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NUCLEOPROTEIN FRACTIONS FROM VARIOUS EMBRYONIC TISSUES

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A Comparison of Physicochemical Characteristics and Biological Activity in Tissue Culture

Roman J. Kutsky, Rodes Trautman, Miriam Lieberman, and Relda M. Cailleau

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ABSTRACT

Nucleoprotein fractions were isolated from extracts of twelve different tissues and organs of the 12-day chick embryo. The streptomycin method used was originally developed to isolate from whole-chick-embryo extract a nucleoprotein fraction that stimulates growth of tissues in culture. Comparison of activities in tissue culture showed that the internal organ nucleoprotein fractions were much less active than those from tissues containing cartilage. Electrophoretic analysis disclosed a definite difference in pattern paralleling the biological activity. No such differences were found in the ultraviolet absorption spectra of the various nucleoprotein fractions. Electrophoretic subfractions were obtained from each nucleoprotein fraction and analyzed spectrophotometrically. Similar families of curves were found for the various sets of subfractions, with maxima ranging from 257 mμ to 275 mμ. Bioassays upon electrophoretic subfractions of the whole-embryo nucleoprotein fraction indicate that the biological activity is contained in a zone occupying approximately the middle third of the electrophoretic pattern.

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INTRODUCTION

Chick embryo extract used to stimulate growth in tissue culture has been found to possess two major active constituents:[2] a dialyzable portion, and a nondialyzable one from which the active ingredients were isolated in the form of a nucleoprotein fraction (NPF).[7] In this study we consider only the latter active fraction. The nucleoprotein fraction (NPF) of 12-day chick embryos was found to be selectively precipitated with streptomycin.[7] Chemical analysis disclosed that this fraction contains approximately 10% ribonucleic acid, and ultraviolet spectrophotometry revealed a typical nucleoprotein curve with maximum at 260 mμ.[7] This fraction exhibited four electrophoretic components, some of which we have recently demonstrated in preliminary experiments[7] to be inactive in tissue culture. When further methods of purification were considered, the heterogeneity of embryonic tissues and organs suggested that a preliminary dissection of the embryos into tissue and organ batches and subsequent isolation of various tissue NPF's might result in purer products and would perhaps segregate the biological activity.

Previous workers had tested saline extracts of chick embryo tissues and organs in culture, but their results are not necessarily applicable to this study because of the above-mentioned low-molecular-weight dialyzable fraction. Correlations of high activity with saline tissue extracts would not necessarily indicate a high content of NPF, but a correlation of low activity would indicate that both NPF and low-molecular-weight materials were

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present in lesser amounts. Hueper,[6] working with 8-day chick embryos, found the eyeless head, eyes, and body to have the same potency as the whole-embryo extract. Fowler[4] compared extracts of 14-day chick embryo head, neck, and trunk with the whole extract; the trunk proved least potent. Fischer,[3] working with 7- to 10-day embryos, found the eyes, brain, and internal organs to be less potent than whole-embryo extract. Davidson and Waymouth[1] found sheep embryo cartilage to have a potency similar to the whole 9-day chick embryo extract. These results suggest that perhaps certain regions of the embryo contain more active materials than others.

It was therefore decided to isolate and test the tissue and organ NP fractions, then to analyze by electrophoresis with subsequent pipetting of subfractions and ultraviolet-spectrum analysis of these subfractions.

METHODS

The following tissues and organs of 12-day chick embryos were cleanly dissected and placed in separate batches on an ice bath prior to chemical isolation: muscle (breast and thigh), liver, lung, kidney, gut (upper and lower), heart, brain, eyes, carcass, cartilage (femur), skin, and spine. A few whole embryos were set aside to furnish whole-embryo control material. The NPF was obtained by use of the streptomycin precipitation method[7] with minor modifications. The final products (approx. 30 to 40 cc) were dialyzed overnight against 7 liters of Gey's solution prior to culture operations, and spun at 105,000g to reduce contaminating microorganisms to a low level, as previously described.[7] Culture methods were similar to those described previously.[1,8,9]

