

# The Ultrastructure of the Nuclear Envelope of Amphibian Oocytes

## IV. On the Chemical Nature of the Nuclear Pore Complex Material\*

U. SCHEER

Department of Cell Biology, Institute of Biology II,  
University of Freiburg i. Br., Germany

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*Summary.* In order to investigate the chemical composition of the nuclear pore complexes isolated nuclei from mature *Xenopus laevis* oocytes were manually fractionated into nucleoplasmic aggregates and the nuclear envelopes. The whole isolation procedure takes no more than 60–90 sec, and the pore complexes of the isolated envelopes are well preserved as demonstrated by electron microscopy. Minor nucleoplasmic and cytoplasmic contaminations associated with the isolated nuclear envelopes were determined with electron microscopic morphometry and were found to be quantitatively negligible as far as their mass and nucleic acid content is concerned. The RNA content of the fractions was determined by direct phosphorus analysis after differential alkaline hydrolysis. Approximately 9% of the total nuclear RNA of the mature *Xenopus* egg was found to be attached to the nuclear envelope. The non-membranous elements of one pore complex contain  $0.41 \times 10^{-16}$  g RNA. This value agrees well with the content estimated from morphometric data. The RNA package density in the pore complexes ( $270 \times 10^{-15}$  g/ $\mu^3$ ) is compared with the nucleolar, nucleoplasmic and cytoplasmic RNA concentration and is discussed in context with the importance of the pore complexes for the nucleo-cytoplasmic transport of RNA-containing macromolecules.

Additionally, the results of the chemical analyses as well as of the  $^3\text{H}$ -actinomycin D autoradiography and of the nucleoprotein staining method of Bernhard (1969) speak against the occurrence of considerable amounts of DNA in the nuclear pore complex structures.

*Key words:* Nuclear envelope — Amphibian oocytes — Nuclear pore complex — Chemical nature — Electron microscopy.

### Introduction

It is widely accepted that the nuclear envelope plays an important role in interactions between nucleus and cytoplasm. The nuclear pore complexes represent at least one of several possible pathways for the nucleocytoplasmic exchange of macromolecules (for other possibilities see, e.g., Szollosi, 1965; Scharrer and Wurzelmann, 1969; Hinsch, 1970). For understanding such transfer mechanisms it is important to know the morphology as well as the biochemical composition of the pore complex. Recent publications on the pore complex sub-architecture in various plant and animal cells showed a good agreement with respect to the basic pore complex elements (e.g., Abelson and Smith, 1970; Franke, 1970a; Franke and Scheer, 1970a; La Cour and Wells, 1972; Roberts and Northcote, 1970). It seems all the more justified to speak of an universality

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of nuclear pore complex architecture among the eukaryotes (Franke, 1970a) as the appearance and arrangement of its substructures is essentially identical after three different electron microscope preparation methods, namely negative staining, freezeetching, and ultrathin sectioning (see, e.g., also Kartenbeck *et al.*, 1971).

In contrast, the chemical nature of the nuclear pore complex constituents is still unknown. Various authors using different cytochemical and enzymatic methods came to divergent results whether these structures contain ribonucleo-protein (RNP), deoxyribonucleoprotein (DNP), or were purely proteinaceous. So, for instance, it was concluded from digestion experiments with various proteolytic enzymes that the material filling the pore interior, mainly fine fibrils and the central granule, consists almost exclusively of pure protein (Merriam, 1961; DuPraw, 1965; Beaulaton, 1968; Clerot, 1968; Koshiba *et al.*, 1970). Additionally, Beaulaton (1968) and Koshiba *et al.* (1970) showed the annulus to be sensitive against pepsin and pronase, respectively. On the other hand, Mentre (1969) concluded that the annulus as well as the central granule of rat hepatic cells contain RNA because she found these structures having less electron contrast after RNase treatment. The application of the "EDTA-method" (Bernhard, 1969) to a variety of animal cells led Monneron and Bernhard (1969) to the suggestion that the ca. 30 Å wide fibrils in the pore interior represent RNP-structures. Furthermore, these authors reported a disappearance of these fibrils when the material had been extracted with 10% cold perchloric acid. Franke and Falk (1970) working with plant as well as with animal cells extended such observations and showed that all known substructures of the pore complex, including the annular subunits, the inner pore structures and the fibrils inserting at the nucleoplasmatic annulus, retain the contrast during the "EDTA-bleaching" which excludes that they are made up of chromatinous material. This conclusion is also in agreement with various results obtained after DNase treatments (e.g. Mentre, 1969; Koshiba *et al.*, 1970). It contrasts, however, to other reports which suggest that annulus-associated fibrils were of chromatin (DNP) nature (e.g. DuPraw, 1965; Comings and Okada, 1970a, b; Maul, 1970, 1971).

In view of these contradictory conclusions of morphological and cytochemical studies it seemed necessary to use biochemical methods to clarify the nature of the pore complex material. The most important prerequisite is to have at hand a nuclear envelope fraction with intact pore complexes but with little cytoplasmic (in particular ribosomes) and nucleoplasmic (e.g. chromatin, nucleoli) contamination. The recently developed techniques for mass isolations of nuclear envelopes from mammalian tissues without application of detergents are not suitable because either the outer nuclear membrane is still studded with ribosomes (Kashnig and Kasper, 1969; Berezney *et al.*, 1970) or the pore complex material is partially dissolved through the extraction in the high salt medium (Franke *et al.*, 1970; compare also the findings of Mentre, 1969).

As described earlier, manually isolated germinal vesicles of mature amphibian oocytes show relatively few ribosomes associated with the outer nuclear membrane (Franke and Scheer, 1970a), and other cytoplasmic contaminations are nearly absent. Moreover, in nuclei of mature *Xenopus laevis* oocytes there is no indication of an electron dense layer underlying the inner nuclear membrane (for details

c.f. Stelly *et al.*, 1970). Therefore single nuclear envelopes with intact pore complexes were collected from *Xenopus laevis* oocytes by hand. A further important advantage of this technique is that one can morphologically determine the number of nuclear pores per surface unit and, consequently, deals with a known total number of pore complexes in the analysis (c.f. Franke and Scheer, 1970b). The limitation of the small mass quantities for analyses, on the other hand, could be overcome by using micromethods for the biochemical determinations.

## Materials and Methods

### *Isolation Procedure*

Adult *Xenopus laevis* females were anaesthetized with 0.1% MS 222 (Fa. Sandoz, Basel), and ovary pieces were withdrawn through a small incision in the ventral body wall and directly placed in amphibian Ringer solution. A mature or nearly mature oocyte (1.0 to 1.2 mm diameter) as characterized by the white equatorial band was brought into the "5:1-medium" (0.1 M KCl and 0.1 M NaCl in a ratio of 5:1), the nucleus was manually isolated under a dissecting microscope according to Callan and Tomlin's technique (1950), and it was cleaned from adherent yolk (Fig. 1a, b). This step lasted no longer than 30 sec. Then the nucleus was transferred to a "5:1-medium" which was 10 mM with respect to  $MgCl_2$ . Hereby, the transparent nucleoplasm "precipitated" and contracted somewhat and thus became opaque within ca. 20 sec. The nuclear envelope separated from the nucleoplasmic aggregate ball. Through sucking the nucleus into a pipette with a bore diameter slightly less than the nuclear diameter the nuclear membrane was locally broken (Fig. 1c), the nucleoplasmic clump slipped out as a whole and was separately stored. Fig. 1d shows the result of this manipulation, i.e. nucleoplasm as a compact ball including the many nucleoli and the nuclear envelope as a membranous "ghost", clearly separated from each other. This separation is particularly facilitated in the mature eggs since here the majority of the nucleoli are located in the nuclear center (Fig. 1b). However, as Fig. 1d demonstrates, this procedure is also successful with not fully mature stages where nucleoli are more accumulated in the periphery of the nucleus (for topological distribution of nucleoli within amphibian oocytes c.f. Callan, 1966; Miller, 1966; Lane, 1967; Wischnitzer, 1967; Franke and Scheer, 1970b). Here the nucleoli stick to the precipitated nucleoplasm and do not remain attached to the nuclear envelope ghost. Storage of both components, nucleoplasm and envelope, was in ice-cold absolute ethanol. The whole procedure from tearing off the oocyte till storage in the alcohol lasted 60–90 sec.

