* This investigation was supported by grants from the National Institute of Neurological Diseases and Blindness, U. S. Public Health Service, aided by a contract with the Office of Naval Research, and by Higgins funds allotted to Princeton University. Permission is granted for reproduction and use by the United States Government.

[1] Killian, G., "Zur vergleichenden Anatomie und vergleichenden Entwickelungsgeschichte der Ohrmuskeln," *Anat. Anz.*, **5**, 226–229 (1890).

[2] Gaupp, E., "Ontogenese und Phylogenese des schalleitenden Apparates bei den Wirbeltieren," *Ergeb. Anat. Entwickl.*, **8**, 990–1149 (1898).

[3] Versluys, J., Jr., "Entwicklung der Columella auris bei den Lacertiliern," *Zool. Jahrb., Abt. Anat.*, **19**, 107–188 (1904).

[4] Wever, E. G., "The cochlear potentials and their relation to hearing," *Ann. Otol. Rhinol. Laryngol.*, **68**, 975–989 (1959).

[5] Wever, E. G., and Jack A. Vernon, "The problem of hearing in snakes," *J. Auditory Res.*, **1**, 77–83 (1960).

[6] Wever, E. G., and Jack A. Vernon, "The sensitivity of the turtle's ear as shown by its electrical potentials," these PROCEEDINGS, **42**, 213–220 (1956); "Auditory responses in the common box turtle," *ibid.*, 962–965.

[7] Wever, E. G., and Jack A. Vernon, "Auditory responses in the spectacled caiman," *J. Cellular Comp. Physiol.*, **50**, 333–339 (1957).

---

*COMPOSITION OF THE NUCLEUS AND CHROMOSOMES IN THE LAMPBRUSH STAGE OF THE NEWT OÖCYTE**

BY MITSUO IZAWA, V. G. ALLFREY, AND A. E. MIRSKY

THE ROCKEFELLER INSTITUTE

Communicated September 4, 1963

A lampbrush chromosome is metabolically active at many sites all along its axis.[1, 2] This is in marked contrast to other interphase chromosomes, in which a few loci are active while most are relatively inactive,[3, 4] and also in striking contrast to mitotic chromosomes, which appear to be metabolically inert, though they are being moved about.[5] Since lampbrush chromosomes are so active metabolically, it is of interest to know about their composition and to compare it with the composition of other chromosomes. In this paper we present determinations of the deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein contents of lampbrush chromosomes and of the nuclei containing these chromosomes.

Lampbrush chromosomes occur in the growing oöcytes of many different animals.[6] In our work we have used oöcytes of the newt, *Triturus viridescens*. Chromosomes of ordinary size are present in the oöcyte both before and after the period of growth; presence of giant lampbrush chromosomes in the oöcyte only at the time of growth points, therefore, to a correlation between the massive synthesis of cytoplasm and the formation of the enormous chromosomes with their lateral extensions, the so-called loops.[7, 8]

Other well-known giant chromosomes are the polytene chromosomes of dipteran larvae.[3] The puffs of these chromosomes are sites of marked accumulation of protein and nucleic acid,[9, 10] and puffs of polytene chromosomes may perhaps be compared with the loops of lampbrush chromosomes, for both puffs and loops are

sites of activity. A difference between these structures is that although loops occur at almost all chromomeres of lampbrush chromosomes,[8] puffs occur at only about 2 per cent of the bands of giant polytene chromosomes.[3] Another difference between these two chromosomes is that the oöcyte chromosomes are destined to replicate after fertilization, whereas the polytene chromosomes of the dipteran larvae are in cells which will not divide and which will indeed be discarded at pupation.

Most interphase chromosomes are of course very much smaller than either lampbrush or dipteran polytene chromosomes. In the ordinary-size interphase chromosomes, DNA is the component which has been determined most often quantitatively. There is a well-known rule according to which the quantity of DNA per set of chromosomes is constant for the different cells of a given organism.[11, 12] The DNA content per set of chromosomes in somatic cells of the newt was determined in the work on which the constancy rule was established, and the constancy rule has led some investigators to assume that newt lampbrush chromosomes have the same DNA content. It may, however, be questioned whether the constancy rule, which applies fairly well to the chromosomes of somatic cells and sperm cells, holds for the strikingly different lampbrush chromosomes. In the generality of interphase chromosomes, as in the giant polytene chromosomes, most loci are relatively inactive; in lampbrush chromosomes, on the contrary, most loci are active. In this paper it will be shown that the DNA content per set of newt lampbrush chromosomes is in fact much higher than the DNA content per set of chromosomes in other cells of the newt. It will be shown also that the protein and RNA contents of lampbrush chromosomes are very different from those of the chromosomes in other cells.

