[5] Watson, J. D., and F. H. C. Crick, in *Viruses*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 18 (1953), p. 123.
[6] Koch, A. L., and C. Miller, *J. Theoret. Biol.*, **8**, 71 (1964).
[7] Fong, P., these PROCEEDINGS, **52**, 641 (1964).
[8] Dounce, A. L., *J. Theoret. Biol.*, **2**, 152 (1962).
[9] Cavalieri, L. F., and B. J. Rosenberg, in *Progress in Nucleic Acid Research*, ed. J. N. Davidson and W. E. Cohn (New York: Academic Press, 1963), vol. 2, p. 1.
[10] Butler, J. A. V., *Nature*, **199**, 68 (1963).
[11] Löwdin, P. O., in *Electronic Aspects of Biochemistry*, ed. B. Pullman (New York: Academic Press, 1964), p. 167.
[12] Sibatani, A., and S. Hiai, *J. Theoret. Biol.*, **7**, 393 (1964).
[13] Stonehill, E. H., *J. Theoret. Biol.*, **9**, 323 (1964).
[14] Jehle, H., these PROCEEDINGS, **53**, 1451 (1965).
[15] Shildkraut, C. L., C. R. Richardson, and A. Kornberg, *J. Mol. Biol.*, **9**, 24 (1964).
[16] Cairns, J., *J. Mol. Biol.*, **6**, 298 (1963).
[17] Cairns, J., in *Synthesis and Structure of Macromolecules*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 28 (1963), p. 43.
[18] Bessman, M. J., I. R. Lehman, E. S. Simms, and A. Kornberg, *J. Biol. Chem.*, **233**, 171 (1958).
[19] de Waard, A., A. V. Paul, and I. R. Lehman, these PROCEEDINGS, **54**, 1241 (1965).
[20] Speyer, J. F., *Biochem. Biophys. Res. Commun.*, **21**, 6 (1965).
[21] Monod, J., J. Wyman, and J. P. Changeux, *J. Mol. Biol.*, **12**, 88 (1965).
[22] Kornberg, A., *Advan. Enzymol.*, **18**, 191 (1957).
[23] Baldwin, R. L., in *The Bacteria*, ed. I. C. Gunsalus and R. Y. Stanier (New York: Academic Press, 1964), p. 327.

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*CHANGING POPULATIONS OF MESSENGER RNA DURING SEA URCHIN DEVELOPMENT**

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Protein synthesis is very rapidly initiated after fertilization of the sea urchin egg.[1, 2] On the other hand, RNA synthesis is negligible before fertilization and increases very little, if at all, at fertilization.[3–5] The suggestion has been made that the onset of protein synthesis represents the activation of pre-existing messenger RNA molecules rather than the *de novo* synthesis of new messages. Support for this view has been provided by experiments in which enucleate egg fragments are found to carry on protein synthesis when artificially activated,[3, 6, 7] and by experiments with inhibitors of RNA synthesis.[8] Further, Maggio *et al.*[9] report that RNA fractions from unfertilized eggs will stimulate amino acid incorporation in rat liver test systems, and Monroy *et al.*[10] provide evidence that dormant protein-synthesizing machinery of the unfertilized egg may be activated *in vitro* without evident concomitant RNA synthesis.

The present communication demonstrates directly the presence of messenger RNA in the unfertilized sea urchin egg and compares the populations of messages present before fertilization with those isolated from early stages of development and

from adult tissues. The attribute of messenger RNA used for its assay is the ability to form specific complexes with single-stranded DNA at a reasonably high RNA:DNA ratio. This property is due to complementarity in sequences of nucleotides between the nucleic acids and, therefore, reflects the primary expression of the genetic material. By injecting labeled phosphate into female sea urchins, Gross *et al.*[11] demonstrated the synthesis, during oögenesis, of labeled RNA capable of binding to DNA. In the present study, similarities in populations of RNA's have been measured by ascertaining the capacity of unlabeled samples from one stage or tissue to compete in the binding of labeled RNA. The labeled RNA was prepared from prism embryos so that the assay is limited to those messenger RNA molecules actively synthesized at that time.