### *RNA and DNA Determination*

The samples pooled from a two days work (i.e. a minimum of 250 nuclear envelopes and 250 nucleoplasmic portions) were centrifuged at 0–2° C for 5 min at 2800 g, and the acid-soluble compounds were removed by washing two times with 10% trichloroacetic acid (TCA) for 20 min each at 0° C. The pellet was suspended in chloroform-methanol (1:2, v/v) for 10 min at room temperature, centrifuged and resuspended in chloroform-methanol (2:1) for 10 min. After sedimenting the material through another centrifuge spin and removal of the supernatant with an aspirator pipette the pellet was dried in a dessicator. It was then suspended in 0.8 ml SSC buffer of pH 7.2 and 0.2 ml pre-treated, RNase-free pronase (5 mg/ml, heated to 80° C for 10 min at pH 5 prior to use; c.f. Hotta and Bassel, 1965) and 0.05 ml 10% SDS was added. (The absence of any RNase activity was routinely controlled by an assay in which yeast RNA was incubated as substrate with the pronase. The activity was read after different times at 260 nm in the supernatant of a perchloric acid precipitation.) After an incubation time of 12 hrs at 37° C, 0.2 ml 60% TCA was added, and the solution was kept for 20 min at 0° C. The pellet then was washed by centrifuging two times in cold 10% TCA. RNA extraction was performed according to the principle of Schmidt-Thannhauser (1945). To the residue 0.2 ml of 0.4 N NaOH was added, and the reaction tube was allowed to stand for 18 hrs at 37° C. After cooling to 0° C the solution was adjusted with 60% TCA to a final TCA concentration of 10% and kept for 20 min at 0° C. Then it was centrifuged in

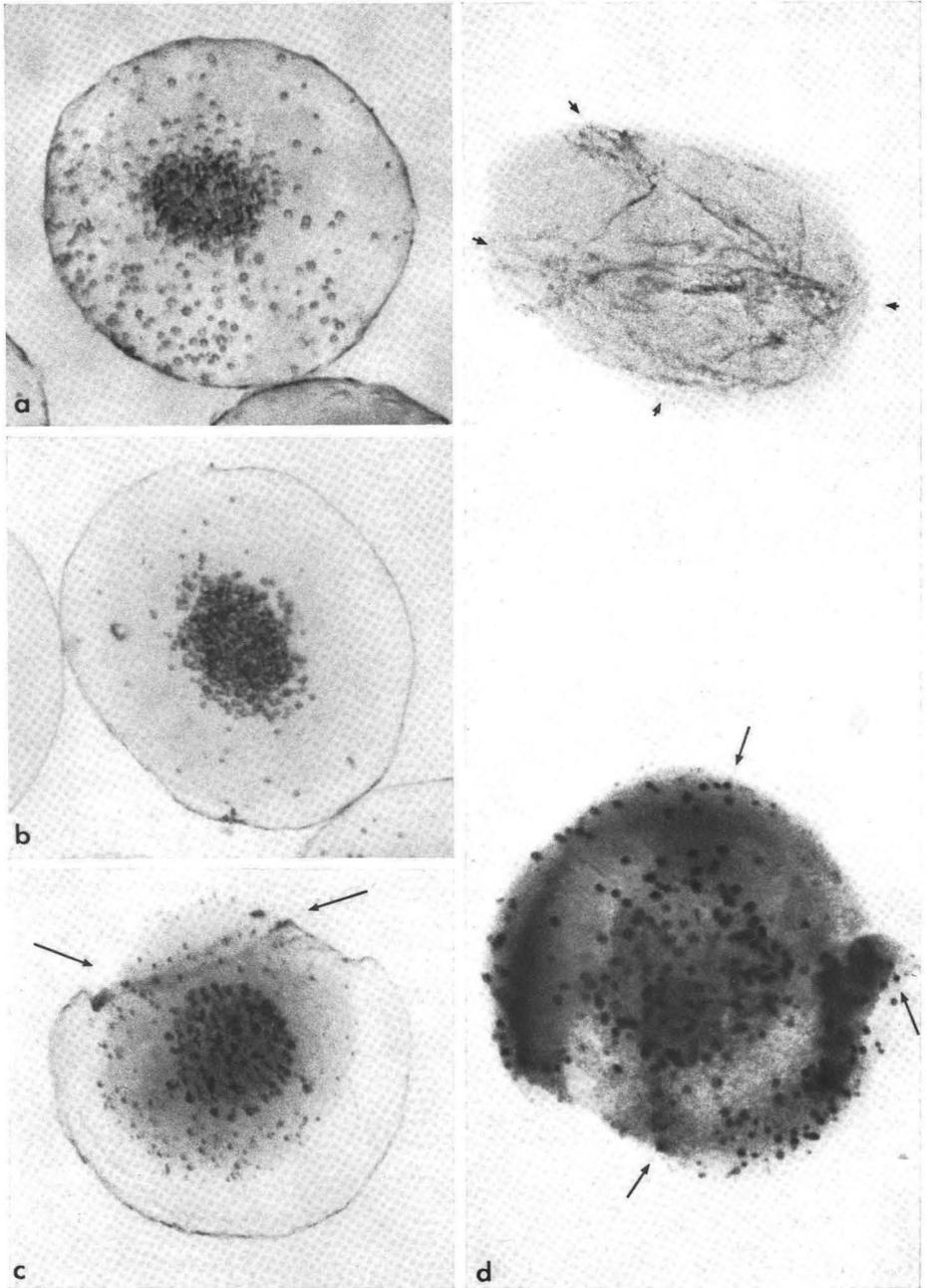


Fig. 1 a-d. Fractionation of an isolated nucleus into nuclear envelope and nucleoplasm. These microphotos were taken from germinal vesicles of *Triturus alpestris* oocytes since they produced a clearer image. (Nuclei from *Xenopus* oocytes, though, have an almost identical appearance.) The nucleus isolated from a nearly mature oocyte shows, besides the centrally located nucleoli, still some nucleoli in the nuclear periphery (a), whereas in a full-grown oocyte all nucleoli lie in the center of the nucleus (b). In the "5:1-medium" with additional  $MgCl_2$  the nucleoplasm clumps into a relatively solid aggregate, and when the nuclear envelope is broken open (c; arrows), both nuclear constituents, nucleoplasm and nuclear envelope, are separated from each other (d). The isolated nuclear envelope then appears as a faint sac-like membranous "ghost" (denoted by the short arrows in Fig. d). Note that all nucleoli, including peripheral ones, remain with the nucleoplasm (long arrows in Fig. d). Figs. a-c,  $\times 80$ ; Fig. d,  $\times 100$

the cold and the supernatant, together with the supernatants of the two subsequent washings in 10% TCA, was collected in small calibrated vials.

In order to hydrolyse remaining DNA, any suspected residue, though not visually detectable, was suspended in 0.2 ml 5% TCA and heated in a boiling water bath for 20 min. After cooling to 0° C and centrifugation as described above the "pellet" was washed in 0.1 ml 5% TCA and both supernatants were combined in a small vial.

The RNA and DNA hydrolysates then were evaporated to dryness using a thermoblock. The further processing in order to determine the nucleic acid phosphorus content with ammonium molybdate was carried out according to the micromethod described by Kleinig and Lempert (1970) which allows to measure as little as 0.5 nmole phosphorus (P; i.e. 0.17 µg RNA). As controls, quantities of the isolation medium required for transferring the nuclear envelopes or nucleoplasm into the ethanol (which might contain some contaminating material not firmly attached to the envelope), were treated in parallel. The difference between the P-content of the controls and the sample was used to calculate the nucleic acid content assuming a P-content of 9% of RNA.

