Presence of Low Molecular Weight Inhibitors of Protein Synthesis in the Developing Embryo[1] (36667)

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Various natural substances have been described which alter protein synthesis, or cell growth, in higher animals. Some of these have been drived from embryos (1–4), while others have originated from adult cells, or tissue components (5–18). During studies of protein synthesis in chick embryos, we encountered previously undescribed low-molecular weight inhibitors of protein synthesis. They were manifest after the first week of embryonic life. Their method of isolation and studies of some of their biochemical and physical properties are the subjects of this report.

Materials and Methods. Embryonated eggs of white leghorn chickens were obtained from Shamrock Poultry Farms, North Brunswick, NJ. The diet of the hens which laid these eggs contained skim milk, dried whey, wheat bran and middlings, yellow corn meal, soy bean oil meal, 3% calcium salts with 1 lb NaCl/ton of feed. The hens had been immunized earlier against Newcastle disease, laryngotracheitis, baby chick bronchitis and fowlpox. They were not exposed to other known chemicals or antibiotic sources. Embryos were gently homogenized with a hand-held glass tissue grinder, and incubated at 37° in medium 199 (Grand Island Biological Co., Grand Island, NY) or in Hanks' balanced salt solution containing glucose (Microbiological Ass., Inc., Bethesda, MD). Embryo extracts from 8-day-old whole embryos were prepared by homogenization, in 0.15 *M* NaCl in Teflon–glass tissue grinders, followed by centrifugation at 7,148*g* for 20 min. Ultrafiltration was performed through Diaflo membranes UM10, UM2, UM05 with an apparatus from Amicon Corp., Lexington, MA. Bovine pancreatic ribonuclease, bovine pancreatic deoxyribonuclease I, bovine pancreatic trypsin (Worthington Biochem. Corp., Freehold, NJ) and pronase (Calbiochem., Los Angeles, CA) were incubated with partially purified embryo extracts, as later described, in Hanks' balanced salt solution (pH 7.4) at concentrations of 0.200 and 0.100 mg/ml for 30 min and 1 hr U-^{14}C-L-lysine 1.9 mCi/mg, and U-^{14}C-L-isoleucine 2 mCi/mg (New England Nuclear Corp., Boston, MA) were used as protein precursors; Methyl-^{3}H-thymidine 11.9 Ci/mmole and uridine-5-^{3}H 2.0 Ci/mmole, (Schwarz Bioresearch, Inc., Orangeburg, NY) were used as precursors of nucleic acids.

Protein synthesis of embryo preparations was measured after their incubation with the isotopically labeled amino acids (19). Incubation was terminated by the addition of trichloroacetic acid (final conc, 10%), along with 0.6 mg/ml of bovine serum albumin, BSA (Pentex, Inc., Kankakee, IL) as carrier. The resulting precipitates were washed, dissolved, and counted by liquid scintillation methods (20). Assay of nucleic acid synthesis was peformed in the same manner on precipitates obtained after incubation of embryo preparations with isotopically labeled thymidine, or uridine. All incubations and analyses were performed in triplicate.

Amino acid analyses after acid hydrolysis (6 *N* HCl, 105°, 20 hr) were done with the assistance of Dr. A. Gold by Mr. W. Schrepel using a Beckman amino acid analyzer (Beckman Instruments Co., Palo Alto, CA). Inhibitor potency was tested by adding 1 ml of solution to be assayed to 1 ml containing the dispersed 3-day-old embryo preparation

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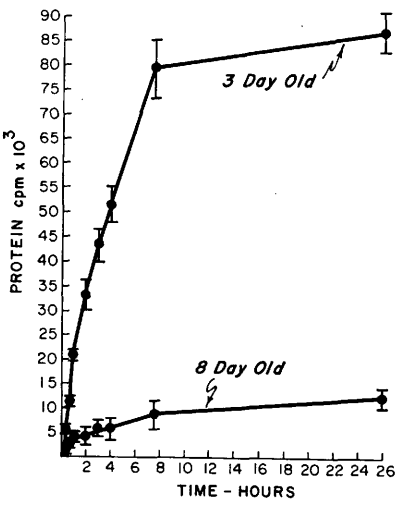

FIG. 1. Incorporation of ^{14}C lysine into protein by preparations derived from 3- and 8-day-old embryos. Whole embryos dispersed by gentle homogenization in 2 ml medium 199 containing 0.25 μCi/ml of ^{14}C-lysine, incubated at 37°, and assayed at times shown. Each point represents an average of 4 replicate experiments.

in buffered medium with isotopically labeled precursor (0.25 μCi/ml). Sugar determinations were done as described in (21–23).

