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THESIS M.Sc.

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INCREASED ACTIVITY OF TRYPTOPHAN PEROXIDASE-OXIDASE IN SUSPENSION CULTURES OF MAMMALIAN CELLS

by

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A Thesis

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Finally, the author is indebted to Miss Maureen Elliott, for her valuable assistance in the preparation of the manuscript.

ABSTRACT

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Human carcinoma (HeLa) cells have been cultured in monodisperse suspensions in contradistinction to the more usual plaque cultures. These monodisperse suspensions have been used for the study of "induced enzyme synthesis" in mammalian cells. The induction of tryptophan peroxidase was found to occur in response to the addition of tryptophan plus RNA to the medium. Tryptophan alone or RNA alone failed to induce the enzyme. The significance of the findings is discussed.

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INTRODUCTION

INTRODUCTION

The interaction between living organisms and their environment is an old but very important problem for biologists. Organisms respond to environmental change in different ways according to time of exposure to the environmental change and to the magnitude of the stress. In living organisms a change in environment which favours survival is said to be adaptive.

Animal physiologists have been concerned mainly with adaptive change in the intact organism or in organ systems. Microbial physiologists, on the other hand, have been primarily concerned with enzymatic changes induced by varying the nutrients in the environment, and have used adaptive enzyme changes as a means of studying protein syntheses.

It seems reasonable to suppose that if a given enzyme can be induced *in vivo*, it might also be induced *in vitro* in cultures of animal cells. If the latter induction proved successful a more precise study of the mechanism of induction would be possible than is possible in the intact animal, since cell cultures could be exposed to the inducing environment while they are maintained in the logarithmic phase of growth. (In animals the effect is lost rapidly due to the constant regeneration of fresh tissue). Further, the tissue cells are monodisperse in suspension culture and each cell is exposed to a known concentration of inductor.

The ability to control the composition of the growth medium is a distinct advantage of this method. Furthermore, the stress caused by hormonal influence is probably minimized. One should therefore be reasonably certain that a specific change found in the enzymatic pattern of the cells represents a direct response to added substrate.

The well-known inducible enzyme system in animals, specifically, the induction of the tryptophan peroxidase-oxidase system in livers, was chosen for this study. The cell cultures selected belonged to an established strain of human epithelial cells (strain HeLa).

HISTORICAL

HISTORICAL

A. The Development of Modern Tissue Culture Techniques

Jolly, in 1903, performed experiments which marked the first detailed observations on cell survival and cell division *in vitro* by the maintenance of salamander leucocytes in hanging drops for up to a month. In 1906, Beebe and Ewing, in a genuine attempt at tissue culture, described the cultivation of an infectious lymphosarcoma in blood from resistant and susceptible animals. In 1907, Harrison demonstrated cultivation of normal function *in vitro* and offered a reproducible technique marking the true beginning of tissue culture. He explanted small pieces of tissue from the medullary tube region of frog embryos into clots of frog lymph. Under aseptic conditions, the fragments survived for some weeks and axones grew out from the cells.

The "traditional" tissue culture techniques developed rapidly from these early beginnings into the late 1940's; but the complexity of the methodology that was developed limited its application to selected areas of study, such as embryology and histology. In virology and biochemistry, however, only the recent development of techniques has allowed the widespread application of the tissue culture method to these fields of study.

The first major difficulty to be overcome was the establishment of a synthetic growth medium. The media derived from natural sources, such as embryo extract, had an unknown and variable composition and it was virtually impossible to reproduce conditions exactly from one experiment to another. In 1946, White reported the first truly synthetic medium. The first defined medium reported that supported both growth, in terms of increase in protoplasmic mass, and maintenance of viability of cultures, was that of Morgan, Morton and Parker (1950). However, one must bear in mind that the roles, if any, that several of the metabolites in defined media possess, have not been determined. The criterion for inclusion is that the cells grow more rapidly if these metabolites are present.

The second major development was the discovery of antibiotics. The incorporation of these substances into the culture medium permitted the abolition of many of the "surgical" techniques previously required to maintain aseptic tissue cultures. The antibiotics used routinely today are usually penicillin, streptomycin and neomycin (Keilova (1948); Cruickshank and Lawburg (1952)).

Sanford _et al_. (1948) derived the first clone strain of cells, L no. 929, from mouse mesenchymal tissue. Individual cells were isolated in capillary tubes and propagated in a medium containing the fluid removed from a large metabolically active culture in progressively larger vessels as it increased. Puck and Marcus (1955) developed the method for plating single cells, which is especially useful for cloning epithelial cell strains, such as HeLa.

The modern culture methods were devised so that pure strains could be established in large numbers of uniform cultures. Syverton _et al_. (1954) developed the procedure for establishing a pure strain of cells by growing them as a monolayer cell sheet on glass. These are transferred into larger numbers of cultures by bringing the cell sheet into suspension followed by dilution to an appropriate size of inoculum. This was done either by scraping the cells from the glass surface or by the use of proteolytic enzymes, usually trypsin.

