Stimulating Effect of Nucleoprotein Fraction of Chick Embryo Extract on Homologous Heart Fibroblasts.* (20368)

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Embryo extract as ordinarily prepared in balanced saline solution(1) stimulates growth in tissue cultures as first reported by Carrel (2). The experiments of Fischer(3) involving dialysis and ultracentrifugation suggest that the growth factors in embryo extract fall into two categories: (a) substances of high molecular weight, probably protein in nature, collectively termed "embryonin" by Fischer, and (b) small molecules readily diffusible through dialysis membranes. Several workers have attempted isolations of the active factors of high molecular weight. Fischer(4) using Hammersten's(5) technique isolated an active nucleoprotein fraction with several electrophoretic components of which 4 were predominant, but he did not test the biological activity of the components. Davidson and Waymouth(6) likewise isolated an active nucleoprotein fraction from chick embryos and Waymouth(7) reported a few electrophoretic peaks in a similar preparation, but their biological activity was not tested. Katsuta and Takaoka(8) claim that a high polymer pentosenucleic acid isolated from chick embryos by Nishioka and Ibuka(9) is Fischer's (4) "embryonin," the growth factor of high molecular weight in chick embryo extract.

Von Euler and Heller(10) have developed an isolation method for liver nucleoproteins by using streptomycin as a specific precipitant. A modification of this method designed for quantity isolation of a chick embryo nucleoprotein fraction will be described here. This fraction will be shown to contain most of the growth factors of high molecular weight in chick embryo extract. Preliminary characterizations of this fraction and tests on the high polymer pentosenucleic acid prepared according to Nishioka and Ibuka(9) will also be presented.

Isolation procedures. The nucleoprotein fraction was obtained from fresh 12-day chick embryos. These 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 borate, pH 7.1, and centrifuged 15 minutes at 24,000 x g in a fluid-cooled Servall centrifuge. Re-extraction of the residue increased the final yield by about 30%. The supernatant was made M/100 in streptomycin sulfate (Lilly) by using a M/4 stock solution, pH 7.5, left undisturbed for 2 hours and the resulting precipitate centrifuged down at 24,000 x g for 10 minutes. The precipitate was gently homogenized with M/3 potassium phosphate buffer, pH 7.2, 4 cc per embryo, yielding a turbid solution which was dialyzed with stirring in ¾ inch Visking tubing for 12 hours against borate, pH 7.1, 120 cc per embryo. The streptomycin precipitation was repeated in the dialyzed solution with resuspension in phosphate as above. Dialysis with rotation of the sac was continued for 12 hours against two changes of M/3 phosphate buffer, 32 cc per embryo. The dense dialyzed suspension was then ultracentrifuged one hour at 105,000 x g in a Spinco refrigerated centrifuge. The free lipids rose to the surface and were removed with a capillary pipet 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 cc per embryo equivalent prior to lyophilization or against the same volume of Gey's saline prior to culture operations. This solution, after ultra-

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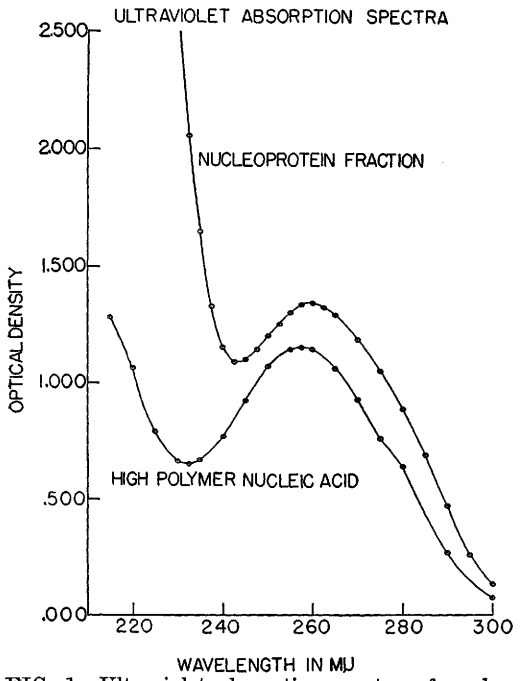

FIG. 1. Ultraviolet absorption spectra of nucleoprotein fraction and high polymer nucleic acid, both in Gey's saline (0.6 mg/cc) at pH 7.5.