Fresh explants of 12- to 14-day chick embryo heart, about 1 mm,[3] were cultivated in carrel flasks at 37° C for 8 days. The basal assay medium consisted of a chicken plasma clot with a supernatant containing 40% horse serum and 60% Gey's saline. Antibiotics were routinely incorporated into the media. Final concentrations of NPF used in culture were approximately 0.3 mg/cc, i.e., 0.7 mg/culture, on a dry-weight basis. All tissues and organs within a series were cultured at similar concentrations of NPF, and two control sets of cultures were carried through in each test series, one(minimum control) containing Gey's solution and the other (maximum control) containing whole-embryo NPF at approximately the same concentration as the tissue and organ

NP fractions. Media were changed at 4 days, and cultures were traced and terminated at 8 days. The areas were determined and statistics calculated as in a previous paper.[9] A Klett moving-boundary apparatus was used for electrophoresis. The conditions for the runs were: 0.1 M potassium phosphate buffer, pH 7.2; 7.4 ma, giving a field strength of 2.9 v/cm; 0.5°C water bath temperature; 110-minute duration of run. Samples of subfractions were pipetted in order from top to bottom with a capillary pipette. A Model DU Beckman spectrophotometer was used for ultraviolet spectrophotometry.

RESULTS

Effects of the Nucleoprotein Fractions in Tissue Culture

The measured 8-day areal outgrowth elicited by the tissue and organ NPF's from the 12-day chick embryo was analyzed statistically for each of the three experiments. The actual areal increase, standard errors, and probability of chance occurrence are given for a sample experiment (March) in Table I. The increases in these three experiments relative to their respective serum controls are comparable on a probability basis; the combined data are shown on a histogram, Fig. 1, illustrating the 1% level of insignificance -- i.e., 99% probability of significant difference -- often used in tissue culture and chosen for this work. The three NP fractions that are as effective as whole-embryo NPF comprise the "highly active" group. For comparative purposes the 3% and 5% levels of insignificance are also shown. In simultaneous experiments at a different laboratory, one of us (R. C.) tested the various NP fractions in roller tube cultures and obtained essentially similar results. The microscopic appearance of the cultures with areas equal to or larger than whole-embryo NPF was comparable to that of the healthy appearance of cells cultured in whole-embryo NPF as previously described,[7] thus furnishing parallel evidence of effectiveness of the various NP fractions.

Electrophoretic Patterns of the Nucleoprotein Fractions

The electrophoretic patterns for the NP fractions obtained from the various tissues and organs showed from two to four distinctly discernible maxima for each NPF, exclusive of the salt boundaries (two in cartilage, four in gut, eye, heart, whole and carcass, three in the other tissues except kidney, which was not done). For the March series, for example, Fig. 2A shows the photographic ascending and descending patterns obtained from gut,

Table I

Sample (March Series) Effects of Embryonic Tissue and Organ NP Fractions on 8-Day Outgrowth of Chick Heart Fibroblasts*

Tissue NPF	Area Increase[a] (mm^2)	S. E.[a]	P[b] (%)	100-P[c] (%)
Serum Controls (Basal medium)	29.4	10.2	--	--
Heart	48.6	7.3	19.8	80.2
Skin	52.4	5.4	8.6	91.4
Kidney	48.9	7.1	16.0	84.0
Liver	38.4	4.0	45.0	55.0
Gut	54.4	2.8	5.0	95.0
Lung	62.2	1.8	2.5	97.5
Muscle	40.9	7.0	39.0	61.0
Brain	48.2	2.5	11.5	88.5
Carcass	82.9	4.4	0.19	99.81
Cartilage	85.2	7.1	0.27	99.73
Spine	75.4	5.6	0.76	99.24
Whole	72.6	3.2	0.98	99.02

* Medium: Chicken Plasma Clot, 30% Horse Serum, 70% Gey's Solution Tissue NPF at 0.30 mg/cc in Gey's Solution.

[a] Mean Value and Standard Error, 4 to 5 Cultures Per Series.

[b] Fischer's t-Test (Snedecor[10]) P = probability that areal difference relative to serum controls would occur by chance alone.

[c] Probability of difference from serum controls = 100-P.