Many investigators, however, observed that the mammalian cells can proliferate in agitated fluid suspensions, i.e. without a supporting framework. The initial techniques were introduced by Earle et al. (1954), who reported successful growth of the "L" strain (no. 929) utilizing a conventional rotary shaker equipped with special flasks, etc. They observed that at 300 r.p.h. some of the cells remained adherent to the walls of the test tube but at 2400 r.p.h. they were all in suspension. Addition of 0.1% methyl-cellulose helped to keep the cells in suspension. Under these conditions the cells suffered no damage (e.g. trypsin shock) and continued to proliferate freely. This method has been further modified by Kelley et al. (1960) who were successful in cultivating swirl cultures without any carbon dioxide enrichment of the atmosphere.

Suspension cultures have also been obtained by using stirrer vessels. (McLimans et al. (1957); Cherry and Hull (1956)).

B. Enzyme Induction in Mammalian Cells Cultured *in vitro*

The phenomenon of substrate-induced enzyme formation is well known in bacteria and other microorganisms. This work has been well reviewed (Stanier, 1951; Cohn, 1957; and Vogel, 1959).

The major contributions for the existence of this phenomenon in mammalian cells have appeared only within the last decade.

Knox and Mehler, in 1951, demonstrated the induction of a ten-fold increase of L-tryptophan oxidase-peroxidase (TPO) in rat liver following administration of L-tryptophan to the animals.

Some evidences of substrate induction have been reported in chick embryos, notably arginase (Roeder, 1957) and alkaline phosphatase (Kato and Moog, 1958). Further references are cited in the review by Knox *et al*. (1956).

Relatively few investigators have used animal cells cultivated *in vitro*. Kato and Moog (1958) found an increase in alkaline phosphatase in duodenal fragments cultivated in the presence of phenylphosphate.

In 1960, Klein was able to induce arginase activity in freshly isolated embryonic chick and mouse cells but could not obtain any activity in established cell strains. In a further work, (Klein, 1961), he studied substrate-induced enzyme synthesis using arginase in embryonic, normal adult, and malignant tissues. He was not able to induce enzyme activity in cultures grown in Morgan's Medium #199, unless, curiously, he incorporated RNA derived from yeast into the medium along with the substrate.

Cox and Pontecorvo (1961) demonstrated the induction of alkaline phosphatase in established cultures of human skin fibroblasts by phenylphosphate and β-glycerophosphate.

It is with this background that the present work, aimed at the induction of enzymes (in particular tryptophan peroxidase) in monodisperse suspension of human carcinoma cells (HeLa) was begun.

MATERIALS AND METHODS

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A. TISSUE CULTURE PROCEDURES

1. Cleansing and Preparation of Glassware

It is a well established fact that nothing is more basic to cell cultures than properly cleaned glassware. A very rigid and thorough procedure must be followed in cleansing because elimination of any step may leave substances adhering to the walls of the vessels that are potentially toxic to the cell cultures.

Any new glassware or equipment was immersed in 1% HCl for three hours followed by a thorough rinsing in running tap water. Used glassware was brushed thoroughly in warm tap water and immersed in a detergent solution ("Micro-solv", from Microbiological Associates, Bethesda, Md.) for twelve hours. Final cleaning was done by an automatic washer set to the following sequence:

(a) An 8 min. wash with detergent using water at a temperature of 180-200° F.

(b) An 8 min. rinse with water of the same temperature.

(c) A rinse of 1 1/2 min. duration with distilled water.

The glassware was then drained for two hours. All flasks were plugged with cotton and gauze capped with tinfoil, and all glassware was sterilized prior to use for four hours at 160° C in a hot air oven.

Immediately after use, all pipettes were immersed in detergent solution for twelve hours followed by a rinse in an automatic pipette rinser for four hours. They were finally rinsed three times with distilled water.

2. Culture Procedures

(a) Monolayer Cell Sheets:

Throughout the work, a stock of HeLa cells was maintained by classical monolayer culture. In such cultures cells attach themselves to the glass, flatten and grow to yield a continuous sheet, or network along the glass surface, usually one cell thick. This technique of cultivating animal cells on a solid surface using a fluid overlay was originated and developed by Earle and co-workers (Earle, Schilling, and Shelton, 1950; Evans, Earle *et al*; 1951).

The culture of HeLa cells[1] used in the present work had been cultured as monolayers in a medium containing 20% human serum in milk dilution bottles. The cultures grew slowly, however, and some cultures failed to establish cell monolayers due to the early onset of non-specific cell degeneration. On the basis of the work by Albano (1959), the cells were successfully adapted to a calf serum supplement[2] in place of human serum. The serum variables were thus eliminated. The advantage of this serum was that non-specific cell degeneration developed at a later time allowing for prolonged culture maintenance and its use was less expensive and time consuming.

The following procedure was employed in the harvest of HeLa cells from a monolayer culture:

(i) The growth medium was removed and replaced by 10 ml of 0.25% trypsin solution. The culture was then re-incubated at 37° C for 10-15 mins. until it could be observed that the cell sheet had detached from the glass surface.

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[1] The original cultures of HeLa cells were kindly provided by Dr. C. K. Hannan, Dept. of Bacteriology, U of M.

[2] From Microbiological Associates, Bethesda, Md.

(ii) The cells were separated in the suspension by pipetting up and down several times. The suspension was then centrifuged at 800-1000 rpm for 10 min. and the supernatant discarded. The cells were washed once with 10 ml of Hank's Balanced Salts Solution (Table II) and recentrifuged.

(iii) The cells were resuspended in Hank's solution and distributed equally to four milk dilution bottles each containing 10 ml of growth medium.

