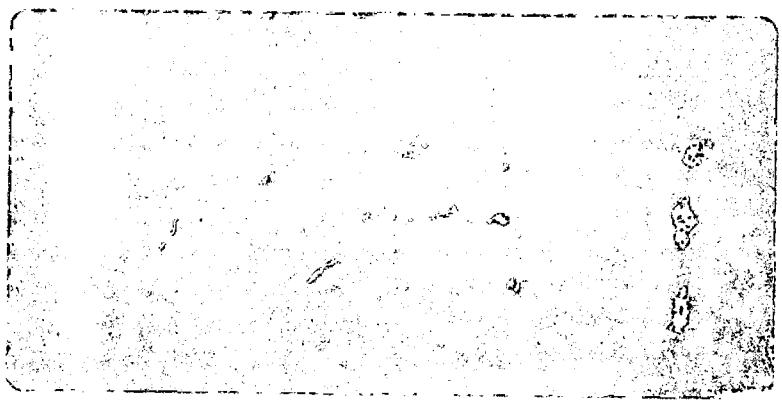
UCRL 2270

UNCLASSIFIED

UNIVERSITY OF CALIFORNIA

Radiation Laboratory



BERKELEY, CALIFORNIA

DISCLAIMER

This document was prepared as an account of work sponsored by the United States Government. While this document is believed to contain correct information, neither the United States Government nor any agency thereof, nor the Regents of the University of California, nor any of their employees, makes any warranty, express or implied, or assumes any legal responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by its trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof, or the Regents of the University of California. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof or the Regents of the University of California.

UCRL-2270
Unclassified-Health and Biology Distribution

UNCLASSIFIED

UNIVERSITY OF CALIFORNIA

Radiation Laboratory

Contract No. W-7405-eng-48

GROWTH-STIMULATING EFFECTS BY NUCLEOPROTEIN AND CELL FRACTIONS ON CHICK HEART FIBROBLASTS IN VITRO

Roman Joseph Kutsky
(Thesis)

July, 1953

Berkeley, California

TABLE OF CONTENTS

	Page No.
INTRODUCTION	3
ISOLATION PROCEDURES	8
Isolation of Cell Fractions	8
Solubilization Procedures with Mitochondria	10
Preparation of Nucleoprotein Fraction	11
Isolations of Nucleic Acid	13
ANALYTICAL METHODS AND RESULTS	15
TISSUE CULTURE TECHNIQUES	19
EFFECTS OF SUPPLEMENTATION WITH CELL FRACTIONS	21
SUPPLEMENTATION WITH NUCLEIC ACIDS AND NUCLEOPROTEIN FRACTIONS	25
DISCUSSION	34
ACKNOWLEDGMENTS	43
SUMMARY	44
BIBLIOGRAPHY	45

GROWTH-STIMULATING EFFECTS BY NUCLEOPROTEIN AND CELL FRACTIONS ON CHICK HEART FIBROBLASTS IN VITRO

Roman Joseph Kutsky

Radiation Laboratory and Department of Zoology,
University of California, Berkeley, California

July, 1953

INTRODUCTION

The development of animal tissue culture in vitro began with Harrison's demonstration (1907) that a nerve fiber could be kept alive and allowed to develop outside the animal body. His technique, however, did not permit indefinite cultivation of various tissues outside the body, mainly because of inadequate media for culturing. Carrel's discovery (1913) of growth factors in saline extracts from embryos, and to a lesser degree in extracts from adult tissues and tumors, was a notable advance. It enabled cultures of chick heart cells or their subcultures, i.e., fibroblasts, to multiply for an indefinite period when embryo extract was included in the medium. Although this discovery opened the possibility of probing into the nature of growth itself, it soon became obvious that an accurate characterization of growth would entail a chemical description of the various biological fluids used in the media, especially embryo extract.

The problem of establishing a well-defined growth-promoting medium was approached in two ways. On the one hand, Carrel, Baker and Ebeling (1925), Baker (1936), Fischer (1941), and recently White (1949) and Morgan, Morton and Parker (1950) attempted to prepare a synthetic growth-promoting medium from pure chemicals such as amino acids, vitamins, coenzymes, etc., but succeeded at best in providing survival media. On the other hand, using an analytical approach, Carrel (1924), Baker and Carrel (1926), and Fischer (1930), all employing similar methods, tried without much success to isolate the active principles from embryo extracts with the aid of various precipitants such as salts, alcohol, acetone, and carbon dioxide and adsorbents such as

charcoal and alumina. They found the active principles to be very labile. In 1913 Carrel had reported that the activity of embryo extract was lost by heating at 70° C for ten minutes and by sterile filtration. Indeed, Carrel and Ebeling (1923) found that embryo extract decreased markedly in potency on standing one week at 0° C, and Carrel (1924) found that the active factors were inactivated by radium and x-ray irradiation. In the early fractionations, Carrel (1924) noted that growth-promoting activity was associated with proteins in embryo extract, and Fischer (1930) found that defatting the embryo extract increased its activity. The problem of maintaining sterility during these fractionations, together with the extreme lability of the active substances to heat, salts, acid, alkali, and any other protein denaturant, made the isolation task doubly difficult and this research lagged.

Fischer's dialysis experiments (1941) following Baker and Carrel's work (1926) on ultrafiltration of embryo extract established the fact that embryo extract possesses active factors in the form of low-molecular-weight substances like amino acids, as well as large molecules such as proteins. Fischer's emphasis on the role of the dialyzable factors and his discovery of a possible synergism in culture between the two classes stimulated a number of investigators to branch into separate lines of research following the two types of factors. The factors of low molecular weight in embryo extract have been actively investigated by Fischer (1946) and his coworkers, by Sanford, Waltz, Shannon, Earle and Evans (1951), and by Harris (1952b).

The research to be described here deals with the nondialyzable constituents of high molecular weight in embryo extract. It should be noted that extracts from various adult tissues have been found by several workers (Carrel, 1913; Hoffman, Tennenbaum and Doljanski, 1939; Davidson and Waymouth, 1945) to have growth-stimulating properties <u>in vitro</u>, but of a much lesser magnitude and of different character from those of embryo extract. Since these have not been conclusively demonstrated to be identical to the embryonic factors they will not be further discussed here. Growth factors from various species of embryos seem

to have similar growth-promoting effects on tissue cultures (Carrel and Ebeling, 1923), although Davidson and Waymouth (1945) have inconclusive evidence that heterologous embryo extracts possess a lower growth-promoting activity than homologous embryo extracts. For purposes of discussion both types will be assumed to be similar in action.

Resuming earlier fractionation attempts, Fischer, associated with Astrup (1943), succeeded in isolating from saline extracts of beef embryos an active nucleoprotein fraction which they called "embryonin". Fischer and Astrup used Hammersten's technique (1920), which consists essentially of drying and defatting a tissue extract and precipitating the nucleoprotein in dilute hydrochloric acid. After reprecipitation this fraction was filtered through Berkefeld filters before being placed in culture, where it demonstrated more growth-promoting activity than any other fraction previously obtained. Heavy losses accompanied the procedure however, and when the fraction was analyzed in the electrophoresis apparatus, the experimenters obtained "several components of which four were predominant" (p. 47, Fischer, 1943). Activity of this fraction was decreased by heat, alkali, acid, and tryptic digestion but was little affected by standing at 0° C or by filtration. Davidson and Waymouth (1945) using a similar method isolated an active nucleoprotein fraction from sheep embryos, but did not analyze for contaminants by electrophoresis. Waymouth (1947) obtained an active preparation from chick embryos containing nucleoprotein by using a modified plasma protein method (Davidson and Waymouth, 1946). This procedure involves a simultaneous defatting and general protein precipitation of a saline tissue extract, using alcohol-ether at -15° C followed by drying <u>in vacuo</u>. The product obtained had "relatively few" electrophoretic components (p. 26, Waymouth, 1947). Unfortunately none of these electrophoretic components was tested for activity in tissue culture. Recently, Nishioka and Ibuka (1952) isolated from chick embryo extract a high-polymer pentose nucleic acid which Katsuta and Takaoka (1952) found to be active in culture and which they state is Fischer's "embryonin". Their method follows that of Kerr and Seraidarian

(1949), and consists of treating an acid precipitate from embryo extract with a cold concentrated salt solution, followed by deproteinization of the concentrated saline extract with chloroform according to a modification of Sevag's procedure (1938).

Concurrent with the resumption of chemical experiments, physical fractionations using the ultracentrifuge were carried out by various workers. Tennant, Liebow and Stern (1941) as well as Fischer and Astrup (1943) subjected chick embryo extracts to ultracentrifugation, and found that both pellet and supernatant stimulated growth of chick fibroblasts. Barski, Wielgosz and Lépine (1951) centrifuged saline extracts of chick embryos at 173,000 g for four hours and assayed the final supernatant in culture. The supernatant was less active than the original extract, although a considerable fraction of the activity was not sedimented under the conditions used. The pellet was not tested directly. Hoffman, Dingwall and Andrus (1952) obtained a pellet and supernatant from saline extracts of chick embryos by ultracentrifugation at 30,000 rpm for 1-3 hours in an air-driven centrifuge. Assays with chick fibroblasts indicated some activity in the sedimentable fraction although the major growth-promoting effect remained with the supernatant phase. Recently Wolken (1952) fractionated chick embryo extract by centrifuging at 33,450 rpm in a Spinco ultracentrifuge for periods up to 12 hours. Although this study was concerned in the main with chemical and physical characterization of the fractions, a few assays were made with cultures of chick heart. No significant differences in surface area at 48 hours were apparent between cultures supplemented with high speed pellet and with supernatant. No attempt was made in any of these investigations to identify and test in culture any specific components of the ultracentrifugal pellet, but Claude (1938) reported the presence of nucleic acid in similar pellets and Brachet and Jeener (1944) determined that 20-40 percent of the nucleic acid was sedimentable from embryo extract by ultracentrifugation.

After a consideration of the history of the problem, a general approach was formulated bearing in mind the fact that a tissue extract consists of a heterogeneous population of molecules and their aggregates. It seemed logical to adopt a systematic process of isolation

from chick embryo extract and testing in culture of various fractions, starting with the largest known units or aggregates and proceeding down the size scale. The best characterized and most widely accepted larger units consist of the cell particulates, namely the cell nuclei, mitochondria, and microsomes, and these seemed to offer the most promising point of departure for the initial investigation.

Separation of cell components by differential centrifugation was introduced by Bensley and Hoerr (1934) and by Claude (1938) using saline extracts. Subsequently, Hogeboom, Schneider and Pallade (1948) found that sucrose solutions gave better preservation of isolated intracellular components, and they developed methods for the separation of nuclei, mitochondria, microsomes (submicroscopic particles) and soluble material (final supernatant). The expanding volume of work on cell fractions has been reviewed recently by Schneider and Hogeboom (1951). For the present work it was planned to isolate cell fractions from chick embryo homogenates in hypertonic sucrose solutions using modifications of the methods originally developed by Hogeboom, Schneider and Pallade (1948) for liver cells.

Considering the soluble non-particulate fraction of chick embryo extract where, as will be shown, most of the activity resides, it became evident that new isolation procedures were desirable because of the heavy losses and complex products sustained in the previous methods. Trials were made with various methods and a successful one was found. A new and relatively mild isolation technique for liver nucleoproteins was developed by von Euler and Heller (1948) following the discovery by Cohen (1947) that the antibiotic streptomycin could be used as a specific precipitant for nucleoproteins and nucleic acids present in saline extracts of tissues. This method which was originally designed for small quantities of adult tissue extracts, was accompanied by large losses and did not maintain mild conditions throughout the procedure. Modifications were anticipated to adapt the method for embryonic protein isolations in large quantity.

When this phase of the work was almost completed, it became inportant to isolate and test chick embryo nucleic acids. Since the streptomycin method would separate nucleic acids as well as nucleoproteins,

it was desirable to obtain chick embryo nucleic acids to check the activity of the nucleic acid component in the nucleoprotein fraction. Moreover, Katsuta and Takaoka (1952) asserted that a nucleic acid was the active growth factor. For purposes of isolation of nucleic acids from the nucleoprotein fraction the method of Grinnan and Mosher (1951) was adopted in modified form, omitting the preliminary guanidine precipitation. The procedure consists of exposing the nucleoprotein fraction to a brief heat treatment followed by a chloroform deproteinization (Sevag, 1938). A method for isolation of high polymer nucleic acids directly from chick embryos was published during the course of this investigation by Nishioka and Ibuka (1952) and was adopted as a second method.

