A SYSTEM FOR STUDYING THE EFFECT OF VITAMIN A ON ^{3}H-URIDINE UPTAKE AND INCORPORATION IN CELL LINE I-407

by

Patricia Kelly

A Dissertation Submitted to
the Faculty of the Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Greensboro

1975

Approved by

Laura G. Anderton

Allen C. Magee

Dissertation Co-Advisers

APPROVAL PAGE

This dissertation has been approved by the following committee of the Faculty of the Graduate School of the University of North Carolina at Greensboro.

Dissertation Co-Advisers

Oral Examination Committee Members

September 7, 1973
Date of Examination

KELLY, PATRICIA. A System for Studying the Effect of Vitamin A on ^{3}H-Uridine Uptake and Incorporation in Cell Line I-407. (1975) Directed by: Dr. Laura Anderton and Dr. Aden Magee. Pp. 92

Vitamin A has been found to be involved in the morphological differentiation of epithelial tissue and to play a role in carcinogenesis in certain instances. Recently, the vitamin has been reported to stimulate RNA synthesis in intestinal epithelial tissue. An in vitro system in which to study the unknown mechanism of action and the effects of the vitamin was developed using a systems analysis approach.

An established line of intestinal cells, I-407, was employed to study the effects of vitamin A as measured by the uptake and incorporation of ^{3}H-uridine. The incorporation of ^{3}H-uridine was assumed to be due to incorporation by RNA. Attempts to deplete the cells of their vitamin A by lowering serum levels, and by the use of a special medium were abandoned in favor of attaining a more constant growth rate. Levels of 5 and 10 µg/ml of vitamin A were found to decrease the uptake and the incorporation of ^{3}H-uridine. A lower level of vitamin A, 1 µg/ml, was found to stimulate the incorporation of ^{3}H-uridine, but to produce no significant change in uptake of the label into the soluble pool fractions. When cold uridine was added to the incubation medium, however, an increase in pool fractions was seen with low levels of the vitamin.

The experimental groups were treated with vitamin A for a specified period of time, usually 10 minutes, before receiving a 10 minute pulse of 2 µCi/ml of ^{3}H-uridine. The cells were immediately washed 3 times, removed from the flasks by trypsinization, and then washed 3 more times by sedimenting and resuspending in cold phosphate buffered saline (PBS) to remove excess label. Next, the cells were homogenized in 5% trichloracetic acid

(TCA), which precipitated the macromolecular fraction, including RNA, and left the smaller molecules, including free ^{3}H-uridine, in solution. Samples of both TCA soluble and TCA insoluble fractions were taken for liquid-scintillation counting.

A variability study was conducted and the system of experimentation was analyzed in detail in order to locate problem areas and develop controls. Variability in growth rates was attributed in part to the glassware and to the serum and other components used in preparing the media. Variability in results from duplicates obtained from the same flask was attributed to sample preparation. Faster and better results were attained by taking aliquots of the freshly washed and resuspended TCA insoluble fraction, rather than drying the fraction and weighing out small samples.

RNA synthesis, as evidenced by the incorporation of ^{3}H-uridine, was effected by the level of vitamin A added; but no reason was found for stipulating that the effect was a direct action on RNA synthesis at the transcriptional level. The fact that vitamin A influenced the uptake of tritiated uridine into soluble pools in most instances is supporting evidence for a permeability change. It was concluded that the system described offers possibilities for additional studies on the effects of vitamin A and other nutrients.

ACKNOWLEDGMENTS

I would like to express my gratitude to all the persons who have aided and supported me in carrying out this research. I would especially like to express my gratitude to Dr. Laura Anderton for her capable guidance and continuous support throughout this study; to Dr. Aden Magee, Chairman of my graduate committee, for his ready assistance and cooperation; and to the other members of my advisory committee: Dr. Mildred Johnson for her encouragement; Dr. Arthur L. Svenson for adding perspective to this study; and Dr. William Bates for help with problems related to scintillation counting. I am also indebted to Dr. Richard Schauer for the use of his laboratory and facilities and for his collaboration on the analytical aspects of this work; to Mrs. Linda Curtis for technical assistance with all aspects of tissue culture and with photography; and to Dr. William Powers for assistance with the statistical analyses. Finally, I would like to acknowledge the support and patience of my husband and children.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX TABLES

APPENDIX A

PROCEDURES AND MATERIALS
FOR CELL CULTURE

LIST OF APPENDIX TABLES

APPENDIX B

DATA

LIST OF APPENDIX TABLES

APPENDIX C

ANALYSES OF VARIATION
VARIABILITY STUDIES

LIST OF FREQUENTLY USED ABBREVIATIONS

RNA - ribonucleic acid

tRNA - transfer ribonucleic acid

MEM - minimal essential medium

FBS - fetal bovine serum

PBS - phosphate buffered saline

TCA - trichloracetic acid

NCS - Nuclear Chicago Solubilizer

DPM - disintegrations per minute

CPM - counts per minute

ESR - external standard ratio

μCi - microcuries

CHAPTER 1

INTRODUCTION

Cancer research continues to receive major emphasis in many medical laboratories and institutions throughout the world. A particular type of cancer that has attracted local interest is familial polyposis, a kind of cancer of the colon, which was discovered to be prevalent in a family residing in the Greensboro, North Carolina area. Because members of the family who develop the cancer do so at different ages, frequently at puberty or menopause, the involvement of hormones and of certain environmental factors, such as additive hormones and vitamins, was suggested.

Previous studies have shown that vitamin A (retinol) in certain instances either increases or decreases the susceptibility of epithelial tissue to cancer. This effect varies with the kind of epithelium and with other factors. There is the possibility that dietary levels and/or additive levels of vitamin A may influence the carcinogenesis in familial polyposis.

An established line of intestinal cells was chosen for the study because of the many advantages it affords. The cells grow rapidly and can be purchased at the same passage number for repeat experiments. In addition, an established line is made up of a single cell type; and interactions of different cell types are avoided.

Because tissue culture systems are individualistic and depend upon equipment, resources, the characteristics of the cell line, and the type

of experiment, an arbitrary system for studying vitamin A *in vitro* was chosen and modified. Hopefully, the basic system with minor modifications can be used for studying other factors and nutrients *in vitro*. Additionally, it is hoped that the work will serve as a model for applying systems analysis to bio-nutritional systems.

A Medline search of literature was obtained from the Department of Health, Education, and Welfare, National Institutes of Health, Bethesda, Maryland. The search was designed to pick up any references to cell line I-407, and to relationships between vitamin A and cells in culture, and vitamin A and carcinogenesis, and to retrieve detailed metabolic studies on vitamin A, from approximately 1100 journals for a period from January 1969 to August 1972. About 50 related and somewhat related references were retrieved, but there were no references to research employing cell line I-407. Although there were several references to research on the effect of vitamin A on organ cultures, the literature concerning the effect of vitamin A on cell cultures was very sparse. Perhaps this work will make a contribution to that small volume of literature.

CHAPTER II

REVIEW OF LITERATURE

The literature pertinent to this research included literature on general functions of vitamin A, and the relationships between vitamin A and ribonucleic acid (RNA) synthesis in epithelial tissue, vitamin A and membranes, vitamin A and cells in culture, and vitamin A and carcinogenesis.

The abundant literature on the general functions of vitamin A was reviewed briefly in a search for possible clues to its molecular mechanism. More attention was devoted to detailed cellular studies than to gross animal experiments.

General Functions of Vitamin A

Although vitamin A functions in vision, reproduction, bone and cartilage, epithelial tissue, and is required for growth, its basic mechanism of action remains unknown. An exception is that the role of vitamin A in vision has been established. The molecular basis of the participation of vitamin A in vision was worked out by Wald and Hubbard (1) who found that the combination of 11-cis retinal with an ε-amino group of lysine in rhodopsin confers the light-labile and stereospecific properties to that compound. Whether the other functions of vitamin A involve the production of such highly-ordered conformations remains to be proven or disproven. Much of the research on vitamin A in the past was directed unsuccessfully toward establishing a conenzyme role for the vitamin.

The effect of vitamin A on epithelium has been demonstrated by the classical experiments of Fell and Mellanby (2) and Fell (3). When 10 I.U./ml of retinol were added to the culture medium of epithelial explants of 7 day old chick embryos, normal keratinization was inhibited; and a mucus-secreting epithelium resulted. An excess of vitamin A has been found to greatly increase the release of lysosomal enzymes, cause resorption of cartilage and bone, and alter membrane systems. In the whole animal, a deficiency of retinol leads to the degeneration of the retina, causes sceretory epithelia to become keratinized, and produces bone malformations.

Vitamin A and RNA Synthesis in Epithelial Tissue

Vitamin A is involved in the growth and morphological differentation of epithelial tissue. In vitro experiments with epithelial tissue show that the production of keratin and mucoprotein depends upon the concentration of vitamin A and the type of epithelium involved. Generally, keratin is produced at lower concentrations of vitamin A and mucoprotein at higher concentrations. Zachman (4) found that vitamin A stimulates the synthesis of RNA in the intestine of vitamin A-deficient rats. He found an average ratio of incorporation of uridine by retinol treated to controls of 1.8 ± .9.

Other workers have also noted effects of vitamin A on RNA synthesis in epithelium. Johnson et al. (5) reported that nuclear RNA synthesis is very rapidly increased when vitamin A is administered to vitamin A-deficient rats. The incorporation of ^{3}H-5-orotic acid into RNA of rat intestinal mucosa was measured by Zile and DeLuca (6) who found a 1.5-3.0

fold stimulation when retinol was administered to the deficient rat. According to DeLuca et al. (7) there is no qualitative difference between normal and vitamin A-deficient iso-accepting species of leucyl transfer ribonucleic acid (tRNA), but the total amount of tRNA in the mucosa was decreased in the vitamin A-deficient rat. DeLuca and workers noted that the difference in uridine incorporation was not due to a dilution effect of the label in the deficient rat or to any changes in cell permeability. DeLuca et al. (8) have also shown a decrease of a specific fucose-containing glycopeptide in the vitamin A-deficient animal, while Kaufman et al. (9) reported that the electrophoretic pattern of RNA molecules synthesized by the vitamin A-deficient epithelium in vitro differed from that synthesized by the normal tracheal epithelium in vitro. In addition, Kaufman found that the normal epithelium produced a greater percentage of RNA species with low electrophoretic mobility. The preceding experiments on the effect of vitamin A seem to indicate the possibility that the vitamin acts at the transcriptional level. In support of this view, Raisz (10) found that actinomycin D blocks the effect of vitamin A on bone resorption. However, none of these studies provide firm evidence to indicate whether the effect of vitamin A on RNA synthesis is a direct or an indirect effect. It is interesting that estrogen stimulates the synthesis of nuclear RNA in the uterus and that this response is lost in the vitamin A-deficient animal. This finding supports evidence that vitamin A is necessary for the action of estrogen (11).

Vitamin A and Membranes

The possibility exists that the multifarious effects of vitamin A are all attributable to its membrane effects. Vitamin A is found to

effect both cellular membranes and the membranes of subcellular fractions. The effect, however, seems to be related to the concentration of vitamin A, because toxic doses of the vitamin cause a rapid breakdown of lysosomes resulting in release of hydrolytic enzymes. Keiser et al. (12) found that the effect of vitamin A on mitochondrial and lysosomal membranes was dependent upon the concentration of the vitamin. Concentrations of 10^{-4}M to 3×10^{-5}M vitamin A inhibited swelling of mitochondria and decreased the release of certain enzymes. From histopathological studies of the nerves of rats suffering from xerophthalmia, Mellanby (13) concluded that a vitamin A deficiency causes a degeneration of myelin. Other studies have been done with artificial membranes to investigate the possible participation of vitamin A as a part of membrane structures (14). Vitamin A was found to increase hydrophilic layer thickness in artificial membranes. While vitamin A has not been shown conclusively to be a structural part of living membranes, it has been shown to regulate the binding of ATPase to the erythrocyte ghost (14). Vitamin A may function at a lipid-water interface and may participate in membranes by enhancing lipid-protein interactions similar to those involved in the formation of the retinol transport complex in serum (15). Other theories hold that vitamin A may possibly function in micelle formation and electron transfer in membranes (16). At this time the possibility that the effect of vitamin A on RNA synthesis may be secondary to changes of a highly-ordered nature in membranes cannot be excluded.

Vitamin A and Cells in Culture

Vitamin A has been studied extensively in organ culture, but not extensively in cell culture (17). Paul (18, 19) stated that the fat

soluble vitamins did not appear to be essential for vertebrate cell survival in vitro. Thomas and Pasternak (20) concluded that vitamin A was not required for the growth of neoplastic mast cells or for the synthesis of heparin by these cells. Kochhar et al. (21) reported that the addition of vitamin A to the medium of an established line of mouse fibroblasts caused the cells to release 2-4 times as much glucosamine-labelled products without reducing the glucosamine content of the treated cells below that of the controls. Their finding that added vitamin A impaired cellular multiplication without producing lysis or death is contrary to reports that vitamin A increases the mitotic index in organ culture.

Experiments designed to establish the absolute vitamin A requirement of human cells in vitro have not been successful, because it has not been possible to grow human cells for an appreciable length of time without serum which normally contains a small amount of vitamin A. Attempts to destroy the vitamin A in serum by ultraviolet irridiation usually result in the destruction of the growth promoting properties of the serum (21).

Vitamin A and Carcinogenesis

Vitamin A has been shown to inhibit the induction and retard the growth of certain experimental tumors. In other instances, it has been shown to potentiate the effect of chemical carcinogens. The effect of vitamin A on carcinogenesis appears to vary with different animals and in different tissues. Crocker and Sanders (22) have demonstrated that vitamin A and benzo(a)pyrene act in a competitive fashion in hamster

respiratory epithelium and in an additive fashion in cartilage in the same animal. Polliack and Levij (23) reported that hamsters which were treated with 9,10 dimethyl-1,2-benzanthracene (DMBA) were found to have a high instance of cervical cancer if killed immediately after treatment with DMBA ceased. If the animals were killed two months after the cessation of treatment with DMBA, the tumors were found to have regressed in size and number. However, if topical vitamin A were introduced after cessation of treatment with DMBA, the size and number of tumors were found to have increased. The effects may have possibly been direct, or vitamin A may have acted indirectly to produce a change in cellular permeability or to alter cellular metabolism.

CHAPTER III

MATERIALS AND METHODS

The inherent variability of the cells and details about their metabolism and growth characteristics were unknown at the beginning of the experiments. It was also unknown whether or not the cells required vitamin A or would respond to its presence in their medium. In the event that the small amount of vitamin A present in serum was enough to satisfy the cellular needs, it was likely that a period of depleting the cells of their vitamin A would be necessary or beneficial in demonstrating the effect of vitamin A on the cells.

Vitamin A depletion has characteristically presented major problems in experiments conducted in vivo because of the length of time required to produce a vitamin A deficiency in the animal and the fact that the vitamin A-deficient animal is very sick and susceptible to infections. Cell culture circumvents these particular problems, but presents a new set of problems due to the fact that the vitamin A requirement of cells in culture has not been established and no criteria for producing a deficiency have been developed. In view of these uncertainties, the strategy used was to reduce the vitamin A level of the medium as low as possible and still maintain adequate growth. An attempt was made to grow the cells without serum on a completely defined medium (24, 25). The composition of the medium is given in Appendix A. Subsequently it was found that the cells could only be adequately maintained on the medium for 5 or 6 days. In the earlier experiments, the cells were depleted by using a medium

containing serum levels of 1% for a period of at least a week prior to being subcultured in preparation for the experiments. No depletion period was used in the later experiments.

