PROPAGATION AND SOME METABOLIC PROPERTIES OF MALIGANT ANIMAL CELLS CULTURED IN BACTERIOLOGICAL MEDIA

by

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A Thesis

submitted to

The Faculty of Graduate Studies and Research

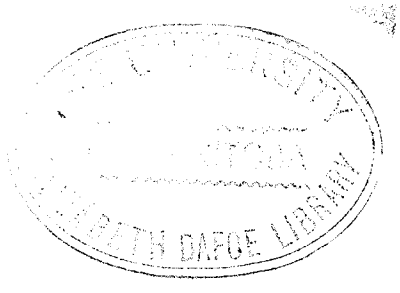
University of Manitoba

In partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

April, 1966



ABSTRACT

HeLa and ascites tumour cells could be cultured in a medium of bacteriological nutrient broth plus yeast RNA. There was no growth if the RNA was omitted from the medium. The growth-promoting property of the RNA was found to reside in a large molecular weight fraction of the RNA ($1\text{-}2 \times 10^5$) and not in any small sequence of nucleotides. This property also seemed to be true for the induction of the enzyme tryptophan pyrrolase in HeLa cells and, in the latter case, the effect could not be attributed to a possible messenger function.

Some properties of tissue cells cultured in this fashion were studied. The oncogenic properties of ascites cells appeared to be lost after a period of culture in the basal medium. The uptake of labelled (C^{14}) amino acids from growth media was affected by RNA. A discrepancy in uniformity of results was noticed in ascites cells depending on the stage of tumour transfer. The significance of these findings is discussed.

ACKNOWLEDGEMENTS

The author is indebted to Dr. Howard Lees, Head of the Department of Microbiology, for his guidance as academic advisor for this investigation.

The author is thankful to Dr. B. D. Sanwal of this Department for many helpful discussions and suggestions related to the problem.

An expression of gratitude is also due to Dr. M. J. Fraser, formerly of the Department of Biochemistry, for helpful suggestions and for supplying the original Ehrlich's ascites tumours used in this study.

The work was supported by a grant from the National Cancer Institute of Canada.

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INTRODUCTION

A living cell is often capable of surviving radical changes in its immediate environment so long as it either possesses the genetic capability to alter its metabolism or receives the aid of some agent foreign to its normal existence such as the nucleic acid involved in "transduction". In the latter case, the transduction may eventually be harmful to the cell, as is frequently the case in a viral invasion; on the other hand, viral invasion may be beneficial if it allows the cell to adapt to its new environment without untoward side effects. In biological science, the experimenter is now able to create these artificial conditions and to study their effects in much greater detail because of advancing knowledge of recent years. As a result, there is now a much better understanding of the phenomenon of adaptation than there used to be. When studies are carried out under *in vitro* conditions, more finely controlled work can be undertaken but the results must be interpreted with more care because of the change in habitat of the cell compared with the *in vivo* situation. Recent work, as outlined in some detail

in the Historical Section (pp. 5-13), has resulted in a much greater understanding of such areas as protein synthesis and the environmental conditions necessary for cell survival.

One example of how a biological material derived from a species completely alien to a cell affects the metabolism of that cell is that of ribonucleic acid (RNA). One active area of study in this field is the effect of infectious RNA derived from various viruses, such as tobacco mosaic virus and the causative agent of poliomyelitis. This thesis is a report of some experiments on the effect of RNA derived from yeast on cells of malignant animal origin.

In a previous work by the author (Kenny, 1962) it was noted that in order to induce activity of the enzyme tryptophan pyrrolase in HeLa cells cultured in Morgan's Medium 199 plus 10% calf serum plus the inducing compound (L-tryptophan), it was necessary to incorporate small amounts of yeast RNA into the culture medium. A similar requirement from RNA was observed (Klein, 1961) in experiments on the induction of arginase.

In the course of subsequent experimental work to determine the nature of the RNA effect on the induction of tryptophan pyrrolase activity we carried out a series of

tests to determine the purity of the commercially-prepared RNA. When it became apparent that the effect was due to the RNA molecule itself and not to any impurity, the question arose as to whether this extracellular RNA incorporation could, in fact, 'initiate' the cells into exhibiting other biochemical or physiological phenomena that they are not capable of doing otherwise. Our initial thoughts were that the yeast RNA was possibly a messenger RNA which was instructing the HeLa cells to synthesize this protein. However, efforts to show tryptophan pyrrolase activity in yeast cells proved fruitless.

During the course of this work, it was thought that the yeast RNA with the demonstrated 'initiating' property might possibly allow tissue culture cells to propagate in serum-free media. Test tubes of nutrient broth which were used for routine sterility testing of reagents used in the cultural procedures were inoculated first with a bacteriological loopful of HeLa cells, and secondly with a similar loopful of sterile yeast RNA solution. After overnight incubation, it was observed that cellular proliferation had indeed occurred (Kenny & Lees, 1963).

Cellular proliferation continued for several

generations in this fashion and attempts were again undertaken to determine which portion of the RNA molecule was responsible. An investigation of whether the growth promoting property was manifested only with HeLa cells was undertaken with the result that similar observations were made for Ehrlich ascites tumour cells cultivated in suspension cultures *in vitro*.

The academic curiosity, as well as the possible practical advantages of growing cell cultures in this fashion, was the stimulus for the work that is presented in this thesis.

HISTORICAL

The culture of animal cells in tissue or cell culture has developed from the first classic experiments of Roux (1885), on the neural plate of a chick embryo; of Jolly (1903), on the division of amphibian leucocytes *in vitro*; and of Harrison (1907) on the living developing nerve fibre.

Except for the fields of cell morphology and embryology, the method received little attention for several years due to the complexity of the methodology until the advent of the antibiotic era. These compounds, when incorporated into culture media, drastically reduced the incidence of microbial contaminations and thus brought tissue culture into the realm of practical methodology.

The first chemically defined medium for animal tissue culture was introduced in 1946 by P. R. White. Since this latter development, great strides in the technology of tissue culture have been made due to the gradual definition of the basic nutritional, chemical and physical requirements necessary for consistent replication

of cell material and similarly, of experimental results. These developments have been well reviewed by several authors (Morgan, 1958; Parker, 1961a).

The use of tissue culture media containing materials designed for bacteriological work has received some acceptance and is often resorted to as an economical substitute for complex amino acid-vitamin mixtures. Melnick and Riordan (1952) propogated polioviruses in testicular tissue of monkeys in a medium consisting of 0.5% lactalbumin hydrolysate, serum ultrafiltrate and balanced saline. Baron and Law (1958) were able to replace the 10% animal sera normally incorporated into Medium 199 (Morgan, Morton, and Parker, 1950) by 20% skimmed milk and maintained Salk's monkey-heart cells, Eagle's KB cells, and rhesus monkey kidney cells in this medium by renewal of medium at seven day intervals. Ginsberg, Gold, and Jordan (1955) maintained HeLa cells in Scherer's maintenance medium supplemented with 7.5% chicken serum and 15-25% tryptose phosphate broth. These experiments and others of similar nature are reviewed by Parker (1961b).

In 1913, Carrel discovered that embryo tissue-extract markedly improved cell growth and multiplication in

his strain of connective tissue cells. Since this time, it has been well established that extracts from tissues such as chick embryo greatly increase proliferation in cell cultures. Fischer et al. (1948) isolated nucleoproteins from calf embryos in which growth-promoting activity appeared to be associated with the ribonucleoproteins. Waymouth (1947) demonstrated a similar activity in sheep embryo extract. Harris and Kutsky (1958) obtained nucleoprotein from adult chicken spleen which induced rapid multiplication of newly-explanted skeletal muscle fibroblasts when added to a basal medium containing chicken serum and Medium 199. Growth did not occur in the unsupplemented basal medium alone or in serum-free Medium 199 supplemented with the nucleoprotein fraction. Moore et al. (1963) successfully grew some lines of human tumour cells as suspension cultures in a chemically defined medium supplemented with albumin or with very small amounts of fetal calf serum. Floss (1964), using a strain of human carcinoma cells derived from ascitic fluid, noted a growth stimulation by lactalbumin hydrolysate in a medium of Earles balanced salt solution plus 10% calf serum. He found, however, that if the concentration of hydrolysate

was raised above 1% an inhibitory effect was observed until at a level of 5% hydrolysate, no growth was observed.

Amos and Kearns (1963) added exogenous P^{32}-labelled RNA derived from the bacterium Escherichia coli B to monolayers of chick embryo fibroblasts in medium that contained 3% horse serum. This exogenous RNA stimulated the synthesis of protein in these cells but only when the concentration was 400 µg/ml RNA and when the RNA was protected, by the addition of protamine, from breakdown by the ribonuclease released by the cells into the medium.

Only in recent years have any extensive investigations been carried out on enzyme activity in cell tissue cultures. Klein, in 1960, using embryonic chick and mouse cells, studied arginase induction and demonstrated that the enzyme occurred only in primary cell strains whereas no enzyme could be found in established strains. In expanding this work, Klein was able to induce the enzyme in established cell-lines when these were grown in Medium 199 with yeast RNA incorporated along with the substrate (Klein, 1961).

Cox and Pontecorvo (1961) observed the induction of alkaline phosphatase, by phenylphosphate and β-glycerophosphate, in established cultures of human skin fibroblasts. Niu et al.

(1962) prepared samples of RNA from mouse, rat, and calf liver and separated each sample into high and low molecular weight fractions. They then incubated mouse ascites cells overnight at 2^{o} C in saline plus RNA from one of these sources, yeast RNA, or tumour RNA. They found that the cells acquired the ability to synthesize liver serum albumin only when the cells were exposed to liver RNA and that they acquired the ability to synthesize tryptophan pyrrolase only in the presence of liver RNA. They concluded that low molecular weight fraction of liver RNA contained some usable genetic information and that the type of specific protein synthesized by the RNA recipient cells was related directly to the tissue source of RNA, i.e. the cells acquired the ability to synthesize typical liver proteins only in the presence of liver RNA. As previously pointed out in the Introduction, we ourselves were also able to demonstrate induction of tryptophan pyrrolase in HeLa cells only in the presence of yeast RNA (Kenny & Lees, 1961).

In a further work Niu used C^{14}-labelled RNA to treat mouse ascites cells and demonstrated that liver RNA was necessary to induce biosynthesis of glucose-6-phosphatase.

As the sedimentation coefficients of the RNA increased upon fractionation, so, correspondingly, did the inducing capability. Once the cells had acquired this ability to synthesize the enzyme the RNA was no longer required. These cells were then injected into mice and the resulting tumour cells, if the mice were injected every 3-5 days with liver RNA, maintained the capacity to synthesize the enzyme (Niu, 1964).

The cytochemical approach to the detection of enzymes in tissue cells in vitro has been reviewed by Fortelius (1963).

Gotto et al. (1964), using ascites cells, found that the addition of nucleotides and nucleosides to Krebs Ringer solution, in which the cells were incubated for two hours after removal from the mouse, enhanced the uptake of C^{14}-labelled amino acids by the cells, and thus promoted protein synthesis.

Watts (1964), using suspension cultures of HeLa cells cultured in 0.25% yeast extract, 0.25% nutrient broth, 5% pig serum, and 95% Earle's saline, found that labelled nucleic acid precursors entered the nuclear RNA much more rapidly than the cytoplasmic RNA and that the rate of

incorporation was dependent on the concentration of labelled precursor in the medium.

Gazet and McKibbin (1965) showed that injections of heterologous (bovine) RNA parenterally into Swiss mice increased the percentage of 'take' of tumours following injection of ascites cells. They suggest that the increased 'take' is due to an interaction of heterologous RNA with both the cancer cells and the host cells.

The nucleic acids are not, of course, the only compounds capable of promoting cellular enzyme synthesis when added to a growing culture. For instance Alpers (1964) observed that HeLa cells harvested from 'high-glucose' medium had over 30 times as much glycogen than did 'low-glucose' cells; they also had higher phosphorylase and glycogen synthetase activity although other glycolytic enzymes were not affected. He suggested that ATP was limiting for phosphorylase activation in low-glucose cells and that added glucose activates phosphorylase by providing materials for ATP synthesis.

While these observations on growth stimulation and enzyme induction were being made, work was proceeding on the fractionation of such growth-promoting or enzyme-

inducing materials as RNA and blood serum. The separation and purification of macromolecules by gel filtration on columns packed with Sephadex, a compound of cross-linked dextran gels, has gained wide usage since its development in the late 1950's. In 1961, Gelotte used a column of Sephadex G-25 to fractionate yeast nucleotides dissolved in NaCl and obtained four main fractions containing nucleic acid, oligonucleotides, nucleoside phosphates, and adenylic acid. By further fractionation on Sephadex G-75 he obtained a separation according to molecular weight of the obviously heterogeneous polynucleotide material. In 1962, Tanaka et al. obtained by partition chromatography on large columns of Sephadex G-25 reasonably pure fractions of soluble RNA, each of which was more-or-less specific for a single amino acid. In 1964, Tozer and Pirt submitted calf serum to gel filtration on Sephadex G-200 and obtained three peaks. The first, or fastest moving peak proved to be α and β-macroglobulins, the second peak was γ-globulin and the third a mixture of albumin and α- and β-globulins. The most effective growth-promoting factor was located in the trough between the first and second peaks, although a considerable amount was also present in the second peak.

The last decade has thus seen the demonstration of growth promoting properties in fractions of several complex materials such as RNA and blood serum. It has also seen the establishment of sound methods for fractionating these materials. Nevertheless, until very recently, there was no really accurate and reliable method for assessing cell growth. Assessments based on the optical density of the cell culture are subject to many interpretations, determinations of cell-nitrogen or cell protein suffer the same disadvantage, microscopic counts are tedious and (to some extent at least) subjective. The advent of an electronic system of cell counting that could also yield data about the size-distribution of cells in the culture was thus a considerable step forward. The use of the Coulter Counter for particle counting in place of the usual visual techniques was first outlined by Mattern et al. in 1957 using blood cells. The accuracy and versatility of this Counter has been proved by the work of Swanton et al. (1962) with bacteria and by Gebicki and Hunter (1963) who successfully counted and measured the size of rat liver mitochondria (to determine the amount of swelling). Almost all the cell counts reported in this thesis have been made with this instrument.

MATERIALS AND METHODS

A. TISSUE CULTURE PROCEDURE

1) The propagation of HeLa cells

HeLa cells were cultivated and maintained during the course of this study by standard tissue culture techniques. These techniques as well as those involved in all preparative work such as the cleaning of glassware and apparatus, sterilization of compounds and media, and the maintenance of aseptic conditions have already been described in some detail (Kenny, 1962).

The HeLa cell stock was built up from one purchased commercially* as a suspension in Eagles Minimal Essential Medium plus 10% calf serum. The cell concentration was 10^6/ml. Monolayer cultures in milk dilution bottles were established infrequently as they were used only to detect any gross morphological changes and to observe the general condition of the strain. HeLa cell stocks were maintained as agitated monodisperse cell suspensions in 100 ml volumes

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*HeLa Clone S3, Microbiological Associates, Bethesda, Md.

contained in 250 ml Erlenmeyer flasks capped with Morton stainless steel closures which were sealed from the atmosphere by three layers of Scotch brand masking tape. The flasks were incubated at 37.5° C on a rotary incubator shaker (New Brunswick Scientific Co., New Brunswick, N. J.) at 120 r.p.m. Stock cultures were grown in a medium consisting of Morgan's Medium 199 (Morgan et al., 1950) plus 10% calf serum* plus antibiotics in the usual fashion. In order to maintain the cultures in the logarithmic phase of growth, the cells were centrifuged out of the old medium at 500 x g for 10 mins. every fourth day. The cell pellets were resuspended in Hank's balanced salts solution (HBSS), enumerated by Coulter Counter (see Section A 7 below) and reinoculated into fresh medium. The composition of the media used were outlined by Merchant and Kahn (1960); the media were the commercial CulturStat* brand. Adjustment of the pH of the culture was accomplished by periodic additions of $NaHCO_3$ solution (1.4%) to maintain the pH at or near pH 7.2.

2) The in vivo propagation of Ehrlich's ascites tumour cells

Female Swiss white mice, strain CF_1 were used.**

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*from Baltimore Biological Labs (usually abbreviated "B.B.L.")
**from the Lemberger Company, Oshkosh, Wisc.

Generally speaking, the tumour, in liquid form, was transferred from one mouse to another after the donor mouse had borne the tumour for seven days.

The donor mouse was killed by chloroform and the body swabbed with 70% ethanol over the entire abdominal region. The animal was held in a vertical position and the ascites fluid was obtained by inserting a 10 ml syringe fitted with a #18 gauge needle into the peritoneal cavity and aspirating. Any ascitic fluid that contained blood was discarded. The needle was then changed to a finer gauge (#24) and 0.3 ml of the suspension was injected into each receptor mouse intraperitoneally. This was a liquid to liquid transfer (L-L). After the 3rd L-L transfer in any series, a liquid to solid tumour was established (L-S) by subcutaneous injection of 0.5 ml ascitic fluid in the upper thigh region of a hind leg of a receptor mouse. After 14 days the solid tumour which had developed on the leg was used to again establish a liquid tumour (S-L). In this procedure, the skin was incised up the mid-line of the tumour-bearing region and laid back to expose the leg tumour, which was then excised and placed into a sterile petri dish. It was finely minced with scissors in a

minimal volume of 0.9% saline and drawn into the barrel of a 10 ml syringe and the volume measured. The tumour suspension was then diluted to 5 times its volume in saline and was further broken up by forcing the suspension through a series of progressively finer needles. Finally, 0.5 ml of this finely-dispersed suspension was injected intraperitoneally into a mouse. The method outlined was a modification of that used by Fraser (Fraser, 1963).

3) The *in vitro* propagation of Ehrlich's ascites tumour cells

All tumour cells used were from second (L-L) transfers. Mice with seven day tumours were sacrificed and the ascitic fluid removed as outlined in (2) above. The fluid was centrifuged at 150 x g for 5 minutes, the supernatant fluid discarded, and the cell pellet washed three times with HBSS after which it was resuspended in the same solution. The cell population was determined by Coulter Counter (see Section A 7). Experimental media were then inoculated from these suspensions. These cultures were established by procedures identical with those used for HeLa cells.