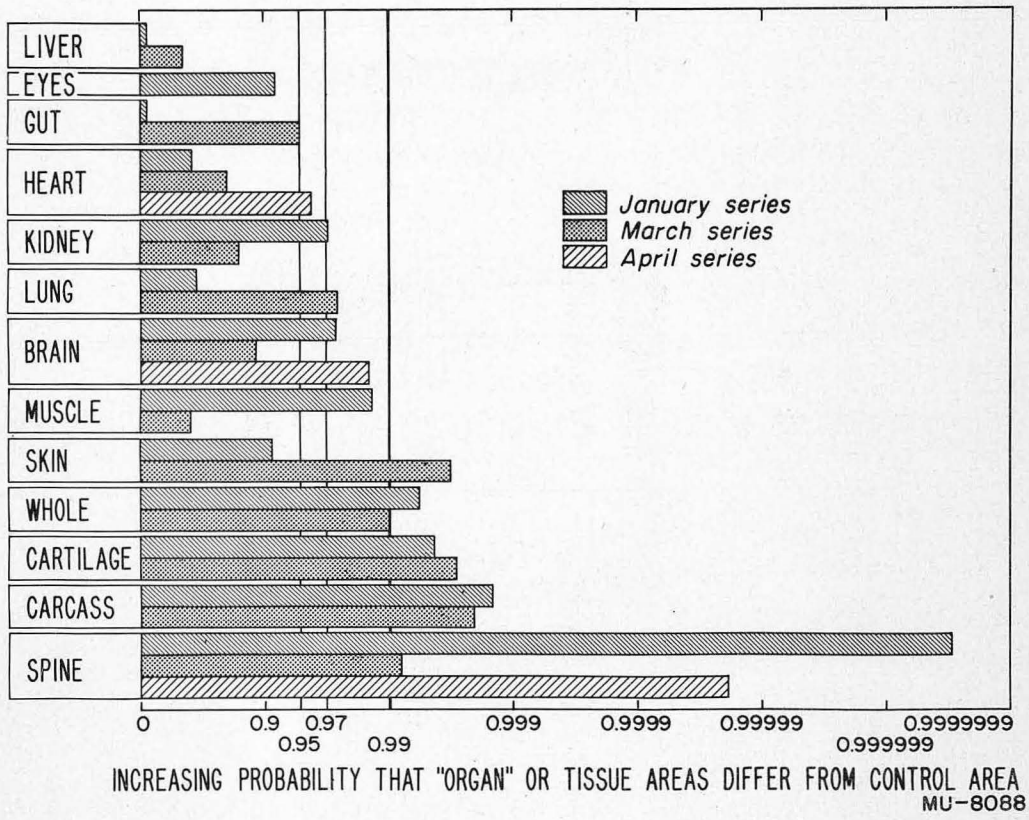

Fig. 1. Combined data from January, March, and April series.

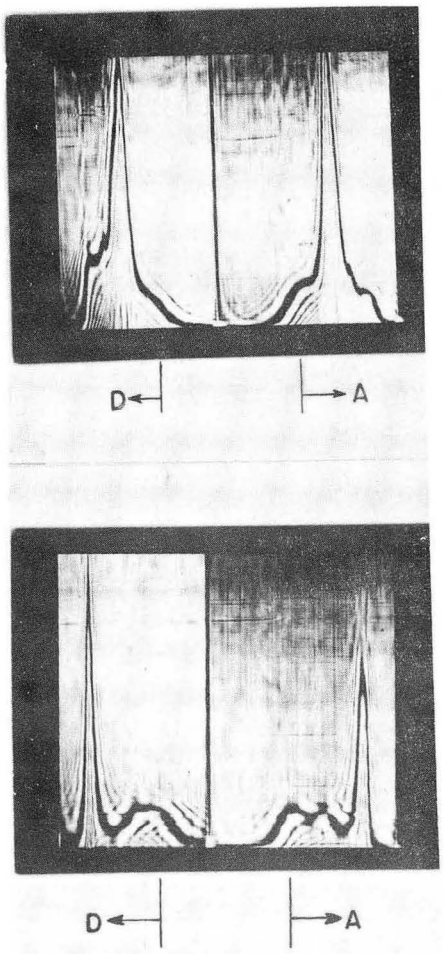

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Fig. 2A. Photographs of electrophoretic patterns of gut NPF. Ascending on right.

Fig. 2B. Photographs of electrophoretic patterns of cartilage NPF. Ascending on right.

and Fig. 2B those from cartilage. On the basis of three separate dissection series it appeared that each tissue or organ yielded a reproducible and characteristic pattern.