Materials and Methods.—Cultivation of embryos: Eggs, obtained from the sea urchin *Strongylocentrotus purpuratus* by injection of isotonic KCl into the coelom or by electrical shock, were inseminated, washed several times with Millipore-filtered sea water, and cultured at 11–13°C in Millipore-filtered sea water containing 0.25 mg/ml streptomycin. The embryos were cultured at less than 0.5% concentration in jars with constant gentle stirring. Only cultures in which fertilization was 98% or better and in which development was morphologically normal were used. The embryos were harvested in a continuous-flow centrifuge at 255 × *g* and washed with filtered sea water containing streptomycin.

Unlabeled RNA was extracted from unfertilized eggs from which the jelly had been removed by brief exposure to sea water at pH 4.5 and from three developing stages. These, as defined by Whiteley and Baltzer,[12] were: 31-hr-old blastulae which were just hatching (hB1), 44-hr-old gastrulae (GaJ 1/4), and 74-hr-old prism embryos (Pr). In the late prism embryos the transverse spicules had not quite met medially and the stomodaeum had not yet broken through to form the mouth. An aliquot of these prisms was also pulse-labeled with P^{32} to label newly synthesized RNA. One female provided the eggs for the unfertilized sample, a second female provided the eggs for the blastulae and gastrulae, and a third female provided the eggs for the prism embryos. Sperm from one male was used for each insemination.

Adult tissue: The gut, including both stomach and intestine, of six adult starved sea urchins and the testes of one unripe mature male were excised, washed in iced sea water, frozen in dry ice, and RNA was extracted.

Pulse labeling of RNA: Three ml of gently packed prism embryos were maintained suspended in a conical centrifuge tube in 20 ml of Millipore-filtered sea water, containing streptomycin, by gently bubbling air through the culture. One mc of P^{32} as orthophosphate was added and after 60 min at 13°C, the embryos were collected by low-speed centrifugation, the insignificant residual unadsorbed P^{32} was removed from the eggs by means of two sea water washes, and the RNA was extracted.

Extraction of RNA: One vol of embryos or tissue was homogenized at 0°C in 2 vol of 0.1 *M* sodium acetate–0.1 *M* NaCl–0.001 *M* $MgCl_2$ at pH 5.2 and containing 3–15 mg bentonite/ml. Two vol of the same solution containing 4% sodium lauryl sulfate were added and the homogenate was shaken for 15 min at 25°C with 5 vol of water-saturated phenol without preservative. The aqueous phase resulting from centrifugation of the emulsion at 25,000 × *g* for 10 min was re-extracted with aqueous phenol and the nucleic acids were precipitated from the aqueous phase at −20°C with 3 vol of ethanol in the presence of 0.1 *M* NaCl. The sample was dissolved in 0.01 *M* sodium acetate–0.002 *M* $MgCl_2$, pH 5.2, and digested for 1 hr with deoxyribonuclease (50 μg/ml, Worthington Biochemical Corp., Freehold, N.J., electrophoretically purified) at 25°C. Residual protein was hydrolyzed for 1 hr at 25°C by adding pronase (50 μg/ml, Calbiochem, Inc., Los Angeles, Calif.) in the presence of 0.05 *M* Tris, pH 7.5.[13] The pronase was first self-digested at 37°C for 2 hr. After two additional phenol extractions, the RNA was reprecipitated with ethanol and dissolved in a minimal volume of 1/10 × SSC (1 × SSC is 0.15 *M* NaCl–0.015 *M* sodium citrate, pH 7.0).

Aliquots were put on a column (1.3 × 30-cm) of Sephadex G-50 overlaid with a 1-cm layer of Dowex-50; the column was equilibrated and eluted with 2 × SSC. Trials with unlabeled *S. purpuratus* RNA showed that with this column approximately 1/3 to 1/2 of the total sRNA was

eluted in 3.0 ml, but that nucleotides were excluded. Bentonite (3–15 mg/ml) was immediately added to the sample which was then stored at −20°C.

RNA prepared as described was susceptible to ribonuclease and alkali digestion. In an experiment with P^{32}-labeled material, exposure for 30 min to 200 μg/ml of ribonuclease (Worthington) at 37°C or to 2 *N* NaOH at 60°C converted 98% of the label to a form not precipitable by 5% TCA.