4) Composition of the basal experimental propagating medium (EPM)

The basal medium used as a starting point for all experiments with both types of cell cultures was made up as follows:-

(a) 8.0 g. bacteriological nutrient broth (B.B.L.) and 10 g. glucose were dissolved in 800 ml distilled deionized water.

(b) 20 mg. phenol red was dissolved in 100 ml distilled deionized water at an alkaline pH and added to (a).

(c) The volume was brought up to 1000 ml and the medium autoclaved at 121° C for 15 mins. at 15 lbs. pressure. It was then dispensed in 90 ml portions in 250 ml. Erlenmeyer flasks.

(d) To each flask 1.0 ml of an antibiotic solution was added (see Section A 5 below for preparation).

(e) The EPM was stored at 4° C until used.

(f) Prior to inoculation of the medium with cells, 1.0 ml of sterile yeast RNA solution * (Section A 5) was incorporated to yield the required final concentration of RNA in the EPM. The amount of RNA used varied considerably

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*RNA, Commercial Grade, from Sigma Chemical Co., St. Louis, Mo.

depending upon the intent of the experiment in question. However, 50 µg RNA/ml EPM represents the final optimal concentration for cell proliferation.

(g) Immediately following inoculation of EPM with cells, 2.0 ml of a 4.0% solution of methylcellulose* was added to the medium to prevent clumping of cellular material during incubation.

5) Preparation of reagents and solutions used in cultural procedures

(a) Preparation of antibiotic solution:

(i) Penicillin G. Potassium - the stock was made to contain 100,000 I.U./ml; 1.0 ml of this stock was removed and diluted 1:10 with distilled water.

(ii) Dihydrostreptomycin sulphate - the stock was made to contain 250 mg/ml; 0.2 ml of this stock was removed and diluted in 18 ml distilled water to give a concentration of 5 mg/ml. Ten ml of this solution was added to (i) above and sterilized by Millipore filtration (pore size 0.3 µ). A 2.0 ml quantity of the final mixed preparation was added to each 90 ml EPM to give a

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*Methocel 15 cps, reagent grade, Dow Chemical Co., Winnipeg, Manitoba.

concentration of 100 I.U. penicillin and 50 µg streptomycin /1.0 ml culture when all culture components had been added. The stock solutions were stored at -20° C until use.

(b) Preparation of yeast RNA solution:

A 0.5 g portion of yeast RNA was dissolved in 100 ml distilled deionized water at a pH of 8.0 and sterilized by Millipore filtration. The solution was stored at -20° C at which the preparation remained quite stable.

(c) Preparation of methylcellulose:

A 4.0 g portion of Methocel was dissolved in 100 ml HBSS and sterilized by autoclaving at 121° C for 15 mins. at 15 lbs. pressure. The solution was stored at 4° C.

(d) Preparation of HBSS:

This solution was prepared according to the method outlined in Merchant and Kahn (1960).

6) Sterility testing

All of the above cultural and preparative procedures outlined in Sections 1 to 5 above were carried out in a sterile room under aseptic conditions throughout.

Sterility tests on all materials used were carried out by inoculation of bacteriological loop specimens into

5.0 ml of Trypticase soy broth (Difco) or Fluid Thioglycollate Medium (Difco) in test tubes. The tubes were incubated at 37.5° C and observed daily for one week. A duplicate set of tubes was incubated at 28° C. If no bacterial growth took place in the broth or thioglycollate, it was assumed that the inoculated material was free from bacterial contamination.

7) Cell enumeration by Coulter electronic cell counter

The instrument used was a Coulter Counter Model A* which was fitted with a 100 micron aperture tube. The aperture current setting was 4 and the threshold setting was 15.

After dilution of the cell sample with twice-filtered (Millipore) physiological saline (to remove any dust particles that may have clogged the aperture) in a 50 ml beaker, the sample was ready for counting. Three counts were taken and the average instrument count was computed. For each count, the vacuum system on the machine draws a 0.5 ml volume of sample through the aperture. Calculation of the cell population in terms of numbers per milliliter was accomplished by the following formula:

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*Coulter Electronics, Chicago, Illinois.

Average instrument count x dilution of sample x 2

The principle of the instrument as related to the counting of tissue culture cells and the determination of the proper machine settings for this work are outlined in the Experimental and Results section under (A).

8) Examination of gross morphology of suspension culture cells

Visual examination of the gross morphology of cell populations was carried out in all experiments to detect any morphological abnormalities that might have occurred. A bacteriological loopful of culture was placed in the centre of a pre-cleaned and acetone-dried lint-free microscopic slide. A pre-cleaned cover slip was then placed over the drop and the preparation was examined by phase microscopy at a magnification of 440 X. In some cases, microphotographs were taken with a 35 mm. camera.

B. ANALYTICAL AND EXPERIMENTAL PROCEDURES

1) Dialysis of yeast RNA

A 50 ml volume of the basic RNA preparation

(concentration of 5 mgm/ml) were placed inside a cellophane dialysis sac and dialysed against distilled deionized water at 4^{o} C for 48 hours under constant agitation. The dialysing liquid was replaced every 8 hours. Samples of 2 ml were removed from both inside and outside the dialysis sac at each 8 hour interval. One ml of each sample was sterilized by Millipore filtration through a hypodermic syringe fitted with a Swinney adapter inside which was the filter membrane. The unit had been previously autoclaved. The filtrate was then inoculated into a culture of HeLa cells in the EPM medium in place of the normal RNA solution. The equivalent final concentration in the culture medium of RNA was 50 µg/ml. The total volume of the cultures was 100 ml and the inoculum in all cases was 2.5×10^{4} cells/ml. The remaining 1.0 ml of sample was analyzed spectrophotometrically at a wavelength of 260 mµ on a Beckman DB spectrophotometer to determine the absorbancy of nucleic acid in the sample. Total cell population in the cultures was determined after each culture had been incubated for 48 hours.

A duplicate experiment was carried out on an RNA preparation (concentration of 25 mgm/ml) and inoculated into

HeLa cell cultures in Morgan's Medium 199 plus 10% calf serum plus 10^{-2} M L-tryptophan to determine the effect of the dialysates on the induction of tryptophan pyrrolase according to the modification of the method of Knox & Auerbach (1955) outlined previously by the author (Kenny & Lees, 1961; and Kenny, 1962c).

2) The assay for tryptophan pyrrolase activity in yeast cells (Saccharomyces cerevisiae)

Inocula of 10^5 yeast cells per ml were made into 250 ml volumes of malt broth* containing either 1% glucose or 1% glycerol as energy sources. To each flask, a solution of L-tryptophan was added to a final concentration of 10^{-2} M. An identical set of flasks also contained 250 µg/ml yeast RNA. A series of cultures identical in composition to the above with the exception that the L-tryptophan concentration was 1.0 M were also inoculated.

The cultures were incubated overnight at 28° C on a rotary shaker. The following day, all cultures were processed and assayed for TPO activity in a fashion identical with that referred to in (1) above by measuring the concentration of L-kynurenine present in the sample at a wavelength of 365 mµ on a Beckman DB spectrophotometer.

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*Baltimore Biological Laboratories

3) Alkaline hydrolysis of yeast RNA

Two 100 ml quantities of the following normalities of NaOH were prepared: 0.1 N, 0.5 N, 1.0 N, and 5.0 N.

A 2.0 g quantity of RNA was then dissolved into each of the two 100 ml volumes of alkali of each normality and 10.0 ml samples of each solution were distributed into nine test tubes and incubated at 37° C; the tenth tube was analyzed immediately as a zero time control. At fifteen minute intervals over a two hour period, one tube in each series of nine was removed from the incubator and its contents immediately neutralized to pH 7.0 with HCl. The hydrolysates were then sterilized by passage through a Millipore filter held in a Swinney adapter as before and incorporated into the EPM in place of the normal RNA constituent after pH adjustment so that the total nucleotide content was equivalent to 50 μg RNA/ml (i.e., the optimal concentration). The inoculum of HeLa cells was 2.5×10^6 cells/ml of each culture. The extent of hydrolysis of the RNA was estimated on an 0.1 ml portion of the hydrolysates by precipitation of the unhydrolyzed RNA with 5.0 ml of a 0.75% solution of uranyl acetate in 25% trichloracetic acid. The precipitate was then centrifuged out and discarded and

the supernatant fluid retained for spectrophotometric analysis at 260 mμ on a Unicam SP 700 Spectrophotometer. The greater the absorbancy, the greater the amount of free oligonucleotides present; the oligonucleotide content served as an index to the degree of hydrolysis that had occurred.

4) Fractionation of RNA hydrolysates by gel filtration

Columns (40 x 2 cm) of washed Sephadex* G-50 were loaded with 200 mg of neutralized hydrolysate supernatant prepared as in (2) above. For reasons to be discussed in the Experimental and Results section, the hydrolysate was that from a 15 min. exposure of RNA to 0.5 N NaOH. The material was eluted by means of density gradient phosphate buffer at pH 7.5 increasing from 0.01 M to 0.2 M. Successive fractions of 5.0 ml volume were collected on a Research Specialties Model D3 fraction collector using the photoelectric drop counting attachment. The absorbancies of the fractions were determined at 260 mμ on the Unicam SP 700 spectrophotometer.

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*Pharmacia Co., Uppsala, Sweden.

Similar procedures were carried out on columns packed with Sephadex G-100 and G-200. All experiments were conducted in duplicate.

The highly-absorbing fractions were pooled and these materials were sterilized as before by Millipore filtration and incorporated into cultures of HeLa cells in EPM medium (with G-50) and ascites tumour cells (with G-100 and G-200). All experiments were conducted in duplicate.

5) <u>The uptake of C^{14}-labelled amino acids by cells cultured in EPM</u>

Ehrlich's ascites tumour cells were used for this study and were prepared in the following fashion:

Ascitic fluid was removed from the mouse as previously described, the cells were centrifuged out, the cell pellet was washed with HBSS and resuspended, and the cell population determined by Coulter Counter. The cultures were inoculated to contain 1×10^4 cells/ml and incubated for 24 hours. The cells were then centrifuged out of the growth medium (basic EPM) and washed again with HBSS. Experimental media were inoculated to a final population of 0.75×10^6 cells/ml.

The cultures were of the following combinations:

(i) EPM without RNA

(ii) EPM plus RNA (50 µg/ml)

(iii) Medium 199 plus RNA (50 µg/ml)

To each of the cultures was added a 1.0 ml volume of a preparation of L-phenylalanine-C^{14} containing a radioactivity of 5 microcuries, so that the actual concentration in the 100 ml volume of culture was 0.05 microcurie/ml. After a period of 10 mins. had elapsed, 10 ml of each culture were removed, one 5 ml aliquot for population determinations and the other for determination of C^{14} uptake. The cultures were then incubated at 37° C on the rotary shaker. At 8 hour intervals over a period of 48 hours, the cultures were examined for both population density and C^{14}-labelled amino acid uptake.

The measurement of C^{14}-amino acid uptake

The 5 ml aliquot that was reserved for this measurement was centrifuged at 120 x g to obtain a cell pellet. A 0.1 ml portion of the resulting supernatant was spread out on a planchet and allowed to dry at room temperature. This was used as a measure of radioactivity in the medium. The cell pellet was resuspended in ice-cold water for 10 minutes

for the purpose of lysing the cells. The protein was then precipitated with ice-cold 5% trichloracetic acid. The resulting material was passed through a Millipore filter membrane of pore size 2 μ and 25 mm in diameter. The membrane was then placed on a planchet and allowed to dry at room temperature.

Determination of the radioactivity present in the samples was made by taking 10 min. counts on a Nuclear-Chicago gas-flow disintegration counter. The results were then calculated in terms of counts per minute and the usual correction factors were applied.

EXPERIMENTAL AND RESULTS

A. CALIBRATION OF THE COULTER COUNTER FOR TISSUE CELL POPULATION COUNTS

The determination of cell populations in cell culture work has always presented a problem in that all known methods have several disadvantages, either in terms of accuracy, or in terms of complexity, or both. To obtain an estimation of the total live cell count, the only reasonably reliable procedure is vital staining of the sample with trypan blue or some similar compound followed by counts of the unstained cells by hemocytometry. This procedure is both time-consuming and imprecise since each count takes about 15 mins. and has an overall error of 20%. Had hemocytometry been the only method of counting cells available to us, the work presented here would have been extraordinarily tedious since the conclusions drawn from the experimental results were almost always based on cell counts. In order to expedite cell counting and at the same time improve its accuracy, an electronic method of cell counting was used throughout. The basic principle of this method is that of "electric gating".

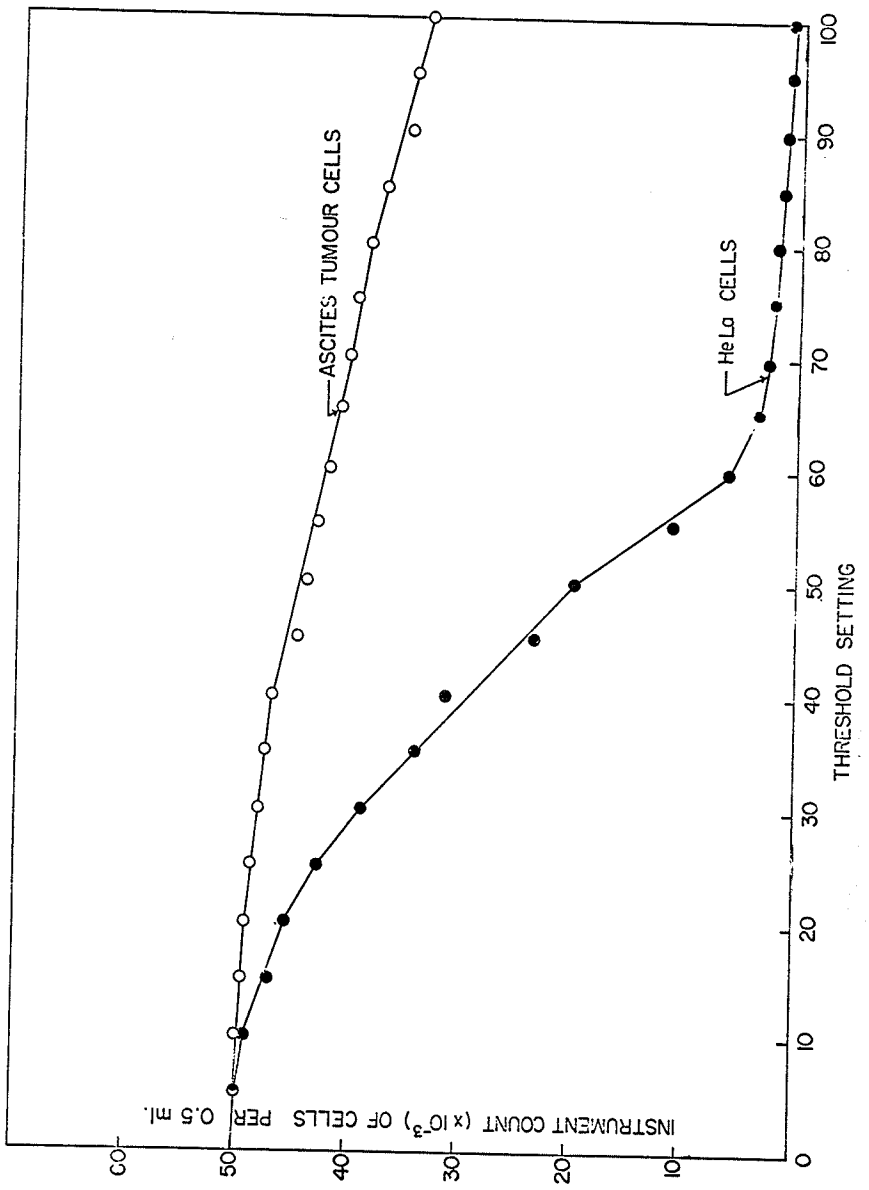
The instrument used was the Coulter Counter and the description given below applies to the theory and operation of this particular instrument.

Cells were suspended in an electrically conductive medium, such as physiological saline, and, by means of a vacuum, exactly 0.5 ml of the suspension was drawn through a small aperture (100 μ diameter x 75 μ depth) which was interposed between two large surface platinum electrodes immersed in the electrolyte. As each cell passed through the aperture it increased the aperture impedance and thus decreased the aperture current; the resulting current pulses were amplified, recorded on a decade counter, and at the same time displayed on an oscilloscope screen. Each pulse appearing on the oscilloscope was a reflection of the size of the cell generating that pulse but only pulses greater than a pre-selected magnitude were amplified and counted by means of a threshold circuit control. As a result a cell size distribution curve could be produced by taking counts at different threshold levels. The magnitude of the pulses could be controlled by adjustment of the voltage delivered to the electrodes.

A threshold curve was plotted for HeLa and ascites

FIGURE 1. Calibration of the Coulter Counter.

All values represent an average of three counts taken at the specified settings. These observations confirmed on three separate suspensions of each of the two strains of cells.


ASCITES TUMOUR CELLS
HeLa CELLS
INSTRUMENT COUNT ($\times 10^{-3}$) OF CELLS PER 0.5 ml.
THRESHOLD SETTING
0
10
20
30
40
50
60
70
80
90
100

tumour cells (Fig. 1). The cells were suspended in HBSS and diluted so that the machine count did not exceed 50,000 per 30 secs (the time required for the 0.5 ml sample to traverse the aperture). If the cell density exceeded 50,000 per 0.5 ml, the pulse frequency began to overlap the dead time of the counter with the result that the cell count diminished in accuracy. The initial threshold setting was 5 which was just above the background noise level of the medium itself. The threshold level was then increased by 5 units at a time until the 100 setting had been reached. At each setting, three counts were taken and the average determined.

The results in Fig. 1 show that, for HeLa cells, a count at a threshold above 20 eliminated some of the smaller cells from the total count, while a threshold setting of 50 did not appreciably reduce the total count for ascites cells. From these results it was concluded that all population determinations could reasonably be made at a threshold level of 15, where all possibilities of spurious counts due to background noise and cellular debris would be virtually eliminated. Background counts of the filtered electrolyte used as diluent proved to be negligible and were

not included in the final computation. It was determined through the course of the above experiment that the aperture current setting that gave the optimal pulse height was 4. This setting was used throughout the work.