The nucleic acid content of isolated whole nuclei or of mature oocytes was also determined with the same method. One sample consisted of either 50 isolated nuclei or 2 oocytes from which the follicle cells had been manually removed with care.

### *<sup>3</sup>H-Actinomycin Autoradiography*

The preparations were made following a modified version of the method as described by Ebstein (1967, 1969) under "nuclear preparation". An isolated nucleus from a lampbrush stage *Triturus alpestris* oocyte was brought into a well slide (constructed according to Gall, 1966) in the "5:1-medium". Herein the nucleus was opened. The nuclear envelope then was directly attached from the forcep tip to the glass surface of the slide. The nucleoplasmic material including the nucleoli here was not especially removed from the envelope. The whole preparation was then allowed to spread for 2 hrs in a vapor chamber. After fixation in ethanol vapor the preparation was dehydrated through graded ethanol steps and air dried. One drop of 50 µCi/ml <sup>3</sup>H-actinomycin D (Schwarz Bioresearch, Orangeburg, New York) in "5:1-medium" was added, and the well slide was kept at room temperature in a vapor chamber for 2 hrs. After rinsing the slide with distilled water the preparation was dehydrated, brought into xylol, then into 100% ethanol, and was finally air dried. Then it was dipped into a solution of 0.5% gelatine and 0.05% chromium potassium sulphate, coated with Kodak AR 10 stripping film and exposed for 20 days at 4° C.

### *Electron Microscopy*

Nuclear envelopes and nucleoplasmic aggregate clumps, isolated according to the above described procedure, were fixed simultaneously in 1% glutaraldehyde and 1% OsO<sub>4</sub>, buffered to pH 7.2 with 0.05 M cacodylate (Franke *et al.*, 1969), postosmicated with 2% OsO<sub>4</sub> for 2 hrs, dehydrated by Sitte's acetone vapor method (1962) and embedded in Epon 812. Thin sections were cut with diamond knives on a Reichert ultramicrotome OmU2 and stained with uranyl acetate and lead citrate. The thickness of the sections used for morphometric determinations were measured with an interferometer microscope. For negative staining preparations the nuclear envelope was pipetted onto a formvar coated grid, fixed with 1% OsO<sub>4</sub>, and stained with 2% neutral phosphotungstic acid as described previously (Scheer and Franke, 1969).

To differentiate between DNP- and RNP-containing structures the method of Bernhard (1969; c.f. also Franke and Falk, 1970) was employed using small oocytes. Micrographs were taken with a Siemens Elmiskop IA.

## **Results**

### *Morphology of the Isolated Nuclear Envelopes*

Survey micrographs of the nuclear envelope fraction (Fig. 2) show largely unfragmented membranes. At higher magnification a dark-light-dark "unit membrane" (70–90 Å thick) appearance is usually resolved with both inner and

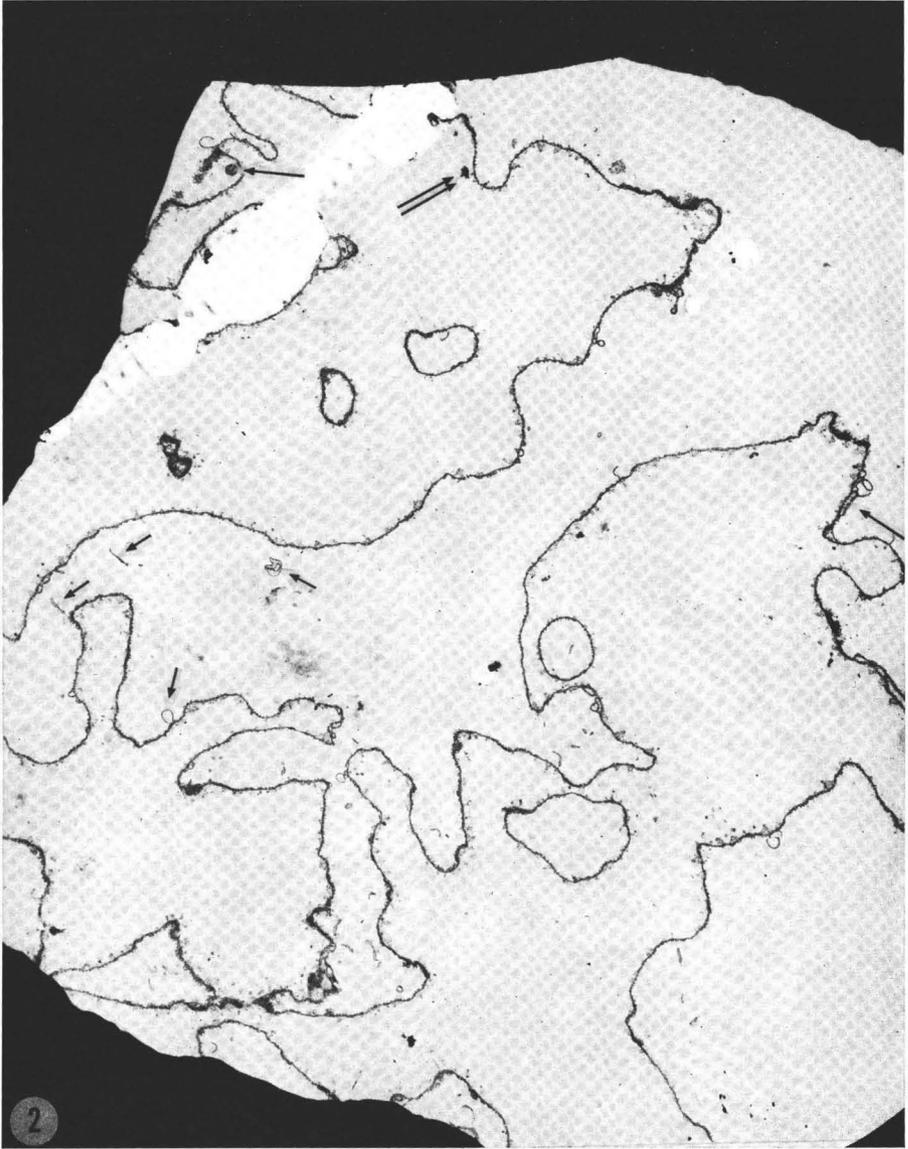


Fig. 2. Survey electron micrograph of an ultrathin section through a nuclear envelope fraction of mature *Xenopus* oocytes, demonstrating the largely unfragmented membranes with their numerous pore complexes. The degree of contamination with nucleoplasmic or cytoplasmic material apparently is very low. The micrograph is bordered by the mesh of the grid. The short arrows in the left denote some short pieces of ER-cisternae. The arrow in the right points to a single annulate lamella. At the long arrow in the upper left a mitochondrion is recognized as being attached to the "outer" side of the nuclear envelope. A small nucleoplasmic aggregate which is indicated by the double arrow lies adjacent to the "inner" side.  $\times 4200$

outer membranes (Fig. 3), in contrast to the predominant appearance after in situ fixation (Franke and Scheer, 1970a; see also Franke *et al.*, 1970). The nuclear pore complex material showed an excellent preservation (Fig. 3). The globular subunits of the annulus, measuring about 160 Å in diameter, lie upon the pore margin on both, the nucleoplasmic ("inner") and the cytoplasmic ("outer") sides of the nuclear envelope. The pore interior is filled with material which has been described in detail in a previous article as being composed of the diverse classes of "internal pore fibrils", of more compact material protruding as "tip-like projections" from the pore margin into the lumen, and of the pronounced "central granule" (Franke and Scheer, 1970a). In addition, fibrils attached to the inner annulus can be seen as extending into the nucleoplasmic direction for a distance of approximately 850 Å. Since these fibrils are tightly connected with the pores and are not removed through the isolation procedures they appear to be integral constituents of the pore complex. Sections tangential to the nuclear envelope revealed, in addition to the above mentioned structures, the highly ordered inner pore fibrils which sometimes appear in a radial "spokes" pattern (Fig. 4). Negative staining of nuclear envelopes isolated in parallel and under exactly identical conditions as for the nucleic acid determinations showed also a good preservation of the annulus material and appeared similar to previously published micrographs (Franke and Scheer, 1970; Figs. 1, 2).

