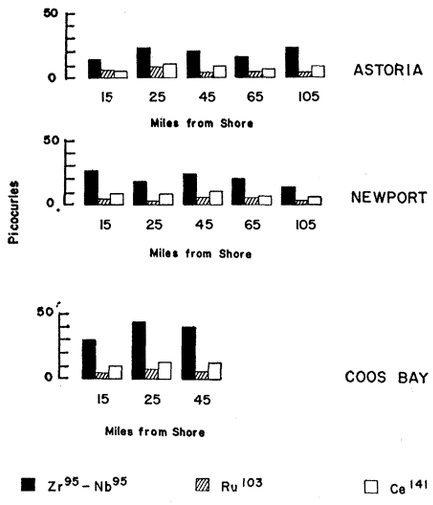

Fig. 2. Euphausiids collected 26 March to 6 April 1962. See legend of Fig. 1 for details.

Radioanalyses of euphausiids taken at the same series of stations (Figs. 1 and 2) in the summer of 1961 (before Russian tests) failed to show any fallout radioactivity at the same level of detection, although Zn^{65} was plentiful. The conclusion is that the three fission products taken up by the euphausiids were part of the airborne fallout associated with the Russian tests (*8*).

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8. This investigation was carried out during the tenure of a predoctoral fellowship from the division of general medical sciences, U.S. Public Health Service. Permission has been received from the graduate school of Oregon State University to publish data which are to appear in a Ph.D. thesis now in progress. The technical assistance of R. W. Perkins of Hanford Laboratories is gratefully acknowledged, as is the cooperation of Edward Brinton of Scripps Institution of Oceanography, who furnished the two plankton samples from off the southern California coast. Special thanks are due N. Kujala and L. Hubbard for collecting the bulk of the biological material, and to Sue Borden for carrying out the electronic data reduction program.

27 July 1962

RNA: A Marker in Embryonic Differentiation

Abstract. A modified fluorochrome technique which identifies nucleic acids differentially in tissues permits following production of RNA in the embryo. Compact primordial chick embryo cells show a sequence to RNA production as differentiation begins: First in the nucleus, then in the perinuclear area, then in the cytoplasm. RNA increases in the cytoplasm with cell growth and continues as the cells merge to form an epithelium.

The incredible complexities of biological systems thwart any simple description of embryonic differentiation. Customarily progressive diversification of the embryo is studied in relation to some "end-point," such as the appearance time and activity of an enzyme or other biological substance, or some change in morphology of cells or tissues. Whatever the milepost chosen, studies should attempt to localize and identify this "end-point" in tissues, to follow it through its lifetime or the organism's (embryo to adult), and to quantitate it biochemically. In the course of studies in a laboratory on the metabolism of nucleic acids and protein in the chick embryo it became necessary to localize RNA in cells and tissues. By means of a modification of a fluorochrome method for differential histochemical identification of nucleic acids, the sequence of early changes seen in primordial chick embryo cells was studied. Intracellular appearance and distribution of RNA was used as the "end-point."

The embryos studied were from a pure strain of chicken, the sex-linked Hallcross (*1*). Cleaving eggs were removed from hens' uteri for study at varying times before organization of the primordial cells into blastoderm epithelium occurred. Stages of cleavage of fertilized eggs which remained in hens' oviducts or uteri were estimated from the times the preceding eggs were laid. It was thus possible to recapitulate the formation of an epithelium by the highly condensed, compact primordial cells, from the time when they were little more than nuclei until they merged into a continuous epithelium.

Conventional staining techniques which localize RNA histochemically lack the sensitivity, reproducibility, and sharp differentiation between RNA and DNA which is afforded by fluorescent histochemical techniques (*2*). The best-known fluorochrome techniques, originated by Strugger (*3*) and studied more recently by Armstrong (*4*) and by von Bertalanffy (*5*), employ acridine orange. Under appropriate conditions acridine orange is conjugated by the nucleic acids and gives a brilliant polychromatic differentiation between DNA, which fluoresces green, and RNA, which fluoresces red. The following method, adapted for embryonic tissues, is a modification of that of von Bertalanffy (*5*) and Bertalanffy (*6*) for detecting malignant cells.