It should be pointed out here, however, that the Coulter Counter has one inherent defect as a cell counting machine; it counts a clump of cells as one cell. Cell clumping, if it occurs to any appreciable extent, can thus introduce serious errors into the electronic count. It was therefore the practice, throughout the work reported in this thesis, to include methyl cellulose in all culture media in order to minimize cell clumping. Regular comparisons of hemocytometer counts and electronic counts showed that the inclusion of the methyl cellulose did in fact reduce clumping to reasonably small proportions except in old, dense, ascites cell cultures. Counts on such cultures are not included in the results presented. It should be stressed, however, that clumping always tends to reduce the electronic count below the value of the "true" count. Since clumping only occurs in older, denser, cultures, and since all the results presented here are based on increases in cell numbers over and above an initial count of cell numbers in a young culture, it follows that electronic counting tends to minimize the "true" increase in cell numbers. Therefore if

electronic counting shows that cell numbers have doubled in a given culture, and if at the same time clumping has taken place in the culture, cell numbers have, in fact, more than doubled. In this sense the defect of electronic counting becomes a virtue because by minimizing the extent of cell proliferation it maximizes its scientific validity. The repeatability of the electronic count is excellent. Five counts of an Escherichia coli culture at a threshold setting of 7 showed an intercount variation of less than 0.05%.

B. THE GROWTH OF HeLa CELLS IN DIFFERENT MEDIA

All these growth experiments were carried out in cultures inoculated to contain 1.0×10^5 cells/ml. It was established at the outset that HeLa cells multiplied well in nutrient broth plus 50 µg RNA for the first four days, provided that the yeast RNA portion was renewed daily to correspond approximately to the generation time of the cells (Kenny & Lees, 1963)*. The natural buffering capacity of nutrient broth initially kept the reaction of the cultures around neutrality but after four days had elapsed, the pH of the medium had dropped to 5.0 and growth levelled off (Curve B, Fig. 2).

In an attempt to combat this fall in pH, $NaHCO_3$ was incorporated into the medium to a final concentration of 10^{-3} M

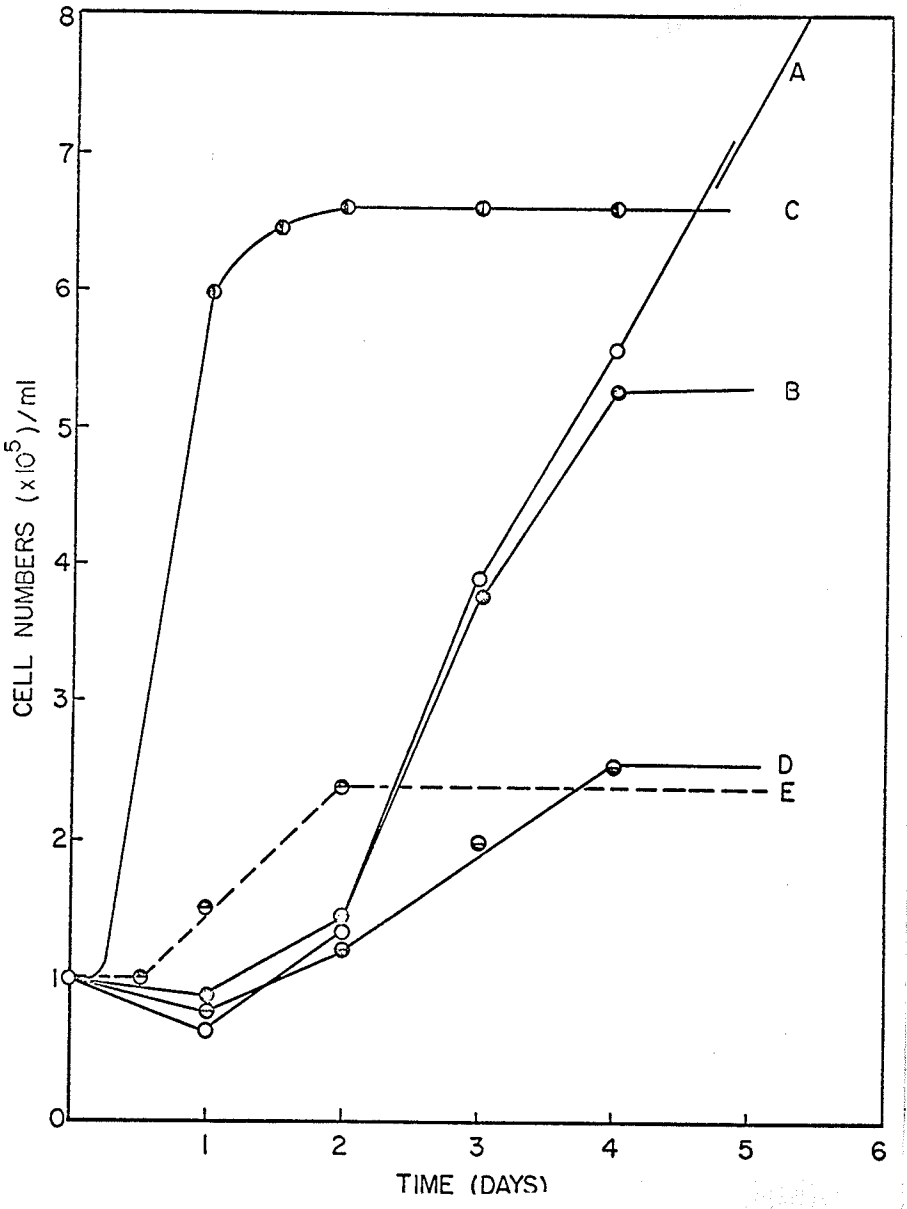
---

*See Appendix, Table II.

FIGURE 2. The growth of HeLa cells in different media.

Curve A: Morgan's 199 plus 10% calf serum (final pH = 7.4). Curve B: Nutrient broth plus 50 µg/ml RNA (final pH = 5.0). Curve C: Nutrient broth plus 50 µg/ml RNA in 10^{-3} M $NaHCO_3$ (final pH = 8.8). Curve D: Nutrient broth plus 25 µg/ml RNA (final pH = 5.2). Curve E: Nutrient broth plus 25 µg/ml RNA in 10^{-3} M $NaHCO_3$ (final pH = 8.5). Initial pH in all cases was 7.5.

Resultant curves confirmed by similar observations in three separate cultures of each experimental condition employed. For growth of HeLa cells in nutrient broth + 10% calf serum, see Appendix, Table I.


CELL NUMBERS (x10^5)/ml
TIME (DAYS)
A
B
C
D
E
0
1
2
3
4
5
6
7
8

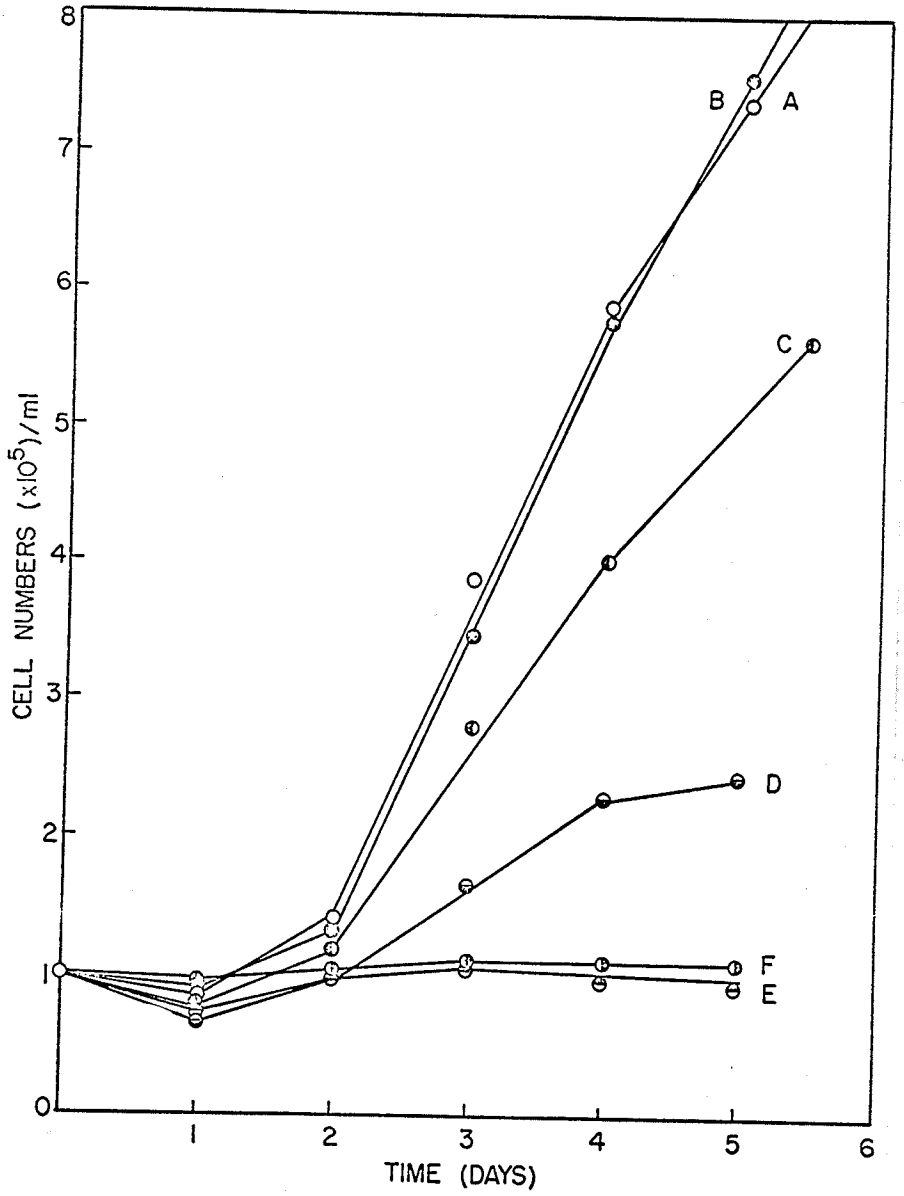
as is the usual procedure with conventional media and the experiments were repeated. Cell proliferation was marked but levelled off after two days when the pH had risen to 8.8 (Curve C, Fig. 2). It was also observed that this $NaHCO_3$ addition noticeably reduced the lag period. After several similar experiments, with different buffers such as phosphate, it was found that the pH of cultures buffered with 10^{-2} M tris hydroxymethylaminomethane (Tris)-HCl buffer (pH 7.4) remained between 6.5 and 7.0 and steady growth was maintained as a result throughout the experimental period (Curve A, Fig. 3).

The RNA concentrations in the nutrient broth were also varied. At 25 µg RNA/ml growth stimulation was observed but at a lower level than with 50 µg RNA/ml. The same pH instabilities were noted in both unbuffered media and in the presence of bicarbonate (Curves D and E, Fig. 2). Concentrations of RNA of 100 µg, 150 µg, 200 µg, and 250 µg/ml were also tested. At concentrations higher than 100 µg RNA/ml in media buffered with Tris, the stimulating effect of the RNA decreased with increasing RNA concentration to the point where the 250 µg RNA/ml, cell proliferation did not occur (Curve E, Fig. 3). Since a concentration of 50 µg RNA/ml was as effective as 100 µg RNA/ml, all subsequent experiments were conducted at the lower concentration. HeLa cells failed to grow in a medium of Morgan's 199 plus 50 µg

FIGURE 3. The growth of HeLa cells in different media.

Curves A-E: Nutrient broth in 10^{-2} M Tris buffer (pH 7.5). Curve A: 50 µg/ml RNA. Curve B: 100 µg/ml RNA. Curve C: 150 µg/ml RNA. Curve D: 200 µg/ml RNA. Curve E: 250 µg/ml RNA. Curve F: Morgan's 199 plus 50 µg/ml RNA.

Resultant curves confirmed by similar observations in three separate cultures of each experimental condition employed.


CELL NUMBERS (x10^5)/ml
TIME (DAYS)
A
B
C
D
E
F
0
1
2
3
4
5
6
7
8

RNA/ml which was incorporated in place of the calf serum (Curve F, Fig. 3).*

C. THE GROWTH OF ASCITES TUMOUR CELLS IN NUTRIENT BROTH

The favourable results obtained by the growth of HeLa cells indicated the possibility that similar results might be obtained with a different type of malignant tissue such as Ehrlich's ascites tumour cells.

The basal EPM medium containing antibiotics and 10^{-2} M Tris buffer (pH 7.4) was inoculated heavily to contain 3.5×10^5 cells/ml. It can be seen from Fig. 4 that the cell numbers increased to 1.11×10^6 cells/ml after 24 hours, i.e. approximately three fold. Similar curves were obtained with successive subcultures over several generations. Cell proliferation was similar to that observed with Morgan's Medium 199 plus 10% calf serum (Fig. 4). The pH of the medium remained steady between 6.8 and 7.2 and the gross morphology of the cells (as revealed by negative phase-contrast microscopy) remained virtually unchanged.

Microscopic examination of ascites cells that had been maintained at -20° in the basal EPM plus 15% glycerol for a period of two months revealed little change in their

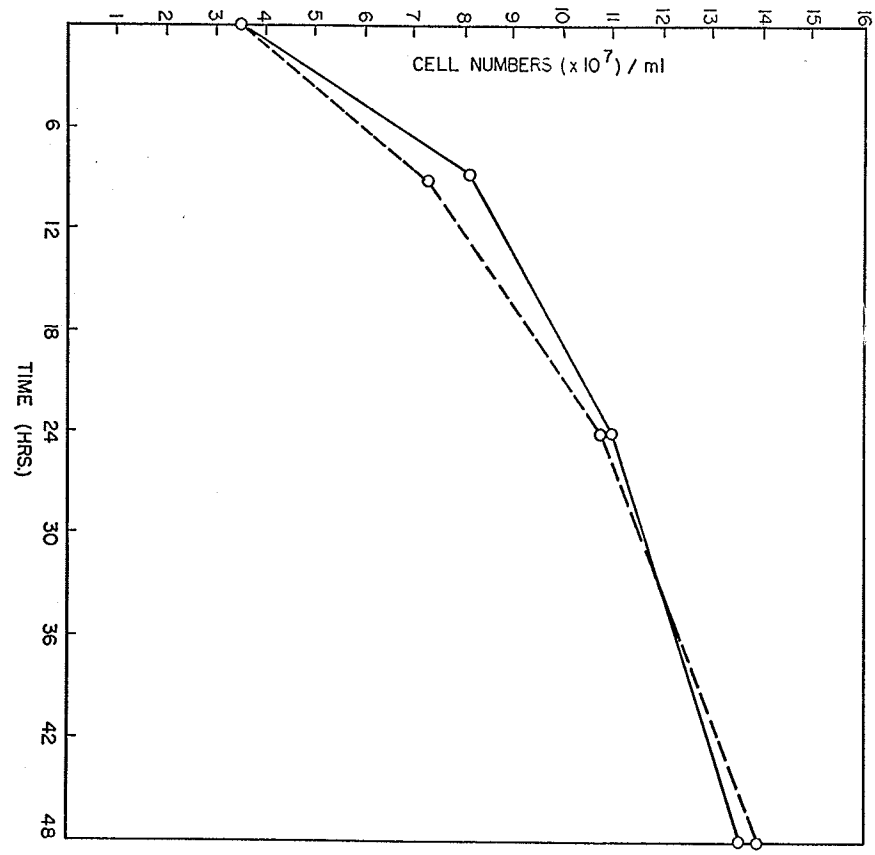
*HeLa cells failed to grow in nutrient broth + 10% calf serum. See Table I, Appendix.

FIGURE 4. The growth of ascites cells in different media.

Morgan's 199 plus 10% calf serum (←--O--→). Nutrient broth plus 50 μg/ml RNA in 10^{-2} M Tris buffer, pH 7.5 (←— O —→).

Resultant curves represent an average of three separate experiments using the conditions outlined above.

ERRATUM. Ordinate description should read cell number ($\times 10^5$) per ml.


CELL NUMBERS (x 10^7) / ml
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
TIME (HRS.)
6
12
18
24
30
36
42
48

gross morphology and subsequent reinoculation into fresh medium was successful in starting new cellular proliferation. Viability after this low temperature storage was approximately 35-40% of the normal value (estimated by dye-exclusion of trypan blue).

D. THE EFFECTS OF DIALYSATES OF YEAST RNA

1) The effects on the growth of HeLa cells

Once that it had been established that incorporation of yeast RNA into the basal EPM medium promoted the growth of malignant cells *in vitro*, dialysis of the RNA was carried out as the first step in identifying the active portion in the RNA molecule.

The results of this experiment are indicated in Table I (p. 41). Generally speaking, the nucleic acid material with an absorbance at 260 mμ remained in the inside portion of the sac, with very little material dialysing through. It can be observed from the table that growth stimulation occurred only when material removed from the inside of the dialysis sac was incorporated into the EPM.

TABLE I

The effect of dialysis on the growth promoting properties of yeast RNA

TIME (hrs)	O.D. (260 mμ)		Cell Numbers at 48 hrs ($\times 10^4$/ml)	
	INSIDE	OUTSIDE	INSIDE	OUTSIDE
Zero	2.00	0.000	4.90	2.40
8	2.00	0.005	4.80	2.25
16	2.00	0.005	4.90	2.25
24	2.00	0.008	4.90	2.20
32	2.00	0.010	4.75	2.25
40	2.00	0.010	4.80	2.25
48	2.00	0.010	4.80	2.30

Yeast RNA was dialysed for 48 hrs at 2° C against distilled water. From time to time, samples were taken from the inside and the outside of the dialysis sac and incorporated into EPM at a level equivalent to 50 μg/ml RNA. The augmented EPM was then inoculated to contain 2.5×10^4 HeLa cells/ml. The right hand side of the Table shows the cell numbers after 48 hrs growth.

2) The effects on the production of tryptophan pyrrolase (TPO)

For this experiment a final concentration of 250 µg RNA/ml was used as this is the optimal concentration for induction of the enzyme in HeLa cells (Kenny, 1962d). The medium used for the cultures was Morgan's Medium 199 plus 10% calf serum plus the inducing compounds (see Materials and Methods). As it required four days culture period in order to observe significant activity of the enzyme, portions from the inside and outside of the dialysis sac were incorporated into the cultures daily at an equivalent concentration of 250 µg/ml. After this four day period, the cultures were then assayed for enzyme activity. The results are outlined in Table II.