Results. Gently homogenized preparations of whole chick embryos incorporated ^{14}C-lysine into protein. It was observed, however, (Fig. 1) that this incorporation was greater in preparations derived from 3-day-old embryos than in those from 8-day-old embryos. Mixtures of these preparations (Fig. 2) demonstrated inhibitory activity to be present in preparations from the older embryos. In the experiment shown only 31% of the expected lysine incorporation of the younger embryo preparation occurred in the mixture. Similar results were obtained with soluble extracts of 8-day-old embryos.

The inhibitory properties of extracts of 8-day-old embryos were studied by observing their effects on lysine incorporation by homogenized preparations of 3-day embryos. Dialysis of these extracts completely removed their inhibitory properties (Table I). Be-

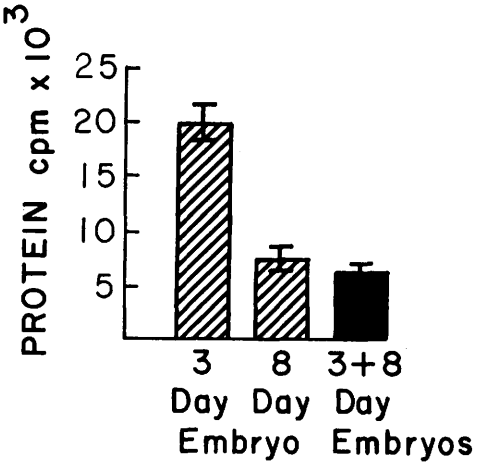

FIG. 2. Incorporation of ^{14}C-lysine into protein by 3- and 8-day-old embryo preparations and mixtures. Whole embryos dispersed by gentle homogenization in 1 ml medium 199. To each of these preparations, 1 ml 0.15 *M* NaCl added, except for the mixture (black bar) where both dispersates were mixed. ^{14}C-Lysine present at 0.25 μCi/ml. Incubated at 37°, for 4 hr. Values are averages of 4 replicate experiments.

TABLE I. Effect of Dialysis upon Inhibitor Potency of Crude Extract Supernatant Fluid Derived from 8-Day-Old Embryos.[a]

	Protein synthesis (cpm, lysine uptake)	Inhibition (% of control)
Control, 3-day embryo preparation	7226 ± 608	—
Undialyzed 8-day-old embryo extract added	1610 ± 142	77.8
Dialyzed 8-day-old embryo extract added	8099 ± 1010	0

[a] (Dialysis for 24 hr against 3 changes of Hanks' balanced salt solution.) Each experiment was done in quadruplicate. Incubation mixture: 1 ml medium 199 containing 3-day-old embryo dispersed by gentle homogenization plus 1 ml 0.15 *M* saline (control) or 1 ml of extract of 8-day-old embryo homogenized in 0.15 *M* saline. ^{14}C-Lysine, 0.25 μCi/ml, incubated at 37° for 2 hr.

