were morphological differences between mutant and normal primary inductor tissue—anterior endoderm.

We obtained fertilized eggs from matings between adult axolotls heterozygous for the cardiac-lethal gene. The normal and mutant siblings were reared in the same containers at 18°C until ready for study, at which time they were removed from their jelly coats and prepared for processing. The embryos were staged by using the system of Schreckenberg and Jacobson (*3*). Normal and mutant sibling embryos at stage 34 (about 165 hours after fertilization) and at stage 37 (about 195 hours after fertilization) were studied. Stage-34 embryos were used since this is the first stage that mutant embryos, because they lack heartbeats, can be distinguished from normal ones. Stage-37 embryos are approximately 1 day older. The embryos were anesthetized in 1:10,000 tricaine methanesulfonate (Finquel from Ayerst Laboratories, New York) and were fixed 12 to 24 hours in 3 percent glutaraldehyde or in a glutaraldehyde–formaldehyde–picric acid mixture buffered with 0.10*M* phosphate to *p*H 7.3. The specimens to be used for transmission electron microscopy were fixed again for 2 hours in 1 percent osmium tetroxide buffered to *p*H 7.2 with 0.10*M* phosphate, dehydrated in ethanol, and infiltrated and embedded in Epon. Thick sections were made for orientation, and thin sections of the endoderm were mounted on bare copper grids, stained with uranyl acetate and lead citrate and viewed at 80 kv on an electron microscope (JEOL 100B or Philips 300). Those specimens to be viewed in the scanning electron microscope were dissected after the initial fixation to expose the anterior endoderm in its entirety, with special care being taken to preserve the spatial relationships between endoderm and heart. The pieces of tissue were then fixed for 30 minutes in 1 percent osmium tetroxide buffered to *p*H 7.2 with 0.10*M* phosphate, dehydrated in graded ethanol solutions, cleared in 100 percent acetone, and prepared with a critical-point drying apparatus (Sorvall). The dried specimens were gold coated and viewed in a Cambridge Stereoscan electron microscope at acceleration voltages of 5 or 10 kv.

Histological examination of the endoderm of stage-35 and stage-37 mutant embryos shows no obvious variation from normal. The one-cell-layer-thick anterior endoderm appears to have a close structural association with the heart tube just as in normal embryos (Fig. 1a). Observations made with a transmission electron microscope of thin sections through various levels of normal and mutant anterior endoderm (gut) at stages 35 and 37 were performed. We were careful to take sections from comparable levels through the endoderm of the normal and mutant siblings; four normal and four mutant specimens at each stage were studied. At stage 35, the mutant endoderm cells have many microvilli on their luminal surfaces while the normal endoderm cells have virtually none (Fig. 1, b and c). By stage 37, the normal cells also had some microvilli but still far fewer than mutants; furthermore, the mutant endoderm microvilli appear to be more highly differentiated (*12*) than they are in normal embryos. Scanning electron microscopy of the anterior endoderm from normal and mutant embryos confirms the observations of the transmission electron microscope. After initial fixation, the embryos were dissected to expose the entire luminal surface of the endoderm, which allowed us to make a complete study and comparison of all areas of the endoderm (Fig. 1d). The normal anterior endoderm (Fig. 1e) has many fewer microvilli than does the mutant endoderm (Fig. 1f). The appearance of microvilli on vertebrate intestinal epithelial (gut) cells is an early indication of their specialized differentiation (*12*, *13*). Thus, our morphological data suggest that the anterior endoderm in cardiac-lethal mutant axolotls has differentiated more rapidly than is normal. This finding could indicate that the mutant endoderm has differentiated beyond the point at which it can induce the precardiac mesoderm to finally become contracting heart tissue; the mutant endoderm may even have an inhibitory effect of some kind.

Whatever the final mechanism turns out to be, gene *c* in axolotl embryos alters the anterior endoderm—the primary heart-inducting tissue. At the same time, it prevents final differentiation of the heart, which fails to begin beating.

