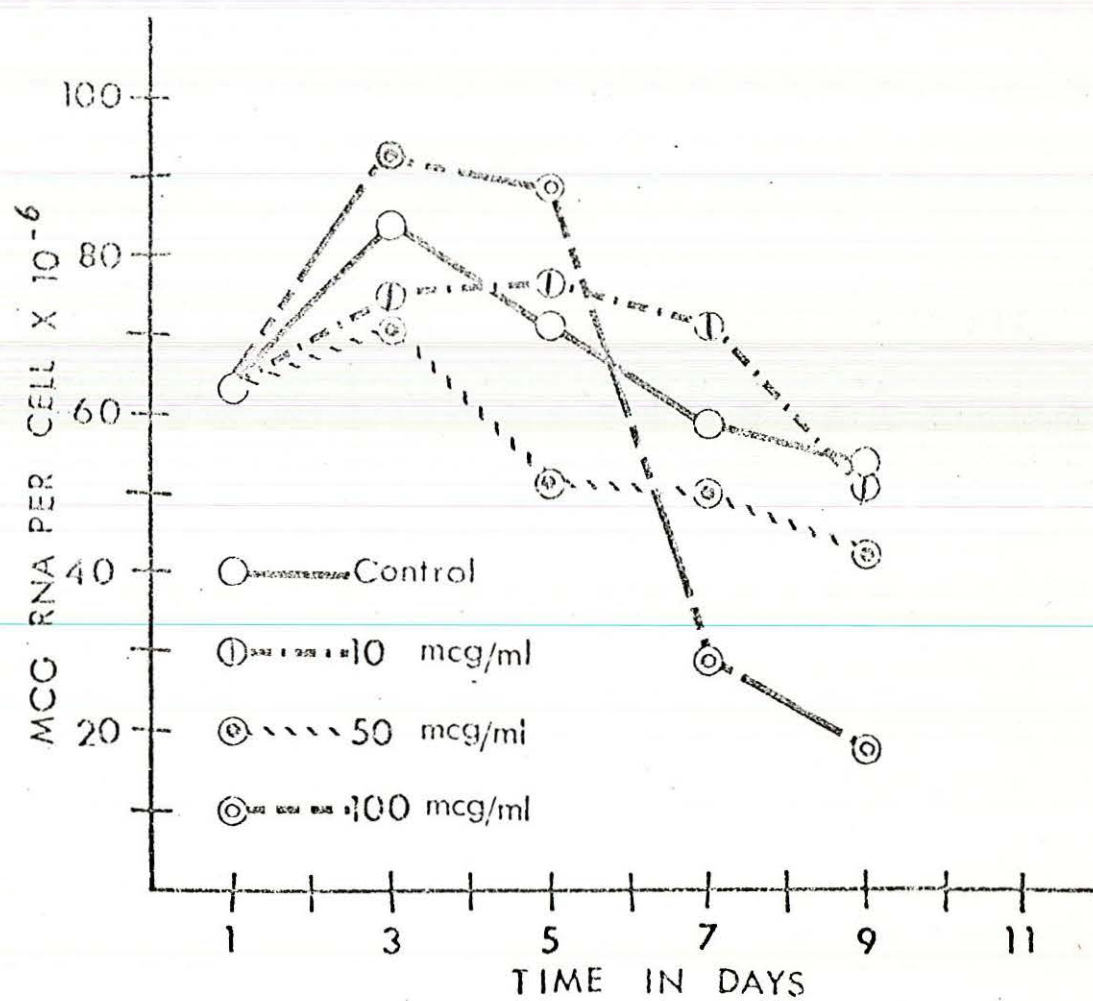


Figure 9

Average of three experiments showing the effects of various concentrations of tetracycline on the RNA content of Low Line cells. (See Appendix III.).

Figure 9



In contrast, the cells exposed to a concentrations of 100 mcg/ml of tetracycline showed a marked increase in the amount of RNA per cell at day 3. This was followed by a very sharp decline to a level considerably lower than the control and other treated cultures.

Succinic Dehydrogenase Activity

Succinic dehydrogenase activity in Low Line cells was evidenced by the presence of reddish-purple granules within the cytoplasm of the cells after exposure to the ditetrazolium reagent (Seligman and Rutenburg, 1951). Representative photomicrographs of cells exposed to various concentrations of tetracycline are shown in Figure 10. It is evident that there was a direct relationship between the amount of succinic dehydrogenase activity and the concentration of the drug to which the cells were exposed. The control cells contained considerable quantities of succinic dehydrogenase. Those treated with 10 mcg/ml of tetracycline seemed to show a slight decrease in enzyme activity. When the cells were subjected to 50 mcg/ml there was a noticeable decrease in activity although it may not be evident in the photograph for the activity appeared to be confined to vacuoles rather than diffuse as seen in the previous photomicrographs. Those cells exposed to a concentration of 100 mcg/ml of tetracycline show very little enzyme

Figure 10

Photomicrographs of Low Line cells treated with ditetrazolium chloride reagent, resulting in a demonstration of succinic dehydrogenase activity. The cells were exposed for four hours to the concentrations of tetracycline listed below.

- A. Control
- B. 10 mcg/ml
- C. 50 mcg/ml
- D. 100 mcg/ml

A.



B.



C.



D.



activity.

ATP was added to the culture medium in a concentration of 0.001 M and the above experiments repeated. The results obtained were identical to those without added ATP.

In order to determine the reversibility of the inhibitory action of tetracycline, cells were exposed to the same concentrations of tetracycline as described above. Four hours after the exposure, the medium was removed, the flasks rinsed 3 times with BSS and fresh medium added. Twenty-four hours later the succinic dehydrogenase activity of the cells was determined. The most evident observation was the great increase in enzyme activity. Figure 11 compares the results seen after a 4 hour exposure to 50 mcg/ml of tetracycline and 24 hours after the removal of the treated medium. Seventy-two hours after the addition of fresh medium, the enzyme activity had returned to normal levels.

Cytochrome Oxidase Activity

The presence of cytochrome oxidase activity was demonstrated cytochemically by the technique of Burstone (1961) which resulted in the formation of a blue-black stain at the site of enzyme activity. It can be seen from the photomicrographs in Figure 12 that the Low Line cells contained considerable quantities of this enzyme. It can also be observed that the 3 concentrations of tetracycline employed during this investigation had little, if

Figure 11

Photomicrographs of Low Line cells treated with ditetrazolium chloride reagent, resulting in the demonstration of succinic dehydrogenase activity. The cells were exposed to various concentrations of tetracycline for 4 hours after which the treated medium was removed, the flask rinsed, and fresh medium added.

-
- A. Cells in control cultures exposed to 50 mcg/ml of tetracycline for 4 hours.
 - B. Cells in control cultures exposed to 50 mcg/ml of tetracycline for 4 hours and then photographed 24 hours after the addition of fresh medium.

A.



B.