Finally it was planned to subject the active products obtained to electrophoretic analysis and separation of components, to biological characterization in tissue culture, and to chemical analysis for ribonucleic acid (RNA) and desoxyribonucleic acid (DNA).

ISOLATION PROCEDURES

Isolation of Cell Fractions

Nuclei

Fresh 12 to 13 day chick embryos were removed aseptically from their membranes, eyes and legs excised, and blotted briefly on sterile filter paper. Each embryo was then placed in a glass homogenizer tube, 12.0 ml sterile 0.88 M sucrose solution (pH 7) was added, and the mixture was ground with a motor-driven pestle. The homogenates were pooled and strained through a double thickness of gauze followed by a single thickness of NuFab (Essential Products, Inc., Chicago) in order to remove larger debris. The strained homogenate was centrifuged at 600 g for 10 minutes using an International Model SB Type 1 centrifuge. To retard breakdown of cell particulates the medium was prechilled and maintained at 8° C or less in this and subsequent operations. The initial pellet obtained contained cellular debris, red blood cells and nuclei. The latter could be demonstrated microscopically in smears

of this material by staining with pyronin-methyl green. The initial sediment was three times resuspended in the original volume of sucrose solution and centrifuged as before, and the nuclear fraction together with some erythrocytes became recognizable as a definite layer at the base of the centrifuge tubes. This layer was then removed for assay and just before being added to the culture medium was washed to remove the sucrose by suspending and centrifuging briefly in Gey's solution.

Mitochondria

The original nuclear supernatant was centrifuged twice at 600 g to remove residual nuclei and debris. Following preliminary clarification as described above the supernatant was centrifuged at 24,000 g for 20 minutes. In this and in the following isolations of cell fractions a Spinco Model "L" refrigerated preparative ultracentrifuge with angle head was used. The plastic centrifuge tubes required for high speed separations were sterilized by autoclaving with internal glass supports to prevent collapse. In operation each tube was sealed with a sterile metal cap. The yellow, opaque pellet obtained represented the mitochondrial fraction as judged by the characteristic color and appearance as described by Claude (1938) and by observations of smears stained with Janus green. The pellet was then resuspended in a volume of fresh sucrose solution equivalent to the original supernatant and centrifuged as before. The final pellet was removed for assay and just before adding to the culture medium was resuspended and centrifuged in Gey's solution to remove sucrose.

Microsomes

A fraction made up of submicroscopic particles (microsomes) was obtained by further processing of the supernatant from the mitochondrial separation. This supernatant was centrifuged for one hour at 105,400 g, resulting in the formation of a reddish, transparent pellet as described by Claude (1938). This pellet was resuspended in the same volume of sucrose solution and again sedimented in the ultracentrifuge. Sucrose was likewise removed from this fraction just prior to testing by suspension in Gey's solution followed by ultracentrifugation.

Final Supernatant

After separation of the various particulates the remaining fluid fraction (final supernatant) was preserved for assay of soluble factors not sedimented by the above procedures. This fraction was dialyzed against sterile Gey's solution to remove sucrose and other dialyzable materials. Dialysis was carried out on a Boerner shaker in the 8° C cold room using 3/4-inch dialysis tubing (Visking Corp., N. Y.) immersed in a two-liter Erlenmeyer flask filled with Gey's solution. The dialysis units were sterilized before use by autoclaving. Two changes of dialyzing fluid were made over a period of 24 hours. The dialyzed supernatant was removed by pipette and stored in rubber-stoppered tubes prior to use.

Solubilization Procedures with Mitochondria

The following procedures were used to obtain additional mitochondrial fractions for assay in culture.

Treatment with Distilled Water

Eight embryo aliquots of mitochondria were washed briefly in double distilled water and then resuspended in 12.0 ml distilled water for 24 hours at 0° C. The suspension was then centrifuged one hour at 105,400 g and the pellet discarded. The supernatant extract was made isotonic by appropriate addition of concentrated Gey's solution for assay in culture. As a variant of this procedure suspensions of mitochondria in distilled water were frozen and thawed one or more times before the final centrifugation.

Treatment with Gey's Solution

A second procedure involved resuspension of washed mitochondria (eight embryo equivalents) in 12.0 ml Gey's solution for 24 hours at 0° C. The suspension was then centrifuged one hour at 105,400 g and the pellet discarded. The supernatant extract was used subsequently for assay.

Extraction with 70 Percent Ethanol

Mitochondrial pellets containing eight embryo equivalents were washed in Gey's solution, resuspended in 25.0 ml of 70 percent ethanol

and placed in the refrigerator at 0° C for 24 hours. This suspension was centrifuged for one hour at 105,400 g and the supernatant was evaporated in vacuo over $CaCl_2$. The residue was reconstituted for assay purposes with Gey's solution

Preparation of Nucleoprotein Fraction

An outline of the fractionation procedure is presented in Fig. 1. Fresh 12-day chick embryos were removed from their membranes and cooled to 0° C. This temperature was maintained throughout the isolation procedure. The embryos were then homogenized in a large Potter homogenizer, stirred one hour with an equal volume of M/8 potassium borate, pH 7.1, and centrifuged 15 minutes at 24,000 g in a fluid-cooled Servall centrifuge. Re-extraction of the residue increased the final yield by about 30 percent. The supernatant was made M/100 in streptomycin sulfate (Lilly) using a M/4 stock solution, pH 7.5, and left undisturbed for two or more hours; the resulting crude nucleoprotein precipitate was centrifuged down at 24,000 g for 10 minutes. To break up the nucleoprotein-streptomycin complex the precipitate was gently homogenized with a M/3 potassium phosphate buffer, pH 7.2, 4 ml per embryo, yielding a turbid solution. This solution was dialyzed with stirring in 3/4-inch Visking tubing for 12 hours against borate, pH 7.1, 120 ml per embryo, to remove streptomycin, phosphate and materials of low molecular weight. The streptomycin precipitation was repeated in the dialyzed solution with resuspension of the precipitate in phosphate as above. Dialysis with rotation of the sac was continued for 12 hours against two changes of M/3 phosphate buffer, 32 ml per embryo, in order to break up any residual streptomycin-nucleoprotein complex. The dense dialyzed suspension was then ultracentrifuged one hour at 105,400 g in a Spinco refrigerated ultracentrifuge. The free lipids rose to the surface and were removed with a capillary pipette, leaving a clear, colorless solution containing soluble nucleoproteins above a pellet of cell particulates and insoluble materials. The solution was stir-dialyzed for 24 hours either against one change of distilled water, 120 ml per embryo, prior to lyophilization or against

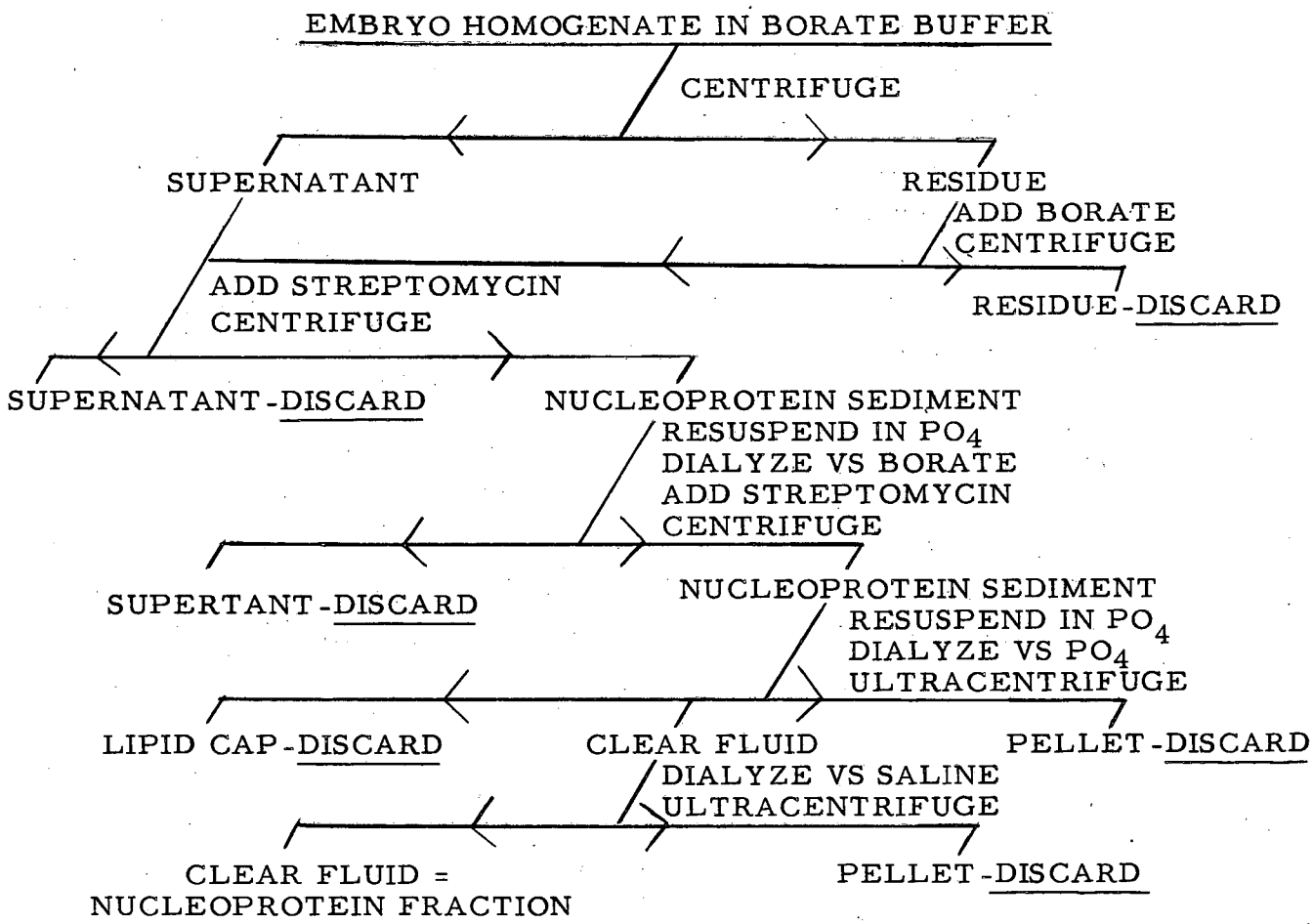


Fig. 1 Outline of Fractionation Procedure

the same volume of Gey's saline prior to culture operations. This solution, after ultracentrifugation again for an hour at 105,400 g, was clear, colorless and viscous and exhibited a typical nucleoprotein absorption spectrum in the ultraviolet, with maximum at 260 mμ and a minimum at 243 mμ (Fig. 2). Concentration of the nucleoprotein fraction in the clear solution was routinely determined by comparing optical densities at 260 mμ against a concentration curve based on dry weight measurements.

Isolations of Nucleic Acid

Nucleic Acid from Nucleoprotein Fraction

A preparation of the nucleoprotein fraction was suspended in a small volume of 1 M NaCl and shaken with an equal volume of 4:1 chloroform: n-butanol. The emulsion was centrifuged at 2000 g for 1/2 hour and the nucleoprotein pad at the chloroform-saline interface was saved. The nucleoprotein pad was suspended in a small volume of saline, brought to 100° C for two minutes, cooled and shaken with an equal volume of chloroform:n-butanol for one hour on a Boerner shaker. After being centrifuged 1/2 hour at 2000 g the aqueous phase containing nucleic acids and proteins was drawn off. The shaking and centrifuging was repeated on the aqueous phase. The aqueous phase was made to 20 percent ethanol and the precipitated proteins were centrifuged off at 2000 g for 1/2 hour. The supernatant was adjusted to 67 percent ethanol and the crude nucleic acid precipitate formed was centrifuged off and redissolved in a small amount of distilled water. Precipitations at 20 percent and 67 percent ethanol were repeated as before and the 67 percent precipitate (nucleic acid) was washed in alcohol and ether and dried *in vacuo*. An intermediate state of polymerization would be expected to result from this procedure. No protein tests were made on the final product. The product was reconstituted with Gey's solution to form a slightly viscous solution before being placed in culture. The ultraviolet spectrum (Fig. 2) was very similar to that of the high-polymer nucleic acid described below.