LARRY F. LEMANSKI, BARRY S. MARX
CRAIG S. HILL
Department of Anatomy, School of Medicine, University of California Medical Center, San Francisco 94143, and *Cell Biology and Cardiology Research Sections, Veterans Administration Hospital, San Francisco 94121*

References and Notes

1. R. R. Humphrey, *Dev. Biol.* **27**, 365 (1972).
2. L. F. Lemanski, *ibid.* **33**, 312 (1973).
3. G. M. Schreckenberg and A. G. Jacobson, *ibid.* **42**, 391 (1975).
4. L. F. Lemanski, *Am. J. Anat.* **136**, 487 (1973).
5. ———, *J. Morphol.* **139**, 301 (1973).
6. ———, M. S. Mooseker, L. D. Peachey, M. R. Iyengar, *J. Cell Biol.* **68**, 375 (1976).
7. L. F. Lemanski, X. Joseph, M. R. Iyengar, *ibid.* **67**, 239a (1975).
8. A. G. Jacobson and J. T. Duncan, *J. Exp. Zool.* **167**, 79 (1968).
9. O. Mangold, *Naturwissenschaften* **43**, 287 (1956); *ibid.* **44**, 289 (1957); H. Amano, *Doshisha Eng. Rev.* **8**, 203 (1958).
10. F. Orts-Llorca, *Arch. Entwicklungsmech. Org.* **154**, 533 (1963); ——— and D. R. Gill, *ibid.* **156**, 368 (1965).
11. O. R. Hommes, *Primary Entodermal Defects. Development of Body Form and Genital Organs of Acardii in Uni-vitelline Twins* (Jacob van Campen, Amsterdam, 1957).
12. T. M. Mukherjee and A. W. Williams, *J. Cell Biol.* **34**, 447 (1967).
13. P. C. Mofey and J. S. Trier, *Gastroenterology* **68**, 1002 (1975); G. DeRitis, Z. M. Folchuk, J. S. Trier, *Dev. Biol.* **45**, 304 (1975).
14. This work was done during the tenure of an established investigatorship from the American Heart Association awarded to L.F.L. The study was supported by grant HL 18480 from the National Institutes of Health and by a grant-in-aid from the American Heart Association. Also supported in part by faculty grants from the Academic Senate and Research Evaluation and Allocation Committee of the University of California, San Francisco. We thank L. Lawrence and M. Nakanishi for technical assistance and T. L. Christianson for typing the manuscript. Dr. L. Peachey permitted us the use of his laboratory for portions of the study.

7 September 1976; revised 17 November 1976

Lymphocyte and Fibroblast Chalones: Some Chemical Properties

Abstract. *Experiments with lymphocyte and fibroblast chalones indicate that they are complexed strongly with RNA in tissue extracts; have a molecular weight of less than 10,000 and more than 1,000 daltons, and probably about 5,000 daltons; are strongly cationically charged; and contain mannose and perhaps other carbohydrates.*

Cell-specific and endogenous proliferation inhibitors have been reported for a number of tissues (*1*). These inhibitors were called chalones by Bullough (*2*). Moorhead *et al.* (*3*) claimed that lymph nodes contained a chalone which inhibited lymphocyte transformation and had a molecular weight of more than 40,000 daltons. We have shown that ultrafiltration in the range 50,000 to 30,000 daltons concentrates most of the cell-specific inhibitory activities (chalone) of aqueous extracts of lymphoid tissue toward lymphocytes (*4*). We have also found that by ultrafiltration within this range, most of the cell-specific inhibitor for fibroblast proliferation may be concentrated from the "used" medium in which WI-38 or other diploid human fibroblasts were cultivated (*5*). Aqueous extracts of lung, which contain a large number of fibroblasts, after concentration by ultrafiltration in this molecular weight range, also demonstrate nonspecies-specific, noncytotoxic, and reversible inhibition of fibroblast proliferation in vitro (*6*).

For the past 3 years we have attempted to purify these inhibitory activities and have been almost totally unsuccessful. We have now determined why so much difficulty was encountered in the application of various purification procedures, and thereby have learned something about the composition of both lymphocyte and fibroblast chalones.