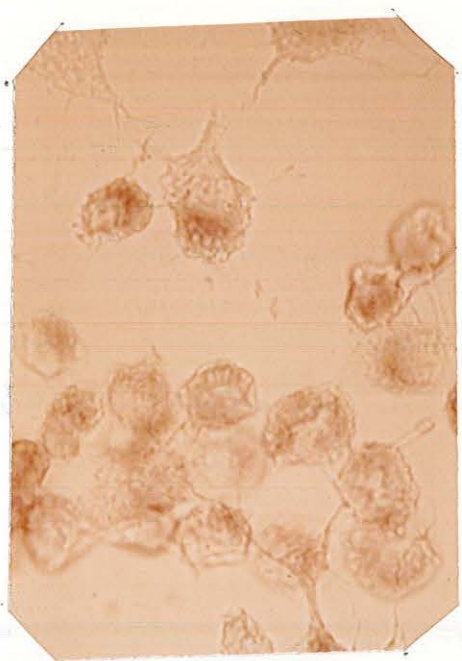


Figure 12

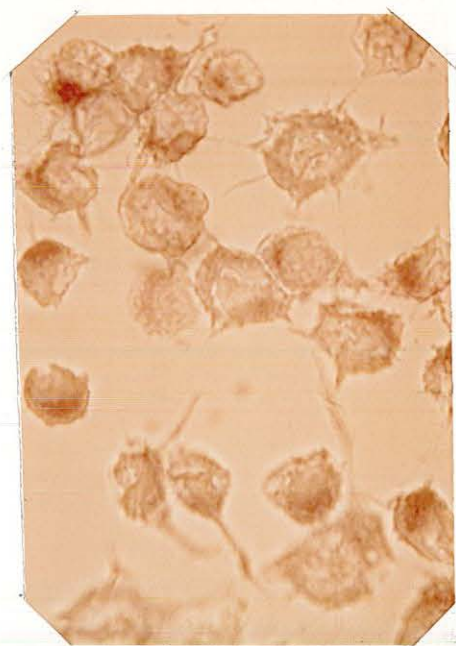
Cytochemical demonstration of cytochrome oxidase activity of Low Line cells exposed to various concentrations of tetracycline for four hours.

- A. Control
- B. 10 mcg/ml
- C. 50 mcg/ml
- D. 100 mcg/ml

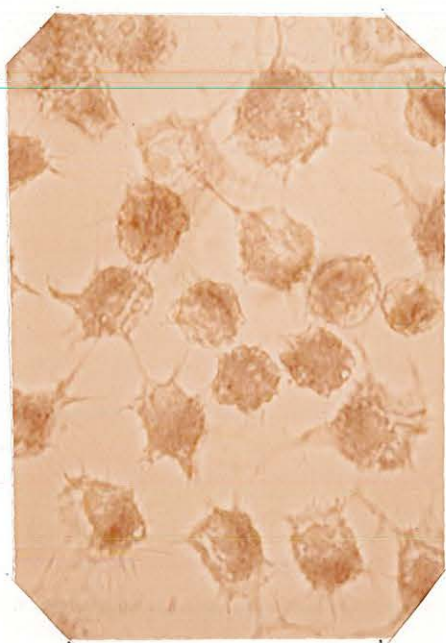
A.



B.



C.



D.



any, effect on the cytochrome oxidase activity of the cells.

Cellular Lipid Accumulation

Cytochemical demonstration of lipid content. Oil-Red O was used to demonstrate the presence of neutral lipids within the cells. When present the lipid droplets stain pink to bright red. The photomicrographs in Figure 13 show that, as with succinic dehydrogenase activity, there was a direct relationship between amount of lipid present and concentration of tetracycline employed. Control cells exhibited a relatively normal amount of lipid. In those cells exposed to a concentration of 10 mcg/ml tetracycline, a slight increase in neutral lipids can be observed. Exposure of Low Line cells to a concentration of 50 mcg/ml resulted in a marked increase in lipid deposits.

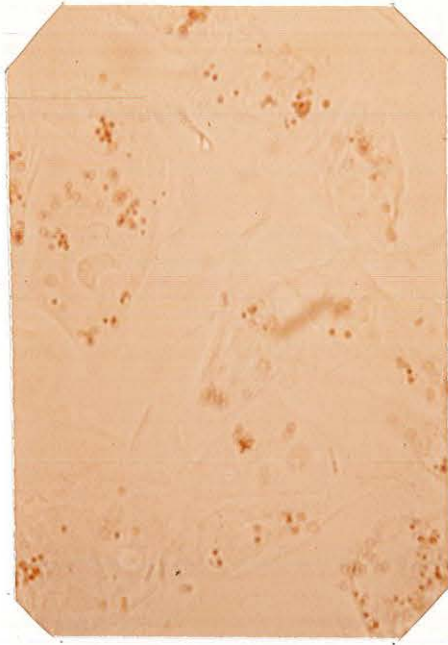
Similar results were obtained when cells were exposed to a concentration of 100 mcg/ml. As in the case of succinic dehydrogenase, it was deemed necessary to study the possible reversibility of these effects. Cells were treated with various concentrations of tetracycline for 24 hours. The treated medium was then removed, the flasks rinsed 3 times with BSS and fresh medium added. Twenty-four hours after the addition of fresh medium the cells were stained with Oil-Red O to determine the amount of neutral lipids present. The results obtained were very similar to those

Figure 13

Cytochemical demonstration of lipid content of Low Line cells exposed to various concentrations of tetracycline.

- A. Control
- B. 10 mcg/ml
- C. 50 mcg/ml
- D. 100 mcg/ml

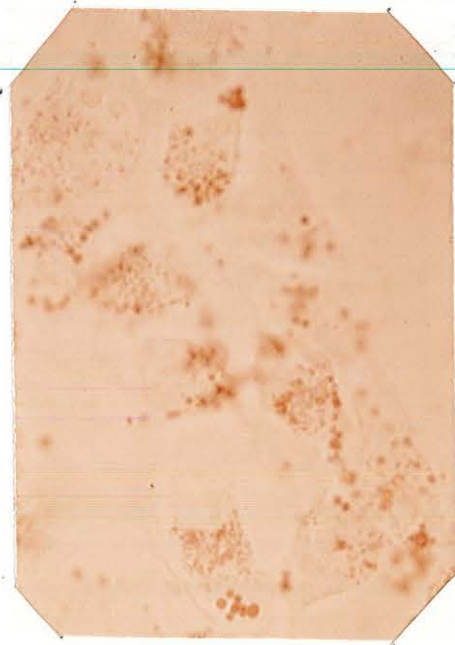
A.



B.



C.



D.



obtained for succinic dehydrogenase. It was quite obvious that the amount of lipid present was decreasing. Photomicrographs demonstrating this effect are shown in Figure 14. It was also observed that 72 hours after the removal of the tetracycline medium the amount of lipid present was nearly back to normal. In order to determine if some of the accumulated lipid was due to an increased uptake of serum lipids already present in the medium, NCTC #2071 clone of Strain L cells were exposed to the same treatment as the Low Line Cells. These cells were maintained on serum-free medium and the results obtained were identical to those observed with the Low Line cells. There was an increase in lipid content in direct proportion to the concentration of tetracycline employed.

Radiotracer studies. In order to express the biochemical results in a more quantitative form, Low Line cells grown in T-60 flasks were treated with 0 or 100 mcg/ml of tetracycline. The medium used for this study also contained 0.025 uCi/ml of carbon-14 labeled sodium pyruvate. The cells were exposed for either 1 or 4 hours to this medium. After exposure, the treated medium was removed and the total cellular lipids extracted as described earlier. The results showing the incorporation of labeled pyruvate into the total lipid fraction are summarized in Table III. Both after 1 hour and 4 hours of exposure, an increase in the uptake of carbon-14 labeled pyruvate into the lipid fraction

Figure 14

Cytochemical demonstration of the lipid content of Low Line cells exposed to a concentration of tetracycline of 50 mcg/ml for four hours after which the treated medium was removed, the flask rinsed, and fresh medium added.

- A. Cells in control cultures exposed to 50 mcg/ml of tetracycline for 4 hours.
- B. Cells in control cultures exposed to 50 mcg/ml of tetracycline for 4 hours and then photographed 24 hours after the addition of fresh medium.

A.



B.



Table III

Compilation of 5 experiments showing the effect of various concentrations of tetracycline on the uptake of carbon-14 labeled pyruvate into cellular lipid.

