The authors would like to express their gratitude to Dr. L. Lindholm, Department of Bacteriology, for his instruction in and assistance with the fluorescent antibody technique. They would also like to thank Mrs. K. Bjurstam for excellent technical assistance.

The antiserum prepared against rabbit protein S100 was kindly given by Dr. L. Levine, Department of Bacteriology, Brandeis University, Waltham, Massachusetts, and by Dr. A. Rubin, Cornell Medical School, New York.

* Economic support was given by the Swedish Medical Research Council and by the U.S. Air Force European Office of Aerospace Research grant no. EOAR 63-28 and by the Wallenberg Foundation, Stockholm.

† Recipient of a U.S. Public Health Service postdoctoral fellowship, 2-F2-SM-22, 097-02. This support is gratefully acknowledged.

[1] Altman, J., *Science*, **135**, 1127 (1962).

[2] Moore, B. W., and D. McGregor, *J. Biol. Chem.*, **240**, 1647 (1965).

[3] Moore, B. W., *Biochem. Biophys. Res. Commun.*, **19**, 739 (1965).

[4] Levine, L., and B. W. Moore, *Neurosci. Res. Progr. Bull.*, **3**, 18 (1964).

[5] Levine, L., personal communication.

[6] Hydén, H., *Nature*, **184**, 433 (1959).

[7] Hydén, H., and A. Pigon, *J. Neurochem.*, **6**, 57 (1960).

[8] Hydén, H., and P. W. Lange, *J. Cell Biol.*, **13**, 233 (1962).

[9] Hamberger, A., and H. Hydén, *J. Cell Biol.*, **16**, 521 (1963).

[10] Hydén, H., and P. W. Lange, *Naturwissenschaften*, in press.

[11] Oudin, J., in *Methods in Medical Research*, ed. M. Cohn (Chicago: The Year Book Publishers, 1951), vol. 5, p. 335.

[12] Weller, T. H., and A. H. Coons, *Proc. Soc. Exptl. Biol. Med.*, **86**, 789 (1954).

[13] Hydén, H., "The neuron," in *The Cell: Biochemistry, Physiology, Morphology*, ed. J. Brachet and A. E. Mirsky (New York: Academic Press, 1960), vol. 4, chap. 5.

[14] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

[15] Rubin, A. L., and K. H. Stenzel, these PROCEEDINGS, **53**, 963 (1965).

[16] McEwen, B. S., and H. Hydén, in preparation.

---

*ACTIVATION OF RNA SYNTHESIS ASSOCIATED WITH GASTRULATION**

BY R. BACHVAROVA,† E. H. DAVIDSON,‡ V. G. ALLFREY, AND A. E. MIRSKY

THE ROCKEFELLER UNIVERSITY

Communicated December 6, 1965

Gastrulation in the amphibian embryo marks the onset of widespread, *de novo* cell differentiation. In this communication we present the results of a radioautographic and biochemical study of gene activity as gastrulation takes place in *Xenopus laevis*. We have found that gene activation occurs abruptly within a 1-hr period preceding the onset of gastrulation and that most of the newly synthesized RNA resulting from this activation appears to be messenger RNA.

Pregastrular and Postgastrular Gene Activity.—From gastrulation onward, development is organized through the controlled activity of the cell-borne genetic apparatus. Although the zygote genome and its blastomeric descendants manifest a slowly rising level of activity all through cleavage and blastulation,[1-6] it is not until gastrulation that the indispensability of embryonic gene action can be demonstrated. This position dates back to the classical sea urchin hybridization studies