The average patterns of the "highly active" and "other" tissues traced to the same scale of total area are compared in Fig. 3. Since the whole and carcass contain mixtures of both groups of tissues and organs, they are not included in the average of the highly active fractions.

Fractions of the whole-embryo NPF corresponding to various areas on the schlieren pattern were pipetted after electrophoretic separation and tested in tissue culture. The preliminary finding[9] of an active subfraction was confirmed in that the activity seemed to be concentrated in an area of intermediate mobility, namely between 2.5 and 4 units on Fig. 3.

Ultraviolet Absorption Spectra of the Nucleoprotein Fractions

Each of the NP fractions showed a nucleoprotein-type uv absorption spectrum with a maximum at 260 mμ, which was very similar for all organs and tissues and resembled the spectrum of the original whole-embryo NPF.[7]

Ultraviolet analyses were performed on various subfractions of tissue NP fractions pipetted from the moving-boundary electrophoresis. All the subfractions were found to exhibit similar families of curves in which the maximum shifts gradually, in going from the fastest to the slowest subfraction, from 257 mμ to 275 mμ. For example, cartilage is shown, Fig. 4, values normalized to 280 mμ, in 0.1M phosphate pH 7.2.

DISCUSSION

The histogram in Fig. 1 indicates that only extracts of spine, cartilage, and carcass have consistently had an activity in terms of areal increase equal to the whole embryo extract. These results might implicate extracts of the major common constituent, cartilage, as being a potent source of activity in the 12-day chick embryo NPF. Other tissue extracts also contain active material, but in smaller concentration or lower unit activity. These results harmonize with those obtained on saline extracts of chick embryos by Fischer[3] and especially those of Davidson and Waymouth[1] who found sheep embryo cartilage extract to be a potent growth factor. Differences between cartilage an and spine may possibly be due either to factors extracted from the spinal cord or to a difference between the stage of development of the femur cartilage and

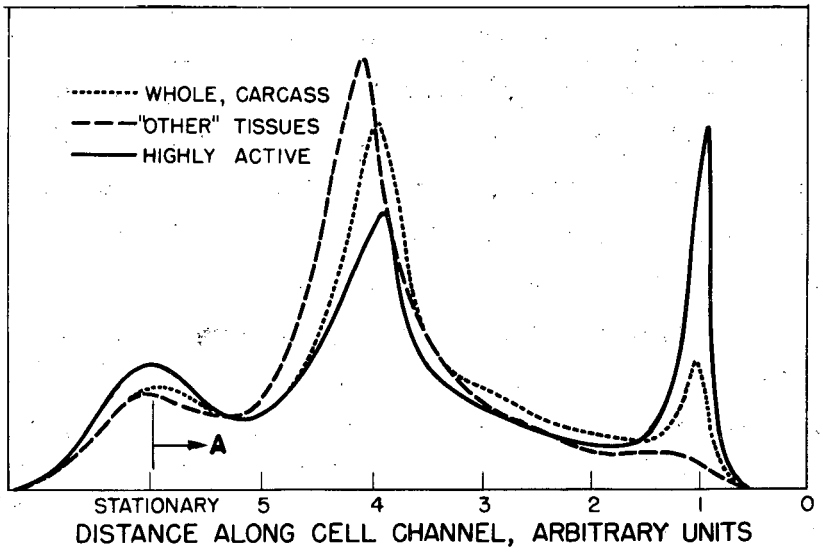

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Fig. 3. Comparison of average electrophoretic NPF patterns (March, ascending limb).

