5. J. B. Moloney, *J. Nat. Cancer Inst.* **24**, 933 (1960).
6. B. S. Wright and J. C. Lasfargues, *ibid.* **35**, 319 (1965).
7. J. G. Sinkovics, B. A. Bertin, C. D. Howe, *Nat. Cancer Inst. Monogr. No. 22*, 349 (1966).
8. J. H. Venable and J. Coggeshall, *J. Cell Biol.* **25**, 407 (1965).
9. G. de Thé and T. E. O'Connor, *Virology* **28**, 713 (1966).
10. T. E. O'Connor, G. de Thé, F. J. Rauscher, M. A. Fink, *Viruses Inducing Cancer* (Univ. of Utah Press, Salt Lake City, 1966), p. 13.
11. J. T. Jacob and V. A. Fulginiti, *Proc. Soc. Exp. Biol. Med.* **121**, 109 (1966).
12. E. Norrby, *Arch. Ges. Virusforsch.* **14**, 306 (1964).
13. We thank F. J. Rauscher, National Cancer Institute, for his advice and suggestions during the preparation of this manuscript and S. A. Mayyasi, Chas. Pfizer & Co., Inc., for interest in this work. Work conducted under contract No. PH 43-66-98 within the special virus leukemia program of the National Cancer Institute.

17 March 1967

RNA and DNA Synthesis in Developing Eggs of the Milkweed Bug, Oncopeltus fasciatus (Dallas)

Abstract. *Ribosomal RNA synthesis in developing eggs of the milkweed bug,* Oncopeltus fasciatus *(Dallas), is turned on at gastrulation and is essentially turned off when organogenesis begins about 72 hours later. The pattern for DNA synthesis is similar although less pronounced.*

The general patterns of RNA and DNA synthesis during early development appear to be similar in amphibians, sea urchins, and fish (*1*). It is of interest to examine other organisms, in which embryological development is well documented, and which might be expected to yield useful information in this area. In earlier papers (*2*) we described changes in the concentrations of two groups of compounds, pteridines and nucleosides, which occur in the egg of the milkweed bug, *Oncopeltus fasciatus*. One group, at least, the nucleosides, might be expected to be involved in nucleic acid synthesis. In fact, we have demonstrated a correlation between nucleic acid synthesis and disappearance of the nucleosides inosine and guanosine in developing eggs. Now, in a more detailed examination of the synthesis of DNA and RNA, we have attempted to correlate their synthesis with embryological developments which are well documented in this organism (*3*). These studies reveal significant points of difference between the synthesis of nucleic acids in *O. fasciatus* and such synthesis in organisms that have been studied intensively heretofore.

Milkweed bugs were raised in plastic dish pans (about 35½ by 30½ by 15 cm) containing a supply of water and milkweed seeds (*4*). Gauze mats were placed in the containers to provide an area for egg-laying and to facilitate collection. Eggs were collected over 10-hour periods from 10 a.m. to 8 p.m. (*5*). They were removed from the gauze mats, divided into batches of 0.30 g (the quantity used in all experiments), and then allowed to develop at 21°C.

Since preliminary experiments (using dyes or radioactive glucose) showed that whole eggs were essentially impermeable, it was found necessary to remove part of the chorion before studies with isotopic tracers could be carried out. This was achieved by floating the eggs (0.3 g) on a 5.25 percent solution of sodium hypochlorite for 9 minutes (both concentration and time are crucial for successful experiments). They were then removed from the solution and thoroughly washed, in a small Buchner funnel (5 cm), with 1 liter of insect Ringer solution (0.15*M* sodium chloride, 0.004*M* potassium chloride, 0.002*M* calcium chloride). The partially dechorionated eggs were incubated at 25°C for 2 hours in 20 ml of Ringer solution without shaking, when they were again collected by filtration and placed in 10 ml of a standard incubation medium containing insect Ringer solution, 0.01*M* tris buffer, *p*H 7.5, and either 50 μc [^{3}H-5T]-uridine (23.0 c/mmole), or 50 μc[^{3}H-6T]-thymidine (14.5 c/mmole). After incubation for 2 hours at 25°C in the dark without shaking, the eggs were collected on a Buchner funnel and rapidly washed with several volumes of insect Ringer solution.