Table III

ONE HOUR EXPOSURE TO TETRACYCLINE

SAMPLE	TOTAL LIPID RADIOACTIVITY (cpm/ 10^6 cells)
Control (no Isotope)	11 \pm 3
Control (Isotope Added)	43 \pm 8
10 mcg/ml TC	80 \pm 4
50 mcg/ml TC	170 \pm 17
100 mcg/ml TC	104 \pm 6

FOUR HOUR EXPOSURE TO TETRACYCLINE

SAMPLE	TOTAL LIPID RADIOACTIVITY (cpm/ 10^6 cells)
Control (No Isotope)	13 \pm 5
Control (Isotope Added)	49 \pm 11
10 mcg/ml TC	157 \pm 13
50 mcg/ml TC	213 \pm 24
100 mcg/ml TC	177 \pm 14

Values are expressed as means \pm standard deviation

is observed with an increase in tetracycline concentration. At both 1 and 4 hours the maximum increase is observed in those cells exposed to 50 mcg/ml and a decrease is observed at 100 mcg/ml.

Protein

Total cellular protein. The results of the effect of various concentrations of tetracycline on the protein content of Low Line cells are shown in Figures 15 and 16. When these results are expressed as mcg of protein per ml of medium in which the cells were growing (Figure 15) the curves are similar to the growth curves. However, when expressed as mcg of protein per cell they are somewhat different (Figure 16). A concentration of 100 mcg/ml of tetracycline seems to have little effect on total cellular protein although there is a slight increase at the termination of the experiment. Concentrations of 10 and 50 mcg/ml seem to cause a slight stimulation in protein synthesis similar to their effect on growth.

Protein synthesis. The results of this phase of the investigation will be expressed in three ways: counts per minute (CPM) per cell, CPM/mcg of protein, and mcg of protein per cell. During the first part of the experiment, the cells were treated 2 hours with medium containing both the desired concentration of the drug and the carbon-14

Figure 15

Average of three experiments showing the effect of various concentrations of tetracycline on the amount of protein per ml of culture medium in which the Low Line cells were grown. (See Appendix IV.).

Figure 15

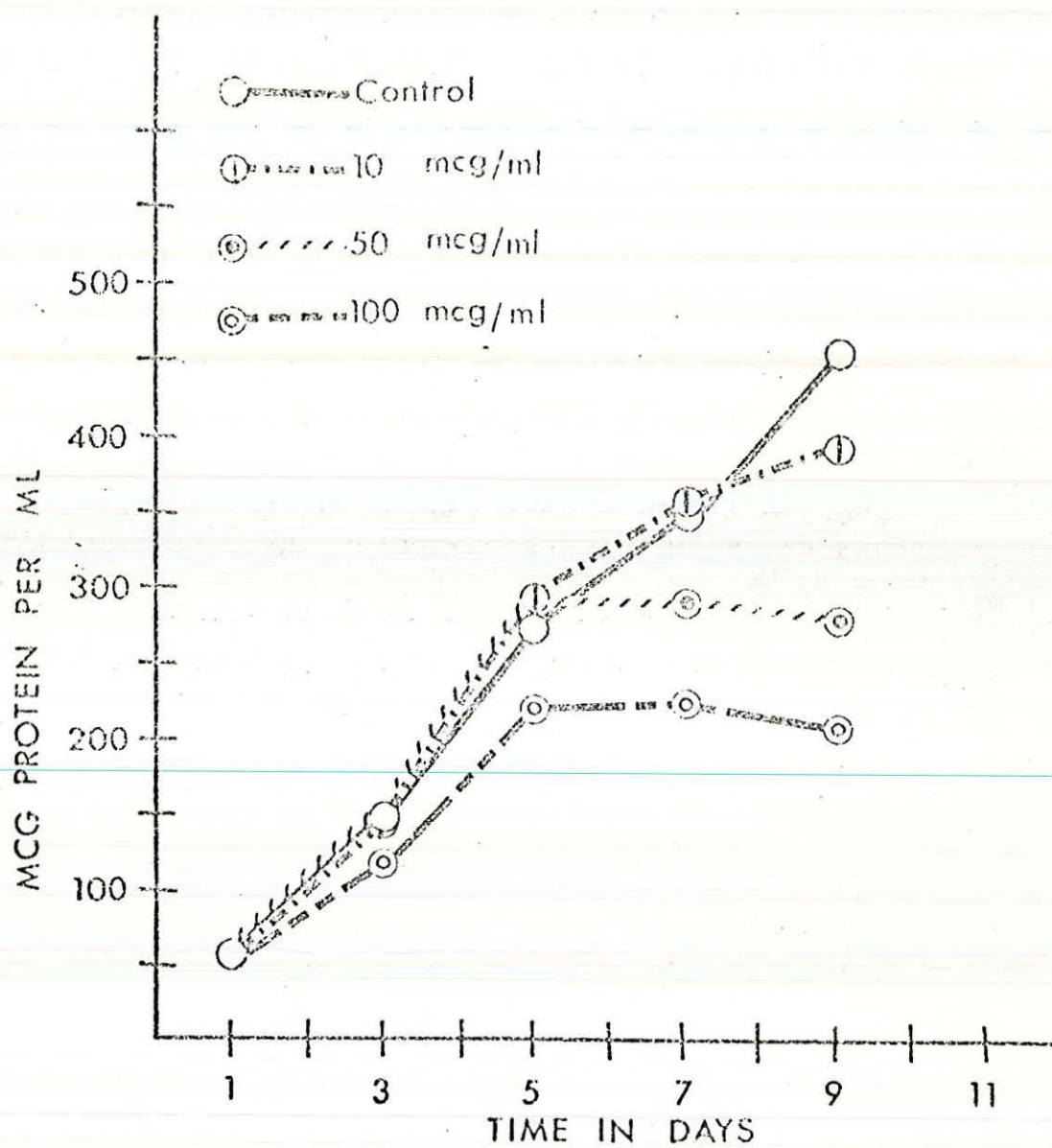
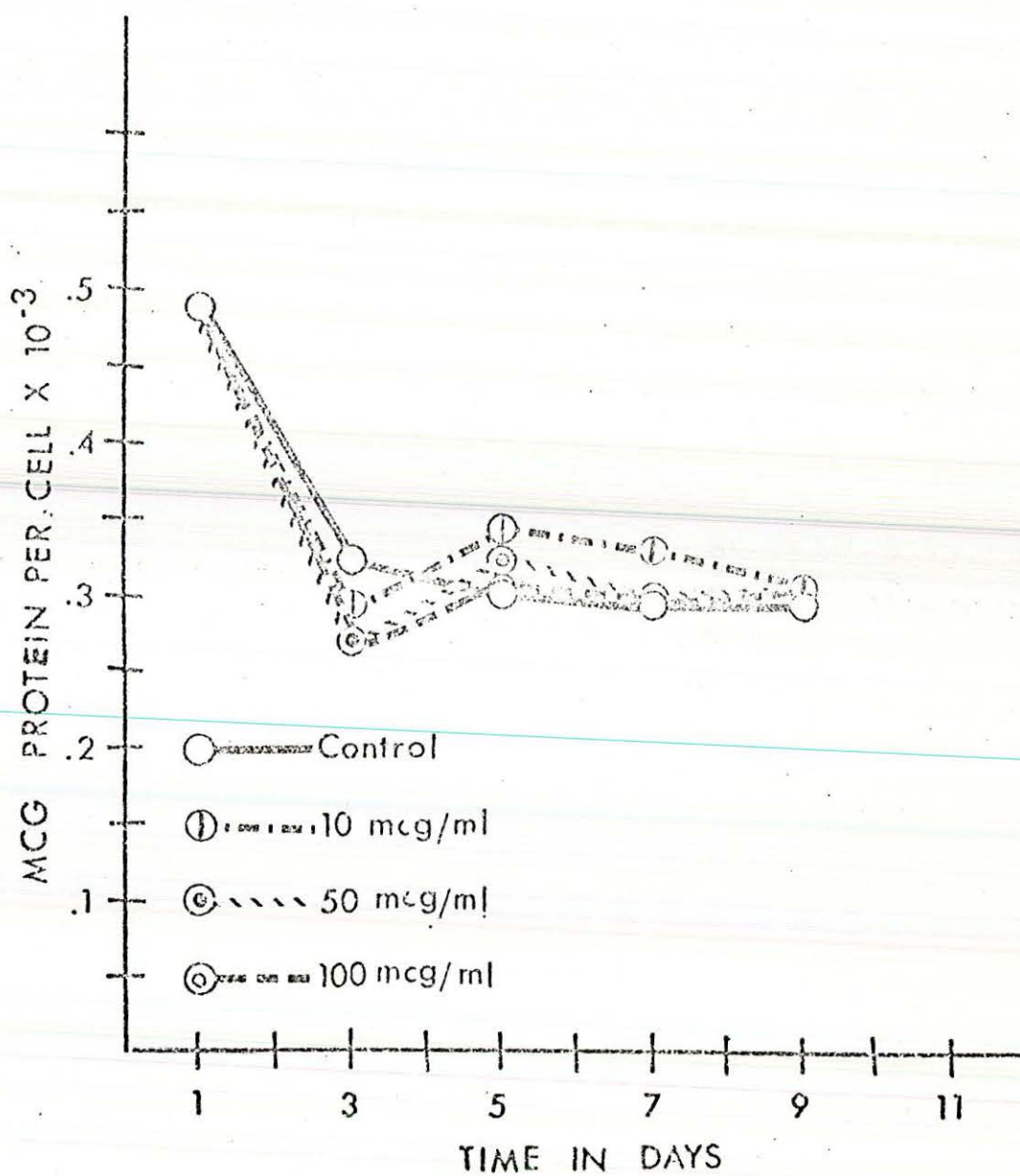