carried out by Boveri, Driesch, and others (1888–1914).[7] Cases were described by these workers in which experimental hybrids bearing lethally incompatible genomic combinations successfully underwent cleavage and blastulation, arresting only at gastrulation. In hybrid merogones expression of the paternal genome is observed only at and after gastrulation; in earlier stages development proceeds according to maternal type, and therefore cannot be considered subject to immediate nuclear control. Similarly, analyses of development in hybrids made from species differing in their mode of primary mesoderm formation revealed a detailed maternal control over the cellular processes through which primary mesoderm arises, although in the developmental events following gastrulation the characteristics of both parental species (i.e., of the hybrid embryo genome) are expressed. Amphibian hybridization experiments carried out by Moore[8] suggest the generality of these classical results. As in the echinoderm, nonviable hybrids develop to early gastrula but then arrest, while in viable amphibian hybrid development paternal genomic influence can be observed only after gastrular differentiation is under way. Totally enucleate echinoderm[9] and amphibian embryos[10] may cleave and undergo some blastulation, but they too arrest by the onset of gastrulation. Recent experiments have shown that echinoderm and amphibian embryos which have been "chemically enucleated" with actinomycin D behave in the same way.[11, 12] The actinomycin experiments, like the older hybridization and enucleation experiments, thus point to the fact that *newly synthesized nuclear gene products are required in order for gastrular morphogenesis to take place*, if not for the preceding morphogenetic events.

Gene Activity and Gastrulation.—These findings pinpoint the period immediately preceding the onset of gastrulation as a critical phase of developmental gene activation, the result of which is the establishment of nuclear control over embryonic organization for the first time in the life of the embryo. The study we now report was undertaken in order to determine precisely when this activation takes place, whether it affects the few cells of each presumptive tissue at different times or large numbers of diverse cell types at the same time, and whether the greater part of the RNA synthesized as a result of pregastrular activation is of messenger or transfer RNA type.

A general picture of the chemistry of gene activity during the period between fertilization and gastrulation in amphibia has emerged from the various recent studies describing RNA synthesis patterns as embryogenesis begins. The RNA synthesized during cleavage and blastulation has appeared to consist of transfer RNA plus small, though significant, amounts of messenger RNA.[6, 13–15] During gastrulation, however, different patterns of RNA synthesis develop. Thus, Brown and Littna have reported the interesting fact that ribosomal RNA synthesis in *Xenopus* embryos occurs for the first time only at the onset of gastrulation,[6] and our results agree with this finding. As we have shown previously, the massive quantity of ribosomal RNA needed during cleavage and blastulation is synthesized much earlier, mainly in the lampbrush phase of oögenesis.[22] By mid- to late gastrula (Nieuwkoop and Faber's stages 11 and 12 in *Xenopus*[17]) the rate of RNA synthesis has increased sharply. According to sedimentation behavior, base composition, and ability to hybridize with DNA, part of this newly synthesized RNA appears to be messenger RNA.[6, 14–16]

Methods.—Adult *Xenopus laevis* were stimulated by injection of chorionic gonadotropin. The

embryos were staged, and the jelly coats removed with papain.[18] To facilitate entry of isotopic precursor, the embryos were oriented and then individually dissected. The dorsal halves were incubated for 1 hr in uridine-H^3, specific activity 1.24–17.4 mc/μM, 0.03–0.2 μM/ml in Barth's Solution X[19] at 18°C. Gastrular movements continued normally in both halves of every embryo. At the termination of labeling, the embryos were either fixed and sectioned in preparation for stripping film radioautography, or subjected to RNA extraction procedures. Assays of the total cold acid-insoluble radioactivity which had been incorporated into newly synthesized embryo RNA were performed on groups of 10 dorsal halves. After homogenization these were washed repeatedly with cold 0.20 *N* PCA, followed by 3:1 ethanol/ether, and then dried from alcohol. The precipitates were then treated for 1 hr at 37° with 3× recrystallized Worthington RNase (1 mg/ml, in 0.01 *M* pH 7.6 tris–0.10 *M* NaCl), and the radioactivity released from the precipitate in acid-soluble form by the enzyme was measured in a scintillation spectrometer; under our conditions over 95% of the cold acid-insoluble RNA radioactivity was rendered acid-soluble by the RNase treatment.

