THE INTRACELLULAR DISTRIBUTION
AND HETEROGENEITY OF RIBONUCLEIC ACID IN STARFISH OOCYTES

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ABSTRACT
A study has been made of the content and composition of RNA in cytoplasm, nucleoplasm, and nucleoli from growing oocytes of the starfish *Asterias rubens*. The determinations were carried out, using ultramicrochemical methods, on units isolated by microdissection from fixed sections. Macrochemical and interferometric control experiments show that RNA can be quantitatively evaluated in this way.

The results show that the growing oocyte represents a system in which the relations between the quantities of nucleolar, nucleoplasmic, and cytoplasmic RNA undergo great changes. These changes are continuous for nucleolar and cytoplasmic RNA so that their amounts may be predicted from the size of the cell. Nucleoplasmic RNA, on the other hand, shows great variations among different cells, independent of cell size.

Purine-pyrimidine analyses show that each cell component contains an RNA which differs significantly from that of the other two. Cytoplasmic and nucleolar RNA are closely related, the only difference being a slightly higher guanine/uracil quotient for the nucleolar RNA. They are both of the usual tissue RNA type, i.e., they show a preponderance of guanine and cytosine over adenine and uracil. Nucleoplasmic RNA deviates grossly from the RNA of the other two components. Here the concentrations of adenine and uracil are higher than those of guanine and cytosine, respectively. This RNA consequently shows some resemblance to the general type of animal DNA although the purine/pyrimidine ratio is far from unity.

Our data favor a nucleolar origin for the stable part of the ribosomal RNA and a nucleoplasmic one for the unstable part (the messenger RNA).

The starfish oocyte has been much used in the study of the RNA chemistry of the nucleolus (1–4). One of the findings obtained by Vincent (4) using this material is that the base composition of the RNA of the nucleolus is not the same as that of the cytoplasm. The difference is marked in contrast to what was found in the spider oocyte (5), where the RNA of the cytoplasm and that of the nucleolus were not significantly different. However, full-grown oocytes were used in the investigation on the starfish, while relatively young, growing oocytes were taken from the spider. Furthermore, the material was collected by bulk isolation using fresh material in the former case and by micro-
dissection of fixed material in the latter. Considering this unsatisfactory state of diverging results with the two kinds of material, we have chosen to investigate the starfish oocyte further using our methods. To make possible an evaluation of the significance of any possible differences in base composition, analyses were also carried out on nucleoplasmic RNA (the RNA of the chromosomal material and the nuclear sap). Finally, determinations of the absolute amounts and the concentrations of RNA were performed for the three cellular compartments.

MATERIALS AND METHODS

Ovaries of the starfish Asterias rubens were used, obtained at the end of November from the Zoological Station of Kristineberg, Sweden. Although the majority of the ovaries contained relatively young and small cells, in a few cases larger cells up to the size of full-grown ones with perivitelline membranes were present. Small tissue pieces were fixed in Carnoy’s fluid for 90 min and embedded in paraffin by means of microelectrophoresis was sectioned at 20 to 30 μ and the nucleolus, a large part of the nucleoplasm and cytoplasm, which are clearly distinguished also in unstained material, will usually allow separation along the inside of the nuclear membrane. The nucleolus can then be isolated and freed from nuclear material, although the latter can, however, be kept small and is unlikely to affect any nuclear or nucleolar values appreciably, particularly since the concentration of RNA in the nucleolus is usually considerably lower than that of the nucleolus. The different stages of isolation are shown in Fig. 1.

Material to be used for pyrimidine-purine analysis by means of microelectrophoresis was sectioned at 20 to 30 μ and the nucleolus, a large part of the nucleoplasm, and a piece of cytoplasm were collected from cells in mid-growth stages (70 to 110 μ diameter in fixed preparations). Parts from thirty cells were pooled and formed the material for extracts giving nucleic acid constituents for about five microelectrophoretic analyses each. (Such a collection of thirty cell parts, e.g., thirty nucleoli, represents one preparation).

The dimensions of cells, nuclei, and nucleoli were determined on units isolated in the oil chamber using fixed and sectioned tissue. With the aid of a measuring eyepiece two horizontal diameters (a and b) were first measured on whole cells and their nuclei, after which the cells were tilted at 90° using the two dissection needles and the third diameter (c) determined. The nucleoli were measured after isolation by determining two horizontal diameters. To calculate the volumes the following approximations were used: abc/π/6 for the volumes of cell bodies and nuclei and ab × √π/6 for the nucleolar volumes. Volume data have a restricted value considering that an appreciable shrinkage occurs during fixation and subsequent embedding (of the order of 50 per cent of the volume), even if the shrinkage is partly reversible in the hydrated state of the tissue in the oil chamber. The volume values are, however, of interest for comparisons within the material.