Figure 16

Average of three experiments showing the effect of various concentrations of tetracycline on the amount of protein per cell. (See Appendix IV.).

Figure 16



labeled medium. Samples were then prepared as described earlier and the appropriate calculations made. The results are presented in Figure 17. They indicate that tetracycline caused a decrease in CPM/cell in all treated cultures. The CPM/mcg of protein increased slightly when the cells were exposed to 10 mcg/ml and decreased when exposed to 50 and 100 mcg/ml of tetracycline. Under the conditions of this experiment there was little, if any, effect of 10 and 50 mcg/ml on the amount of protein per cell. However, those cells exposed to a concentration of 100 mcg/ml did show a decrease in the amount of protein per cell.

The second part of this experiment involved pre-treating the cells with the desired concentration of tetracycline for 24 hours. After the 24 hour period, fresh medium containing tetracycline as well as 0.01 uCi/ml of carbon-14 labeled leucine, was added. Two hours later this medium was removed and the cell samples prepared as described earlier. The results of this phase of the investigation are given in Figure 18. A concentration of 10 mcg/ml did not have the same effect on the CPM/cell as it had in the first part of this experiment. However, in contrast to the previous experiment, tetracycline concentrations of 50 and 100 mcg/ml did cause a marked decrease in CPM/cell. All concentrations of the drug caused a slight proportional decrease in CPM/mcg of protein. The amount of protein per

Figure 17

Average of two experiments showing the effect of various concentrations of tetracycline on protein synthesis by Low Line cells. The cell cultures were exposed to the medium containing the desired concentration of tetracycline and 0.01 uCi/ml of carbon-14 labeled leucine for 2 hours. (See Appendix V.).