Extraction of DNA: One gm of washed *S. purpuratus* sperm was suspended in 250 ml of 0.1 *M* EDTA–0.04 *M* Tris, pH 8.2, pronase (50 μg/ml) was added, and the preparation was incubated at 37°C for 8 hr. This preparation was shaken with 1 vol of water-saturated phenol and centrifuged. There was essentially no interphase pad. The DNA was precipitated from the aqueous phase with 3 vol of ethanol, washed, dissolved in 1/10 × SSC at a concentration of 500 μg/ml, heated at 100°C for 10 min, and cooled quickly to convert the DNA to the single-stranded form. The preparation was adjusted to a concentration of 1 × SSC, and the DNA was precipitated with alcohol and redissolved at the desired concentration in 1/10 × SSC.

The concentrations of DNA and RNA were estimated from the absorbancy at 260 mμ.

RNA-DNA binding: Aliquots (0.025 ml) of the P^{32}-labeled RNA, 0.05 ml of a bentonite suspension (20 mg/ml), competing RNA, and sufficient 2 × SSC to make a total volume of 0.475 ml, were mixed in a small test tube. The contents of the tube were transferred with a Pasteur pipette to a screw-cap siliconed vial containing 0.30 gm of DNA-agar prepared as described by McCarthy and Hoyer.[14] A single preparation of DNA-agar was used throughout. The contents of the vial were mixed with a Vortex mixer, and the vial was incubated for 48–60 hr in a 60°C water bath.

After incubation, the DNA-agar was washed at 60°C in 2 × SSC to remove unbound RNA, and then at 73°C in 1/100 × SSC to remove RNA bound to the DNA. Details are described by McCarthy and Hoyer.[14] The labeled RNA's were precipitated with TCA, collected on membrane filters, and assayed with a liquid scintillation counter. Per cent of binding of RNA to DNA is defined as the ratio of radioactivity eluted at 73°C to the total radioactivity precipitated from the two washes.

Results.—Properties of RNA and binding to DNA: The sedimentation profile of RNA obtained from prisms after a 1-hr exposure to radioactive phosphate is shown in Figure 1. Much of the incorporated label appears as a 4*S* component—presumably sRNA. Radioactivity appears not only in the regions of the bulk ribosomal and sRNA components, but also at intermediate positions and in a region indicating a size greater than 30*S*.

This labeled RNA preparation was incubated with DNA-agar to determine the amount required for optimal binding. Various amounts of RNA were incubated for 48 hr at 60°C with aliquots of the same DNA-agar preparation (Fig. 2). Four μg of labeled RNA, which gave an optimal binding of about 22 per cent, were used in most of the competition experiments to be described.

Figure 3 illustrates the kinetics of the binding reaction. The optimal period of incubation at 60°C is about 48 hr. Periods of incubation longer than 90 hr result in lower values, probably reflecting loss of DNA from the agar gel.[15]

Comparison of RNA's from developmental stages: Populations of RNA molecules isolated from four stages in the early development of the sea urchin were compared in competition experiments summarized in Figure 4. Each assay was performed with the same amount of P^{32}-labeled prism-stage RNA, a constant amount of DNA-agar, and various amounts of unlabeled RNA from one of the developmental stages. Each of the sea urchin RNA preparations compete in the binding, but to different extents. As would be expected, unlabeled RNA prepared from the stage identical to that of the pulse-labeled RNA is most effective in this competition. Competition by RNA extracted from unfertilized eggs is not inconsiderable. Simi-

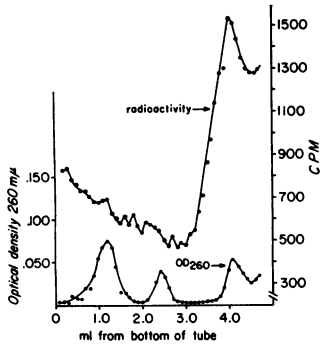

FIG. 1.—Sedimentation properties of P^{32}-labeled *S. purpuratus* prism RNA. Eight μg of RNA were layered on 4.6 ml of a 5-20% sucrose gradient and centrifuged in the cold at 38,000 rpm in a SW39 rotor of a model L Spinco centrifuge for 5 hr. Optical density at 260 mμ and radioactivity were determined.