(iv) The bottles were incubated statically for a minimum of two days at 37° C to allow for the formation of the new cell sheet on the glass surface.

(v) The cell monolayers were fed by removal of half of the old medium and replacement with fresh medium every 2 days.

(b) <u>Agitated Monodisperse Cell Suspensions</u>:

Throughout the work, all experimental studies were performed on cultures grown in monodisperse suspension for reasons already outlined in the introduction. The important feature is that in these suspensions, each cell exists as a separate organic entity.

The original culture was obtained from a monolayer culture by trypsinization as in 2(a) above and enumerated by the haemocytometer method (see below). HeLa cells were dispersed into 90 ml of growth medium in a 250 ml Erlenmeyer flask in a concentration of 4.0×10^5 cells/ml and placed on the shaker (New Brunswick Gyratory Water Bath Shaker, Series G 76) set at 37.5° C and a rotation speed of 120 rpm.

The following procedure was then employed in subsequent culture of HeLa cells in agitated suspension:

(i) Subculturing was performed after 24 hrs. using inocula of 2.0×10^5 cells/ml in 60 ml of growth medium in a 250 ml Erlenmeyer flask.

(ii) The flasks were then placed on the shaker.

(iii) Centrifugation was carried out at 2000 rpm in order to obtain cells from the suspension.

All subculturing and experimental procedures could now be performed by simple aseptic bacteriological techniques. Upon microscopic observation (by hanging drop preparations) the cells appeared to be monodisperse, i.e. they existed as

TABLE I(a)

Growth Medium for HeLa Cell Cultures

20% Calf Serum

80% Morgan's Mixture #199

50 μg/ml Dihydrostreptomycin sulphate

100 I.U./ml Penicillin G. Potassium

or

50 μg/ml Neomycin sulphate

TABLE I(b)

Morgan's Mixture #199

Ingredients per liter:

L-arginine	70 mg	DL-alanine	50 mg
L-histidine	20 mg	L-proline	40 mg
L-lysine	70 mg	L-hydroxyproline	10 mg
L-tyrosine	40 mg	glycine	50 mg
DL-tryptophan	20 mg	pantothenate	0.01 mg
DL-phenylalanine	50 mg	biotin	0.01 mg
L-cystine	20 mg	folic acid	0.01 mg
DL-methionine	30 mg	choline	0.5 mg
DL-serine	50 mg	inositol	0.05 mg
DL-threonine	60 mg	p-aminobenzoic acid	0.05 mg
DL-leucine	120 mg	vitamin A	0.1 mg
DL-isoleucine	40 mg	calciferol	0.1 mg

Table I(b) contd.

DL-valine	50	mg	menadione	0.01	mg
DL-glutamic acid	150	mg	α-tocapherol phosphate	0.01	mg
DL-aspartic acid	60	mg	ascorbic acid	0.05	mg
glutathione	0.05	mg	riboflavin	0.01	mg
cholesterol	0.2	mg	pyridoxine	0.025	mg
sodium acetate	50	mg	pyridoxal	0.025	mg
L-glutamine	100	mg	niacinamide	0.025	mg
adenosinetriphosphate	1	mg	niacin	0.025	mg
adenylic acid	0.2	mg	desoxyribose	0.5	mg
ferric nitrate	0.1	mg	Tween 80	0.005	mg
ribose	0.5	mg	sodium chloride	8	g
L-cysteine	0.1	mg	potassium chloride	0.4	g
adenine	10	mg	calcium chloride	0.14	g

Table I(b) contd.

guanine	0.3	mg	magnesium sulphate	0.2	g
xanthine	0.3	mg	disodium phosphate	0.06	g
thymine	0.3	mg	monopotassium phosphate	0.06	g
hypoxanthine	0.3	mg	sodium bicarbonate	0.35	g
uracil	0.3	mg	dextrose	1	g
thiamin	0.01	mg	phenol red	0.02	g

Final pH 7.0 ± 0.2

Ref: Morgan, J. F. Morton, and Parker, 1950.

TABLE II

Hank's Balanced Salts Solution (BSS)

A. 10X solution

	Material	Amount	Preparation
Unit #1	$NaHCO_3$	3.5 gm	Dissolvedin 250 ml dis-water and dispensed in a convenient bottle (screw-cap) and autoclaved at 120° C for 15 mins.
Unit #2	NaCl	80.0 gm	Dissolved in 800 ml distilled water.
	KCl	4.0 gm	
	$MgSO_4 \cdot 7H_2O$	2.0 gm	
	$Na_2HPO_4 \cdot 2H_2O$	0.6 gm	
	Glucose	10.0 gm	
	KH_2PO_4	0.6 gm	
Unit #3	$CaCl_2$	1.4 gm	Dissolved in 100 ml distilled water.
Unit #4	Phenol red	0.4 gm	Mixed in small amount of water until a paste, diluted to 150 ml with distilled water, titrated to pH 7 with N/20 NaOH. Made up to final volume of 200 ml and preserved with 1-2 ml chloroform.

Table II contd.

100 ml of Unit #2 were added to Unit #2 and then Unit #3 was added to make 1000 ml. The solution was poured into a glass stoppered bottle and 3-4 ml chloroform were added as a preservative. The solution was stored at room temperature.

