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*ON THE RNA SYNTHESIZED DURING THE LAMPBRUSH PHASE OF AMPHIBIAN OÖGENESIS**

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Lampbrush chromosomes have been observed in the growing oöcytes of animals ranging from mollusks to mammals. They are present throughout the diplotene of the first meiotic division. These elongate, paired structures bear many thousands of loops projecting laterally from the main chromosomal axis, and they are characteristically accompanied in the nuclear sap by several thousand small nucleoli. It was long ago suggested that the organization of the egg and the early processes of embryogenesis were the result of nuclear activity occurring during ovarian oögenesis,[1] and we now know the lampbrush chromosomes of amphibian oöcytes are in a state of intense genetic activity. The visible evidence of this activity is provided by autoradiographic studies of RNA synthesis, which have demonstrated newly synthesized RNA in all regions of the chromosomes[2, 3] as well as in the nucleoli. A significant comparison has been drawn by Izawa, Allfrey, and Mirsky to illustrate the unique, high level of genic activity characteristic of lampbrush chromosomes.[4] Their measurements show that the ratio of RNA to DNA in lampbrush chromosomes is over 100 times greater than the comparable figure for the chromatin of a typical differentiated cell such as a liver cell. The experiments to be reported here throw some light on the nature of this intense gene activity. It is shown that most or all of the RNA synthesized during the lampbrush stage of oögenesis in the anuran *Xenopus laevis* is conserved, at least until ovulation, and that over 90 per cent of this RNA is of ribosomal type. The remaining RNA synthesized in these oöcytes appears to be of a different type, and possesses unusual characteristics.

Materials and Methods.—Total acid-insoluble RNA: Mature *Xenopus* females were injected with pituitary extract to stimulate oögenesis, and three days later the RNA of the oöcytes was labeled by injection into the dorsal lymph sac of a total of 1 mC of uridine-H^3/animal, administered in three equal doses over the succeeding 3 days. Three days following the termination of labeling, the ovaries were removed, and oöcytes of the desired stages were dissected free from their follicles with watchmaker's forceps, under Barth and Barth's solution X,[5] containing 2 × NaCl. It is essential that the follicles be removed, since autoradiographs of Ficq and others have shown that follicle cells are very active in RNA synthesis.[6, 7] The oöcytes were washed in cold acid and fat solvents, and the RNA extracted with hot 0.5 *N* PCA.[8] RNA content was

measured by the highly specific phloroglucinol method of Dische and Borenfreund.[9] Over 95% of the counts scored as RNA could be rendered acid-soluble with RNAse.

Phenol extraction of RNA: The homogenate of several hundred defollicled oöcytes was phenol-extracted at pH 7.6 and pH 9.2, as described by Brawerman *et al.*[10] All solutions were tris-buffered and contained 4 μg/ml polyvinylsulfonate (PVS) or 0.015 *M* naphthalene disulfonate as RNAse inhibitor. The RNA of each fraction was twice precipitated with 67% ethanol–10% NaCl. Further purification was carried out with Sephadex G25 and G50 columns. Linear, 4.0 cc 9–30% sucrose gradients centrifuged at 39,000 rpm for 5 hr in the Spinco SW39 head were employed in sedimentation analyses.

No DNA synthesis occurs at the lampbrush stage of oögenesis and, therefore, the presence of DNA could not affect gradient cpm profiles. Furthermore, gradient analysis of the RNA preparations after RNAse treatment gave little evidence of DNA in the RNA preparations by optical density criteria.

Nucleotide analysis of RNA: RNA base compositions were determined by optical density and P^{32}-count distributions in hydrolysates of Sephadex-purified, phenol-extracted, P^{32}-labeled RNA. To separate the nucleotides, the Dowex-50 and Dowex-1 method of Katz and Comb,[11] and the Dowex-1 system of Osawa *et al.*[12] were employed interchangeably.

RNA Synthesis in Oöcytes at Progressive Stages of Oögenesis.—The ovary of *Xenopus* contains oöcytes in all stages of maturation, in this study classified according to Duryee.[13] Table 1 shows that most of the RNA finally present in the

TABLF 1

RNA SYNTHESIS IN *Xenopus* OÖCYTES

Stage	State of nuclear apparatus	Diameter of oöcyte (mm)	μg RNA*	Relative specific activity of RNA 3–7 days after labeling†	Average specific Activity (cpm/μg RNA) of RNA after Labeling with 1 mC Uridine-H^3 in One Representative Experiment‡ 1 day	3 days	14 days	42 days	75 days
6	Lampbrush retracted	1.0–1.2	2.6	16	14	10	30	53	60
5	Late lampbrush	0.6–0.8	2.8	77	40	65	100	38	57
4	Lampbrush maximal	0.5–0.6	1.9	100	37	81	108	32	34
3	Beginning lampbrush	0.4–0.5	0.7	49					
Total cpm in the RNA of an average Stage 4 + an —average Stage 5 + an average Stage 6 oöcyte					226	417	441	382	392

* Values based on 20–30 determinations for each stage of oöcyte.