RNA was extracted from the eggs (0.3 g) by a modification of the phenol procedure of Kirby (*6*). The precipitated RNA was redissolved in 1.5 ml of acetate buffer and analyzed on a linear sucrose gradient (5 to 20 percent wt/vol in acetate buffer) with a Spinco centrifuge (SW 25 rotor for 17 hours at 25,000 rev/min and 4°C). Fractions (0.8 ml) were collected, diluted with water (0.6 ml), and analyzed for radioactivity (0.5-ml aliquot in 15 ml of Bray's solution, assayed on an Ansitron liquid scintillation counter) and optical density at 260 mμ (remainder of sample).

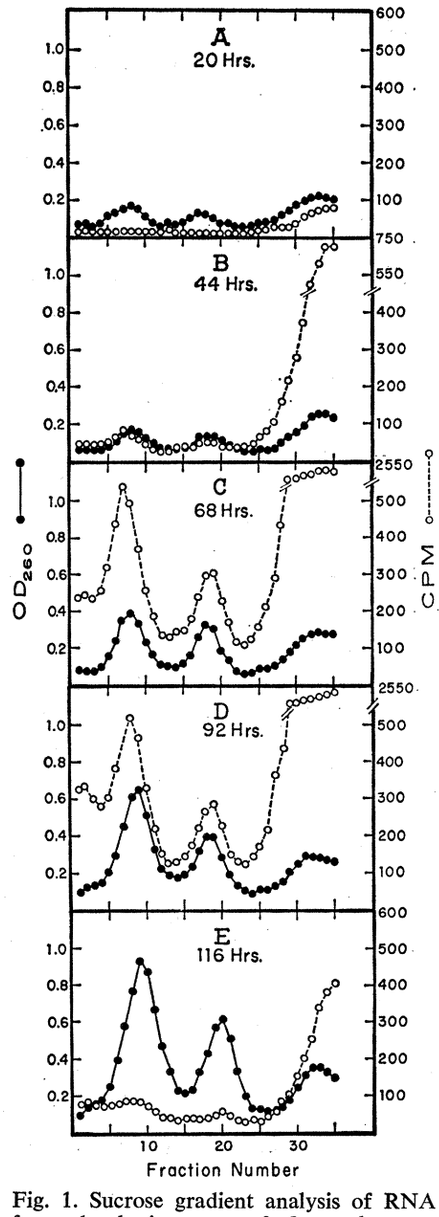

Fig. 1. Sucrose gradient analysis of RNA from developing eggs of *Oncopeltus*.

The specific activities of the two ribosomal RNA peaks were thus determined by using the equivalence, 41 μg RNA in 1 ml of fluid has an O.D.$_{260}$ of 1.0. Total tissue radioactivity was measured by using a 0.1-ml aliquot of the original homogenate, and radioactivity in ethanol-insoluble material was measured by summing all of the radioactivity measured from the sucrose gradients. Ribosomal RNA peaks were characterized by analysis on a model E analytical ultracentrifuge (*7*). They had S values of 24 and 16. The base ratios

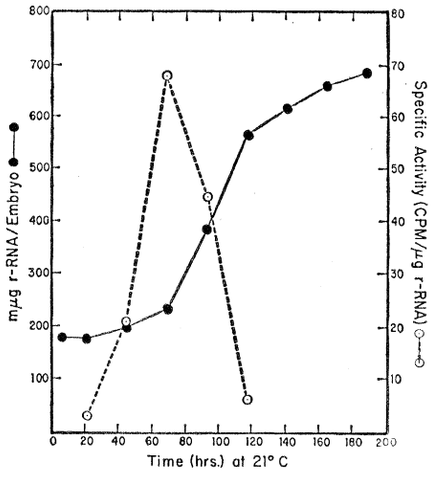

Fig. 2. Amounts of ribosomal RNA in developing *Oncopeltus* eggs, compared with its rate of synthesis.

of samples of both types, collected on three different days (3rd, 4th, and 5th) of development, were determined by standard procedures (*8*). They were not substantially different. The average values were: 24S RNA: U, 12; G, 39; A, 22; C, 27; 16S RNA: U, 16; G, 34; A, 26; C, 25. As with ribosomal RNA from *Xenopus laevis* and the chick embryo (*9*), the G + C content is high in the 24S RNA, and only slightly lower in the 16S material.

