*ACCUMULATION OF NEWLY SYNTHESIZED RNA TEMPLATES IN A UNIQUE CLASS OF POLYRIBOSOMES DURING EMBRYOGENESIS**

By Anthony A. Infante and Martin Nemer

THE INSTITUTE FOR CANCER RESEARCH, FOX CHASE, PHILADELPHIA

Communicated by Thomas F. Anderson, June 12, 1967

The production of RNA templates well in advance of their use in protein synthesis may be a characteristic event in cellular differentiation. Evidence supporting this proposition can be drawn from experiments on early developing embryos,[1, 2] tissues in the terminal stages of differentiation,[3] and developing unicellular organisms.[4] In all these cases actinomycin was used to block RNA synthesis, and either specific protein synthesis or cellular differentiation could be observed to continue afterwards. In the starfish the results of actinomycin treatment at different times suggest that only the RNA templates synthesized before the swimming blastula stage may be required to promote subsequent gastrulation.[1] The present experiments with sea urchin embryos reveal a class of polyribosomes (polysomes) that accumulate newly synthesized RNA during the early stages. One interpretation of our results is that this class of polysomes provides a structure responsible for delaying the translation of RNA templates, thus allowing a programing of genetic information at the cytoplasmic level through the course of embryonic development.

Spirin and Nemer[5] proposed that a unique class of polysomes might be responsible for holding the activity of newly synthesized RNA templates in abeyance. They noted two distinct classes of polysomes in the early-cleaving sea urchin embryo strictly on the basis of radioactive incorporation; a rapidly sedimenting class (r-polysomes) was predominant in the incorporation of amino acids in nascent protein, whereas a slowly sedimenting class (s-polysomes) appeared to be inactive or minimally active. The slowly sedimenting s-polysomes were detected in the early-cleaving embryo solely by virtue of their content of newly synthesized RNA. This RNA, which was not associated with the r-polysomes, had messenger RNA properties, namely nonribosomal RNA sedimentation behavior and extensive hybridization with DNA.[6] In the present studies the continued incorporation of new RNA in the s-polysomes has been shown to result in their accumulation to amounts that can eventually be detected spectrophotometrically. The amounts of absorbance at 260 mμ (A_{260}) attributed to the s-polysomes and r-polysomes change markedly during development. Various demonstrations[7] that new RNA synthesis is not involved in the activation of protein synthesis after fertilization and its maintenance through part of early development raise the questions of how and when new RNA does contribute to protein synthesis. We have approached this problem by examining the two sedimentation classes of polysomes in embryos developing in the presence and absence of actinomycin, to determine the consequences of a block in RNA synthesis.

Materials and Methods.—Embryos and cell-free extracts: Eggs of *Strongylocentrotus purpuratus* (Pacific Bio-Marine Supply Company, Venice, California) were fertilized in 400 times their volume of synthetic sea water (SSW) and allowed to develop in 25 times their volume of SSW at 18°C.[8] Other eggs from the same females were incubated for one hour in actinomycin D (from Merck, Sharp and Dohme,

Rahway, New Jersey) at 25 μg/ml prior to fertilization.[9] These actinomycin-treated eggs were allowed to develop in actinomycin at 25 μg/ml. Eggs or embryos were collected by centrifugation for one minute at 500 $\times$ g and washed once with 100 volumes of homogenization medium (0.05 M triethanolamine-HCl at pH 7.8, 0.24 M KCl, 0.005 M $MgCl_2$, 0.25 M sucrose). The embryos were resuspended in 4 volumes of homogenization medium containing bentonite at 2 mg/ml, and homogenized with a Dounce size B homogenizer, using only two strokes to avoid mechanical disruption of polysomes. This homogenate was centrifuged at 15,000 $\times$ g for five minutes to obtain a supernatant fluid (S15) free of nuclei and mitochondria. The high concentration of bentonite, the brief homogenization, and the maintenance of the temperature below 4°C were necessary to reduce the activity of endogenous RNase and to protect the integrity of polysomes.

Sucrose gradient centrifugation: Approximately 0.4 ml of the S15 extracts was layered onto 4.6 ml of linear 15–30 per cent (w/w) sucrose gradients made up in homogenization medium. Centrifugation was in the Spinco SW39 rotor for 48 minutes at 0–1°C. After centrifugation, 0.4 ml was discarded from the top and gradients were displaced by careful addition of 60 per cent sucrose to the bottom of the centrifuge tube. The contents were passed through a 0.065-ml capacity-flow cell (2-mm light path), and the absorbance at 260 mμ (A_{260}) was monitored continuously.