Objectives

The objectives of this work were: (1) to determine if vitamin A had any effect on RNA synthesis in cell line I-407 as evidenced by ^{3}H-uridine incorporation into the macromolecular fraction and uptake into soluble pools; and (2) to use systems analysis in order to determine and evaluate the kinds of problems involved in the work and establish controls for the experimental procedures.

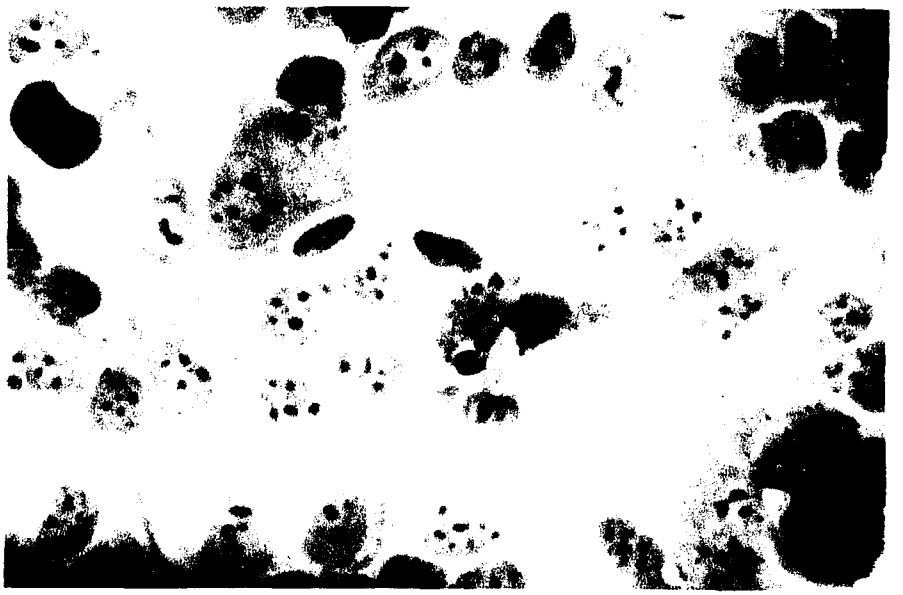
Cell Line

Cell line I-407, also called CCL 6, was purchased from the American Type Culture Collection[1] and used throughout the experiments. These epithelial-like cells were originally derived from human embryonic intestine (26). Appendix A gives detailed information about I-407 and Figure 1 shows various photomicrographs of slides of I-407 stained with Alcian blue (27). The procedure for preparing slides is given in Appendix A.

In theory it would be more desirable to work with diploid cells; however, in practice there are fewer problems associated with growing an established line for extended periods of time. Cell line I-407 is 100% aneuploid and can be maintained and subcultured indefinitely. Cells with

---

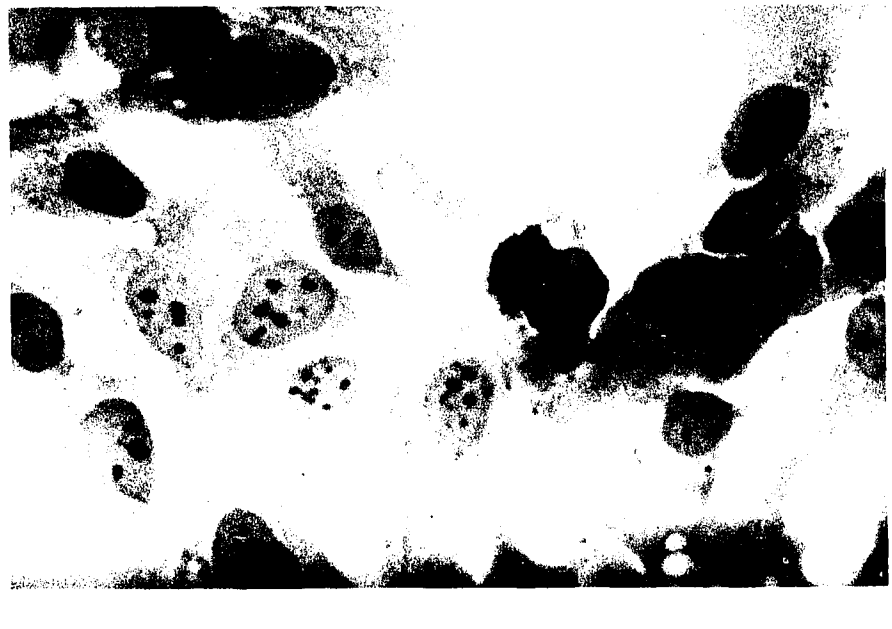
[1]American Type Culture Collection, 2301 Parklawn Drive, Rockville, Md.



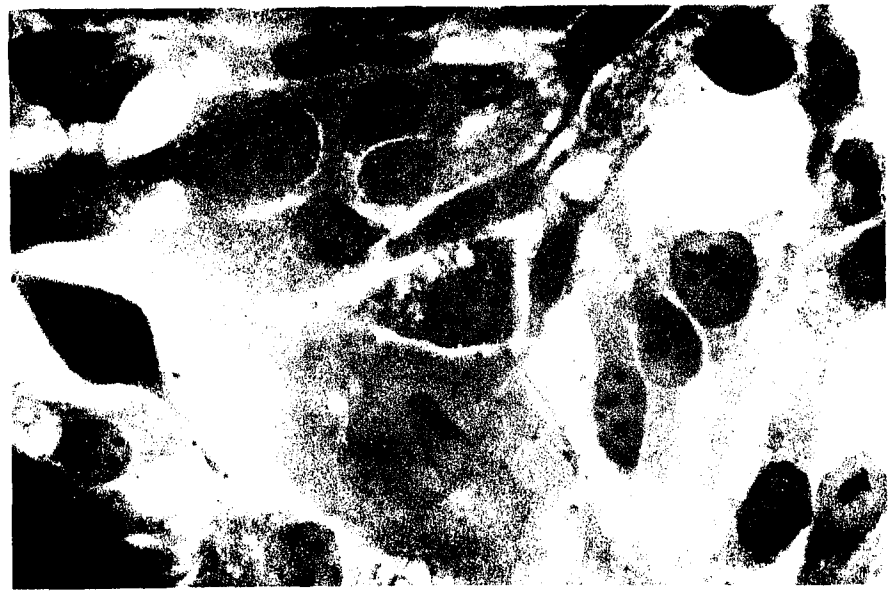
1a. The difference in nuclear and cellular size is typical of the morphology of cell line I-407. Note typical anaphase in the upper left and atypical multipolar spindles in the lower right.

1b. Also note difference in size of cells. There is an abnormal mitotic figure at the left which includes a multipolar spindle.

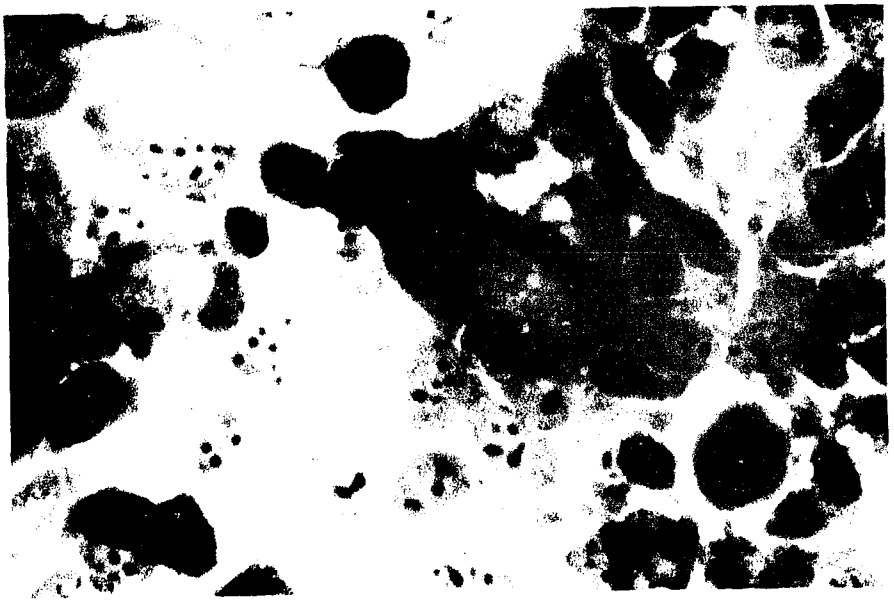
Figure 1. Photomicrographs of Cell Line I-407



1c. There is a large nucleus at the left top. At the far right, a mitotic cell appears to be in telophase.

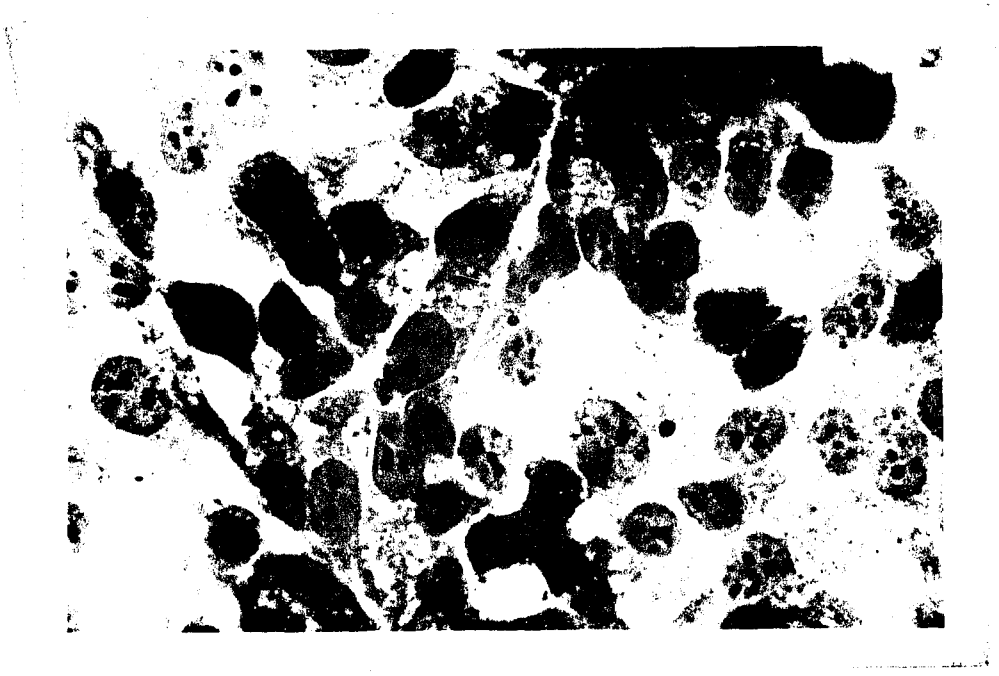


1d. At the far left, observe a mitotic figure having microspikes which anchor it to the substrate for cellular division. There is a dividing cell in anaphase in the upper right.

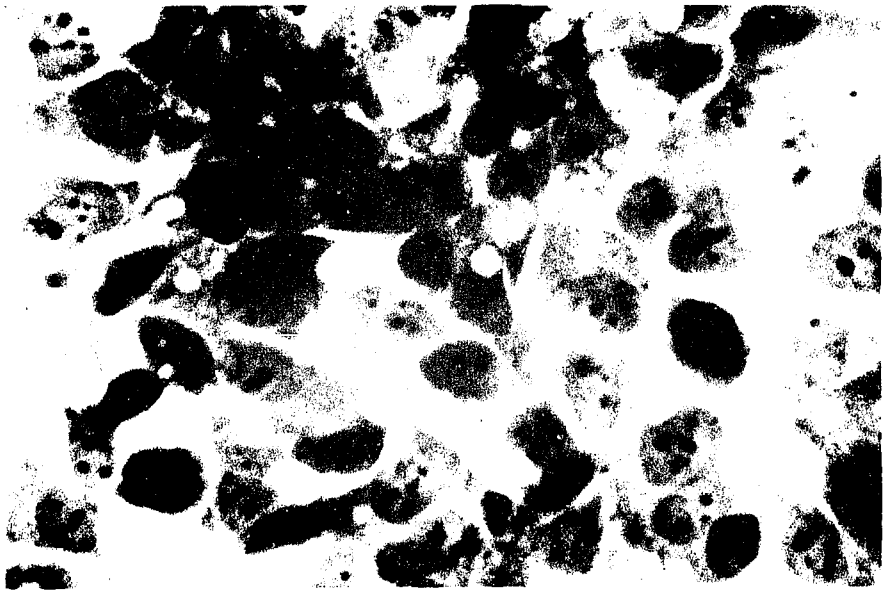


1e. Two cells in metaphase are located in the upper central and lower right. There is a mitotic figure in telophase at the left of center.

1f. In the center is a large polyploid cell in mitosis. There is a cell in anaphase which is located below the center.



1g. In the left center is a normal appearing metaphase cell with two microspikes holding it to the substrate. There is an abberant tripolar mitotic figure located below center.



1h. At the left appears to be a dead cell with a disintegrating nucleus. A cell in telophase appears in the lower left.

the normal diploid number grow more slowly and divide only for a limited number of times before beginning to decline.

Conclusions drawn from results with I-407, or with any cells in culture, must be interpreted cautiously, because the conclusions hold primarily for the cell line and conditions used. Although cell line I-407 was derived from intestinal cells, the cells cannot be spoken of as normal adult intestinal cells. In the process of adapting to life in the test tube and also in becoming an established cell line, certain metabolic pathways become preferred over others in a manner which has not been fully investigated. Cell line I-407 resembles tumor cells or perhaps, precancerous cells that have escaped normal control mechanisms to a certain extent. These cells have the ability to produce tumors in mice when injected in sufficient numbers.

Population dynamics is involved in the cultivation of cells such as I-407. There is a minimum number of cells per volume of medium which is required for growth and survival. Since different cells in the population have vastly different chromosome numbers, it could be assumed that different metabolic pathways are favored in different cells and that there is a certain amount of cross-feeding of metabolic intermediates. It could also be assumed that changing conditions of the culture medium could shift the population dynamics.

The cells were observed under the tissue culture microscope a minimum of 3 times a week to check for a healthy, normal appearance. In a normal, healthy culture of I-407, a large percentage of the cells spread out on the surface of the flask and give a flat, epithelial-like appearance. Unhealthy cells round up and do not spread out on the surface by

the first day following subculture. This condition may be encountered if the cells do not have the proper nutrients, if a noxious chemical substance is present, or if they are contaminated by viruses, bacteria, mold or any other organisms which may be growing in their medium. Cells in mitosis also round up, but are accompanied by a large number of cells spread out on the surface if the cultures are unsynchronized.

Cell line I-407 grows as a monolayer and reaches confluency in less than 1 week when grown on minimal essential medium (MEM), Table 1, supplemented by 5-10% fetal bovine serum (FBS), non-essential amino acids, sodium pyruvate, and glutamine, Table 2. In addition, 50 units/ml of both penicillin and streptomycin were added. Supplies used for preparing the media were obtained from GIBCO[2].