† Values based on 10–12 determinations for each stage of oöcyte. Value for Stage 4 set at 100.

‡ Each lot of toads tends to label at a different over-all rate, in our experience, and it is consequently difficult to pool absolute specific activity data. However, *relative* synthetic activities of the different stages of oöcyte agree well in different experiments. The animals were labeled with 3, 0.33 mC injections of uridine-H^3 (sp. ac. 3.73 C/mM) given on the 3rd, 4th, and 5th days following stimulation with bovine pituitary extract. The animals were caused to ovulate again at 30 days, to remove previously ripened Stage 6 oöcytes and thus prevent dilution of the newly maturing Stage 6 oöcyte population.

mature oöcyte is synthesized during the lampbrush phase, i.e., in Stages 4 and 5. Between the end of Stage 5 and the end of Stage 6, no additional accumulation of RNA is detected, though the mass of the oöcyte increases by a factor of 4. The level of RNA labeling 3 days after an injection of uridine-H^3 is far lower in Stage 6 oöcytes than in oöcytes of the lampbrush Stages 4 and 5 (Table 1). Stage 3 oöcytes also synthesize RNA at less rapid rates than do Stages 4 and 5 oöcytes. Thus Stage 4, the most pronounced lampbrush phase, is the phase of maximal RNA synthesis. It should be noted that the Stage 6 Xenopus oöcyte population sampled does not include those oöcytes which were already sufficiently mature to have been ovulated when the animals were stimulated with pituitary extract at the start of the experiment.

The right-hand columns of Table 1 describe the distribution of RNA counts at various times after a three-day pulse of uridine-H^3. As time progresses, oöcytes which had been in the lampbrush phase when the labeled precursor was given, mature and disappear from the Stage 4 population. Thus, the specific activity drops in Stage 4 oöcytes after 14 days, and more intense labeling begins to appear in the Stage 6 population. The specific activity of Stage 6 oöcytes increases until, after 6 weeks from the time of labeling, these are the most highly labeled oöcytes. This interpretation of the data is supported by a study of the acid-soluble nucleotide pool in oöcytes during maturation. Figure 1 shows the parallel clearance of label from the acid-soluble pool in all classes of oöcyte and from the blood of the uridine-H^3-labeled animals. The radioactivity of RNA precursors in the oöcytes falls steeply in close equilibrium with blood radioactivity, and it can be concluded that the observed labeling of the RNA in Stage 6 oöcytes after long time intervals is not due to late labeling from "stored" nucleotides.

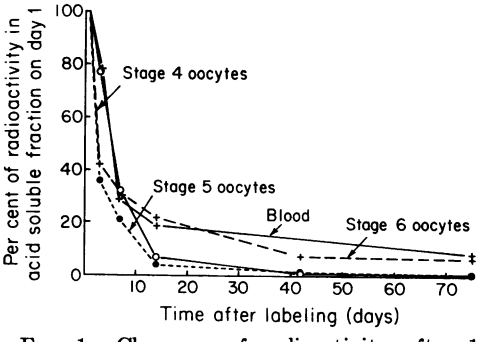

FIG. 1.—Clearance of radioactivity after 1 mC uridine-H^3/animal in blood and in oöcytes of Stages 4, 5, and 6. Radioactivity was assayed in the acid-soluble fraction of oöcyte homogenates and of blood serum at the times noted on the abscissa.

The total counts incorporated into oöcyte RNA during and shortly after a 3-day pulse of uridine-H^3 are conserved throughout the maturation of the oöcyte, as shown in the last line of Table 1. Due to the variability of the animals, experiments such as that of Table 1 cannot be expected to reveal small increases or losses in total RNA label. It is nonetheless clear that most, or perhaps all, of the RNA synthesized during the lampbrush phase is conserved for several months or more, i.e., through the whole period of oögenesis, rather than being used and turned over. Ovulated eggs bear the same label as Stage 6 oöcytes. Thus, it can be stated that at least 75 per cent of the RNA present in the egg when it is fertilized had been synthesized during the lampbrush phase of oögenesis.