Phenol extraction of embryo RNA was carried out according to Brown and Littna,[6] except that the DNase treatment was omitted. Extractions were carried out on groups of 20–40 embryo halves, with whole unlabeled embryos sometimes added as carrier. Yeast carrier RNA was also added during the extraction in some experiments. Sephadex G-100 columns (0.9 × 35 cm) were used to separate transfer RNA from both heavier and lighter components, as described by Schleich and Goldstein.[20] This method has advantages over the sucrose gradient method, since it clearly distinguishes between transfer RNA and various other labeled compounds of lower molecular weight. Base ratios were determined by assaying P^{32} distribution among the four 2′,3′-ribonucleotides after alkaline hydrolysis of purified phenol-extracted RNA. The nucleotide separation was carried out by electrophoresis on cellulose acetate in pH 3.42 0.04 *M* citrate buffer.

Radioautographic Study of Pregastrular Activation.—A widespread activation of nuclear RNA synthesis was found to occur abruptly during a stage in blastulation corresponding to Nieuwkoop and Faber's stage 8. This is shown in our radioautographic studies,[21] some of which are briefly summarized in Table 1. Only 1 hr separates stage 8 and stage $8^1/_2$. It is within this short space that the embryo nuclei achieve a new, stepped-up level of gene transcription. It can be seen from Table 1 that nuclear activation affects many diverse presumptive tissue areas

TABLE 1

GRAINS PER NUCLEUS REPRESENTING RNA SYNTHESIS IN VARIOUS REGIONS OF DORSAL HALVES OF *Xenopus* EMBRYOS FROM MORULA TO LATE GASTRULA STAGE

Stage	Presumptive ectoderm		Equatorial region			Presumptive endoderm
$6^1/_2$	0.1					0.1
7	1.5					0
8	0					0.7
$8^1/_2$	1.8		17.3			10.5
			Outer	Inner		
9	6.5		2.4	21.9		23.2
	Presumptive neural tissue (1)	Outer mesoderm (2)	Blastopore lip (3)	Inner mesoderm (4)	Prechordal and anterior mesoderm (5)	Endoderm (6)
10	10.6	1.1	2.1	19.7	46.9	25.1
$10^1/_2$	0.6	0	2.5	16.2	41.2	32.3
11	4.5	3.1	5.8	11.5	26.0	28.0
$11^1/_2$	15.5	1.8	3.9	19.2	33.7	23.5
12	18.1	13.2	10.2	29.8	34.7	13.2

Grains were almost completely localized in the cell nuclei. All grain counts tabulated here represent the difference in grain counts between alternate sections, one of which had been treated with RNase previous to the application of stripping film. The embryos were labeled as described in *Methods*, fixed in Smith's fixative, washed with cold 0.20 *N* PCA, dehydrated, and embedded. All radioautographs were exposed for 7 days, and results were quantitatively homogeneous from experiment to experiment. Additional evidence that the radioautographic grains quantitatively represent RNA synthesis comes from the finding that no grains appear in sections from actinomycin-treated embryos. Numbers in parentheses refer to map areas in Fig. 1.

(see Fig. 1); counts indicate that over half of all the nuclei in the dorsal hemisphere participate in stage 8–9 gene activation. The further increases in activity per nucleus as gastrulation progresses are small compared with those occurring between the beginning and the end of stage 8. By the beginning of stage 10 the embryo is fully engaged in gastrulation and the newly synthesized RNA present must result in large part from the activity of the recently aroused genes.