Figure 17

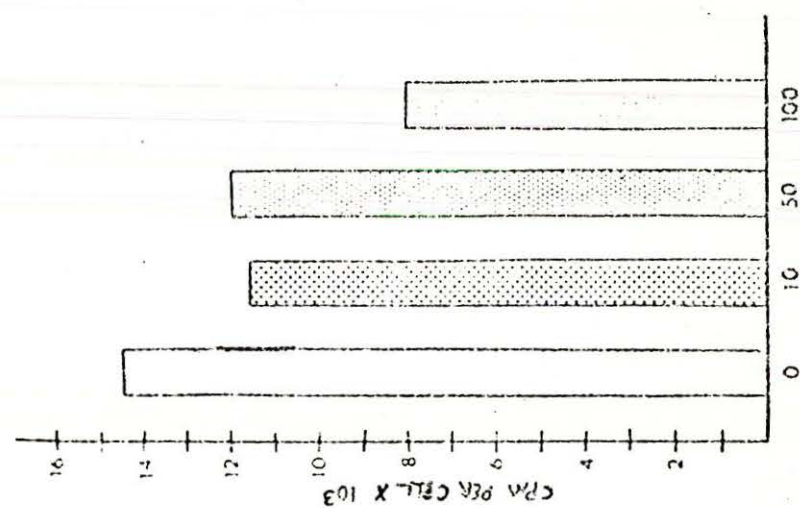
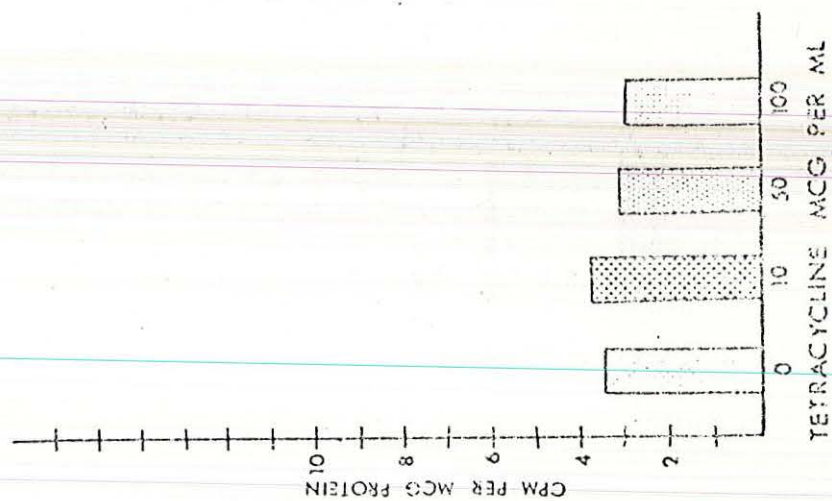
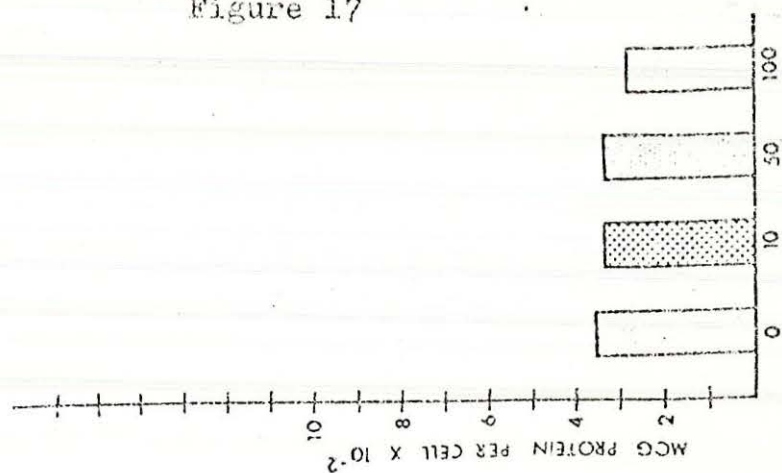
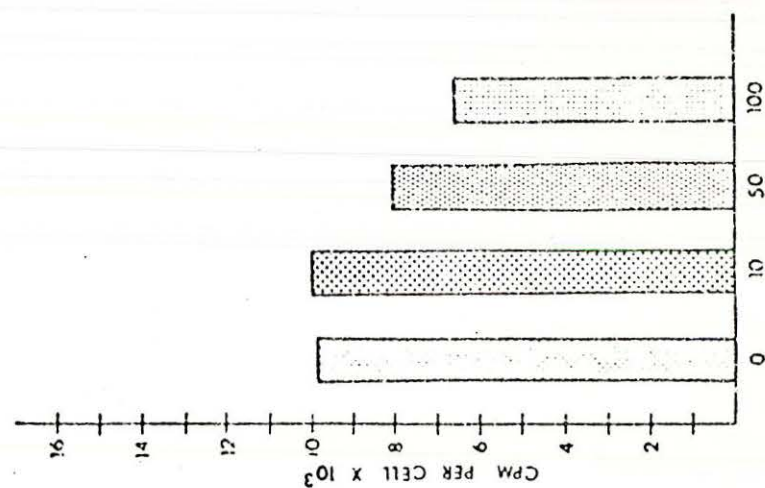
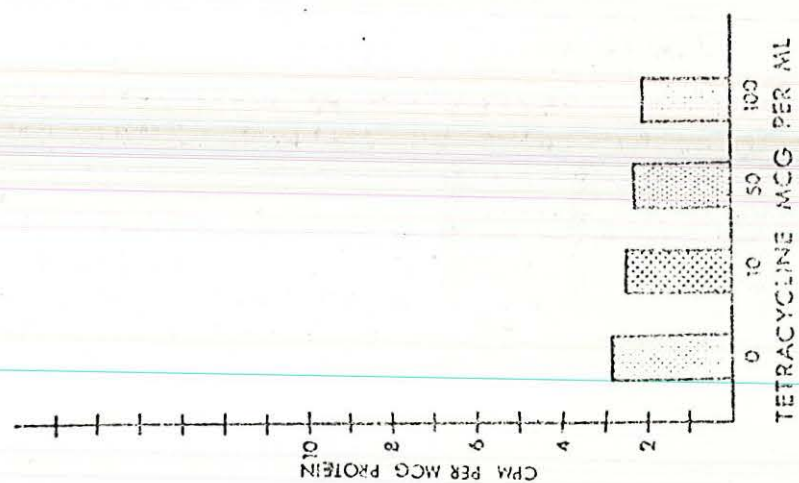
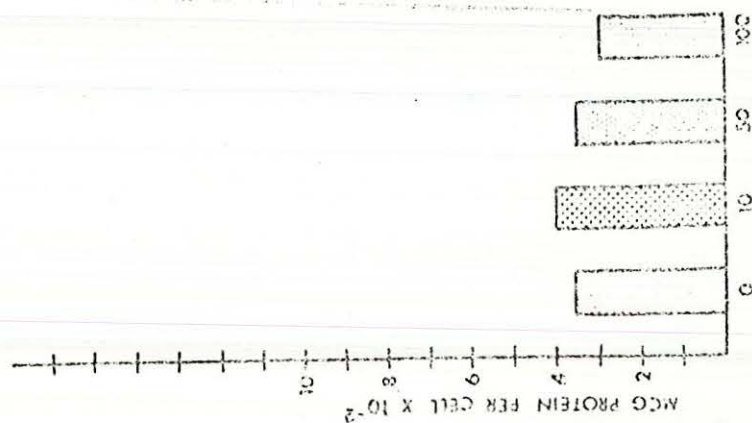


Figure 18

Average of two experiments showing the effect of various concentrations of tetracycline on protein synthesis by Low Line cells. The cell cultures were pre-treated with the chosen concentration of tetracycline for 24 hours. At the end of this period fresh medium containing tetracycline and 0.01 $\mu\text{Ci/ml}$ of carbon-14 labeled leucine was added and the cells incubated for 2 additional hours. (See Appendix VI.).

Figure 18



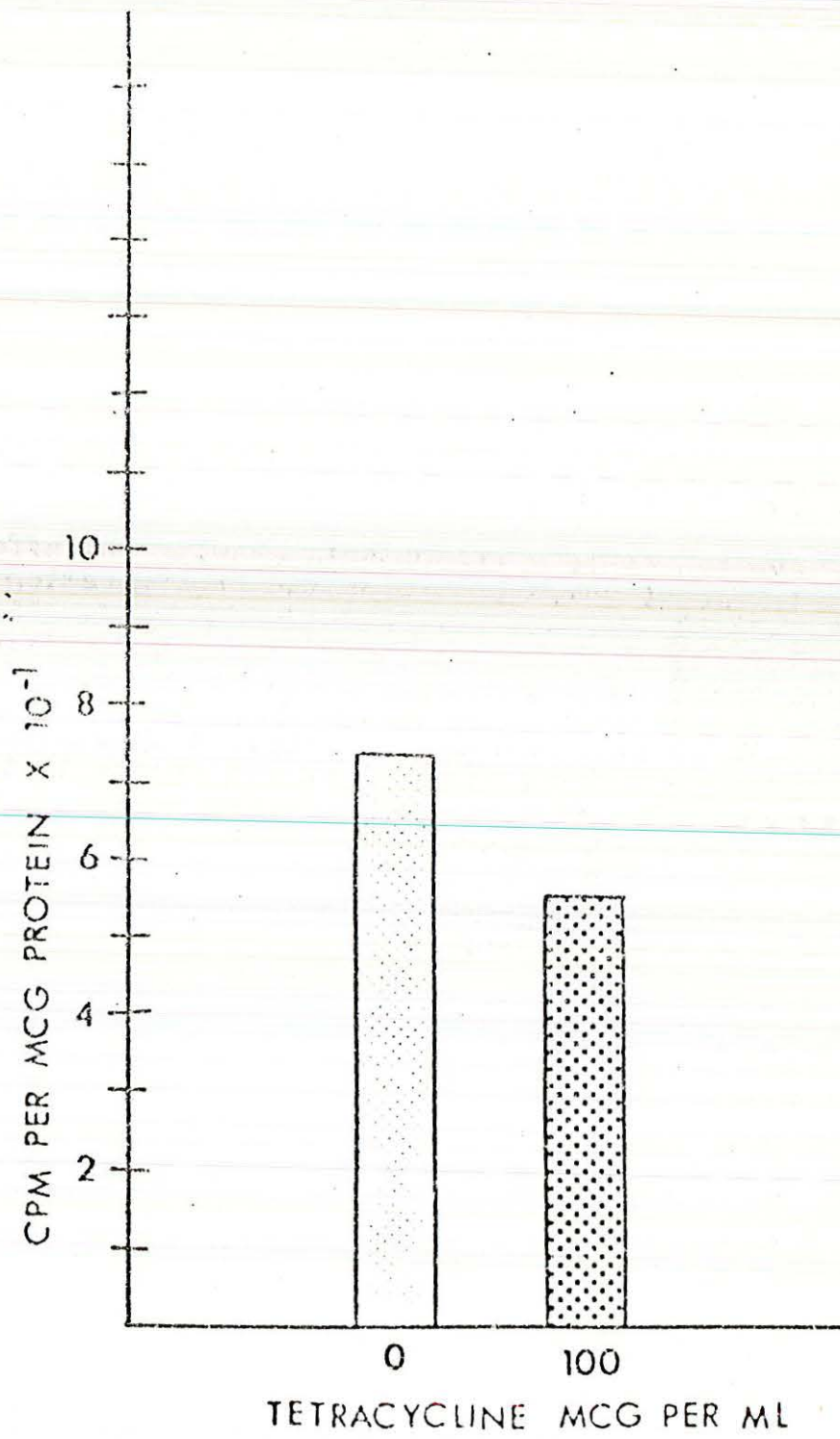
cell was affected differently by the various concentrations used. Tetracycline at 10 mcg/ml resulted in a slight increase in mcg of protein per cell; 50 mcg/ml had no effect; and 100 mcg/ml caused a decrease in mcg of protein per cell.