Qualitative Identification of the RNA Synthesized in Stage 4 Oöcytes.—All RNA synthesis in amphibian oöcytes is nuclear, according to autoradiographic studies,[6, 14] and this nuclear RNA synthesis, as in other cells, appears to be DNA-dependent.[3] Since the mechanism of primary gene expression is the DNA-dependent synthesis of RNA, an important step toward understanding the embryological function of the active DNA in the lampbrush would be the identification of the types of RNA being synthesized in such a nucleus. We have extracted the RNA's of Stage 4 oöcytes using the phenol method of Brawerman *et al.*,[10] and have studied the rates of labeling, base composition, and sedimentation behavior of the RNA's. In the Brawerman method an initial phenol extraction is carried out at pH 7.6, and the interfacial residue is then re-extracted at pH 9.2. Brawerman *et al.* found that RNA possessing "template" activity in cell-free protein synthesis systems is refractory to the initial extraction at pH 7.6, but is released into the aqueous phase at higher pH. Ribosomal and sRNA, on the other hand, were obtained in the pH 7.6 extract.[10] In our experiments the RNA's extracted from oöcytes at the two pH

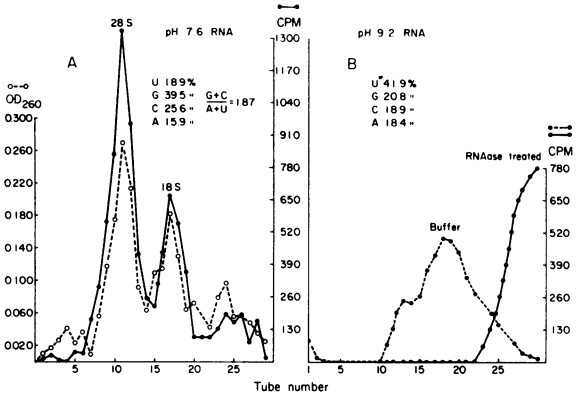

Fig. 2.—Sucrose gradient sedimentation pattern, and base composition, of uridine-H^3-labeled Stage 4 RNA. The RNA was extracted at pH 7.6 (*A*) and pH 9.2 (*B*) as described in text. In (*A*), OD_{260} and cpm profiles are shown for pH 7.6 RNA. This RNA is clearly of ribosomal type since it is composed of components sedimenting at 28 S and 18 S, a property specifically characteristic of ribosomal RNA's. Furthermore, the high G + C/A + U base ratio of this RNA is typical of ribosomal RNA, and in composition it resembles closely the RNA obtained by Brown and Gurdon from post-fertilization *Xenopus* ribosomes.[16] In (*B*), the cpm profiles of pH 9.2 RNA with and without previous RNAse treatment are presented. This RNA sediments heterogeneously, and the same radioactivity profile is obtained regardless of whether the label is phosphate-P^{32} or uridine-H^3. As with pH 7.6 RNA, the cpm profile parallels the OD_{260} profile of the bulk RNA in the fraction. Gradient preparation of pH 9.2 RNA reveals no low-molecular-weight components, and sedimentation patterns obtained with this RNA are the same irrespective of whether the fraction is further purified by passage through Sephadex G25 and G50 columns. The fraction contains some ribosomal RNA contaminant, as evidenced by the small shoulder in the 26–28 S region of the gradient profiles (Figs. 2*B* and 4*B*). The fraction as a whole bands broadly between the 5 S and 25 S regions. That pH 9.2 RNA is not merely degraded ribosomal RNA is shown by the facts (1) that its specific activity is always different from that of ribosomal RNA extracted from the same oöcytes, and (2) that its base composition is distinct from that of ribosomal RNA. In nucleotide composition studies it was found that alkaline hydrolysates of Sephadex-purified pH 9.2 RNA contain a highly radioactive, ultraviolet-absorbing compound eluted with uridylic acid in both the Dowex-1 and Dowex-50 systems. The relative specific activity of this minor component is 3–6 times that of the other nucleotides. Optical density and P^{32}-count distributions yield similar values for the A, G, and C content of pH 9.2 RNA, but only OD can be used for estimating the nucleotide content of the uridylic acid region of the effluent. The high specific activity component can be separated from uridylic acid by paper electrophoresis and on Dowex-1, and is probably another nucleotide. The value given for U* in 2*B* represents uridylic acid (UMP) plus the unidentified nucleotide, calculated by OD_{260} from the molar extinction coefficient of the major component, UMP. Current experiments, in which pH 9.2 RNA is purified of all ribosomal RNA and the high specific activity component separated before nucleotide analysis, indicate that the base composition of this RNA is of high A-U, messenger RNA type.