The mean pore diameter measured in sections grazing the nuclear envelope and in negative staining preparations was nearly identical:  $710 \pm 32$  Å and  $725 \pm 21$  Å, respectively. The same held true for the numbers of pores per square micron of envelope surface which were  $48 \pm 6.6$  and  $47 \pm 4.8$ , respectively. Such quantitative data of the germinal vesicle pore complexes are independent of the preparation method as recently confirmed through freeze-etch studies of oocytes (Kartenbeck *et al.*, 1971). The central granule frequency, i.e. the number of pores containing a central dot, was determined from tangential sections (Fig. 4) of the nuclei analyzed as 69%.

#### *Cytoplasmic and Nucleoplasmic Contaminations*

The survey micrograph in Fig. 2, which is representative for the total nuclear envelope fraction, demonstrates the low degree of cytoplasmic or nucleoplasmic contaminations. A quantitative evaluation of the contaminations adhering to the nuclear envelope fraction was performed using morphometric methods. The mean thickness of the thin sections was determined interferometrically as  $60 \pm 6$  nm (for methodical discussions see, e.g., Silverman *et al.*, 1969; Gillis, 1971). On micrographs with a total magnification of 20000:1 the nuclear envelope profile was traced and ribosomes, mitochondria, endoplasmic reticulum (ER), annulate lamellae (AL), and occasional "nucleoplasmic clumps" were measured and counted over a total nuclear envelope length of 1 mm. Eight different preparations from different animals were examined. This contour length corresponds to  $60 \pm 6 \mu^2$  envelope surface and the visible contaminations associated therewith. In most cases, the observable ER was recognized in continuity with the perinuclear cisterna but sometimes there was no connection apparent (Fig. 2). Ribosomes were associated with the ER-elements but were very rarely attached to the outer nuclear membrane or to the AL-cisternae. The AL also showed a cisternal con-



Fig. 3. Partial magnification from Fig. 2. Note the good preservation of the nuclear pore complexes (marked by arrows), especially of the annular subunits lying upon both sides of the pore margins, and of the annulus-attached fibrils. These fibrils which in situ extend into the nucleoplasm, clearly mark the nucleoplasmic ("inner", *i*) aspect of the nuclear envelope, since they do not occur on the cytoplasmic ("outer", *o*) side. "Unit membrane" character of the nuclear membrane is apparent.  $\times 70000$

Fig. 4. Section grazing the isolated nuclear envelope of a mature *Xenopus* oocyte. Most of the pores show a central granule. Some ribosomes are associated with the outer nuclear membrane (short arrows). Long arrows denote the annulus-associated fibrils which often form more or less compact tube-like structures. The inset shows some ultrastructural aspects of the pore complexes: the double arrow marks two pores with a unit membrane appearance of the pore walls. The inner pore material can appear either as largely non-substructured electron dense material in the pore periphery or as more distinct, tip-like projections at the pore wall (both upper arrows). Sometimes the central granule is connected by radial fibrils with the pore wall ("spokes pattern", right arrow).  $\times 40000$ ; inset,  $\times 80000$

Table 1. *Morphometric data of the contaminations per average isolated nuclear envelope<sup>a</sup>*

	Per total surface of an average nucleus = 940800 $\mu^2$ (nuclear diameter 480 $\mu\text{m}$ , surface value corrected for all deviations from spherical shape)
Total membrane area of the nuclear envelope <sup>b</sup>	$1.88 \times 10^6 \mu^2$
Total number of nuclear pore complexes (47.5 pores/ $\mu^2$ )	$44.7 \times 10^6$
Contaminating endoplasmic reticulum (total area)	215600 $\mu^2$
Total surface of contaminating annulate lamellae cisternae	27400 $\mu^2$
Total membrane area of AL <sup>b</sup>	54873 $\mu^2$
Total number of contaminating AL- pore complexes (determined by counting)	$2.2 \times 10^6$
Total number of contaminating ribosomes	$23.4 \times 10^6$
Total number of contaminating mitochondria	7336
Total volume of the contaminating "dense aggregates"	1300 $\mu^3$

<sup>a</sup> Calculated on the basis of 60 nm thick sections (for details see text).

<sup>b</sup> These values are also corrected for the membranous portion of the pores, i.e. the interruptions constituted by the pores have been subtracted and the pore walls have been added.

tinuity with the nuclear envelope and usually appeared as single porous cisternae parallel to the nuclear envelope (Fig. 2; c.f. also Kessel, 1968; Scheer and Franke, 1969). Stacks of AL were only rarely found adhering to the envelope after the above outlined type of preparation: they were probably sheared off during the pipetting in the course of the isolation procedures. The pore frequency of the AL amounted to 79 pores per square micron. This markedly increased pore frequency in relation to the nuclear envelope of the same cell (47.5 pores/ $\mu^2$ ) seems to be a feature characteristic of amphibian AL (Scheer and Franke, 1969). The only rarely observed clumps of electron-dense material subjacent to the inner side of the envelope, though without an apparent connection with the pore complexes or the annulus-associated fibrils, were regarded as nucleoplasmic contaminations (Fig. 2) which are likely to be identical to the nucleolus derived dense RNP aggregates known to occur in such oocyte nuclei (e.g. Lane, 1967; Scheer and Franke, 1970b). It should be emphasized that throughout the whole study no nucleolus was found in the membrane fraction, which is in accordance with the light microscopical observations on the nucleolar behaviour during the separation of nucleoplasm and envelope ghost (see Material and Methods, p. 129, and Fig. 1d).

The morphometrically determined contaminations were then calculated for the total nuclear surface of one average nucleus with a mean diameter of 480  $\mu\text{m}$  (Table 1). Since it has been shown that the nucleus in a growing, transparent oocyte, due to outfoldings and invaginations of the nuclear envelope, has a surface

Table 2. RNA determination of the two nuclear subfractions from mature *Xenopus laevis* oocytes

Sample No.	RNA content ( $10^{-9}$ g) of					Nuclear envelope RNA (% of total nuclear RNA)	
	Total nucleus <sup>a</sup>		nucleoplasm	nuclear envelope		measured value	corrected value
	measured value	corrected value	measured value	measured value	corrected value		
1	24.00	23.89	21.0	3.00	2.89	12.5	12.1
2	29.02	28.91	27.6	1.42	1.31	4.9	4.5
3	18.74	18.63	16.5	2.24	2.13	12.0	11.4
4	19.32	19.21	17.2	2.12	2.01	11.0	10.5
Mean	22.77	22.66	20.57	2.20	2.09	9.7	9.2

<sup>a</sup> These values represent the sum of nuclear envelope and nucleoplasmic RNA each.

250 nuclei each were collected and fractionated. The values are listed as RNA content per *one* nucleus, *one* nuclear envelope, and *one* nucleoplasm. The corrected values represent the measured RNA contents minus RNA content of the morphometrically determined contaminations.

area which is about 30% redundant as compared with that of a sphere enwrapping the nucleus (c.f. Scheer, 1970, 1972) the nuclear surface value "corrected" for this is given in Table 1. The error of measurement in determining the contaminations of one isolated nuclear envelope, chiefly introduced by variations in section thickness and by slightly different nuclear diameters (460 to 500  $\mu\text{m}$ ), can be calculated as approximately  $\pm 17\%$ .