As before, the absorbancy of nucleic acid material at 260 mµ showed that the majority of this material remained in the inside portion of the dialysis sac. Enzyme activity was observed only in cultures into which material from the inside of the sac had been incorporated into the medium.

E. THE GROWTH OF HeLa CELLS IN ALKALINE HYDROLYSATES OF RNA

Once it had been established that the growth stimulating property of the RNA resided in the non-dialysable fraction of

TABLE II

The effect of dialysis on the enzyme-inducing properties of yeast RNA

TIME (hrs)	O.D. (260 mμ)		TPO activity in Knox units (at 4 days)	
	INSIDE	OUTSIDE	INSIDE	OUTSIDE
Zero	2.00	0.000	1.74	0.00
8	2.00	0.005	1.68	0.00
16	2.00	0.008	1.71	0.00
24	2.00	0.010	1.72	0.00
32	2.00	0.010	1.67	0.00
40	2.00	0.012	1.70	0.00
48	2.00	0.015	1.71	0.00

Dialysis was carried out as in Table I. Samples from inside and outside the sac were added to Morgan's 199 plus 10% calf serum plus L-tryptophan. The augmented media were then inoculated with HeLa cells. The tryptophan pyrrolase (TPO) activity of the cells was measured (Kenny, 1962) after 4 days growth. One Knox unit = micromoles of L-kynurenine formed per gram dry weight of cell homogenate per hour.

FIGURE 5. The growth of HeLa cells in nutrient broth plus hydrolysed RNA.

Hydrolysis was in 0.5 M NaOH at 37° C. The hydrolysed RNA was added to EPM inoculated with HeLa cells (see Materials and Methods p. 25). All growth media were buffered with 10^{-2} M Tris buffer (pH 7.5). Increase in cell numbers during 24 hours growth (—○—). Optical density of uranyl acetate supernatant (—●—).

The curves represent an average result of three experiments.

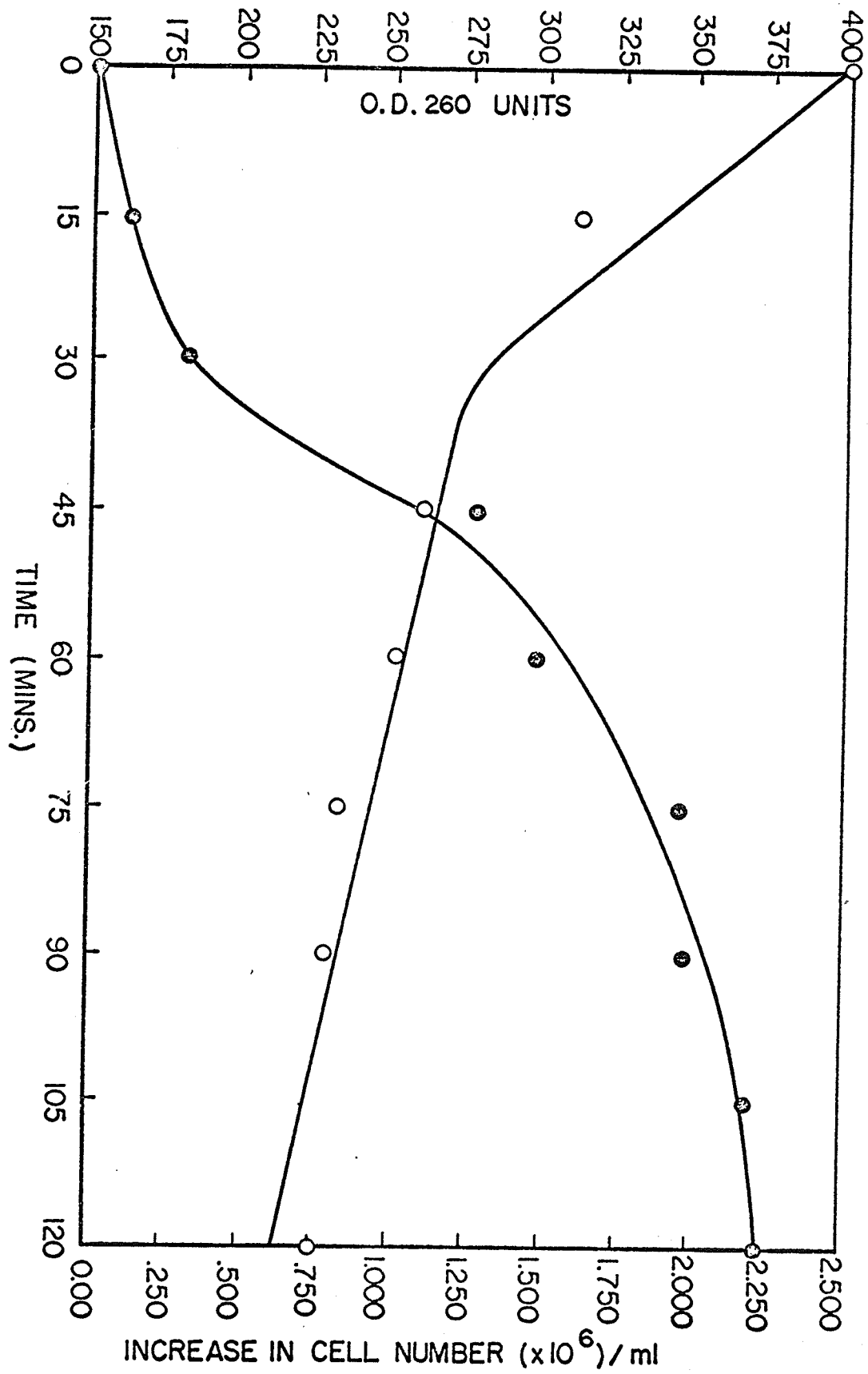
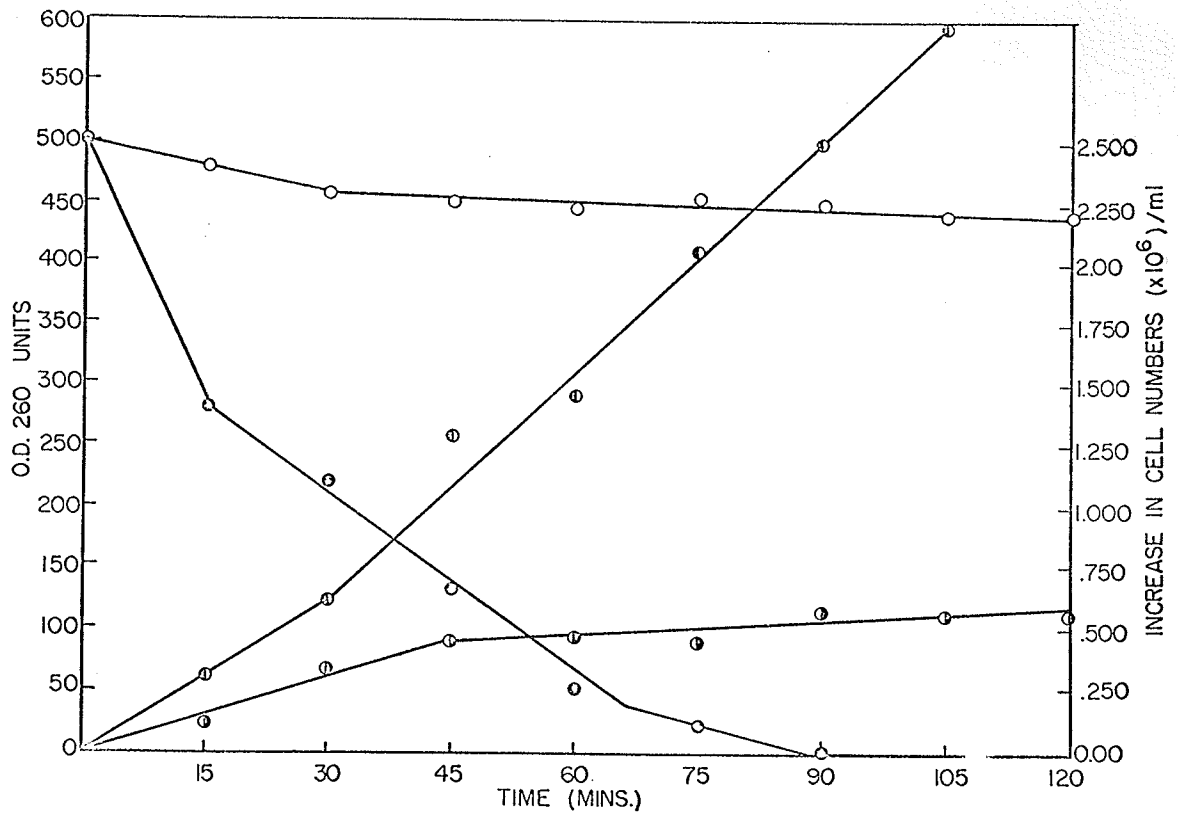

O.D. 260 UNITS
150
175
200
225
250
275
300
325
350
375
400
TIME (MINS.)
0
15
30
45
60
75
90
105
120
INCREASE IN CELL NUMBER (x10^6)/ml
0.00
.250
.500
.750
1.000
1.250
1.500
1.750
2.000
2.250
2.500

FIGURE 6. The growth of HeLa cells in nutrient broth plus hydrolysed RNA.

Experimental details were parallel to those shown in Fig. 5. Increase in cell numbers during 24 hours growth (— ○ —), 0.1 M NaOH; (— ● —), 5 M NaOH). Optical density of uranyl acetate supernatant (— ◑ —), 0.1 M NaOH; (— ◐ —), 5.0 M NaOH).

The curves represent an average result of three experiments.


600
550
500
450
400
350
300
250
200
150
100
50
0
O.D. 260 UNITS
2.500
2.250
2.00
1.750
1.500
1.250
1.000
.750
.500
.250
0.00
INCREASE IN CELL NUMBERS (x10^6)/ml
15
30
45
60
75
90
105
120
TIME (MINS.)

the material, alkaline hydrolysis of the RNA was carried out in a further attempt to isolate the active portion.

Hydrolysed RNA was added to the EPM medium as outlined in Material and Methods and the results of these experiments are indicated in Figures 5 and 6. Generally speaking, as the absorbancy at 260 mμ increased (i.e. as the hydrolytic breakdown increased) cell proliferation in the nutrient broth plus hydrolysate decreased.

Figures 5 and 6 are plotted in terms of cell number increase after 24 hours growth from the time of the incorporation of the hydrolytic fractions. Figure 6 has shown that the RNA from the 0.1 N NaOH hydrolysate supported growth from all stages of the two-hour hydrolysis with little change in increase of cell numbers throughout.

On the basis of this result and the others depicted in the curves, a 15 minute 0.5 N NaOH hydrolysate of yeast RNA was chosen for further fractionation as it showed a significant breakdown of the RNA while still retaining the power to stimulate cell proliferation.

F. CELL GROWTH IN RNA HYDROLYSATES FRACTIONATED BY GEL FILTRATION

Fractionation of the 15 minute 0.5 N NaOH hydrolysate of RNA was carried out on columns packed with Sephadex G-50. This particular grade of Sephadex, which does not retain molecules of M.W. greater than about 50,000, was chosen to allow the smaller, inactive portions of the hydrolysate to be retained by the column.

Fig. 7 shows that a fraction absorbing strongly at 260 mμ appeared for the first 150 ml of elution and was followed by a small, non-absorbing fraction. Elution then proceeded with the appearance of a series of small peaks until 450 ml of eluate had been obtained. These three general groups were pooled, as indicated in the Figure, into three fractions, sterilized by Millipore filtration, and incorporated into the basal EPM medium in place of intact RNA.

The flasks were inoculated to contain 6.0×10^4 HeLa cells/ml and growth curves followed over 48 hours (Fig. 8). It can be seen that pooled Fraction I promoted growth very well over the two days of study; Fraction II, which had no absorption at 260 mμ did not, and Fraction III,

FIGURE 7. Sephadex G-50 fractionation of hydrolysed RNA.

RNA was hydrolysed for 15 mins in 0.5 M NaOH at 37° C. Ordinate gives the optical density of the eluate.

The curve represents an average of two fractionations on separate columns.

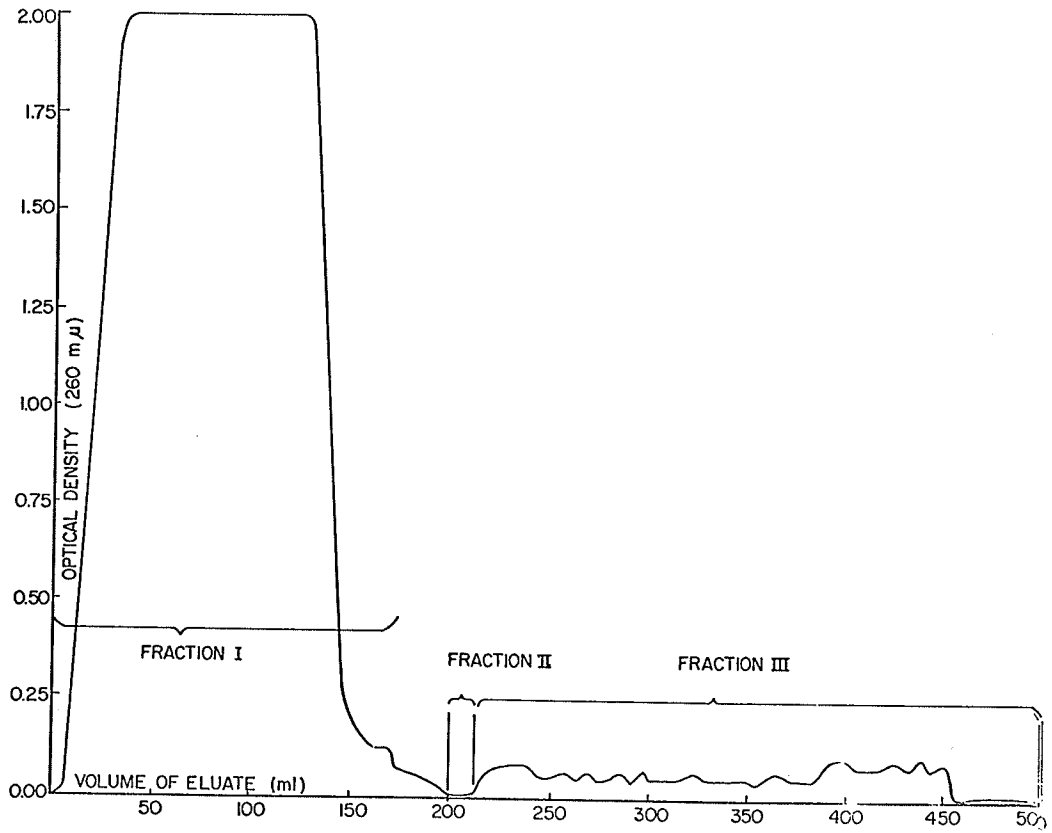
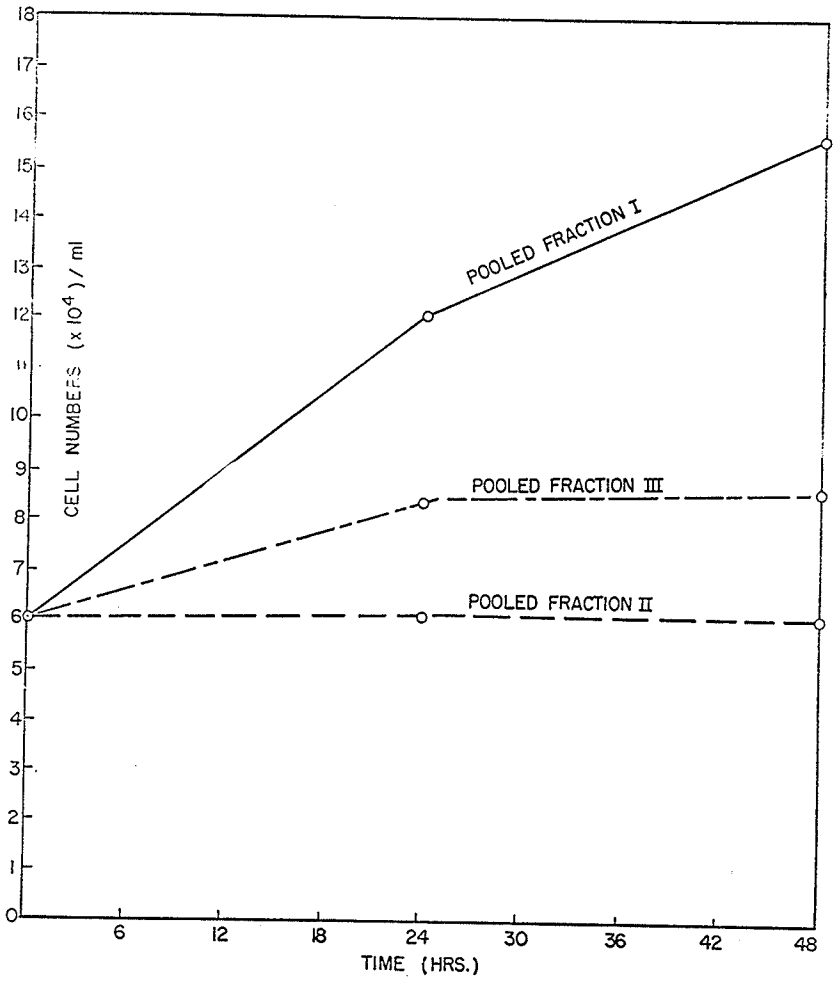

2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
OPTICAL DENSITY (260 mμ)
FRACTION I
FRACTION II
FRACTION III
VOLUME OF ELUATE (ml)
50
100
150
200
250
300
350
400
450
500

FIGURE 8. The effect of hydrolysed RNA on the growth of HeLa cells

Cells were grown in EPM buffered with 10^{-2} M Tris (pH 7.5) supplemented with the eluate fractions shown in Fig. 7.

Growth curves represent an average of three cultures containing the fractions.

(See Appendix, Table III, for actual experimental data).


CELL NUMBERS (x 10⁴) / ml
TIME (HRS.)
POOLED FRACTION I
POOLED FRACTION III
POOLED FRACTION II
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
6
12
18
24
30
36
42
48

comprised of the series of small peaks, had low growth-promoting activity.