High-Polymer Nucleic Acid

The method of Nishioka and Ibuka (1952) requires that the temperature be maintained at 0° C throughout the procedure. Fresh 11-day

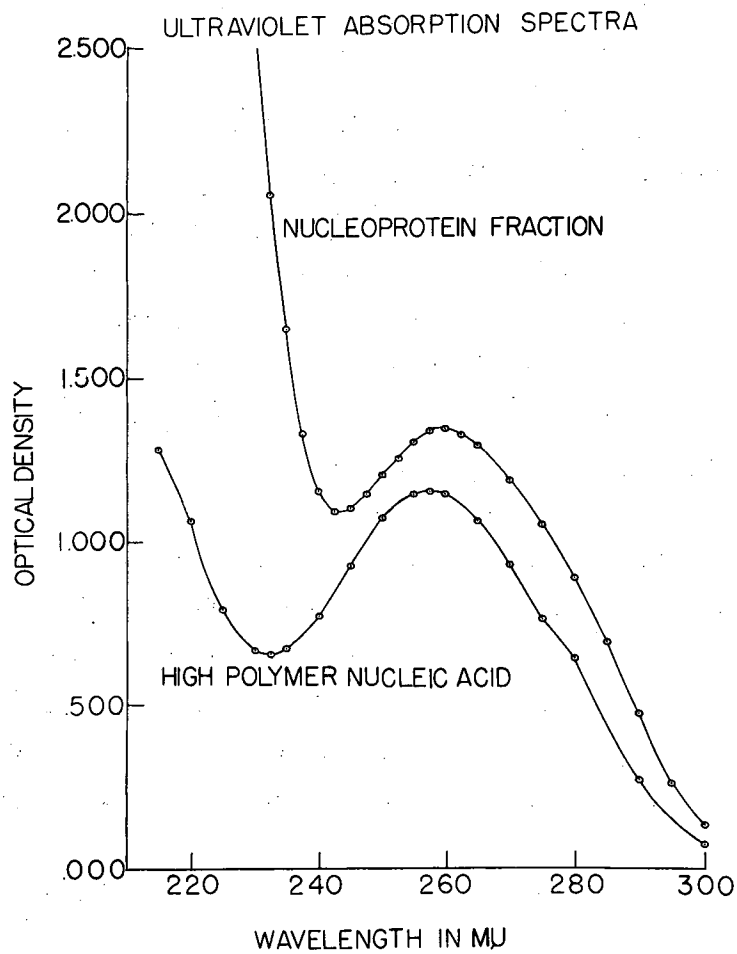


Fig. 2 Ultraviolet absorption spectra of nucleoprotein fraction and high polymer nucleic acid, both in Gey's saline (0.6 mg/cc) at pH 7.5.

chick embryos were homogenized and then extracted with 1-1/2 volumes of physiological saline solution. Nucleoproteins were precipitated from the centrifuged extract by lowering the pH to 4.2, sedimented at 3000 g for 1/2 hour, and resuspended in 1-1/2 volumes of saline at pH 7.0. The solution was made 3 M in NaCl by adding crystalline NaCl, and allowed to stand 48 hours. Some of the proteins were precipitated at pH 4.2 and centrifugally removed and 3 volumes of 95 percent ethanol were added to the supernatant. The nucleic acid-protein precipitate was centrifuged down at 3000 g for 1/2 hour and resuspended in physiological saline at pH 7.0. This suspension was shaken with 2-1/2:1 chloroform: amyl alcohol, the emulsion centrifuged, and the aqueous phase drawn off. This shaking process was repeated until the aqueous layer showed a negative biuret test (described below) for proteins. Three volumes of 95 percent alcohol were added to the final aqueous layer, the resulting nucleic acid precipitate was centrifuged off, washed in alcohol and ether, and dried *in vacuo*. This product was reconstituted either in Gey's solution prior to culturing or in distilled water prior to relative-viscosity determinations in an Ostwald viscosimeter. The viscosity found was very close to that given by Katsuta and Takaoka, namely 2.4. The ultraviolet absorption spectrum shown in Fig. 2 resembles that of a nucleic acid with a typical maximum at 258 mμ.

ANALYTICAL METHODS AND RESULTS

Electrophoretic Technique

Preliminary electrophoretic analysis were run by Dr. R. Trautman using the Klett Analytical Electrophoresis Apparatus at the Donner Laboratory of Medical Physics. This apparatus was equipped with a schlieren optical scanning apparatus and special compensating devices. An 11 cc cell, 3 mm by 25 mm cross section, with two sliding horizontal sections, was used in a water bath maintained at 1.8° C. Sterile technique was maintained throughout the electrophoretic separations. An exploratory run was followed by two preparatory runs as follows: The first run was made in 0.02 M phosphate buffer, pH 7.2, at a concentration of 0.4 gm/100 cc of nucleoprotein fraction, with current at 10

milliamperes and field strength at 10 volts/cm. The second run was made with the same buffer, 0.92 gm/100 cc of nucleoprotein fraction, current 13 milliamperes and field strength as before. After running 6 to 8 hours, the current was turned off, each cell section isolated by horizontal displacement relative to adjoining sections, and subfractions removed. These were drawn off with a capillary pipette at vertical distances along the cell lumen corresponding to the location of the peaks as measured in the optical patterns. Because of the small volume of some components it was advisable to remove these in combination with an adjoining fraction. The component nearest the parent material could not be separated, owing to admixture with the parent fraction. The ultraviolet absorption spectrum of each isolated subfraction was determined before testing in culture. In general, four components were found: two successive small and fast leading components were followed by a large component, followed by a small component. Ultraviolet spectra in the order mentioned resembled two nucleic acids with absorption maxima at the typical nucleic acid maximum at 260 mμ, next a nucleoprotein with a maximum at 260 mμ and a typical nucleoprotein curve similar to the parent nucleoprotein fraction, and lastly a component without an absorption maximum at 260 mμ.

Protein Tests

Qualitative tests for the presence of protein were made using the biuret test (Hawk, Oser, and Summerson, 1949) and the more sensitive Sakaguchi test (Sakaguchi, 1925). The biuret test was made by mixing one drop of solution to be tested with a drop of 40 percent NaOH-$CuSO_4$ reagent, a positive test for protein being the formation of a violet color. The Sakaguchi test was performed by adding one drop of 40 percent NaOH to 1/2 ml of test fraction, followed by one drop of 0.1 percent alpha naphthol in 70 percent alcohol and one drop of 5 percent sodium hypochlorite. A resulting red color indicated a positive test for protein. Both tests gave positive results on the nucleoprotein fraction, while the high-polymer nucleic acid isolated according to Nishioka and Ibuka (1952) gave a negative biuret but a positive Sakaguchi test.

Nucleic Acid Analyses

Quantitative analyses for nucleic acids in the nucleoprotein fraction were made as follows: the method of Schmidt and Thannhauser (1945) employing an incubation in 1N NaOH for 18 hours at 37° C was used to separate both RNA and DNA from the nucleoprotein fraction. To precipitate out the DNA plus the soluble proteins, 5 percent trichloroacetic acid (cold) was added and the precipitate was centrifuged down. The supernatant containing RNA was saved for analysis. The DNA in the precipitate was solubilized using Schneider's technique (1945), which employs a 5 percent trichloroacetic acid extraction at 90° C for 15 minutes. The protein remains insoluble and is centrifuged off. Analysis for RNA in the supernatant from the first extraction was made using a modification of Mejbaum (1939): one ml of a sample was mixed with 1-1/2 ml of 0.1 percent $FeCl_3$ in concentrated HCl and containing 0.3 percent orcinol. The mixture was heated in a boiling water bath 20 minutes, cooled and made up to 3 ml, and the absorption at 660 mμ was determined in a Beckman Spectrophotometer using appropriate reagent blanks. The RNA concentration was determined from a standard curve made with known quantities of RNA. Using Stumpf's method (1947), DNA in the second supernatant was determined as follows: a 1/2-ml sample was mixed with 0.05 ml of 5 percent cysteine hydrochloride followed by 5 ml of 70 percent H_2SO_4. After being mixed, then standing ten minutes, samples were read at 490 mμ using appropriate reagent blanks. The concentration of DNA was determined from a standard curve made with known quantities of chick erythrocyte DNA (kindly donated by Dr. Mazia). Results of these analyses are shown in Table I.

Test for Streptomycin

A test to determine streptomycin in the nucleoprotein fraction was based on the observation that streptomycin or a derivative of it is released from combination with the nucleoprotein fraction by incubating in 1N NaOH for 18 hours at 37° C. A similar assay method was later found to have been proposed by Waksman (1947). Streptomycin has an absorption maximum at 325 mμ which is enhanced some

Table I Nucleic Acid Determinations on Nucleoprotein Fraction

Analysis	Sample wt. mg.	Nucleic acid found, mg.*	Percent nucleic acid	Av. percent nucleic acid
PNA	10	1.05	10.5	
	10	1.06	10.6	10.3
	10	0.99	9.9	
DNA	100	0.33	0.33	0.33
	100	0.32	0.32	

* Each value represents the average of two aliquot determinations.

200 times by incubation with alkali. The alkaline incubation breaks down the streptomycin to form maltol (Waksman, 1947), which has an absorption maximum very close to 325 mμ. The nucleoprotein fraction alone has a very low, steadily decreasing absorption in this region. Thus by incubating a sample of nucleoprotein fraction with alkali and determining the absorption at 325 mμ, one can estimate concentrations of streptomycin from a standard curve made with known quantities of alkali-incubated streptomycin. Very little, if any, streptomycin could be detected in the final product of the nucleoprotein fraction; i.e., any amount present was at the lower limit of sensitivity of this method.

Test for Heme-Type Proteins

A test for contaminating proteins similar to heme proteins in the nucleoprotein fraction was based on the general presence of a reddish color and of an absorption maximum at 405 mμ in heme proteins. Crude nucleoprotein fraction was reddish in color and possessed an absorption maximum at 405 mμ. The final nucleoprotein fraction was colorless, and possessed no absorption maximum at 405 mμ, within limits of sensitivity of the method. Hence, there was probably very little, if any, heme protein present.

TISSUE CULTURE TECHNIQUES

The method of tissue culture described below is a modification of that developed by Carrel (1913). All cultures were made in Carrel flasks using fresh explants from 12 to 15-day chick embryo heart. The basal unsupplemented medium included a solid phase of undialyzed chicken plasma and a supernatant of dilute undialyzed serum (40 percent horse serum, 60 percent Gey's saline). The following were incorporated into the saline fraction so as to give the stated levels in the completed medium: 100 units/ml sodium penicillin G (Merck); 50 units/ml dihydrostreptomycin (Pfizer); and 0.002 percent phenol red as a pH indicator. The concentration of whole saline embryo extract (EE_{50}) in the medium was restricted to a minimum required for the initial clotting process (approximately 0.01 ml EE_{50} per flask).

Supplements to be tested were added in an appropriate volume of Gey's saline, which ordinarily consisted of one fourth of the saline fraction. Each supplemented series of six flasks was matched with a group cultivated in unsupplemented basal assay medium. In evaluating effects of specific cell fractions, concentrations were expressed in terms of corresponding amounts of starting material (embryo equivalents) added per series of six flasks. In the case of nucleoprotein fraction or nucleic acid preparations the relative concentrations were expressed in terms of mg/cc of the total volume of the medium. The cultures were incubated at 38° C and supernatants replaced biweekly without washing the plasma clot. Outgrowths were examined microscopically at 7 days, and areal tracings were made biweekly with a projectoscope. Areal increases between zero and ten days were determined with a planimeter and the results were treated statistically (Snedecor, 1946). The effectiveness of a particular fraction was judged by the areal increase of the culture at ten days, by the characteristic microscopic morphology of the cells in the culture, and in certain cases, by measurements of pH in the supernatant fluids at ten days. Cell fractions and the original nucleoprotein fractions were prepared using sterile technique throughout. When this sterile technique was omitted in the later nucleoprotein preparations, there was no sign of alteration in the final product and it may be emphasized that the temperature was maintained at 0° C throughout the entire isolation procedure. Antiobiotics were routinely incorporated into the media and the nucleoprotein fractions were routinely subjected to one hour's ultracentrifugation at 105, 400 g just prior to culturing so as to reduce contaminating microorganisms to a low level. As a result of these precautions, nucleoprotein fractions even though prepared under non-sterile conditions did not in general produce contaminations in tissue culture. Nucleic acid preparations did not require sterilization since they were dried with ether and the dry product was reconstituted with sterile Gey's solution.