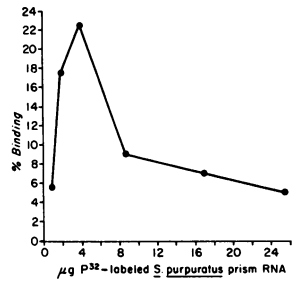

FIG. 2.—Binding of different amounts of P^{32}-labeled *S. purpuratus* prism RNA to a constant amount of *S. purpuratus* DNA-agar. DNA-agar (0.30 gm) containing 110 μg DNA, varying amounts of labeled RNA, total volume = 0.475 ml, incubated at 60°C for 48 hr.

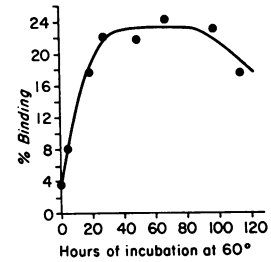

FIG. 3.—Binding of P^{32}-labeled *S. purpuratus* prism RNA to *S. purpuratus* DNA-agar as a function of time. Four μg P^{32}-labeled RNA, 0.30 gm DNA-agar containing 110 μg DNA, total volume = 0.475 ml, incubated at 60°C for time shown.

larly, RNA from blastulae at the time of hatching competes rather strongly and, as seen in Figure 4, this RNA is not clearly distinguishable from that of unfertilized eggs. RNA extracted from early gastrulae is a very effective competitor, nearly as effective as RNA of the homologous stage. In quantitative terms, depression of the binding by 50 per cent requires about 600 μg of unfertilized egg or blastula RNA, compared with about 150 μg of gastrula RNA and about 100 μg of homologous prism RNA.

The kinds of messenger RNA molecules present in different populations may be compared by a variant of the binding approach other than direct competition. Since the formation of DNA-RNA complexes is essentially irreversible at 60°C, it is possible to incubate the DNA sequentially with different RNA preparations. Molecules present in one sample may preempt sites on the DNA with which labeled molecules, subsequently added, may otherwise combine. This type of experiment was used to obtain the data of Table 1 which compares the four unlabeled RNA samples from the embryonic stages. It is clear that each group of RNA molecules is able to occupy positions on the DNA complementary to some P^{32}-labeled molecules. However, the blastula and unfertilized egg RNA's are considerably less effective than the RNA from prism and from gastrula. In addition, there is little difference between RNA's from blastulae and unfertilized eggs, and RNA from gas-

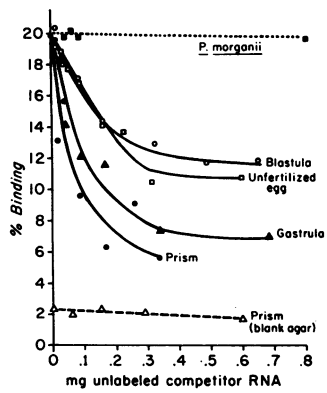

FIG. 4.—Competition by unlabeled RNA from developmental stages in the binding of P^{32}-labeled prism RNA to DNA-agar. Four μg P^{32}-labeled *S. purpuratus* prism RNA, unlabeled competitor RNA as shown, 0.30 gm of *S. purpuratus* DNA-agar containing 110 μg DNA, or 0.30 gm agar lacking DNA (*bottom curve*), total volume = 0.475 ml, incubated at 60°C for 48 hr.

TABLE 1

REACTION OF DNA-AGAR WITH COMPETITOR RNA BEFORE ADDITION OF LABELED DNA

RNA added initially	RNA added after 36 hr, 60°C	Per cent binding	Per cent control
P^{32}-prism	None	9.6	100
P^{32}-prism + unlabeled prism	None	5.6	58
None	P^{32}-prism	7.1	100
Unlabeled prism	P^{32}-prism	4.2	59
Unlabeled gastrula	P^{32}-prism	4.2	59
Unlabeled blastula	P^{32}-prism	5.4	76
Unlabeled unfertilized	P^{32}-prism	5.8	82

P^{32}-labeled *S. purpuratus* prism RNA (8.5 μg), 0.6 mg each unlabeled competitor RNA, 0.30 gm of *S. purpuratus* DNA agar containing 110 μg DNA, total volume = 0.475 ml, incubated at 60°C for 60 hr as shown above.

trulae and prisms are not distinguishable. Therefore, this experiment confirms the results shown in Figure 4. As seen in Table 1, preincubation of the DNA-agar with unlabeled prism RNA gives the same degree of competition (59% of the control) with subsequent binding of P^{32}-prism RNA as does simultaneous incubation with both labeled and unlabeled RNA (58% of the control), indicating that there is no destruction of nucleic acids during preincubation, and that complete saturation by unlabeled RNA was not achieved. It should be noted that this experiment was performed with 8.5 μg of P^{32}-RNA rather than 4.0 μg, so the per cent of binding is not the same as in the experiments of Figure 4.