B. Working Solution

The working BSS was prepared by dilution of 10X stock 1:10 with distilled water. It was dispensed in screw cap bottles and autoclaved at 120° C for 15 mins. 2.5 ml of sterile sodium bicarbonate solution (Unit #1) were added aseptically to each 100 ml of BSS. The pH was adjusted to 7.4.

separate entities in the suspension. Cells obtained from monodisperse suspensions could be cultured as monolayers with no apparent reduction of their glass-attaching property.

In the above culture procedures, all cultures and reagents were tested for bacterial sterility as follows:

(i) Aerobic microorganisms - inoculation of tubes of Difco brain heart infusion broth followed by incubation for one week at 28^{o} and 37^{o} C.

(ii) Anaerobic microorganisms - inoculation of tubes of thioglycollate agar followed by incubation for one week at 28^{o} and 37^{o} C.

3. Cell Enumeration Procedure

Although the direct count of cells is tedious in comparison with the indirect methods, (total N_2 by micro-Kjeldahl, determination of cellular RNA, etc.) the individual existence of the cells in agitated suspension favoured a direct count. Such a method was used because it allowed for a more accurate determination than is often possible.

The method used was the haemocytometer technique adapted from that of Merchant *et al*. (1960).

After the haemocytometer had been thoroughly cleansed, 0.5 ml of cell suspension was diluted to a final cell concentration of $1 \times 10^5 - 2 \times 10^5$ cells per ml. The diluent was a solution of 0.01% crystal violet in 0.1 M citric acid. This diluent also stained the nuclei which facilitated enumeration and tended to minimize any clumping that can occur in the chamber.

Both chambers of the haemocytometer were filled and the cells counted were those in the four corner squares and the centre square of each chamber in the haemocytometer.

B. L-TRYPTOPHAN PEROXIDASE ASSAY PROCEDURE

The following programme was carried out for all assay procedures. The method was modified from that of W. E. Knox (1955). The assay is based on these reactions:

(a) Tryptophan peroxidase (TPO) catalyzes the oxidation of L-tryptophan to L-formylkynurenine. This is the first step in the major pathway of tryptophan degradation, leading eventually to the formation of nicotinic acid, to intermediates like kynurenine and xanthurenic acid, and to certain eye pigments in insects.

(b) L-formylkynurenine is hydrolyzed to L-kynurenine by excess kynurenine formamidase present in the homogenate. The L-kynurenine is then determined by its absorption at 365 mμ.

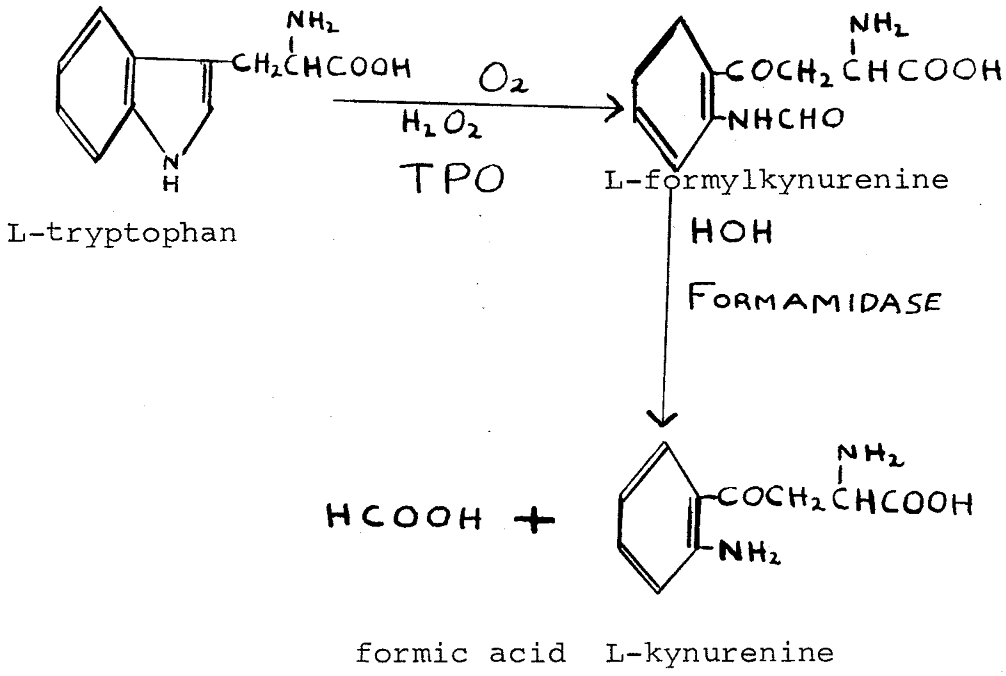


Fig. 3. The basic reactions of the TPO system.

Deionized water was used in all reagents and tests because of the inhibitory effects of Cu^{++} and Fe^{+++} ions. All operations were carried out at 0-5° C. All determinations were performed in duplicate.

(i) The cell suspensions were centrifuged at 10,000 x g for 5 mins. This was followed by two washes with Hanks Balanced Salts Solution (Table II) to remove all traces of the medium.

(ii) All assays for tryptophan peroxidase (TPO) were performed on a cell sample having a wet weight of 0.45 g.

(iii) The cells were resuspended by trituration (effected by repeatedly drawing the material into a pipette and expelling it again) with 3 volumes of alkaline KCl. The suspensions were allowed to stand for 5 mins.