EFFECTS OF SUPPLEMENTATION WITH CELL FRACTIONS

Controls

Outgrowths in unsupplemented media provided a basal level of reference for the various experimental series. In such series of controls, the increase in surface area (Table II) was minimal over the ten-day period of assay. Although occasional explants exhibited thin, widely spreading mesothelial sheets, the usual pattern was a characteristic compact outgrowth zone. The cells were massed closely, became granulated and refractile after a few days, and did not show radial orientation with respect to the explant.

Nuclei

Washed nuclear fractions were added to assay cultures at levels of 5, 7 and 15 embryo equivalents per series of six cultures. None of these concentrations produced significant changes in surface area (Table II), and the cell picture microscopically was identical with that of the control cultures. These results suggest that the nuclear fraction is inert but not inhibiting in culture.

Mitochondria

Washed mitochondrial fractions exhibited a clear-cut growth-stimulating effect when tested alone in assay cultures. This could be verified by measurements of surface area (Table II) as well as by correlated microscopic changes. Supplemented cultures showed a predictable difference from controls in cell picture and colony pattern. The cells in the outgrowth zone were visibly more numerous, did not mass closely together, and showed a more regular, radial alignment, particularly at the periphery of the outgrowth zone. After a few days the outgrowths became refractile and vacuolated but continued to expand. In general the outgrowths closely resembled those previously obtained with dialyzed whole embryo extract (Harris, 1952a).

The effects of supplementation with mitochondrial suspensions were most clearly evident at dosage levels corresponding to eight embryos per series, falling off below this point (Table II). High concentrations of mitochondria produced lesser increases in surface area with

Table II Effect of Cell Fractions from Chick Embryos on Areal Spread of Chick Heart Fibroblasts*

Cell fraction added	Concentration level, embryo equivalents†	Areal increase†† at 10 days, mm^2	Difference from unsupplemented paired controls	Significance of difference, P†††
None (controls)	--	13.2 ± 1.7	--	--
	--	16.0 ± 2.7	--	--
	--	16.0 ± 5.7	--	--
	--	10.8 ± 1.7	--	--
	--	18.0 ± 2.3	--	--
Nuclei	5	14.5 ± 1.0	-1.5 ± 2.9	>0.5
	7	16.7 ± 0.9	-0.5 ± 1.9	>0.5
	15	18.1 ± 1.8	0.9 ± 2.5	>0.5
Mitochondria	3	25.1 ± 1.6	14.3 ± 2.4	<0.001
	5	37.8 ± 3.7	21.8 ± 4.6	<0.001
	5	33.7 ± 5.3	22.9 ± 5.6	<0.01
	8	54.9 ± 0.9	44.1 ± 1.9	<0.001
	8	51.4 ± 1.3	35.4 ± 5.8	<0.001
	8	40.7 ± 1.0	22.7 ± 3.0	<0.001
	14	21.9 ± 0.6	8.7 ± 1.8	<0.01
Microsomes	4	55.7 ± 1.9	43.1 ± 7.5	<0.001
	8	56.0 ± 2.4	44.4 ± 7.7	< 0.001
	13	29.9 ± 2.3	16.7 ± 2.9	<0.001
Dialyzed final supernatant	0.5	55.7 ± 4.0	42.5 ± 4.4	<0.001
	0.3	59.6 ± 3.2	50.5 ± 2.9	<0.001

* Medium: Chicken plasma clot + 40 percent horse serum, 60 percent Gey's solution.

† Total amount added per series of six cultures

†† Mean value and standard error (Snedecor, 1946). Six cultures per series.

††† Fischer's t test (Snedecor, 1946).

the cells becoming increasingly refractile and heavily loaded with vacuoles. These inclusions may reflect the relative abundance of lipids in the cytoplasmic particulates of chick embryos as reported by Claude (1940).

Microsomes

The addition of washed suspensions of microsomes produced large increases in surface area (Table II) and cellular changes similar to those described for cultures supplemented with mitochondria. No significant differences were observed in comparisons of cultures supplemented with the two particulates respectively. As with mitochondrial suspensions, high levels of microsomes were inhibiting.

Soluble Cell Fraction (Dialyzed Final Supernatant)

The final supernatant retained a conspicuous level of activity after separation of the particulate fractions and dialysis to remove sucrose and other components of low molecular weight. Even relatively minor additions of this fraction produced substantial increases in surface area (Table II) and altered growth pattern. In general these changes were similar to those produced by suspensions of mitochondria or microsomes, but the outgrowths showed fewer inclusions, were less refractile, and the cells were more regular in arrangement. It is apparent that while suspensions of cytoplasmic particulates exhibit growth-promoting effects, the largest part of the activity of whole embryo extract resides in the soluble cell fraction.

Extracts of Mitochondria

Table III summarizes the results obtained from assays with the extracts of mitochondria. In general, changes in surface area were paralleled by alterations in cell picture as described for intact suspensions of mitochondria. It appears that the active principle in mitochondrial fractions can be partially solubilized by treatment with either distilled water or Gey's solution, although there was no clear indication that extraction was aided by freezing or thawing. Freezing and thawing twice seems to decrease the activity of the extract, possibly because of inactivation due to this treatment. Extraction with 70 percent ethanol did not solubilize active materials and this result is particularly interesting since 70 percent alcoholic extracts of entire embryos are rich in dialyzable growth factors (Harris, 1952b).

Table III Effect of Extracts Obtained from Washed Mitochondrial Suspensions on Areal Spread of Chick Heart Fibroblasts.*

Extraction medium	Additional treatment	Areal increase† at 10 days in cultures supplemented with final centrifuged extract†† mm^2	Difference from unsupplemented paired controls	Significance of difference P†††
Distilled Water	--	36.5 ± 4.9	18.5 ± 5.4	<0.01
	frozen and thawed once	49.4 ± 2.5	38.6 ± 3.0	<0.001
	frozen and thawed twice	30.0 ± 3.0	19.6 ± 3.2	<0.001
Gey's solution	--	33.3 ± 2.1	22.9 ± 2.2	<0.001
70 percent ethanol	Evaporated; reconstituted in Gey's solution	17.6 ± 2.8	-0.4 ± 3.6	>0.5

* Medium: Chicken plasma clot plus supernatant of 40 percent horse serum, 60 percent Gey's solution.

† Mean value and standard error (Snedecor, 1946). Six cultures per series.

†† Added at levels corresponding to eight embryo equivalent per series.

††† Fischer's t test (Snedecor, 1946).

SUPPLEMENTATION WITH NUCLEIC ACIDS AND NUCLEOPROTEIN FRACTIONS

Effects of Nucleoprotein Fraction

In Table IV may be found the relative increases in surface area at 10 days in cultures containing three concentrations of the nucleoprotein fraction as compared to outgrowth of similar cultures in the unsupplemented basal assay medium. The obvious increases in the cultures containing the nucleoprotein fraction are further documented by the photomicrographs shown in Figs. 3 and 4. Increases in surface area were also assoicated with characteristic alterations in cell morphology. In the unsupplemented control cultures the cells were heavily vacuolated and densely massed without conspicuous orientation (Fig. 5). In cultures containing the nucleoprotein fraction the outgrowth zone was much less granular, with the marginal cells broad and flat, tending toward a much looser, more radial arrangement (Fig. 6). It should be emphasized that the basal assay medium contained undialyzed chicken plasma and horse serum. Consequently it has not been possible to separate the relative role of nucleoprotein and dialyzable factors in the total effect obtained. Synergism between these factors has recently been demonstrated by Harris and Kutsky (1953) and will be described later in more detail.

Table IV also includes data on areal increases in cultures supplemented with the supernatant (thoroughly dialyzed against Gey's saline) from the first of the two streptomycin precipitations. The supernatant fraction, essentially a 50 percent borate extract minus nucleoprotein fraction, was incorporated at a level normally used to obtain large outgrowths with 50 percent saline embryo extract (EE_{50}), i.e., 15 percent of the total volume of the medium, replacing a like amount of Gey's saline. It is apparent that the residual activity in the supernatant is small in comparison to that of the streptomycin precipitate.

Nucleic Acids

Assays of the high polymer PNA prepared according to the method of Nishioka and Ibuka (1952) were uniformly negative (Table IV), as were similar experiments with nucleic acid isolated directly from the

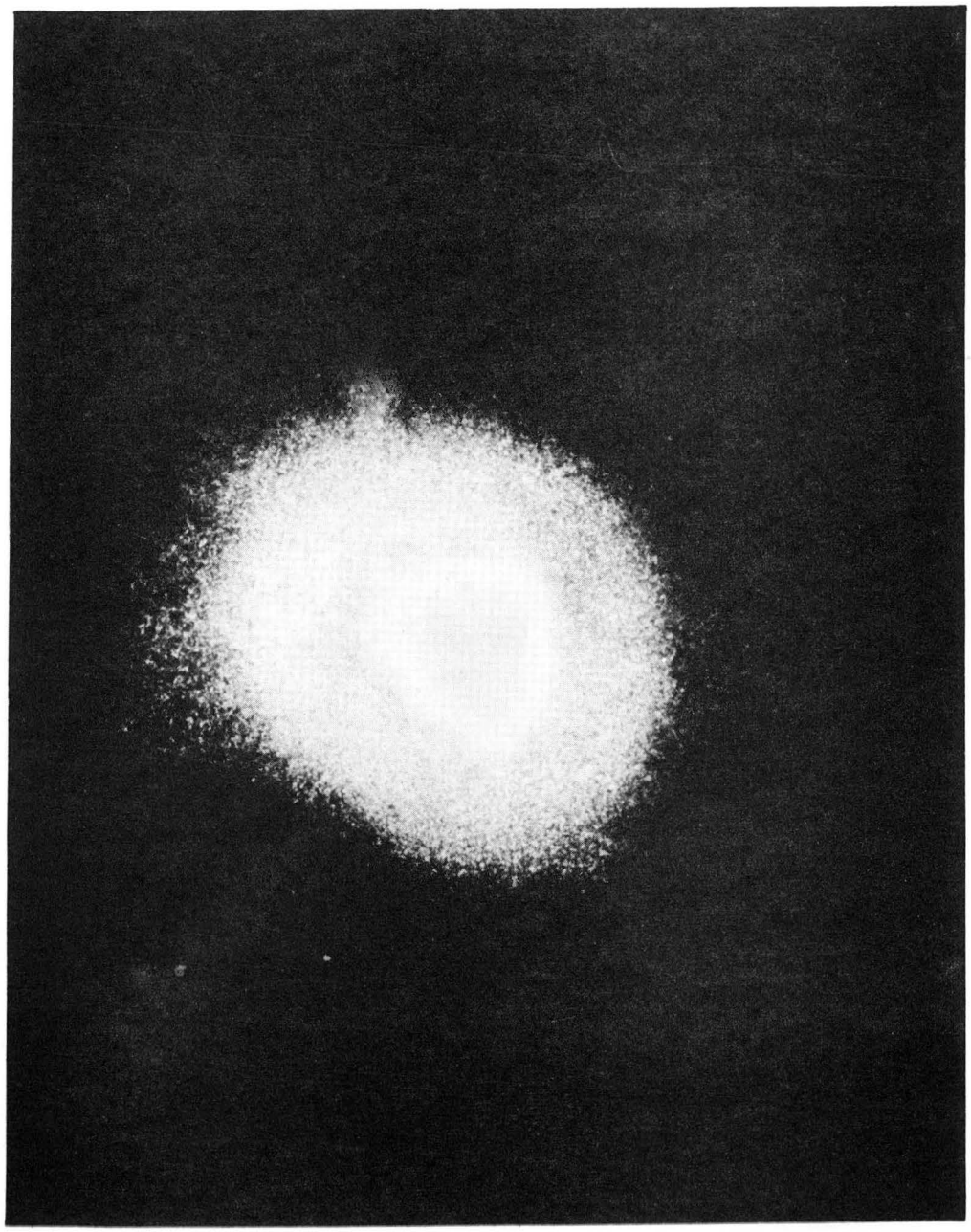
Table IV Outgrowth of Chick Heart Fibroblasts in Undialyzed Media Supplemented with Fractions from Chick Embryo Extract

Supplement to basal medium*	Areal increase 10 days mm^2** supplemented	Areal increase 10 days mm^2** controls	Difference from unsupplemented controls	Significance of difference, P
Nucleoprotein fraction				
0.2 mg/cc	34.7 ± 2.3	14.3 ± 1.4	20.4 ± 2.7	<0.001
0.4 mg/cc	53.4 ± 0.9	14.3 ± 1.4	39.1 ± 1.7	<0.001
0.7 mg/cc	65.0 ± 1.2	14.3 ± 1.4	50.7 ± 1.8	<0.001
Supernatant dialyzed*** 15 percent	22.8 ± 1.3	13.9 ± 1.3	8.9 ± 1.9	<0.01
High polymer nucleic acid 1 mg/cc	10.0 ± 0.6	13.8 ± 3.9	3.8 ± 4.0	>0.3
Nucleic acid from nucleo-protein 0.3 mg/cc	17.0 ± 3.0	19.9 ± 4.6	2.9 ± 5.5	> 0.5

* Basal Medium: Plasma clot, 40 percent horse serum, 60 percent Gey's solution.