Data on the RNA content of developing eggs as determined on batches of eggs (not dechorionated) by sucrose gradient centrifugation are presented in Table 1. At 21°C, *Oncopeltus* eggs hatch into first-instar nymphs approximately 225 hours after laying. Therefore, assays were carried out on eggs at 24-hour intervals, from 0 to 4 hours ("4-hour eggs") up to 182 to 192 hours ("188 hours") of development.

The rate of RNA synthesis was measured at the five stages of development at which changes were most likely on the basis of the above measurements. The results were then as follows:

1) Newly fertilized eggs ("4-hour eggs"). The total RNA content is about 300 mμg (average weight of the egg, 0.32 mg). About two-thirds of this is ribosomal RNA and one-third is "low-molecular-weight" RNA. This latter figure is of doubtful significance because of possible contamination of the gradient with material of low molecular weight.

2) "20-hour eggs." The embryo is involved at this stage in the formation of the blastoderm. Cell walls have formed around the nuclei, and the blastoderm is going through a rapid period of growth. During this period there is no detectable synthesis of ribosomal RNA, nor does the amount (180 mμg per egg) alter. However, it is possible that a small amount of RNA of low molecular weight is being synthesized. In labeling experiments, this region of the gradient has the highest specific activity at this time. This could represent s-RNA synthesis, turnover of CCA terminal groups of s-RNA, or contamination from isotope precursor pools.

3) "44-hour eggs." Gastrulation begins after 35 hours of development and is completed, with formation of the germ bands, at about 54 hours. In 44-hour eggs, involved in gastrulation, ribosomal RNA synthesis is first detected, and the amount of ribosomal RNA has increased slightly (200 mμg per egg).

4) "68-hour eggs." The development of neural tissue from the ectoderm is proceeding, and the ventral nerve cord has taken on some definitive organization. The ectoderm shows signs of segmentation. During this stage of development there is a very marked increase in the rate of ribosomal RNA synthesis, although the net amount (237 mμg per egg) has not increased much over the 44-hour level.

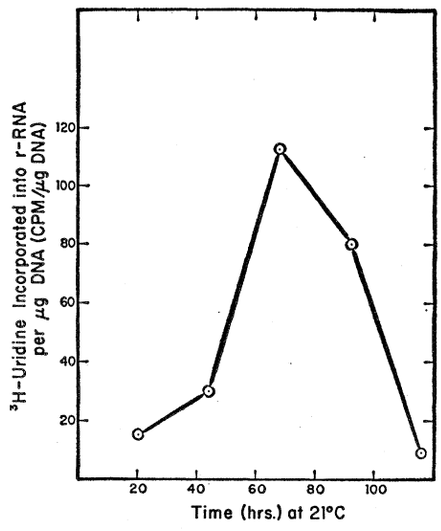

Fig. 3. Rate of RNA synthesis per unit of DNA.

5) "92-hour eggs." The complex process of organogenesis has begun. Stomodeum and proctodeum rudiments have formed, and there is considerable segmentation and nervous tissue differentiation. The rate of ribosomal RNA synthesis is still high (although now only two-thirds of the rate in 68-hour eggs), and there is a very considerable increase in the amount (383 mμg/per embryo).

6) "116-hour eggs." Considerable differentiation and organogenesis are taking place. The rate of r-RNA synthesis has now decreased to about 10 percent of the maximum level (in 68-hour-old embryos), but the amount (about 569 mμg per embryo) has increased considerably.

7) "140-, 164-, and 188-hour eggs." There is a gradual rise in ribosomal RNA over this period to about 690 mμg per embryo at 188 hours, but the rate of synthesis is now very slow (barely detectable).

From these results, illustrated in Fig.

Table 1. Analysis of s-RNA and r-RNA synthesis at various stages of development of *Oncopeltus fasciatus* eggs.

Measure	Time								
	4 hr	20 hr	44 hr	68 hr	92 hr	116 hr	140 hr	164 hr	188 hr
Low-molecular-weight RNA*	112	105	140	133	106	161	128	135	152
r-RNA*	183	180	202	237	383	569	619	662	686
Total RNA*	295	285	342	370	489	730	747	797	838
Total tissue radioactivity (count/min × 10^{-5})		1.13	1.51	2.70	2.71	1.34			
Ethanol insoluble radioactivity (count/min × 10^{-5})		0.017	0.132	0.441	0.449	0.093			
Percent ethanol-insoluble		1.5	8.73	16.3	16.6	7.6			
Specific activity†									
r-RNA		3.5	23.2	68.8	45.0	6.5			
Low-molecular-weight RNA		14.0	107.0	229.0	283.0	38.0			
Total RNA		6.9	50.1	122.0	124.0	17.0			

* Millimicrograms per embryo. † Count/min per microgram of RNA.