Materials and Methods.—Ovarian eggs of the newt, *Triturus viridescens*, were used for all experiments on determination of chemical constituents.

Preparation of the whole nucleus: Procedures for dissecting the oöcytes were those developed by Duryee,[13] Gall,[7] and Callan and Lloyd.[8] A detailed description is given by Callan and Lloyd. Since it was recently found that newt oöcyte nuclei isolated in saline swell and lose dry weight,[14, 15] the medium for isolating nuclei to be used for DNA, RNA, and protein analyses contained 3% polyvinylpyrolidone (PVP) in 0.1 *M* NaCl adjusted to pH 6.8 with NaOH. In this medium an isolated nucleus was of the same size as a nucleus *in situ*. The cell membrane of the oöcyte in PVP solution was broken with the fine tip of tweezers under the dissecting microscope. The nucleus was squeezed out, and yolk platelets attached to the nuclear membrane were removed with the tweezers. After collection of a certain number of the nuc'ei into a conical 1-ml centrifuge tube using a fine-tipped pipette, PVP was washed out with cold absolute ethanol. The chemical analyses described below were done on this preparation.

Preparation of chromosomes: In this case nuclei were isolated in 0.1 *M* NaCl, pH 6.8 (*not* containing PVP), and nuclear membranes were peeled off with tweezers under the dissecting microscope, the membranes remaining attached to the tweezers. After a certain number of nuclei without their membranes were collected, they were transferred to the conical tube using a fine-tipped pipette. Nuclear materials were centrifuged down in an International Clinical Centrifuge. The supernatant was discarded and 0.5 ml of medium B of Callan and Lloyd[8] (0.1 *M* 5:1 KCl-NaCl, 1 vol, + 0.001 *M* KH_2PO_4, 4 vol) was added to disperse the nucleoplasm. Nuclear materials were stirred gently with a fine glass rod and then slowly centrifuged down at 50 × *g* for 2 min. The supernatant was again discarded, and the sediment washed once again in the same way. The packed materials were examined with an inverted microscope (a Zeiss "plankton" microscope) with phase contrast optics. The preparation consisted of lampbrush chromosomes and a few nucleoli.

DNA determination: Before chemical analyses of the chromosomes were undertaken, the ma-

terial was washed once with cold 5% TCA or 0.2 *N* PCA to remove acid-soluble materials. DNA was determined by a modified version of the fluorometric method of Kissane and Robins.[16] This method is far more sensitive than two others commonly used for DNA measurement, i.e., the Hoff-Jørgensen microbiological assay[17] and the Burton modification of the diphenylamine procedure.[18] The determination of DNA in the diphenylamine procedure and also in the fluorometric procedure of Kissane and Robins depends in the final analytical step on a reaction of desoxyribose—colorimetric in the former and fluorometric in the latter. Kissane and Robins showed that their method agreed well with the diphenylamine procedure for determination of DNA in rabbit brain tissue. We also compared values given by these two methods, using a newt liver homogenate, and got good agreement. Our experience is that when applied to the amphibian oöcyte, however, the fluorometric procedure gives a result for "DNA" about 10 times higher than does the diphenylamine procedure. This result was obtained using the Turner Fluorometer (and, with the exception to be mentioned presently, all of our determinations were done with this instrument) in which filters are used for both the exciting and emitted light. It seemed possible that measurement of the maximum wavelengths for excitation and emission might distinguish between DNA and other fluorescent material present in relatively high concentration in the oöcyte. These measurements were kindly made for us by Dr. Gerald Edelman. The maximum wavelengths were the same for both DNA and the fluorescent material of the oöcyte: 409 mμ for excitation and 532 mμ for emission. The component in DNA which fluoresces is the aldehydic group of desoxyribose, and the other fluorescent material in the oöcyte may well be an aldehyde.