Preparation of Cells

The frozen cells were shipped from the repository packed in dry ice. They were thawed quickly, diluted to give 10^6 cells per ml, and then placed in flasks which had been pre-equilibrated with the culture medium and a sterile air mixture containing 5% carbon dioxide. Methods for freezing and thawing the cells are given in Appendix A. Stock cultures were carried in either 30 ml or 150 ml plastic disposable flasks[3] and the experiments were run in the smaller flasks unless otherwise indicated.

---

[2]Grand Island Biological Company, 3175 Staley Road, Grand Island, N.Y.
[3]Falcon Plastics, Los Angeles, Calif.

TABLE 1

COMPOSITION[a] OF MEM, MINIMUM ESSENTIAL MEDIUM (EAGLE)
WITH EARLE'S BALANCED SALT SOLLUTION
FOR MONOLAYER CULTURES[b]

Component	mg/L	Component	mg/L
NaCl	6800.00	L-Threonine	48.00
KCl	400.00	L-Tryptophan	10.00
$NaH_2PO4.H_2O$	140.00	L-Tyrosine	36.00
$MgSO4.7H_2O$	200.00	Valine	46.00
$CaCl_2$ (anhydrous)	200.00	Choline Cl	1.00
Glucose	1000.00	Folic acid	1.00
L-Arginine	105.00	i-inositol	2.00
L-Cystine	24.00	Nicotinamide	1.00
L-Glutamine	292.00	D-Ca pantothenate	1.00
L-Histidine	31.00	Pyridoxal HCl	1.00
L-Isoleucine	52.50	Riboflavin	0.10
L-Leucine	52.40	Thiamin HCl	1.00
L-Lysine	58.00	Phenol red	10.00
L-Methionine	15.00	$NaHCO_3$	2200.00
L-Phenylalanine	32.00		

[a]GIBCO catalogue (1974), p. 121
[b]Reference: Eagle, H. 1959. Science 130:432

TABLE 2

COMPOSITION OF SUPPLEMENTED MEM + 5% FBS

Component	ml/100 ml medium
MEM, Earle's 1X	90
MEM Non essential amino acids* (100X)	1
L-Glutamine (29.2 mg/ml)	1
Sodium Pyruvate Solution (100 mM)	1
Fungizone (Amphotericin B, 250 mcg/ml)	1
Penicillin-Streptomycin Solution (5,000 units penicillin and 5,000 mcg streptomycin/ml)	1
Fetal Bovine Serum	5

*Composition[a] of Non Essential Amino Acid Solution (100X)

Component	mg/L
L-alanine	890.00
L-Asparagine.H_2O	1500.00
L-Aspartic acid	1330.00
L-Glutamic acid	1470.00
L-Proline	1150.00
L-Serine	1050.00
Glycine	750.00

[a]GIBCO catalogue (1974), p. 121

Subculturing

When the cells reached confluency in the flasks, they were subcultured in a ratio of 2:1. The procedure of subculturing consisted of suctioning off the medium and washing the cells twice with Ca^{++} and Mg^{++} free Dulbecco's phosphate-buffered saline (PBS), before removing the cells from the flask with .25% trypsin in PBS. The cells were allowed to remain in the trypsin solution until they began to detach from the surface (about 10 minutes). Steps in the subculture procedure are given in Appendix A. Aliquots of the cell suspension containing approximately 10^6 cells were placed in the flasks which had been adjusted to approximately pH 7.2 by bubbling a sterile mixture of 5% carbon dioxide and air into the medium. Further action by trypsin was prevented by a trypsin inhibitor contained by serum, a constituent of the medium. The medium was changed on the day following subculture and at regular intervals thereafter.

Cell Counts

The haemocytometer technique combined with the trypan blue dye exclusion technique was used to count cells (28). The dye exclusion technique differentiates living from dead cells, because dead cells do not have the ability to exclude the dye. Since the use of the haemocytometer results in an error of at least 10%, estimation of cell numbers, a technique commonly used by many laboratories, was used routinely.

The estimation of cell numbers is possible because the number of cells contained in a confluent monolayer of the same line of cells is roughly equivalent in all flasks of the same size. For this reason, cell numbers could be estimated quite conveniently and accurately by estimating

the percentage of confluency. The fact that the experiments were begun when the monolayers approached confluency resulted in approximately the same number of cells being used in all experiments. Furthermore, all the flasks used in a given experiment were prepared by taking equal aliquots of the same cell suspension. Repeated use of the haemocytometer technique indicated that a confluent monolayer of the cells in the 30 ml flask contained $2.0 - 2.5 \times 10^6$ cells. The counting procedure is included in Appendix A.

Sterility Precautions

The most stringent sterile techniques were observed in growing the cells, in order to prevent the growth of bacteria and other micro-organisms. Although penicillin and streptomycin were used, sterile techniques could not be disregarded because resistant strains may be encountered and certain organisms, in particular pleuropneumonia-like organisms (PPLO), are not inhibited by penicillin and streptomycin. In the event of contamination, the inhibition of cellular wall formation by penicillin makes it possible for L-forms of bacteria, bacterial protoplasts without cellular walls, to become stabilized in the culture medium. PPLO may grow inside the cells without producing morphological changes, or any readily observable change other than slowing down the growth rate of the cells somewhat. The presence of PPLO would be likely to distort the results of metabolic studies.

Plates for the detection of PPLO were streaked with .1 ml of cell suspension and placed in a carbon dioxide incubator. After a period of 1 week, the plates were examined for the presence of minute PPLO colonies,

which have a fried-egg appearance. Autoradiography employing ^{3}H-thymidine may be used to test for PPLO. The presence of large numbers of radioactive granules in the cytoplasm is evidence for the presence of PPLO. Kanamycin and other antibiotic agents which inhibit PPLO growth are available. However, PPLO were not detected as contaminants during the experiments.

Gram stains and other routine bacteriological procedures were used to test for bacteria when contamination was suspected. Since PBS and MEM were frequently prepared in the laboratory by diluting the 10X stock solution, samples from each preparation were incubated for several days to check for bacterial growth. The procedures for preparing the 1X solutions from the 10X concentrates are outlined in Appendix A. It was found that carbon dioxide tends to be driven off when the sodium bicarbonate buffer solution is autoclaved, but can be added back by bubbling sterile carbon dioxide through the solution. However, a sterile sodium bicarbonate solution may be purchased in the diluted form.

Careful attention was paid to autoclaving times in order to eliminate bacterial spore formers as a source of contamination. A list of autoclaving conditions which were used is given in Appendix A. Materials sterilized in paper bags were re-autoclaved if not used in a period of 2 weeks.

The laboratory was ideally equipped with a small room containing the tissue culture supplies, hood, and incubator; and the room was used exclusively for tissue culture. The room was built to maintain a positive air pressure, which helped to prevent the flow of dust into the room. Routine cleaning was done by laboratory personnel in order to prevent

contamination of the laboratory. Floors and counter tops were washed with Lysol weekly, and the hood was washed with a 10% Chlorox solution before and after each use. The hood under which the tissue culture procedures were performed also maintained positive pressure and was equipped with ultraviolet and fluorescent lighting and outlets for a gas burner and vacuum hose.

Glasswashing Procedure

Proper care was given to the glassware because of its extreme importance in the tissue culture laboratory. Cells in culture are very sensitive to chemical contamination and will not grow in glassware which has been in contact with formaldehyde. In order to avoid chemical contamination, the tissue culture glassware was kept separate from all other glassware and was rinsed and filled with water immediately after using. All tissue culture glassware was boiled for 5 minutes in a 1:100 dilution of "7X" detergent[4], scrubbed and rinsed 10 times in tap water, 2 times in distilled water, and then oven dried before autoclaving. Any glassware used for growing cells was rinsed additionally in glass distilled water. The glassware was not allowed to stand for extended periods of time in the detergent solution, and the rinsing procedure was followed closely. Appendix A contains additional information about the glasswashing procedure.

---

[4]Linbro Chemical Company, New Haven, Conn.

Physiological Levels of Vitamin A

Since abnormally high levels of vitamins may produce effects which have nothing to do with the normal roles of the vitamins, levels of vitamin A which were within the normal physiological range were desirable for the experiments. It was not clear what level of vitamin A would constitute a normal level in cell culture, but a working hypothesis was that levels approximating human serum levels were normal levels. In addition, vitamin A in the serum was known to be bound in a complex while the vitamin A used in the experiments was in a free form. Whether the free form or the bound has greater entry into the cell has not been established. Presumably, there would be a small amount of the binding agent contributed by the serum in the medium. It should be pointed out that considerable variability in serum levels of vitamin A is found in apparently healthy animals, with 1 µg/ml being more or less average, and levels of .4 µg/ml being sometimes associated with deficiency symptoms, such as night blindness (29). Because the length of treatment with vitamin A was to be very short, usually 10 minutes, a level somewhat higher, 5-10 µg/ml, was chosen for the experiments. This was the level found to be the most effective in producing mucous secretion in organ culture (2, 3).

Preparation of Vitamin A Dilutions

Due to the fact that vitamin A is more stable in nonaqueous solvents and in concentrated solutions, a concentrated stock solution of vitamin A was prepared by dissolving 100 mg of the vitamin (trans-retinol)[5]

---

[5]Sigma Chemical Company, St. Louis, Mo.

in 1 ml of absolute ethanol. This stock solution was kept in the freezer at -20°C and fresh dilutions were made on the day of each experiment. The final concentration of ethanol in the medium did not exceed .5% in any experiment and was about .25% in most experiments. All dilutions of the vitamin were shielded from light to prevent chemical breakdown. The experimentals and controls were treated with equal amounts of ethanol. The vitamin A solutions used in experiments lasting for longer than 2 hours were sterilized by passing the solution through a millipore filter[6].

The first dilution of the 100 mg/ml stock solution was prepared by taking .1 ml of the stock solution plus 4.9 ml ethanol, to give a vitamin A concentration of 2 mg/ml. The second dilution of the stock was prepared by taking .5 ml of the first dilution and 4.5 ml of the medium, to give a concentration of .2 mg/ml. When .05 ml of the second dilution was added to the 2 ml of medium in the flask, a final concentration of 5 µg/ml of vitamin A and .25% concentration of ethanol were achieved.

Other Chemicals

Uridine-5-^{3}H had a specific activity ranging from 25.7-28.5 Ci/mM.[7] Nuclear Chicago Solubilizer (NCS)[8] was used for digestion of samples, and all samples were counted in 10 ml of scintillation fluid (Packard Permaflour)[9] containing .1 g P-bis {2-5 (phenyloxazolyl)} benzene (POPOP) and 5.0 g 2,5 diphenyloxazole (PPO) per liter of toluene.

---

[6] Millipore Filter Corp., Bedford, Mass.
[7] New England Nuclear Corp., Boston, Mass.
[8] Packard Inst. Co., Downers Grove, Ill.
[9] Amersham/Searle Corp., Arlington Heights, Ill.

Equipment

An Olympus tissue culture microscope model CK[10] with phase contrast attachment was used to observe the cells. A refrigerated International Centrifuge, model B-20A; a clinical centrifuge, International model CL[11]; and a motor-driven[12] ground glass homogenizer were used in the analytical procedures. Counting was done with a Beckman liquid scintillation counter, model LS-100.[13]

Experimental Procedure

The basic experimental procedures consisted of preparing the cells for experimentation; treatment of the cells with vitamin A and ^{3}H-uridine; centrifuging, washing, and homogenizing the cells; and preparing and counting the samples.

The cells were subcultured 2 or 3 days in advance of the experiments by pooling the contents of 5 small flasks and dividing the cells into 10 new flasks. The 8 most uniform appearing flasks were selected. The occasional failure of a flask to hold pH was attributed to loss of carbon dioxide by defective flasks and could be recognized by a color change in the medium. A color comparator[14] was used to judge the pH of the flasks. The optimum pH, 7.2 for cell line I-407, was a salmon pink color due to the presence of a phenol red indicator. A more acidic condition resulted in a yellowish color and a more alkaline condition in a purplish color.

---

[10] C.P. Hall Co., Akron, Ohio
[11] International Equipment Co., Needham, Mass.
[12] Talboy's Engineering Corp., Emerson, N.J.
[13] Beckman Instruments Inc., Atlanta, Ga.
[14] LaMotte Chemical Products Co., Chestertown, Md.

The flasks were observed for uniformity on the day of the experiment. The experiments were begun when the cells were judged to be in logarithmic growth, usually the second or third day following subculture.

On the day of the experiment, the prescribed amount of vitamin A, in most instances .05 ml of the second dilution, was added to the experimentals, and an equivalent amount of ethanol was added to the controls during the prescribed treatment period of 10 minutes. The culture flasks contained 2 ml of media, so the addition of .05 ml of the second dilution of the vitamin brought the final concentration of vitamin A to 5 µg/ml and the concentration of ethanol to .25%. For experiments using a level of 1 µg/ml vitamin A, a 1:5 dilution of the second dilution was prepared. Comparable dilutions were made for the other levels. For the 10 µg/ml level of vitamin A, .1 ml of the second dilution was added to the flasks.

The addition of the isotope was timed carefully so that all flasks received the same pulse length of 10 minutes, except in the pulse length study. To each flask was added .5 ml of the ^{3}H-uridine solution to achieve a level of 2 µCi/ml. At the end of the incubation period, the supernatant medium was quickly removed, and the cells were washed twice with 3 ml PBS before being removed from the flasks with a .25% trypsin solution, as in the subculturing procedure. Immediately after removal, the cells were placed in centrifuge tubes at 0^{o}C and sedimented at 1500 g in a refrigerated centrifuge. The supernatant was decanted and the cells were resuspended and washed 3 times in 3 ml of ice cold PBS, in order to bring the isotope count of the final wash near the background count.

The washed cell pellets were transferred with .2 ml PBS to a ground glass homogenizer. The addition of .2 ml 10% trichloracetic acid (TCA)

and 1.6 ml 5% TCA gave a final concentration of 5% TCA in a volume of 2 ml. The homogenization process required 1-2 minutes, after which the homogenate was sedimented at 1400 g for 10 minutes in a clinical centrifuge. Aliquots (.1 ml) of the soluble fractions were taken for scintillation counting. The TCA insoluble fraction was washed 3 times with 5 ml of 5% TCA, so that the final wash was near the background count. In the original procedure, the pellets were dried for at least 2 days in a 37°C drying oven and samples of 1 mg were taken for scintillation counting.

The samples for liquid-scintillation counting were prepared by carefully weighing out 1.0 mg samples of the dried TCA insoluble pellets, adding 20 µl of water, .5 ml NCS, and allowing the sample to digest overnight. The samples were counted in 10 ml of scintillation fluid.

The method of quench correction used was the external standard ratio (ESR), a method which employs an external standard in conjunction with the channels ratio technique. The liquid scintillation counter used, Beckman Model LS-100, employs cesium^{137} as the external standard, and is fully automated. The Beckman module for counting tritium was used. Four separate counts were automatically taken--the sample alone in each channel and the sample plus the external standard in two channels simultaneously. The net count in each channel was determined and a ratio of net counts in the upper channel to net counts in the lower channel (the ESR value) was printed out. All counting was done at 2% standard error. A gain setting of 2.5 was used.