RNA from adult tissues: The results of the experiments described suggest that there is a class of messenger RNA molecules which exist in the unfertilized egg and which are continually synthesized at least to the prism stage. These may be concerned with the synthesis of proteins of value to all types of cells as, for example, structural proteins and the enzymes of intermediary metabolism. In this connection, it would be of value to compare the RNA of adult tissues with the labeled prism RNA preparation. This comparison was undertaken by means of competition experiments with unlabeled RNA from adult gut and testis (Fig. 5). Again, these preparations act as competitors for at least some of the P^{32}-labeled messenger RNA molecules, but the level of competition is much less than with comparable amounts of prism RNA and, in fact, is less than that with RNA from unfertilized eggs and blastulae.

The competition curves presented in Figures 4 and 5 show a rapid decrease in binding at low levels of competitor RNA followed by a more gradual decrease with larger amounts of competitor. The form of this curve suggests the existence of two or more populations of RNA molecules in each preparation.[16] A similar effect may be seen in Figure 2 where the per cent of binding decreases rapidly with an increasing RNA:DNA ratio. Thus, one of these classes of molecules is either very abundant or represented by only a small fraction of sites on the DNA. This degree of reaction may, therefore, be attributed either to frequent messen-

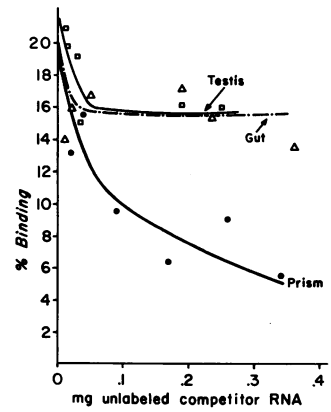

FIG. 5.—Competition by unlabeled RNA from adult tissues in the binding of P^{32}-labeled prism RNA to DNA-agar. Conditions and amounts as for Fig. 4.

ger RNA molecules or ribosomal RNA. As shown in Figures 2, 4, and 5, other classes include RNA molecules at low concentrations so that large amounts of competitor must be added in order to decrease their binding. It may be that the frequent class is relatively less abundant in the gut and testis RNA than in the embryo RNA.

Specificity of RNA-DNA binding: RNA from a completely unrelated source, the bacterium *Proteus morganii*, does not bind to *S. purpuratus* DNA, as shown by its failure to compete in the binding of *S. purpuratus* prism RNA, even when present in large quantities (Fig. 4). Likewise, little reaction occurs with the agar gel alone and, as shown in the bottom curve of Figure 4, when larger amounts of RNA are present, this nonspecific retention of radioactivity is not affected.

Discussion.—The present report demonstrates the existence of mRNA in the unfertilized sea urchin egg. It has been shown that some of this RNA is able to recognize sites in the DNA which are active in the synthesis of RNA at later stages of development. The former molecules compete in the binding of such newly synthesized RNA to DNA. Therefore, it is possible to conclude not only that this is messenger RNA but also that it belongs to a particular category of messages which are made both in oögenesis and during embryogenesis. By the nature of the experimental design it is, of course, impossible to say how much of the total message population of unfertilized eggs has this characteristic. It is not surprising, however, that such a core of messages exists, for all cells share a number of functions and structures requiring the synthesis of large groups of proteins held in common. Thus, it is to be expected that the populations of messenger RNA from groups of cells within an animal would overlap to some extent. This is apparently the case for messenger RNA isolated from various adult mouse tissues.[14]

The results also provide good evidence for the existence of messenger RNA molecules specific for a given developmental stage in accordance with the view that genes are sequentially activated during embryogenesis. For example, it is clear that many of the RNA molecules labeled at the prism stage are essentially unrepresented very early in development (unfertilized eggs and blastulae) or in two adult tissues. A similar conclusion has been reached for *Xenopus laevis*.[17]