TABLE II. Effect of Ultrafiltration Through Diaflo Membrane Filters on Inhibitory Potency of Undialyzed Supernatant Fluids from 8-Day Embryos.[a]

	Protein synthesis (cpm, lysine uptake)	Inhibition (% of control)
Control, 3-day embryo preparation	8416 ± 628	—
Crude 8-day-old embryo extract added	1612 ± 128	80.8
UM10 diffusate from 8-day-old embryo extracts added	1110 ± 87	86.8
UM2 diffusate from 8-day-old embryo extracts added	1428 ± 84	83.0

[a] Each experiment was performed in quadruplicate. Incubation mixture: 1 ml Hanks' balanced salt solution containing 3-day-old embryo dispersed by gentle homogenization plus 1 ml 0.15 *M* saline (control) or 1 ml of test solutions in saline. ^{14}C-Lysine, 0.25 μCi/ml, incubated at 37° for 2 hr.

cause this suggested that the inhibitors might be of relatively low molecular weight, ultrafiltration of undialyzed extracts was performed. Full inhibitory activity was present in diffusates obtained after passage through both UM10 and UM2 filters, indicating that the inhibitory factor could pass through filters designed to retain molecules greater in molecular weight than 10,000 and 2000 (Table II). The concentrated material retained by the filters lost inhibitory activity after redilution to original volumes.

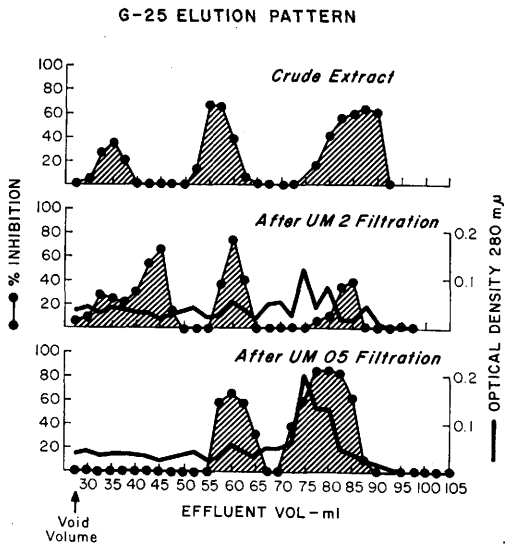

Fig. 3. Gel filtration chromatography of extract of 8-day-old embryo: G-25 Sephadex column, 103 × 1.5 cm; void volume (dextran blue), 25 ml; sample size, 4 ml. Eluates (1 ml) added to 1 ml containing 3-day-old embryo dispersed by homogenization and ^{14}C-lysine (0.25 μ Ci/ml final conc). Assays done in duplicate after incubation 37° for 4 hr and expressed as percentage inhibition compared to control 3-day-old embryo preparations to which only 1 ml 0.15 *M* NaCl had been added. Inhibitory zones shown in shaded areas.

Gel filtration chromatography of extracts over Sephadex G-25 (Fig. 3, upper panel) demonstrated inhibitory activity to be present in three eluate zones. Each of these inhibitory zones appeared in a volume greater than that of the void volume. As shown in the lower two panels of Fig. 3, all three zones of inhibitory activity were demonstrable in the UM2 diffusate, while only zones II and III were present in the diffusate obtained from UM05 filters. These filters are designed to retain molecules greater in mol wt than 500. Approximate molecular size of the inhibitory factors present in zones I, II, and III was determined by gel filtration on columns of G-15 Sephadex. Inhibitor I had an approximate mol wt of 600–700, II of 450, while III was eluted in a volume greater than that for sodium chloride, suggesting that it was retarded, or bound to the column, for reasons other than those of size alone (Table III).

Each of the three inhibitor factors blocked the uptake of isoleucine as well as lysine by chick embryo preparations in an identical fashion (Table IV). None of the three factors interfered with thymidine uptake, although preparations of inhibitor III blocked

TABLE III. Elution Characteristic of Inhibitors I, II, III from G-15 Sephadex.[a]

	Elution vol (ml)	
Standard substances		Mol wt (e.n.i.)
glucose	182	180
lactose	164	360
raffinose	145	504
Inhibitor preparation		Determined mol wt—approx
I	125	600–700
II	157	450
III	265	Under 500

[a] Column: 60 × 3 cm, void volume (dextran blue), 111 ml.

uridine incorporation. The three factors were stable to heating at 100° for 30 min, and treatment of them with RNAse, DNAse, trypsin and pronase did not diminish their activity. In these experiments enzymes (0.2 and 0.1 mg/ml) were added to the inhibitors in Hanks' balanced salt solution at pH 7.4 for 30 min and 1 hr at 37°. These mixtures were then chromatographed over G-15 Sephadex to remove residual enzyme or other products from the inhibitors, which were recovered in the expected elution positions, and then tested in the assay system.