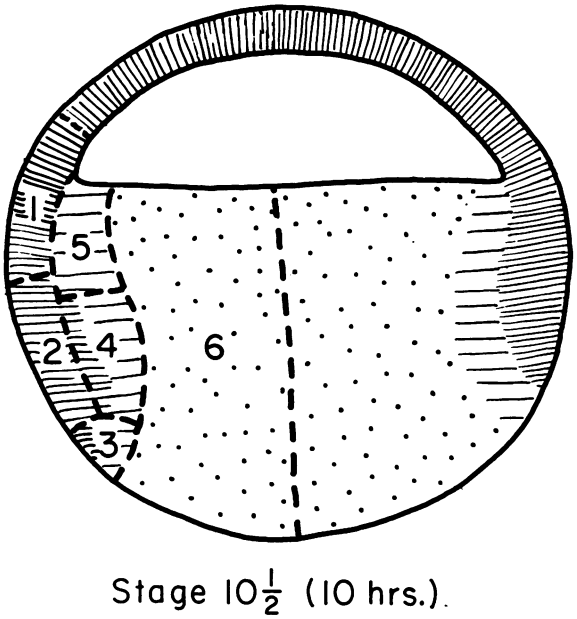

FIG. 1.—Map of stage 10, early gastrula, denoting the areas in which grains have been counted in the experiments summarized in Table 1. The numbers in parentheses in Table 1 refer to the equivalently numbered regions in this figure.

Molecular Species of Newly Synthesized RNA.—In order to determine what part of the massive RNA synthesis occurring as a result of late blastular gene activation is transfer RNA synthesis, embryos were labeled for 1 hr at stage 7 (preactivation blastula), stage 9 (just-activated late blastula), and stage 10 (early gastrula). The RNA was extracted and the preparations were placed on G-100 columns together with authentic transfer RNA marker. The results of one such experiment are presented in Figure 2. A *marked increase* in the amount of newly synthesized high-molecular-weight RNA is observed by stage 10 even in comparison to the immediately preceding stage 9 embryos, but the major change in high-molecular-weight RNA synthesis occurs between stage 7 and stage 9, as expected from the radioautographic study. Preactivation (stage 7) embryos formed only a small, though significant, amount of high-molecular-weight RNA. In contrast to the findings of Brown and Littna,[6] we could detect no *de novo* transfer RNA synthesis at this stage at all. By stage 9 transfer RNA synthesis has begun, however, and the rate of this synthesis remains the same in stage 10. Quantitatively, the impressive increase in high-molecular-weight RNA synthesis is the primary result of the gene activation occurring in stage 8–9 embryos. By stage 10 the transfer

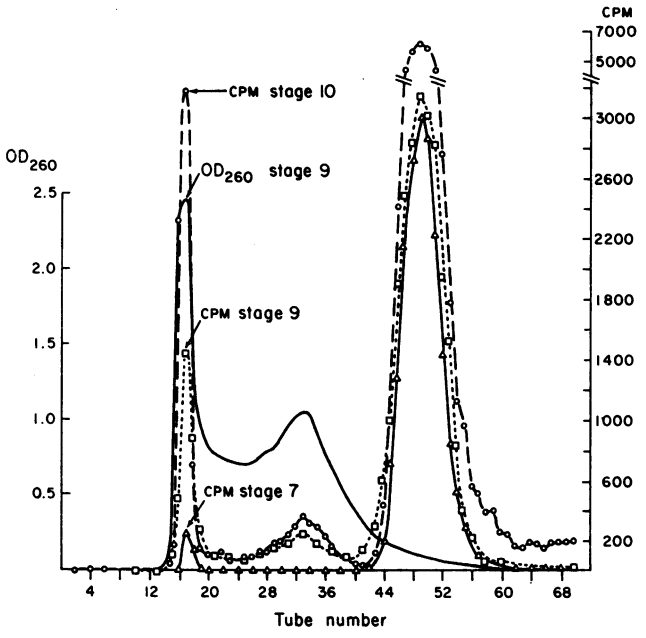

FIG. 2.—Sephadex G-100 elution pattern of uridine-H^3-labeled RNA from embryos of different stages. The first optical density peak is primarily due to carrier yeast bulk RNA, and the second to yeast transfer RNA marker which had been added to the final RNA solution.

RNA counts represent only 29 per cent of the combined transfer and high-molecular-weight RNA counts (this value is an average from six experiments). Similar experiments in which the embryos were labeled with adenosine-H^3 or cytidine-H^3 rather than uridine-H^3 yielded similar elution patterns on G-100, except that 60 and 53 per cent of the counts, respectively, occurred in the transfer RNA peak. These higher values probably reflect —C—C—A end-group turnover and are therefore to be expected. (Transfer RNA isolated after labeling with uridine-H^3 contained only 3.5% of the counts as cytidine-H^3.)