The highly-absorbing Fraction I was then subjected to a further fractionation on columns packed with Sephadex G-100. Figure 9 shows that there was no further significant fractionation of the molecule, as only one highly absorbing peak again appeared. Figure 10 shows that growth of ascites tumour cells occurred only in this fraction to any degree.

Pooled Fraction I was then again subjected to a further fractionation on columns packed with Sephadex G-200 and the absorbancy at 260 mμ followed as before (Fig. 11). The pooled fractions indicated were again sterilized and incorporated into the EPM medium. The effect of these fractions on the growth of ascites tumour cells are depicted in Fig. 12; fraction II had some growth-promoting activity; the other fractions were inactive. It is clear from Fig. 12 however that much of the growth-promoting fraction was retained by the column since the growth-stimulation observed was very small.

FIGURE 9. The second Sephadex fractionation of hydrolysed RNA.

Fraction I of Fig. 7 refractionated on Sephadex G-100.

The curve represents an average of two fractionations from separate columns.

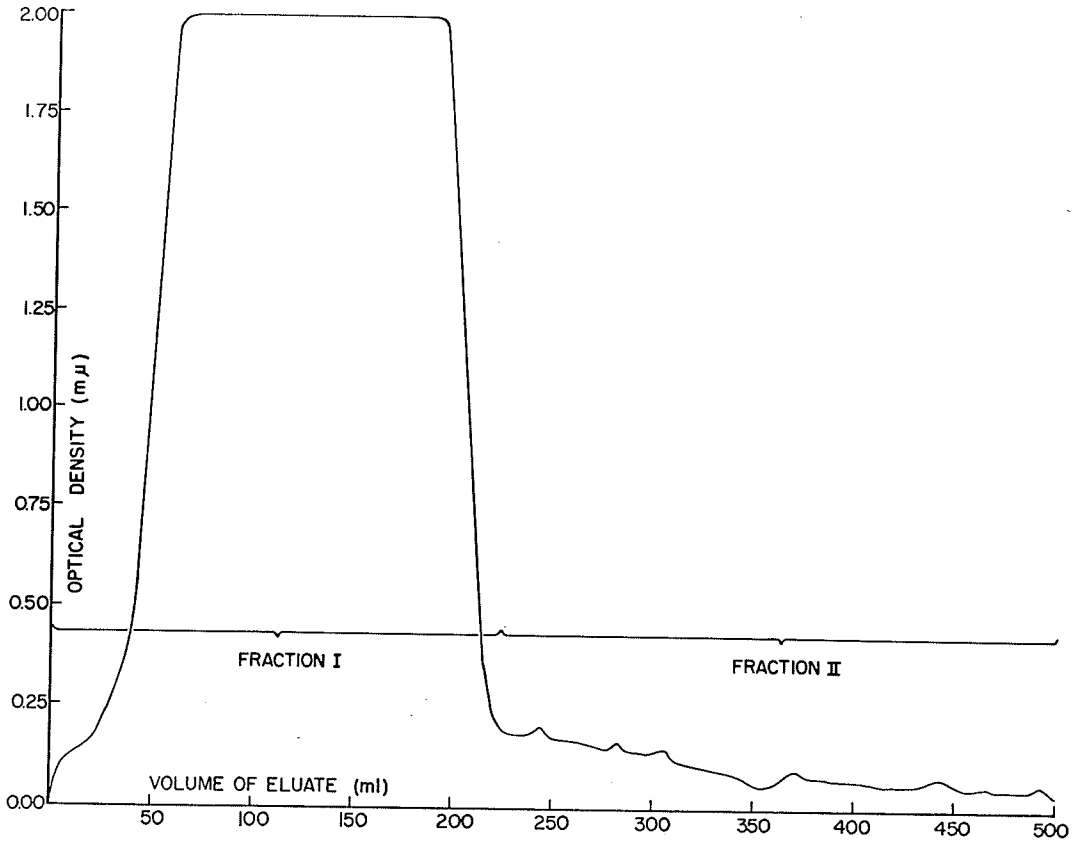

2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
OPTICAL DENSITY (mμ)
FRACTION I
FRACTION II
VOLUME OF ELUATE (ml)
50
100
150
200
250
300
350
400
450
500

FIGURE 10. The effect of hydrolysed RNA on the growth of ascites cells.

Cells were grown in EPM buffered with 10^{-2} M Tris (pH 7.5) supplemented with the eluate fractions shown in Fig. 9.

Growth curves represent an average of three cultures containing the fractions.

(See Appendix, Table IV, for actual experimental data).

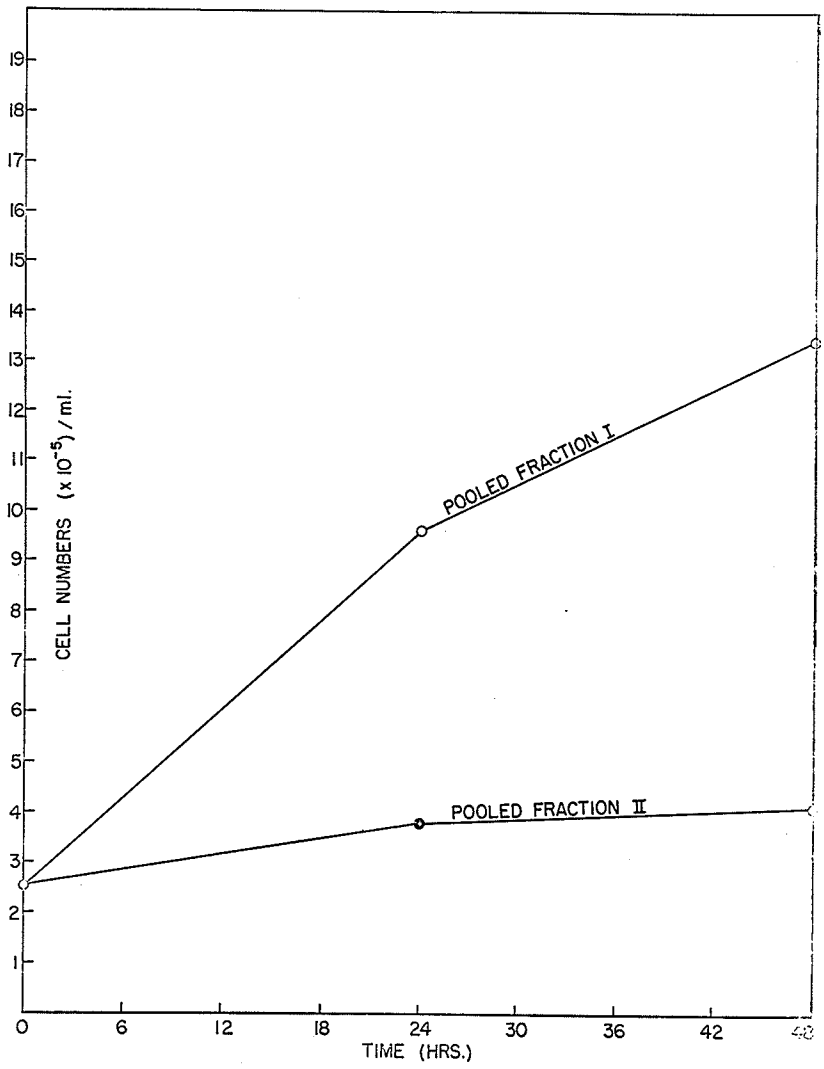

CELL NUMBERS (x 10⁻⁵) / ml.
TIME (HRS.)
POOLED FRACTION I
POOLED FRACTION II
0
6
12
18
24
30
36
42
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19

FIGURE 11. The third Sephadex fractionation of hydrolysed RNA.

Fraction I of Figure 7 refractionated on Sephadex G-200.

The curve represents an average of three fractionations from separate columns.

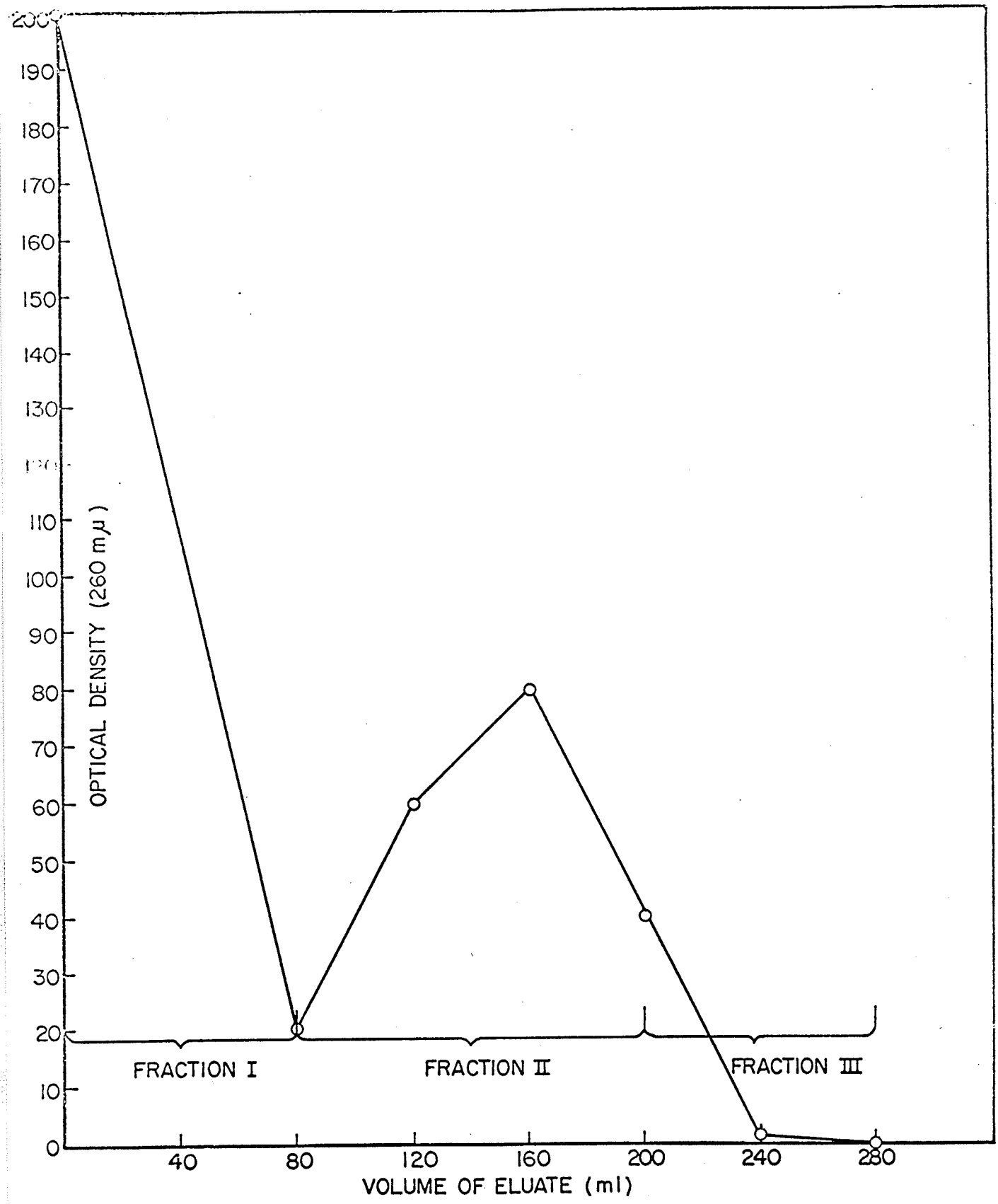
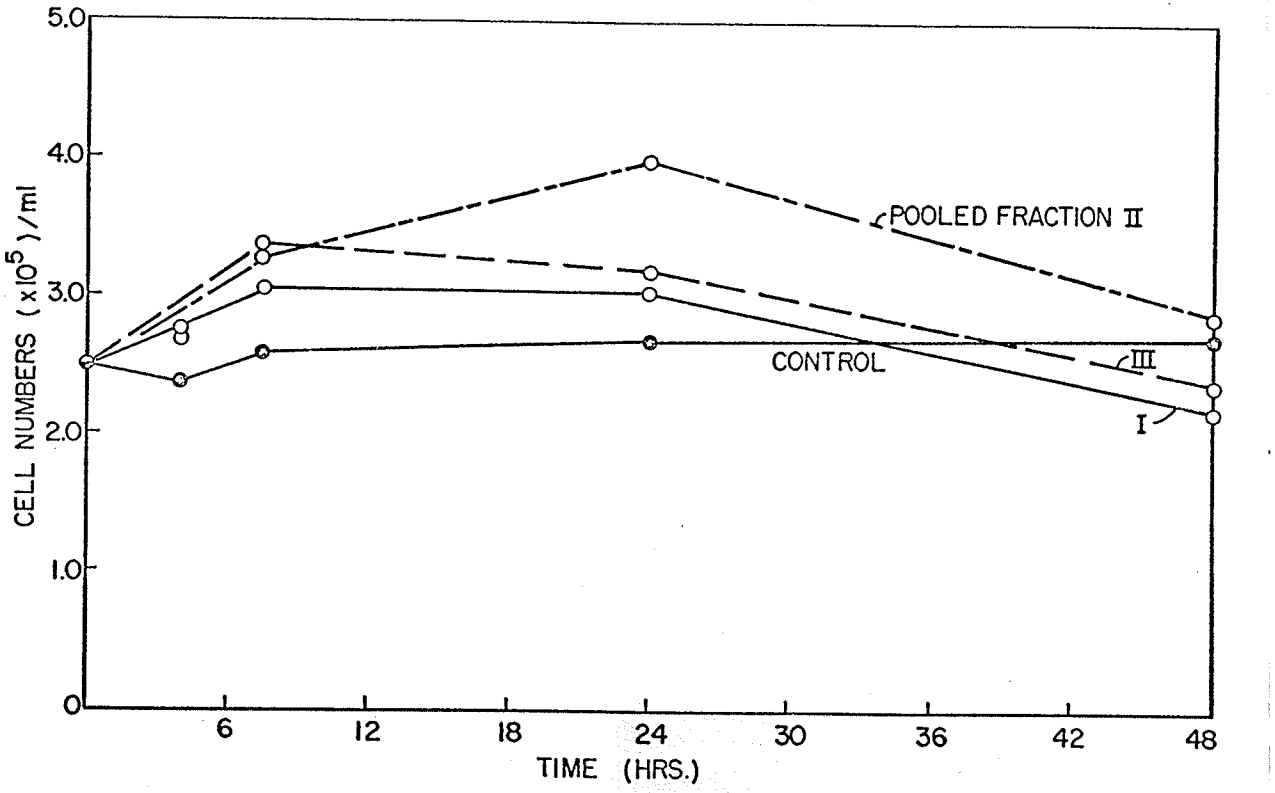

200
190
180
170
160
150
140
130
110
100
90
80
70
60
50
40
30
20
10
0
OPTICAL DENSITY (260 mμ)
FRACTION I
FRACTION II
FRACTION III
40
80
120
160
200
240
280
VOLUME OF ELUATE (ml)

FIGURE 12. The effect of hydrolysed RNA on the growth of ascites cells.

Cells were grown in EPM buffered with 10^{-2} M Tris (pH 7.5) supplemented with the eluate fractions shown in Fig. 11.

Growth curves represent an average of three cultures containing the fractions. The control curves represent cultures of ascites cells in nutrient broth alone. (See Appendix, Table V, for actual experimental data).


5.0
4.0
3.0
2.0
1.0
0
CELL NUMBERS (x10^5) / ml
6
12
18
24
30
36
42
48
TIME (HRS.)
POOLED FRACTION II
III
CONTROL
I

G. THE UPTAKE OF C^{14}-LABELLED AMINO ACIDS BY CELLS CULTURED IN EPM

A series of experiments was undertaken in which cultures of ascites tumour cells which had been growing in EPM were subsequently placed in various growth media in order to detect any changes that might occur in their amino acid uptake as a result of a change in their nutritional environment. The use of C^{14}-labelled amino acids might possibly reveal differences in the pattern of protein synthesis or, at least, in the requirement for the particular labelled amino acid used. The amino acid selected was C^{14}-phenylalanine.

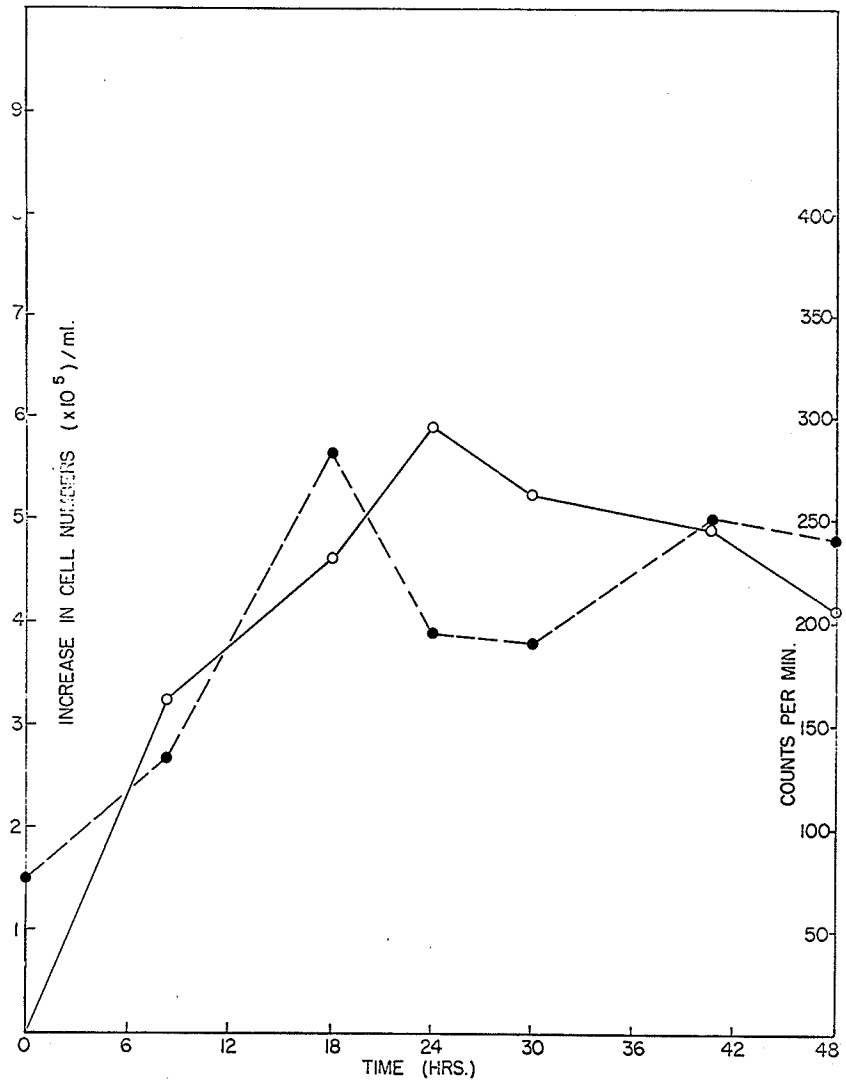
It can be observed from Figure 13, that ascites cells cultured in nutrient broth only, i.e., without the customary RNA supplement, proliferated for a period of 24 hours. After this period, cell numbers began to decline steadily. The uptake of phenylalanine rose steadily for 18 hours and then decreased although cell proliferation still continued for a further 8 hours. Then at 30 hours, when the population was steadily declining, phenylalanine uptake again increased, indicating a possible alteration in metabolic activity at

FIGURE 13. The uptake of C^{14}-phenylalanine by ascites cells.