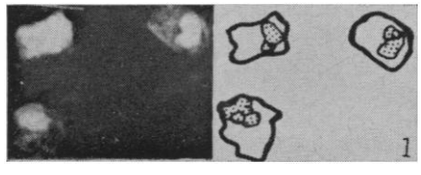
Tissues were fixed in Carnoy's solution at 4°C for 6 to 12 hours, washed in three changes of absolute alcohol over 2 hours at 4°C, cleared in cedarwood oil, and embedded in paraffin; sections were mounted and cut at 3 microns' thickness, and the paraffin was removed with xylol. The sections were hydrated rapidly, in 80, 70, and 50 percent alcohols, successively and then they were placed in 0.002*M* $MgSO_4$. Once the sections were hydrated, the presence of 0.002*M* $MgSO_4$ in all subsequent solutions resulted in better polychromatic differentiation, cell integrity, reproducibility, and stability. Thus, every solution in the following steps contained 0.002*M* $MgSO_4$. Sections were then placed in 1 percent acetic acid for 1 minute, rinsed in $MgSO_4$ solution, stained for 3 minutes in 0.01 percent acridine orange in .067*M* Na_2HPO_4 and KH_2PO_4 buffer (*p*H 6), and rinsed 5 minutes or more (until excess aridine orange had been removed) in the *p*H 6, .067*M* phosphate buffer. After 2 minutes in 0.1*M* $CaCl_2$ solution, sections were again rinsed in phosphate buffer, mounted in a drop of buffer with a thin coverslip, and sealed with paraffin.

Observations were made with a Leitz (Ortholux) microscope and fluorescence equipment. Light from a 200-watt, high-pressure mercury lamp was passed through a blue BG-12 (Schott) filter to transmit light of 4150 Å wavelength, and fluorescence was observed through a suppression filter OG-1 (Schott) mounted in the barrel of the microscope. Photomicrographs were taken on 35-mm high-speed Ektachrome color film with an Exacta single-lens reflex camara.

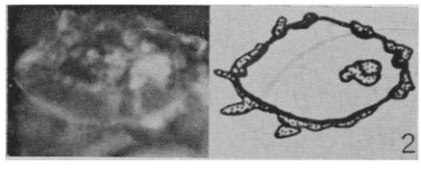
Companion control sections for every section examined were treated with ribonuclease (*7*). Any fluorescence present after ribonuclease treatment was presumed to be due to substances (notably mucopolysaccharides)

other than RNA. Other specificity studies were done: Treatment with deoxyribonuclease left RNA intact, but DNA fluorescence of nuclei disappeared; trypsin digestion did not alter DNA or RNA fluorescence; pepsin digestion was not completely satisfactory because of the low tissue *p*H necessary, but under these conditions no change in RNA or DNA occurred; hot trichloracetic acid extracted all detectable RNA and DNA; a methylene blue extinction series corresponded to RNA fluorescence. The possibility of diffusion artifacts during fixation was eliminated by comparing the appearance and distribution of the polychromatic fluorescence with embryonic tissues which had been prepared by the freeze-dry technique (*7*).

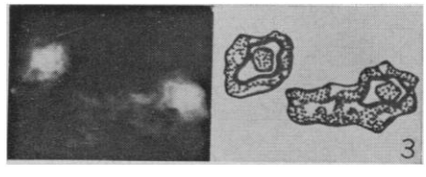
The following six figures show the successive steps in differentiation of primordial embryonic cells into epithelia. These are halftone reproductions of fluorescent color photomicrographs and do not show the marked polychromatic differentiation which is observed directly through the microscope or which is seen in great contrast on color film. The line tracings adjoining the prints are camera lucida tracings from the color slides. RNA fluoresence is stippled, and the green fluorescing nuclei are drawn in heavy line.