Since individual types of biological samples serve as quenching agents, decreasing the observed counts per minute (CPM), it was necessary to do preliminary studies to determine quench produced by samples of I-407.

By using ^{3}H-toluene with specific activity of 1.68 X 10^{6} disintegrations per minute (DPM) and observing the CPM in the presence of different quantities of the TCA insoluble samples, information was obtained for establishing the relationship between ESR values and efficiency in counting for the individual experiments. A 1:10 dilution of the stock ^{3}H-toluene[15] was prepared. One tenth ml of the dilution, or 16,800 DPM, were added to a blank and samples containing .25, .5, .75, 1.0, 2.0, and 2.5 mg of TCA insoluble samples, giving the results shown in Table 1 of Appendix B and in Figure 2.

That the TCA insoluble sample was not a strong quenching agent was indicated by good efficiency in counting. The ESR values were not altered greatly by the larger samples, but a significant factor in efficiency in counting was the amount of water used to disperse the sample. Better efficiency in counting was attained by dispersing the sample in .1 ml of water rather than .02 ml. The amount of NCS used could accommodate .1 ml of water. Figure 2 shows the difference in efficiency attained using the different levels of water. Average efficiency ratings were used for individual experiments because the ESR values of the individual samples within a given experiment were numerically close together. The range of efficiency in counting among different experiments was between 29-45%.

---

[15] contributed by Dr. William Bates

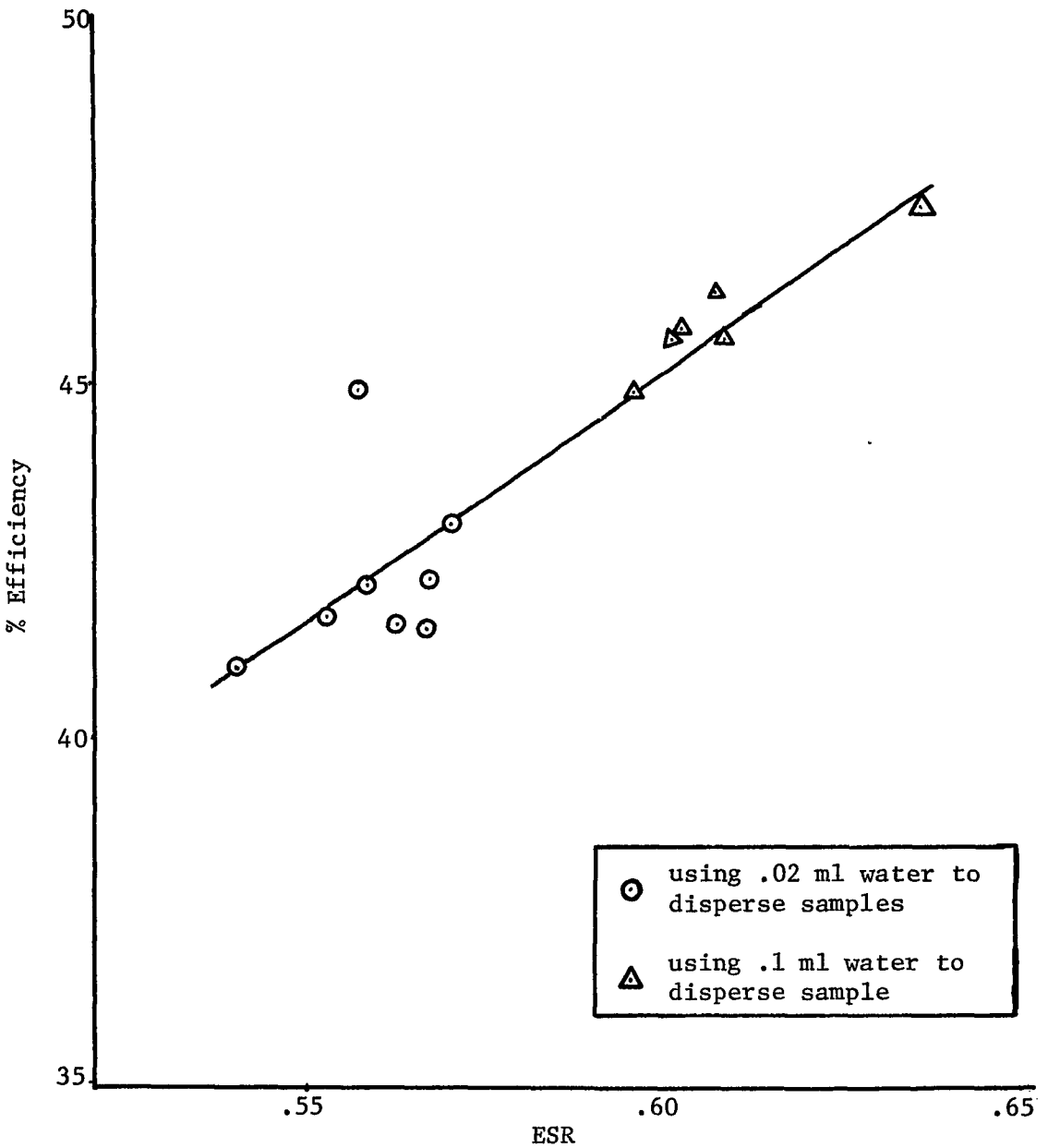


Figure 2. The Relationship Between ESR and Efficiency in Counting for TCA Insoluble Pellets (I-407) Ranging from .25 to 2.5 mg.

CHAPTER IV

RESULTS AND DISCUSSION

Group I Experiments

The first group of experiments was designed to determine if vitamin A (5 µg/ml) influenced the uptake of ^{3}H-uridine into cellular pools and/or incorporation of the ^{3}H-uridine into the macromolecular fraction of the cells at 10 minutes, 30 minutes, and 1-2 hours after treatment. Physical limitations of time and equipment necessitated 3 replications (A,B, and C) of the experiment. The experiments showed that vitamin A at the 5 µg/ml level produced a significant decrease in the incorporation of ^{3}H-uridine into the macromolecular fraction without changing the uptake into the pool fraction. The results of the 3 experiments were combined for purpose of statistical analysis. Since the average control values varied greatly on the different days on which the experiments were run, a value of 1.000 was assigned to the average of the controls of each separate experiment. All data were expressed as fractional parts of the average control value. Therefore, the treatments for each particular experiment were assigned values based on their uptake of isotope in relation to the uptake by controls on that particular day. The cells used for experiment A had been kept on the supplemented MEM containing only 1% FBS for several weeks before the experiment, in an attempt to deplete the cells of vitamin A as much as possible. Two days before the cells were to be subcultured for the experiment, the serum level of the medium was increased to 5% and kept at that level throughout the experiment. This

was done to ensure adequate uptake of isotope at the time of the experiment. Experiment A was run on the second day following subculture. Results of Experiment A are listed in Table 2 of Appendix B. The ESR value was .555, or 41.8% efficiency. In Experiment A, the values for uptake into cellular pools and incorporation into the macromolecular fraction were all lower in the experimentals than in the controls. However, the results of Experiments A, B, and C will be combined and discussed collectively.

The cells for Experiment B were prepared as in Experiment A, with the exception that the cells were not placed on 5% serum until the time of subculture. From this data, it appears that the cells were not growing as rapidly as the cells used for the previous experiment and that the duplicate values are more variable. The ESR value for Experiment B was .569, or 42.2% efficiency. A new stock of cells was introduced prior to Experiment C, and the cells were kept on media supplemented with 5% FBS throughout. No depletion period was used this time. Experiment C was run on the fourth day following subculture, at a time when the cells had almost reached a confluent monolayer. Dispensing with the depletion period apparently led to a better growth rate, and the same general trend was seen in all 3 experiments. The flasks treated with 5 µg/ml of vitamin A took up and incorporated less ^{3}H-uridine at 10 minutes, 30 minutes, and 1-2 hours after treatment with the vitamin. Results of Experiment C are found in Table 2 of Appendix B. The ESR value for Experiment C was .454 (about 30% efficiency), a value which could not be read from the efficiency curve for the sample. Therefore data from Experiment C were left in CPM. That the low ESR values obtained in several experiments were not

due to sample quench, was evidenced by the fact that the blanks were equally effected. The low values were most likely due to the preparation of the counting fluid. In experiments in which the ESR values were below .55, the results were left in CPM.

The combined results of Experiments A, B, and C indicated that vitamin A at the level of 5 µg/ml was associated with a significant decrease ($p \leq .05$) in the incorporation of ^{3}H-uridine into the macromolecular fraction of the cells. The combined results are shown in Table 3 and in Table 3 of Appendix B. By using Duncan's multiple Range ($\alpha = .05$), it was found that the treatment means differed from the control mean, but not among themselves, indicating that the effect of vitamin A was evidenced at 10 minutes, 30 minutes, and 1-2 hours after treatment. Although there seemed to be a general trend of decreased uptake into the pool fraction, especially in Experiment C, no significant difference was found in the relative uptake into the soluble fractions by the vitamin A treated and by the controls. The analysis of variance for Group I Experiments is given in Table 1 of Appendix C.

Group II Experiments

In order to discover if other levels of vitamin A also decreased the incorporation of ^{3}H-uridine, levels of 1, 5, and 10 µg/ml of vitamin A were tested. A treatment period of 10 minutes was used consistently in succeeding experiments. Two experiments, D and E, were designed. Cells for Experiment D were placed on medium A2 plus APG (Holmes) specially prepared without vitamin A and without serum 5 days before subculturing into the same medium to which 1% FBS had been added. An almost confluent

TABLE 3

THE EFFECT OF VITAMIN A (5 µg/ml) ON THE UPTAKE AND INCORPORATION OF ^{3}H-URIDINE AT DIFFERENT TIMES AFTER TREATMENT (GROUP I EXPERIMENTS)

Time after treatment	Level of vitamin A µg/ml	Relative Incorporation into Insoluble Fraction	Relative Uptake into Soluble Fraction
0	0	1.00000	1.00000
10 minutes	5	.39680	.51166
30 minutes	5	.49563	.51156
1-2 hours	5	.41335	.57131

monolayer had been attained on the second day following subculture, at which time the experiment was begun. Cells for Experiment E were maintained on MEM plus 1% FBS for a period of 1 week prior to subculturing into MEM plus 5% FBS. On the day of the experiment, the cells were placed on medium A2 plus APG (Holmes) without vitamin A or serum. Judged by the controls, cells for Experiment D grew slowly and for E grew very well. Results of Experiments D and E appear in Table 4 of Appendix B. The combined results are given in Table 4 and in Table 5 of Appendix B.

The results showed that vitamin A at the levels of 5 and 10 µg/ml appeared to decrease significantly ($p \leq .05$) the incorporation of ^{3}H-uridine into the macromolecular fraction, and to decrease highly significantly ($p \leq .01$) the uptake into the soluble pool fraction. No significant difference was found between the means of the controls and the 1 µg/ml treatment groups. (Duncan's Multiple Range, $\alpha = .05$ and .01, respectively). Experiments D and E had ESR values of .466 (about 30% efficiency) and .452 (about 29% efficiency), respectively. Analysis of variance for Group II is given in Table 2 of Appendix C.

The second group of experiments further confirmed the previous finding that 5 µg/ml vitamin A decreased the incorporation of ^{3}H-uridine into the macromolecular fraction. Furthermore, a level of 10 µg/ml produced a decrease as well, but the effect of lower levels, such as 1 µg/ml, was not certain.

The dramatic decrease of ^{3}H-uridine into cellular pools confirmed the suspicion about the decrease in Group I. Although uptake into soluble pools was highly significantly effected in Group II Experiments and not significantly effected in Group I, it is probable that the variability

TABLE 4

THE EFFECT OF DIFFERENT LEVELS OF VITAMIN A ON THE UPTAKE AND INCORPORATION OF ^{3}H-URIDINE (GROUP II EXPERIMENTS)

Level of vitamin A µg/ml	Time after treatment	Relative Incorporation into Insoluble Fraction	Relative Incorporation into Soluble Fraction
0	——	1.00000	1.00000
1	10 minutes	.94695	.84008
5	10 minutes	.55918	.36670
10	10 minutes	.45585	.26410

in Group I masked the effect. Attempts to deplete the cells of vitamin A altered the growth rate and perhaps led to a disequilibrium of precursors in the pool fraction.

The immediate problem suggested was the clarification of the effect of lower levels of vitamin A. The levels of vitamin A which produced an increase in RNA synthesis in organ culture could possibly be too high for a sensitive monolayer of cells.

Group III Experiments

Lower levels of vitamin A (.01, .1, and 1.0 µg/ml) were tested for their effect on the uptake and incorporation of ^{3}H-uridine by the cells. It was found that the 1.0 µg/ml level produced a significant increase in the incorporation of ^{3}H-uridine, but did not effect the uptake into cellular pools. Three experiments (F, G, and H) were run. For Experiment F, 6 flasks of cells were used. The cells had been kept for 5 days on the special medium A2 plus APG (Holmes) and 1% FBS, and did not appear to be growing rapidly. The slow growth rate was confirmed. Cells for Experiment G were maintained on supplemented MEM plus 5% FBS until the day of the experiment when they were placed on special medium A2 plus APG (Holmes) without serum and without vitamin A. The cultures were 5 days old when confluency was approached and the experiment was begun. The cells were growing well and an appreciable increase in incorporation by the flasks treated with 1 µg/ml of vitamin A was seen. The results of the treatment with the lower levels appeared inconclusive. Results of Experiments F and G are found in Table 6 of Appendix B.

Cells used in Experiment H were 4 days old and were on supplemented MEM plus 10% FBS. This level of serum was used to attain an adequate

growth rate. Previously, the cells had been showing a tendency to detach following subculture, but they appeared healthy at the time of the experiment, and their good growth rate was confirmed by the data. Twelve flasks were used to determine the effects of adding cold uridine to the incubation medium and simultaneously subjecting the flasks to the same experiment as the preceding experiment, using lower levels. The cold uridine aspect of Experiment H is included in the general discussion. Results of Experiment H appear in Table 6 of Appendix B, and the combined results of Group III Experiments appear in Table 5 and in Table 7 of Appendix B.

The results of these experiments indicate that vitamin A at the 1 μg/ml level was associated with a significant <u>increase</u> ($p \leq .05$, Duncan's Multiple Range, $\alpha = .05$) in the incorporation of ^{3}H-uridine. No significant difference was found in the uptake of ^{3}H-uridine into the soluble cell fraction. See Table 3 of Appendix C for the analysis of variance for Group III data. The ESR values for Experiments F, G, and H were .497 (33% efficiency), .587 (44% efficiency), and .603 (45% efficiency), respectively.

Group III Experiments clarified the effect of adding 1 μg/ml level of vitamin A. All the data for the 1 μg/ml level of vitamin A in Group II and III were combined into a single group. A t-test was employed and the means were found to be unequal ($\alpha = .05$). The conclusion based on the combined data is that the 1 μg/ml level of vitamin A is associated with a significant increase in ^{3}H-uridine incorporation into RNA. The uptake into cellular pools was not statistically different between the vitamin A treated and the controls.