values also proved to be strikingly dissimilar.

pH 7.6 RNA: Figure 2*A* demonstrates that the oöcyte RNA extracted at pH 7.6 is ribosomal RNA. Further evidence that pH 7.6 RNA is of ribosomal type was provided by an experiment in which a ribosomal pellet was prepared from a labeled Stage 4 oöcyte homogenate by ultracentrifugal fractionation. The RNA present in this and in heavier cell fractions was then extracted and analyzed. Though the oöcytes had been labeled only 3 days before, 75 per cent of the radioactive pH 7.6 RNA was already localized in the ribosomes of the 105,000 *g* pellet. Only 67 per cent of the total ribosomal RNA was recovered in the pellet, and it follows that the 75 per cent value is an underestimate of the true proportion of newly synthesized pH 7.6 RNA which is localized in the ribosomes after 3 days.

If the pH 7.6 extract is not first purified on Sephadex, a light highly radioactive polynucleotide component is also present, banding sharply at the top of the gradients. This material is discussed further below.

pH 9.2 RNA: Figure 2*B* shows that the RNA extracted at pH 9.2 is of high molecular weight but differs from ribosomal RNA in both base composition and sedimentation pattern.

TABLE 2

DISTRIBUTION OF COUNTS IN STAGE 4 OÖCYTE RNA, THREE DAYS AFTER LABELING WITH 1 mC OF URIDINE-H^3*

RNA fraction	Total counts in fraction	Total RNA in fraction (μg)	Specific activity of fraction
pH 7.6 Acid-insoluble	379,900		554
Acid-soluble	81,511		2264
Total	461,420	721	640
pH 9.2 total	31,172	51	611
Total acid-insoluble + acid-soluble RNA of whole homogenate (before phenol extraction)	472,236	867	544

* Data averaged from several extractions, after normalizing to the median total homogenate acid-insoluble cpm. The counts in the acid-soluble pH 7.6 fraction are obtained by subtraction of the acid-insoluble counts from the total counts in the fraction. The efficiency of the extractions averaged here varied from 85 to 91%, pressed as

$$\left(\frac{\text{total acid-insoluble RNA counts extracted}}{\text{total acid-insoluble RNA counts in homogenate before extraction}}\right).$$

Quantitative Distribution of Newly Synthesized RNA.—The newly synthesized RNA is distributed among the various RNA fractions as shown in Table 2. The pH 9.2 fraction accounts for only 5–10 per cent of the total RNA, judged either by total counts or by direct measurements using the phloroglucinol or OD_{260} methods. Specific activity of the pH 9.2 fraction is always of the same order of magnitude as that of the pH 7.6 fraction, though the actual values are never exactly the same. In contrast, the 28 S and 18 S RNA's of the pH 7.6 fraction possess identical specific activities in uridine or P^{32}-labeling experiments. The acid-soluble component of the pH 7.6 extract listed in Table 2 is the low-molecular-weight polynucleotide alluded to above. It is ethanol-precipitable, and is probably analogous to the "acid-soluble RNA" reported by Finamore and Volkin in *Rana*.[15] Figure 3 shows a separation of this RNA from the ribosomal RNA on Sephadex G 50, which retards molecules under 8,000–10,000 in molecular weight. In the Stage 4 oöcyte, the mass of this low-molecular-weight fraction varies about an average value of about 5 per cent of the mass of the ribosomal RNA, though it may contain more than 20 per cent of the total pH 7.6 counts. The relation between the specific activity of the acid-soluble and the acid-insoluble pH 7.6 RNA is remarkably constant in various experiments: specific activity of the light RNA fraction is always 3–4 times that of the ribosomal RNA whether the label used is uridine-H^3, as in Table 2, or P^{32}-phosphate (Fig. 4). When the specific activity of the whole pH 7.6 extract is thus subdivided, it is seen (Table 2) that the specific activity of the acid-insoluble fraction approximately equals that of the bulk acid-insoluble RNA of the whole homogenate, before phenol extraction.