centrifuging again for an hour at 105,000 x g, was clear, colorless, viscous and exhibited a typical nucleoprotein absorption spectrum in the ultraviolet with a maximum at 260 mμ and a minimum at 243 (Fig. 1). 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. Duplicate analyses for PNA and DNA in the streptomycin-precipitated fraction are seen in Table I. For purposes of analysis, PNA and DNA were separated and protein removed by the method of Schmidt and Thannhauser(13) followed by Schneider's procedure(14). DNA was determined by the method of Stumpf(15), PNA by the orcinol method of Mejbaum(16). Direct preparation of high polymer pentose-nucleic acid from chick embryo extract followed the method of Nishioka and Ibuka(9). The ultraviolet absorption spectrum of the final product is shown in Fig. 1. The slight inflection at 280 mμ suggests residual protein. In addition, a preparation of nucleic acid was obtained from the streptomycin-precipitated nucleoprotein fraction according to Grinnan and Mosher (12) omitting the preliminary guanidine precipitation.

Tissue culture technic. Fresh explants of 13-16 day chick heart were cultivated in Carrel flasks at 37°C for a 10-day period. The basal assay medium consisted of a chicken plasma clot with a supernatant containing 40% horse serum and 60% Gey's saline. Supernatants were changed twice a week without washing the cultures. The media were not dialyzed. Details are given in a previous paper by Kutsky and Harris(11). The experimental cultures were supplemented with the nucleoprotein fraction in saline solution, each series being matched with a corresponding group of non-supplemented control cultures. The effectiveness of a particular fraction was judged by the increase in area of the culture at ten days, by the characteristic microscopic morphology of the cells in the culture and in certain cases by measurements of the pH levels in the supernatant fluids. Fractions were originally prepared by using sterile technics, but when this was omitted there was no sign of alteration in the final product since the temperature was maintained at 0°C throughout the isolation procedure. Fractions prepared under non-sterile conditions did not in general produce contaminations in tissue culture. Antibiotics were routinely incorporated in the media and the fractions were routinely subjected to one-hour's ultracentrifugation at 105,000 x g just prior to use to reduce contaminating microorganisms to a low level.

Results. Table II shows the relative increases in surface area, at 10 days, of cultures containing 3 concentrations of the nucleopro-

TABLE I. Nucleic Acid Determinations on Nucleoprotein Fraction.

Analysis	Sample wt, mg	Nucleic acid found, mg*	% nucleic acid	Avg % NA
PNA	10	1.05	10.5	10.3
	"	1.06	10.6	
	"	.99	9.9	
DNA	100	.33	.33	0.33
	"	.32	.32	

* Each value represents avg of two aliquot determinations.

TABLE II. Outgrowth of Chick Heart Fibroblasts in Undialyzed Media Supplemented with Fractions from Chick Embryo Extract.

Supplement to basal medium*	Areal increase 10 days mm²†		Diff. from unsupplemented controls	Signif. of diff. P
	Supplemented	Controls		
Nucleoprotein fraction				
.2 mg/cc	34.7 ± 2.3	14.3 ± 1.4	20.4 ± 2.7	<.001
.4 mg/cc	53.4 ± .9	14.3 ± 1.4	39.1 ± 1.7	<.001
.7 mg/cc	65.0 ± 1.2	14.3 ± 1.4	50.7 ± 1.8	<.001
Supernatant dialyzed‡				
15%	22.8 ± 1.3	13.9 ± 1.3	8.9 ± 1.9	<.01
High polymer nucleic acid				
1 mg/cc	10 ± .6	13.8 ± 3.9	3.8 ± 4.0	>.3
Nucleic acid from nucleoprotein				
.3 mg/cc	17 ± 3	19.9 ± 4.6	2.9 ± 5.5	>.5

* Basal medium: Plasma clot, 40% horse serum, 60% Gey's solution.

† Mean value and stand. error (Snedecor(17)). Six cultures/series except last supplement where 3 used.

‡ From first streptomycin precipitation, replacing ¼ of Gey's solution in culture.

tein fraction as compared to outgrowth of similar cultures in the unsupplemented basal assay medium. The obvious increases in cultures containing the nucleoprotein fraction are further documented by the photomicrographs shown in Fig. 2, 3. Increases in surface area were also associated with characteristic alterations in cell morphology. In the unsupplemented control cultures the cells were heavily granulated and densely massed without conspicuous orientation (Fig. 4). In cultures containing the nucleoprotein fraction the outgrowth zone was much less granular, with the marginal cells broad and flat, tending toward a looser, more radial arrangement (Fig. 5). 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 in dialyzed assay media by Harris and Kutsky(18) and will be described later in more detail.