TABLE 5

THE EFFECT OF LOWER LEVELS OF VITAMIN A ON THE UPTAKE AND INCORPORATION OF ^{3}H-URIDINE (GROUP III EXPERIMENTS)

Level of vitamin A µg/ml	Time after treatment	Relative Incorporation into Insoluble Fraction	Relative Incorporation into Soluble Fraction
0	——	1.00000	1.00000
.01	10 minutes	1.40966	1.44282
.1	10 minutes	1.12170	1.29023
1.0	10 minutes	1.73052	1.47068

Table 8 of Appendix B gives a compilation of the average control values, the kind of media used, and other experimental information. From these data it would appear that the lower uptake of the controls obtained in Experiments B, D, and F was due to a reduced growth rate caused by the reduction of serum levels in an attempt to deplete the cells of vitamin A.

The overall results indicated that the depletion period was not necessary for the observed response to vitamin A. In experiments by Rasmussen et al. (30), Syrian hamster cells cultured on media supplemented with 15% FBS responded to levels of 1 μg/ml. Perhaps I-407 cells as well as Syrian hamster cells become deficient in vitamin A after repeated passage in vitro. Studies are being done by others in the laboratory to determine whether low levels of vitamin A stimulate cell growth and proliferation in I-407.

After it became apparent that the depletion period was not producing a significant effect, it was discontinued. Dispensing with the depletion period and adopting a steady growth medium, such as supplemented MEM plus 5% FBS, favored the attainment of a more constant growth rate. The adoption of confluency as a criterion for determining the best time at which to begin the experiment was another refinement which was made in experimental procedures.

General Discussion

The level of 1 µg/ml of vitamin A apparently effected the I-407 cells differently than did levels of 5-10 µg/ml. Zachman (4) concluded that vitamin A increased the incorporation of ^{3}H-uridine without changing pool sizes *in vivo* and *in vitro* (organ culture). Results with the lower levels are in agreement with his findings. However, the findings at the 5 and 10 µg/ml level did not agree with the report by Zachman concerning the absence of an effect on the pool fraction. A definite decrease in the pool fraction and incorporation was seen with the 5 and 10 µg/ml levels of the vitamin. Incorporation paralleled uptake in most experiments. When cold uridine was added to the incubation medium, an increase in uptake of ^{3}H-uridine into the soluble fraction was encountered with the lower vitamin levels. In case of I-407 cells, the increase in incorporation of ^{3}H-uridine into the macromolecular fraction at the lower levels of the vitamin and the decrease in incorporation at the higher levels are probably due to changes in cell permeability. The discrepancy in the effect on pools found here and reported by Zachman may be due to differences inherent in cell culture and organ culture. However, the results published by Zachman were preliminary and the effect on pools may have been obscured by other variables.

Since the uptake of ^{3}H-uridine into cellular pools closely paralleled the incorporation into the macromolecular fraction in most of the experiments, there is no reason to postulate a direct effect on RNA synthesis in I-407. It appears more likely that vitamin A is effecting membrane permeability, with the lower levels of vitamin A leading to an increased uptake of uridine, and the higher levels resulting in less uptake. If vitamin A serves as a

cell regulator, perhaps it regulates permeability of the membranes to various cell nutrients. Differences in uptake of vitamin A by various tissues and subsequent differences in membrane permeability could be postulated as a mechanism leading to morphological differentiation. This proposal is also in keeping with the established facts about the membrane effects of vitamin A.

Since the consensus of opinion heretofore has been that cells in culture do not require the fat soluble vitamins, it was surprising to find an effect produced by physiological levels of the vitamin. In preliminary studies, cells were grown in the presence of various levels (1, 5, and 10 μg/ml) of the vitamin, and appeared healthy. It is possible that more definitive studies may show that levels used in these experiments are somewhat toxic to the I-407 cells. Showing that the cells respond to vitamin A does not prove that the cells require the vitamin or that the effect is related to the natural function of the vitamin.

In experiments on uridine uptake, Stambrook and Siskin (31) added very high levels of cold uridine to the medium, reasoning that at high levels of uridine, the chief pathway of entry of uridine into the cell would be by diffusion and an effect mediated by the uridine transport system could be ruled out. In Experiment H, cold uridine (200 μM) was added and it was noteworthy that while the lower levels of the vitamin still evoked an increase in uridine incorporation, that a clear effect on pools was seen as a response to levels of the vitamin in the presence of the cold uridine.

It is interesting to speculate how vitamin A may be acting in changing membrane permeability. A possibility suggested by the structure

and chemical properties of the vitamin is that it acts to bind certain enzymes to cellular membranes. Perhaps vitamin A may regulate membrane porosity by reversibly joining "mobile modular units" (32) within the membrane. Cell culture would provide a refined system in which to study membrane action. An understanding of the normal molecular action of vitamin A would serve as a basis on which to use the vitamin therapeutically in the treatment of cancer.

CHAPTER V

SYSTEMS ANALYSIS OF LABORATORY METHODS

Introduction

Systems analysis is an approach which has been used chiefly by certain governmental and business organizations, and is a technique which is generally adaptable to studying complex problems. It is an attempt to look at a situation in its entirety and identify problem areas. Various alternatives to the problems are considered and evaluated with the help of models. Some situations are amenable to reduction to mathematical models or to a series of equations and may rely on a computer basis for evaluation of the alternatives. The type of model must be appropriate to the problem under investigation.

There are several principles of a good analysis, which have been discussed by Quade (33). The objectives of the analysis must be defined and the right problem tackled. The system must be viewed in a broad scope and the interdependencies with other areas or systems must be identified. This may necessitate bringing in specialists from different disciplines. Uncertainty is prevalent in problem situations and must be acknowledged. The effect of the uncertainty must be taken into account. Also, an attempt should be made to discover new alternatives. Scientific principles and methodology should be observed throughout.

The application of systems analysis to laboratory procedures forces the investigator to take a cold, objective look at the research. The changes needed to be made may come to attention through judgmental and

intuitive thinking, however. Viewing the system as a whole helps to prevent the pitfall of becoming so involved in one problem area that other problem areas are ignored. However, no analysis is ever complete, since all the interdependencies in a complex situation cannot be identified.

Due to the complex nature of the art and science of tissue culture experimentation and to the stepwise procedures used in the analysis, this research seemed particularly well suited for systems analysis. The analysis was not a separate study entirely, because it was used as an approach throughout the study.

The chief purpose of applying systems analysis techniques was to evaluate procedures employed in the laboratory in order to determine where there were problems and what controls could be introduced for better management of the quality and quantity of results. For purposes of analysis, the experimentation was divided into 4 systems: namely, the preparation of the cells for experimentation; the treatment of the cells with vitamin A and ^{3}H-uridine; the washing and preparation of the sample for liquid-scintillation counting; and the actual liquid-scintillation counting process.

An interdisciplinary team of 2 faculty members and several graduate students cooperated in designing and setting up the experiments. The disciplines represented were physiology, biochemistry, histology, genetics, and nutrition. Other support and fresh ideas were obtained from faculty committee members. Although the person or persons best qualified in each area contributed the most to the part of the system related to his specialty, agreement was attained on the basic approach used. Frequent meetings were held to discuss problems and voice different view points.

Figure 3 is a diagrammatic scheme which shows the system of experimentation. Outside influences and interrelationships are indicated, but the analysis was confined chiefly to the immediate physical procedures in the laboratory which influenced the outcome of the work.

Preparing the Cells for Experimentation

The chief problem encountered in carrying out the research was a downtime of about 50% caused by having to grow new stock cultures from 1 small vial when contamination occurred. Due to contamination, 3 separate stocks of cells were grown up during this experimentation. This delay amounted to about 3 or 4 months, since over 1 month was required for growing up enough cells for the experiments. A faster recovery from contamination is needed. The laboratory needs a low temperature ($-70^{o}C$) freezer, so that stock can be frozen and multiple vials brought out immediately when needed, speeding the recovery time to about 1 or 2 weeks. Being able to freeze stock would also allow the laboratory to grow cells only when needed and when experiments were planned, saving greatly on supplies and effort necessary to keep cells growing continuously.

The precautions taken to prevent bacterial contamination were discussed previously, but other ideas were considered. Some of these were placing overhead lights in the tissue culture room, and coating the walls and counter tops with a thin layer of oil to settle dust. More scrupulous attention to certain details may prove beneficial; for example, the suction flasks should be emptied of media immediately after use and the lines rinsed with alcohol so that bacteria do not grow under the hood. Mold should be prevented from growing in the water bath, because it will be

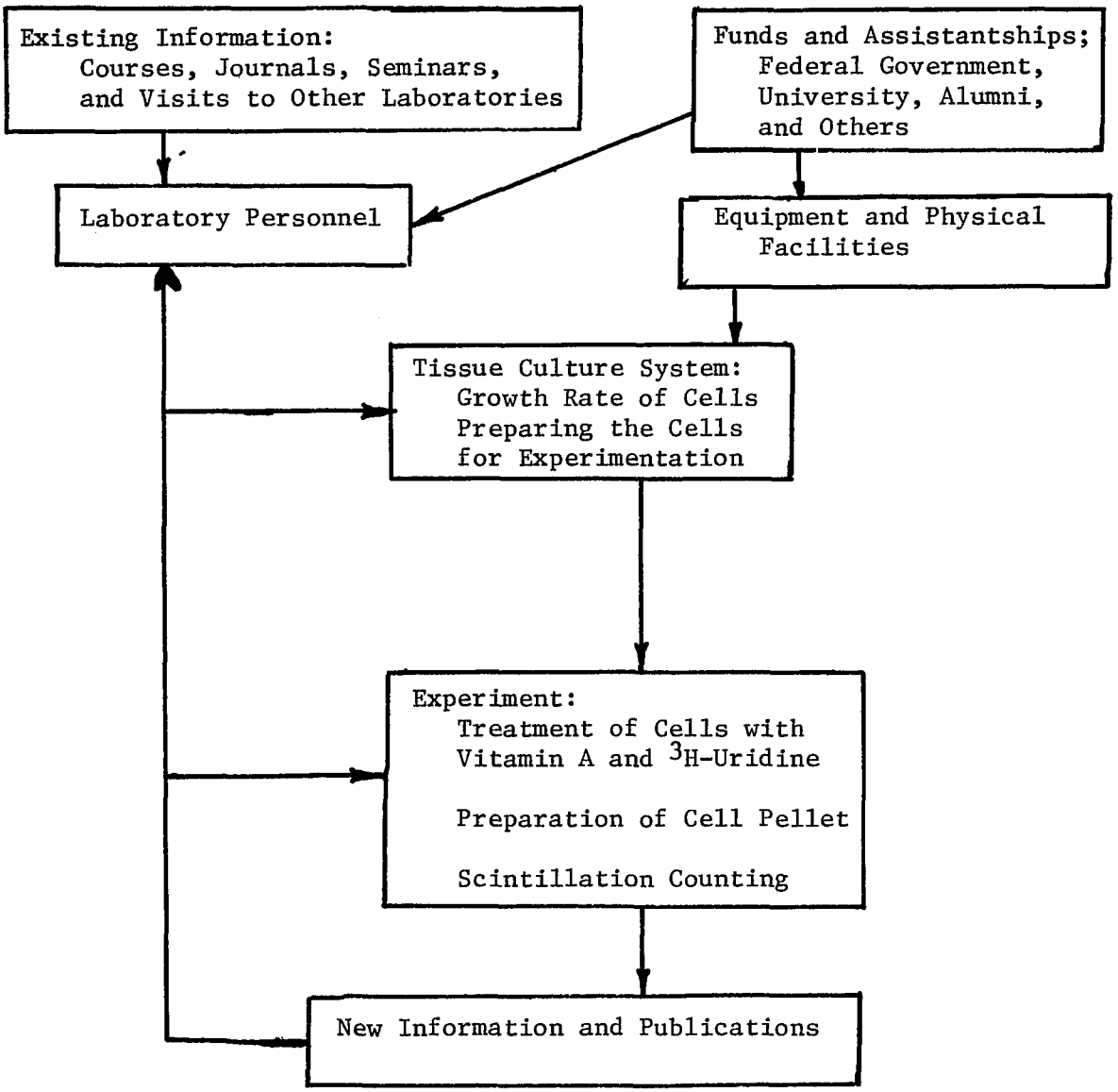


Figure 3. The System of Experimentation

transferred directly to the hood on the supplies. Everything which goes under the hood should be wiped off with an antiseptic solution, such as a 10% Chlorox solution, and only a clean laboratory coat should be worn in the tissue culture room.

Chemical contamination of glassware was also a major concern because tissue culture glassware must be scrupulously clean and free of detergent and other chemicals, in order for the cells to grow. Problems may have been associated with the division of labor in the laboratory; 5 persons shared 5 different chores on a rotating schedule. It may have been better to have had 1 person responsible for glassware, and also 1 person responsible for autoclaving materials. Assigning chores on a rotating basis was logical because there were 5 persons working and 5 major chores to be done regularly: glassware to be washed; pipettes to be washed and plugged with cotton; materials to be autoclaved; media to be prepared; and cleaning and scrubbing to be done. However, a careless glasswasher can ruin the whole operation; so it is better if some division of duties is established so that everyone does not have to be penalized for mistakes, even if it means each person washing his own glassware. It is good to use as many disposable items as the economic circumstances of the laboratory will permit, when doing tissue culture on a small scale; but even disposable items are not 100% reliable. Some instances of batches of flasks contaminated by metals or chemicals used in the manufacturing process have been reported.

Growth Rate

An attempt was made to do the experiments when the cells were in the logarithmic (log) phase of growth, but no procedures had been developed

to determine exactly when log phase occurred. Since no literature is available yet on growth studies of I-407, these studies need to be made before further metabolic work is done. A quick and reliable method is needed for determining mitotic index, so that the experimenters can have some way of knowing whether to do the studies on one particular day or wait a day or so. It would be desirable to have a certain mitotic index which would be a "go no-go" type of control. Also, growth studies would reveal what happens to the mitotic index as the cultures become confluent. From the data, it can be assumed that the log phase was not always attained at the time of the experiment. It might be particularly undesirable to hit a point of inflection between lag and log phase, because variable results among flasks would be expected at that time. The theoretical aspects of taking cells in log phase were re-examined, and it was suggested that it might be possible to attain more consistent results by taking the cells after a state of confluency has been reached. At what rate the cells continue to divide after reaching confluency was not known.

In any event, it should be the aim of the laboratory to standardize the growth rate of the cells by standardizing the procedures as much as possible, using chemically clean glassware, avoiding and detecting contamination, and holding the composition of the medium as constant as possible. In standardizing the composition of the medium, it is of greatest importance that none of the ingredients used in preparing the medium have expired their shelf lives. All bottles should be marked not only with the date at which they were received, but the date at which they expire; and all shelf-life data should be interpreted very conservatively, particularly if the ingredients have been repeatedly thawed and refrozen.

The number of entries into a bottle should be marked and the bottle should bear the initial of the person opening it. The shelf-lives of the MEM, glutamine, and serum are particularly important to observe; and it should be remembered that different batches of sera may have very different growth-promoting effects. To obtain a reasonably constant growth rate, it is necessary to test each bottle of serum received until a desirable lot has been found and to order more bottles of the same lot. Although it is more expensive to purchase the MEM than to prepare it from the 10 X, for a small laboratory it is probably worth the expense in order to decrease the chances of contamination. A carbon dioxide incubator would help greatly in maintaining a constant pH in the culture medium and would prevent the loss which occurs when flasks are not airtight. A constant pH is necessary in maintaining a constant growth rate. A set-up for filter sterilizing large quantities of solutions, such as the bicarbonate solution, would also be a good investment in the event that it became necessary or desirable to prepare media and PBS.