None of the three inhibitors demonstrated proteolytic activity. This was determined by incubating preparations of 3-day-old embryos with ^{14}C-lysine, and after 6 hr separating the supernatant fluid, which contained newly synthesized, radioactive soluble proteins. The inhibitor preparations were added to these fluids, incubated at 37°, and at intervals, the radioactivity of precipitates formed with trichloroacetic acid was determined. There was no loss of radioactivity with time.

Preparations of these three inhibitors were found on amino acid analysis to contain a variety of amino acids and peptides. There were small amounts of lysine present (0.25–8 μg/ml); however, addition of these amounts of lysine did not produce artifactual inhibition of radioactive amino acid uptake in the assay system. Pentose and deoxypentose were not detected in active inhibitor preparations.

Discussion. The precise chemical nature of these embryonic low molecular weight inhibitors of protein synthesis remains unknown. They were dialyzable, heat stable and of three apparent molecular sizes or weights (I, 600–700; II, approx 450; III, under 500). They were unaffected by incubation with DNAse, RNAse, trypsin or pronase.

Low molecular weight substances, such as polyamines (15, 16), purine and pyrimidine bases and their derivatives (12–14) and ketoaldehydes (5, 6, 7), as well as a variety of other substances, have been encountered in nature which interfere with protein synthesis or disturb normal cell functions. Evidence, thus far, indicates that the inhibitors described here are different from other embryonic substances. One such, found in frog embryos, was of high molecular weight and was heat labile (4). Coogan and co-workers

TABLE IV. Effect of Partially Purified Inhibitors on Incorporation of ^{14}C-Lysine and ^{14}C-Isoleucine by 3-Day-Old Embryo Preparations.[a]

	Protein synthesis (cpm)		Inhibition of incorporation (%) of	
	Lysine	Isoleucine	Lysine	Isoleucine
Control (3-day-old embryo preparation)	7418 ± 806	4300 ± 286	—	—
+ I	3126 ± 212	2100 ± 171	57.7	51.3
+ II	3541 ± 382	1885 ± 176	52.2	56.2
+ III	2001 ± 304	1072 ± 93	73.0	75.0

[a] Incubation mixture: 1 ml medium 199 containing 3-day-old embryo dispersed by gentle homogenization plus 1 ml 0.15 *M* saline (control) or inhibitor solutions prepared by chromatography of saline extracts of 8-day-old homogenized embryos. ^{14}C-Lysine, 0.25 μCi/ml, incubated at 37° for 2 hr.

(3) noted inhibition of fibroblast growth by an ultrafiltrate of 7-day-old chick embryos. This material was heat stable, but further characterization was not developed.

The mechanism of action of these low molecular weight substances is not known. They do not possess proteolytic activity, and the amount of amino acid present in the preparations was not sufficient to induce artifactual inhibiton by competition with the radioactive lysine. Moreover the inhibition of the uptake of radioactive isoleucine was in close agreement with that of lysine, suggesting action of these substances on protein synthesis. The inhibitors did not, however, cause cessation of all cell function since the incorporation of precursors into nucleic acids occurred normally in the presence of I and II, and the incorporation of thymidine into nucleic acid occurred normally in the presence of III.

The role of these low molecular weight substances, which were noted by the second week of embryonic life, as possible modulators of the growth process is under study.

Summary. Low molecular weight substances isolated from 8-day-old chick embryos inhibited incorporation of lysine and isoleucine into TCA-precipitable protein. One of these, moreover, blocked the uptake of uridine into nucleic acid.

They were heat stable and dialyzable. Inhibitor I had an approximate mol wt of 600–700; II had an approximate mol wt of 450; III was under 500. Treatment with RNAse, DNAse, trypsin and pronase did not result in loss of activity of the inhibitors. None of the inhibitors manifested proteolytic activity.

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