For determinations of DNA in the nucleus and chromosomes of the newt oöcyte, the specificity of the procedure of Kissane and Robins was increased by using crystalline pancreatic DNAase. The sediment, in a conical centrifuge tube, of nuclear or chromosomal material isolated by the method mentioned above was treated with 0.5 ml of cold 5% TCA to remove acid-soluble materials and centrifuged down. After discarding the supernatant, the residue was washed with 0.5 ml of absolute ethanol once at room temperature and once at 60° C to remove lipids. Then one sample of the material was treated with 0.04 ml of DNAase-buffer solution (0.2 *M* phosphate buffer, pH 7.0, containing 0.003 *M* $MgCl_2$ and 0.4 mg DNAase/ml), and another sample was treated with 0.04 ml of the buffer-$MgCl_2$ solution for 5 hr at 37° C. At the end of the treatment the suspensions were centrifuged. Each supernatant was transferred to a conical tube using 50-μl micropipettes and dried in a centrifuge desiccator.[19] Fluorescence determinations were made on the residue in each tube. The DNA content of the material was taken to be the difference between the supernatant obtained by DNAase treatment and that simply after treatment with buffer. Calf thymus DNA was used as a DNA standard for fluorometry. The residues obtained after DNAase (or simply buffer) treatment and then centrifuging were washed twice with 80% ethanol, dried in the centrifuge desiccator, and the fluorescent values determined. The DNA content was taken to be the value of the residue after treatment with buffer minus the value of the residue after DNAase treatment. It can be seen from Table 1 that DNA determinations on the extracts and residues, as just described, check very well. It is also evident that if the material which is acted on by DNAase is taken to be DNA, most of the fluorescent material in nucleus and chromosomes is DNA. In contrast to the oöcyte cytoplasm, only 23–40% of the fluorescent material in the nucleus and only 10% of that in the chromosomes is not DNA.

DNA is a polynucleotide ordinarily containing a mixture of four or more different nucleotides. Whether the material we are determining is DNA in this sense is not known. The material we have determined is acid-insoluble, gives a reaction for desoxyribose, and is acted on by DNAase. At the present time there is no reason to consider the "DNA material" we have determined in newt oöcyte chromosomes to be essentially different in composition from the DNA ordinarily found in chromosomes.

RNA measurements: RNA contents of the nucleus and the chromosomes were calculated by subtraction of DNA content measured with fluorometry from total nucleic acid content measured by ultraviolet absorption. For total nucleic acid measurement, each material was first treated with cold 0.2 *N* PCA to remove acid-soluble materials. The residue was then extracted with 3:1 ethanol-ether at 60°C for 5 min to remove lipids. Finally, total nucleic acid was extracted in 0.2–0.3 ml of 0.5 *N* PCA at 70°C for 20 min, and measured by UV absorption at 260 mμ. Total nucleic acid content was calculated from the following formula: $E_{260}/ml = 3.3 \times 10^{-6}$ gm nucleic acid/ml.

Protein measurement: Protein analysis was done by the colorimetric procedure using the phenol reagent.[20] Isolated nuclei or chromosomes were treated with 5% cold TCA to remove acid-soluble materials. The residue was hydrolyzed with 0.1 ml of 0.6 *N* NaOH at room temperature for 3 hr. This hydrolysate was the starting material for colorimetry. Beef serum albumin (Armour) was used as a protein standard.

Results and Discussion.—(1) *DNA content of newt lampbrush chromosomes:* The result of the determination is given in Table 1. To compare the DNA content of

TABLE 1

DNA CONTENT OF NEWT OÖCYTE NUCLEUS AND LAMPBRUSH CHROMOSOMES

	(1) DNA equivalent in residue after being in buffer	(2) DNA equivalent in residue after DNAase treatment	(3) DNA content (1) minus (2)	(4) DNA in extract of buffer	(5) DNA in extract after DNAase treatment	(6) DNA content (5) minus (4)
Nucleus	(*a*) 2.5	(*a*) 1.0	(*a*) 1.5	0.5	1.8	1.3
	(*b*) 2.2	(*b*) 0.5	(*b*) 1.7	0.7	2.0	1.3
Chromosomes	0.88	0.10	0.78	less than 5×10^{-11}	0.65	0.65