Cells were grown in EPM buffered with 10^{-2} M Tris (pH 7.5) supplemented with C^{14}-phenylalanine. Increase in cell numbers (—○—). Radioactive counts/minute (--●--).

Curves represent an average of three experiments under the described conditions.

(See Appendix, Table VI, for actual experimental data).


INCREASE IN CELL NUMBERS (x10 5) / ml.
COUNTS PER MIN.
TIME (HRS.)
0
6
12
18
24
30
36
42
48
1
2
3
4
5
6
7
9
50
100
150
200
250
300
350
400

this stage of the growth pattern.

On the other hand as is shown in Figure 14, a culture of EPM with the usual RNA component, showed a levelling off in cell numbers at 24 hours, but after 30 hours, again showed an increase. The amino acid pattern differed, however, in that after the 18 hour increase, a decline set in as before (Figure 13) but after 30 hours, uptake declined even though the population had begun to increase again.

Figure 15 gives results obtained with a culture which was established in Medium 199 plus 50 µg RNA/ml. Cell numbers increased, then decreased to the original level at 24 hours following which there was a gradual increase over the next 18 hours and then a sharper increase towards 48 hours. However, in this medium, uptake of the amino acid was markedly over the uptake in EPM. The same pattern seemed to persist, in that once the culture was in its second stage of proliferation after the first 24 hours, uptake began to decrease as cell numbers increased.

In similar experiments carried out with L-leucine-C^{14}, a similar pattern of uptake was observed in all cases, but the amount of leucine taken up was approximately one-fifth that of L-phenylalanine.

FIGURE 14. The uptake of C^{14}-phenylalanine by ascites cells.

Cells were grown in EPM buffered with 10^{-2} M Tris (pH 7.5) supplemented with 50 μg/ml RNA and C^{14}-phenylalanine. Increase in cell numbers (— ○ —). Radioactive counts /minute (- - ● - -).

Curves represent an average of three experiments under the described conditions.

(See Appendix, Table VII, for actual experimental data).

ERRATUM. Ordinate should read "0, 1, 2, ... "

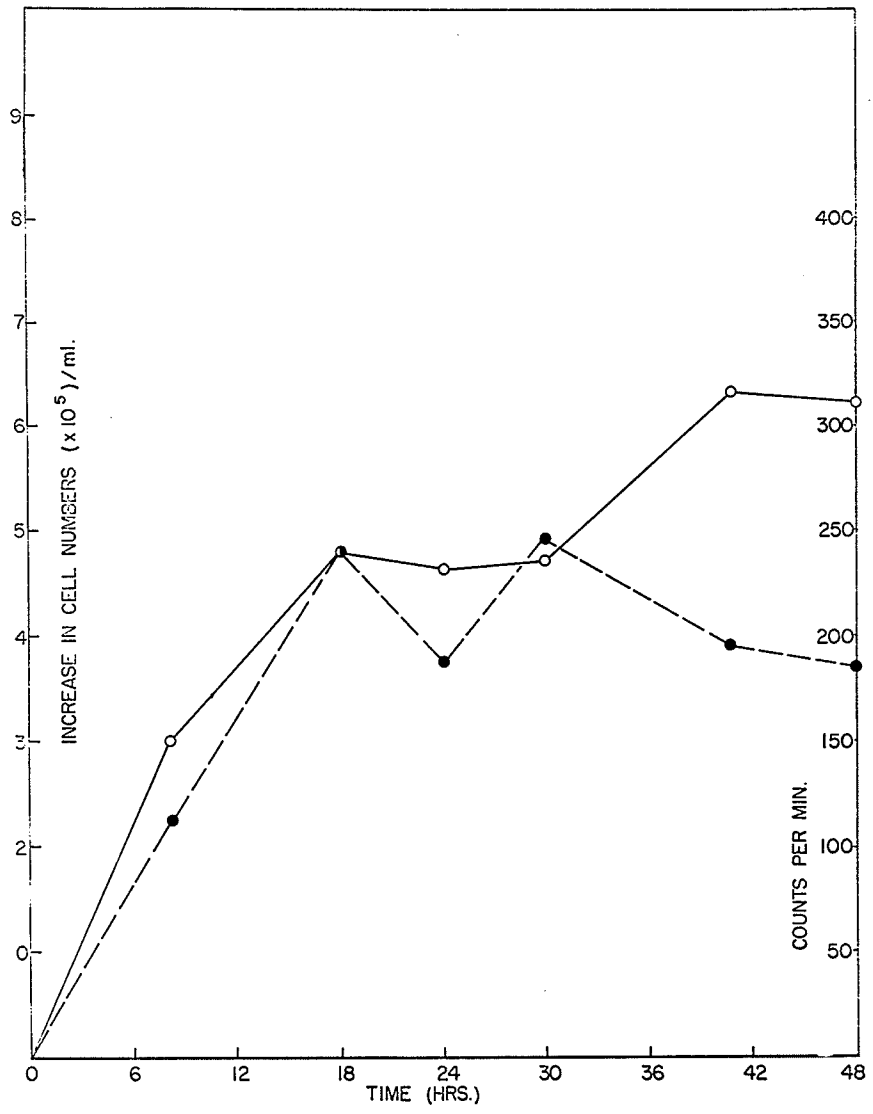
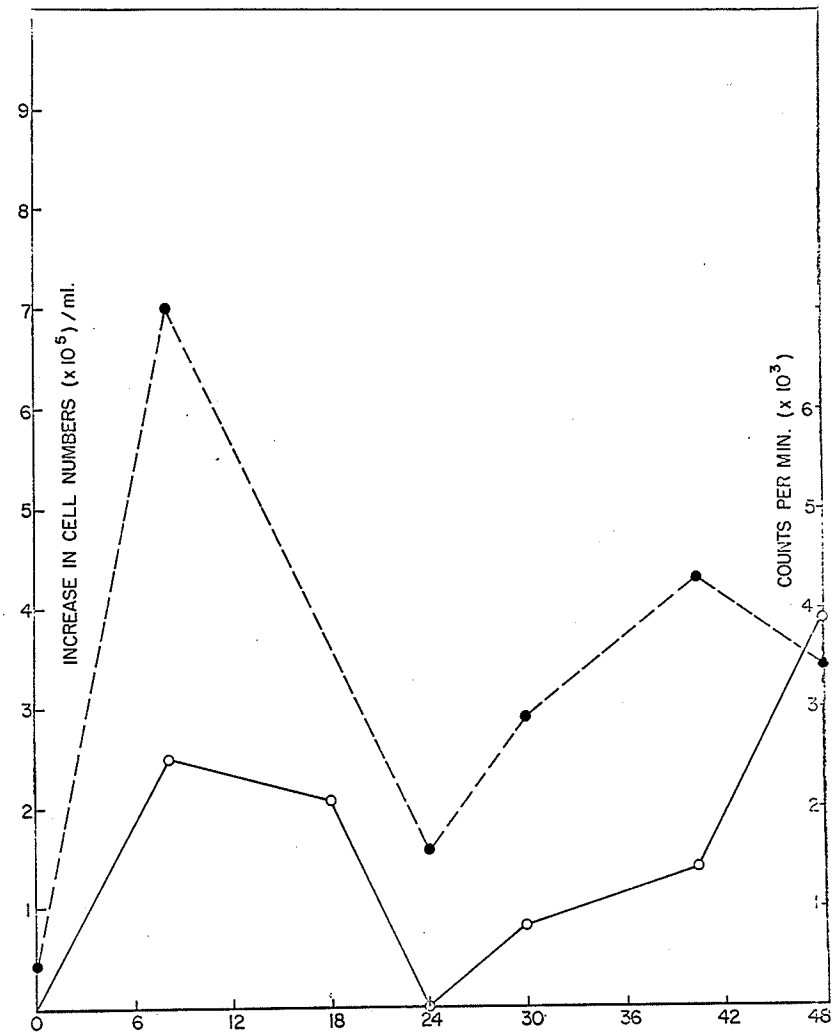

INCREASE IN CELL NUMBERS (x 10⁵)/ml.
COUNTS PER MIN.
TIME (HRS.)
0
2
4
6
8
9
50
100
150
200
250
300
350
400
0
6
12
18
24
30
36
42
48

FIGURE 15. The uptake of C^{14}-phenylalanine by ascites cells.

Cells were grown in Medium 199 supplemented with 50 µg/ml RNA and C^{14}-phenylalanine. Increase in cell numbers (—O—). Radioactive counts/minute (– –●– –).

Curves represent an average of three experiments under the described conditions.

(See Appendix, Table VIII, for actual experimental data).


INCREASE IN CELL NUMBERS (x 10⁵) / ml.
COUNTS PER MIN. (x 10³)
0
6
12
18
24
30
36
42
48
1
2
3
4
5
6
7
8
9

H. SOME OBSERVATIONS ON THE GROSS MORPHOLOGY OF EHRLICH'S ASCITES TUMOUR CELLS CULTURED IN EPM

It was the policy throughout this study, as mentioned in the Materials and Methods, to examine microscopically all cell cultures used at all stages of experimentation in order to detect possible differences in gross morphology of the population which may have been a result of the conditions imposed upon the cells. It was also mentioned that all cultures of Ehrlich's ascites tumour cells were established *in vitro* from a 2nd L-L tumour of the peritoneal cavity, the reason being to develop as nearly identical conditions as possible. Cells from later L-L transfers did not proliferate as well *in vitro* due to a general lessening of overall activity which eventually indicates the need to establish a solid muscular tumour in order to 'regenerate' the strain. This fact was confirmed by preliminary trials. As a consequence it was thought that more vigorous *in vitro* growth might be observed if cell transfer from the animal was performed at an earlier stage; specifically at the first liquid tumour established in a mouse after solid tumour development (1st S-L).

Ascitic fluid from such a mouse was processed in the

usual fashion and a culture was established in EPM plus 50 µg RNA/ml plus antibiotics with an initial count of 1.0×10^6 cells/ml. The experiment was repeated in its entirety on three separate occasions. Proliferation was no greater than that observed in the usual 2nd L-L transfer; after 24 hours culture contained 2.5×10^6 cells/ml. Microscopic observations showed however that the gross morphology of the cells at this time was significantly different from those previously seen (Plates 1-10).

In contrast to the more normal general ovoid shape of ascites cells, the cells cultured from a 1st S-L transfer were somewhat amorphous with no definite arrangement in the cell clusters, as illustrated in Plate 1. Several structures were seen at the periphery of some cells which could be loosely termed 'pseudopodia'; the nuclei varied in size; there were many more lipid granules than is usual. Several cells (Plate 2) were quite large in size and contained a large nucleus above which there appeared a structure at one end of the cell that was ciliated in appearance. This structure permeated the cytoplasm almost to the nuclear membrane. These cell types may have been similar to the 'balloon' cells of Morgan & Tolnai (Tolnai, 1964). The

All photomicrographs were taken by negative phase microscopy.

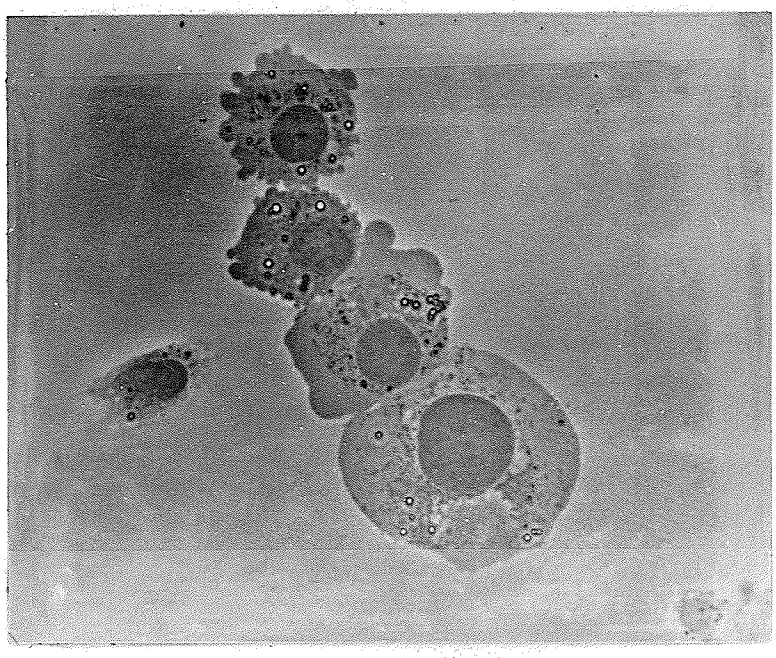


Plate 1. Ascites cells from first S-L transfer cultured in EPM. General appearance; 16 hrs (x 1000).

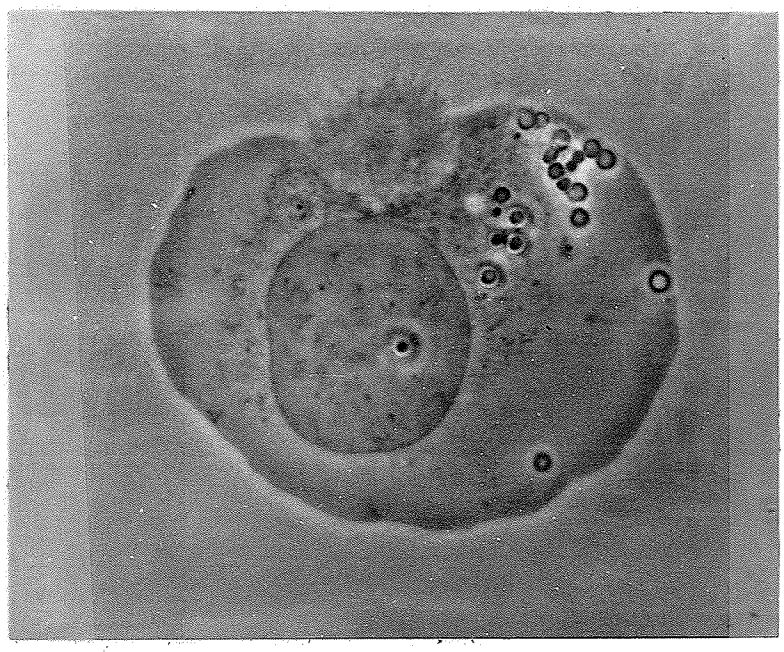


Plate 2. Giant EPM ascites cell with ciliated structure invaginating the cytoplasm to the edge of the nucleus (x 1000).

ciliated structure (Plate 3) sometimes spread out from the outer edge of the cell. The cell shown in Plate 3 had four nuclei and a large round structure which appeared to have been shed from the cell. The latter had no inclusions or other visible internal detail and almost equalled the dimensions of the parent cell. For purposes of description, this portion was loosely termed as an 'expulsion body'. Under higher magnification, this amorphous cell type showed the many lipid granules present in the cytoplasm and the irregular cell margin (Plate 4). Cells which appeared ciliated at the periphery and contained a large nuclei were sometimes observed (Plate 5). Along one of the longitudinal edges of the cell in Plate 5 four structures were seen which appeared to be the forerunners of the 'expulsion bodies'. There were three of these latter structures on the edge of the cell which seemed to be surrounded by a "capsular layer" visually similar to those seen in some bacteria. Again no interior detail was present. The apparent ejection of an 'expulsion body' from the parent cell was observed (Plate 6). Some very large cells were sometimes seen (Plate 7) which contained two well-separated nuclei similar in appearance to a double-yolked fried egg.

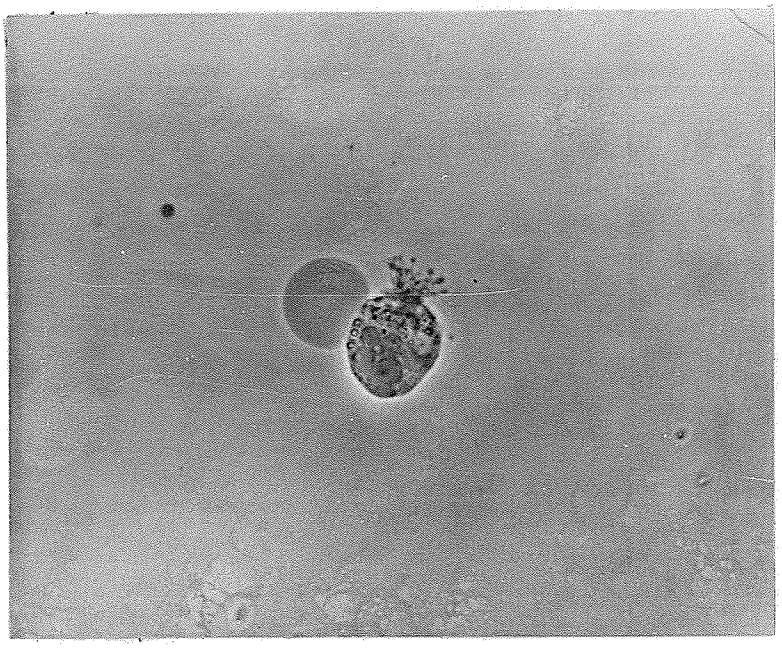


Plate 3. Multinucleated cell with nearby "expulsion body" (x 450).