(iv) The suspensions were then homogenized in a glass tissue grinder for 5 mins.

(v) 1.5 ml of homogenate were pipetted into each reaction tube. The reaction tube contained 1.0 ml of 0.2 M phosphate buffer, pH 7.0, 0.3 ml of 0.03 M L-tryptophan, and deionized water to make a total volume of 4.0 ml. The

L-tryptophan was omitted from the control tube.

(vi) The tubes were then shaken for one hour at 38° C in an aerobic atmosphere. The reaction was stopped, the material deproteinized, and neutralized in a manner identical with that of Knox.

(vii) The determination of TPO activity was accomplished by the measurement of the L-kynurenine present in the sample by means of a Beckman DB spectrophotometer at a wave length of 365 mμ.

Calculation of Activity

The optical density difference between the blank and the experimental was the measure of the L-kynurenine formed. This is proportional to the TPO concentration under the assay conditions.

The units of Knox were used in the calculations, i.e. the number of micromoles of kynurenine formed per gram of dry weight of homogenate per hour.

The dry weight was determined from 2 ml of fresh homogenate dried for 2 hours at 100° C, and corrected for the weight of the contaminating KCl.

The molar extinction coefficient of kynurenine under these conditions is $\epsilon = 4540$ at a wavelength of 365 mμ.

C. THE INDUCTION OF TPO ACTIVITY

Once it had been established that the TPO system did not exist in the normal HeLa cell, an attempt was made to induce TPO activity by raising the L-tryptophan concentration of the growth medium from 10^{-4} M to 10^{-2} M.

This attempt was unsuccessful, no TPO being induced in response to the increase in tryptophan concentration. The next step, in conformity with the findings of Klein (1961), was to increase both the tryptophan and the RNA concentrations of the medium.

Yeast RNA (from Sigma Chemical Co.) was dissolved in 9 parts of deionized water to 1 part of 0.2 M phosphate buffer, pH 8.0. The solution was then filtered through a Millipore membrane filter (pore size 0.45 μ) and aseptically incorporated into the culture medium to give a final concentration of 250 μg RNA/ml medium. Under these conditions, TPO activity was induced in the HeLa cells.

In the presentation of the Results and the Discussion, the term "basal culture" will be used to describe one in which the tryptophan concentration alone was increased; the term "RNA culture" will be used to describe one in which both RNA and tryptophan concentrations were increased.

The experimental design consisted of ten "basal cultures" and ten "RNA cultures" of HeLa cells. These cultures were then assayed at two day intervals for the activity of TPO.

Five "normal" cultures (in which neither the tryptophan or RNA concentrations were augmented) were assayed. Five cultures in which the RNA concentration alone was augmented were also assayed.

RESULTS

RESULTS

A. The Growth of HeLa Cells in Monodisperse Suspensions.

The HeLa cell strain had been established in monolayer cultures. Trypsinization of the cell sheets produced a suspension in which the cells were monodisperse, i.e. they existed individually with no evidence of aggregation or clumping.

Once the fact had been established that the cells could exist and multiply individually in the agitated suspensions, a growth curve was determined (Fig. 1). Haemocytometer counts of cell population were made every two days until the population entered the maximum stationary phase at the end of 16 days.

A closer picture of the growth pattern during the lag phase is provided in Fig. 1(a).

During the period of accelerated growth, the medium was virtually free of noncellular debris and the pH of the medium (phenol red) remained stable at 7.2. At the beginning of the stationary phase, some non-specific degeneration and debris was evident and the pH of the medium decreased slightly.

The mean generation time of the HeLa cells under these conditions was 24.83 hours.

B. Increased Activity of TPO

All of the "normal" cultures tested at two day intervals over a ten day period showed no activity of TPO.

The "basal" cultures, i.e. those containing an increased L-tryptophan concentration, were also lacking in TPO activity.

The five cultures containing extra RNA but no tryptophan increment were devoid of measurable activity.

The positive results that were obtained occured only in the "RNA" cultures, i.e. those containing extra tryptophan and extra RNA.

These latter observations are summarized in Table III. A curve of the TPO activity obtained has been correlated with the growth curve of the HeLa cells in Fig. 2.

From Fig. 2, it can be observed that the activity of the TPO system occuring in the "RNA" cultures increases as the culture age increases until the population goes into the stationary phase of growth. At this point, TPO activity