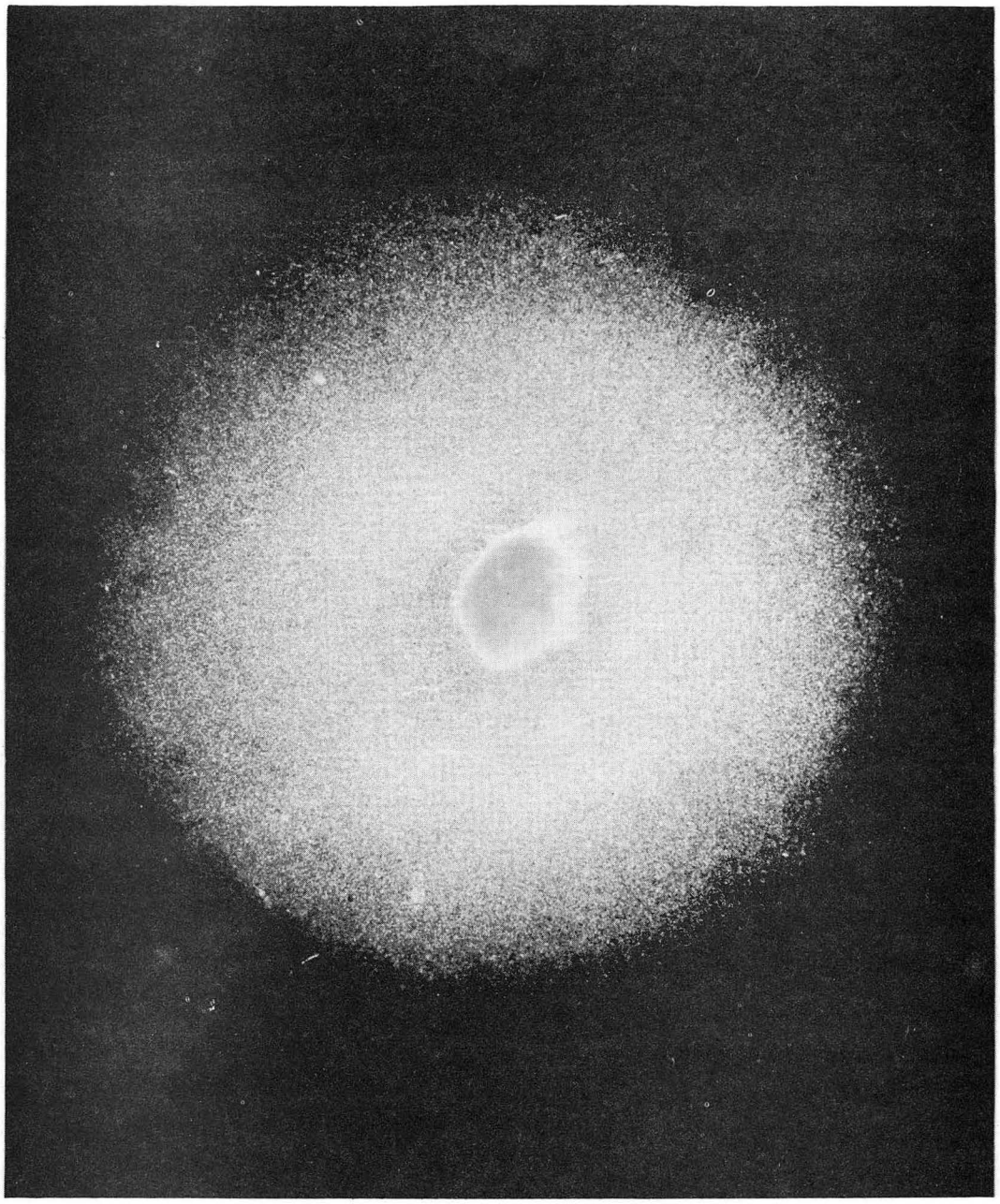
** Mean value and standard error (Snedecor, 1946). Six cultures per series except last supplement where three used.

*** From first streptomycin precipitation, replacing one fourth of the Gey's solution in culture.



ZN-657

Fig. 3 Outgrowth of chick heart fibroblasts after 10 days in unsupplemented basal assay medium. x 7-1/2.



ZN-656

Fig. 4 Outgrowth at 10 days in basal medium supplemented with nucleoprotein fraction (0.7 mg/cc) in Gey's saline. x 7-1/2.

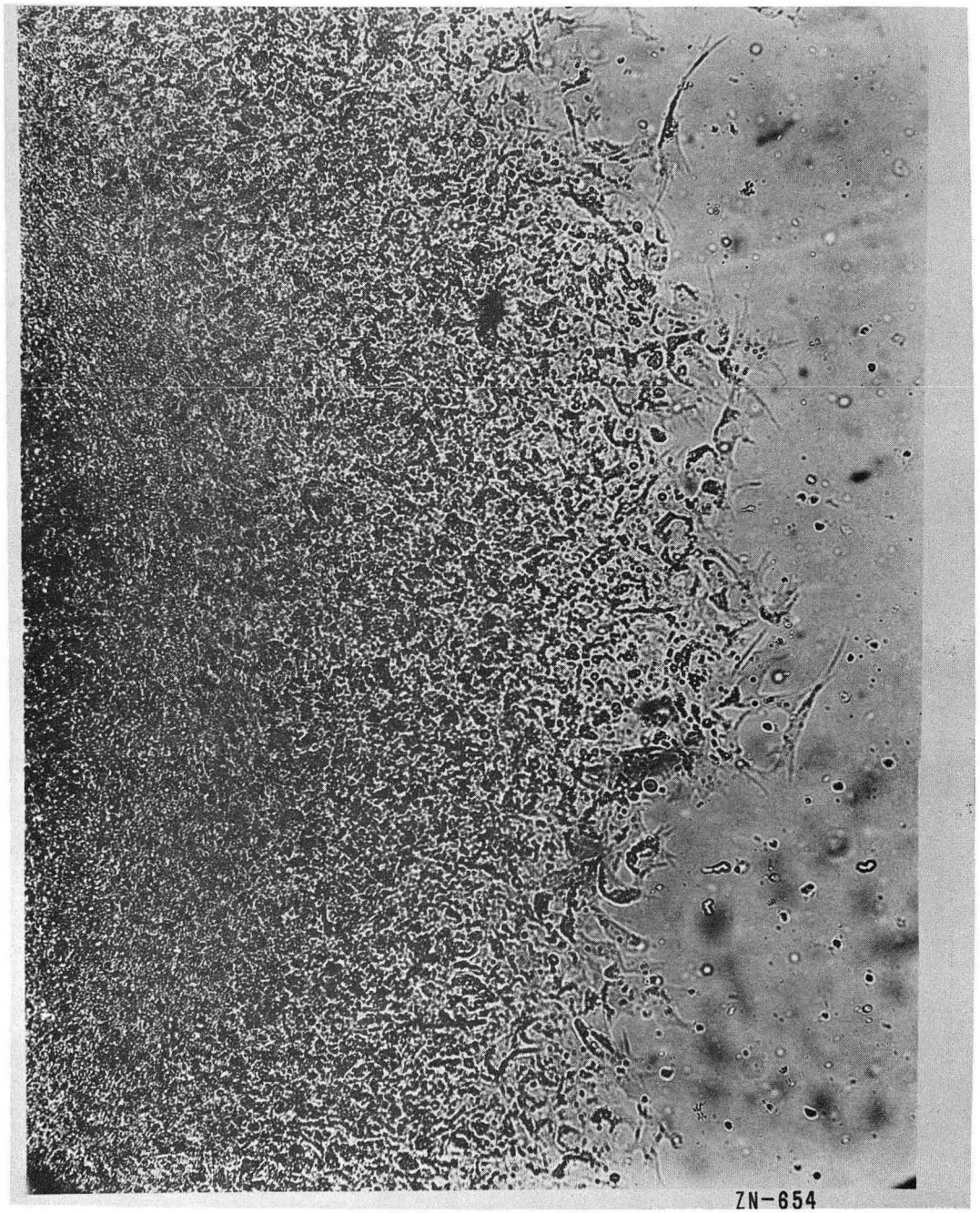


Fig. 5 Cellular detail at 10 days in culture of chick heart fibroblasts in unsupplemented basal medium. x122.

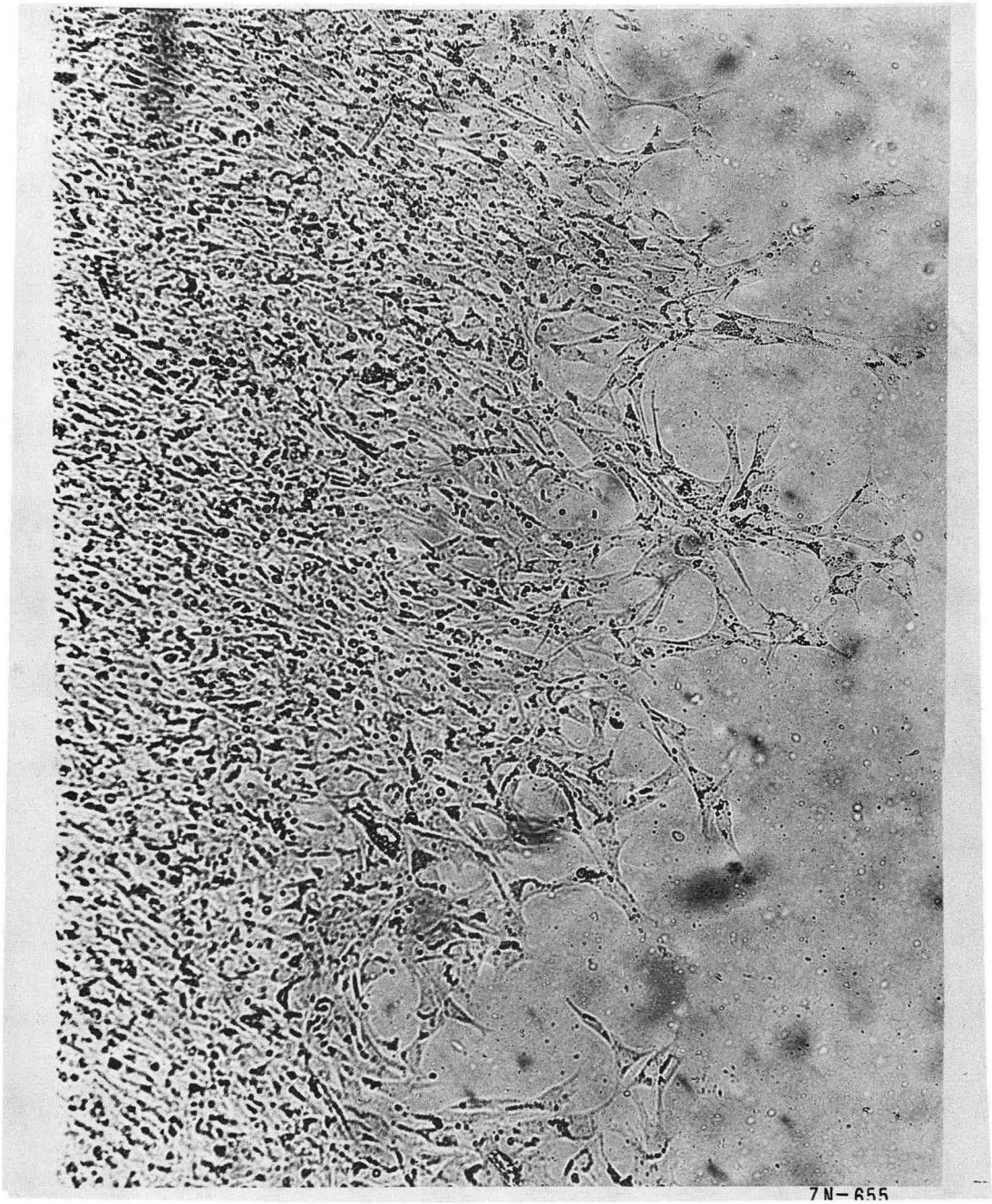


Fig. 6 Similar view of culture containing supplementary nucleoprotein fraction (0.7 mg/cc) in Gey's saline. x122.

nucleoprotein fraction as described above. Neither of these fractions produced increases in surface area or alterations in cell morphology similar to those seen with the nucleoprotein fraction.