#### RNA Determinations

In order to test the RNA determination method employed in this study, the RNA content of nearly mature *Xenopus* oocytes (1.0 to 1.2 mm diameter) from which the follicle cells had been manually removed was measured. The acid-insoluble RNA of one oocyte of this size class amounted to 3.9  $\mu\text{g}$  and this value agrees well with comparable data of other authors using different methods (e.g. Brown, 1966; Hanocq-Quertier *et al.*, 1968; Mairy and Denis, 1971).

The results of the RNA analyses of the two nuclear fractions, namely "nucleoplasm" and nuclear envelopes, are represented in Table 2. Values of nuclear RNA content from direct measurements in whole nuclei were in good agreement with corresponding values calculated as the sum of the RNA recovered in the nuclear envelope and the nucleoplasmic fraction (Table 2). This indicates that during the fractionating procedure no considerable loss of material takes place. They are in the same order of magnitude as those reported from other amphibian oocyte nuclei (e.g. Izawa *et al.*, 1963).

In the previous chapter it was demonstrated that some nucleoplasmic and cytoplasmic contaminations are associated with the nuclear envelope fraction. It is important to know the amount of non-nuclear envelope RNA which can as such introduced into the preparation. The RNA content of these contaminating structures can be calculated using the values of Table 1:

(a) *Ribosomes*. The RNA of one *Xenopus*-ribosome is represented by the molecular weight sum of  $1.5 \times 10^6$ ,  $0.7 \times 10^6$ , and 30000 daltons (e.g. Brown and Littna, 1966; Loening *et al.*,

1969). This corresponds to a RNA content per ribosome of  $3.7 \times 10^{-18}$  g. *The total ribosomal RNA contamination of one nuclear envelope thus amounts to  $86.6 \times 10^{-12}$  g, i.e. no more than about 4.1% of the RNA content of the nuclear envelope.*

(b) *Endoplasmic Reticulum.* The average nuclear envelope appears to be associated with  $215600 \mu^2$  ER-membranes which, using a mean thickness of 70 Å, represent  $1500 \mu^3$  ER-membrane volume. Using a physical density of  $1.16 \text{ g/cm}^3$  for such relatively smooth ER-membranes (e.g. Moule, 1968), the ER-membranes associated with a nuclear envelope can be calculated to weigh  $1750 \text{ pg}$  ( $10^{-12}$  g). If one assumes from the studies on the mammalian liver ER-membranes that about 1% of the dry weight of smooth ER consists of "truly membrane bound RNA" (for a discussion see Deumling, 1972), *one nuclear envelope therefore could be contaminated with 17.5 pg "ER-RNA" which, however, represents only a total of 0.8% of the nuclear envelope RNA.*

(c) *Mitochondria.* These organelles contain  $2.4 \times 10^{-9}$  g DNA per *Xenopus* egg (Dawid, 1966) in the form of circular molecules with a mean contour length of 5.4 to 5.8  $\mu\text{m}$  (Wolstenholme and Dawid, 1967, 1968). The DNA concentration is  $0.52 \mu\text{g}$  per milligram mitochondrial protein (Dawid, 1966). Swanson and Dawid (1970) have demonstrated ribosomal RNA in mitochondria of *Xenopus* oocytes. Assuming that the RNA content of amphibian oocyte mitochondria is comparable to that of, e.g., rat liver mitochondria (ca.  $2 \mu\text{g}$  RNA per mg protein; Kroon, 1969) then the DNA/RNA ratio in a mitochondrion is about 1:4. So one mitochondrion having  $1 \times 10^{-16}$  g DNA (c.f. Nass, 1969) then contains ca.  $4 \times 10^{-16}$  g RNA. *Consequently, one nuclear envelope could be contaminated by 2.9 pg mitochondrial RNA, which is no more than 0.14% of the total nuclear envelope RNA content.*

(d) *"Nucleoplasmic clumps"*. The nucleoplasm of a mature *Xenopus* nucleus with 480  $\mu\text{m}$  diameter has a volume of ca.  $58 \times 10^6 \mu^3$ . Thus one whole nuclear envelope is contaminated with 0.0022% or  $1300 \mu^3$  of the nucleoplasm, i.e.  $0.46 \text{ pg}$  "nucleoplasmic RNA", as calculated from the nucleoplasmic RNA values in Table 2. *This sums up to no more than 0.022% of the nuclear envelope RNA content.*

Annulate lamellae can be regarded as a structural and possibly also functional amplification of the nuclear envelope (compare, e.g., Kessel, 1968; Scheer and Franke, 1969), with the only obvious difference being the higher pore frequency in the AL. The determinations of the nuclear envelope contamination show both, an increase in membrane material as well as in the number of pore complexes (Table 1). Consequently, the RNA determination of the nuclear envelope is based upon the presence of an additional  $27440 \mu^2$  AL-membrane surface, which corresponds to an additional  $2.2 \times 10^6$  AL-pore complexes per one nuclear envelope. To simplify the following calculations, the total pore complex containing membrane structures are summarized under the term "nuclear envelope". Anyway, such a consideration of the adhering AL is of minor importance since they amount to only 3% on the basis of membrane area and to 5% on the basis of the pore complex number.

Taken together,  $968240 \mu^2$  of nuclear envelope (including the few AL) with a total of  $46.9 \times 10^6$  pore complexes (Table 1) are associated with  $2.09 \times 10^{-9}$  g RNA (Table 2, corrected value). It is a noteworthy result of this study that as much as 9% of the total nuclear RNA content is bound to the lipoprotein of the nuclear membrane.

At least a part of this nuclear envelope RNA may be constituted by "truly membrane-embedded RNA", as this is the case with the morphologically and biochemically related ER-membranes. One nuclear envelope consists of  $1.93 \times 10^6 \mu^2$  membrane area (Table 1), and the mean membrane thickness is 80 Å. Using a mean density of  $1.18 \text{ g/cm}^3$  (from sucrose gradient isopycnic point determinations, Scheer, unpublished) the membrane material of one nuclear envelope with a total volume of  $15440 \mu^3$  has a total weight of  $18.2 \times 10^{-9}$  g. One percent of this weight may be constituted by the "membrane-embedded" RNA, i.e. one nuclear envelope could contain maximally  $0.18 \times 10^{-9}$  g RNA of this type, corresponding to 9% of the measured total nuclear envelope-attached RNA.

The nuclear envelope fraction shows only two constituents, i.e. the membrane envelope and the non-membranous pore complex material (c.f. Figs. 3 and 4). After correction for the small fraction of the membrane-embedded RNA there

remains a total of  $1.91 \times 10^{-9}$  g RNA per nuclear envelope which thus must be necessarily located in the pore complex structures. One "average pore complex" therefore contains  $0.41 \times 10^{-16}$  g RNA.

#### *Some Calculations Based upon the Morphological Data*

It has been shown that the non-membranous pore complex material contains RNA. This nucleic acid is most likely complexed with protein as has also been suggested by several authors (see Introduction). The RNA content of the various cellular RNP particles ranges from 50% (ribosomes, with a density of  $1.55 \text{ g/cm}^3$ ) to ca. 17% (lowest RNA content, reported for informosomes with  $1.35 \text{ g/cm}^3$ ; Spirin, 1969). So it is possible to roughly estimate the range of the RNA content of a pore complex, if one knows the volume of the pore complex constituents (for terminology compare Franke, 1970a; Franke and Scherr, 1970a). Combining the data from the diverse electron microscopic techniques, freeze-etching included (Kartenbeck *et al.*, 1971), one can make approximations on the dimensions of the pore complex components:

(a) One *globular subunit of the annulus* with a mean diameter of 16 nm, has a volume of  $2.15 \times 10^3 \text{ nm}^3$ . The 16 annular subunits of a pore complex then comprise a total of  $3.44 \times 10^4 \text{ nm}^3$ .