Evidence suggesting that the high-molecular-weight RNA of Figure 2 is of messenger RNA type is presented in Table 2 and Figure 3. Table 2 gives the base

TABLE 2

NUCLEOTIDE COMPOSITION OF P^{32}-LABELED, HIGH-MOLECULAR-WEIGHT STAGE 10 RNA

Preparation	% G + C	Mole % Nucleotide U(T)	G	C	A
G-100-excluded, stage 10 RNA	50	25	30	20	25
Xenopus total ribosomal RNA[22]	65	19	39	26	16
Xenopus DNA (liver)[23]	41	29	22	19	29

composition of a P^{32}-labeled high-molecular-weight RNA which had been excluded from a G-100 column in an experiment similar to that of Figure 2. The G—C content of this RNA is closer to that of *Xenopus* DNA than to *Xenopus* ribosomal RNA, and the over-all composition resembles that of *Xenopus* messenger RNA from other stages of development.[6] Figure 3 shows a sucrose gradient analysis of the RNA extracted from stage 10 embryos. It is apparent that the labeled high-molecular-weight RNA sediments heterogeneously as is expected of a hetero-

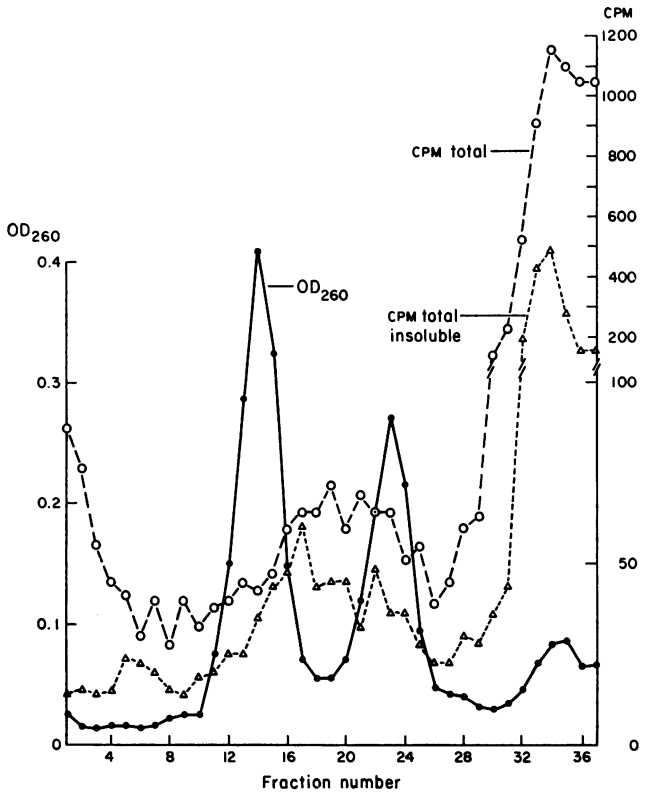

FIG. 3.—Sedimentation pattern of uridine-H^3-labeled RNA from early gastrulas (stage 10), in a 5–20% sucrose gradient. The procedures of Brown and Littna[6] were used in carrying out the gradient analyses, including final precipitation of the effluent fractions with cold acid.

geneous messenger fraction, and is neither of ribosomal nor transfer RNA type. Synthesis of high-molecular-weight RNA in stage 10 is a DNA-dependent process, as shown in Figure 4. Here it is demonstrated that a 10-min pretreatment with actinomycin D obliterates over 98 per cent of the uridine-H^3 incorporation into embryo RNA.