Treatment of Cells with Vitamin A and ^{3}H-Uridine

Since the treatment of the cells with vitamin A has been dealt with previously, no further analysis will be made here. There were several questions related to the treatment of the cells with ^{3}H-uridine: Was the uptake of label linear over the period of 1 hour? Was the pulse length too short? Was the process of ^{3}H-uridine uptake extremely temperature sensitive?

A preliminary experiment was designed to answer these questions. The cells for the experiment had been maintained on supplemented MEM plus

5% FBS for 6 days and were almost confluent on the day of the experiment when they were placed on special medium A2 plus APG (Holmes) without serum and without vitamin A. Four flasks were treated for pulse lengths of 10, 15, 30, and 60 minutes at room temperature (19°C) and 4 flasks were treated similarly at 38°C. Results from this experiment can be seen in Table 9 of Appendix B. It appeared that neither the uptake of ^{3}H-uridine into cellular pools nor the incorporation into macromolecular fractions were linear with respect to time over the period tested at 38°C. At 19°C, the entry of uridine into the cell was slower, as was expected; and the pools appeared to accumulate the label. Although no extreme temperature sensitivity was found, the temperature of the flasks should be controlled carefully during the treatment period so that the metabolic activity will be normal. The 10 minute incubation period was judged adequate. A suggestion for improving the accuracy in adding the isotope was to use a micropipette and add the label in a smaller quantity, .1 ml rather than .5 ml, because foaming of the medium produced error in reading the meniscus when larger quantities were added.

Preparation of the Cell Pellet

The homogenization step needed to be monitored more carefully, particularly as the ground glass homogenizers became worn and more time was needed for the homogenization. Microscopic inspection revealed that some whole cells remained, and indicated a need for some control values of permissible quantities of whole cells allowed to remain. Either the ground glass homogenizers should be replaced more frequently or another process, such as sonication, should be considered for rupturing the cells.

Variability studies were run to determine the amount and source of the variability in the results. The variation in average uptake values among the experiments could be ascribed chiefly to growth rates, but differences in duplicate values in the same experiment were due to flask variation and to experimental error. Experiments were designed to determine which of these was the source of the greater amount of variation. Six large flasks (75 cm^2 surface---250 ml volume) were used for the experiments. Two of the flasks were removed for experimentation on the second day following subculture, and 2 were removed on each of the following days. After adding the isotope to the large flasks, the contents of each flask were subdivided into 3 centrifuge tubes; and duplicate determinations were run on the pellets from each centrifuge tube. The results of this study are given in Table 4 of Appendix C and show that duplicate values from the same centrifuge tube are more like each other, but that in isolated cases the duplicate values are astonishingly different. On the first day, in particular, there was a great amount of variation among the 3 tubes from the same flask, indicating problems in experimental technique. Results from duplicate analyses performed on Experiment G in Group III are given in Table 5 of Appendix C.

A new method of sample preparation was devised in an attempt to improve results. Rather than drying the TCA pellet and weighing out a sample, the TCA pellet was washed as usual after homogenization. It was resuspended in 1 ml of 5% TCA; a .1 ml sample was taken, and NCS was added immediately. The TCA pellet was dried and the total weight was used to determine the size of sample contained in the aliquot taken previously.

Experiment H of Group III was run by the new method in which duplicate aliquots were taken from each flask (Table 6 of Appendix C).

Several reasons can be postulated for the vast improvements achieved by this method; first of all, weighing out 1.00 mg samples of a dried, powdery TCA pellet is an undesirable and inaccurate procedure. Additionally, since a dried TCA pellet is very compact and difficult to digest, it was found that 100 µl (.1 ml) water gave better results (Table 7 of Appendix C) than the 20 µl used in the original procedure. That adding the .1 ml water to the dried pellet did not solve all the problems was evidenced by the results of adding .1 ml of water in Experiment G (Table 5 of Appendix C). It was found to be much better and faster to take suspended aliquots. It was also possible that improperly digested material in combination with the NCS produced chemiluminesence which gives high, unpredictable results. The results of the variability studies are obvious, although a limited number of experiments were performed. The same type of experiment with the large flasks should be rerun using the new method of sample preparation to study variability.

Scintillation Counting

After the improvements were made in sample preparation, it was possible to attain ESR values in the range of 5.8-6.1. Careful attention was needed in the preparation and storage of the scintillation counting fluid. Preferably, the same person should prepare the scintillation counting fluid each time, taking care to use a grade of toluene manufactured specifically for scintillation counting, and storing the fluid in an airtight container away from light. Occasionally, scintillation vials which are not airtight are encountered; but if NCS does crystallize out during the digestion process, the addition of 1 or 2 drops of fresh NCS

is usually enough to put the crystals back into solution. The gain setting on the liquid scintillation counter should be checked to be certain that it has not been changed and the value recorded.

CHAPTER VI

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS FOR FURTHER INVESTIGATIONS

Summary

An *in vitro* system was devised to study the effect of vitamin A on the uptake and incorporation of ^{3}H-uridine in cell line I-407. Traditionally, in order to study the effect of a vitamin on an animal, the animal is first depleted of that vitamin. Likewise, attempts were made to deplete the cells of vitamin A. One approach was to place the cells on a synthetic medium which was specially compounded without vitamin A. Because the medium did not contain serum, the cells could not be grown for extended periods of time on the synthetic medium.

Another approach was to grow the cells on MEM plus a reduced amount of serum, since the only vitamin A in the medium was contained by the serum. Because serum levels are closely related to growth rates of human cells *in vitro*, these efforts were abandoned when it was found that the same type of response was observed with or without a depletion period. Perhaps, the cells were already very low in vitamin A since they had been subcultured repeatedly *in vitro*.

Systems analysis was used to locate problem areas and to develop controls for the laboratory procedures. This method of approach represents a somewhat unique meshing of ideas, applying business management principles to the solution of bionutritional problems.

The original plan was as follows:

1. Deplete the cells of vitamin A.

2. Add vitamin A (1, 5, and 10 µg/ml).

3. Ten minutes later, follow by a 10 minute pulse with ^{3}H-uridine (2 µCi/ml).

4. Wash cells, precipitate the TCA insoluble fraction, and take a sample of both the TCA soluble and TCA insoluble fractions. The TCA insoluble fraction contained large molecules, including the RNA. The smaller molecules, including the unincorporated ^{3}H-uridine, remained in the TCA soluble fraction.

Conclusions

From the experimental work, the following conclusions were drawn:

1. The addition of 5 and 10 µg/ml vitamin A to cultures of cell line I-407 resulted in a significant decrease in the incorporation of ^{3}H-uridine into the macromolecular fraction containing RNA.

2. The addition of 5 and 10 µg/ml vitamin A resulted in a very significant decrease in the uptake of ^{3}H-uridine into the soluble cell fraction. This effect was masked by variability when the cells were subjected to a depletion period.

3. The decrease in uptake and incorporation of ^{3}H-uridine was evidenced at all times tested; i.e., 10 minutes, 30 minutes, and 1-2 hours after treatment with vitamin A.

4. However, the addition of 1 µg/ml vitamin A resulted in a significant increase in ^{3}H-uridine incorporation, but produced no statistical change in the uptake into cellular pool fractions.

5. The depletion period did not appear to be necessary for the observed responses to occur, but rather led to lower growth rates and erratic levels of uptake and incorporation of the isotope.

6. Confluency was selected as a criterion for judging when the cells were ready for experimentation.

7. Preliminary experiments with the addition of high levels of cold uridine indicated that vitamin A produced a permeability change rather than an effect mediated by the uridine transport system.

8. It appeared that cell line I-407 could be maintained adequately on a special synthetic medium void of serum (A2 plus APG Holmes) for about 5 days.

From the systems analysis, the following recommendations and observations were made:

1. More growth studies on cell line I-407 are needed before further metabolic work is done.

2. The growth rate of the cells should be standardized as much as possible and an adequate growth rate index established. Cultures not attaining such a rate should be discarded.

3. The cells did not exhibit an extreme temperature sensitivity; but nevertheless, careful temperature control should be considered an integral part of the experimental procedure.

4. A low temperature freezer for cell storage would greatly increase the efficient use of time in the laboratory and would decrease the cost of continuous cultivation of the cells.

5. A pulse length of 10 minutes was found to be an adequate exposure time of the cells to the ^{3}H-uridine.

6. Each batch of serum which is obtained should be tested to determine its growth promoting properties.

7. Careful attention should be given to the shelf life of each of the chemicals used in media preparation. In addition, careful attention is needed in the preparation and storage of the scintillation counting fluid.

8. Since the experiments were done as a small scale operation, it was more feasible to purchase disposable equipment and commercially prepared solutions in most instances.

9. The following refinements in experimental procedures are recommended:

a. The TCA insoluble fraction can be prepared by resuspending the wet pellet and taking aliquots of the suspension.

b. A micropipette can be used for adding the radioactive label.

c. The homogenization step should be monitored microscopically. The maximum number of whole cells allowed to remain should be established.

Recommendations for Further Investigations

Growth studies on I-407 could be done and a certain mitotic index established as a control to determine whether the cells are growing optimally. In addition, histological studies to determine the effect of different levels of vitamin A on mitotic index and morphology would be very helpful correlations to the biochemical studies. These studies would help to establish whether or not the levels used have any degree of toxicity for the cells. Any morphological differences in cells treated with vitamin A would be of great interest.

Additional data is needed for the effect of lower levels of vitamin A, using standardized growth conditions. Also, it would be informative to discover whether or not high levels of cold uridine reverse the effects of 5 and 10 μg/ml of vitamin A. A vitamin A inhibitor, such as citral (34), could be tested to determine if it blocked both the entry of uridine into the cells and the incorporation into the macromolecular fractions.

The logical direction indicated by the present results is in the area of membrane studies using I-407 cells and different levels of vitamin A. Sophisticated techniques and equipment would be required for such experiments. Although electron micrographic pictures have failed to show any differences in membranes from vitamin A-deficient and normal animals, other techniques could be employed. Cell culture would provide a refined system for studying membranes.

The relationship between vitamin A and benzo(a) pyrene could be investigated in cell line I-407. The influence of vitamin A and citral on the effect of benzo(a) pyrene in hamster cells has been investigated (35).

A spin-off of this research was the discovery that serum is not needed for the uptake of uridine by cell line I-407. Since this line of cells can be grown for 5 or 6 days on defined medium A2 plus APG (Holmes), uridine uptake _per se_ could be investigated. Stambrook and Siskin did related studies (31).

BIBLIOGRAPHY

1. Hubbard, R., D. Bownds, and T. Yoshizawa. 1965. The Chemistry of Photoreception. Cold Spring Harbor Symp. Quant. Biol. 30:301.

2. Fell, H.B., and E. Mellanby. 1953. Metaplasia Produced in Cultures of Chick Ectoderm by High Vitamin A. J. Physiol. 119:470.

3. Fell, H.B. 1957. The Effect of Excess Vitamin A on Cultures of Embryonic Chicken Skin Explanted at Different Stages of Differentiation. Proc. Roy. Soc. B. 146:242.

4. Zachman, R. 1967. The Stimulation of RNA Synthesis In Vivo and In Vitro by Retinol (Vitamin A) in the Intestine of Vitamin A-Deficient Rats. Life Sciences 6:2207.

5. Johnson, B., M. Kennedy, and N. Chiba. 1969. Vitamin A and Nuclear RNA Synthesis. Am. J. Clin. Nutr. 22(8):1048.

6. Zile, M., and H.F. DeLuca. 1970. Vitamin A and Ribonucleic Acid Synthesis in Rat Intestine. Arch. Biochem. Biophys. 140:210.

7. DeLuca, L., H.K. Kleinman, E.P. Little, and G. Wolf. 1971. RNA Metabolism in Rat Intestinal Mucosa of Normal and Vitamin A-Deficient Rats. Arch. Biochem. Biophys. 145:332.

8. DeLuca, L., M. Schumacher, and G. Wolf. 1970. Biosynthesis of a Fucose-Containing Glycopeptide from Rat Small Intestine in Normal and Vitamin A-Deficient Conditions. J. Biol. Chem. 245:4551.

9. Kaufman, D., S. Baker, M. Smith, W. Henderson, C. Harris, M. Sporn, and U. Saffiotti. 1972. RNA Metabolism in Tracheal Epithelium: Alteration in Hamsters Deficient in Vitamin A. Science 177:1105.

10. Raisz, L.G. 1965. Inhibition by Actinomycin D of Bone Resorption Induced by Parathyroid Hormone on Vitamin A. Proc. Soc. Exptl. Biol. Med. 119:614.

11. Coward, W.A., J.M. Howell, G.A. Pitt, and J.N. Thompson. 1966. Effects of Hormones on Reproduction in Rats Fed a Diet Deficient in Retinol but Containing Methyl Retinoate. J. Reprod. Fertility 12:309.

12. Keiser, H., G. Weissmann, and A.W. Bernheimer. 1964. Studies on Lysosomes IV. Solubilization of Enzymes During Mitochondrial Swelling and Disruption of Lysosomes by Streptolysin-S and Other Hemolytic Agents. J. Cell Biol. 22:101.

13. Mellanby, E. 1934. Xerophthalmia, Trigiminal Degeneration and Vitamin A Deficiency. J. Pathol. Bacteriol. 38:391.

14. Roels, O.A., O.R. Anderson, N.S.T. Lui, D.O. Shah, and M.E. Trout. 1969. Vitamin A and Membranes. Am. J. Clin. Nutr. 22(8):1020.

15. Goodman, D.S. 1969. Retinol Transport in Human Plasma. Am. J. Clin. Nutr. 22(7):911.

16. Lucy, J.A. 1969. Some Possible Roles for Vitamin A in Membranes: Micelle Formation and Electron Transfer. Am. J. Clin. Nutr. 22(8):1033.

17. Fell, H.B. 1965. The Effects of Vitamin A and C on Cells and Tissues in Culture, in Cells and Tissues in Culture. Ed. E. Willmer. New York: Academic Press.

18. Paul, J. 1970. Cell and Tissue Culture. Baltimore: Williams and Wilkins.

19. Paul, J. 1960. The Nutrition of Cells In Vitro. Proc. Nutr. Soc. 19(11):45.

20. Thomas, D.B., and C.A. Pasternak. 1969. Vitamin A and the Biosynthesis of Sulphated Mucopolysaccharides. Biochem. J. 111:407.

21. Kochhar, D.M., J.T. Dingle, and J.A. Lucy. 1968. The Effects of Vitamin A (Retinol) on Cell Growth and Incorporation of Labelled Glucosamine and Prline by Mouse Fibroblasts in Culture. Exp. Cell Research 52:591.

22. Crocker, T.T., and L.L. Sanders. 1970. Influence of Vitamin A and 3,7-Dimethyl-2, 6-octadienal (Citral) on the Effect of Benzo(a) pyrene on Hamster Trachea in Organ Culture. Cancer Research 30:1312.

23. Polliack, A., and I.S. Levij. 1969. The Effect of Topical Vitamin A on Papillomas and Intraepithelial Carcinomas Induced in Hamster Cheek Pouches with 9,10-Dimethyl-1,2-benzanthracene. Cancer Research 29:327.

