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Effect of Macromolecular Material from Chick Embryos on Growth Rate of Mouse Heart Fibroblast Cultures.*

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(Introduced by M. C. Winternitz.)

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Twenty-seven years ago Carrel and his coworkers[1] observed that extracts of embryonic tissues promote the proliferation of connective tissue cells *in vitro*. It was later found that extracts prepared from the organs of adult animals possess a similar activity. It has remained common practice to incorporate crude tissue extracts or organ emulsions into the blood plasma media employed for culturing tissues *in vitro*.[2]

Recently, Claude[3] isolated, by means of differential centrifugation at high speed, from normal 8-day-old chick embryos a macromolecular material possessing chemical and physical properties[3, 4, 5] similar to those of the purified Rous chicken sarcoma agent[6] and other materials obtained from normal and malignant tissues.[7] The purified chick embryo fraction was tested for possible tumor-producing activity in Plymouth Rock hens with negative results.[8]

The present experiments were undertaken to test the hypothesis that Claude's chick embryo fraction might be responsible for the growth-stimulating activity of embryo extracts.

Experimental. Preparation of Chick Embryo Fraction. The procedure employed in the present work was similar to that described by Claude,[3] but an air-driven ultracentrifuge[8] was substituted for the high-speed laboratory centrifuge attachment used by Claude.

Eight-day-old normal chick embryos (White Leghorn or Plymouth Rock) were freed from accessory material with aseptic precautions, ground to a paste with mortar and pestle and stored frozen at about —8° for from one to 3 days. The material was then weighed, finely

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1 Carrel, A., *J. Exp. Med.*, 1913, **17**, 14; Ebeling, A. H., *ibid.*, 1913, **17**, 273.

2 Parker, R. C., *Methods of Tissue Culture*, P. B. Hoeber, N. Y., 1938, p. 67.

3 Claude, A., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 398.

4 Claude, A., *Science*, 1939, **90**, 213; 1940, **91**, 77.

5 Stern, K. G., and Duran-Reynals, F., *Science*, 1939, **89**, 609.

6 Claude, A., *Science*, 1938, **87**, 467.

7 Stern, K. G., and Kirschbaum, A., *Science*, 1939, **89**, 610.

8 Beams, J. W., Linke, F. W., and Sommer, P., *Sci. Instr.*, 1938, **9**, 248.

ground with sterile sand and extracted with 6 times its volume of Tyrode[9] solution of pH 7.2. In order to eliminate tissue debris, the extract was centrifuged for 20 minutes at 2500 RPM in the horizontal head of a size 1 centrifuge (type S. B.) of the International Centrifuge Company. The opalescent supernatant fluid was then spun in sterilized lusteroid tubes in the concentration rotor of the air-driven high-speed centrifuge for 30 minutes at 30,000 RPM (67,000 G.). The resulting closely packed pellets, formed at the bottom of the tubes, were then resuspended in Tyrode solution. The suspension was freed from aggregated, coarse particles by a low-speed run in the laboratory centrifuge. The opalescent supernatant solution was withdrawn and subjected again to sedimentation in the ultracentrifuge. In all, the macromolecular material was purified by 3 or 4 cycles comprising a low- and a high-speed centrifugation. The final yellowish ultracentrifuge pellets, which had a translucent appearance and a gelatinous consistency were suspended in the same volume of Tyrode solution as the original embryo extract from which they were derived. In some experiments, samples of the solutions of the purified fraction were placed for 15 minutes in a boiling water bath, in order to determine the stability of the active principle. The solutions of the purified fraction were invariably opalescent and colorless.

Evaluation of Chick Embryo Fraction in Tissue Cultures. The methods of tissue culture employed in this laboratory have been presented in detail.[10] The explants of the hearts of newborn mice in Carrel flasks were allowed to grow for approximately 72 hours in washed chicken plasma clots with a nutrient of rat serum diluted with 2 volumes of Tyrode. The colonies were then divided into control and experimental groups of at least 30 colonies each, and 0.3 cc of Tyrode, 0.3 cc of pellet, and 0.3 cc of pellet solution heated in a boiling water bath for 15 minutes were added to the respective flasks. The areas (A) of the explants were recorded and measured with a planimeter. The colonies were then allowed to grow for an interval of 30 hours and their areas (B) were again determined. The expansion rate factor (ERF) is defined as $B - A/B \times 100{,}000$ divided by the number of hours in the experiment interval (30). The means of the ERF of the groups with their standard errors were compared for statistical significance by use of Fisher's[11] "t" factor.