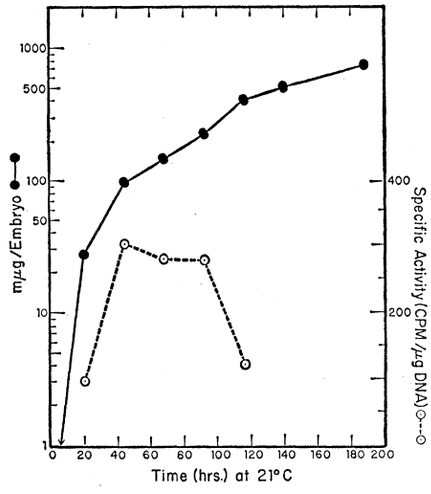

Fig. 4. Amount of DNA in developing *Oncopeltus* eggs compared with its rate of synthesis.

1, the following conclusions can be drawn: The amount of ribosomal RNA (in ribosomes?) in the newly fertilized egg (180 mμg per egg) constitutes 30 percent of the total ribosomal RNA of the fully developed egg. The existence of maternal ribosomes has been reported in a number of other organisms. In *Oncopeltus*, the maternal ribosomal RNA constitutes 0.06 percent of the weight of the egg.

In the sea urchin egg, synthesis of RNA of high molecular weight can be detected in early cleavage stages. In *Oncopeltus* eggs, on the other hand, at least in the conditions of our experiments, there is no evidence for the synthesis of comparable material at the 20-hour blastula stage.

The onset of ribosomal RNA synthesis is concurrent with the onset of gastrulation (44 hours), and the subsequent pattern seems to be quite different from that observed in other organisms. Instead of a slowly increasing amount and rate of synthesis characteristic of the sea urchin or *X. laevis*, there is a rapid burst of ribosomal RNA synthesis during the 68- to 118-hour period, when almost all of this type of RNA found in the 188-hour-old egg is synthesized; following this, there is an abrupt but not complete shutdown in synthesis. The relation between amount and rate of synthesis is shown in Fig. 2. Furthermore, this burst of ribosomal RNA synthesis, when compared, on a unit basis, with DNA synthesis (Fig. 3) indicates a high degree of differential gene activity with respect to the *rate* of ribosomal RNA synthesis. This is confirmed by an analysis of the increments of r-RNA synthesized over the range of time periods (*10*). The question then arises as to the nature of the controlling factor or factors involved in the activation and subsequent shutdown of this synthetic process.

DNA was estimated at different stages in the developing egg, using the diphenylamine procedure (*11*). The results are presented in Fig. 4. At the earliest time period (4-hour-old eggs) the results are not reliable because of the very small amount of DNA in these eggs. However, in a 6-hour-old egg of *Oncopeltus*, the periplasm contains several nuclei and thus DNA must be present in some quantity. Roughly, a 4-hour-old egg has about 10 to 100 μμg of DNA.

The rate of DNA synthesis (as determined by thymidine labeling and assay of radioactivity in perchloric acid-insoluble material) in the 20-hour blastula stage is high but it does not account for the increase in amount of DNA and cell numbers which occurs at about this stage. Thus it appears that DNA made at this early stage of development may arise, at least partially, from preformed precursors. From gastrulation (44 hours) up to 92 hours of development, DNA is synthesized at a constant rate which then decreases in the later stages of development. This is reflected also in the total amount of DNA in eggs at these stages. If this is, at least roughly, an indication of the rate of cell division, it is interesting to note that the control of ribosomal RNA synthesis persists through successive cell generations. Between the 92nd and 116th hour of development there is a decrease in the rate of DNA synthesis, which parallels the decrease in RNA synthesis, and which, in turn, is correlated embryologically with the onset of organogenesis and complex differentiation.