(b) The volume of the *inner pore material*, i.e. the tip-like projections, the inner pore fibrils and the amorphous material approximates a centrally perforated ("central channel") cylinder with a mean height of 14 nm and a diameter equivalent to the pore diameter (71.7 nm). Assuming that the diameter of the central channel is 15 nm then this cylinder measures  $5.41 \times 10^4 \text{ nm}^3$ .

(c) The *central "granule"* is a rod-like massive cylinder with a diameter of 15 nm and a mean length of 50 nm. In sections grazing the nuclear membrane 70% of the pores contained a central granule. Therefore the volume of the centrally located material amounts to  $0.62 \times 10^4 \text{ nm}^3$  in an "average" pore complex.

(d) The *annulus-associated fibrils* extend about 85 nm into the nucleoplasmic direction forming more or less compact tube-like structures. The 30 Å-fibrils appear often coiled up into more electron dense aggregates. Therefore, it appears to be the best approximation that the volume of the annulus-associated fibrils is equivalent to a compact 30 Å thick tube wall volume (inner tube diameter 68.7 nm, outer diameter 74.7 nm). Its total volume is then  $5.74 \times 10^4 \text{ nm}^3$ .

In summary, the non-membranous material which builds up the nuclear pore complex of a mature *Xenopus* oocyte has a total volume of  $15.2 \times 10^4 \text{ nm}^3$ . Assuming a density range of the RNP-material from  $1.35$  to  $1.55 \text{ g/cm}^3$ , the constituents of one pore complex have a weight of  $2.1$  to  $2.4 \times 10^{-16}$  g. The RNA content of one pore complex can then be calculated as ranging from  $0.35 \times 10^{-16}$  g (17% RNA) to  $1.2 \times 10^{-16}$  g (50% RNA). These results are in good accordance with the nuclear pore complex RNA values of the present determination ( $0.41 \times 10^{-16}$  g).

#### *On the Search for DNA*

DNA-P could not be detected, neither in whole nuclei nor in the nucleoplasm and nuclear fraction. The sensitivity limit of the method applied was  $0.17 \mu\text{g}$  DNA per sample, i.e. the DNA content of one nucleoplasm must be lower than 680 pg using 250 nucleoplasmic portions per sample. This estimation is in the line with the values from *Xenopus* germinal vesicles as reported by other authors (95 pg, Brown and Dawid, 1968; 42.6 pg, MacGregor, 1968). Although obviously the bulk of the DNA is located in the nucleoplasm (chromosomes and nucleoli) it could not be excluded that some DNA remained in the nuclear envelope fraction as "annulus associated chromatin fibers" (compare, e.g., Comings and

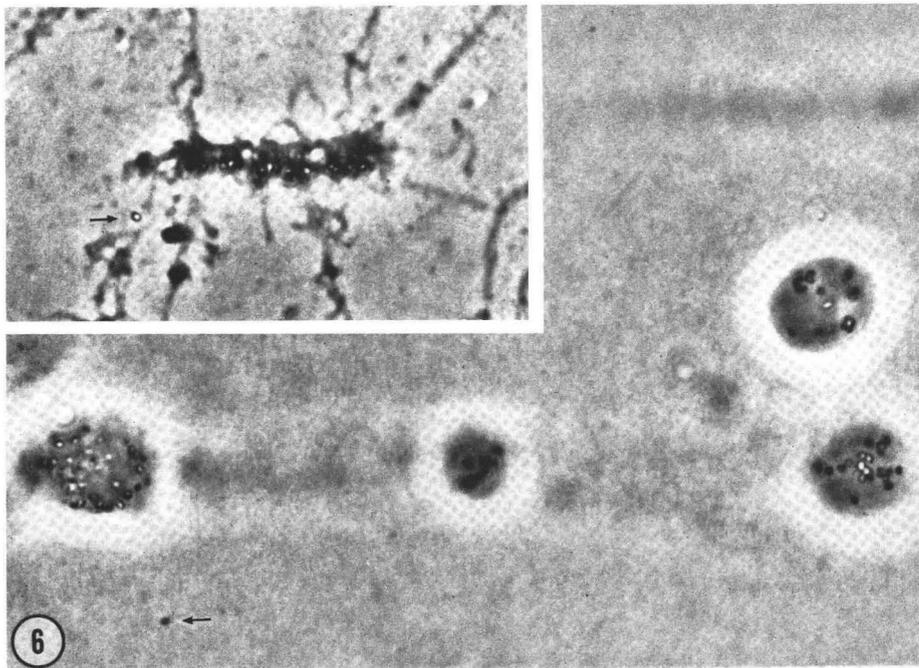
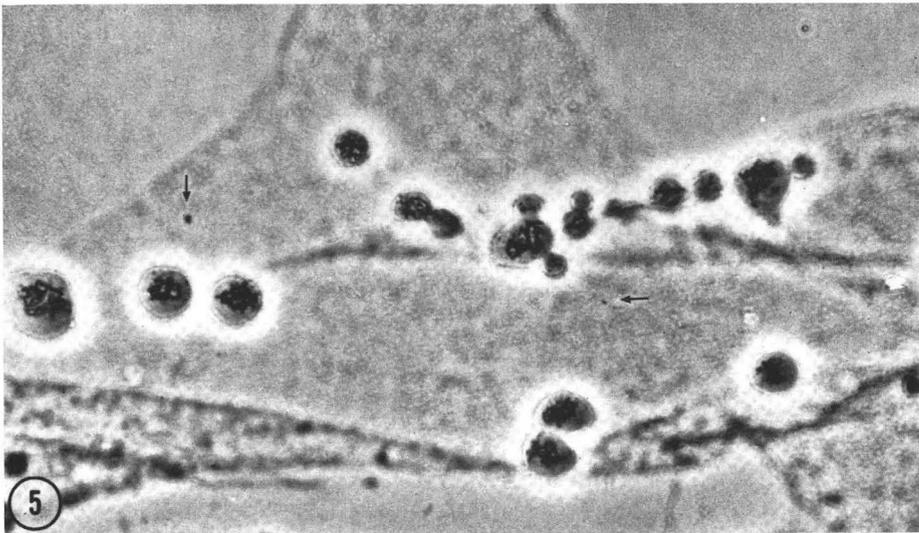


Fig. 5. Autoradiographic spread preparation of the nuclear envelope from a lampbrush stage *Triturus alpestris* oocyte which is overlaid by numerous nucleoli.  $^3\text{H}$ -actinomycin D binding to the nucleolar DNA causes a heavy concentration of silver grains in these structures, whereas the nuclear envelope (arrows point to a very few grains present) is not labeled above background.  $\times 800$

Fig. 6. Same autoradiographic preparation as in Fig. 5. The nuclear envelope is nearly free of grains (the only one single grain is detectable at the arrow) whereas the nucleoli are clearly labeled. Inset shows the binding of  $^3\text{H}$ -actinomycin D to the chromomeres of a lampbrush chromosome, one silver grain (arrow) is located over a chromosomal loop.  $\times 2400$ ; inset,  $\times 2000$