Results.—Formation of polysomal classes: Polysomes are barely detectable in the mature unfertilized egg.[10] They appear to be present at a level commensurate with the small extent of protein synthesis.[11] The A_{260} material sedimenting more rapidly than the 74S monoribosomes that is eliminated by mild RNase treatment

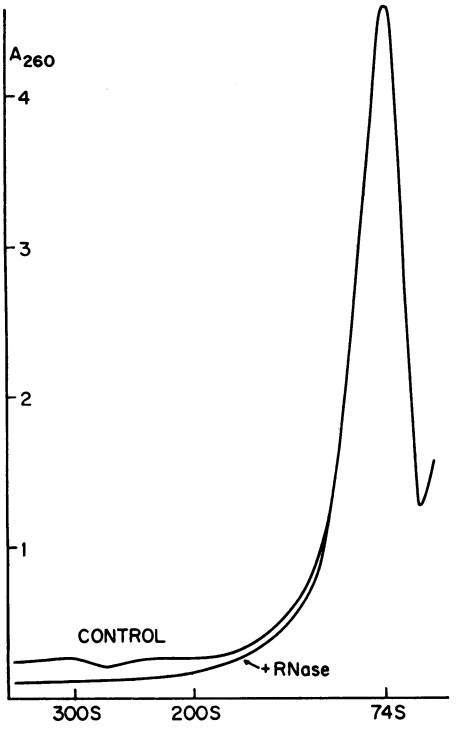

FIG. 1.—Sedimentation of ribosomes and polysomes of the unfertilized egg. Unfertilized eggs were used for the preparation of S15 extracts (text). Half of the S15 preparation was incubated with 10 μg/ml of pancreatic ribonuclease (Worthington Biochemical Corporation, Freehold, New Jersey) at 25°C for 1 min. The S15 extracts were layered onto linear 15–30 per cent (w/w) sucrose gradients and centrifuged in the Spinco SW39 rotor for 48 min at 0–1°C. Experimental details are given in the text (*Methods*). Each curve is a tracing of the absorbance throughout the gradient monitored continuously with a recording spectrophotometer.

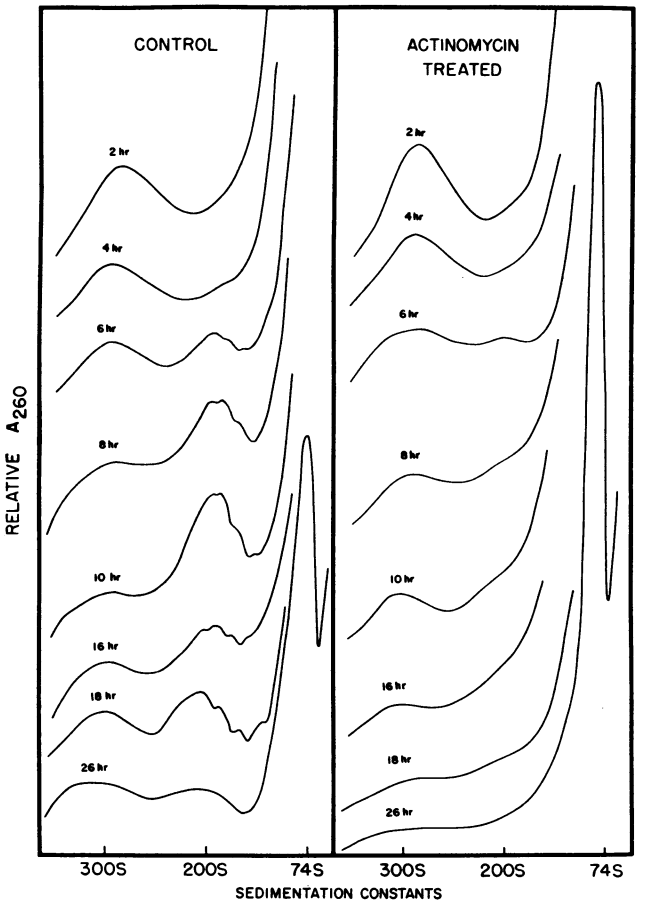

FIG. 2.—Sedimentation profiles of polyribosomes from embryos of *S. purpuratus* developing in the presence and absence of actinomycin. S15 extracts from embryos of the indicated stages were prepared and sedimented as in Fig. 1. The series of continuous tracings by the recording spectrophotometer has been drawn from a single batch of eggs. The controls were repeated five times with close agreement and the actinomycin treatment was studied three times. Variability in response to actinomycin was noted, as depicted in Fig. 4. We have noted similar qualitative changes in the embryonic development of two other species, *Lytechinus pictus* and *Arbacia punctulata*.