All figures are grams $\times 10^{-9}$ per nucleus. 100 nuclei were used for each determination.

lampbrush chromosomes with that of the somatic chromosomes of *Triturus viridescens*, we take the value for this animal's erythrocyte nucleus given by Edström and Gall.[21] The erythrocyte nucleus is diploid, and the oöcyte at this stage is tetraploid.[22] To compare the DNA contents per set of diploid chromosomes, we take the DNA content of the erythrocyte nucleus and one half the DNA content of the chromosomes present in the oöcyte nucleus. From the figures given in the last column of Table 3, it can be seen that in lampbrush chromosomes the DNA content per chromosome set is more than four times that in erythrocyte chromosomes.

The DNA content per set of chromosomes in the erythrocyte is within narrow limits the same as that in other cell types of a given organism. This rule of constancy does not hold for lampbrush chromosomes. Since these are probably the only chromosomes which are active at all loci, it may be supposed that a large excess of DNA and activity are associated properties. The chromosomal activity which we are considering is the synthesis of RNA on a DNA template. Apparently, synthesis of RNA in lampbrush chromosomes is stepped up by increasing the number of DNA templates. This increase in DNA may well occur at active loci of other interphase chromosomes. If the percentage of active loci in them were about the same as the percentage of puffs (about 2 per cent) in the giant polytene chromosomes in Chironomus, the increase in DNA content of a set of ordinary interphase chromosomes would be approximately 10 per cent, hardly a significant departure from "constancy." Indeed the increase in DNA content in nuclei of the adrenal cortex observed in heightened cell activity is said to be about 10 per cent,[23] but the possibility has not been eliminated that this increase in DNA is not due to incipient mitosis.

The high rate of RNA synthesis in lampbrush chromosomes is associated with high DNA content, but there is no evidence for a continual replication of DNA. No incorporation of thymidine could be detected in autoradiographs made after exposing isolated oöcytes to labeled thymidine.[1, 2]

(2) *DNA in the nucleoplasm:* It can be seen from Table 1 that the nucleus contains twice as much DNA as is found in the chromosomes. There is as much DNA in the

nucleoplasm as in the chromosomes. Part of the nucleoplasmic DNA seems to be in the nucleoli. The evidence for this is as follows: the oöcyte nucleus in the lampbrush stage contains hundreds of nucleoli which are not attached to chromosomes, and because they are not attached, there has been some doubt whether these bodies are comparable to the nucleoli which in other nuclei are found attached to chromosomes, indeed to particular sites on chromosomes. In autoradiographic experiments with labeled RNA precursors (usually tritiated uridine), the RNA in free nucleoli of oöcyte nuclei, like that in the attached nucleoli of other nuclei, becomes intensely radioactive. This is prevented by actinomycin D,[2, 24] indicating that the process is DNA-dependent. Knowing that the oöcyte nucleoplasm contains DNA, one suspects that the DNA responsible for RNA synthesis in the nucleoli is located in the nucleoli themselves. It is, however, possible that the DNA is located elsewhere (perhaps in the chromosomes), whence the RNA passes to the nucleoli. In these experiments the labeled RNA precursors were supplied to isolated oöcytes. After RNA synthesis had occurred, the nuclei were dissected out and autoradiographs were made.

RNA synthesis in subnuclear organelles has been observed after removal of the nuclear membrane. The nucleus was first dissected out, the nuclear membrane torn off, and the gel-like nucleoplasm liquified and dispersed by adding salt solution C of Callan. Now chromosomes and nucleoli were not in an organized nucleus but merely present, and not associated, in the same salt solution. To promote RNA synthesis, the triphosphates of uridine, cytidine, guanosine, and adenosine (the last as H^3-ATP) were added. After 5 hr the material was fixed in acetic acid-formaldehyde, one preparation was treated with ribonuclease, and finally autoradiographs were made. In the preparation treated with ribonuclease nothing could be seen. In the others it was evident that RNA had been synthesized in the chromosomes and, in much higher concentration, in the nucleoli. If the synthesis is DNA-dependent, the experiment points to the presence of DNA in the nucleoli.