Table II also includes data on areal increases in cultures supplemented with the supernatant (thoroughly dialyzed against Gey's saline) from the first streptomycin precipitation. The supernatant fraction was incorporated at the level of 15% of the 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.

Assays of the high polymer PNA prepared according to the method of Nishioka and Ibuka(9) were uniformly negative as were similar experiments with nucleic acid isolated directly from the nucleoprotein fraction(12). Neither of these preparations produced increases in surface area (Table II) or alterations in cell morphology similar to those seen with the nucleoprotein fraction.

Discussion. The original isolation technic of von Euler and Heller(10) proved unsuitable for chick embryo extract and was therefore 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(19), 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

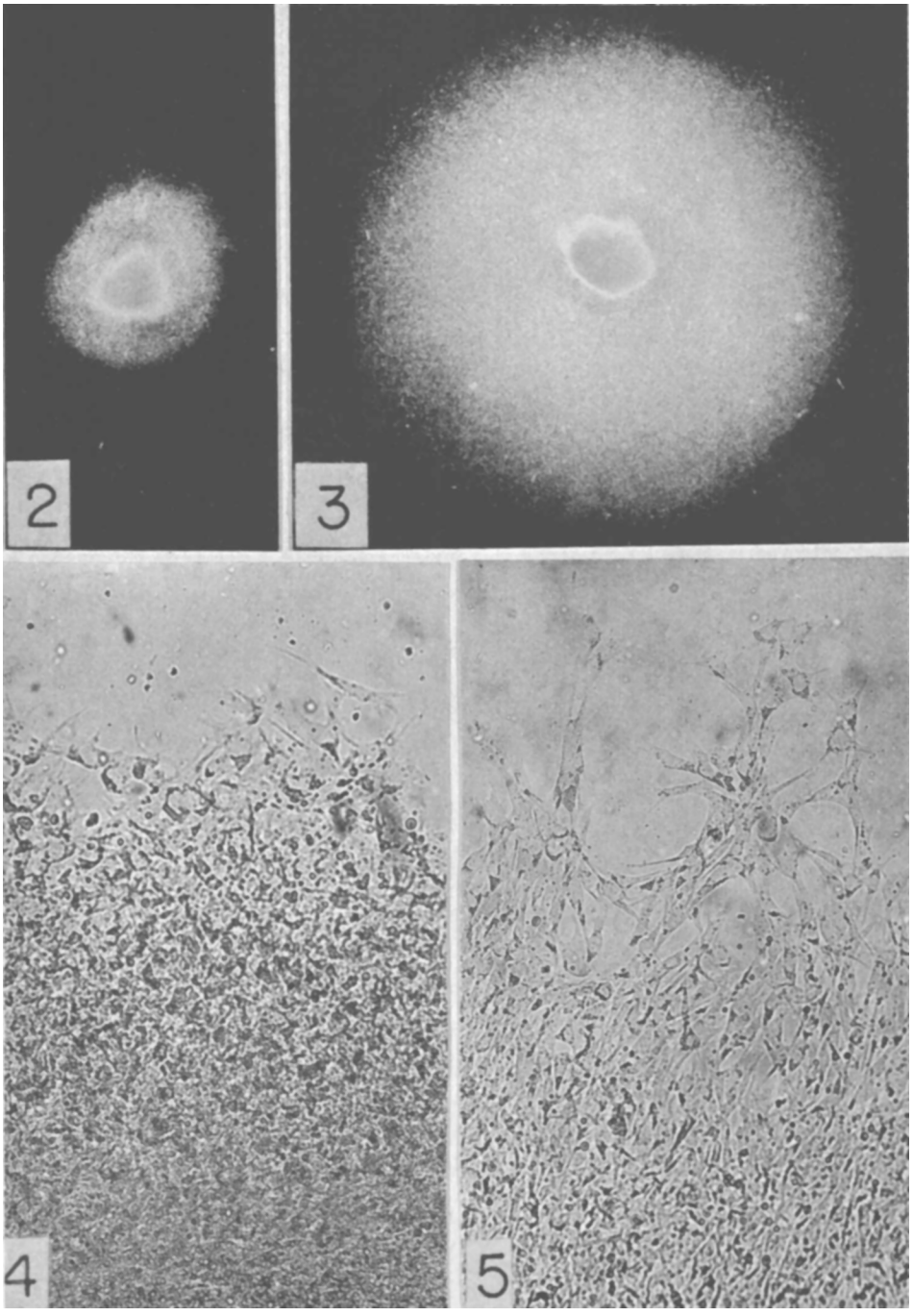

FIG. 2. Outgrowth of chick heart fibroblasts after 10 days in unsupplemented basal assay medium. × 7½.