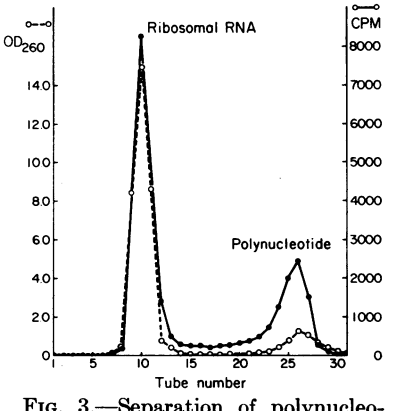

FIG. 3.—Separation of polynucleotide fraction of pH 7.6 RNA extract from ribosomal RNA on a Sephadex G50 column. The RNA, which was labeled with phosphate-P^{32}, was placed on the column dissolved in 0.5 cc water, and successive 0.5-cc fractions were collected.

The amount of ribosomal RNA in Stage 4 oöcytes is 14–24 times as great as the amount of pH 9.2 RNA in our extractions, and the likelihood that some ribosomal RNA contami-

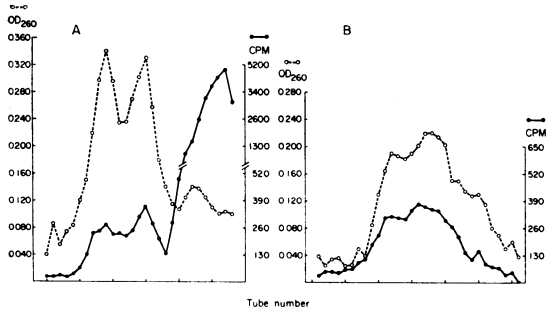

FIG. 4.—Sucrose gradient sedimentation pattern of uridine-H^3-labeled RNA extracted at pH 7.6 (*A*) and pH 9.2 (*B*). The counts represent RNA synthesized within a 60-min period of exposure to the radioactive precursor (3 mC/animal).

nant is present in the pH 9.2 extract makes this estimate a conservative one. Since the amounts of RNA of each type are so unequal, comparison of the specific activities of the two fractions is less meaningful than comparison of the total counts incorporated in each. Assuming that the ribosomal and the pH 9.2 species of RNA are both synthesized from the same labeled precursor pools, the total count data of Table 2 leads to the surprising conclusion that at any one time, an average 93 per cent of the acid-insoluble RNA synthesis occurring in the lampbrush-stage oöcyte nucleus is ribosomal RNA synthesis. In some experiments this figure is as high as 97 per cent. Even in HeLa cells, which are growing and dividing at optimal rates, only 60–70 per cent of the RNA synthesized at any one time is ribosomal in type.[17]

RNA Synthesis after 60-Min Pulse Labeling.—It is desirable to know whether there exists a species of RNA with a high rate of synthesis and turnover in Stage 4 oöcyte nuclei. A short-lived RNA might not be identified as such after a period of labeling as long as 3 days. However, we have been able to rule out this possibility by characterizing the radioactive RNA extracted from Stage 4 oöcytes after only 60 min exposure to uridine-H^3 (Fig. 4). As before, the pH 7.6 ribosomal RNA and the pH 9.2 RNA are both labeled in close parallel to their OD_{260} profiles. The ratios of pH 7.6 ribosomal RNA specific activity to pH 9.2 RNA specific activity in two experiments was 1.7 and 1.9. These values are indistinguishable from the corresponding values after 3 days of labeling. Thus, we can conclude that the relationship between ribosomal and pH 9.2 RNA are the same irrespective of the length of the synthesis period monitored.

The gradient of Figure 4*A*, however, does show an extremely radioactive light peak, containing, at 60 min, most of the radioactivity in the extract, and the question arises whether this could be the labeled material observed in lampbrush autoradiographs. This material is the cold acid-soluble polynucleotide component discussed above, and it is unlikely that such a uridine-labeled polynucleotide would remain insoluble in the cold acid washes to which autoradiographs are exposed. However, a very high local concentration *in situ* and/or binding to proteins might render such a molecule insoluble in cold acid. If this is the case, the polynucleotide is made in the nucleus. If not, it could be synthesized in the cytoplasm of the oöcyte since the evidence for nuclear localization of RNA synthesis is solely autoradiographic.