Presence of DNA in the nucleoli is a source of error in the determination of DNA in the chromosomes, for preparations of isolated chromosomes contain some nucleoli enmeshed in the chromosomal strands.

There have been no previous DNA determinations on the newt oöcyte nucleus. Finamore *et al.*[25] have reported very high DNA contents—several hundred times the tetraploid value—for the nuclei of mature frog oöcytes.

(3) *Protein, RNA, and DNA content:* Determinations of these components are given in Table 2. The ratios of protein to DNA and RNA to DNA are very much higher than in somatic nuclei and chromosomes. A comparison of these ratios for newt lampbrush chromosomes and the chromatin of beef liver is shown in Table 3.

The relative masses of newt lampbrush and newt liver chromosomes can be computed if it is supposed the protein:DNA ratio is the same in newt and beef liver chromosomes. Considering that lampbrush chromosomes have four times as much DNA as do newt liver chromosomes and that the ratio of protein to DNA is 200 times greater in lampbrush than in liver chromosomes, lampbrush chromosomes have 800 times the mass of liver chromosomes. The large amount of protein present in lampbrush chromosomes is at least in part the enzyme machinery needed for synthesis of RNA, a synthesis which is very active, considering the high RNA con-

TABLE 2

DNA, RNA, AND PROTEIN CONTENT OF THE NEWT OÖCYTE NUCLEUS AND LAMPBRUSH CHROMOSOMES

	Protein gm/nucleus	Total nucleic acid‡ gm/nucleus	DNA‡ gm/nucleus	RNA gm/nucleus	Protein/DNA	RNA/DNA
Nucleus	*14×10^{-7}	29×10^{-9}	1.5×10^{-9}	26.4×10^{-9}	1060	16.5
	17×10^{-7}	27×10^{-9}	1.7×10^{-9}			
	20×10^{-7}					
	Av.	Av.	Av.			
	17×10^{-7}	28×10^{-9}	1.6×10^{-9}			
Chromosomes†	3.3×10^{-7}	8.2×10^{-9}	0.78×10^{-9}	7.0×10^{-9}	550	9
	5.2×10^{-7}	7.3×10^{-9}				
		8.0×10^{-9}				
	Av.	Av.				
	4.3×10^{-7}	7.8×10^{-9}				

* 10 nuclei used per analysis.
† 20 nuclei used per analysis.
‡ 100 nuclei used per analysis.

TABLE 3

COMPARISON OF RATIOS OF PROTEIN:DNA AND RNA:DNA IN NEWT LAMPBRUSH CHROMOSOMES AND BEEF LIVER CHROMATIN

	Protein/DNA	RNA/DNA	DNA per diploid set
Beef Liver Chromatin	2.8	0.062	
Newt Lampbrush Chromosomes	550	9	0.39×10^{-9} gm
Newt Erythrocyte Chromosomes			0.089×10^{-9} gm*

* Value taken from paper by Edström and Gall (ref. 21).

tent and the rapid incorporation of labeled RNA precursors shown in autoradiographs.

The high RNA:DNA ratio of lampbrush chromosomes, about 100 times larger than the ratio for liver chromosomes, is of the right order, if it is supposed (by analogy with giant polytene chromosomes) that about 2 per cent of the loci are active in liver chromosomes, whereas all of them are active in lampbrush chromosomes. The RNA:DNA ratios of puffs and other regions in Chironomus polytene chromosomes were measured by Edström and Beermann.[26] They found that the puffs (active sites) had a higher RNA:DNA ratio than other parts of the giant chromosomes. High as this ratio was in the puffs, it was much lower than in lampbrush chromosomes. The ratio in the puff itself was probably higher than would appear from the analyses, for as the puff forms it extends over neighboring loci, so that when the puff is dissected out, neighboring, inactive loci are included.

The absolute amount of RNA in both nucleus and chromosomes given in Table 2 is greater than values reported by Edström and Gall.[21] One reason for the difference is that before analysis their preparations were fixed, and consequently some chromosomal loops may have been lost.