Conclusions.—The use of hemisected embryos in this study has made it possible to circumvent the problems posed by the low permeability of the early amphibian embryo, and to avoid the cumulative long-term labeling procedures employed in previous radioautographic and biochemical studies.[6, 24, 25] We have thus been able to monitor RNA synthesis at precisely determined developmental intervals as the embryo is moving rapidly into gastrulation. Gastrular morphogenesis continues in normal fashion in dorsal embryo halves, and analysis of RNA synthesis during gastrular onset in the dorsal-half preparation has made it possible to obtain a sharper picture than has previously been available. Our experiments have shown that large areas of the stage 8 *Xenopus* blastula suddenly undergo gene activation resulting in a dramatic multiplication of nuclear RNA synthesis rates. The properties of the newly synthesized high-molecular-weight RNA are found to be those of a heterogeneous mixture of messenger RNA's, but this identification cannot be considered definite until template activity is demonstrated for this

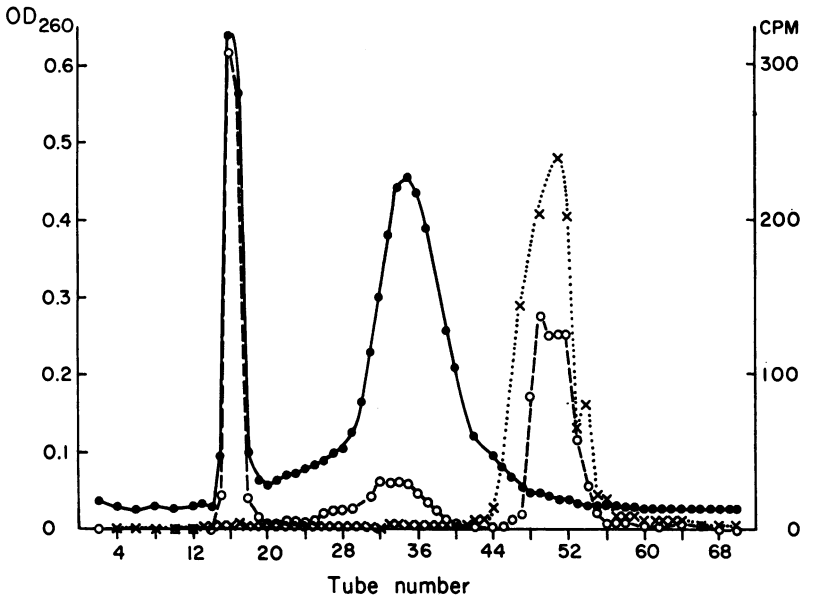

FIG. 4.—Sephadex G-100 elution pattern of uridine-H^3-labeled RNA from control and actinomycin-treated early gastrulas (stage 10). Thirty-five dorsal halves were preincubated for 10 min in 90 μg/ml of actinomycin D in Solution X.[19] Uridine-5-H^3 was added and the incubation continued for 1 hr. The control consisted of a duplicate group of embryos incubated without actinomycin. Thirty-five whole unlabeled embryos were added as carrier before the extraction. One fifth of the extracted RNA from each group was placed on each column. The second optical density peak is due to yeast transfer RNA marker. The third peak contains little UV-absorbing material but includes counts in the form of unidentified lower-molecular-weight substances which label with both uridine-H^3 and P^{32}, and which tend to appear in sucrose gradients in the same place as does transfer RNA.

RNA (experiments now in progress). Through the use of Sephadex for quantitative separation of transfer RNA, the onset of pregastrular activation has also been found to be the time when transfer RNA synthesis is initiated, in that no transfer RNA synthesis appears to exist in the immediately preceding stage 7 embryo.

According to our findings, the only RNA synthesis antecedent to stage 8–9 activation appears to be a small amount of high-molecular-weight RNA synthesis, probably messenger RNA. A qualitatively minor, but definite, early synthesis of messenger RNA is a general characteristic of cleavage and blastulation, having been identified in teleosts[26] and echinoderms[27, 28] as well as in amphibia. Blockage of early messenger synthesis does not interfere with development until the late blastula or early gastrula stages, and it is possible that this early message synthesis is required to set up the biochemical machinery for the massive gene activation to follow. Analogous systems in which a small, early RNA and protein synthesis obligatorily precede a massive subsequent gene activation include cases of hormonal stimulation (e.g., refs. 29 and 30), and the response of liver cells to hepatectomy.[31] In these cases, as in gastrulation, the underlying mechanism of gene activation remains completely obscure since we do not understand the basis for the correct selection of the genes which are to undergo activation.