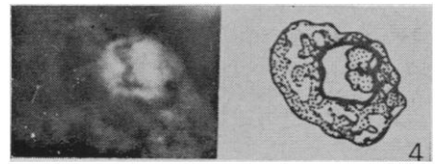
The first change seen is the production of nucleolar RNA in the nucleus of the highly condensed primordial embryonic cell (about × 1000).



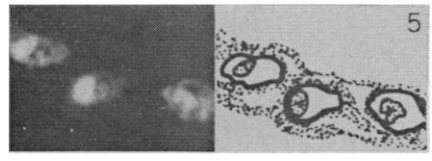
RNA is promptly seen at various areas along the periphery of the nucleus (about × 2000).



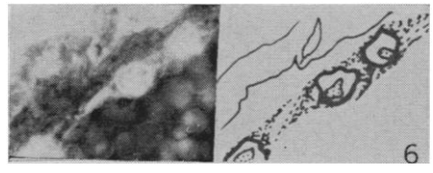
RNA is then seen in the cytoplasm (× 1000).



RNA in the cytoplasm increases as the cell takes shape (× 1000).



Finally cells coalesce as an epithelium emerges (× 1000).



In Fig. 6 is seen a deeper epithelial layer, approximately 24 hours after fertilization and after the blastoderm has been established. The coalescence is similar to Fig. 5. The thin lines in the upper left are outlines of epithelia, purposely left unstippled (× 1000).

The RNA in the unfertilized ovum is currently under investigation as a possible source of early "structural information" (*8*). Most RNA is found toward the animal pole of the egg, the bulk nearest the blastodisc, but fanning out from here in a diminishing arc well beyond the blastodisc. It has been observed repeatedly that early embryonic cells seem to "arrange" themselves in this RNA which is no longer found by about 24 hours after fertilization when the blastoderm has formed. In local areas, RNA seems to disappear earlier, perhaps depending upon the thickness of the forming cell layers.

The significance of these observations is, of course, yet to be established, but current investigation is proceeding along temptingly speculative lines as if this "free RNA" in the ovum carries information for primordial structure and perhaps is a contribution solely from the maternal organism.

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8. With this modified method studies have been in progress of both qualitative and correlative quantitative aspects of organ development and embryo development. Of special interest has been the "life cycle" of RNA in particular cell lines, the manuscript of which is now in preparation.
9. Supported by grants from the Association for the Aid of Crippled Children and the James Hudson Brown Memorial Fund of the Yale University School of Medicine. Presented in part before the Society for Pediatric Research, Atlantic City, N.J., 8–10 May 1962. We are grateful to Dr. Russell J. Barnett, Department of Anatomy, Yale University School of Medicine, for advice and criticism.

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Amino Acids in Deep-Sea Water

Abstract. Approximately 17 different amino acids in acid hydrolyzates of dissolved organic material in sea water to depths of 3500 meters from the central Gulf of Mexico have been identified by ion-exchange resin chromatography. They are glutamic acid, lysine, glycine, aspartic acid, serine, alanine, leucine, valine plus cystine, isoleucine, ornithine, methionine sulfoxide, threonine, tyrosine and phenylalanine, histidine, arginine, proline, and methionine. The results indicate that the dissolved organic material in deep-sea water may make an important contribution to the organic budget of the sea.

Parsons and Strickland have determined the concentration of organic material in oceanic detritus in the northeastern Pacific Ocean (*1*). They found that the amount of particulate carbon at 3000 m was not appreciably different from that at 300 to 500 m. They detected glycine, alanine, glutamic acid, aspartic acid, lysine, arginine, and proline in the oceanic detritus by paper chromatography. Erdman *et al* have reported the presence of ten amino acids in sediments from the Gulf of Mexico (*2*).

To identify dissolved organic material in sea water one must deal with the presence of about 10^4 times by weight more inorganic salts than organic material. Various techniques which have been evaluated for the isolation of dissolved organic material include dialysis, adsorption, ion exchange, sol-