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

by

C. PAUL KENNY, B.Sc., M.Sc.

A Thesis

submitted to

The Faculty of Graduate Studies and Research

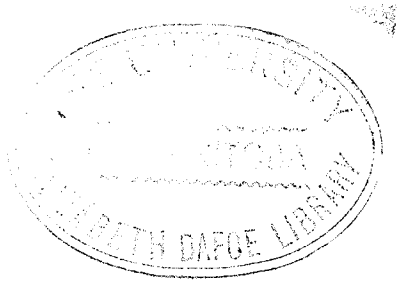
University of Manitoba

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.

TABLE OF CONTENTS

	PAGE
INTRODUCTION	1
HISTORICAL	5
MATERIALS AND METHODS	14
Tissue Culture Procedure	14
Analytical and Experimental Procedures	22
EXPERIMENTAL AND RESULTS	30
Calibration of the Coulter Counter	30
The Growth of HeLa Cells in Different Media	34
The Growth of Ascites Tumour Cells in Nutrient Broth	38
The Effects of Dialysates of Yeast RNA	40
The Growth of HeLa Cells in Alkaline Hydrolysates of RNA	42
Cell Growth in RNA Hydrolysate Fractionated by Gel Filtration	47
The Uptake of C^{14}-Labelled Amino Acid by Cells Cultured in EPM	55
The Gross Morphology of Ascites Cells Cultured in EPM	60
Tumour-Producing Ability of Ascites Cells Cultured in EPM	66
Tryptophan Pyrrolase Activity in Yeast Cells	69

TABLE OF CONTENTS CONT'D

	PAGE
DISCUSSION	71
SUMMARY	85
REFERENCES	87
APPENDIX	94

INDEX OF TABLES AND FIGURES

TABLE		PAGE
I	The Effect of Dialysis on the Growth-Promoting Properties of yeast RNA	41
II	The Effect of Dialysis on the Enzyme-Inducing Properties of yeast RNA	43

FIGURE		
1	Calibration of the Coulter Counter	32
2	The Growth of HeLa Cells in Different Media ..	35
3	The Growth of HeLa Cells in Different Media ..	37
4	The Growth of Ascites Cells in Different Media	39
5	The Growth of HeLa Cells in Nutrient Broth plus Hydrolyzed RNA	44
6	The Growth of HeLa Cells in Nutrient Broth plus Hydrolyzed RNA	45
7	Sephadex Fractionation of Hydrolyzed RNA	48
8	The Effect of Hydrolyzed RNA on the Growth of HeLa Cells	49
9	The Second Sephadex Fractionation of Hydrolyzed RNA	51
10	The Effect of Hydrolyzed RNA on the Growth of Ascites Cells	52
11	The Third Sephadex Fractionation of Hydrolyzed RNA	53

LIST OF TABLES AND FIGURES CONT'D

FIGURE		PAGE
12	The Effect of Hydrolyzed RNA on the Growth of Ascites Cells	54
13	The Uptake of C^{14}-Phenylalanine by Ascites Cells	56
14	The Uptake of C^{14}-Phenylalanine by Ascites Cells	58
15	The Uptake of C^{14}-Phenylalanine by Ascites Cells	59

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

---

*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.**

---

*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

---

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