FIG. 3. Outgrowth at 10 days in basal medium supplemented with nucleoprotein fraction in Gey's saline. × 7½.

FIG. 4. Cellular detail at 10 days in culture of chick heart fibroblasts in unsupplemented basal medium. × 122.

FIG. 5. Similar view of culture containing supplementary nucleoprotein fraction in Gey's saline. × 122.

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. The presence of contaminating heme-proteins was followed at their absorption maximum at 405 mμ. 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 volumes 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 the streptomycin maximum at 325 mμ after incubation with alkali. The streptomycin when bound to the nucleoprotein loses its characteristic absorption maximum at 325 mμ. Von Euler and Heller's procedure made no provision for removal of the free lipids or the microsomes, whereas they are here removed by ultracentrifugation. Lyophilization preserves most of the activity of the redissolved nucleoprotein fraction but the material is slow to redissolve, about 30% remaining insoluble. The material retains its biological activity in the dissolved state for 3 weeks when kept in the refrigerator.

To check the possibility that streptomycin itself may have a stimulating effect in tissue culture, various concentrations (M/40 to M/4000) were tested in culture in the same manner as was the nucleoprotein fraction. None of the concentrations were stimulating, indeed the higher ones were inhibitory. A similar inhibition was recently reported by Fusillo, Metzger, and Kuhns(20).

High polymer pentosenucleic acid (relative viscosity 2.4) isolated according to Nishioka and Ibuka(9) had no effect in tissue culture at 1 mg/cc and although biuret negative, showed a definite positive Sakaguchi test for protein. (See also slight inflection at 280 mμ, Fig. 1). Similar material was also tested in cultures in the absence of horse serum at a level of .3 mg/cc with embryo extract present, likewise with no positive results. It is possible that the positive results obtained by Katsuta and Takaoka(8) could be explained by a larger concentration of the contaminating active nucleoprotein fraction in their preparations than in the present experiments where more sensitive protein tests were used. The nucleic acid isolated from the nucleoprotein fraction by the method of Grinnan and Mosher(12) also showed no activity (Table II).

A preliminary electrophoretic separation was performed on an early preparation of the nucleoprotein fraction through the courtesy of Dr. R. Trautman of the Donner Laboratory of Medical Physics. This separation resulted in 4 subfractions of which only the major component was active when tested in culture. This component had an ultraviolet absorption spectrum similar to a nucleoprotein. Culture tests of the minor subfractions showed no activity but these may have been too dilute to show an effect. Two of the minor subfractions resembled nucleic acids in their ultraviolet absorption spectra while the third subfraction gave no spectral evidence of an absorption maximum at 260 mμ.

The small residual activity in the supernatant from the first streptomycin precipitation indicates that the nucleoprotein fraction contains most of the outgrowth-promoting substances of high molecular weight in chick embryo extract. The concentration series shows that a relatively large amount (0.7 mg/cc) is required to elicit an optimal effect.

A yield of approximately 2 g of lyophilized material has been obtained from 130 embryos. The nucleoprotein fraction gives positive biuret and Sakaguchi tests for protein. Analyses for nucleic acids (Table I) revealed that 0.3% DNA and 10.3% PNA were pres-

ent in 2 samples of lyophilized nucleoprotein fraction. The DNA is probably an inactive contaminant in the procedure, since culture tests on both low and high polymer DNA from various sources showed no stimulating effect.

Summary. 1. A quantity isolation method is described for a chick embryo nucleoprotein fraction based on von Euler and Heller's(10) procedure for liver nucleoproteins. 2. When this fraction was tested in an assay medium of undialyzd chicken plasma and dilute undialyzed horse serum large outgrowths of chick heart fibroblasts were obtained with well-defined alterations in cell morphology and mode of outgrowth. 3. The nucleoprotein fraction described contains protein, 10.3% PNA, 0.3% DNA and has a characteristic nucleoprotein absorption spectrum. 4. High polymer pentose nucleic acid isolated according to Nishioka and Ibuka(9) produced no changes in size or appearance of assay cultures.

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L-Lyxoflavin in the Starting Diets of Turkeys and Chickens.* (20369)

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Since Pallares and Garza(1) described the isolation of L-lyxoflavin there has been considerable interest in this compound in nutrition although there is still comparatively little published information concerning its essentiality in the diet. Emerson and Folkers(2),

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