[9] Gey, G. O., and Gey, M. K., *Am. J. Cancer*, 1936, **27**, 45.

[10] Tennant, R., and Liebow, A. A., *Yale J. Biol. and Med.*, 1940, **13**, 39.

[11] Fisher, R. A., *Statistical Methods for Research Workers*, 5th Ed., Oliver & Boyd, London, 1934.

The results are recorded in Table I. In 9 experiments with material derived from 5 different batches of embryo extract the ERF of the pellet treated cultures was always greater than that of the Tyrode controls, in 5 instances with statistical significance.

The ERF of the cultures treated with the heated pellet materials even exceeded that of the unheated pellets, once in 5 instances with statistical significance (Exp. 296). The ERF of the former was in all 5 cases significantly greater than that of the controls.

Discussion. The association of a growth-stimulating principle present in embryo extracts with the macromolecular fraction isolated

TABLE I.
Effect of Macromolecular Material from Chick Embryo Extracts on Expansion Rate of Mouse Heart Fibroblast Cultures.

Source material and date	Exp. No.	1940	Tyrode control*	Pellet	Heated pellet	t	P
274	274	1/16	963±27.5 (35)	1059±32.2 (35)		2.28 (ty, P)	.02
1/16							
287	300	5/3	1433	1477		(ty, P)	
2/28					1523	(ty, HP)	
296	296	4/11	1482±20.8 (37)	1596±23.2 (38)		3.67 (ty, P)	<.01
4/11	302	5/8	1727±24.5 (39)	1803±25.1 (38)	1695±24.6 (32)	2.91 (P, HP)	<.01
	308	6/1	1266±29.4 (42)	1390±28.2 (39)		2.15 (ty, P)	<.05
					1855±31.2 (36)	3.21 (ty, HP)	<.01
						3.04 (ty, P)	<.01
313	313	6/29	1234±25.9 (38)	1312±29.5 (45)		2.00 (ty, P)	
6/29	316	7/8	1068±40.1 (39)	1381±30.6 (46)	1392±32.3 (41)	3.82 (ty, HP)	<.01
						6.29 (ty, P)	<.01
					1393±38.4 (44)		
315	317	7/29	1316±39.4 (35)	1316±28.1 (33)			N.S.
7/12	318	9/8	1377±25.3 (33)	1451±20.6 (44)		2.27 (ty, P)	

*Expansion rate ractors are given with their standard errors. The number of colonies in the group is included between parentheses. t—Fisher's factor; P—probability of error; ty—Tyrode solution; P—pellet; HP—heated pellet; N.S.—not significant.

by Claude from such preparations by differential high-speed centrifuging which is indicated by the experiments here described opens the approach for an inquiry into its chemical nature. According to Claude[3, 4] this material, like similar fractions obtained from other tissues, consists essentially of a phospholipid portion associated with a nucleoprotein of the ribose type. However, this does not necessarily mean that the *intact* complex is required for the effect of this material on explanted cells. The observation that the heated pellet material exhibits even greater growth stimulation indicates that the heat-stable portion of the material (possibly the lipoid or the nucleic acid component) is sufficient to produce the effect. Moreover, the macromolecular nature of the intact complex makes it highly improbable that it could enter the cells by diffusion without preceding dissociation into smaller units.

Investigations concerning this increase in potency after heating are now in progress particularly in relation to possible liberation of nucleic acid[12] and of vitamin H.[13] The supernatant after the first ultracentrifugation is also under study since it has been found to be variable in its properties in the experiments conducted to date.

While this work was in progress, the attention of the writers was directed to a brief communication by A. Fischer[14] dealing with the purification of the active fraction present in beef embryo extracts. This worker finds that the activity appears to be associated with a nucleoprotein of the ribose type. The heat-lability and the destruction of the biological activity by tryptic digestion in his experience indicate that a protein forms an integral part of the active principle. Mention is also made of the fact that it is rather easily sedimented in the ultracentrifuge.

Summary. It is shown that a *macromolecular* fraction isolated from chick embryo extracts by differential high-speed centrifugation essentially according to Claude, exerts a distinct growth-stimulating effect on cultures of mouse heart fibroblasts.

The writers wish to acknowledge the technical assistance of Miss E. Carter and of Mr. H. Nelson in these experiments.

[12] Claude, A., and Rothen, A., *J. Exp. Med.*, 1940, **71**, 619.

[13] György, P., Melville, D. B., Burk, D., and duVigneaud, V., *Science*, 1940, **91**, 243.

[14] Fischer, A., *Nature*, 1939, **144**, 113.