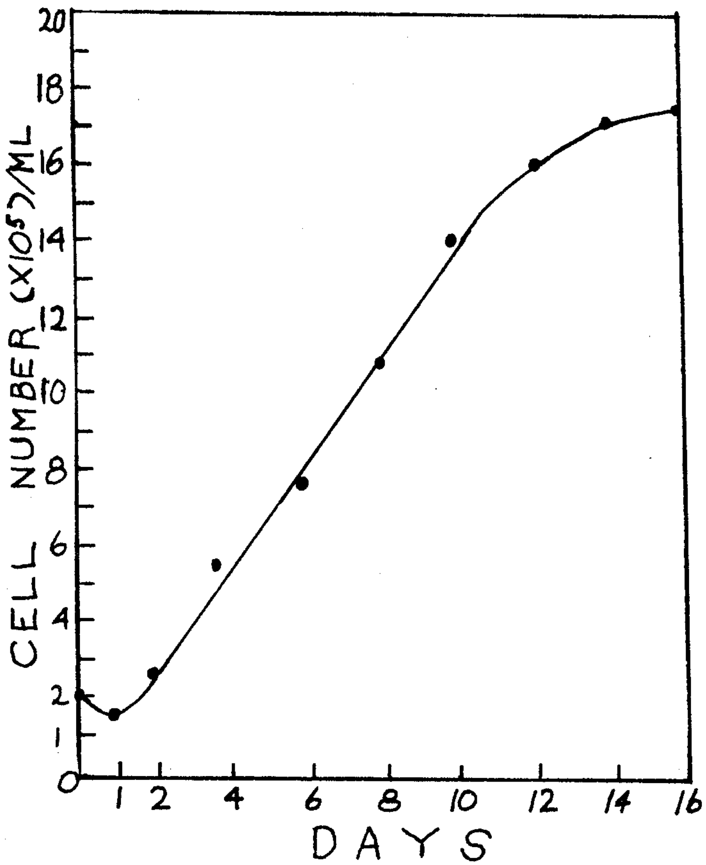


Fig. 1. Growth of HeLa cells in monodisperse suspensions cultured in normal growth medium (Table I(a)).

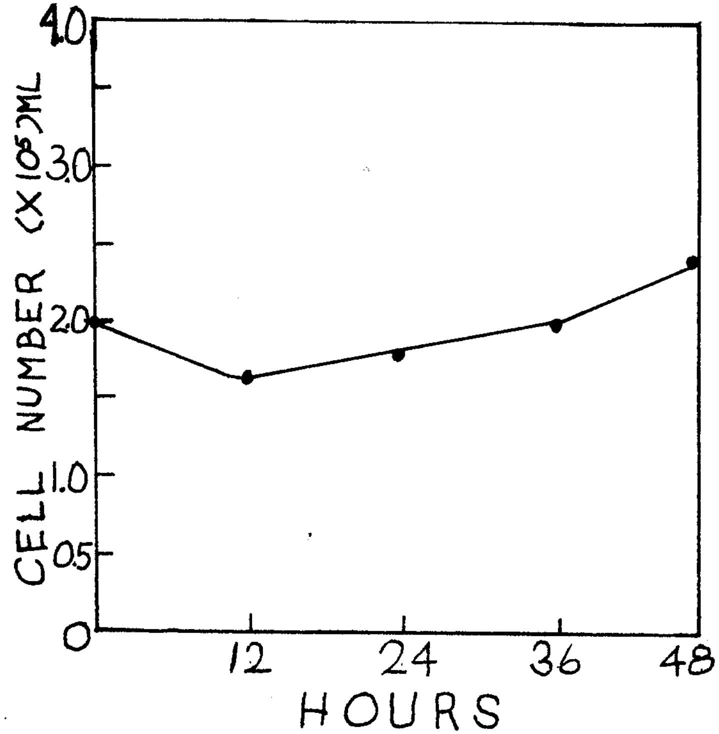


Fig. 1(a). Growth of HeLa cells in monodisperse suspensions during lag phase.

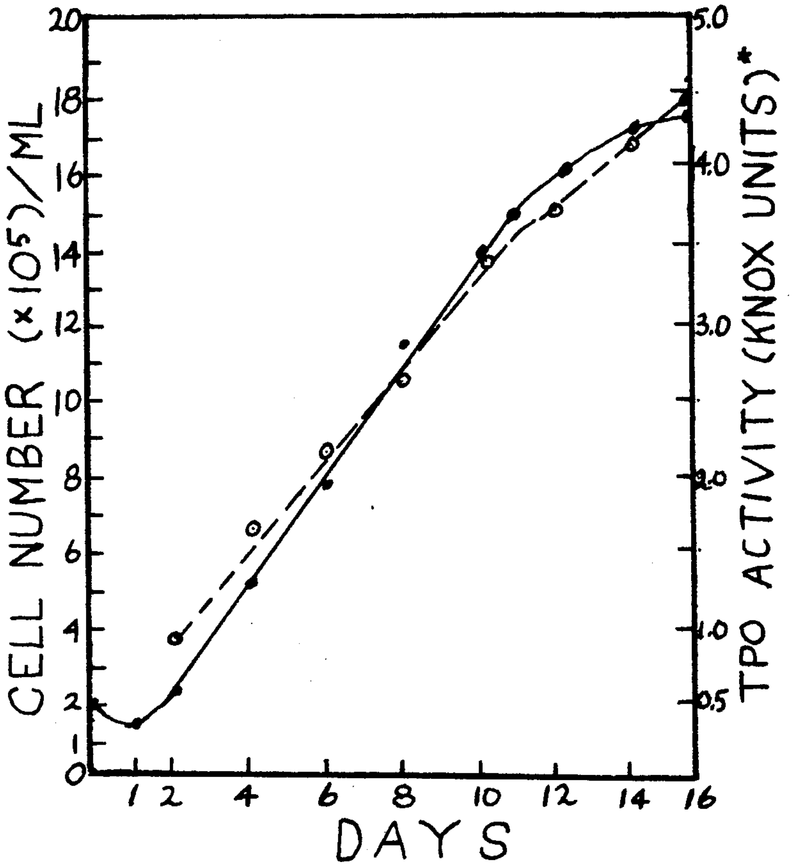
also levels off.