constitutes only 5–10 per cent of the total ribosomes in the egg (Fig. 1). However, after fertilization considerably more of this material has been reported to arise.[10] Figure 2 represents one of several series of experiments in which embryos were allowed to develop in the presence and absence of actinomycin and their polysomes and monoribosomes examined. During the course of development to the gastrula stage, sedimentation modes of A_{260} appear at approximately 300*S* and 200*S*, representing the r-polysomes and s-polysomes, respectively (Fig. 2). The evidence that these are polysomes rests on the conversion of both of these sedimentation modes into 74*S* monoribosomes by mild RNase treatment, under conditions similar to those of Figure 1,[12] and the observation by electron microscopy that they are

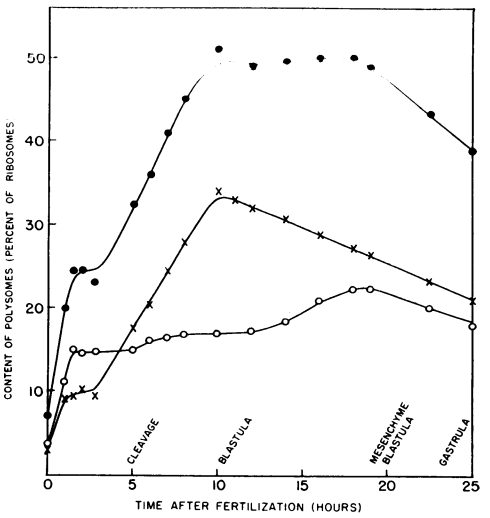

FIG. 3.—Relative amounts of ribosomes present in polysome classes at different embryonic stages. The amounts of polysomes (*closed circles*) and the subclasses of s-polysomes (X's) and r-polysomes (*open circles*) were calculated as the percentage of total ribosomes by integrating the areas under the A_{260} curves of monoribosomes and polysomes, as represented in the control diagram of Fig. 2.

structures containing an average of 23 and 7 ribosomes, respectively.[12] Each embryonic stage displays a characteristic sedimentation profile containing these two polysomal classes. The sedimentation profiles of the actinomycin-treated embryos diverge considerably during development from those of the controls. As can be seen in Figure 2, striking changes in the relative amounts of the two polysomal classes occur in both the normal and actinomycin-treated embryos. In order to evaluate these changes, we have estimated the proportion of total ribosomes associated with each class, and in the following sections these estimations have been analyzed as a function of embryogenesis.

The content of polysomes in normal embryos: The relative amounts of polysomes and monoribosomes were determined by measuring the areas under the curves of Figure 2, and the results for normal embryos are given in Figure 3. The polysomes formed in the first 2 hours after fertilization are predominantly r-polysomes. To a lesser extent some s-polysomes are apparently also formed. The kinetics of formation of the two classes appear to be the same, with both reaching a plateau value within the first 2 hours after fertilization. A difference in the relative formation of the two polysomal classes begins to be evident at about the 16-cell stage. Further development brings a striking buildup in the amount of s-polysomes, with a sharp rise in concentration occurring between 4 and 10 hours of development. However, during this period there is little change, if any, in the content of r-polysomes. In the 10-hour early blastula a maximal level of s-polysomes is reached at 32–35 per cent of the total amount of ribosomes. This accumulation of s-polysomes accounts for the increase in total polysomes to a level of slightly more than 50 per cent. The amount of total polysomes remains constant at this level during the period from the 10-hour blastula to the 20-hour mesenchyme blastula. However, during this period of constancy of total polysomal concentration, the prominent content of s-polysomes declines and concomitantly the amount of r-polysomes increases. These changes are consistent with a possible conversion of s-polysomes to r-polysomes.

