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TISSUE-CULTURE GROWTH STIMULATION BY NUCLEIC ACID -AND LIPID-RICH SUBFRACTIONS OF THE EMBRYONIC NUCLEO-PROTEIN FRACTION

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TISSUE-CULTURE GROWTH STIMULATION
BY NUCLEIC ACID- AND LIPID-RICH
SUBFRACTIONS OF THE EMBRYONIC
NUCLEOPROTEIN FRACTION

BERKELEY, CALIFORNIA

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ABSTRACT

The nucleoprotein fraction (NPF) from chick embryos that stimulates growth in tissue culture has been fractionated by ultracentrifugation and electroconvection. Various subfractions were obtained that were rich in nucleic acids or rich in lipids. When tested in tissue culture the various subfractions exhibited essentially specific activities the same as or lower than the parent NPF. These data indicate that the presence in the subfractions of nucleic acid and lipid has no correlation with growth-stimulating activity, and consequently these substances are probably inert. The data indicate that the major constituent of NPF --namely, protein--is probably the seat of biological activity of the NPF.

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INTRODUCTION

The growth-stimulating activity in tissue culture of embryo extracts was originally reported by Carrel in 1913.¹ Fischer first described and non-dialyzable, high-molecular-weight characteristics of some of the growth stimulating constituents of embryonic extract.² A method using streptomycin has been reported by Kutsky for isolating from embryonic extract these high-molecular-weight growth substances as a nucleoprotein fraction (NPF).³ The NPF was biologically active but was found to be heterogeneous in composition, and contains lipids and nucleic acids.^{3,4} The effects of its constituent nucleic acid- and lipid-rich subfractions had not been fully evaluated in tissue culture. The nucleic acids isolated chemically from the NPF had been tested and found inert in tissue culture.³ In view of the instability of the NPF,⁵ the rigorous chemical isolation treatment including the use of organic solvents may well have inactivated any biologically active nucleic acid molecules. So far as the lipid constituents are concerned, no previous tests have been made on them in tissue culture.

In view of the instability and small yields of the NPF it was necessary to consider efficient fractionation methods that were amenable to maintenance of sterile conditions and proper control of ionic environment. The method of density separation in the ultracentrifuge, which has been successfully used for isolation of serum lipoproteins,⁶ seemed well suited for simultaneous concentration of lipid-containing constituents in the top subfractions and denser nucleic-acid-containing constituents in the bottom subfractions.

Previous studies had already shown that electrophoretic fractions high in nucleic acid could be separated in very small quantities from the NPF by an applied electrical field.⁵ Electroconvection can be considered a type of continuous semiquantitative electrophoresis,⁷ and it therefore offers a more efficient means of concentrating electrophoretically migrating subfractions such as those containing nucleic acids.

This paper investigates the effects on biological activity in tissue culture of lipid-enriched and nucleic-acid-enriched fractions of the NPF obtained through ultracentrifugation and electroconvection.

MATERIALS AND METHODS

The NPF was isolated from borate extracts of 12-day chick embryos as described by Kutsky.³ Briefly, $M/100$ streptomycin sulfate is used to precipitate the nucleoproteins, which are suspended and dialyzed in 1 M NaCl, and clarified at 105,000 g for 2 hours in the ultracentrifuge. The lipid-rich fraction, which underwent flotation, was removed and discarded as usual. The clear NPF at this stage was used for further ultracentrifugal fractionation studies. For the electroconvection studies, the NPF was subjected to an additional dialysis against 0.1 M phosphate buffer of the desired pH followed by clarification as above. Following the separations by either method the sub-fractions were dialyzed against Gey's solution and clarified in the ultracentrifuge as usual prior to testing in culture.

Density separations were made at 0°C, using angle-head rotors in a Spinco preparative ultracentrifuge. The following experiments were performed:

(a) In the initial experiments the NPF was centrifuged in 1 M NaCl (density at 20°C, 1.038) for 14 hours at 79,000 g, but later runs were made in media of higher density.

(b) The NPF was centrifuged 17.5 hours at 79,000 g in a medium (density at 20°C, 1.115) achieved by substituting 1 M NaCl-D₂O for the 1 M NaCl used for the resuspension of the last streptomycin precipitate.

(c) The NPF was centrifuged 26 hours at 114,000 g in a medium (density at 20°C, 1.215) achieved by adding an equal volume of 6.1 M NaNO₃-D₂O to the NPF in 1 M NaCl. Prior tests in tissue culture disclosed no deleterious effects of the resultant salt concentration upon the biological activity of the NPF.

Electroconvection studies were made in the apparatus described by Raymond,⁸ using double-channel operation. The runs were made for 24-hour periods at 3°C in 0.1 M phosphate buffer at pH values of 5.6, 6.4, 7.2, and 8.0. The field strength used was similar to that in previous electrophoretic studies;⁵ it was 3 volts/cm, and the current was 0.5 amp, both being constant for all experiments. Only two fractions were taken: the top reservoir and the rest of the tube.