TABLE III

The Activity of TPO in "RNA" Cultures
with Relation to Culture Age

Age (days)	O. D. diff.	Knox Units*
0	0.0	0.0
1	0.0	0.0
2	0.044	0.97
4	0.074	1.62
6	0.097	2.14
8	0.119	2.62
10	0.155	3.41
12	0.169	3.74
14	0.195	4.29
16	0.21	4.61

* Knox Units - micromoles of L-kynurenine formed per gram dry weight of homogenate per hour.



●——● Growth curve of HeLa cells.

o- - -o Activity of TPO.

*Micromoles of kynurenine per gram dry weight of homogenate per hour.

Fig. 2. Increased activity of TPO concomitant with growth.

DISCUSSION

DISCUSSION

The success in the development of a method of culturing mammalian cells in agitated monodisperse suspensions offered cellular material that has provided relatively tractable for biochemical and physiological studies of cell behaviour. In particular, the homogeneity of the cell suspensions has proved rather suitable for a study of "induced enzyme synthesis".

The phrase, "the induction of TPO activity" in mammalian cells cultured in vitro must be interpreted with caution. Knox (1958) points out that activity assays are always a ratio of two factors:

(a) the measured activity and

(b) the amount of tissue used in the assay.

Since our experiments were carried out using the same weight of cellular material in each instance, specifically 0.45 gm wet weight, then the condition in (b) has been fulfilled and the interpretation of results is dependent on (a) only. Firm conclusions about enzyme concentration changes can be reached only if the changes observed are large in comparison with the mass of material in the tissue because extraction procedures are not always replicable with high accuracy.

The results that we have obtained are based upon the uniform amount of tissue used thus indicate a significant increase in enzyme activity under the inducing conditions. (Fig. 2). This activity increase is concomitant with growth of the cells and levels off when the population reaches the maximum stationary phase of growth. There is also a lack of any activity during the lag phase of growth.

Knox (1955) offers considerable evidence that the assay procedure is valid for experiments performed on rat livers. Thus the amount of L-kynurenine accumulated under his conditions and measured adequately indicated the amount of tryptophan oxidized in the TPO reaction. Deproteiniziation with metaphosphoric acid gives superior recoveries of kynurenine as compared with the former method using 5% zinc acetate and 0.18 N NaOH (Knox, 1950). The immediate oxidation product of the reaction, L-formylkynurenine, does not accumulate because a large excess of kynurenine formamidase is present in the preparation.

However, all such conclusions that the TPO system is inducible are drawn on the basis of indirect *in vivo* evidences that Knox (1955) outlines. Now that the system has been found to occur in mammalian cells cultured *in vitro*, the first indication of this work would seem to be the determination of actual enzyme synthesis, specifically, changes in actual enzyme concentration. If this proved to be negative, then the activities obtained would probably be due to activation of TPO protein already present in the HeLa cell.

The unusual aspect of the results obtained in this work is that the inducing substance, L-tryptophan, was not active alone but required the extra addition of RNA to the culture. This problem also arose in the work of Klein (1960, 1961). Curiously enough, the addition of RNA derived from yeast promoted the increase in enzyme activity in the presence of tryptophan. However, our results differed from those of Klein in which the addition of RNA alone gave minimal activity of arginase. In this work, no such activity was observed with TPO. The addition of animal RNA was not necessary. Klein suggests that there is a competition for available RNA precursors and amino acids between different enzyme-forming templates in a cell. The increased concentration of added RNA then facilitates the production of templates that are poor precursors. He bases this assumption on a theory proposed by Spiegelman _et al._ (1956).

This is the most interesting aspect of our results in that it promises further investigation. What is the effect of the added RNA? Incorporation of single bases into the medium, e.g. uracil, might prove to be the regulating factor. Dialyzed and hydrolyzed RNA preparations should offer interesting conclusions.

In animals, TPO activity is also increased by the administration of hormones, specifically, hydrocortisone. If this and other related substances were also studied by the techniques outlined here, some insight into the mechanism of "induced" TPO synthesis may be gained.

There appear to be advantages in the growth of tissue cultures in agitated monodisperse suspensions. If other cell systems can be adapted to this procedure, it would become possible to carry out important biochemical and physiological investigations with the advantage of reduced time and materials. It also appears that the cellular material thus obtained would provide for more accurate investigations because of the greater control the worker would have over the conditions of growth and the medium. The homogeneity of the cells in the suspensions is a distinct improvement in this control because the great majority of the population are now exposed to identical conditions affording a greater degree of accuracy in the interpretation of experimental results.

The obvious "stress" and "shock" imposed upon the cell by the proteolytic procedure of trypsinization has been eliminated. The ability to obtain successive cell cultures and to feed existing populations by the use of simple bacteriological techniques is very useful.

SUMMARY

(1) A method for the *in vitro* culture of mammalian cells in agitated monodisperse suspensions was developed.

(2) Activity of the enzyme system, tryptophan peroxidase-oxidase (TPO), was increased in monodisperse suspensions by the inducer, L-tryptophan and added RNA derived from yeast.

(3) The activity of TPO increased as culture age increased until the maximum stationary phase of the population growth had been achieved.

SUMMARY

REFERENCES

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Albano, A., (1959); The cultivation of HeLa cells in calf serum and the susceptibility of such cells to polio and adenoviruses. Acta. Pathol. et Microbiol. Scand., 45:307.