Chalone activities concentrated by membrane ultrafiltration from aqueous extracts of both lung and spleen were determined by inhibition of [^{3}H]thymidine incorporation into acid-insoluble DNA in phytohemagglutinin-stimulated human lymphocytes (*4*) or by inhibition of the proliferation rate of 3T3 fibroblasts (*7*). These activities were destroyed by incubation with trypsin or chymotrypsin, but not by incubation with either ribonuclease or dseoxyribonuclease (*1*, *4*). In view of the large amount of nucleic acids absorbing at 260 nm in these aqueous bovine tissue extracts after ultrafiltration, we decided to treat them with trypsin-free ribonuclease (Worthington Biochemical Corp.) and then ultrafilter them through Amicon membranes to remove the small reaction products of the action of this enzyme. As shown in Table 1, none of the chalone activity of either type would pass through a 10,000-dalton Amicon filter (UM-10). After ribonuclease treatment (10 μg/ml for 4 hours at 37°C and *p*H 7.0), however, all of both chalone activities were found exclusively in the range 1,000 to 10,000 daltons.

Ribonuclease passes completely through the UM-10 membrane and might inhibit fibroblast and lymphocyte proliferation directly. Separate experiments indicated that ribonuclease at 10 μg/ml did not inhibit the proliferation rate of 3T3 cells in vitro. Also, a 20-fold dilution of splenic ultrafiltrate after ribonuclease treatment for assay (from 1.0 to 0.05 mg) reduced the ribonuclease concentration to 0.5 μg/ml; at this concentration no inhibition of phytohemagglutinin-stimulated lymphocyte transformation was found. Further, it should be noted that the total quantitative inhibitory activity was essentially the same before and after ribonuclease treatment and that after ribonuclease treatment all of the inhibitory activity for both systems moved from the fraction heavier than 10,000 daltons into the fraction lighter than 10,000 daltons. These data suggest that the inhibitors had formed a very strong complex with relatively small molecules of RNA. They further suggest that the molecular weight of both chalone inhibitors was considerably less than 30,000 daltons. If the bulk of the RNA of this size is transfer RNA (which has a molecular weight of approximately 25,000 daltons), and since the complex moves rapidly through a 50,000-dalton filter and very slowly through a 30,000-dalton filter, then each chalone activity had a molecular weight of about 5,000 daltons. These findings also suggest that the chalone polypeptide must be cationic in order to form such a strong complex with the anionic polyelectrolyte RNA.

It is also possible that RNA contributed to the cell specificity of chalone action. Therefore, the ribonuclease-treated splenic ultrafiltrate was tested for inhibitory activity against 3T3 cell proliferation at doses ten times higher than that required for 50 percent inhibition of phytohemagglutinin-stimulated lymphocyte transformation. No such inhibition could be demonstrated.

A 10-mg portion of each ultrafiltrate (50,000 to 30,000 daltons) prepared from aqueous extracts of spleen or lung was dissolved in 20 ml of 0.1*M* acetate buffer (*p*H 6) containing 1*M* NaCl, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, and 1 m*M* $MnCl_2$. This material was introduced into a 100-ml concanavalin A–Sepharose (Pharmacia) column and eluted with the buffer until no further material absorbing at 280 nm could be removed. At this time the column was washed with a solution of 0.1*M* methyl-α-D-glucopyranoside (*8*). The bulk of both inhibitory activities was found to be adsorbed to the column and to be released from it at this concentration of sugar. This evidence suggests that both chalone activities involved covalently bound mannose.

From the experiments described above we conclude that (i) both lymphocyte and fibroblast chalones have a molecular weight of less than 10,000 and more than 1,000 daltons, and probably about 5,000 daltons; (ii) these polypeptides must be strongly cationically charged; and (iii) they contain mannose and perhaps other carbohydrates. If two such different chalone activities share these three properties, it is tempting to conclude that other chalones might be generally described as cationic glycopeptides of this general size.