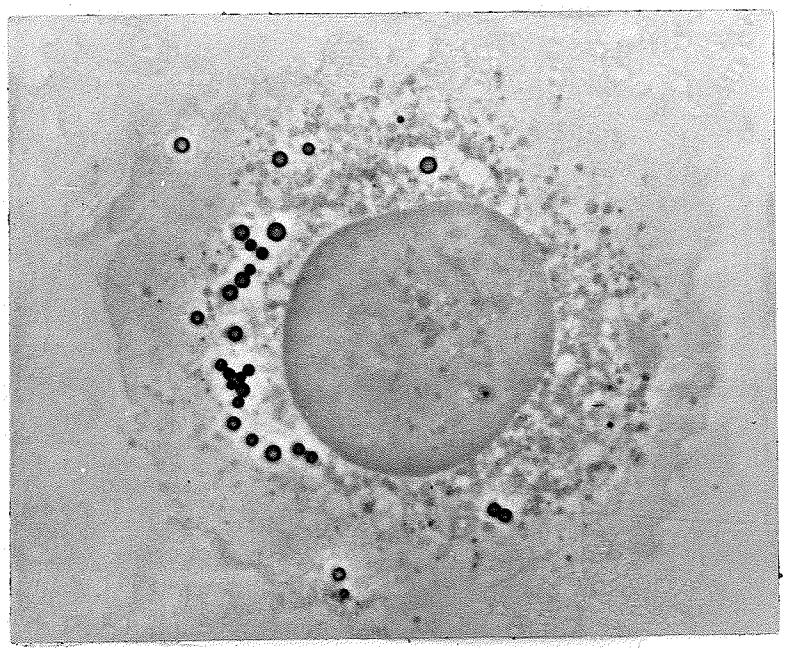


Plate 4. Giant cell showing large numbers of lipid granules in the cytoplasm (x 1000).

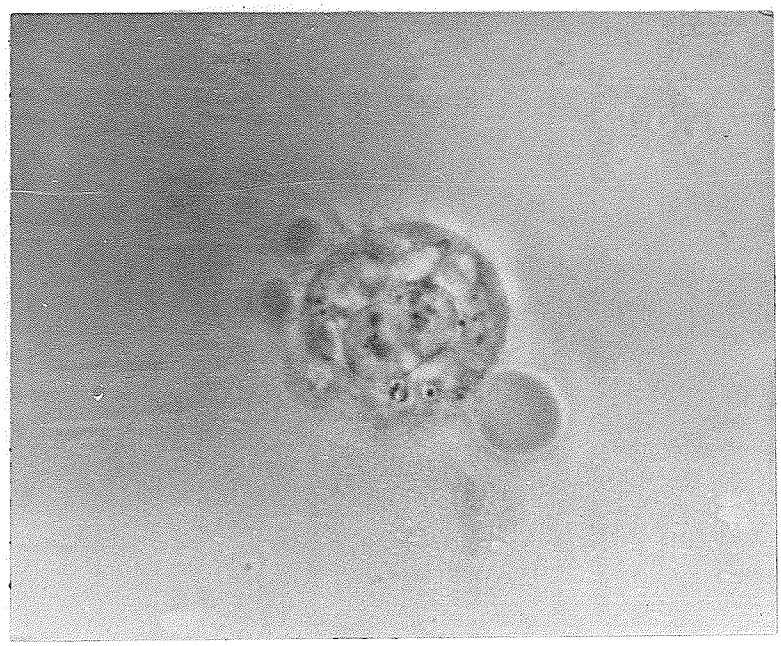


Plate 5. Ciliated cell showing possible "expulsion body" formation and release (x 1000).

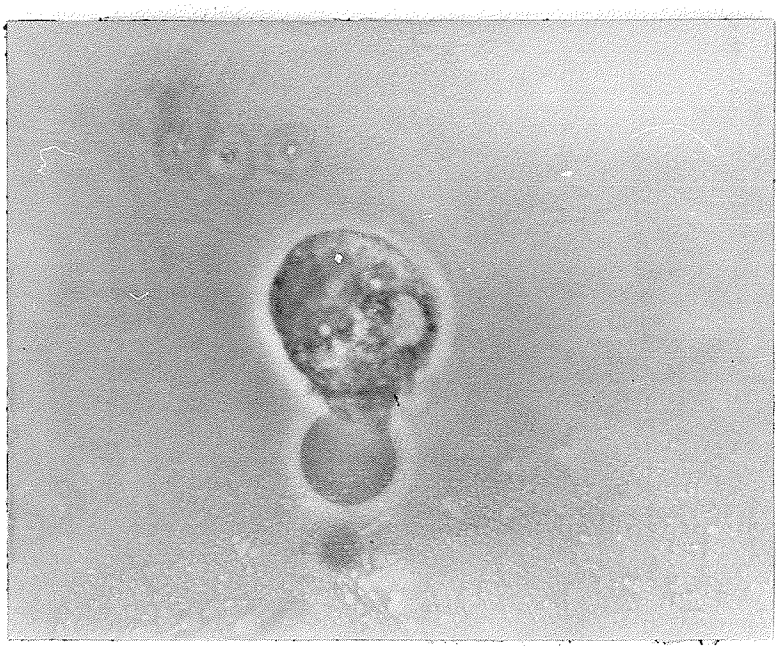


Plate 6. Apparent ejection of "expulsion body" from an ascites cell (x 1000).

After 24 hours had elapsed, the cells began taking on a more normal appearance. The periphery were no longer ciliated and the margin was regular in shape (Plate 8). The general size of the cells had decreased and the cytoplasm contained far fewer inclusions and these now appeared to be mainly lipid granules, the numbers of which had also decreased. The occasional large cell was still present, however (Plate 9). After 48 hours growth, the population was subcultured into fresh EPM medium and gave rise to cells of entirely 'normal' appearance. The morphology of the 'normal' cells is shown in Plate 10 which was derived from a culture grown from a 2nd L-L transfer and grown in EPM for 24 hours.

I. THE MAINTENANCE OF TUMOUR-PRODUCING ABILITY IN ASCITES CELLS CULTURED IN EPM.

A culture was established in the usual fashion in EPM of ascites cells and incubated. A zero control was used and at periods of 2, 8, 16, and 24 hours, 0.5 ml of a suspension of the culture in which the cells were centrifuged out of the medium and resuspended in HBSS to a concentration of 1 x 10^6 cells/ml was inoculated intra-

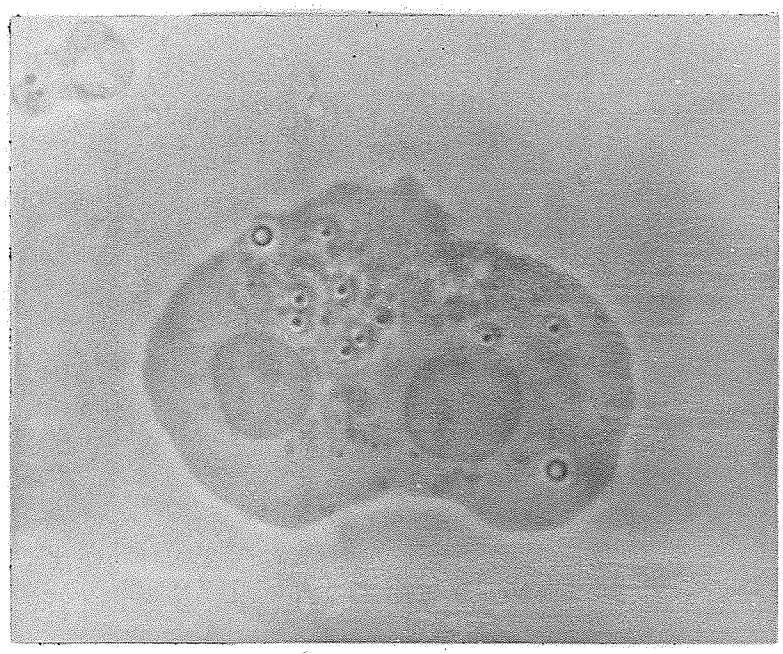


Plate 7. Binucleated giant cell (x 1000).

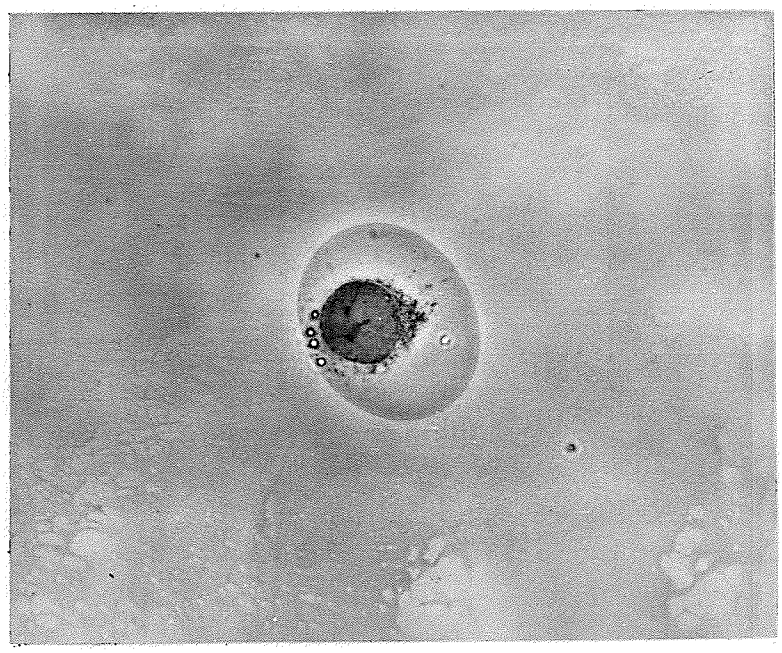


Plate 8. Ascites cells from first S-L transfer cultured in EPM. General appearance; 24 hours (x 1000).

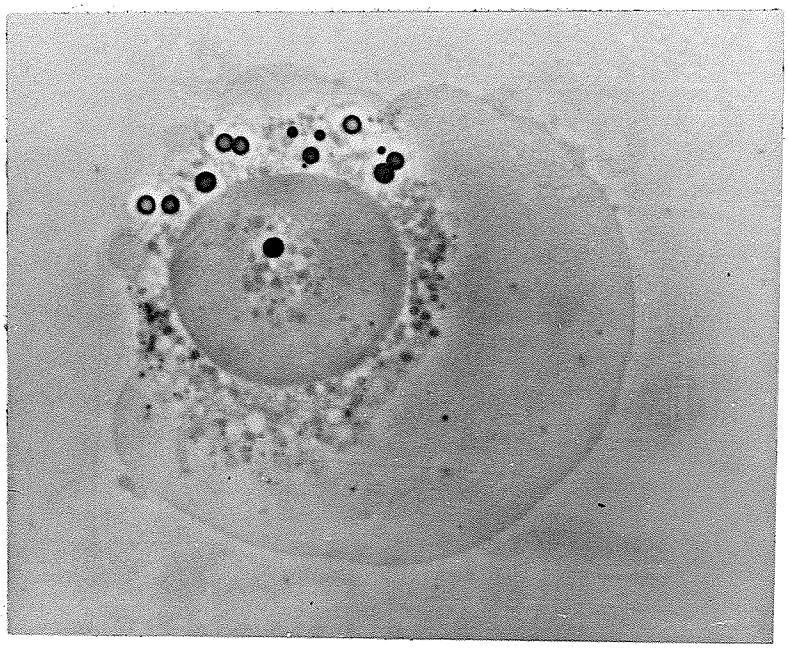


Plate 9. Giant cell from EPM culture after 24 hours' growth (x 1000).

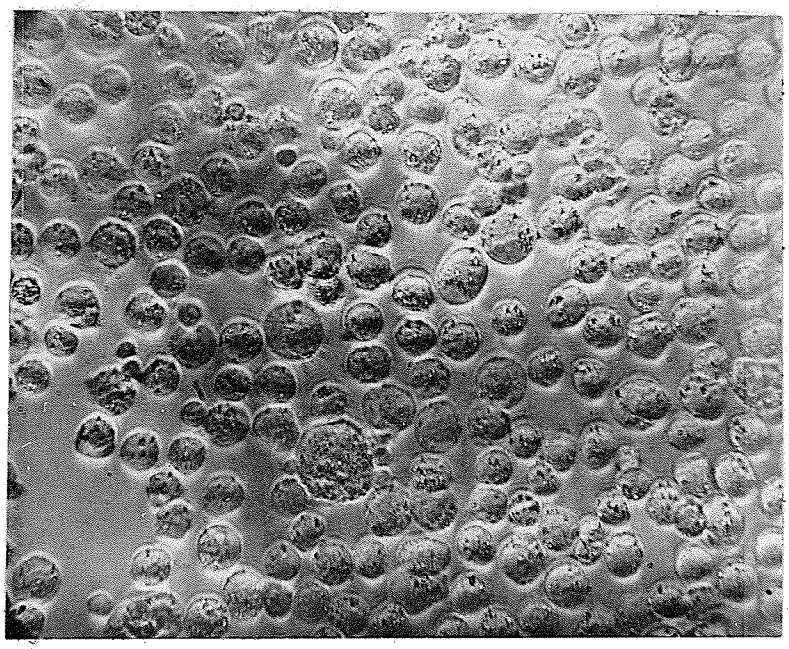


Plate 10. Normal ascites cells from second L-L transfer cultured in EPM; 24 hours.

peritoneally into a mouse. A sham inoculation of HBSS alone was carried out at each period. A duplicate experiment was carried out with a separate culture established from another mouse. Three mice were used for each period from each culture.

Tumour development appeared after the usual seven day period in the mouse injected after the two hour period and yielded ascites cells of the usual morphology. The suspension was very thick (approx. 10^9 cells/ml). The volume of ascitic fluid obtained was 9.5 ml. The mouse injected with an 8 hour culture showed some distress only after a period of 12 days post-injection had elapsed and yielded ascitic fluid of only 2.5 ml volume. No tumours were evident in mice injected after 16 and 24 hour EPM culture periods.

J. TRYPTOPHAN PYRROLASE ACTIVITY IN YEAST CELLS

The attempt to demonstrate TPO activity in yeast cells as a possible indication that yeast RNA may be carrying a messenger function to enable HeLa cells to synthesize the enzyme was not successful. All cultures were TPO-negative regardless of the concentration of

inducing amino acid, L-tryptophan, and regardless of whether or not yeast RNA was also incorporated into the culture medium. The replacement of glucose by glycerol as an energy source (in order to circumvent the well-known anti-induction effect of glucose) was equally unsuccessful in eliciting TPO synthesis. It thus appeared that yeast does not possess the ability to metabolize L-tryptophan in this fashion and consequently it seems unlikely that the active portion of the yeast RNA which does allow synthesis in HeLa cells of the enzyme is carrying a 'message'.

DISCUSSION

The incorporation of heterologous ribonucleic acid (yeast RNA) into the complex medium used for the culture of malignant animal cells caused these cells to propagate and metabolize in a fashion different from that which is customarily observed. These extracellular additions of RNA have demonstrated that they can exert a metabolic control because they can cause HeLa cells to synthesize tryptophan pyrrolase in the presence of its substrate, L-tryptophan; without these additions of L-tryptophan and yeast RNA, the HeLa cells are unable to synthesize tryptophan pyrrolase (Kenny & Lees, 1961, and Kenny, 1962).

Extension of this work on tryptophan pyrrolase (TPO) induction has demonstrated that the induction process depends on an RNA fraction of fairly large molecular weight. This is shown by the results of RNA dialysis experiments (Table II) in which it was found that only non-dialysable material was capable of stimulating enzyme synthesis. The optimal final concentration of RNA for enzyme induction had previously been assessed at about 250 µg RNA/ml (Kenny, 1962).

The possibility that TPO induction might have occurred because of uptake of a messenger yeast RNA by the HeLa cultures would, of course, be supported by a demonstration that yeast contained TPO and therefore contained a messenger RNA appropriate to its synthesis. However, our inability to detect TPO activity in any yeast culture on our part seems to indicate that the HeLa cells are not producing TPO protein in response to messenger RNA but rather their "own" enzyme. This observation negates any suggestion that HeLa cells are gradually becoming "yeast cells" under these conditions in a fashion similar to a proposal by Niu et al. (1962).

Subsequent to our primary study previously referred to, Niu et al. (1962) were able to demonstrate TPO biosynthesis in ascites cells which had been extracted from a mouse and incubated in a liver RNA-saline mixture in the cold for 15 hours. They concluded that this induction was due to some coding or messenger material in the RNA that was taken up by the ascites cells and which enabled the cells to synthesize TPO. The experimental conditions used by Niu et al. differ in two important respects from ours. First, TPO activity has been observed in mouse liver in vivo

whereas it is absent from yeast and secondly, their ascites cells were in a non-dividing state. No useful comparison between their experiments and ours is therefore possible.

Much of the work embodied in this thesis is however based on a different, albeit related, property of RNA. This is the ability of heterologous RNA to bestow on nutrient broth (a complex medium suitable for the growth of bacteria but totally unsuited to the growth of animal cells) the ability to support the growth of HeLa and ascites cells (Kenny & Lees, Can. J. Biochem. In press). The nutrient broth, supplemented with the appropriate amount of yeast RNA has, indeed, proved a perfectly satisfactory medium for the propagation of these cells and will subsequently be referred to in this discussion as EPM (experimental propagating medium).

The growth properties of HeLa cells in EPM may be described as being similar in most respects to cultures propagated in conventional tissue culture media. The cells were able to form monolayers on a glass substratum and appeared to show no marked differences in gross morphology. The requirement for the yeast RNA portion is immediate upon inoculation of the cells into EPM. The cells may cease to

proliferate unless the RNA is renewed at intervals that approximate the generation time of the HeLa population. When these optimal conditions were maintained, the cultures could be propagated over several generations and kept in the logarithmic phase of growth. Some cultures of HeLa cells were kept in this manner for a six month period with no apparent loss of normal morphological characteristics.