The dry weight of isolated nucleoli was measured using the interference microscope of Johansson (7). The nucleoli were measured in the dry state resulting from keeping them under liquid paraffin for some time after the isolation from fixed tissue. The mass (or weight) was calculated from the formula, m = φA/χ, where m is the mass, φ the optical path difference (o.p.d.), A the area and χ the refractive increment (8).

The nucleoli were flattened out between the slide and the coverslip in liquid paraffin in order to obtain discs of uniform thickness. The areas of these discs were determined by planimetric measurements of projections.

In order to determine the value of χ in liquid paraffin for the nucleolar substance, which for practical purposes can be considered to be the same as that for protein, the o.p.d. of fourteen nucleoli were measured both in liquid paraffin and silicone oil. The value of the refractive index of the material in the nucleoli could then be calculated according to the formula: φp/φs = (npp - n𝑝)/(n𝑝 − n𝑠), where n and the indices p, s and f refer to refractive index, liquid paraffin, silicone oil, and protein respectively, n𝑝 is 1.4812 and n, 1.4044. The value of n𝑝 was found to be 1.563 ± 0.009. The value of χ could then be calculated from the formula χp = (npp − np)/ep, where ep = 1.333, the density of dry protein. According to this formula χ will be 0.062 ± 0.006.

RNA was extracted with a buffered ribonuclease solution and the extracts were measured in ultraviolet light as previously described (9). For purine-
FIGURE 1

Isolated oocytes, oocyte nuclei, and nucleoli from fixed starfish ovaries as seen in the oil chamber with phase contrast optics. × 500.
pyrimidine analysis (10) the pooled RNA extracts were hydrolyzed in a micropipette and the hydrolysate applied to an alkali-treated cellulose fiber containing a solution of extremely high viscosity and high acidity, in which the RNA constituents are separated by means of high voltage electrophoresis (2000 to 4000 V/cm). 200 to 500 μg of RNA can be analyzed in this way. After the electrophoretic separation the resulting bands are measured quantitatively by means of a photographic-photometric procedure in ultraviolet light. (Two modifications have been introduced into the original procedure: the concentration of the sulfuric acid used in the electrophoresis medium has been doubled and the fibers are kept in this medium in the refrigerator for several days before use.)

RESULTS

Macrochemical Control Experiments

It has been reported that RNA may be partly removed as a result of fixation with Carnoy’s fluid even after as short a time as 2 hours (11). On the other hand most workers have found that RNA is completely preserved during fixation times below 2 hours (12, for ref.). Since, however, the question does not seem to be completely settled yet and since soluble RNA has been found to be extractable with cold ethanol after treatment of the tissue with trichloroacetic acid (13, 14), a series of experiments were undertaken to determine whether RNA is dissolved during tissue fixation and subsequent processing. Pieces of ovaries were fixed for varying times between 90 min. and 24 hrs in a total of eight experiments and the volumes of fixative used were investigated spectrophotometrically, either directly or following extraction with 1N HCl, which removed the nucleotides from Carnoy’s fluid (15). In no case could anything but insignificant amounts of ultraviolet-absorbing compounds be eluted. The fixation with Carnoy’s fluid was followed by absolute ethanol for 90 min. In this case the fluids contained even less ultraviolet-absorbing compounds. The benzene used in the tissue processing was shaken out with distilled water which was found to be free from ultraviolet-absorbing compounds. It is to be observed that the free nucleotides are removed only in moderate amounts by the fixation fluids, in agreement with earlier findings on nervous tissue (15). The compounds eluted constitute 10 to 20 per cent of the free nucleotides present but only 1 to 2 per cent of the RNA when calculated on an identical ultraviolet absorption basis. Thus the fixation preserves not only the bulk of RNA but also the greater part of the free nucleotides.