Serum proteins. Low Line cells were incubated in medium containing tetracycline for 8 hours and then for 4 more hours after carbon-14 labeled leucine was added to bring the final concentration to 0.5 uCi/ml. The medium was then removed from both the control and the treated cultures and fractionated by ultracentrifugation. Repeated examination of the top fraction (density less than 1.063) failed to show any incorporation of carbon-14 labeled amino acid. No activity higher than the background was recorded. A protein precipitate was observed in this fraction, which averaged less than 5 mcg total, however it apparently was unlabeled. The bottom fraction, containing the balance of the serum proteins, did contain significant amounts of incorporated labeled leucine. In all instances the tetracycline-treated samples showed a lower level of activity than the controls. The results are compiled in Figure 19.

Figure 19

Average of four experiments showing the effect of 100 mcg/ml of tetracycline on the incorporation of carbon-¹⁴ labeled leucine into serum proteins. The cells were pre-treated for 8 hours with tetracycline. At this time a quantity of labeled leucine was added to each flask to bring the final concentration to 0.5 uCi/ml. After 4 additional hours of incubation the samples were prepared as described in the text. (See Appendix VII.).

Figure 19



DISCUSSION

CHAPTER IV

DISCUSSION

It was found that Low Line cells exposed to tetracycline at concentrations up to 10 mcg/ml exhibited no apparent cytotoxic effects. It was also found that a concentration of 250 mcg/ml was toxic to the cells almost immediately. These results are in partial agreement with Journey and Goldstein (1963) who used the homologue oxytetracycline. They reported that HeLa cells exposed to 300 mcg/ml of oxytetracycline for two hours resulted in degeneration of the mitochondria and other cytoplasmic elements. They also found that 100 mcg/ml produced no toxic effects and that 1000 mcg/ml was lethal to all cells. James (1969) has reported similar results when studying the effects of tetracycline on Ehrlich ascites cells. The results of the present study would seem to be in conflict with the two above mentioned reports for it was found that 100 mcg/ml of tetracycline did result in toxic effects on Low Line cells. It will be noted, however, that the studies of the above investigations were of short duration (less than 24 hours) and the toxic effects reported here did not become apparent until 48 hours after the administration of the drug. The lowest concentration used in the current study was approximately twice as high as the average blood level following therapeutic oral adminis-

tration (Welch et al., 1957; Whalley, 1964). It is, however, less than half the concentration suggested for use in tissue culture medium in order to prevent bacterial contamination (Parker, 1961).

The effect of tetracycline on growth of Low Line cells was consistent with the morphological observations. The slight stimulation in growth rate observed when the cells were exposed to a concentration of 10 mcg/ml was not wholly unexpected. The use of several different antibiotics in animal feeds to stimulate growth was introduced after investigations, such as those of Migicovsky et al., (1951), showed that inculsion of penicillin in the diet of infant chicks led to a marked increase in the index of calcium absorption. In 1954, Rusoff et al., published results demonstrating that weekly intramuscular injections of chlor-tetracycline in young calves increased growth by 30% over control animals. The possible mechanisms to explain these findings are still unknown, however, it is obvious that a correlation exists between the effect on growth and concentration of drug administered.

Another part of this investigation dealt with the effect of tetracycline on glucose utilization and lactic acid production. Control cultures exhibited a high rate of glucose utilization during the initial phase of the treatment corresponding to the logarithmic growth phase. This

initial increase was followed by a gradual decline during the later growth phases. This pattern of glucose utilization is similar to those reported for other cell types (Fulton et al., 1956; Munyon and Merchant, 1959). All concentrations of tetracycline employed during this investigation caused increases in glucose utilization. A concentration of 10 mcg/ml did not seem to totally alter the metabolic functions of the cells since glucose utilization at this level parallels that of the controls. In contrast to this observation, the higher concentrations differ markedly from the normal pattern. There is a sharp increase at day 5. It is possible that this is an attempt to return to the more "normal" pattern. The tetracyclines have been shown to uncouple oxidative phosphorylation (Loomis, 1950; Van Meter et al., 1951) which would result in a decrease in the amount of ATP available. The net effect would be an increase in glucose metabolism as demonstrated by this study.

In many instances the lactic acid production observed during this investigation exceed glucose utilization. This was especially evident when the cells were exposed to 50 and 100 mcg/ml of tetracycline. It is apparent that the increase in lactic acid must result from metabolic pathways other than glucose metabolism. It is been found that glucose is not necessary for the production of lactic acid. Wilson et al., (1942), working with chick embryo tissue, and Bailey et al., (1958) using mouse lymphoblasts found that lactic

acid was formed both in the presence and absence of glucose. One possible source of this excess lactic acid may have been lipid material. An increase in lipids has been reported in this study and will be discussed later. Because free fatty acids and triglycerides supply energy through the oxidation of fatty acids (Rothblat, 1969), an increase in metabolism of this lipid might be a possible source of the excess lactic acid reported in the current investigation. It is also possible that glucose utilization was actually decreased in relation to lactic acid production. In other words, there may have been a decrease in the amount of glucose available to the cell possibly attributable to either the interference of glucose transport or some enzyme system in the conversion of glucose to pyruvate. Thus, the lactic acid produced was probably from some other source such as the oxidation of lipid. This could be possible since Bailey et al., (1958) found that cells survived in the absence of glucose for 48 hours, apparently utilizing lactic acid.

The fact that lactic acid can be produced from sources other than glucose, and also can be utilized by cells, could perhaps account for the discrepancy between glucose utilization and lactic acid production.

The lack of alkaline phosphatase activity reported in this study is in agreement with Cox and Macleod (1962)

and Shaw (1969). Reported histochemical and colorimetric determinations failed to show any activity of this enzyme. Even though the enzyme can be found in many tissues, its function remains poorly understood. As has been observed by others, its localization in the cellular membrane area (Melnykovich et al., 1967) and its increase, during neonatal development, in proportion to the absorptive capacity of the intestinal mucosa (Clark, 1957), would suggest an involvement in cellular absorption. Since Cox and Macleod (1962) did find alkaline phosphatase activity in epithelial cells but not in human or mouse fibroblasts it would also appear that the activity is limited to certain cell types.

In contrast to the lack of alkaline phosphatase activity, Low Line cells did exhibit considerable acid phosphatase activity. Acid phosphatase is usually considered to be associated with the cellular lysosomes and hence is used as an indication of the stability of the lysosomal membrane. Cristofalo et al., (1967) have reported an increase in acid phosphatase activity in human lung cells during aging. The present study however, which demonstrated a decrease in enzyme activity of Low Line cells during the growth cycle is in agreement with Shaw (1969). It would be purely speculative to suggest that the increase in acid phosphatase activity seen with tetracycline administration might be due to similar metabolic effects as those which

result in the increase observed during aging as reported by Cristofalo et al., (1967). It is obvious that further research is indicated in both of these areas. Nevertheless, the end results are similar. The increase in acid phosphatase activity tends to lower the concentrations of metabolically important phosphatase esters and shifts the equilibrium away from synthesis towards catabolism, thus resulting in a general deterioration of the cells (Cristofalo et al., 1967).