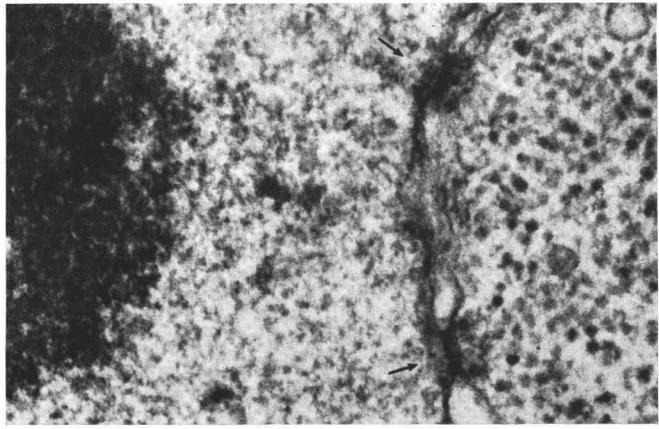
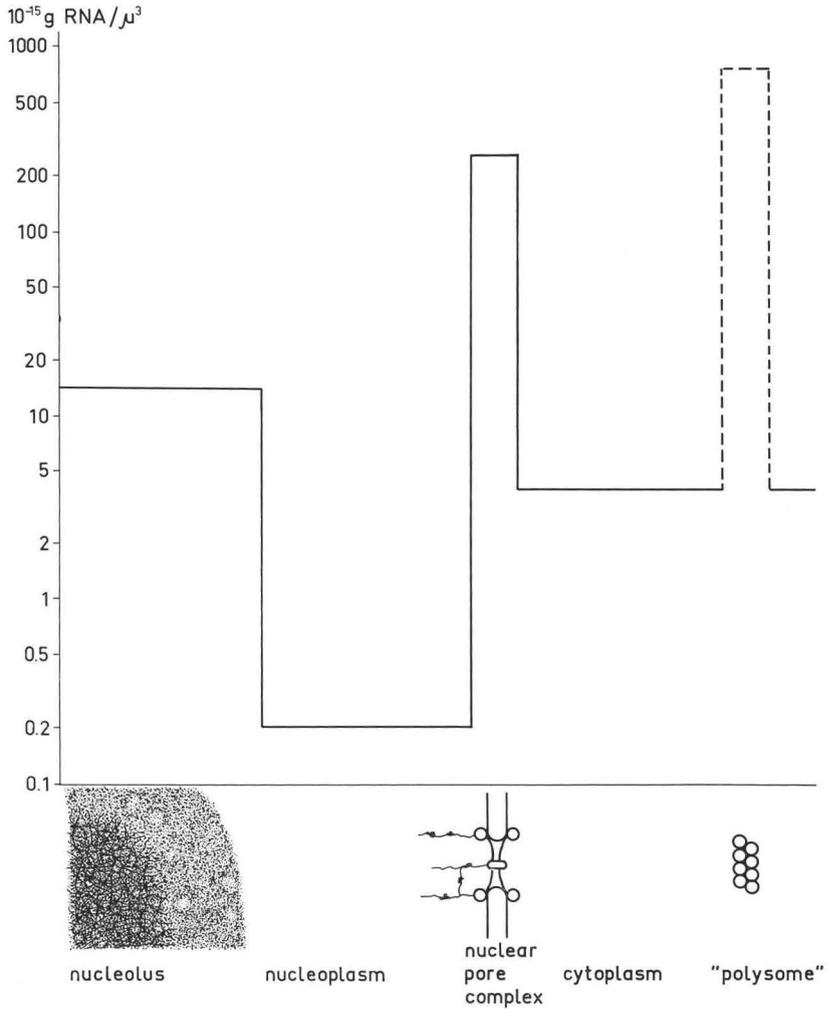


Fig. 7. Graphical presentation of the comparison of the RNA concentration in the nuclear pore complexes with other intranuclear and extranuclear RNA concentrations in a mature *Xenopus* oocyte. The value of a "polysome" represents the RNA concentration in a ribosome, i.e. the RNP particle of a cell with the highest RNA concentration known. The ultrathin section through the nuclear envelope region of an oocyte illustrates the cytological situation of the scheme (arrows mark the pore complexes).  $\times 100000$

Okada, 1970b; see also DuPraw, 1965). An attempt was made in this study to examine the presence of DNA on the nuclear envelope using the sensitive autoradiographic method of Ebstein (1967, 1969). Since in nuclei of *Triturus alpestris* oocytes the annulus-associated fibrils are especially well pronounced and can be in some cases as long as 1  $\mu\text{m}$  (Franke and Scheer, 1970a), germinal vesicles of this species were preferentially used. Figs. 5 and 6 present the spread preparation of a nuclear envelope together with some nucleoli and a segment of a lampbrush chromosome:  $^3\text{H}$ -actinomycin D marks clearly the nucleolar DNA as well as the chromomeric DNA whereas the nuclear envelope is almost totally free of grains. This result is taken as an indication that the nuclear membrane contains no considerable amounts of DNA and that the fibrils associated with the annuli represent rather RNP than DNP. The same conclusion was drawn from the staining behaviour of the pore complexes in ultrathin sections through lampbrush oocytes treated according to Bernhard (1969). The pore complex elements maintained the uranyl-staining contrast during the EDTA treatment and this suggested a RNP-nature as described in detail by Franke and Falk (1970).

#### *RNA Concentration of the Pore Complex as Compared with Other Cellular RNA Concentrations*

The RNA concentration (w/v) of a pore complex as calculated from the above given values amounts to  $270 \times 10^{-15} \text{ g}/\mu^3$ . This RNA package value is lower than that of a ribosome, the cellular RNP-particle with the highest RNA packing known ( $780 \times 10^{-15} \text{ g}/\mu^3$ ). However, the RNA concentrations of other cellular RNP structures are much lower than that of the pore complex:

(a) *Nucleolar RNA* concentration. According to Edström and Gall (1963) and MacGregor and Moon (1971) one oocyte nucleolus of *Triturus cristatus* contains 4.6 to 10 pg RNA. One oocyte nucleus of *Xenopus* contains about 1500 nucleoli (Perkowska *et al.*, 1968) with a similar size of 10  $\mu\text{m}$  diameter (Van Gansen and Schram, 1968). In the total nucleolar volume of  $7.8 \times 10^5 \mu^3$  there is 6900–15000 pg RNA or 8.9 to  $19.2 \times 10^{-15} \text{ g}/\mu^3$ .

(b) *Nucleoplasmic (=nuclear sap plus chromosomal) RNA* concentration. The nucleoplasm contains 5400 to 13500 pg RNA (calculated from the values of Table 2, compare also the data of Gall, 1966). The RNA concentration then amounts to 0.1 to  $0.24 \times 10^{-15} \text{ g}/\mu^3$  (nuclear diameter 480  $\mu\text{m}$ ; nuclear volume =  $57.91 \times 10^6 \mu^3$ ; nuclear volume minus nucleolar volume =  $57.13 \times 10^6 \mu^3$ ).

(c) *Cytoplasmic RNA* concentration. The cytoplasm of a mature *Xenopus* oocyte has a volume of  $846.9 \times 10^6 \mu^3$  and a RNA content of 3.9  $\mu\text{g}$ . The average cytoplasmic concentration (neglecting a "basophilia gradient") then is  $4.6 \times 10^{-15} \text{ g RNA}/\mu^3$ .

These data, summarized in Fig. 7, confirm the view of the nucleoplasm as having a very low concentration and of the nucleolar RNA concentration being markedly higher than that of the cytoplasm or nucleoplasm, respectively (c.f., e.g., Caspersson and Schultz, 1940; Edström *et al.*, 1961). Furthermore they indicate a relatively high RNA package concentration in the nuclear pore complexes.

#### Discussion

With the technique described above one obtains within a very short time (60–90 sec) isolated nuclear envelopes in a structurally intact state. The quantities obtained are sufficient for chemical analysis, since the huge germinal vesicle provides a nuclear envelope surface equivalent to that of, e.g., ca. 4500 liver

cell nuclei. The nucleoplasmic and cytoplasmic contamination of such a nuclear envelope fraction is very low, the RNA of the contaminants sums up to no more than about 5% of the total envelope-associated RNA<sup>1</sup>. Essentially, the isolated nuclear envelopes do not show any structures other than membranes and pore complexes. Since RNP structures are generally well visible with the present days' electron microscopic techniques and since the isolated nuclear envelopes show no non-membrane-structures other than the pore complex constituents one can conclude that the nuclear envelope-associated RNA measured is localized at or in the pore elements. One average nuclear envelope contains  $1.9 \times 10^{-9}$  g RNA. It is noteworthy that even in a non-RNA synthesizing nuclear type like the mature *Xenopus* germinal vesicle as much as 9% of the nuclear RNA is present in a membrane-attached state.