When they are to be used for microdissection, tissue sections are deparaffinized with chloroform and diethyl ether and brought to 0.01 N acetic acid via 70 per cent ethanol. Several analyses were carried out to investigate the amounts and the nature of the ultraviolet-absorbing compounds dissolved by the ethanol and the acetic acid. For this purpose tissue sections of known wet weight were put into test tubes where they were subjected to extractions. The eluate was found to contain ultraviolet-absorbing compounds in amounts corresponding to about 10 per cent of the RNA present when calculated on the same ultraviolet absorption basis (see Table I). Increasing the time of incubation with acetic acid up to 15 hours did not increase these amounts. Upon hot acid hydrolysis (1 N HCl, 100°C, 60 min.) and subsequent chromatography the fraction was found to contain adenine, guanine, and uridine as well as a few unidentified spots, two of which also showed fluorescence. It could be estimated that the compounds containing adenine must have been responsible for 50 to 60 per cent of the absorption in the extract, and those containing uridine and guanine for 10 to 20 and 5 to 10 per cent respectively. This shows that they could not have been present in the acetic acid as a result of dissolution of RNA which would have given the two purine bases and the two pyrimidine nucleotides after hot acid hydrolysis. It has been shown earlier, for nervous tissue, that the free nucleotides are removed completely by the acetic acid (15). In agreement with this, similar values were found for free nucleotides when freeze-dried ovaries were extracted with cold perchloric acid as shown in Table I.

It has been much debated whether ribonuclease is able to remove all RNA from fixed tissue. This question was studied in two ways. In repeated experiments sections in test tubes were extracted with alkali according to the method of Elson et al. (16) after they had been extracted with ribonuclease. Such second extracts were practically free from compounds exhibiting the ultraviolet absorption characteristics of RNA. Finally, the amounts of RNA present in ovary pieces were determined from the nucleotide content using the method of Davidson and Smellie (17) as modified by Elson et al. Other pieces were fixed and treated as in the microchemical procedure using sectioned material...
in the test tube. The amount of RNA extractable by ribonuclease digestion was determined both from its nucleotide content and from its optical density at 260 m\(\mu\) (since the ultraviolet absorption curve is essentially that of an uncontaminated nucleic acid preparation). The RNA contents were calculated from the absorption data using either the expression \((E_{260}-E_{290}):0.03\) or the expression \(E_{257}:0.0293\) (15) to give the RNA concentration in \(\mu\)g/ml at 1 cm cuvette depth. The first alternative was found to give an average value for the RNA content of 95 per cent of that obtained by nucleotide analysis, while the latter gave an average value of 122 per cent. Although determination of RNA content from the sum of the constituent nucleotides is a relatively accurate method, it probably tends to give values below 100 per cent recovery. Thus the first expression used probably gives somewhat too low values and the latter expression, which was also used in the present microchemical determinations of RNA (on grounds that 10 \(\mu\)g of digested yeast RNA per ml at 1 cm cuvette depth was found to give an absorption at 257 m\(\mu\) of 0.293, (15), may give rise to a moderate positive systematic error.

Finally a comparison of the base composition of RNA obtained by the two treatments was made. Table II shows that there is a discrepancy in the cytosine content, not too pronounced but still significant. It has not been possible to determine the cause of this deviation. Its presence does not, of course, invalidate the analytical microchemical work, but one should keep it in mind during comparisons with works in which the RNA base composition is determined with methods of the Davidson and Smellie type.

**Interferometric Measurements on Isolated Nucleoli**

As a further check on the RNA determinations as well as to establish the relation of the RNA content to the dry weight, interferometric mass determinations were carried out on isolated nucleoli before and after two types of ribonuclease digestion and the weight differences were compared with the RNA values obtained through direct photometrical measurements. The results are given in Table III, from which the following conclusions can be drawn. When RNA is extracted with ribonuclease in distilled water at pH 6.0 according to Brachet (18), the weight loss corresponds relatively well to the amounts of RNA measured in the extract (RNA constituting 85 per cent of the weight loss). Using ribonuclease in the volatile buffer at pH 7.6 which is employed in the microchemical extractions of RNA for the determination of RNA content and composition, it can be seen that a considerably higher weight loss is obtained, of which RNA constitutes as little as 50 per cent. The values for the RNA content and concentration are the same in the two groups of nucleoli, confirming the known fact that in the optical determination of RNA a relatively high admixture of protein can be tolerated since its specific optical density at 260 m\(\mu\) is only about 2 per cent of that of RNA. It is,

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**Table I**

<table>
<thead>
<tr>
<th>Recovery of Nucleotides in Fixed as Compared to Freeze-Dried Starfish Ovaries</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Free nucleotides* g/100 g wet weight (±SEM)</td>
</tr>
<tr>
<td>RNA g/100 g wet weight (±SEM)</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

* Calculated from ultraviolet absorption data using the expression \(E_{260}-E_{290}\) to give the concentration of free or RNA nucleotides in \(\mu\)g per ml at 1 cm cuvette depth.
† Nucleotides extracted by the fixation fluids included. Incubation with 0.01 N HAc during 2 hrs.
§ As for † but incubation time 15 hrs.
¶ Determined from nucleotide content.
†† Calculated as for * but using the expression \(E_{257}:0.0293\).