Discussion.—This study has demonstrated quantitatively that the predominant gene product of the lampbrush-stage oöcyte nucleus is ribosomal RNA. The outstanding function of the nuclear apparatus of the oöcyte at this stage has been shown to be the synthesis of huge quantities of ribosomal RNA, which is conserved

throughout oögenesis, and of ribosomes themselves. It is known from the recent work of Brown and Gurdon[16] that in *Xenopus* further ribosomal RNA synthesis does not occur until late in gastrulation. The protein synthesis required for the initial processes of embryogenesis is carried out completely on pre-existent ribosomal components. Our findings dovetail with this view, and indicate the source of these preformed ribosomal components. At least one of the essential functions of the unique lampbrush-stage nucleus is thus elucidated, and we have thereby provided an explanation for the remarkably large amount of RNA synthesis: this nucleus is assigned the task of building the major fraction of the massive protein-synthesizing machinery with which the future embryo will begin life.

However, these results pose an interesting problem. If indeed 90–95 per cent of the RNA synthesis in a lampbrush nucleus is ribosomal, what is the cytogenetic meaning of the autoradiographs showing rapid RNA synthesis occurring all along the linear lampbrush structure itself? Edstrom and Gall[18] have analyzed the RNA of these chromosomes, and found it to be of messenger type in base composition. However, their analyses concern the bulk RNA of the chromosomes, not necessarily the RNA momentarily being synthesized. Brown and Gurdon[16] have identified a nucleolar organizer region in the *Xenopus* genome, and believe ribosomal RNA synthesis to be restricted to polygenic sites in this area, amounting to about 10 per cent of one chromosome. In the urodele lampbrush chromosome, Gall has similarly found a localized nucleolar organizer area which he believes responsible for the production of the several thousand nucleoli present in the nuclear sap.[19] Now these nucleoli contain RNA of ribosomal type, and in radioautographs they demonstrate actinomycin-sensitive RNA synthesis themselves[3] at a rate roughly equivalent to that of chromosomal RNA synthesis. Painter and Taylor[20] have reported that such nucleoli contain Feulgen-positive areas. Since the nucleolar RNA synthesis is DNA-dependent, and the nucleolar RNA is ribosomal, it could well be that the lampbrush nucleolar organizer region produces free ribosomal RNA synthesis units (nucleoli) each containing its own packet of DNA coding for ribosomal RNA and active in the synthesis of ribosomal RNA. One objection to such a hypothesis is that in the urodele *Triturus* the total mass of the nucleolar RNA is only about 2 times the mass of the total chromosomal RNA,[18] and unless there are large species differences in RNA distribution or synthesis rates, the 90–95 per cent ribosomal RNA synthesis occurring in the *Xenopus* nucleus could not easily be explained as mainly nonchromosomal. These are scarcely the sole alternatives. however, for many unexplained facts remain. For example, one would like to known what is the nature and disposition of the excess chromosomal DNA which has been shown by Izawa, Allfrey, and Mirsky[4] to be present in amounts 4 times greater than can be predicted on the basis of diplotene tetraploidy.

The possible function of the heterogeneous pH 9.2 RNA and the reason for its peculiar base composition are at present obscure. It is interesting to note that Finamore and Volkin[21] reported an RNA fraction localized in the nuclei of mature *Rana* oöcytes which contained 43 per cent uridylic acid, using a Dowex-1 system for the analysis. Experiments are now in progress to determine whether an RNA similar to the pH 9.2 RNA can be extracted from mature oöcytes. Aside from tiny peaks heavier and lighter than the ribosomal RNA in the gradient preparations

(Fig. 2A), the pH 9.2 RNA is the only nonribosomal, acid-insoluble RNA synthesized in the lampbrush nucleus that we have been able to detect.

Summary.—The RNA synthesized in lampbrush-stage oöcytes of *Xenopus laevis* has been extracted and characterized. Most of the RNA present in the mature oöcyte is synthesized in the lampbrush stage, and over 90 per cent of this is ribosomal RNA.

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*THE BIOSYNTHESIS OF APOFERRITIN BY RETICULOCYTES**

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Ferritin has been found in erythroblasts by electron microscopy (Bessis and Breton-Gorius),[1] and hemosiderin, a derivative of ferritin, has been detected in reticulocytes by histochemical techniques (Matioli).[2]

The presence of ferritin in hemoglobinopoietic cells opened the question of its origin. A widely accepted view, based on electron microscopy, suggests that ferritin is transferred through a process similar to pinocytosis from macrophages to the surrounding erythroblasts.[1] It follows from this theory that the gene for apoferritin synthesis in erythroblasts should be constantly repressed. Further, the destiny of the iron absorbed by the erythropoietic cells, regardless of the stage of maturation, would serve mainly for hemoglobin synthesis.