24. Holmes, R. 1967. Preparation from Human Serum on an Alpha-One Protein which Induces the Immediate Growth of Unadapted Cells In Vitro. J. of Cell Biol. 32(2):297.

25. Holmes, R. 1961. Serum Fractionation and the Effects of Bovine Serum Fractions on Human Cells Grown in a Chemically Defined Medium. J. Biophys. Biochem. Cytol. 10:389.

26. Henle, G. and F. Deinhardt. 1957. The Establishment of Strains of Human Cells in Tissue Culture. J. of Immunology 79:54.

27. Lehman, H.E. 1965. A Cytochemical Screening Method for Demonstrating General Molecular Groups in Single-Tissue Sections: II. Polychrome Stain. J. Elisha Mitchell Scientific Society 81(2):90.

28. Merchant, D., R. Kahn, and W. Murphy. 1960. Handbook of Cell and Organ Culture. Minneapolis, Minnesota: Burgess Publishing Co.

29. Moore, T.A. 1957. Vitamin A. New York: Elsevier Publishing.

30. Rasmussen, R., I. Wang, and T. Crocker. 1972. Vitamin A-Induced Modification of Benzo-(a)pyrene Metabolism in Syrian Hamster Cell Cultures. Journal of the National Cancer Institute 49(3):693.

31. Stambrook, P.J., and J.E. Sisken. 1972. Induced Changes in the Rates of Uridine-^{3}H Uptake and Incorporation During the G_1 and S Periods of Synchronized Chinese Hamster Cells. Cell Biol. 52:514.

32. Gingell, D. 1972. Membrane Permeability Change by Aggregation of Mobile Glycoprotein Units. J. Theor. Biol. 38(3):677.

33. Quade, E.S. 1969. Systems Analysis Techniques for Planning-Programming-Budgeting, in Systems, Organizations, Analysis, Management: A Book of Readings. Eds. D.I. Cleland and W.R. King. New York: McGraw-Hill.

34. Aydellote, M.B. 1963. The Effects of Vitamin A and Citral on Epithelial Differentiation In Vitro. I. The Chick Tracheal Epithelium. J. Embryol. Morph. II(1):279.

35. Crocker, T., and L. Sanders. 1970. Influence of Vitamin A and 3,7-Dimethyl-2, 6-octadienal (Citral) on the Effect of Benzo(a)-pyrene on Hamster Trachea in Organ Culture. Cancer Research 30:1312.

APPENDIX A

PROCEDURES AND MATERIALS FOR CELL CULTURE

COMPOSITION[a] OF DEFINED MEDIUM A2 + APG (HOLMES)[b]

Component	mg/L	Component	mg/L
NaCl	6000.000	Citric acid	1.000
KCl	400.000	5-Methyldeoxycytidylic acid	0.200
$MgSO_4.7H_2O$	200.000	Deoxycytidine HCl	10.000
$CaCl_2.2H_2O$	140.000	Deoxyguanosine	10.000
$Na_2HPO_4.7H_2O$	113.000	Deoxyadenosine	10.000
KH_2PO_4	60.000	Thymidine	10.000
$FeNH_4(SO_4)_2.12H_2O$	0.860	Niacin	0.025
Dextrose	1000.000	Niacinamide	1.000
NaAcetate·$3H_2O$	83.000	Pyridoxal HCl	1.200
Glycine	100.000	Pyridoxine HCl	0.025
L-Arginine HCL	140.000	Thiamine HCl	1.000
L-Histidine	40.000	Riboflavin	1.000
L-Lysine HCl	140.000	D-Ca. Pantothenate	0.010
L-Tryptophan	20.000	i-Inositol	11.000
L-Phenylalanine	50.000	Menadione	0.010
L-Methionine	30.000	Para-Aminobenzoic acid	0.050
L-Serine	50.000	Choline - Cl	2.000
L-Threonine	60.000	Na Tocopherol (PO_4)	0.010
L-Leucine	120.000	Folic Acid	1.000
L-Isoleucine	40.000	Biotin	1.000
L-Valine	50.000	Ascorbic acid	17.500
L-Glutamic acid	134.000	Vitamin B_{12}	2.500
L-Aspartic acid	60.000	Vitamin A	0.100
L-Alanine	50.000	Lipoic acid	1.500
L-Proline	80.000	Adenosine - 5 - (PO_4)	0.020
L-Hydroxyproline	20.000	D-Ribose	0.200
L-Tyrosine	40.000	D 2 Deoxyribose	0.200
L-Cystine	20.000	Galactosamine	100.000
L-Cysteine	57.000	Sodium Pyruvate	100.000
Glutathione	15.000	Acetyl choline - Cl	10.000
L-Glutamine	400.000	Diphosphopyridine nucleotide	7.000
L-α-amino-n-butyric acid	2.500	Triphosphopyridine nucleotide	1.000
L-Asparagine	4.100	Coenzyme A	2.500
L-Ornithine HCl	3.500	Cocarboxylase	1.000
L-Taurine	2.000	Flavin adenine dinucleotide	1.000
L-Homocystine	1.000	Uridine triphosphate	1.000
L-Ergothionine	0.800	Adenosine triphosphate	1.080
D-Glucosamine HCl	3.200	Cholesterol	0.200
Glucuronolactone	1.000	Calciferol	0.100
Na Glucuronate	1.000	Beta estradiol	0.100
Xanthine	1.000	Tween 80*	5.000
Hypoxanthine	1.000	Cholic acid	20.000

Component	mg/L	Component	mg/L
Glucose - 1 - PO_4(K).....	1.000	Phenol red.....	10.000
Fructose - 1,6 - PO_4(Mg)...	100.000	$NaHCO_3$.....	1400.000

*Trademark of Atlas Powder Co.

[a]GIBCO catalogue (1974) p. 103

[b]References: J. Biophys. and Biochem. Cytol. 1961. 10:389
J. Cell Biol. 1967. 32:297

CHARACTERISTICS OF I-407 (CCL 6)[a]

HISTORY:

Intestine 407 was isolated by G. Henle and F. Deinhardt in January, 1955, from the jejunum and ileum of a two-month-old Caucasian embryo (J. Immunol., 79:54, 1957). The cells were first grown in Scherer's maintenance medium, 75%; acetone chick embryo extract, 5%; and human serum, 20%. The original cells were fibroblasts and histiocytes; however by the fourth passage epithelial-like cells were observed. The culture medium was changed to basal medium (Eagle), 90%; and human serum, 10%; and the cells became completely epithelial-like. In 1958, the line was adapted to calf serum.

Henle and Deinhardt found that Intestine 407 had a broad viral spectrum but was not as sensitive to most viruses as HeLa. Transmissible CPE effects were observed with herpes simplex, pseudorabies, vaccinia, vesicular stomatitis, poliovirus type 1, 2 and 3, all types of Coxsackie B viruses, adenovirus type 3, Newcastle disease virus, and some strains of mumps virus. Influenza virus produced CPE with large inocula but was not transmissible. Rabies virus could not be propagated.

DESCRIPTION OF REPOSITORY REFERENCE SEED STOCK:

Number of Serial Subcultures from Tissue of Origin: 273
Freeze Medium: Culture medium, 95%; glycerol, 5%; antibiotic-free.
Viability: Approximately 94% (dye exclusion).
Culture Medium: Basal medium (Eagle) with Hanks' BSS, 85%; calf serum, 15%; antibiotic-free.
Growth Characteristics of Thawed Cells: An inoculum of 10^5 viable cells/ml, in the above culture medium, multiplies 10-fold in 7 days.
Plating Efficiency: Approximately 55% in MEM (Eagle), 80%; calf serum, 20%.
Morphology: Epithelial-like.
Karyology: Chromosome Frequency Distribution 47 Cells: 2n = 46

Cells:	1	1	1	1	1	1	1	4	2	7	2	6	3	2	1	1	2
Chromosomes:	56-	60-	65-	68-	71	72	73	74	75	76	77	78	79	80	81-	84-	86-

	1	1	1	1	1	1	1	2	1
	91-	108-	112-	115-	121-	136-	142-	143-	153

No marker chromosomes noted. Aneuploidy 100%.
Sterility Tests: Free of mycoplasmas, bacteria, and fungi.
Species: Confirmed as human by hemagglutination technique, immunofluorescence, agar immunodiffusion, cytotoxic-antibody plating, and cytotoxic-antibody dye-exclusion techniques.

Virus Susceptibility: Susceptible to virulent polioviruses types 1,2,3, adenovirus type 3, and vesicular stomatitis (Indiana Strain) virus.

Tumorigenicity: Forms intraperitoneal tumors in irradiated and cortisonized rats with 1 x 10^6 cells. Forms tumors in the cheek pouch of normal hamsters with 1 x 10^4 cells.

Isoenzymes: G6PD type A.

Submitted by: W. Henle, Virus Laboratory, Children's Hospital of Philadelphia, and the University of Pennsylvania, Philadelphia, Pennsylvania.

Prepared and characterized by: Institute for Medical Research, Camden, New Jersey.

---

[a]American Type Culture Collection catalogue (1973).

ALCIAN BLUE WITH LEHMAN'S POLYCHROME

Fixatives: 10% neutral buffered formalin, NOT Bouin's or Zenker's.

1. Remove coverslips from neutral buffered formalin; place in 2 changes of tap water; then leave in distilled water for 30 minutes.

2. Stain in alcian blue for 30 minutes. Remember to filter the alcian blue.

3. Wash in running tap water 2 minutes; then rinse in distilled water.

4. Place in 0.5% acid celestine blue, pH 1.6 for 5-7 minutes. Prepare by mixing 100 cc distilled water, 5 gm iron alum and 0.5 gm celestine blue. Boil 3 minutes, cool, and filter; add 14 cc glycerine and 1 cc conc. H_2SO_4.

5. Wash in distilled water for 1 minute.

6. Differentiate in 1% chromalum for 30 seconds. (pH 3)

7. Wash in distilled water for 1 minute. (pH 4)

8. Place in 1% acid naphthol yellow S (in 1% acetic acid) for 1 minute. (pH 3)

9. Differentiate in 1% acetic acid for 1 minute. (pH 3)

10. Mordant in 1% phosphomolybdic acid-1% phosphotungstic acid (1:3) for 30 seconds. (pH 1.5)

11. Place slides in acid aniline blue-chromotrope 2R for 1.5 minutes. Prepare on day of use by mixing: 1 part 1% aniline blue in 0.1 N HCl, 1 part 1% chromotrope 2R in 0.1 N HCl, 1 part 1% citric acid, 1 part 1% phosphomolybdic acid, and 3 parts 1% phosphotungstic acid. Prepare citric acid fresh each time.

12. Give the slide one quick dip in 1% acetic acid. (pH 3)

13. Differentiate with 95% acid ethanol-butanol for 3-5 dips. (Prepare by mixing 45 cc absolute ethanol, 45 cc absolute tertiary butanol and 5 cc 1% acetic acid.)

14. Dehydrate in 2 changes of absolute triethylphosphate or absolute ethanol. (pH 3)

15. Clear in toluene or xylol. (pH 2.5)

16. Mount in permount.

Results: Celestine blue stains the nuclei steel blue and the cytoplasmic RNA lavender. Histones, hemoglobin, keratin, and other basic proteins or proteins rich in -SH groups are yellow. Aniline blue stains collagen and basement membranes clear blue. Alcian blue stains mucopolysaccharides bright blue. Acid and neutral proteins, including muscle and fibrin, are scarlet. Mitotic chromosomes are occasionally green. Nucleoli are green on some slides and lavendar on others.

THE PRESERVATION OF CELLS BY FREEZING

1. Select a confluent or nearly confluent flask of cells.
2. Remove the cells with trypsin as in the subculturing process.
3. Centrifuge the cell suspension and discard the supernatant fluid. Resuspend the cells in the usual growth medium at a cell density of 10^5 to 10^6 cells/ml. Adjust the pH with carbon dioxide to pH 7.
4. Add sterile glycerol to the medium to a final concentration of 8% by volume. Mix and set at room temperature for 10 minutes.
5. Dispense .5 to 1.0 ml into small tubes with teflon lined caps.
6. Place the tubes in an insulated container in a deep-freeze at -70°C, so they will freeze slowly.
7. Store cells at -70°C.

HANDLING OF FROZEN CELLS

1. Thaw the cells as rapidly as possible in a 37°C water bath by rapidly swirling the tube. Thawing should take from 40-60 seconds.
2. As soon as the ice is melted, remove the tube from the water bath and place in 70% ethanol at room temperature.
3. Add enough growth medium to give the desired concentration of cells. After opening the tube, all techniques should be done aseptically.
4. If it is desired to remove the glycerol, the cells may be centrifuged. It is not necessary to remove the glycerol with I-407 until the media change the following day.
5. Place the cells in flasks and treat as freshly subcultured cells.

STEPS IN THE SUBCULTURE PROCESS

1. Prepare dilutions of trypsin by mixing .2 ml of stock trypsin and .6 ml of PBS. Add .1 ml of this dilution to 9.9 ml PBS. (Final concentration of trypsin-0.25%)

2. Aspirate old media with Pasteur pipet.

3. Rinse cells twice with PBS.

4. Add 2 ml of trypsin solution to each flask.

5. Incubate at $37^{o}C$ for 10 minutes, or until the cells start to come off the bottom of the flask.

6. Agitate the flasks until the cells are removed from the bottom of the flask.

7. Split the contents between 2 flasks, adding 1 ml of the cell suspension and 2 ml of media to each flask. The media may be placed in the flasks before transferring in the cells. The contents of several flasks may be pooled before splitting if an experiment is planned.

8. Gas with 5% carbon dioxide for a predetermined number of seconds. It takes a few minutes for the color of the media to change.

9. Change media the next day, if possible, to remove toxic products.

HAEMOCYTOMETER TECHNIQUE FOR COUNTING LIVING CELLS[a]

(Dye Exclusion Method)

1. Add 1.0 ml cell suspension to .5 ml trypan blue.
2. Mix well.
3. Carefully fill both chambers under the cover glass.
4. The living cells have the ability to exclude the dye and remain clear. Count the number of clear living cells in the 4 corners and 1 center square on each of the 2 grids.
5. Multiply the total number of cells in the 10 squares by 1000 and again by 1.5 to give the total number of living cells per ml.

---

[a]Modification of Merchant et al.

THE PROCEDURE FOR PREPARING 1X MEM AND 1X PBS FROM THE 10X CONCENTRATES

1. To prepare 1X PBS from the 10X concentrate, aseptically add 450 ml of sterile glass distilled water plus 50 ml of 10X PBS and store at room temperature.

2. To prepare 1X MEM from the 10X concentrate, aseptically add 400 ml of sterile glass distilled water, 50 ml of 10X MEM and 50 ml of sterile 7.5% sodium bicarbonate solution to a sterile glass bottle. Filter sterilize or purchase the sodium bicarbonate solution. Incubate a sample for several days to check for sterility.

CONDITIONS USED FOR AUTOCLAVING

	TEMPERATURE	TIME
All bottles and glassware	121°C	30 minutes
All wrapped materials	121°C	30 minutes
2 liter flasks with fluids	121°C	35-40 minutes

DIRECTIONS FOR WASHING TISSUE CULTURE GLASSWARE[a]

1. New glassware is immersed in 1% hydrochloric acid for 18 hours and rinsed several times in running tap water before washing. New stoppers are boiled in 0.5 N NaOH before washing.