The tissue-culture assay method is described below. For further details see Kutsky and Harris.⁹ Fresh explants of 10- to 14-day chick-embryo heart were cultivated in D-3.5 Carrel flasks at 37°C for an 8-day period. The basal assay medium consisted of a chicken plasma clot in which a single explant (1 mm³) was embedded, with a fluid supernatant consisting of 40% horse serum and 60% Gey's solution, or 20% horse serum and 80% Gey's solution in the electroconvection experiments. Each test fraction was assayed in a set of six Carrel flasks, and each series of tests included a background control set of six flasks. Supernatant fluids were changed at 4 days, and cultures were traced and terminated at 8 days. Areal increases at 8 days were determined and statistics calculated for each set of six cultures, including the controls.

Dry weights were obtained (samples were dried overnight in a vacuum oven at 102°C), and were corrected for salt background. These values were used as a basis for determination of concentrations in tissue culture as well as the percentage compositions by dry weight. Ultraviolet spectra were

obtained with a recording spectrophotometer, and total nucleic acids were estimated by comparison of ultraviolet absorption at 260μ with standards of Schwartz RNA. DNA accounts for less than one-tenth of the total nucleic acid of the 12-day chick-embryo NPF.³

The method of silicic acid chromatography and infrared analysis¹⁰ was used to determine the lipid content. The total amount of lipid was small, and the content of fatty acids and triglycerides was found to be relatively low compared with the cholesteryl esters and phospholipids. The lipids were therefore estimated and reported as cholesteryl esters and phospholipids.

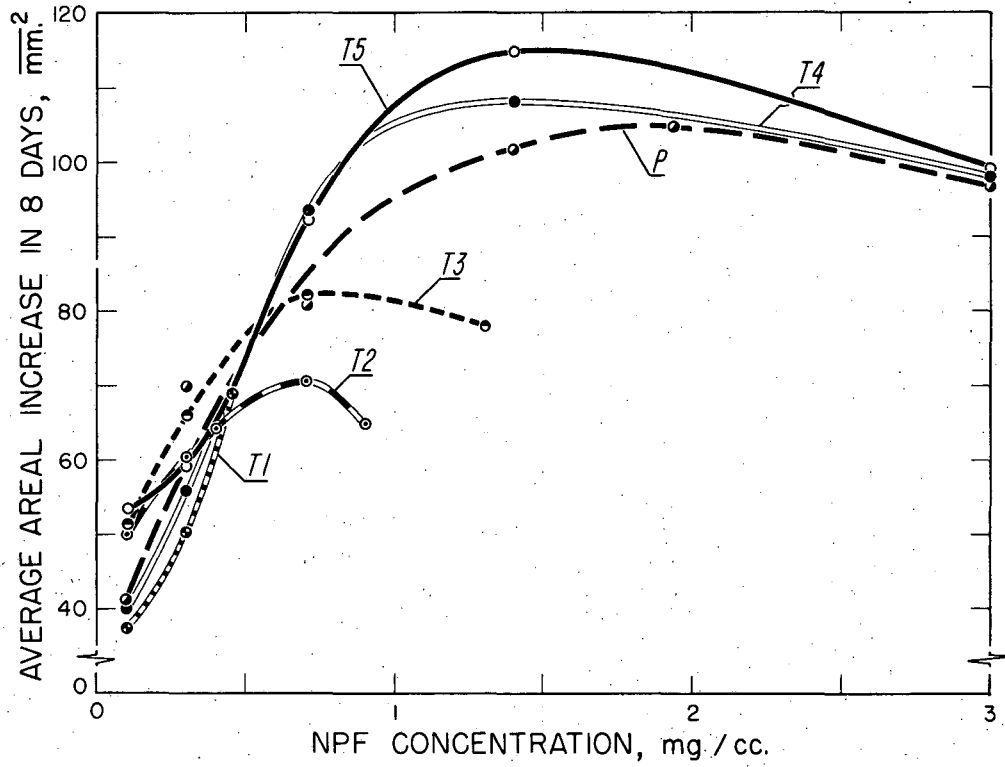
RESULTS

Two ultracentrifuge runs were made with the NPF in 1 M NaCl, with similar results in culture. The data obtained from the second run (Fig. 1) show the biological activity as a function of concentration. T1-T4 represent successive 1.5-ml fractions starting at the top of the 6-ml preparative tube. T5 is the resuspended bottom pellet, P is the parent NPF before spinning; essentially the same curve was obtained for the NPF remixed after spinning. It should be noted that all subfractions gave essentially the same response below 0.5 mg/cc.

Table I presents a comparison of the estimated nucleic acid and lipid content of the subfractions with the biological activity for the above series at a concentration of 0.3 mg/cc in culture. The data indicate a progressive increase of nucleic acid content going down the tube, but a reverse trend for the lipids, while the specific biological activity remains essentially constant. The ultraviolet spectra reflect the nucleic acid content. T4 and T5 show a typical nucleoprotein absorption curve with absorption maximum at 260μ similar to that of the parent NPF, already published.³ The $260\text{-}\mu$ maximum gradually decreases in size in T3 and T2 and shifts toward 280μ . T1 exhibits a plateau from 260 to 280μ .