An interesting speculation on the necessity for renewal of the RNA portion after each generation time is that, because of the very fact that the number of cells in culture has doubled, coupled with the utilization of some of this compound by the culture, a dilution factor may be involved. The total yeast RNA concentration has probably been reduced to a level below that which is critical for initiation of further proliferation. Therefore, as each generation period arrives, the RNA component must be restored to its original concentration as there are now twice as many cells capable of division.

Cultures inoculated into nutrient broth supplemented with calf serum in lieu of RNA were unable to grow. This fact further substantiated the necessity of regular additions of RNA to EPM in order to obtain growth promotion.

An interesting feature of the growth of HeLa cells in EPM was that the buffering capacities of bicarbonate and phosphate added to the medium in the concentrations usual in tissue culture media could not cope with the fluctuations in pH caused by the metabolism of the cells. This observation is depicted in Fig. 2 (p. 35). Curve C of this figure illustrates an unusual feature in that addition of sodium bicarbonate to the EPM caused a marked cellular proliferation accompanied by an increase in pH to 8.8. The pH level observed in more conventional conditions is a shift towards acidity due to the gradual release of CO_2 by the cells as an end-product of their metabolism. We have no explanation of this phenomenon at the present time. However, it was noted that Tris-HCl buffer could stabilize the pH at a point close to neutrality after a slight increase of acidity. This may be an indication that the natural buffering capacities of nutrient broth are not as efficient as those of blood sera for animal cell cultivation or that the potential pH fluctuations in EPM were much greater than those encountered when HeLa cells are grown in more conventional media. These experiments also determined that the optimal final concentration of RNA in EPM was 50-100 μg RNA/ml. for cell

proliferation.

This latter property made it impossible to demonstrate whether tryptophan pyrrolase induction could be observed in HeLa cells cultured in EPM due to their inability to propagate in EPM containing 250 µg RNA/ml. (Curve E, Fig. 2). This latter concentration, as previously mentioned, was necessary in order to obtain TPO synthesis.

The characteristics of growth exhibited by Ehrlich's ascites cells propagated in EPM are different in some respects from HeLa cells. The primary difference is that they show some initial ability to multiply in nutrient broth alone without the incorporation of RNA, even though they had been thoroughly washed free of the ascitic fluid upon removal from the mouse and resuspended in nutrient broth (Fig. 4, p. 39). It is therefore possible to obtain a bell-shaped growth curve for ascites cells grown in nutrient broth medium alone. However, if one wishes to obtain continued cell proliferation yeast RNA must eventually be incorporated into the medium. If this increment is continued on a regular basis, as with HeLa cells, ascites cells may also be kept in a logarithmic increase phase of growth; indeed,

one culture was maintained for a period covering 72 generations.

Another major difference is that ascites cells cultured in EPM do not form a monolayer on glass as HeLa cells do and consequently they must be propagated and maintained in suspension culture. After several cell generations, ascites cells also tend to clump in EPM which makes assessment of cell numbers somewhat difficult. The critical size of inoculum of ascites cells under different culture conditions was not determined. Stock cultures were usually inoculated with 1×10^4 cells/ml medium. It was generally observed that response in the hydrolytic and fractionation experiments was augmented in cultures whose initial population was approximately 1×10^3 cells/ml. It was assumed that "carry-over" was not a factor, however, since experimental cultures were usually derived from stock cultures in EPM.

Ascites cells for experimental study were obtained from the 2nd L-L transfer in the <u>in vivo</u> tumour cycle, as mentioned in the Experimental and Results Section and cells

of normal size and uniform gross morphology were observed. The pattern of the *in vivo* propagation cycle indicated that cells derived from different stages in the cycle may show variation in either form or metabolism or both. This indication was borne out in the growth studies noted in the Experimental and Results Section and illustrated in Plates 1-10. Ascites cells from a 3rd L-L transfer did not proliferate as well as those used for major experimental work (2nd L-L). Cells from an earlier stage, however, grew no more rapidly than cells from 2nd L-L tumours. This latter observation seemed to indicate that the cells' metabolic activities were oriented for purposes other than replication only. Tolnai, Scantland & Morgan (1963) and Tolnai (1964) noted the presence of similar anomalous morphological forms in newly extracted ascites cells which did not sediment upon centrifugation in spite of their larger size. Their reports, however, did not mention the stage of tumour transfer involved. Their "balloon" cultures appeared to possess a transmissable agent capable of producing tumours in mice. Thus it would appear that

ascites cells derived from different stages of *in vivo* propagation are different in structure and function.

The conclusion may therefore be drawn that ascites cells derived from a 1st S-L transfer and subsequently cultured in EPM are morphologically and probably metabolically, distinct from cells derived from 2nd L-L transfers treated in an identical fashion. There also appears to be an indication that their process of replication may be unusual and may involve "explusion bodies". The numbers of nuclei, ranging randomly from one to four, indicate that the variability between cells in the same population is probably large. This may also be illustrated by the increase in numbers of lipid granules in the cytoplasm. A final observation was that, although the morphology of the cells return to a more "normal" appearance following 24 hrs. in culture, the nuclei of the cells are somewhat small in many cases until the culture age increases.

These observations suggest that when an investigator is working with a type of cell similar to ascites, it would

probably be wise to indicate precisely from what particular stage of the cell's history experimental cells were derived. Unless this information is available, workers in another laboratory may have difficulties in repeating the results.

The original intent of the experiments involving the fractionation of the RNA molecule was to isolate the active or "initiating" portion of the RNA in the hope of identifying nucleotide sequences. However, in light of the evidence obtained, this goal seemed an impractical one for the present because all our results suggest that the active portion is a lengthy sequence of nucleotides that would not be amenable to analysis.

The dialysis of the RNA, as previously mentioned, indicated that a relatively high molecular weight portion of the RNA was probably responsible for induction of the enzyme, TPO (Table II, p. 43), and for stimulation of HeLa cell growth in EPM (Table I). The results obtained by fractionation of the RNA molecule by means of alkaline hydrolysis and gel filtration appeared to support the supposition that the active growth-promoting portion is equally large.

A preparation of NaOH, whose molarity was 0.1, was

too weak to break down the nucleic acid to any degree during a two hour hydrolysis period (Fig. 6, p. 45). At the opposite extreme, 1.0 M and 5.0 M NaOH were too strong as hydrolytic agents in that the active portion of the RNA was rapidly destroyed and little cellular proliferation resulted when HeLa cells were inoculated into nutrient broth plus the hydrolysed RNA. The optimal hydrolytic breakdown was the exposure of RNA to 0.5 M alkali for a 15 minute period at 37° C (Fig. 5, p. 44). Under these conditions, maximal breakdown of RNA was obtained compatible with the retention of a reasonable amount of the growth-promoting properties of the RNA.

When this 15 minute hydrolysate was further fractionated by means of the Sephadex gel column procedure, the pooled fraction that yielded the maximum increase in cell numbers was the first one to emerge from columns packed with the G-50 and G-100 grades of the gel (Figs. 7-10). Subsequent application of this fraction to the Sephadex G-200 column (in which particles of a molecular weight of 2.0×10^5 or less are retained) yielded three fractions, the second of which permitted a slight growth of ascites cells when added to nutrient broth (Fig. 11, p. 53). The other two fractions had

no growth promoting activity (Fig. 12, p. 54). It is evident that a good deal of the growth-promoting material was retained by the G-200 column.

From these results it was apparent that we were probably dealing with a fairly high molecular weight portion of the RNA (M.W. 1.0 - 2.0 x 10^5) rather than a short chain of nucleotides which would have been more amenable to further analysis.

However, there is currently no practical means of determining the base-sequence of a nucleotide chain of this length; moreover, if the sequence were determinable, it is doubtful whether the information it contained could be decoded. At this point, therefore, further analysis of the yeast RNA was abandoned. The one firm conclusion to be drawn from this phase of the work is that some fairly large base-sequence in yeast RNA can so influence the metabolism of HeLa cells that these cells are able to proliferate in nutrient broth -- a medium normally quite incapable of even sustaining them, and certainly not capable of supporting their proliferation. According to present theories of RNA action, this activity of yeast RNA can best be explained on the grounds that the RNA penetrates the HeLa cell more-or-less

intact and there promotes the synthesis of some enzyme system, or systems, that enables the HeLa cell to convert the raw materials of the nutrient broth into building blocks suitable for the HeLa cell synthesis of new HeLa cell material. A decision as to whether this is the true explanation or not must await further work on RNA action.

However, it was thought that some insight into RNA action might be gained from a study of amino acid uptake by tumour cells from various media in the presence and absence of RNA since amino acid uptake is some indication of protein synthesis. For this purpose ascites cells, which can survive for a period in nutrient broth alone were used in preference to HeLa cells.

The study of the uptake by the cells of the C^{14}-labelled amino acid, L-phenylalanine, demonstrated differences in the requirement for this amino acid dependent on the composition of the culture medium. Although ascites cells exposed to nutrient broth alone cannot proliferate after a 24 hour period, the amino acid uptake increases as the population is in a state of decline indicating a possible attempt by the cells to adapt to the adverse environment in an effort to survive (Fig. 13, p. 56). Conversely, in the

favourable atmosphere of nutrient broth plus RNA, the L-phenylalanine uptake begins to decrease as the cell numbers increase (Fig. 14, p. 58). The uptake pattern in the favourable environment of Medium 199 + serum resembles that observed in nutrient broth plus RNA except that the overall uptake is greater, by a power of 10^3 (Fig. 15, p. 59). This additional uptake compared to that in EPM is probably due to the fact that serum is a poorer source of phenylalanine than the nutrient broth.

Although no immediate importance attaches to these results with phenylalanine, the results do suggest that a fuller investigation of amino-acid-uptake patterns under various culture conditions might be helpful in deciding the gross nutritional effect of RNA if not the precise mechanism of its action. Such studies are therefore now under way in this laboratory.

Another aspect of the physiology of tumour cells that was investigated was their oncogeny. After exposure of ascites cells to EPM for a few hours, their ability to induce tumours appears to be lost. This indicates that the oncogenic agent in the cells whether it be chemical, genetic, or viral in nature, cannot be maintained in EPM. The fact

that the cells cultured over several generations maintain a stable gross morphology but nevertheless do lose their oncogenic characteristics, indicates that the ability to induce tumours is not a necessary characteristic of the ascites cell. It should be stressed that the cells grown in EPM were absolutely normal in morphology when compared with virulent cells grown in vivo; what they had lost was, in fact, simply the ability to grow in vivo. However, these are no more than preliminary observations; the evidence is as yet not strong enough to suggest that any genetic change is brought about in the ascites cells as a result of exposure to EPM. Any future studies should encompass exposure of ascites cells for various periods of time, and for different numbers of cell generations, to EPM to determine the genetic stability of the apparent change in virulence. Moreover, dye-exclusion tests should be conducted to check on cell-viability. It is possible that such experiments, thoroughly replicated and incorporating the proper controls, might yield useful information about the basic oncogenic properties of ascites cells.

The results presented in this thesis suggest several further lines of investigation. Conditions have been

established that permit the growth of malignant cells in simple bacteriological media provided the media are supplemented with yeast RNA. It would be of interest to determine whether the effect of yeast RNA is peculiar to this particular RNA or whether the effect could be replicated if RNA from other sources were used instead. If the effect can be replicated, then analyses of the RNA from other sources, by the methods of hydrolysis and gel-separation described in the thesis, may yield useful comparative results. The ultimate aim of such studies would be to reveal, if possible, a particular nucleotide sequence, common to all "active" RNA molecules, that is responsible for the "growth promoting" effect of the RNA. It is possible, of course, that no such sequence exists and that many different nucleotide sequences have similar growth promoting effects; a firm demonstration of this fact would be equally interesting. The observations on the uptake of labelled amino acids might also be profitably extended; it is important to compare in some detail the amino acid uptake patterns, and patterns of protein synthesis, of cells grown in the supplemented bacteriological media with the corresponding patterns in cells grown under more normal tissue culture conditions.

The work reported here was, in effect, a series of preliminary experiments on certain environmental features which allow a living cell to remain a living cell. The author feels that amplification, modification, and perfection of these avenues of investigation may help to further understand what conditions are necessary for malignant cells to survive in the tissues of their host.

SUMMARY

(1) A medium, normally utilized for the propagation of bacterial cultures, has been adapted to promote cellular proliferation of malignant animal cells in agitated monodisperse suspension culture over several generations. The medium was composed of nutrient broth fortified with 50 micrograms per milliliter of yeast ribonucleic acid.

(2) HeLa cells and Ehrlich's ascites tumour cells of mice have been successfully cultured in this fashion.

(3) It was established that the portion of the yeast RNA molecule causing this stimulation was between 1 - 2 x 10^5 molecular weight. It was also shown that a large molecular weight was necessary for stimulation of tryptophan pyrrolase enzyme synthesis by HeLa cells.

(4) It would appear that the RNA does not fulfill a 'messenger' function.

(5) Differences in gross morphology as observed upon culturing ascites cells under these conditions from different stages in the mouse to mouse tumour transfer cycle caused these cells to show differences in activity of growth.

(6) The uptake of the C^{14}-labelled amino acid L-phenylalanine after culture in nutrient broth for 24 hours was greater in cultures which did not contain RNA. The uptake was much greater in cells cultured in Medium 199 than in nutrient broth.

(7) Mouse ascites cells, in a pilot study, were shown to gradually lose their ability to establish a peritoneal tumour *in vivo* after cultured in nutrient broth for a few hours.

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APPENDIX

APPENDIX

TABLE I

The growth of HeLa cells in nutrient broth + 10% calf serum

	*Cell Numbers (x 10^5)/ml					
Exp't No.	Day 1	2	3	4	5	6
1	0.80	0.81	0.63	0.22	0.21	0.18
2	0.91	0.85	0.72	0.43	0.28	0.27
3	0.75	0.69	0.60	0.24	0.24	0.21

*Original inoculum to population of 1 x 10^5 cells/ml.

TABLE II

The requirement for daily renewal of RNA in EPM for growth of HeLa cells

Exp't No.	Supplement	*Cell Numbers (x 10^5)/ml					
		Day 1	2	3	4	5	6
1	None	0.90	0.86	0.71	0.30	0.21	0.14
2		0.81	0.80	0.66	0.24	0.17	0.10
3	RNA (50 µg/ml)	0.93	1.42	1.44	1.10	0.84	0.63
4	Added 1st 24 hrs	0.88	1.49	1.55	1.09	0.90	0.68
5	RNA added	0.91	1.58	3.36	5.95	7.94	11.51
6	Every 24 hrs	0.94	1.48	3.35	6.08	8.11	10.97

*Original inoculum to population of 1 x 10^5 cells/ml.

NOTE: EPM was buffered with 10^{-2} M Tris-HCl buffer.

TABLE III

The effect of hydrolyzed RNA on the growth of HeLa cells (Fig. 8)

Exp't No.	Pooled Fraction No.	*Cell Numbers (x 10^4)/ml	
		24 hrs	48 hrs
1	I	12.04	15.70
	II	6.06	6.00
	III	8.41	8.75
2	I	12.08	15.76
	II	6.02	6.01
	III	8.39	8.73
3	I	12.03	15.77
	II	6.02	6.00
	III	8.44	8.79

*Original inoculum to population of 6.0 x 10^4 HeLa cells/ml.

Figure 8 represents the average of these experiments.

TABLE IV

The effect of hydrolyzed RNA on the growth of ascites cells (Fig. 10)

Exp't No.	Pooled Fraction No.	*Cell Numbers (x 10^5)/ml	
		24 hrs	48 hrs
1	I	9.70	13.25
	II	3.85	4.03
2	I	9.63	13.16
	II	3.94	4.04
3	I	9.77	13.33
	II	3.87	3.96

*Original inoculum to population of 2.5 x 10^5 ascites cells/ml.

Figure 10 represents the average of these experiments.

TABLE V

The effect of hydrolyzed RNA on the growth of ascites cells (Fig. 12)

Exp't No.	Pooled Fraction	*Cell Numbers (x 10^5)/ml		
		8 hrs	24 hrs	48 hrs
1	I	3.05	3.04	2.51
	II	3.21	3.85	3.11
	III	3.40	3.20	2.51
	control	2.60	2.65	2.68
2	I	2.94	2.97	2.23
	II	3.33	3.96	2.92
	III	3.34	3.17	2.47
	control	2.52	2.52	2.60
3	I	2.99	2.96	2.28
	II	3.29	3.99	2.74
	III	3.42	3.21	2.39
	control	2.53	2.56	2.58

*Original inoculum to population of 2.5 x 10^5 ascites cells/ml.

Figure 12 represents the average of these experiments.

Control figures represent cultures in unsupplemented nutrient broth.

TABLE VI

The uptake of C^{14}-phenylalanine by ascites cells in EPM

	*Increase in Cell Numbers (x 10^5)/ml					
Exp't No.	8 hrs	18 hrs	24 hrs	30 hrs	40 hrs	48 hrs
1	3.30	4.44	6.96	6.36	5.02	4.18
2	3.21	4.38	6.80	6.26	4.88	3.97
3	3.40	4.50	6.92	6.28	5.08	4.09

*Original inoculum to population of 0.75 x 10^6 ascites cells/ml.

Figure 13 represents the average of these experiments.

TABLE VII

The uptake of C^{14}-phenylalanine by ascites cells in EPM + RNA (50 μg/ml)

	*Increase in Cell Numbers (x 10^5)/ml					
Exp't No.	8 hrs	18 hrs	24 hrs	30 hrs	40 hrs	48 hrs
1	3.15	4.82	4.71	4.73	6.15	6.10
2	3.04	4.73	4.69	4.75	6.27	6.14
3	2.96	4.78	4.66	4.80	6.21	6.06

*Original inoculum to population of 0.75 x 10^6 ascites cell/ml.

Figure 14 represents the average of these experiments.

TABLE VIII

The uptake of C^{14}-phenylalanine by ascites cells in 199 + RNA (50 µg/ml).

	*Increase in Cell Numbers (x 10^5)/ml					
Exp't No.	8 hrs	18 hrs	24 hrs	30 hrs	40 hrs	48 hrs
1	2.21	2.19	0.06	0.82	1.40	3.84
2	2.63	2.28	0.00	0.69	1.31	3.42
3	2.49	2.09	0.00	0.78	1.30	4.31

*Original inoculum to population of 0.75 x 10^6 ascites cells/ml.

Figure 15 represents the average of these experiments.