2. Glassware is washed according to directions of the detergent used. A 1% solution of 7X or a .5% solution of Microsolve (Microbiological Associates) is used. Glassware is boiled for 5 minutes, scrubbed with a nylon brush, rinsed 10 times in running tap water, and 2 times in distilled water. The glassware is then oven-dried.

3. Accumulation of proteinic material in glass culture flasks can be removed by overnight soaking in a solution of potassium dichromate and sulfuric acid. (A solution may be prepared by mixing 500 ml H_2O, 45.0 g $K_2Cr_2O_7$, and 48 ml H_2SO_4. This is much weaker than the sulfa-chromic solution suggested by Penso and Balducci which contained 50 g of $K_2Cr_2O_7$ dissolved in 1 L of H_2SO_4.)

4. Washing is facilitated if media bottles are rinsed several times and left full of water while waiting for washing. Instruments should be rinsed thoroughly and then wiped dry to prevent rusting. Discarded cultures should be autoclaved before washing. Discarded media should be autoclaved immediately as a safety precaution.

5. Tissue culture glassware and general glassware must be kept separate since general glassware is also used for staining and formalin associated work. Pans used for washing tissue culture glassware should not be used for other purposes.

6. Several means of packaging for sterilization by autoclaving are used: Screw cap tubes for forceps must have strings to be sure that hot steam can enter and moisture escape with the following oven drying. Cotton-plugged, foil-covered large culture tubes are used for scapels and other instruments. Cotton-plugged disposable Pasteur pipets are packaged in bags, as are T-flasks and Leighton tubes with stopper closures.

---

[a]Summarized and adapted for the laboratory from Penso, G. and Balducci, D. 1963. Tissue Cultures in Biological Research. Chapter 3. New York: Elsevier Publishing Co.

APPENDIX B

DATA

TABLE 1

THE EFFECT OF SAMPLE SIZE OF I-407 TCA INSOLUBLE FRACTIONS ON THE COUNTING EFFICIENCY AND ESR VALUES USING ^{3}H-TOLUENE

Sample Size in mg	DPM	CPM	ESR	% Efficiency
.00	16,800	7274	.558	43.30
.25	"	6908	.563	41.10
.50	"	6936	.567	41.40
.75	"	6846	.550	40.70
1.00	"	6875	.571	41.00
1.50	"	6472	.553	38.50
2.00	"	6795	.558	40.40
2.50	"	6553	.565	39.00

TABLE 2

THE EFFECT OF VITAMIN A (5 μg/ml) ON THE UPTAKE AND INCORPORATION OF ^{3}H-URIDINE

Length of treatment with vitamin A	DPM/mg TCA precipitate (incorporation)	DPM/ equivalent soluble sample (uptake)*
	EXPERIMENT A	
0	11,786	16,200
	31,725	68,600
10 minutes	4,752	5,340
	4,652	5,340
30 minutes	2,299	3,680
	1,470	2,920
2 hours	8,799	12,540
	5,017	10,760
	EXPERIMENT B	
0	2,016	840
	670	100
10 minutes	196	200
	245	540
30 minutes	503	460
	1,036	100
1 hour	434	220
	614	420
	EXPERIMENT C	
Length of treatment with vitamin A	CPM/mg TCA precipitate	CPM/ equivalent soluble sample
0	11,581	17,732
	19,414	21,103
10 minutes	9,366	10,850
	11,252	19,042
30 minutes	9,549	15,193
	16,090	18,154
2 hours	8,781	12,803
	7,722	11,207

*The DPM/equivalent soluble sample were found by dividing the DPM per .1 ml sample by the size of the TCA precipitate and multiplying by 20, since the .1 ml sample was taken from a 2 ml volume homogenate.

TABLE 3

GROUP I EXPERIMENTS COMBINED
THE EFFECT OF VITAMIN A (5 μg/ml) ON THE UPTAKE
AND INCORPORATION OF ^{3}H-URIDINE

Length of treatment with vitamin A	Relative incorporation into TCA precipitate	Relative uptake into soluble fraction
0	.5418	.3812
	1.4582	1.6179
	1.5013	1.7628
	.4987	.2372
	.7473	.9132
	1.2527	1.0862
mean	1.0000	1.0000
10 minutes	.2138	.1262
	.2184	.1263
	.1457	.4261
	.1821	1.1541
	.6043	.6594
	.7271	.5772
mean	.3484	.5116
30 minutes	.1057	.0867
	.0676	.0689
	.3747	.9825
	.7714	.2021
	.6162	.7825
	1.0382	.9351
mean	.4955	.5096
1-2 hours	.4044	.2956
	.2306	.2536
	.3229	.4650
	.4574	.8742
	.5666	.5588
	.4982	.9807
mean	.4200	.5713

TABLE 4

THE EFFECT OF DIFFERENT LEVELS OF VITAMIN A ON THE UPTAKE AND INCORPORATION OF ^{3}H-URIDINE

Level of vitamin A (µg/ml)	CPM/mg TCA precipitate (incorporation)	CPM/equivalent soluble sample (uptake)
	EXPERIMENT D	
0	390	664
	611	458
1	265	301
	452	614
5	254	143
	206	176
10	195	126
	360	217
	EXPERIMENT E	
0	28,298	32,618
	16,442	27,860
1	33,834	35,000
	18,839	17,272
5	15,705	13,780
	13,753	13,346
10	10,576	10,032
	5,383	3,380

TABLE 5

GROUP II EXPERIMENTS COMBINED
THE EFFECT OF DIFFERENT LEVELS OF VITAMIN A ON
THE UPTAKE AND INCORPORATION OF ^{3}H-URIDINE

Level of vitamin A (µg/ml)	Relative incorporation into TCA precipitate	Relative uptake into soluble fraction
0	.7795	1.1837
	1.2205	.8164
	1.2650	1.0787
	.7350	.9213
mean	1.0000	1.0000
1	.5290	.5369
	.9041	1.0958
	1.5125	1.1574
	.8422	.5712
mean	.9470	.8401
5	.5078	.2553
	.4120	.3144
	.7021	.4557
	.6148	.4414
mean	.5592	.3667
10	.3899	.2250
	.7201	.3875
	.4728	.3318
	.2406	.1121
mean	.4559	.2641

TABLE 6

THE EFFECT OF LOWER LEVELS OF VITAMIN A ON THE UPTAKE AND INCORPORATION OF ^{3}H-URIDINE

Level of vitamin A (µg/ml)	CPM/mg TCA precipitate (incorporation)	CPM/equivalent soluble soluble
EXPERIMENT F		
0	484	355
.01	883	336
.1	402	231
1.0	1,039	247
EXPERIMENT G*		
Level of vitamin A (µg/ml)	DPM/mg TCA precipitate (incorporation	DPM/equivalent soluble sample (uptake)
0	44,402	62,340
	45,457	84,120
.01	23,489	69,558
	65,676	107,584
.1	40,214	58,650
	69,213	140,933
1.0	63,740	132,239
	90,924	80,892
EXPERIMENT H		
0 (no cold uridine)	27,063	55,864
	29,722	54,306
0 (with cold uridine)	1,458	3,131
	1,850	4,473
.01	2,435	7,231
	2,927	7,261
.1	2,102	5,839
	1,773	6,815
1.0	2,627	8,385
	2,455	7,399
5.0	1,744	3,773
	1,966	6,949

*Each value in this experiment represents the average of two determinations performed on each TCA pellet.

TABLE 7

GROUP III EXPERIMENTS COMBINED
THE EFFECT OF LOWER LEVELS OF VITAMIN A ON
THE UPTAKE AND INCORPORATION OF ^{3}H-URIDINE

Level of vitamin A (µg/ml)	Relative incorporation into TCA precipitate	Relative uptake into soluble fraction
0	1.0000	1.0000
	.9883	.8513
	1.0117	1.1487
	.8813	.8235
	1.1187	1.1765
mean	1.0000	1.0000
.01	1.8218	.9443
	.5228	.9499
	1.4618	1.4691
	1.4720	1.9020
	1.7699	1.9102
mean	1.4097	1.4351
.1	.8294	.6488
	.8951	.8009
	1.5405	1.9245
	1.2713	1.5358
	1.0720	1.7927
mean	1.1217	1.3405
1.0	2.1440	.6950
	1.4187	1.8058
	2.0170	1.5197
	1.5882	2.2057
	1.4847	1.9461
mean	1.7305	1.6344

TABLE 8

COMPILATION OF DATA

Experiment	ESR	% Efficiency	Approximate average CPM of controls	Media used for experiment	Depletion Period
A	.555	41.8	4,800	supplemented MEM + 5% FBS	yes
B	.569	42.4	550	" "	yes
C	.454	30.0	15,400	" "	no
D	.466	30.0	500	A2 + APG(Holmes)	no special medium A2 + APG(Holmes)
E	.452	29.0	22,400	" "	yes
F	.497	33.0	600	" "	no
G	.587	44.0	20,000	supplemented MEM + 10% FBS	no
H	.603	45.0	5,000	supplemented MEM + 5% FBS	no
Variability Study	.579	43.5	3,000 7,000 4,000	" "	no
Pulse length Temperature Study	.548	41.2	9,000	A2 + APG(Holmes)	no

TABLE 9

TIME-TEMPERATURE STUDY

Temperature (°C)	Pulse length (minutes)	Incorporation into TCA insoluble pellet (DPM/mg TCA pellet)	Uptake into TCA soluble fraction (DPM/ equivalent soluble sample)
19	10	9,216	12,900
	15	13,187	22,480
	30	12,192	31,400
	60	27,513	37,320
38	10	23,410	42,940
	15	14,789	15,820
	30	26,998	21,160
	60	16,681	22,320

APPENDIX C

ANALYSES OF VARIANCE

VARIABILITY STUDIES

TABLE 1

ANALYSIS OF VARIANCE FOR GROUP I EXPERIMENTS
(INSOLUBLE FRACTION)

Source	df	SS	MS
Total	23	3.754935	
Treatments	3	1.583689	.527896 *
Replications	5	.712672	.142534
Expt. Error	15	1.458574	.097238

(SOLUBLE FRACTION)

Source	df	SS	MS
Total	23	5.085471	
Treatments	3	1.005289	.335096
Replications	5	1.339078	.267815
Expt. Error	15	2.741104	.182740

*Sig. at 0.05 level

TABLE 2

ANALYSIS OF VARIANCE FOR GROUP II EXPERIMENTS
(INSOLUBLE FRACTION)

Source	df	SS	MS
Total	15	1.809110	
Treatments	3	.895464	.298488 *
Replications	3	.479809	.159936
Expt. Error	9	.433837	.048204

(SOLUBLE FRACTION)

Source	df	SS	MS
Total	15	2.018302	
Treatments	3	1.535072	.511690 **
Replications	3	.144855	.048285
Expt. Error	9	.338375	.037597

*Sig. at 0.05 level
**Sig. at 0.01 level

TABLE 3

ANALYSIS OF VARIANCE FOR GROUP III EXPERIMENTS
(INSOLUBLE FRACTION)

Source	df	SS	MS
Total	19	3.483596	
Treatments	3	1.594267	.531422
Replications	4	.747832	.186958
Expt. Error	12	1.141496	.095124

(SOLUBLE FRACTION)

Source	df	SS	MS
Total	19	4.367354	
Treatments	3	.694869	.231623
Replications	4	1.964002	.491000
Expt. Error	12	1.708482	.142373

*Sig. at 0.05 level

TABLE 4

VARIABILITY EXPERIMENT ON UPTAKE AND INCORPORATION OF ^{3}H-URIDINE (OLD METHOD)

Day	Flask #	Tube #	DPM/µg TCA precipitate	DPM/equivalent soluble sample
1	1	1.	363 596	2,560
		2.	1,033 1,153	2,620
		3.	2,111 2,208	3,860
	2	1.	256 8,150	2,540
		2.	1,857 2,218	6,060
		3.	2,917 4,120	1,940
2	3	1.	16,526 12,992	52,580
		2.	6,114 5,547	27,880
		3.	7,750 9,356	34,200
	4	1.	6,719 6,047	17,640
		2.	9,263 4,407	16,720
		3.	9,484 5,548	20,360
3	5	1.	5,197 3,930	12,380
		2.	4,695 3,614	15,540
		3.	4,480 7,221	14,220

TABLE 4 Continued

Day	Flask #	Tube #	DPM/µg TCA precipitate	DPM/equivalent soluble sample
		1.	4,098 3,611	19,360
	6	2.	3,248 3,407	9,920
		3.	4,700 5,585	25,280

TABLE 5

VARIABILITY STUDY CONTINUED: VALUES OF DUPLICATES OBTAINED IN EXPERIMENT G (OLD METHOD)

Level of vitamin A (µg/ml)	DPM/mg TCA precipitate	% Difference	DPM/equivalent soluble sample
0	35,470		62,340
	53,333	33.49	
	44,628		84,120
	46,287	3.58	
.01	31,624		69,558
	15,351	51.46	
	75,121		
	56,231	25.15	107,584
.1	40,894		
	39,533	3.33	58,650
	66,699		
	71,727	7.01	140,933
1.0	48,837		
	78,642	37.9	132,239
	116,685		
	64,652	44.59	80,892
Average % Difference in duplicates		25.87	

TABLE 6

VARIABILITY STUDY CONTINUED: VALUES OF DUPLICATES OBTAINED IN EXPERIMENT H (NEW METHOD)

Level of vitamin A (µg/ml)	DPM/mg TCA precipitate	% Difference	DPM/equivalent soluble sample
0	27,071		56,806
	27,056	.06	54,922
(no cold uridine)	29,271		53,892
	30,174	2.98	54,720
0	1,459		3,021
	1,456	.21	3,241
(with cold uridine)	1,850		4,426
	1,850	.00	4,519
.01	2,357		7,369
	2,512	6.29	7,093
	2,935		7,159
	2,918	.58	7,364
.1	2,037		5,766
	2,168	5.04	5,915
	1,758		7,943
	1,788	3.81	5,687
1.0	2,456		8,201
	2,789	11.94	8,569
	2,432		7,296
	2,479	1.90	7,501
5.0	1,712		3,832
	1,776	3.60	3,714
	1,953		6,909
	1,978	1.26	6,989
Average % Difference in duplicates		3.13	

TABLE 7

INFLUENCE OF DISPERSING SAMPLE
IN .1 ml AND .02 ml WATER

	Dispersed in .02 ml water				Dispersed in .1 ml water			
Sample size	DPM	CPM	ESR	% efficiency	DPM	CPM	ESR	% efficiency
.00	16,880	7,274	.568	43.1	16,880	8,046	.637	47.5
.25	"	7,008	.563	41.5	"	7,738	.609	45.6
.50	"	7,136	.567	42.3	"	7,891	.607	46.7
.75	"	7,146	.556	42.3	"	7,788	.604	46.0
1.00	"	7,275	.571	43.1	"	7.665	.604	45.4
1.50	"	7,072	.553	41.7	"	7,783	.604	46.1
2.00	"	7,595	.558	44.9	"	7,703	.602	45.7
2.50	"	7,553	.565	44.8	"	7,579	.596	44.9
Average			.563	42.9			.608	46.0