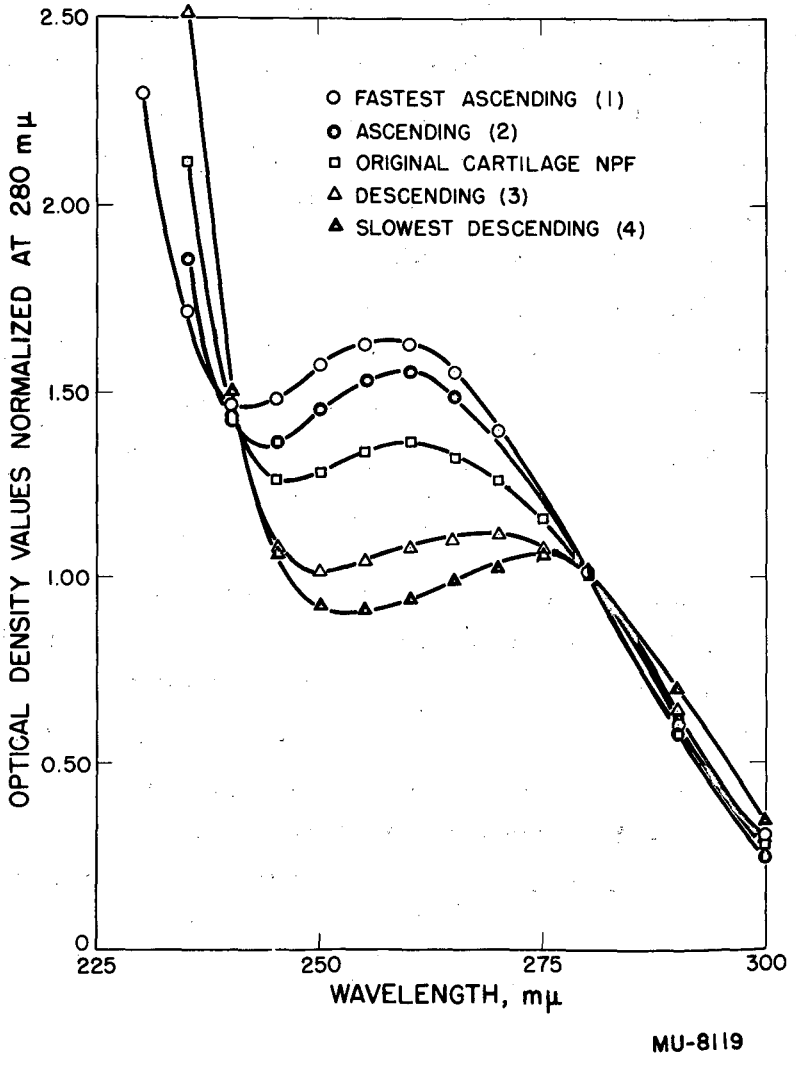

Fig. 4. Ultraviolet spectra of pipetted subfractions. January cartilage NPF electrophoresis.

that of the spinal cord cartilage.

Various tissue NP fractions also show definite differences in electrophoretic pattern. The more active fractions exhibit a much greater leading peak than the other fractions. The close reproducibility of the tissue electrophoretic patterns from month to month argues well for the reproducibility of the streptomycin method. The only biologically (in vitro) active subfraction of the whole-embryo NPF corresponds to a zone on the electrophoretic pattern that is poorly defined. It would therefore appear that the active compounds in this active subfraction are either polydisperse or are masked on the electrophoretic pattern by other inactive components. It should be noted that none of the tissue NPF electrophoretic patterns exhibits peaks in a zone corresponding to the active zone in the whole-embryo NPF pattern; furthermore the areas under the curves (i.e., total amounts of dissolved material) in the various corresponding active zones are about the same when the more active tissue NP fractions are compared with the other NP fractions. This might indicate either that each tissue NPF has a different constitution and potency or that varying amounts of the same active material are present, mixed with inactive material. The presence of inhibitors might complicate the picture, but this possibility should not be excluded.

The first ascending electrophoretic component of the whole-embryo NPF has a high content of nucleic acid-like materials, as shown by its ultraviolet spectrum, but is inactive. An increase of this first peak is noted when the NPF is denatured, aged, or further processed. It is not clear why the more active NP fractions possess an enhanced leading peak which presumably is inactive, but perhaps the more active NP fractions are more labile and release their nucleic acid moiety more rapidly than the less active NP fractions.

SUMMARY

1. A nucleoprotein fraction (NPF) was isolated by streptomycin precipitation from twelve different chick embryo tissues.
2. Bioassays in tissue culture revealed maximum growth-promoting activity in those NPF's from material containing cartilage.
3. Electrophoretic analyses indicate that the leading peaks of the highly active NPF's are much greater than those of the other NPF's.
4. Ultraviolet analyses of tissue NPF's indicate similar nucleoprotein absorption spectra, while the electrophoretic subfractions of these NPF's exhibit families of curves which are similar and have maxima from 257 mμ to 275 mμ.

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