The RNA content of the pore complex as estimated from the electron microscopic morphometric data and the reasonable assumption that it is within the range of that of other cellular RNP particles, is close to the value obtained in the present RNA determinations. The total weight of a pore complex estimated by the morphometric methods ( $2.1$  to  $2.4 \times 10^{-16}$  g) is further in good agreement with the calculations of DuPraw and Bahr (1969) from honeybee embryonic cells using a quantitative evaluation of the electron-scattering properties ( $1.8$  to  $8.8 \times 10^{-16}$  g). It should be kept in mind, however, that the values of these authors also include the membranous portion of the pore complex.

Biochemical and ultrastructural investigations showed some nuclear DNA to be tightly connected with the inner nuclear membrane in various cell types (e.g. Moses, 1958; Bornens, 1968; Beams and Mueller, 1970; Berezney *et al.*, 1970; Franke *et al.*, 1970; Mizuno *et al.*, 1971; Zentgraf *et al.*, 1971). Moreover, an association of chromatin fibers with the nuclear pore complexes was first reported by DuPraw (1965) in honeybee embryonic cells and was extended later on by other authors studying whole mounts of mitotic, male meiotic, and interphase nuclei (Comings, 1968; Comings and Okada, 1970a, b). Such an attachment of nuclear DNA with the nuclear membrane has been discussed as being of importance in replication, condensation, orientation, distribution and pairing processes of the chromosomes (c.f. Sved, 1966; Comings, 1968; Comings and Okada, 1970b; King, 1970; Soyer, 1971). The observations of a structural continuity of chromatin fibers with the annulus and the central granule, respectively, then might be interpreted as an indication for a DNP-nature of the annulus-attached fibrils (see also Maul, 1970, 1971). At least in the special case of the amphibian oocyte some points strictly argue against such a conclusion:

(i) During the lampbrush chromosome preparation according to Gall (1966), the nuclear envelope is removed but the chromosomes spread as undamaged units onto the glass surface of the slide. This behaviour speaks for no or at least

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<sup>1</sup> Arguments against the significance of the measured nuclear envelope-RNA might be especially derived from the difficulties of avoiding contamination with cytoplasmic ribosomes. But this can be ruled out on the basis of the following consideration: The assumption that the nuclear envelope-RNA is caused by contaminating ribosomes would necessarily mean that the envelope surface is covered with 35 ribosomes per micron length (assuming 60 nm section thickness). This, however, is not the case as is demonstrated through the morphometrical determination which shows only 1.5 ribosomes per micron envelope length.

a very weak association between chromosomes and nuclear membrane (in contrast to the findings of Comings and Okada, 1970b). In addition, the chromosomes of the mature oocyte have contracted their loops and lie already in the center of the nucleus, separated from the nuclear envelope by a distance of 100  $\mu\text{m}$  or more. The annulus-attached fibrils nevertheless are still present.

(ii) The sensitive  $^3\text{H}$ -actinomycin D radioautographic method fails to detect any DNA associated with isolated nuclear envelopes whereas the nucleolar DNA as well as the chromosomal DNA in the chromomeres was clearly labeled (Figs. 5 and 6). If there is any DNA in the pore-attached fibrils, then the local DNA concentration must be much lower than in the nucleolus or the chromosomes.

(iii) The annulate lamellae pore complexes have an identical subarchitecture as compared to the nuclear pores, including the annulus-associated fibrils (e.g. Scheer and Franke, 1969). From the autoradiographic study of Ganion and Kessel (1965) and from the observation that in sea urchin oocytes the AL pore complex material can be in direct structural continuity with the RNP-containing (Conway and Metz, 1970) "heavy bodies", it is somewhat unlikely that they contain DNA.

(iv) As shown by Brown and Dawid (1968) one nucleus from a mature *Xenopus laevis* oocyte contains maximally 70 pg chromosomal DNA. Since these authors could not exclude a contamination by mitochondrial DNA, they suggested that the chromosomes of a germinal vesicle contain no more than the tetraploid amount of DNA (i.e. 12 pg, see also MacGregor, 1968). The maximal DNA content of one pore complex could then amount to  $0.3\text{--}1.6 \times 10^{-18}$  g by making the extreme assumption that the total chromosomal DNA would be in association with the pores. Even in this extreme estimation, the DNA would comprise no more than 0.8 to 4.5% of the pore complex nucleic acid content. From such a calculation a considerable DNA content appears excluded not only from the pore complex but also from the annulus-attached fibrils, since nearly 40% of the whole pore complex volume is represented by these fibrils. In this context an abstract of Flaumenhaft (1960) should be mentioned, who found the nuclear envelope of mature amphibian oocytes to be composed of proteins, lipids and RNA.

Since the similarity in the subarchitecture of the nuclear pore complexes throughout the eukaryota is well established (e.g. Franke, 1970a; Franke and Falk, 1970) the demonstration of the by far predominant RNP-nature of the germinal vesicle pore complex elements might be generalized and extended to nuclei of other cell types. The RNA concentration of the pore complex material is approximately 60 times higher than that of the cytoplasm and 150 times higher than in the nucleoplasm (see Fig. 7). Since a mature *Xenopus* oocyte has ceased to synthesize RNA, including the ribosomal RNA (rRNA), the main transcriptional product during oogenesis (Davidson *et al.*, 1964), it is concluded that the pore complex elements in a fully grown oocyte represent rather stable structures. More than 90% of the RNA present in a mature oocyte is made up of 28 S and 18 S rRNA (Mairy and Denis, 1971). If both molecule species build up a pore complex in a 1:1 ratio then it could contain ca. 20 of such rRNA molecules (taking a mean approximate molecular weight of  $1.1 \times 10^6$  daltons; Loening *et al.*, 1969) in a membrane bound state. The concept of a primarily static behaviour of the pore complex structures in certain physiological states is also confirmed by the findings of Harris (1961), Daniels and Roth (1964), Stevens (1967), and Bajer

and Mole-Bajer (1969) that nuclear envelope-derived cisternae with typical pore complexes can be preserved during plant and animal mitoses. Likewise, the subarchitecture of AL pore complexes, which are supposed to have a storage rather than a nucleocytoplasmic transport function (e.g. Bal *et al.*, 1968; Babbage and King, 1970; Franke and Scheer, 1971), do not differ from the nuclear pores. In contrast to the mature oocyte, the lampbrush stage shows an intense transcriptional activity. 90–95% of this RNA synthesis have been shown to be ribosomal (Davidson *et al.*, 1964). During the lampbrush stage the nuclear pore complex structures suggest a more dynamic nature (Scheer, 1972). Here they might represent nucleocytoplasmic “transit structures” (Stevens, 1967; Stevens and Swift, 1966), in which RNP-material is supplied to the nuclear part of the pore complex, remains there for a certain time, and finally is released into the cytoplasm. So in the lampbrush stage, as in any other cell types or states active in transcription, the pore complex structures might be the expression of an input = output equilibrium. Both functional states of the pore complexes, the static and the dynamic one, can apparently change into each other depending on the transcriptional activity of the nucleus. The changing parameter during such transitions might then be the velocity of RNP-translocation through the pore, i.e. the Nuclear Pore Flow Rate (Franke, 1970b; Scheer, 1972). In this view, the mature oocyte nuclear pore complex then might represent something like a “frozen” conveyor-belt. The finding that the pore subarchitecture in both cases does not significantly differ, suggests that the pore complex structures are integrated constituents of the morphologically intricate pore complex as such, but are not only matter being en route through the nucleocytoplasmic “gateway”.

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Dr. Ulrich Scheer  
Institut für Biologie II der Universität Freiburg  
Lehrstuhl für Zellbiologie  
D-7800 Freiburg i. Br.  
Schänzlestr. 9  
Germany