Effect of actinomycin: Continuous exposure to actinomycin before and after fertilization does not block the early formation of either polysome class. This result is consistent with the various demonstrations that protein synthesis, necessarily supported by polysomes, is independent of new RNA synthesis during this early period.[7] On the other hand, the increase in content of the s-polysomes is drastically inhibited by actinomycin after the early period. Therefore, the generation of these polysomes depends upon the synthesis of RNA. The aggregation of ribosomes into s-polysomes, presumably by newly synthesized mRNA,[6] is the earliest indication of a large-scale contribution of RNA synthesis to the protein-synthesizing system. Little if any RNA synthesized at this time is ribosomal.[13] After extended incubation in actinomycin, the embryos experience a decline in their total amount of ribosomes associated with polysomes.

(a) *s-Polysomes:* The concentration of s-polysomes in both the control and actinomycin-treated embryos rose to a maximum at the same time, e.g., 10 hours after fertilization. However, as could be seen in Figure 2, the formation of these polysomes was markedly inhibited by the antibiotic. This inhibition was never observed to be complete, nor did it occur to the same extent from batch to batch. The maximal, 10-hour amounts in the three batches allowed to develop in actinomycin, *a*, *b*, and *c*, respectively, were 60 per cent, 45 per cent, and 38 per cent of the maximal, 10-hour control values (Fig. 4). Furthermore, although the same concentration of the antibiotic was present, the different batches of embryos developed at different rates and were arrested in development at different stages. Batch *a* went on to reach the hatched blastula stage at 20 hours. Normally, hatching occurs at 15 hours. Batch *b* of embryos consisted of unhatched blastulae at 20 hours. Batch *c*, which attained the lowest concentration of s-polysomes, suffered 50 per cent embryonic death at 16 hours. Therefore, a direct relationship

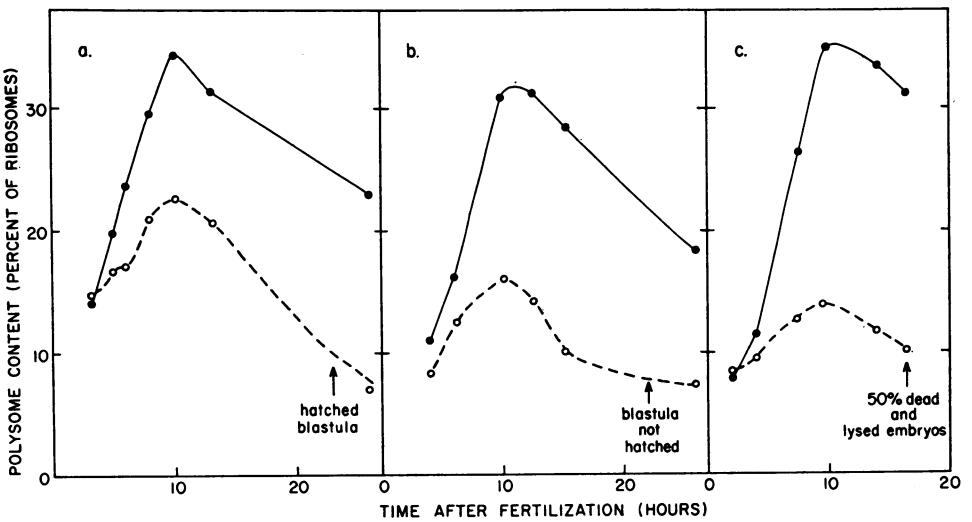

FIG. 4.—Effect of actinomycin in the content of s-polysomes and the extent of development. The amounts of s-polysomes were calculated as the percentage of total ribosomes in the control and actinomycin-treated embryos, as in Fig. 3, in three series of experiments from three separate batches of eggs, *a*, *b*, and *c*. *Closed circles*, controls; *open circles*, actinomycin-treated. It should be noted that the control values did not vary by more than 10% in the three cases.

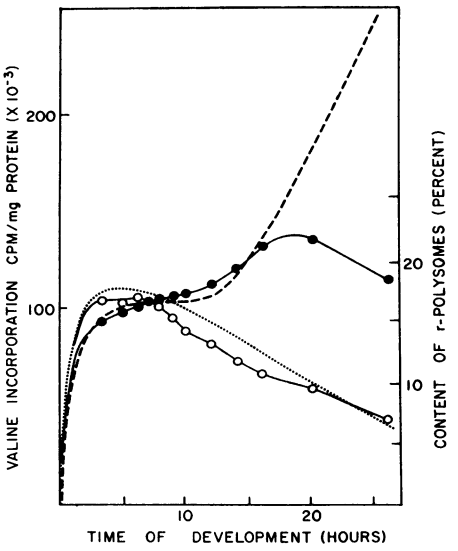

FIG. 5.—Content of r-polysomes and the rate of protein synthesis of embryos developing in the presence and absence of actinomycin. The same method as in Fig. 3 was used to calculate the content of r-polysomes. *Closed circles,* control; *open circles,* actinomycin-treated. The rates of valine incorporation in protein were measured by Gross[14] under the similar conditions of development. The concentration of actinomycin was 20 μg/ml. His values of valine incorporation have been replotted here: controls, *dashed line;* actinomycin-treated, *dotted line.*

is indicated between the amount of s-polysome formation and the extent of development.