Table 1. Percentage inhibition of 3T3 fibroblast proliferation or [^{3}H]thymidine incorporation into acid-insoluble DNA of normal, phytohemagglutinin-stimulated lymphocytes by various-sized ultrafiltrates of aqueous extracts of calf lung or spleen, respectively, before or after ribonuclease treatment for 4 hours at 37°C (trypsin-free enzyme, 10 μg/ml, *p*H 7.0). Each value in column 2 is the mean of four areas (0.19 mm^2) in each of three Leighton tubes, 48 hours after seeding with 5×10^4 3T3 cells in 10 percent calf serum and minimal Eagle medium (*7*). Each value in column 3 is the mean uptake by triplicate cultures of 5×10^5 human peripheral lymphocytes of [^{3}H]thymidine into acid-insoluble material 6 hours after isotope addition and 72 hours after phytohemagglutinin stimulation in 10 percent fetal calf serum and medium 199 (*4*). Ultrafiltrates were prepared by extracting, in a Waring blender at 4°C, 100 g of calf spleen or lung with 1000 ml of distilled water and storing with stirring for 18 hours at 4°C. After centrifugation, 24-hour dialysis against 200 volumes of water, and lyophilization, the extracted materials were redissolved in distilled water (2 to 5 mg/ml), passed through an Amicon XM-50 membrane (50,000 daltons), and concentrated and washed on a UM-10 membrane (10,000 daltons). The materials passing through the UM-10 membrane were in turn washed and concentrated on a 1,000-dalton filter. All ultrafiltered materials were then lyophilized and stored at 4°C.

Molecular weight (daltons)	Inhibition (%)	
	Lung (3T3 proliferation)	Spleen ([^{3}H]thymidine uptake)
Untreated		
50,000 to 10,000		
1.0 mg/ml	68	
0.5 mg/ml	33	
0.05 mg/ml	0	55
10,000 to 1,000		
1.0 mg/ml	9	
0.5 mg/ml	0	
0.05 mg/ml	0	0
After ribonuclease treatment		
50,000 to 10,000		
1.0 mg/ml	7	
0.5 mg/ml	0	
0.05 mg/ml	0	0
10,000 to 1,000		
1.0 mg/ml	73	
0.5 mg/ml	53	
0.05 mg/ml	0	68

J. C. HOUCK
Virginia Mason Research Center, Seattle, Washington 98101
K. KANAGALINGAM
C. HUNT
A. ATTALLAH
Children's Hospital, Washington, D.C. 20009
A. CHUNG
Department of Surgery, Georgetown University Medical School, Washington, D.C. 20007

References and Notes

1. J. C. Houck and H. Hennings, *FEBS Lett.* **32**, 1 (1973); J. C. Houck, Ed., *Chalones* (American Elsevier, New York, 1976).
2. W. S. Bullough, *Biol. Rev.* **37**, 307 (1962).
3. J. F. Moorhead, E. Paraskova-Tchernozenska, A. Pirrie, C. Hayes, *Nature (London)* **224**, 1207 (1969).
4. J. C. Houck and S. Leikin, *Science* **173**, 1139 (1971); A. Attallah, G. Sunshine, C. Hunt, J. C. Houck, *Exp. Cell Res.* **93**, 282 (1975); A. Attallah and J. C. Houck, in *Chalones*, J. C. Houck, Ed. (American Elsevier, New York, 1976), p. 355.
5. J. C. Houck, R. Weill, V. Sharma, *Nature (London) New Biol.* **240**, 210 (1972).
6. J. C. Houck, in *Chalones*, J. C. Houck, Ed. (American Elsevier, New York, 1976), pp. 247–264.
7. E. Raff and J. C. Houck, *J. Cell. Physiol.* **74**, 235 (1969).
8. E. Heidemann, A. Jung, K. Wilm, *Klin. Wochenschr.* **54**, 221 (1976).
9. Supported in part by NIH grant CA-14,484 and by a contract from the Office of Naval Research.

29 October 1976