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TABLE II
Base Analyses of RNA from Starfish Ovaries Treated as for Cell Chemical Work Compared to Standard Macrochemical Treatment

The bases are determined as nucleotides after ionophoresis in the macroscale according to Davidson and Smellie (17). Mean values of molar proportions in per cent of the sum ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Freeze-dried, digested with alkali according to Eison et al. (16)</th>
<th>Carnoy-fixed, digested with ribonuclease as for microextractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>19.9 ± 0.6</td>
<td>20.6 ± 0.4</td>
</tr>
<tr>
<td>Guanine</td>
<td>31.6 ± 0.7</td>
<td>31.9 ± 0.3</td>
</tr>
<tr>
<td>Cytosine</td>
<td>28.4 ± 0.2</td>
<td>25.6 ± 0.3</td>
</tr>
<tr>
<td>Uracil</td>
<td>20.3 ± 0.2</td>
<td>21.8 ± 0.4</td>
</tr>
<tr>
<td>Number of</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>preparations</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Relation between the Quantities of RNA in the Cytoplasm, Nucleoplasm, and Nucleolus

15 oocytes of varying size, ranging up to full-grown cells with a well developed perivitelline membrane, were isolated and the nuclei and nucleoli were dissected out. Volume and RNA values are shown in Table IV, in which the cells have been arranged according to increasing cell size. The following conclusions can be drawn from the data of the table.

Cytoplasmic RNA is correlated in amount with cytoplasmic volume as expected. It does not, however, increase proportionately, but there is a concentration decrease during growth.

Also the nucleoplasmic volume is correlated with the volume of the cytoplasm (or cell volume) but shows a smaller relative growth. Thus, when the cytoplasmic volume increases 100 times, the nucleoplasmic volume increases only twenty times.

Nucleoplasmic RNA shows an irregular behavior. On the whole there is no correlation with nucleoplasmic or cell volume and one can find values between 50 and 300 μg among small as well as big cells. The relative contribution of nucleoplasmic RNA to the total cell RNA is consequently, as a rule, many times higher in young than in old cells.

Also the concentration of RNA (w/v) in the nucleoplasm falls from relatively high values in small cells (around 3 per cent, w/v) to low values in the bigger oocytes (around 0.5 per cent, w/v). The nucleoplasm usually shows the lowest concentration of RNA in the cell.

The nuclear volume increases with cell size but,
### TABLE IV

**Volume, RNA Content, and Concentration in Cellular Compartments of Individual Starfish Oocytes**

<table>
<thead>
<tr>
<th>Whole cell volume</th>
<th>Cytoplasm</th>
<th>Nucleoplasm</th>
<th>Nucleolus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cell RNA volume</td>
<td>RNA (w/v)</td>
<td>RNA (w/v)</td>
<td>RNA (w/v)</td>
</tr>
<tr>
<td>~3</td>
<td>DDg per cent</td>
<td>~ #Ng per cent</td>
<td>~ #Dg per cent</td>
</tr>
<tr>
<td>7,730</td>
<td>6,040</td>
<td>306</td>
<td>5.1</td>
</tr>
<tr>
<td>16,500</td>
<td>13,100</td>
<td>779</td>
<td>6.0</td>
</tr>
<tr>
<td>22,500</td>
<td>17,300</td>
<td>933</td>
<td>5.4</td>
</tr>
<tr>
<td>23,200</td>
<td>18,200</td>
<td>665</td>
<td>3.6</td>
</tr>
<tr>
<td>28,600</td>
<td>19,400</td>
<td>1,140</td>
<td>5.9</td>
</tr>
<tr>
<td>69,000</td>
<td>61,900</td>
<td>2,730</td>
<td>4.4</td>
</tr>
<tr>
<td>76,900</td>
<td>66,100</td>
<td>3,280</td>
<td>5.0</td>
</tr>
<tr>
<td>202,000</td>
<td>180,000</td>
<td>5,950</td>
<td>3.3</td>
</tr>
<tr>
<td>255,000</td>
<td>242,000</td>
<td>6,250</td>
<td>2.6</td>
</tr>
<tr>
<td>458,000</td>
<td>428,000</td>
<td>12,300</td>
<td>2.9</td>
</tr>
<tr>
<td>323,000</td>
<td>502,000</td>
<td>13,500</td>
<td>2.7</td>
</tr>
<tr>
<td>586,000</td>
<td>555,000</td>
<td>11,400</td>
<td>2.1</td>
</tr>
<tr>
<td>645,000</td>
<td>602,000</td>
<td>11,600</td>
<td>1.9</td>
</tr>
<tr>
<td>703,000</td>
<td>669,000</td>
<td>15,100</td>
<td>2.3</td>
</tr>
</tbody>
</table>