RNA's from unfertilized eggs and hatching blastulae cannot be clearly distinguished. It is possible that different populations of messages exist at these two times and that the equal competitions are due to fortuitous proportions of these, but our experiments do not require this conclusion. The more direct interpretation is that, for the most part, relatively few new genes are transcribed in this developmental interval which is instead supported by masked messages already present before fertilization. This interpretation agrees with the host of observations,[18] dating from Boveri's early studies, that pregastrular development is maternal and that new nuclear influences begin to appear in the period between blastulation and gastrulation. Many biosyntheses are accelerated then. Between fertilization and hatching, few new differentiations appear: cilia, desmosomes, a few enzymes including the hatching enzyme, and phosphate transport carrier.[19] The formation of the gastrula, however, involves the differentiation of many specialized features in all three germ layers and the synthesis of numerous new enzymes. It also entails form changes. The early features may be supported by the utilization of pre-existing

messages which would continue to be produced subsequent to hatching for maintenance purposes.

The latter differentiation may be expected to require new messages. Quantitatively we cannot distinguish clearly between the messenger populations at the onset of gastrulation and at the onset of the pluteus stage. Forty hr have elapsed between these two stages, whereas 10–13 hr elapse between hatching blastulae and early gastrulae. If it is established that the RNA at the onset of gastrulation has the same qualitative composition as that at the onset of the pluteus stage, it would follow that a burst of production of new messages occurs during the 10-hr interval just prior to gastrulation and that these are either activated at one time or stored and sequentially activated throughout this long interval.

Summary.—Experiments on the binding of RNA to DNA and competition in this binding by RNA's from various developmental stages have shown: (1) messenger RNA is present in unfertilized eggs of *Strongylocentrotus purpuratus;* (2) some of these kinds of molecules of mRNA continue to be synthesized as late as the prism stage; (3) mRNA from unfertilized eggs and blastulae were not distinguished; (4) adult tissues share some of the same molecules with prism embryos; and (5) other molecules, assembled at the prism stage, are much less abundant or absent at both earlier and later stages of development.

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[1] Hultin, T., *Exptl. Cell Res.*, **3**, 494 (1952).

[2] Nakano, E., and A. Monroy, *Exptl. Cell Res.*, **14**, 236 (1958).

[3] Brachet, J., M. Decroly, A. Ficq, and J. Quertier, *Biochim. Biophys. Acta*, **72**, 660 (1963).

[4] Nemer, M., these PROCEEDINGS, **50**, 230 (1963).

[5] Wilt, F. M., *Biochem. Biophys. Res. Commun.*, **11**, 447 (1963).

[6] Denny, P. C., and A. Tyler, *Biochem. Biophys. Res. Commun.*, **14**, 245 (1964).

[7] Tyler, A., *Am. Zool.*, **3**, 109 (1963).

[8] Gross, P. R., L. I. Malkin, and W. A. Moyer, these PROCEEDINGS, **51**, 407 (1964).

[9] Maggio, R., M. L. Vittorelli, A. M. Rinaldi, and A. Monroy, *Biochem. Biophys. Res. Commun.*, **15**, 436 (1964).

[10] Monroy, A., R. Maggio, and A. M. Rinaldi, these PROCEEDINGS, **54**, 107 (1965).

[11] Gross, P. R., L. I. Malkin, and M. Hubbard, *J. Mol. Biol.*, **13**, 463 (1965).

[12] Whiteley, A. H., and F. Baltzer, *Pubbl. Staz. Zool. Napoli*, **30**, 402 (1958).

[13] Gillespie, D., and S. Spiegelman, *J. Mol. Biol.*, **12**, 829 (1965).

[14] McCarthy, B. J., and B. H. Hoyer, these PROCEEDINGS, **52**, 915 (1964).

[15] Hoyer, B. H., B. J. McCarthy, and E. T. Bolton, *Science*, **140**, 1408 (1963).

[16] McCarthy, B. J., and E. T. Bolton, *J. Mol. Biol.*, **8**, 184 (1964).

[17] Denis, H., *Ann. Rep. Carnegie Inst. Wash.*, **63**, 505 (1964).

[18] For a review, see J. Brachet, in *Biochemical Cytology* (New York: Academic Press, 1957), chap. 8.

[19] Whiteley, A. H., and E. L. Chambers, in *Symposium on Germ Cells and Development* (Institut. Intern. d'Embryologie and Fondazione A. Baselli, 1960), p. 387.