The variable response to actinomycin from batch to batch is more likely attributable to a variable degree of inhibition of RNA synthesis, rather than to a variable content of preexisting template RNA in the unfertilized egg. We have noted variable degrees of inhibition of RNA synthesis, even at the level of 25 μg/ml used in all these studies.[12] It was at 40 μg/ml that complete blockage of *de novo* RNA synthesis could be consistently achieved. However, the risk of nonspecific damage at this higher concentration was too great for developmental correlations to be made.

(b) *r-Polysomes:* The initial rapid accumulation of r-polysomes in the first two hours after fertilization was not reduced by actinomycin. In fact, there was a slight but highly reproducible increase, about 10 per cent greater than the control value at two hours (Fig. 5). Whereas RNA synthesis at this time has been shown not to be involved in the promotion of protein synthesis,[7] newly synthesized RNA may, conversely, impede some polysome formation, and thus have a regulatory role. Furthermore, the results with actinomycin indicate that r-polysome formation at this time is brought about by the mobilization of template RNA stored in the egg.[5, 7, 9]

In the control embryos the content of r-polysomes remains approximately constant until about ten hours, then there is an increase which coincides with the decrease in s-polysome content at that time. This increase in r-polysomes starting at ten hours was prevented by actinomycin treatment, just as the rise in s-polysome content that preceded it was also inhibited. It is a reasonable hypothesis that the block in s-polysome formation led to the failure in the subsequent formation of r-polysomes.

In Figure 5 we have superimposed the rates of protein synthesis, measured by Gross[14] with this species under the same conditions of development, upon our curves describing the content of r-polysomes. In the control embryos both the rate of *in vivo* incorporation in protein and the concentration of r-polysomes rise sharply

during the first 2–3 hours after fertilization, then maintain constant levels until about 12–15 hours of development, at which time they both experience a second increase 4–5 hours prior to the onset of gastrulation. The coincidence of protein synthesis and r-polysome concentration was just as striking in the respective actinomycin-treated embryos. In this case both coincided with or, in fact, were slightly higher than the controls, until approximately 6–8 hours. Then both decayed at remarkably similar rates. The half time of this decline was roughly 20 hours. The close correlation between r-polysome formation and the rate of protein synthesis is in marked contrast to the lack of association between s-polysome concentration and protein synthesis. We may then conclude that the r-polysomes are responsible for the major part of protein synthesis measured *in vivo*, in agreement with previous suggestions,[5] whereas the s-polysome class includes members which do not participate in protein synthesis until they are activated in the period prior to gastrulation.

Discussion.—Although the *de novo* synthesis of RNA may start soon after fertilization,[6] the newly formed messengers appear not to contribute substantially to polysome formation, protein synthesis, or embryonic development for several hours. Rather, they become to an increasing extent associated with special polysomal structures of the s-polysome class. A unique role of the s-polysomes in embryonic development is indicated by two aspects of their behavior. First, they may be minimally active in synthesizing protein or may contain members that are inactive. Further studies have now shown that the *in vivo* rate of incorporation of amino acid per ribosome is considerably greater for the r-polysomes than for the s-polysomes.[15] Second, the s-polysomes may be converted to r-polysomes. The activation of s-polysomes may permit more ribosomes to enter the structure, and the greater number of ribosomes in the now-active polysomes would thus account for the more rapid sedimentation, characteristic of the r-polysomes. After incorporation of RNA label in the s-polysomes, we have observed that during subsequent incubation in the presence of a concentration of actinomycin high enough to block further RNA synthesis there is a shift of RNA label out of the s-polysomes and a concomitant buildup of RNA label in the r-polysomes.[15]

The s-polysomes accumulate substantially during the cleavage and very early blastula stages, a period of "predifferentiation."[16] It is during this period that a block in RNA synthesis results in the subsequent inhibition of gastrulation in the starfish and sea urchin embryos. It is our proposal that the RNA templates synthesized at this time are accumulated in the form of inactive polysomes, and that the extensive cellular changes, associated with the formation of the mesenchyme blastula and early gastrula, are brought about by the activation and use of these accumulated polysomal templates.