Beebe, S. P. & Ewing, J. (1906); A study of the biology of tumour cells. Brit. Med. J., ii, 1559.

Cherry, W. R. & Hull, R. N. (1956); Studies on the growth of mammalian cells in agitated fluid media. Proc. Tissue Culture Assoc. Meeting, Milwaukee, Wisc.

Cohn, M. (1957); Contributions of studies on the β-galactosidase of Escherichia coli to our understanding of enzyme synthesis. Bact. Rev., 21; 140.

Cox, R. P. & Pontecorvo, G. (1960); Induction of alkaline phosphatase by substrates in established cultures of cells from individual human donors. Proc. Nat. Acad. Sci., 47:839.

Cruickshank, C.N.D. & Lowbury, E.J.L. (1952); Effect of antibiotics on tissue cultures of human skin. Brit. Med. J., 2:1070.

Earle, W. R., Schilling, E. L., & Shelton, E. (1950); Production of malignancy in vitro. X. Continued description of cells at the glass interface of the cultures. J. Nat. Cancer Inst., 10:1067.

Earle, W. R., Bryant, J. C. & Schilling, E. L. (1954); Certain factors limiting the size of the tissue culture and the development of massive cultures. Ann. N.Y. Acad. of Sci., 58:1000.

Evans, V. J. & Earle, W. R. et al. (1951); The preparation and handling of replicate tissue cultures for quantitative studies, J. Nat. Cancer Inst., 11:907.

Harrison, R. (1907); Observations on the living developing nerve fibre. Proc. Soc. Exp. Biol., 4:140.

Jolly, J. (1903); Sur la duree de la vie et de la multiplication des cellules animals en dehas l'organisme. C. R. Soc. Biol. (Paris), 55:1266.

Kato, Y. & Moog, F. (1958); Difference in response of phosphatases in chick embryo to injection of substrate. Science, 127:812.

Keilova, H. (1948); The effect of streptomycin on tissue cultures. Experientia, 4:483.

Kelley, G. C., Adamson, D. J., & Vail, M. N. (1960); Further studies on the growth of human tissue cells in the absence of an added gas phase. Amer. J. Hyg., 72:275.

Klein, E. (1960); On the substrate-induced enzyme formation in animal cells cultured _in vitro_. Expt'l. Cell Res., 21:421.

Klein, E. (1961); Studies on the substrate-induced arginase synthesis in animal cell strains cultured _in vitro_. Expt'l. Cell Res., 22:226.

Knox, W. E. & Mehler, A. H. (1950); The conversion of tryptophan to kynurenine in liver: I. The coupled tryptophan peroxidase-oxidase system forming

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formylkynurenine. J. Biol. Chem., 187:419.

Knox, W. E. & Mehler, A. H. (1951); The adaptive increase of the tryptophan peroxidase-oxidase system of liver. Science, 113:237.

Knox, W. E. & Auerbach, V. H. (1955); The hormonal control of tryptophan peroxidase in the rat. J. Biol. Chem., 218:307.

Knox, W. E., Auerbach, V. H. & Lin, E.C.C. (1956); Enzymatic and metabolic adaptations in animals. Physiol. Rev., 36:164.

Knox, W. E. (1958); Adaptive enzymes in the regulation of animal metabolism, in Physiological Adaptation, Prosser, C. L. (ed.) Lord Baltimore Press, Baltimore, Md. p. 107.

McLimans, W. F., Davis, E. V., Glover, F. L. & Rake, G. W. (1957); The submerged culture of mammalian cells: The spinner culture. J. Immunol., 79:428.

Merchant, D. J., Kahn, R. H. & Murphy, W. H. (1960); in

Handbook of Cell and Organ Culture, Burgess Publishing Co., Minneapolis, Minn., p. 118.

Morgan, J. F., Morton, H. J. & Parker, R. C. (1950); Proc. Soc. Expt'l. Biol. Med., 73:1.

Puck, T. T. & Marcus, P. (1955); A rapid method for viable cell titration and clone production with HeLa cells in tissue culture. Proc. Nat. Acad. Sci., 41:432.

Roeder, M. (1957); The induction of arginase activity in the chick embryo. J. Cell. & Comp. Physiol., 50:241.

Sanford, K. K., Earle, W. R. & Likely, G. D. (1948); The growth in vitro of single isolated tissue cells. J. Nat. Cancer Inst., 9:229.

Spiegelman, S., Halvorson, H. O. & Ben-Ishai, R. (1955); Amino Acid Metabolism, McElroy, W. D. & Glass, B. (eds.), Johns Hopkins Press, Baltimore, Md., p. 124.

Stanier, R. Y. (1951); Enzymatic adaptation in bacteria. Ann. Rev. Microbiol., 5:35.

Syverton, J. G., Scherer, W. F. & Ellwood, P. N. (1954); Studies on the propogation *in vitro* of poliomyelitis. V. The application of strain HeLa human epithelial cells for isolation and typing. J. Lab. & Clin. Med., 43:286.

Vogel, H. J. (1959); Induction and repression of enzyme formation, in Handbuch der Pflanzenphysiologie, Ruhland, W. (ed.), 11:117.

White, P. R. (1946); Cultivation of animal tissues *in vitro* in nutrients of precisely known constitution. Growth, 10:231.