The decrease in the amount of DNA as compared to concentrations of the tetracycline employed paralleled the observations on growth with one notable exception. Although the decrease in amount of DNA and the concentration of drug used were in agreement, all treated cultures evidenced a lesser amount of DNA than the controls. This was especially evident during the early stages of the experiment where the concentrations of 10 and 50 mcg/ml actually stimulated growth. However, after this initial period it would appear that the higher concentrations of tetracycline were inhibitors of mitosis.

When studying the effects of tetracycline on the amount of RNA as compared to DNA, one is immediately cognizant of the differences in the 10 and 100 mcg/ml concentrations. It was found during these investigations that the inhibition of protein synthesis was most pronounced after 48 to 72 hours of exposure to the drug. In reference to the large increase

in RNA production early during exposure to a concentration of 100 mcg/ml it would appear that there was a decrease in mitosis (as seen in the DNA study). At the same time there was not an inhibition of RNA synthesis thus there was an increase in amount of RNA per cell and also a concomitant slight increase in the amount of protein. After the initial phase (day 3 to 5) a rapid decrease in RNA content of the cells was noted, probably a manifestation of other cytotoxic effects. The increase in RNA content observed when the cells were exposed to 10 mcg/ml may be related to the growth stimulatory effect which was also observed at this concentration.

It was stated earlier in this discussion that the increase in glycolytic activity which was observed might be attributed to the uncoupling of oxidative phosphorylation. It is also possible that such an affect may be due to an inhibition of the tricarboxylic acid cycle. The complete oxidation of glucose via the pentose shunt and tricarboxylic acid cycle yields 38 high energy bonds in the form of ATP, while only two such bonds result from the anaerobic metabolism of glucose. It is obvious then that a decrease in activity of the tricarboxylic acid cycle would result in an increase in glycolytic activity. The results of this investigation indicated that tetracycline had a definite inhibitory action on the enzyme succinic dehydrogenase (see also James and Pace, 1970). These findings were in agreement with the

earlier work indicating inhibition of certain isolated enzyme systems by chlortetracycline and other antibiotic agents (Arora and Murti, 1951).

The actual effect of the tetracyclines on cellular respiration has not been resolved. Loomis (1950) first reported an uncoupling of oxidative phosphorylation without effect on respiration. In contrast to this, VanMeter and Oleson (1951) reported a definite inhibition of cellular respiration along with uncoupling of oxidative phosphorylation. During the present investigation we have found no effect on cytochrome oxidase. However, if a more extensive study were made it is possible that a correlation between dose, length of exposure, and type of cell might be found.

Accumulation of lipid material in cells exposed to certain concentrations of tetracycline was observed as early as one hour after administration. This was not due to "shock" from media change since control cultures were changed at the same time and did not exhibit lipid accumulation. Nor was it due to changes in medium pH. Although reduction of pH levels from 7.4 to 6.9 have been reported to result in an increase in lipids in cells (Mackenzie et al., 1967a), it was reported in the current study that the concentrations used resulted in no change of pH (pH = 7.4). Journey and Goldstein (1963) also reported pH values of 7.1 to 7.4 when using even higher concentrations. Increases in lipid content have also been shown to be related to the amount of lipids

present in the serum of the culture medium. Howard (1968) and Mackenzie et al., (1967b) using labeled exogenous fatty acids, have shown that under standard conditions exogenous fatty acids carried in the serum contributed from 80 to 95% of the fatty acid carbon incorporated into cellular glycerides and phospholipid. The observed accumulation of lipid material from tetracycline administration is apparently not due to an increased uptake of fatty acid precursors from the medium, since the present study demonstrated that identical lipid accumulation occurred in Strain L cells grown on serum-free medium. One suggested mechanism of the cause of tetracycline-induced fatty liver has been that the antibiotics in high concentrations interfere with hepatic cellular energy (ATP) synthesis (Wruble et al., 1965), resulting in impaired fat metabolism. This assumption would seem valid since other substances which cause a fatty liver (ethionine, carbon tetrachloride, and alcohol) also depress hepatic ATP (Hyams and Isselbacher, 1964). It has been found, however, that the hepatic ATP levels of pregnant and non-pregnant rats treated with tetracycline were not lowered below the level of the control animals. In the current study ATP was added to cultures of Low Line cells in order to ascertain the effect of additional ATP on the lipid accumulation. It was found that both the action on the enzyme succinic dehydrogenase and the accumulation of lipid were the same in ATP-treated and control cultures. These results become difficult to

interpret when one considers the fact that one of the internal controls of fatty acid synthesis is the amount of ATP (White et al., 1968). Increases in ATP will inhibit the enzymes citrate synthetase and iso-citric dehydrogenase. An inhibition of citrate synthetase will reduce the activity of the tricarboxylic acid cycle resulting in an accumulation of acetyl co-enzyme A which will be shunted into fatty acid synthesis. When this is considered it becomes apparent that more work is necessary before the decrease in ATP observed in certain liver pathologies can be adequately understood in terms of lipid accumulation.

The observations of Lewis et al., (1967) and Mukherjee and Mukherjee (1969) suggest that the increase in lipids resulting from tetracycline administration are mainly due to increases in triglyceride content. The reversible nature of cellular lipid accumulation has been demonstrated by several investigators. Geyer (1967) has shown that lipid droplets begin decreasing after the conditions resulting in the accumulation are changed. He suggests that the decrease in triglyceride content is accompanied by an increase in phospholipid synthesis. Moskowitz (1967) claims that the disappearance of lipid droplets may not only result from changes in the conditions which originally resulted in lipid accumulation, but may also result because of an adaptive response of the cell.

Gale and Folkes (1953) found that the tetracyclines inhibited protein synthesis in bacteria at bacteriostatic concentrations. Since that time, numerous reports on the exact mechanism concerned with inhibition of protein synthesis have appeared in the literature. Connamacher and Mandel (1964) first reported that tetracycline could bind to the 30-S subunit of Escherichia coli. This observation has been confirmed by Day (1966 a,b,c) who also noted binding to the 50-S subunit although to a much smaller degree. More recently, Connamacher and Mandel (1968) have presented evidence from which they postulate that the tetracyclines bind specifically to the 30-S subunit of the ribosome at the point of attachment of tRNA, thus preventing the formation of a tRNA-mRNA-ribosome complex.

The present investigation confirms the inhibition of protein synthesis by tetracycline. A concentration of 100 mcg/ml definitely results in a decrease in the uptake of labeled amino acid into protein. However, the results obtained when cells are exposed to 50 mcg/ml do not demonstrate a decrease in the amount of protein per cell. There is a decrease in CPM/cell and CPM/mcg of protein. The effects on rate of cellular division by tetracycline must also be considered. It is possible that the rate of division has been slowed which probably results in an accumulation of protein within the cell even though the rate of protein synthesis has decreased.

The effect of tetracycline on serum protein synthesis is considered an important factor in regard to the accumulated lipid material noted earlier. The inhibition of total cellular protein synthesis by tetracycline may also apply to serum proteins synthesized by the cells. In this way synthesis of the protein moiety of the B-lipoprotein would be decreased. Since triglycerides are transported out of cells in the form of lipoproteins, a decrease in synthesis of the lipid-acceptor protein moiety may result in an accumulation of lipid material within the cells. It has been suggested that fatty liver resulting from ethionine and carbon tetrachloride administration may be due to a reduction in the rate of synthesis of plasma lipoproteins by the liver (Robinson and Harris, 1961). An inhibition of plasma lipoprotein production in liver slices exposed to puromycin has been shown by Robinson and Seakins (1962). The results of the present investigation, indicating an apparent decrease in synthesis of serum proteins by Low Line cells exposed to tetracycline, would seem to confirm this as a possible mechanism of tetracycline-induced lipid accumulation. Another factor which should be considered is the apparent binding of the tetracycline molecule to the lipoprotein complex. Lacko et al. (1959) first reported that tetracycline formed a complex with lipoprotein and actually used this property to isolate serum lipoproteins.