* This investigation was supported in part by American Cancer Society grant E-334.

† Current address: Institute of Biological and Medical Chemistry of the Academy of Medical Science of U.S.S.R., Pogodinskaya 10, Moscow.

‡ Reprint requests should be addressed to E. H. Davidson, The Rockefeller University, New York 21, New York.

[1] Wilt, F. H., *Develop. Biol.*, **9**, 299 (1964).

[2] Comb, D. G., and R. Brown, *Exptl. Cell Res.*, **34**, 360 (1964).

[3] Davidson, E. H., G. W. Haslett, R. J. Finney, V. G. Allfrey, and A. E. Mirsky, these PROCEEDINGS, **54**, 696 (1965).

[4] Mintz, B., in *Differentiation and Development*, New York Heart Association Symposium (1964), p. 85.

[5] Aitkozhin, M. A., N. V. Belitsina, and A. S. Spirin, *Biokhimya*, **29**, 169 (1964).

[6] Brown, D. D., and E. Littna, *J. Mol. Biol.*, **8**, 669 (1964).

[7] These experiments are summarized in Morgan, T. H., *Experimental Embryology* (New York: Columbia University Press, 1927), pp. 608–656.

[8] Moore, J. A., *J. Exptl. Zool.*, **86**, 405 (1941).

[9] Harvey, E. B., *Biol. Bull.*, **79**, 166 (1940).

[10] Briggs, R., E. V. Green, and T. J. King, *J. Exptl. Zool.*, **116**, 455 (1951).

[11] Brachet, J., H. Denis, and F. deVitry, *Develop. Biol.*, **9**, 398 (1964).

[12] Gross, P. R., and G. H. Cousineau, *Exptl. Cell Res.*, **33**, 368 (1964).

[13] Brown, D. D., and E. Littna, *J. Mol. Biol.*, **8**, 688 (1964).

[14] Decroly, M., M. Cape, and J. Brachet, *Biochim. Biophys. Acta*, **87**, 34 (1965).

[15] Denis, H., in *Carnegie Institution of Washington Year Book* (1964), vol. 63, p. 505.

[16] Mariano, E. E., and A. Schram-Doumont, *Biochim. Biophys. Acta*, **103**, 610 (1965).

[17] Nieuwkoop, P. D., and J. Faber, *Normal Table of Xenopus laevis (Daudin)* (Amsterdam: North-Holland Publishing Company, 1956).

[18] Spiegel, M., *Anat. Rec.*, **111**, 544 (1951).

[19] Barth, L. G., and L. J. Barth, *J. Embryol. Exptl. Morphol.*, **7**, 210 (1959).

[20] Schleich, T., and J. Goldstein, these PROCEEDINGS, **52**, 744 (1964).

[21] Bachvarova, R., E. H. Davidson, V. G. Allfrey, and A. E. Mirsky, in preparation.

[22] Davidson, E. H., V. G. Allfrey, and A. E. Mirsky, these PROCEEDINGS, **52**, 501 (1964).

[23] Dawid, I. B., *J. Mol. Biol.*, **12**, 581 (1965).

[24] Denis, H., *Devel. Biol.*, **9**, 458 (1964).

[25] Sirlin, J. L., and C. H. Waddington, *Nature*, **174**, 309 (1954).

[26] Spirin, A. S., N. V. Belitsina, and M. A. Aitkozhin, *Federation Proc.*, **24** (5) part II, T907 (1965).

[27] Wilt, F., *Devel. Biol.*, **9**, 299 (1964).

[28] Spirin, A. S., and M. Nemer, *Science*, **150**, 215 (1965).

[29] Begg, D. J., and H. N. Munro, *Nature*, **207**, 483 (1965).

[30] Kidson, C., and K. S. Kirby, *Nature*, **203**, 599 (1964).

[31] Tsukada, K., and I. Lieberman, *J. Biol. Chem.*, **240**, 1731 (1965).