Electrophoretic Fractions

Assays of the four subfractions obtained after electrophoresis of the nucleoprotein fraction are shown in Table V. Details of the technique were described in the previous section. Two separate experiments are summarized in the table. It will be noted that in the first experiment only one subfraction corresponding to the third ascending component was active. The third descending component was cultured at half the level used for the third ascending component, and its inactivity may be due to this fact. In the second experiment the data show that the starting material was twice as concentrated; three subfractions were active in culture, corresponding to the combined first two ascending components, to the third ascending component and to its analogue on the descending limb of the cell. A small amount of contaminating material from adjoining subfractions may be included in a particular subfraction during mechanical separation. It is therefore quite possible that the subfraction consisting of the first two ascending components may owe its activity to the presence of the third ascending component adjoining it, which is much more active. The appearance of the cells in culture containing the active subfractions was similar to that in the nucleoprotein-fraction-supplemented cultures described above. These data definitely suggest that one subfraction is active, but in view of the small amount of the other subfractions obtained, judgment should be reserved on their effect in culture. The concentrations of the minor components may have been below the threshold of activity in tissue culture. In any case, the active subfraction does account for more of the activity of the parent nucleoprotein fraction than any of the other subfractions.

Effect of Streptomycin in Culture

Streptomycin sulfate (Lilly) was added at three concentration levels in assay cultures, to check the possibility that combined streptomycin was the active factor in the nucleoprotein fraction. The data shown in

Table V Preliminary Tests of Electrophoretic Subfractions of Nucleoprotein Fraction in Tissue Culture

Electrophoresis Run No.	Supplement to basal medium*		Areal increase** at 10 days mm^2	Difference from unsupplemented controls	Significance of difference, P
First	None (controls)	--	19.2 ± 1.8	--	--
Preparative	Peaks 1 and 2 ascending	12 percent	11.7 ± 0.9	-7.5 ± 2.0	<0.01 (inhibitory)
Run	Peak 3 - ascending	22 percent	64.3 ± 9.5	44.1 ± 9.7	<0.01
Parent	Peak 3 - descending	11 percent	14.0 ± 0.9	-5.2 ± 2.0	<0.05 (inhibitory)
Material	Peak 4 - descending	12 percent	15.5 ± 1.6	-3.7 ± 2.4	>0.1
0.40 percent protein	Parent material	15 percent	70.8 ± 12.3	51.6 ± 12.4	<0.01
Second	None (controls)	--	15.5 ± 3.7	--	--
Preparative	Peaks 1 and 2 ascending	25 percent	34.9 ± 1.2	19.4 ± 3.9	<0.01
Run	Peak 3 - ascending	20 percent	84.4 ± 26.0	68.9 ± 26.2	<0.05
Parent	Peak 3 - descending	15 percent	74.3 ± 11.1	58.8 ± 11.7	<0.01
Material	Peak 4 - descending	22 percent	21.8 ± 3.8	6.3 ± 5.3	>0.2
0.92 percent protein	Parent material	15 percent	63.5 ± 6.1	48.0 ± 7.1	<0.001

* Basal Medium: Chicken plasma clot, 40 percent horse serum, 60 percent Gey's saline. Levels of fluid subfractions listed correspond to percent of total volume of medium.

** Mean value and standard error (Snedecor, 1946). Three cultures per series.

Table VI Effects on Areal Outgrowth of Chick Heart Fibroblasts of Various Concentrations of Streptomycin Sulfate

Supplement to basal medium*		Areal increase** at 10 days mm^2	Difference from unsupplemented controls	Significance of difference, P
None (controls)	--	12.8 ± 0.5	--	--
Streptomycin - SO_4	M/40	2.9 ± 0.2	-9.9 ± 0.53	<0.001 (inhibitory)
Streptomycin - SO_4	M/400	8.5 ± 0.3	-4.3 ± 0.58	<0.001 (inhibitory)
Streptomycin - SO_4	M/4000	11.0 ± 1.1	-1.8 ± 1.21	>0.1

* Basal Medium: Plasma clot, 40 percent horse serum, 60 percent Gey's solution. Concentration of streptomycin (Lilly) calculated for total volume of medium.

** Mean value and standard error (Snedecor, 1946). Six cultures per series except last supplement where three used.

Table VI indicate that no stimulation was obtained at streptomycin concentrations which might be present in the nucleoprotein fraction; indeed, higher concentrations were inhibitory. Appearance of the cells in culture was not improved over that found in the control cultures. Similar data have been reported by Fusillo, Metzger, and Kuhns (1952).

DISCUSSION

It seems desirable to preface discussion of the experimental results with a brief consideration of the basic techniques for assay of growth-promoting activity in tissue culture. Brachet (1950) states that growth is difficult to define but that it consists of the transformations of foods into protoplasm. Needham (1942) defines growth as an increase in spatial dimensions and in weight. Cunningham and Kirk (1942) define growth in tissue culture as an increase in the mass of the living substance of the culture. In any case, a general criterion of growth in tissue culture should take into account the following processes involved in tissue growth: (1) cell migration and outgrowth patterns, (2) cell division rate, (3) cell diminution and enlargement, (including syncitial development) (4) cell longevity and disintegration, (5) deposition of extracellular materials, (6) turnover and reorganization of intracellular materials (including visible cytoplasmic changes), (7) cell metabolism, and perhaps other unknown processes of equal importance. Since no method has yet been found that integrates all criteria into a single measurement of growth, one must seek a suitable combination of methods. This should measure simultaneously as many of these factors as possible, preferably without sacrificing the culture, should be simple and inexpensive and should not require an inordinate amount of time and effort, and should keep sampling, procedural and measuring errors at a minimum.

The principal methods that have been used by previous workers to measure growth in tissue culture, together with some major defects inherent in each method, are as follows. Five optical methods have been used, the first three requiring sacrifice of the culture.

(1) Measurement of mitotic index (Willmer and Kendal, 1932) has a large sample and procedural error owing to variability of mitoses throughout the culture, is tedious, and fails to consider growth processes other than cell division.

(2) Enumeration of cells in culture (Tompkins, 1942) is defective due to a variability of cell density throughout the cultures, is tedious, and ignores changes in cell size among other processes.

(3) An optical measurement of protoplasmic increase by determining cell number, cell size, and volume of the culture (Tompkins, Cunningham, and Kirk, 1947) suffers from the extreme variability in measurements of cell volume and irregular distribution of mitoses throughout the culture and is tedious.

(4) Enumeration of cell nuclei (Sanford et. al. 1951) is tedious and very expensive and fails to consider processes other than cell division.

(5) Measurement of areal increase (Ebeling, 1921) has the disadvantage of some variability of areas among cultures, gives more weight to cell migration than cell division and fails to consider metabolism and deposition.

Attempts to find a more fundamental measure of growth include five chemical and three physical methods respectively, of which the first three and the last two require a sacrifice of the cultures:

(1) Microanalyses of total nitrogen (Tompkins and Kirk, 1942) are tedious and interfered with by inert materials difficult to remove.

(2) DNA and RNA phosphorus analyses (Davidson, Leslie and Waymouth, 1949) are difficult and tedious; a direct relationship to growth needs to be proven.

(3) Total nucleoprotein phosphorus analyses (Willmer, 1942) have the same disadvantages as the preceding method.

(4) Measurements of metabolic factors such as oxygen uptake (Meier, 1931a), carbon dioxide formation (Laser, 1932),

or lactic acid formation (Demuth and Meier, 1929), are difficult to standardize and control adequately; in all cases a direct relationship to growth needs to be proven.

(5) Measurements of depletion of constituents in the medium such as phosphorus (Brues, Rathbun and Cohn, 1944) or sulfhydryl groups (Ephrussi, 1931) have the same disadvantages as the preceding method.

(6) Measurements of radioactive phosphorus uptake (Hull and Kirk, 1950) are subject to errors of adsorption and absorption by various surfaces and possible interference by turnover reactions not directly related to growth.

(7) Measurements of dry weight (Meier, 1931b) are subject to error from the presence of inert materials very difficult to remove from the cultures.

(8) Measurements of wet weight (Schaffer, 1950) have difficulties similar to the dry weight measurements plus the difficulty of rapid drying out of the cultures during weighing.

As can be seen, all the methods have inherent difficulties and sources of error. In addition, each measures a different set of the growth processes. It is therefore necessary to use several methods in conjunction to obtain sufficient information on a number of criteria. Davidson, Leslie, Smellie and Thomson (1950) demonstrated a constant content of DNA per nucleus in various tissues of chick embryos and Davidson and Leslie (1950) have therefore used DNA phosphorus as a basis of reference in tissue culture experiments. Basically, this method affords an excellent measure of cell division but it suffers from interference due to contaminating cell debris and inert materials in cultures, and from contaminating phosphorus compounds. If only the viable nuclei were isolated (Sanford et al, 1951) and the DNA analyzed via sufficiently sensitive methods for desoxyribose (Ceriotti, 1952) or thymine (Woodhouse, 1949), a much more accurate estimate might be obtained. If successful, it would still be desirable to obtain parallel measurements using an independent method to obtain information on other criteria such as cell size, migration and metabolism.

The method of areal measurements chosen for this work represents a combined measure of cell migration, cell division, and cell enlargement. Variability in surface area can be controlled by a thorough statistical treatment of data. With all its limitations, correlation of this method with growth has had no well-documented general disagreement, particularly if combined with other indexes. This has been coupled with an inspection of microscopic morphology, which furnishes information on cell enlargement, cell reorganization, growth patterns, and extracellular deposition. A low-power magnification inspection of the cultures furnished additional clues as to density of outgrowth and cell migration. In addition to this, pH measurements on the 10-day final supernatant fluids indicated the amount of acid production and therefore give indications as to cell metabolism.

Turning now to the results obtained in the tissue culture assays, work on the particulates suggests the following: The inertness of the nuclei in culture suggests that the various components of the nuclei are without growth-promoting activity. This would eliminate nuclear DNA and nuclear desoxyribonucleoprotein as active substances. Because of the large amount of surface involved in the mitochondrial and microsomal fractions it is possible that their activity may be dependent on active factors adsorbed from the supernatant. However, in view of the thorough washing of the particulates and the decrease in activity of both mitochondria and microsomes noted at the 14-embryo level (no such decrease was noted with high concentration of purified nucleoprotein fraction from the active supernatant), it seems more likely that actual components of the particulates are involved. The decrease may not necessarily be due to lipids, because when isolated by flotation in preliminary ultracentrifugal studies the lipids were inert but not inhibiting. Failure of 70 percent ethanol to extract active factors from the mitochondria indicates that substances of high molecular weight are probably responsible for mitochondrial activity since the active factors of low molecular weight as isolated by Harris (1952b) are soluble in 70 percent alcohol. Since evidence has been presented that the soluble active large molecule may be a ribonucleoprotein while RNA is inactive, and since both mitochondria and microsomes are rich in ribonucleoprotein, the most probable common active constituent is ribonucleoprotein. Indeed,

the possibility suggests itself that the mitochondria and microsomes may have other common properties because of their common nucleoprotein and lipid constituents and similar activity in tissue culture. Brachet and Jeener (1944) found that the particulates account for 20 to 40 percent of the embryonic ribonucleoprotein while the remaining 60 to 80 percent is found free as soluble ribonucleoprotein in the cytoplasmic fraction. The trends of activity in tissue culture and distribution of ribonucleoprotein in embryo extract seem to be parallel, suggesting that the free-ribonucleoprotein plays the primary role in immediate growth stimulation while the particulates may conceivably be the source of the nucleoprotein growth factor (Brachet 1950).