These results confirm and extend the results reported by Lockshin (*12*) for Coleoptera, by use of radioautography. In addition they reveal new phenomena (in particular, the shutdown of ribosomal RNA synthesis) which would be difficult to detect with Lockshin's technique. Further exploration in this system may lead to an understanding of the factors controlling these phenomena.

STEPHEN E. HARRIS
HUGH S. FORREST
Zoology Department,
University of Texas, Austin 78712

References and Notes

1. D. D. Brown, in *Developmental and Metabolic Control Mechanisms and Neoplasia* (Williams and Wilkins, Baltimore, 1965), p. 219.
2. H. S. Forrest, M. Menaker, J. Alexander, *J. Insect Physiol.* **12**, 1411 (1966); H. S. Forrest, S. E. Harris, L. J. Morton, *ibid.* **13**, 359 (1967).
3. F. H. Butt, *Cornell Univ. Agr. Expt. Sta. Mem. 283* (1949).
4. S. D. Beck, C. A. Edwards, J. T. Medler, *Ann. Entomol. Soc. Am.* **51**, 283 (1958).
5. Although eggs were collected over a 10-hour period, the great majority are deposited in a 2-hour period between 2 p.m. and 4 p.m. Their age is therefore much more precise than the collection time would indicate; for convenience, eggs laid between 10 a.m. and 8 p.m. are referred to by a single age (4-hour-old eggs).
6. K. S. Kirby, *Biochem. J.* **64**, 405 (1956). After homogenization in a cold glass/Teflon homogenizer, 4.5 ml of cold acetate buffer (0.01*M* sodium acetate, 0.1*M* sodium chloride, 0.001*M* magnesium chloride, *p*H, 5.0 at 25°C), containing 250 μg of polyvinyl sulfate per milliliter, was added, followed by 0.6 ml of 10 percent sodium dodecyl sulfate. The mixture was extracted twice with water-saturated phenol, and 2 volumes of cold, 95 percent ethanol were then added to the aqueous layer. After 30 minutes at —20°C the precipitated RNA was collected, redissolved to 3 ml of the same buffer, and reprecipitated in the same way with ethanol. The pellet was gently washed with acetate buffer.
7. We are grateful to Dale H. Henning for carrying out these determinations.
8. B. Magasanik, in *The Nucleic Acids*, E. Chargaff and J. N. Davidson, Eds. (Academic Press, New York, 1955), vol. 1, p. 373.
9. E. Bell, in *Molecular and Cellular Aspects of Development*, E. Bell, Ed. (Harper and Row, New York, 1965), p. 314.
10. The question of pool size, which might distort the results presented in this paper, is a difficult one. It can be assumed that pool size of immediate precursor (uridine triphosphate) is larger during rapid synthesis and smaller during slow synthesis (such a situation is suggested by the work of Bucher and Swaffield on hepatectomized liver) [N. L. R. Bucher and M. N. Swaffield, *Biochim. Biophys. Acta* **108**, 55 (1965)]. Thus the problem is in comparing situations in which more isotope is entering a large pool, on the one hand, and less isotope is entering a small pool, on the other hand. Because of the correlation between the isotope experiments, and the direct measurements of incremental r-RNA synthesis in relation to accumulated RNA or DNA, it seems reasonable to suppose that the two opposing effects resulting from this situation cancel each other out. A referee has also pointed out that endogenous nucleosides decrease during development, and that this must therefore affect precursor pool size. However, only purine nucleosides have been demonstrated to be present in young eggs and to decrease during development. Pyrimidine nucleosides (or immediate precursor) have not been found in eggs, and a large, changing uridine pool is therefore not a complicating factor.
11. K. Burton, *Biochem. J.* **62**, 315 (1956). DNA was extracted by homogenizing 0.3-g egg samples with 0.3 ml of insect Ringer solution, treating 1.5 ml of the homogenate with 5 ml of 0.4*M* perchloric acid, collecting the precipitate after 1 hour at 0°C, washing it with 5 ml of 0.4*M* perchloric acid, and extracting the ether-dried precipitate twice with 2-ml portions of 0.4*M* perchloric acid at 85°C for 30 minutes.
12. R. A. Lockshin, *Science* **154**, 775 (1966).
13. Supported in part by NIH grant GM-12323 and training grants 2T1 GM-337-06 and GM 00337, and by a grant from the Robert A. Welch Foundation, Houston, Texas.

18 April 1967