The results of the other two ultracentrifuge runs made in higher-density media were similar in biological activity and distribution of nucleic acid and lipid composition to the runs made in 1 M NaCl. In no case was there noted any increase of specific activity over that of the parent NPF.

The data from the electroconvection experiments at pH values of 5.6, 6.4, 7.2, and 8.0 were very similar among themselves in biological activity and distribution of nucleic acid composition. No lipid analyses were done. Table II gives, for example, the results obtained at pH 7.2 after a 24-hour run. Nucleic acid estimates for each of the electroconvected subfractions are also presented. The data show a great increase (approximately 20-fold) of nucleic acid content in the bottom fraction relative to the top, while the specific activity of the subfractions did not increase significantly above the parent NPF. At 3 hours at pH 7.2 a larger content (20%) of nucleic acid was found in the bottom fraction than that reported here. This subfraction did not show any significant increase in specific activity relative to the parent NPF. The ultraviolet spectra of the electroconvected subfractions reflect their nucleic acid content in a manner similar to the ultracentrifugal subfractions.



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Fig. 1. Biological activity of NPF as a function of concentration. T1 through T4: successive ultracentrifuged fractions from chick-embryo extracts; T5: resuspended bottom pellet; P: parent NPF before centrifugation.

Table I

Chemical composition and biological activity of ultracentrifugal subfractions of the NPF			
Subfraction	Areal increase at 8 days*† (mm ²)	Nucleic acid** (%)	Lipid** (%)
T1	50.3 ± 2.5	0.9	18.6
T2	60.5 ± 4.7	0.9	6.9
T3	66.2 ± 8.7	2.3	7.3
T4	55.8 ± 11.1	4.5	3.3
T5	59.6 ± 7.4	6.6	--
P	70.0 ± 17.1	4.8	4.1
Background Control	13.0 ± 1.1	--	--

† Mean value and standard error. Six flasks per series.

* All samples cultured at 0.3 mg/cc.

** Estimated percentages of dry weight.

Table II

Biological activity and nucleic acid content
of subfractions of the NPF from the electroconvector

Subfraction	Areal increase at 8 days*† (mm ²)	Nucleic acid** (%)
Top	66.8 ± 12.1	0.1
Bottom	99.0 ± 11.6	8.2
Parent	96.7 ± 10.5	7.9
Background control	43.6 ± 5.6	--

* All samples cultured at 0.3 mg/cc.

** Estimated percentages of dry weight.

† Mean value and standard error. Six flasks per series.

DISCUSSION

It is interesting to note from these experiments that the nucleic acid- and lipid-rich subfractions of the NPF, which migrate during the two types of fractionation to produce subfractions as high as 18% in lipid or 20% in nucleic acid content, seem to have no effect on the stimulation of growth as measured here. Although T4 and T5 at concentrations of 1 to 3 mg/ml show a greater specific activity than the parent NPF, this increase is not significant. No other subfraction of the NPF exhibited activity greater than that of the parent NPF. In both experiments, fractionation did not result in any subfraction completely free of lipid or nucleic acid content. It is possible this may be the result of incomplete fractionation. The data suggest a slightly decreased biological activity in the upper ultracentrifugal subfractions T1 (Table I), and at higher concentrations of T2, T3 (Fig. 1) than in the other subfractions. However, since the cells in cultures containing T1, T2, and T3 did not show morphological signs of inhibition, it is not necessary to invoke the hypothesis of lipid inhibitors. The decrease in growth can be easily accounted for as an increased concentration of inert lipid ballast displacing an equivalent amount of active materials.

No increase in specific activity was noted in any of the subfractions of the NPF after fractionation under a wide range of densities and pH values. In consideration of the results here presented, the possibility arises that after removal of nucleic acid and lipid-rich subfractions it may be extremely difficult to separate the active material further by physical means. The electrophoretic studies⁵ also show that this physical method will separate nucleic acid-rich components from NPF, but the major active component remains unresolved. Chemical means may therefore offer greater promise, in view of these results. Our chemical analyses indicate the embryonic NPF to contain 80% to 85% protein, 5% to 10% nucleic acid, and 3% to 5% lipid; but since the latter two components seem to be largely inactive in culture, it appears that the protein portion may play the major role in growth stimulation. This possibility has been given further weight by preliminary experiments in which the protein moiety of the NPF has been chemically split from the nucleic acid and found to be active in tissue culture. ¹¹

SUMMARY

1. The nucleoprotein fraction (NPF) from chick embryos that stimulates growth in tissue culture has been fractionated into various lipid-rich and nucleic-acid-rich subfractions by ultracentrifugation and electroconvection.
2. All subfractions when tested in tissue culture had specific activity essentially the same as or lower than the parent NPF.
3. The data indicate that the presence of both nucleic acids and lipids in the subfractions has no positive correlation with growth-stimulating activity.
4. The possibility is discussed that protein is the biologically active material and that nucleic acid and lipid are inert.

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