In the attempt to develop an appropriate isolation technique for this nucleoprotein factor, the original nucleoprotein isolation technique of von Euler and Heller (1948) proved unsuitable for chick embryo extract and was modified in the present experiments. Dialysis against distilled water to remove interfering salts, as specified in the original procedure, partially denatured the chick embryo proteins. Homogenization in and precipitation from borate, suggested by experiments of Gros and Rybak (1948), eliminated the initial dialysis and doubled the effectiveness of the first streptomycin precipitation. The original streptomycin sulfate concentration of M/800 resulted in only a partial precipitation of chick embryo nucleoproteins, but when increased 8-fold to M/100 gave a maximum yield. In general, owing to the extremely fine precipitates formed by embryonic nucleoproteins, much higher centrifugal speeds were necessary than in the original method. The small residue of activity remaining in the supernatant after the first streptomycin precipitation may be due to the gradual nature of the precipitation of the chick nucleoprotein in contrast to the liver nucleoprotein. On standing, this precipitate develops a coarse texture which sediments at lower speeds and fortunately occludes less heme protein than the initial fine precipitate. Development of the coarse texture made it unnecessary to wash the precipitate and eliminated the third and fourth reprecipitations of von Euler and Heller's method, which resulted in heavy losses. Trials with various salts revealed that substitution of M/3 potassium phosphate for M sodium chloride to break up the streptomycin-nucleoprotein complex and to dissolve the nucleoprotein fraction reduced the large amounts of solvent originally required.

Residual streptomycin associated with the nucleoprotein fraction could be split off by dialyzing against the phosphate, and was checked by determining the increase in optical density at 325 mμ as already described. Von Euler and Heller's procedure made no provision for removal of the free lipids or the microsomes, whereas they were here removed by ultracentrifugation. Lyophilization preserves most of the activity of the redissolved nucleoprotein fraction but the material is slow to dissolve, about 30 percent remaining insoluble. The material retains its biological activity in the dissolved state for three weeks when kept in the refrigerator. Streptomycin alone when tested in culture at various concentrations (as described above) showed no activity and hence the activity of the nucleoprotein fraction was not due to any residual streptomycin.

To recapitulate, the method of preparing a nucleoprotein fraction with streptomycin as outlined here offers the advantages of very mild conditions, a high efficiency, a simplified technique omitting the sterile precautions, a product more stable in purer form, and potency ten times as great as dialyzed embryo extract on a weight basis (i.e. resulting in equal areal outgrowth). This product has been found to react synergistically with the factors of low molecular weight (Harris and Kutsky, 1952) when tested in dialyzed media. Although the nucleoprotein fraction is still complex electrophoretically, the preliminary culture tests on the subfractions and other negative tests on RNA and DNA point to the fact that the major nucleoprotein subfraction and possibly the fourth minor component contain active factors. It is conceivable that the major nucleoprotein subfraction may not be one ribonucleoprotein but a family of closely related ribonucleoproteins which migrate similarly during electrophoresis, one or more of which may be active.

It would be interesting to compare electrophoretic results, potency, and activity levels in culture of the various preparations of the active nucleoprotein fraction made to date. The present nucleoprotein fraction with 10.3 percent RNA, 0.3 percent DNA, a tenfold milligram potency over dialyzed embryo extract, and active at levels of 0.4 to 1.0 mg/cc in culture compares well with the preparations of previous workers. Fischer's (1943) beef embryo nucleoprotein fraction

had 7.4 percent RNA, 5.3 percent - 8.1 percent DNA, a potency (nitrogen basis) of three times that of the parent extract, and a complex electrophoretic pattern with four major components, and was active at a level of 12 mg/cc in culture. Davidson and Waymouth's (1945) preparation from sheep embryos had 5.9 percent RNA, 0.7 percent DNA, and was active in culture at levels of 0.5 to 1.0 mg/cc, but with an unknown potency and number of electrophoretic components since no analysis for these was made. Waymouth's (1947) active preparation from chick embryos had RNA and DNA in unreported amounts. Activity levels in culture and potency were likewise unreported but the electrophoretic analysis revealed relatively few components. The RNA content of the present nucleoprotein preparation is seen to be higher than that of the others, and may be due to the presence of free RNA in residual quantities precipitated by the streptomycin along the with nucleoprotein. This residual RNA and the small quantity of DNA demonstrated by analysis could account for the two small leading peaks in the electrophoresis analysis.

The high polymer pentose nucleic acid isolated by Nishioka and Ibuka (1952) may have contained some undetected nucleoprotein, since they relied solely on the insensitive biuret test for proteins and did not use a more sensitive test such as the Sakaguchi reaction. Apparently it is not feasible to obtain preparations completely protein-free by their method since 21 chloroform extractions still left the preparation with a Sakaguchi-positive reaction. This contaminating nucleoprotein could account for the activity noted in their nucleic acid preparation. The nucleic acid preparation obtained from the active nucleoprotein fraction was probably not as highly polymerized as the above preparation (which was 2.4 relative viscosity units), because a very short heat treatment was used to split the nucleoproteins and the resulting preparation was only slightly viscous. Tennant, Stern and Liebow (1942) tested various nucleic acids in culture and asserted that certain nucleic acids were active at low concentrations. However, their culture periods were only 30 hours, whereas three to five days are usually necessary to observe a real effect. Moreover, their data were presented as complex functions of the areas and when these were recalculated in a simplified form, few if any significant differences were noted. Fischer (1946) and Waymouth (1947) both tried various nucleic acids in culture with negative

results. The present work on the nuclear fractions suggests that chick DNA is inactive and the tests on the electrophoretic fractions with nucleic acid spectra again suggest that nucleic acids are inactive. Preliminary to this research various samples of commercial RNA and DNA and high polymer DNA were tested and found inactive. The evidence therefore points to the fact that both types of nucleic acid even in high polymer form are inactive in culture.

If the active principle in embryo extract is a nucleoprotein, then on the basis of its greater stability in purer form (as found in this work) as compared to the known lability of embryo extract (Carrel and Ebeling, 1923) one might conclude that we are here dealing with a mutually stabilized system, sometimes found in complex proteins, of a labile nucleic acid in combination with a labile protein which is disrupted and inactivated by the action of the enzymes of the parent extract. Considering the action of this principle, Fischer (1946) thinks it to be catalytic at the cell surface; against this idea is the fact that large amounts are needed in culture (as confirmed in this work) and the fact that he could not demonstrate any enzymatic activity in embryonin. A more promising view is held by Needham (1931), who states that the growth-promoting factor is a right conjunction of nutrient materials and the capacities to use them. Continuing along this line, Davidson and Waymouth (1945) believe in a nutritive role for the nucleoprotein factor possibly to supply energy and materials for the nucleoprotein synthesis which they demonstrated to precede cell division. The large quantity requirement and the need for constant replenishment of the embryo extract (Tompkins, Cunningham and Kirk, 1947) in culture would be in harmony with this view. Brachet (1942), Ludford (1948), and Lansing and Rosenthal (1952) give evidence that ribonucleoprotein exists on cell surfaces, which Lansing and Rosenthal believe may regulate the calcium metabolism of the cell and control aging by this mechanism. However that may be, the results of the present research, indicating a quantity requirement, are not incompatible with the idea of a surface combination phenomenon, i.e., that each new unit of surface formed requires a certain amount of nucleoprotein in order to function. It might also be possible that this factor acts as a structural component of new cell surfaces forming in culture with special chemical properties due to the nucleic acid moiety

of the molecule (Neuberg and Roberts, 1949). It is known that the ribonucleoprotein of tobacco mosaic virus can travel into the interiors of cells (Bawden, 1950); perhaps this active nucleoprotein can likewise be transported into the cell interior there to become incorporated into new cellular structures. In this connection it is interesting to note that the mitochondria and microsomes shown here to be active in culture are known to contain ribonucleoprotein (Schneider and Hogeboom, 1951).

ACKNOWLEDGMENTS

I am greatly indebted to Dr. Morgan Harris for helpful advice as well as for the provision of tissue culture and special facilities used in this research. Without his material aid and valuable consultation, this research would have been impossible. I am also indebted to Dr. Daniel Mazia for consultation, use of the Spinco "L" ultracentrifuge, lyophil apparatus and samples of high polymer DNA, to Drs. Hardin Jones and Rodes Trautman of the Donner Laboratory of Medical Physics for the electrophoretic separations, and to Miss Carolyn Brode, Mr. Lawrence Resseguie and Mr. Donald Farquhar for technical assistance in tissue culture operations. A generous gift of streptomycin sulfate was made by the Eli Lilly Company. The photomicrographs were taken by Dr. Morgan Harris and Mr. Victor Duran.

I wish to gratefully acknowledge the generous support of this research by the Oak Ridge Institute of Nuclear Studies which awarded me an A. E. C. Predoctoral Fellowship in the Biological Sciences for 1951-1953. This research was also supported by grants to Dr. Morgan Harris from the Cancer Research Coordinating Committee, University of California.

SUMMARY

1. Chick embryo extract has been fractionated chemically and physically and the resulting products tested for growth promoting activity in tissue culture, using an appropriate statistical treatment of data. Cell particulates (cell nuclei, mitochondria, and microsomes) have been isolated by differential centrifugation in hypertonic sucrose solutions. When tested in tissue culture, the nuclei were inert but not inhibiting in effect, and the mitochondria and microsomes both gave a small increase in outgrowth (decreasing at higher concentrations); but the soluble, particulate-free extract (final supernatant) gave a maximal outgrowth response. Some activity could be extracted from the mitochondria by distilled water and Gey's solution but not by 70 percent ethanol.

2. A nucleoprotein fraction precipitated from chick embryo extract by streptomycin and freed of particulates by ultracentrifugation was found to possess most of the nondialyzable growth-promoting activity of the embryo extract in tissue culture. Analysis of the fraction disclosed 10.3 percent RNA and 0.3 percent DNA, protein, and a typical nucleoprotein ultraviolet absorption spectrum with a maximum at 260 mμ and a minimum at 243 mμ.

3. Preliminary subfractionation of the active nucleoprotein fraction in the electrophoresis apparatus resulted in four components. Three small components were inactive in culture. The major component had an ultraviolet absorption spectrum similar to that of the parent nucleoprotein fraction and was active in culture.

4. Nucleic acids were isolated from chick embryos using two different chemical procedures. A nucleic acid split from the active nucleoprotein fraction and a high polymer pentose nucleic acid in chick embryo extract were both isolated. Neither proved to be active in tissue culture.

5. Methods of measuring growth in tissue culture and possible modes of action of the active nucleoprotein fraction are discussed.

BIBLIOGRAPHY

1. L. E. Baker, 1936. Artificial media for cultivation of fibroblasts, epithelial cells, and monocytes. Science 83, 605-606.

2. L. E. Baker and A. Carrel, 1926. Action on fibroblasts of the protein fraction of embryonic tissue extract. J. Exp. Med. 44, 387-395.

3. G. Barski, L. Maurin, G. Wielgosz, and P. Lépine, 1951. Conditions de nutrition cellulaire in vitro en culture sans support plasmatique. Rôle des fractions micro- et macromoléculaires. Ann. Inst. Pasteur 81, 9-33.

4. F. C. Bawden, 1950. Plant viruses and virus diseases, 3rd ed., Waltham, Chronica Botanica Co., 335 pp.

5. R. R. Bensley and N. L. Hoerr, 1934. Studies on cell structure by the freezing drying method. VI The preparation and properties of mitochondria. Anat. Rec. 60, 449-455.

6. J. Brachet, 1942. La localisation des acides pentosenucléiques dans les tissus animaux et les oeufs d'amphibiens en voie de dévelloppement. Arch. Biol. 53, 207-257.

7. J. Brachet and R. Jeener, 1944. Recherches sur des particules cytoplasmiques de dimensions macromoléculaires riches en acide pentosenucléique. Enzymologia 11, 196-212.

8. J. Brachet, 1950. Chemical embryology. New York, Interscience Publishers, 533 pp.

9. A. M. Brues, E. N. Rathbun, and W. E. Cohn, 1944. Metabolism of tissue cultures. II Estimation of total growth in tissue culture: Growth in incomplete media as a steady state. Jour. Cell. and Comp. Physiol. 24, 155-158.

10. A. Carrel, 1913. Artificial activation of the growth in vitro of connective tissue. J. Exp. Med. 17, 14-19.

11. A. Carrel, 1924. The method of tissue culture and its bearing on pathological problems. Brit. Med. Jour. 2, 140-145.

12. A. Carrel and A. H. Ebeling, 1923. Action on fibroblasts of extracts of homologous and heterologous tissues. J. Exp. Med. 38, 499-511.