Summary.—The concentrations of two classes of polyribosomes, sedimenting at modes of about 200*S* and 300*S*, respectively, have been measured in the early developing sea urchin embryo. Soon after fertilization the 300*S* class of polyribosomes (r-polysomes) appears and increases in amount for 2 to 3 hours, then maintains a constant level until the early blastula stage at 12 hours, at which time a further increase occurs. These changes are closely correlated with the changes in the rate of protein synthesis described by others. In contrast, the 200*S* class (s-polysomes) increases in amount most rapidly between 4 and 10 hours, then decreases during the

period of the second increase in the r-polysomes. Actinomycin did not block the first increase in the r-polysomes, but did inhibit the formation of the s-polysomes and the later increase in r-polysomes. The results suggest that the r-polysomes are active in protein synthesis and that the s-polysomes include inactive forms which accumulate during a period preparatory to differentiation, and are subsequently converted to active r-polysomes prior to gastrulation.

* This investigation was supported by U.S. Public Health Service research grants CA-05936 and CA-06927 from the National Cancer Institute and 1-SO1-FR-05539 from General Research Support.

[1] Barros, C., G. S. Hand, and A. Monroy, *Exptl. Cell Res.*, **43**, 167 (1966).

[2] Wilt, F. H., *J. Mol. Biol.*, **12**, 331 (1965); Wessels, N., and F. H. Wilt, *J. Mol. Biol.*, **13**, 767 (1965); Wilde, C. E., Jr., and R. B. Crawford, *Exptl. Cell Res.*, **44**, 471 (1966).

[3] DeBellis, R. H., N. Gluck, and P. A. Marks, *J. Clin. Invest.*, **43**, 1329 (1964); Papaconstantinou, J., J. A. Stewart, and P. V. Koehn, *Biochim. Biophys. Acta*, **114**, 428 (1966); Rutter, W. J., N. K. Wessels, and C. Grobstein, *Natl. Cancer Inst. Monograph*, **13**, 51 (1964).

[4] Sussman, M., and R. R. Sussman, *Biochim. Biophys. Acta*, **108**, 463 (1965); Sussman, M. W. F. Loomis, Jr., J. M. Ashworth, and R. R. Sussman, *Biochm. Biophys. Res. Commun.*, **26**, 353 (1967); Rosas del Valle, M., and A. I. Aronson, *Biochem. Biophys. Res. Commun.*, **9**, 421 (1962); Lovett, J. S., in *The Molecular Aspects of Biological Development*, ed. R. A. Deering and M. Trask (NASA CR-673, 1967), p. 165.

[5] Spirin, A. S., and M. Nemer, *Science*, **150**, 214 (1965).

[6] Nemer, M., and A. A. Infante, *Science*, **150**, 217 (1965).

[7] Gross, P. R., L. I. Malkin, and W. A. Moyer, these PROCEEDINGS, **51**, 407 (1964); Brachet, J., A. Ficq, and R. Tencer, *Exptl. Cell Res.*, **32**, 168 (1963); Denny, P. C., and A. Tyler, *Biochem. Biophys. Res. Commun.*, **14**, 245 (1964).

[8] Nemer, M., and A. A. Infante, *J. Mol. Biol.*, in press.

[9] Gross, P. R., and G. H. Cousineau, *Biochem. Biophys. Res. Commun.*, **10**, 321 (1963).

[10] Monroy, A., and A. Tyler, *Arch. Biochem. Biophys.*, **103**, 431 (1963).

[11] Hultin, T., *Exptl. Cell Res.*, **25**, 405 (1961); Piatigorsky, J., H. Ozaki, and A. Tyler, *Develop. Biol.*, **15**, 1 (1967); Epel, D., these PROCEEDINGS, **57**, 899 (1967).

[12] Nemer, M., A. A. Infante, and M. Bayer, unpublished.

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

[14] Gross, P. R., *J. Exptl. Zool.*, **157**, 21 (1964).

[15] Nemer, M., and A. A. Infante, in preparation.

[16] Runnström, J., in *Acidi nucleici e loro funzione biologica*, ed. A. Baselli (Instituto Lombardo, 1964), p. 342.