This was later confirmed by Berquist et al., (1963) who found that the tetracyclines were bound to the B-lipoprotein fraction of the serum proteins. Whether it is the effect of inhibition of protein synthesis demonstrated by the present study or a possible disruption of the transport mechanism by binding with the B-lipoproteins is still debatable.

SUMMARY AND CONCLUSIONS

CHAPTER V

SUMMARY AND CONCLUSIONS

Tetracycline in concentrations of 50 mcg/ml and above caused cytotoxic effects on Low Line cells as evidenced by morphological changes and changes in normal aerobic metabolism. Concentrations less than 10 mcg/ml resulted in no observable morphological changes. A concentration of 250 mcg/ml was almost immediately toxic to the cells.

The growth of the cells was stimulated by a concentration of 10 mcg/ml. Those exposed to 50 mcg/ml appeared to show a slight increase in growth rate from days 4 through 7 followed by a marked decline after day 7. The growth rate of those subjected to 100 mcg/ml was considerably decreased until day 7 after which a very sharp decline occurred.

The acid phosphatase activity of Low Line cells exposed to 10 or 50 mcg/ml of tetracycline was less than the control cells through the 5th day of the experiment. The curve representing the results obtained when the cells were exposed to 10 mcg/ml approximated that of the controls, while those cells exposed to 50 mcg/ml showed a definite increase in enzyme activity. At no time during these experiments was alkaline phosphatase activity detected in these cells.

DNA content of Low Line cells was decreased by all

concentrations of tetracycline employed. Ten mcg/ml resulted in a slight decrease through day 6 following which the curve representing DNA content per cell approximated that of the controls. After the 3rd day of treatment the cells exposed to 50 or 100 mcg/ml of tetracycline evidenced a leveling off of the content per cell. This was followed by a steady decline throughout the duration of the experiment. The RNA content of the cells was increased sharply by 100 mcg/ml of tetracycline through the 3rd day after which a rapid decline occurred. There was a decrease in RNA concentration of the cells exposed to 50 mcg/ml throughout the experiment, while 10 mcg/ml caused a increase from day 4 through 8.

The enzyme succinic dehydrogenase was inhibited by all concentrations of the drug used during these investigations. The greatest inhibition seemed to occur in those cells exposed to 100 mcg/ml of tetracycline. This effect was reversible after a four hour exposure and the activity was greatly increased after the drug was removed.

The cytochrome oxidase enzyme of Low Line cells appeared to be unaffected by tetracycline under the conditions of this investigation.

An accumulation of lipid droplets occurred in cells treated with 50 or 100 mcg/ml of tetracycline. This accumulation was reversible after a four hour exposure to the drug.

Low Line cells exposed to tetracycline exhibited a

decreased rate of synthesis of both total cellular protein and serum proteins.

It can be concluded that lipid accumulation occurring after tetracycline administration is the result of several factors. Two of the most important appear to be an inhibition of serum lipoprotein synthesis and enzyme inhibition. A decrease in B-Lipoprotein synthesis could result in a decrease in removal of synthesized triglycerides from the cells. At the same time, there appeared to be a decrease in the activity of the tricarboxylic acid cycle which would result in the availability of more acetyl co-enzyme A for synthesis of lipid material.

In attempting to relate these findings to the clinical situation, it could be said that the high blood levels of tetracycline resulting from large and rapid intravenous injections of the drug into normal patients or those suffering from kidney impairment could cause the above mentioned effects resulting in fatty degeneration of the liver.

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APPENDIXES

Appendixes

I. Data for Growth and Glucose Utilization

Day	Tetracycline mcg/ml	Cells/ml	Glucose Utilized/cell mcg x 10 ⁻³
1	0	125,000	0.68
	10	125,000	0.59
	50	125,000	0.50
	100	125,000	0.36
3	0	510,000	0.64
	10	560,000	0.69
	50	375,000	0.72
	100	510,000	1.10
5	0	915,000	0.52
	10	1,010,000	0.66
	50	965,000	0.59
	100	645,000	1.02
7	0	1,165,000	0.47
	10	1,495,000	0.62
	50	1,460,000	0.84
	100	760,000	1.21
9	0	1,570,000	0.42
	10	1,590,000	0.57
	50	620,000	0.95
	100	175,000	1.29

II. Lactate Produced and Acid Phosphatase Activity

Day	Tetracycline mcg/ml	Lactate Produced/cell mcg x 10 ⁻³	Acid Phosphatase Sigma Units/ mcg protein
1	0	0.36	6.9
	10	0.25	7.8
	50	0.25	9.1
	100	0.18	-
3	0	0.38	13.8
	10	0.54	11.2
	50	0.62	11.2
	100	0.78	-
5	0	0.36	15.5
	10	0.50	14.6
	50	0.48	16.8
	100	0.74	-
7	0	0.24	13.9
	10	0.57	14.7
	50	1.50	18.7
	100	1.86	-
9	0	0.19	12.1
	10	0.39	13.7
	50	1.71	19.2
	100	4.10	-

III. DNA and RNA Content

Day	Tetracycline mcg/ml	DNA mcg/cell x 10 ⁻⁶	RNA mcg/cell x 10 ⁻⁶
1	0	4.5	63
	10	4.5	63
	50	4.5	63
	100	4.5	63
3	0	12.8	84
	10	10.6	75
	50	9.1	71
	100	5.9	92
5	0	13.1	72
	10	12.5	77
	50	8.9	52
	100	4.4	89
7	0	13.4	59
	10	14.0	73
	50	8.3	51
	100	4.3	28
9	0	11.7	56
	10	11.0	52
	50	7.9	43
	100	3.2	18

IV. Protein Content

Day	Tetracycline mcg/ml	Protein mcg/ml	Protein mcg/cell x 10 ⁻³
1	0	57	0.48
	10	57	0.48
	50	57	0.48
	100	57	0.48
3	0	143	0.33
	10	139	0.29
	50	144	0.27
	100	121	0.27
5	0	276	0.31
	10	293	0.35
	50	291	0.33
	100	224	0.31
7	0	348	0.29
	10	363	0.34
	50	288	0.31
	100	227	0.30
9	0	461	0.29
	10	390	0.32
	50	281	0.32
	100	218	0.31

V. Effect on Protein Synthesis of a two Hour Exposure to Tetracycline. Average of two Experiments.

Tetracycline mcg/ml	CPM/cell x 10^3	CPM/mcg Protein	Mcg Protein/cell x 10^{-2}
0	14.4	3.4	3.6
10	11.6	3.8	3.4
50	11.9	3.1	3.4
100	8.1	2.9	2.8

VI. Effect on Protein Synthesis of a twenty-four hour Exposure to Tetracycline. Average of two Experiments

Tetracycline mcg/ml	CPM/cell x 10^3	CPM/mcg Protein	Mcg Protein/cell x 10^{-2}
0	9.8	2.9	3.6
10	10.1	2.7	4.1
50	8.1	2.4	3.6
100	6.6	2.1	3.0

VII. Effect of Tetracycline on Serum Protein Synthesis.

Tetracycline mcg/ml	CPM/mcg Protein x 10^{-1}
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0	7.4
100	5.6