13. A. Carrel, L. E. Baker, and A. H. Ebeling, 1925. The effect of certain pure chemical substances on the multiplication of sacromatous rat fibroblasts. Arch. für Zellf. 5, 125-127.

14. G. Ceriotti, 1952. A microchemical determination of desoxyribonucleic acid. J.B.C. 198, 297-303.

15. S. S. Cohen, 1947. Streptomycin and desoxyribonuclease in the study of variations in the properties of the bacterial virus. J.B.C. 168, 511-526.

16. A. Claude, 1938. A fraction from normal chick embryo similar to the tumor producing fraction of Chicken Tumor I. Proc. Soc. Exp. Biol. Med. 39, 398-403.

17. A. Claude, 1940. Particulate componenets of normal and tumor cells. Science 91, 77-78.

18. B. Cunningham and P. L. Kirk, 1942. Measure of growth in tissue culture. Jour. Cell. and Comp. Physiol. 20, 343-358.

19. J. N. Davidson and C. Waymouth, 1945. The nucleoprotein content of fibroblasts growing in vitro. II The effect of tissue extracts. Biochem. Jour. 39, 188-199.

20. J. N. Davidson and C. Waymouth, 1946. The nucleoprotein content of fibroblasts growing in vitro. III The use of defatted media. Biochem. Jour. 40, 568-575.

21. J. N. Davidson, I. Leslie, and C. Waymouth, 1949. The nucleoprotein content of fibroblasts growing in vitro. IV Changes in the ribonucleic acid phosphorus and desoxyribonucleic acid content. Biochem. Jour. 44, 5-17.

22. J. N. Davidson and I. Leslie, 1950. A new approach in the biochemistry of growth and development. Nature 165, 49-53.

23. J. N. Davidson, I. Leslie, R. M. S. Smellie, and R. Y. Thomson, 1950. Chemical changes in the developing chick embryo related to the desoxyribonucleic acid content of the nucleus. Biochem. Jour. 46, proc. xl.

24. F. Demuth and R. Meier, 1929. Milchsäurebildung in Gewebekulturen. Biochem. Zeit. 212, 399-418.

25. A. H. Ebeling, 1921. Measurement of the growth of tissues in vitro. J. Exp. Med. 34, 231-243.

26. B. Ephrussi, 1931. Sur les facteurs limitant l'accroissement des cultures tissus in vitro. Signification de l'energie residuelle. Compt. Rend. de l'Acad. des Sci. 192, 1763-1765.

27. H. von Euler and L. Heller, 1948. Schonende Darstellung der zytoplasmatischen Nukleoprotein: mittels Streptomycin und anderen Fällungsmitteln. I Darstellung aus Leber. Arkiv. Kemi, Mineral., Geol. 26A (No. 14), 1-12.

28. A. Fisher, 1930. Gewebezüchtung, 3rd ed. Munich, Müller and Steinicke, 661 pp.

29. A. Fischer, 1941. Die Bedeutung der Aminosäuren für die Gewebezellen in vitro. Acta Physiol. Scand. 2, 143-188.

30. A. Fischer, 1946. Biology of tissues cells. New York, Hafner Publishing Co., 348 pp.

31. A. Fischer and T. Astrup, 1943. Untersuchungen über die chemischen Eigenschaften des tierischen Wuchsstoffes aus Embryonen. Pflüger's Arch. für ges. Physiol., 247, 34-52.

32. M. H. Fusillo, J. F. Metzger and D. N. Kuhns, 1952. Effect of chloromycetin and streptomycin on embryonic tissue growth in tissue culture. Proc. Soc. Exp. Biol. Med. 79, 376-377.

33. E. L. Grinnan and W. A. Mosher, 1951. Highly polymerized ribonucleic acid. Preparation from liver and depolymerization. J.B.C. 191, 719-726.

34. F. Gros and B. Rybak, 1948. Action de la pénicilline et de la streptomycine sur le catabolisme de l'acide ribonucléique. Helvetica Chim. Acta 31, 1855-1863.

35. E. Hammersten, 1920. Eine "gekoppelte" Nucleinsäure aus Pancreas. I Mitteilung. Hoppe-Selyer's Zeitschr. für Physiol. Chem. 109, 141-165.

36. M. H. Harris, 1952a. The use of dialyzed media for studies in cell nutrition. Jour. Cell. and Comp. Physiol. 40, 279-302.

37. M. H. Harris, 1952b. Growth factors in alcoholic extracts of chick embryos. Growth 16, 215-230.

38. M. H. Harris and R. J. Kutsky, 1953. Interaction of growth factors in chick embryo extract. (Abst.) Anat. Rec. 115, 452.

39. R. G. Harrison, 1907. Observations on the living developing nerve fiber. Proc. Soc. Exp. Biol. Med. 4, 140-143.

40. P. B. Hawk, B. L. Oser, and W. H. Summerson, 1949. Practical physiological chemistry. 12th ed. Philadelphia, The Blakiston Co., 1323 pp.

41. R. S. Hoffman, E. Tennenbaum, and L. Doljanski, 1940. The growth activating effect of extracts of adult tissue on fibroblast colonies in vitro. Growth 4, 207-221.

42. R. S. Hoffman, J. A. Dingwall, and W. deW. Andrus, 1952. Growth effects on chick fibroblast cultures of fractions of adult and embryonic tissue extracts following differential ultracentrifugation. Science 113, 268-269.

43. G. H. Hogeboom, W. C. Schneider, and G. E. Pallade, 1948. Cytochemical studies of mammalian tissues. I Isolation of intact mitochondria from rat liver; Some biochemical properties of mitochondria and submicroscopic particulate material. J. B. C. 172, 619-636.

44. G. H. Hogeboom, 1951. Separation and properties of cell components. Fed. Proc. 10, 640-645.

45. W. C. Hueper, A. Allen, M. Russell, G. Woodward, and M. Platt, 1933. Studies of growth factors of embryo extract. Am. Jour. Cancer. 17, 74-105.

46. W. Hull and P. L. Kirk, 1950. Tissue culture studies. IV Growth of tissue in vitro as related to the direct measurement of P^{32} uptake. Jour. Gen. Physiol. 33, 343-348.

47. H. Katsuta and T. Takoaka, 1952. Pentose nucleic acid (PNA) as a growth promoting substance for tissue culture. Jap. J. Exp. Med. 22, 173-180.

48. S. E. Kerr and K. Seraidarian, 1949. Studies on ribonucleic acid. I Preparation from the pancreas. J. B. C. 180, 1203-1208.

49. A. I. Lansing and T. B. Rosenthal, 1952. The relation between ribonucleic acid and ionic transport across the cell surface. Jour. Cell. and Comp. Physiol. 40, 337-345.

50. H. Laser, 1932. Eine Methode zur manometrischen Messung des Stoffwechsels von Gewebekulturen während des Wachstums. Bioch. Zeit. 251, 1-9.

51. R. J. Ludford, J. Smiles and F. V. Welch, 1948. The study of living malignant cells by phase contrast and ultraviolet microscopy. J. Roy. Micros. Soc. 68, 1-9.

52. W. Mejbaum, 1939. Über die Bestimmung kleiner Pentosemengen, insbesondere in Derivaten der Adenylsäure. Hoppe-Selyer's Zeitschr. für Physiol. Chem. 258, 117-120.

53. R. Meier, 1931a. Zur methodik der Stoffwechseluntersuchungen an Gewebekulturen. I Atmungmessung an Gewebekulturen, Biochem. Zeit. 231, 247-252.

54. R. Meier, 1931b. Zur methodik der Stoffwechseluntersuchungen an Gewebekulturen. II Gewichtsbestimmung an einzelnen Gewebekulturen. Biochem. Zeit. 231, 253-259.

55. J. F. Morgan, H. J. Morton, and R. C. Parker, 1950. Nutrition of animal cells in culture. I Initial studies on a synthetic medium. Proc. Soc. Exp. Biol. Med. 73, 1-8.

56. J. Needham, 1931. Chemical embryology. Cambridge, University Press, 3 vols.

57. J. Needham, 1942. Biochemistry and morphogenesis. Cambridge, University Press, 785 pp.

58. C. Neuberg and I. S. Roberts, 1949. Remarkable properties of nucleic acids and nucleotides. Arch. Bioch. 20, 185-210.

59. K. Nishioka and K. Ibuka, 1952. Extraction of high polymer pentose-nucleic acid and its interaction with protein. Jap. J. Exp. Med. 22, 163-171.

60. S. Sakaguchi, 1925. Über eine neue Farbenreaktion von Proteine und Arginin. J. Biochem. (Japan) 5, 25-31.

61. K. K. Sanford, W. R. Earle, V. J. Evans, H. K. Waltz, and J. E. Shannon, 1951. The measurement of proliferation in tissue cultures by enumeration of cell nuclei. J. Nat. Canc. Inst. 11, 773-795.

62. K. K. Sanford, H. K. Waltz, J. E. Shannon, W. R. Earle, and V. J. Evans, 1952. The effects of ultrafiltrates and residues of horse serum and chick embryo extract on proliferation of cells in vitro. J. Nat. Canc. Inst. 13, 121-137.

63. F. L. Schaffer, 1950. Growth of chick embryo heart cultures in vitro as related to changes in mass. Ph.D. Thesis, University of California, Berkeley, 94 pp.

64. G. Schmidt and S. J. Thannhauser, 1945. A method for the determination of desoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissues. J.B.C. 161, 83-89.

65. W. C. Schneider, 1945. Phosphorus compounds in animal tissues. I Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. J.B.C. 161, 293-303.

66. W. C. Schneider and G. H. Hogeboom, 1951. Cytochemical studies of mammalian tissues. The isolation of cell components by differential centrifugation. A review. Cancer Res. 11, 1-22.

67. M. G. Sevag, D. B. Lackman, and J. Smolens, 1938. The isolation of the components of streptococcal nucleoproteins in serologically active form. J.B.C. 124, 425-436.

68. G. W. Snedecor, 1946. Statistical methods applied to experiments in agriculture and biology. Ames, The Collegiate Press, Inc., 485 pp.

69. P. K. Stumpf, 1947. A colorimetric method for the determination of desoxyribonucleic acid. J.B.C. 169, 367-371.

70. R. A. Tennant, A. A. Liebow, and K. G. Stern, 1941. Effect of macromolecular material from chick embryos on the growth rate of mouse heart fibroblast cultures. Proc. Soc. Exp. Biol. Med. 46, 18-21.

71. R. A. Tennant, K. G. Stern, and A. A. Liebow, 1942. The stimulating action of nucleic acids upon mouse heart fibroblasts. Canc. Res. 2, 218-222.

72 E. R. Tompkins, 1942. Factors affecting growth in tissue culture. Ph.D. Thesis, University of California, Berkeley, 108 pp.

73. E. R. Tompkins and P. L. Kirk, 1942. Quantitative drop analysis. XVI An improved diffusion method for total nitrogen. J.B.C. 142, 477-485.

74. E. R. Tompkins, B. Cunningham, and P. L. Kirk, 1947. Mitosis, cell size and growth in culture of embryonic chick heart. Jour. Cell. and Comp. Physiol. 30, 1-14.

75. S. A. Waksman, 1947. Microbial antagonisms and antibiotic substances, 2nd ed. New York, Commonwealth Fund, 415 pp.

76. C. Waymouth, 1947. Proteins with growth promoting action on tissue cells in vitro. Proc. Soc. Exp. Biol. Med. 64, 25-26.

77. P. R. White, 1949. Prolonged survival of excised animal tissue in vitro in nutrients of known constitution. Jour. Cell. and Comp. Physiol. 34, 221-241.

78. E. N. Willmer and L. P. Kendal, 1932. The utilization of proteoses by chicken heart fibroblasts growing in vitro. J. Exp. Biol. 9, 149-179.

79. E. N. Willmer, 1942. Carbohydrate metabolism of chick fibroblasts in vitro. J. Exp. Biol. 18, 237-256.

80. J. J. Wolken, 1952. Fractionation of embryo extract by ultracentrifugation. I Analysis of fractions. Jour. Cell. and Comp. Physiol. 40, 243-253.

81. D. P. Woodhouse, 1949. A method for the colorimetric estimation of thymine. Biochem. Jour. 44, 185-187.