Summary.—The DNA content of lampbrush chromosomes in the newt oöcyte nucleus is about four times that of chromosomes in nuclei of other cell types in the newt. The nucleoplasm of the oöcyte contains as much DNA as is present in the chromosomes. Some of the nucleoplasmic DNA is located in nucleoli, which in this nucleus are not attached to the chromosomes. The protein:DNA and RNA:DNA ratios in lampbrush chromosomes are very much higher than in other chromosomes. The unusual characteristics of the composition of lampbrush chromosomes are related to the fact that these chromosomes, unlike others, are active at all loci.

* This research was supported in part by a grant (GM 04919) from the U.S. Public Health Service.

[1] Gall, J. G., and H. G. Callan, these PROCEEDINGS, **48**, 562 (1962).
[2] Izawa, M., V. G. Allfrey, and A. E. Mirsky, these PROCEEDINGS, **49**, 544 (1963).
[3] Beermann, W., *Chromosoma*, **5**, 139 (1952).
[4] Allfrey, V. G., and A. E. Mirsky, these PROCEEDINGS, **48**, 1590 (1962).
[5] Prescott, D. M., and M. A. Bender, *Exptl. Cell Research*, **26**, 260 (1962).
[6] Wilson, E. B., *The Cell in Development and Heredity* (New York: Macmillan Co., 1925), p. 350 (1953).
[7] Gall, J. G., *J. Morphol.*, **94**, 283 (1954).
[8] Callan, H. G., and L. Lloyd, *Phil. Trans. Roy. Soc. London, Ser. B*, **243**, 135 (1960).
[9] Ficq, A., and C. Pavan, *Nature*, **180**, 983 (1957).
[10] Pelling, C., *Nature*, **184**, 655 (1959).
[11] Boivin, A., R. Vendrely, and C. Vendrely, *Compt. rend.*, **226**, 1061 (1948).
[12] Mirsky, A. E., and H. Ris, *Nature*, **163**, 666 (1949).
[13] Duryee, W. R., *Ann. N. Y. Acad. Sci.*, **50**, 920 (1950).
[14] Battin, W. T., *Exptl. Cell Research*, **17**, 59 (1958).
[15] MacGregor, M. C., *Exptl. Cell Research*, **26**, 520 (1962).
[16] Kissane, J. M., and E. Robins, *J. Biol. Chem.*, **233**, 184 (1958).
[17] Hoff-Jørgensen, E., *Biochem. J.*, **50**, 400 (1952).
[18] Burton, K., *Biochem. J.*, **62**, 315 (1956).
[19] Albers, R. W., and O. H. Lowry, *Anal. Chem.*, **27**, 1829 (1955).
[20] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
[21] Edström, J. K., and J. G. Gall, *J. Cell Biol.*, in press.
[22] Alfert, M., *J. Cellular Comp. Physiol.*, **36**, 381 (1950).
[23] Roels, H., *Nature*, **182**, 873 (1958).
[24] In this paper, when referring to our observation that actinomycin D causes the chromosomal loops to be retracted, we said that "the results suggest that the morphology of an active chromosomal site is not only closely related to its capacity to synthesize RNA, but is dependent upon it." A better statement would be: the results suggest that the morphology of an active chromosomal site is closely related to its capacity to synthesize RNA.
[25] Finamore, F. J., D. J. Thomas, G. T. Crouse, and B. Lloyd, *Arch. Biochem. Biophys.*, **88**, 10–16 (1960).
[26] Edström, J. K., and W. Beermann, *J. Cell Biol.*, **14**, 371 (1962).

---

*VARIATION OF THE CONFORMATION OF THE ACTIVE SITE OF α-CHYMOTRYPSIN WITH HYDROGEN ION CONCENTRATION**

BY ALAN PLATT AND CARL NIEMANN

GATES AND CRELLIN LABORATORIES OF CHEMISTRY,† CALIFORNIA INSTITUTE OF TECHNOLOGY

Communicated September 23, 1963

It is common knowledge that α-chymotrypsin-catalyzed hydrolyses of many model substrates are inhibited by structurally related compounds incapable of functioning as substrates of this enzyme. It also is known that the *nature* of the inhibition process is dependent not only upon the structure of the inhibitor (modifier) molecule but also upon the structure of the substrate molecule.[1–3] We now find that for a given uncharged substrate-inhibitor (modifier) pair the *nature* of the inhibition (modification) process also is dependent upon the hydrogen ion concentration of the reaction system.