### TABLE V

**Base Composition of RNA from Different Parts of Starfish Oocytes**

<table>
<thead>
<tr>
<th></th>
<th>Cytoplasm</th>
<th>Nucleoplasm</th>
<th>Nucleolus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean values of molar proportions in per cent of the sum, ± SEM.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenine</td>
<td>23.5 ± 0.19</td>
<td>35.7 ± 1.3</td>
<td>23.7 ± 0.28</td>
</tr>
<tr>
<td>Guanine</td>
<td>31.9 ± 0.29</td>
<td>24.8 ± 0.90</td>
<td>33.4 ± 0.25</td>
</tr>
<tr>
<td>Cytosine</td>
<td>24.8 ± 0.33</td>
<td>16.5 ± 0.54</td>
<td>24.3 ± 0.44</td>
</tr>
<tr>
<td>Uracil</td>
<td>19.7 ± 0.28</td>
<td>23.0 ± 0.80</td>
<td>18.5 ± 0.46</td>
</tr>
<tr>
<td>Number of preparations</td>
<td>7</td>
<td>4</td>
<td>7</td>
</tr>
</tbody>
</table>

$P$ for differences: cytoplasm-nucleoplasm <0.001; cytoplasm-nucleolus <0.01; nucleoplasm-nucleolus <0.001.

like the nucleoplasmic volume, to a smaller extent than the cytoplasmic volume. On the other hand, the nucleolar volume constitutes a rather constant fraction of the total nuclear volume in cells of varying sizes (mean value 7.2 per cent, v/v).

The nucleolar RNA content increases with cell size, but the relative increase is small (about five times when the cell volume increases 100 times and the total cellular RNA twenty times). About 5 per cent of the total RNA is nucleolar in the youngest of the oocytes investigated, while the corresponding figure for the largest cells lies below 1 per cent.

Also the concentration of RNA (w/v) in the nucleoli decreases (from 15 to 4 per cent). The concentration of RNA in the nucleoli is, however, always markedly higher than those of the cytoplasm and nucleoplasm, in agreement with the histological picture.

### Purine-Pyrimidine Composition of the RNA in Cytoplasm, Nucleoplasm, and Nucleolus

Growing oocytes (diameter in the fixed state 70 to 110 μ) from one animal were used for this investigation. As can be seen from Table V the RNA of the nucleoplasm differs markedly and significantly from the RNA of the cytoplasm and the nucleoli. It is characterized by relatively high adenine and uracil and low guanine and cytosome values and shows in this respect some resemblance...
to the general type of animal DNA (uracil substituted for thymine).

Nucleolar and cytoplasmic RNA are very similar but still show a difference which is of statistical significance. In the nucleolus the guanine content is slightly higher and the uracil content slightly lower than in the cytoplasm.

**DISCUSSION**

The macrochemical control experiments indicate that tissue processing of the kind used in this work is ideal in that it conserves the RNA and also makes it digestible so that it can be completely extracted with ribonuclease. These results are in good agreement with the experience of most students of this question (12, 19, 20).

A great deal of uncertainty exists regarding the quantitative relationships between the RNA in the cytoplasm, nucleoplasm, and nucleoli. One fact shown by the present investigation is that these relationships are not fixed for a cell which can grow to large dimensions but vary during growth. While the RNA in the nucleolus and the cytoplasm in the present material is quantitatively well correlated with the size of these compartments, the RNA of the nucleoplasm seems to be independent of cell or nuclear size. In early stages an appreciable fraction of the total RNA is intranuclear (about 3/4) but later on only 2 per cent of it is contained in the nucleus.

Direct evidence has been furnished for a transfer of RNA from the nucleus to the cytoplasm (21). Autoradiographic and microirradiation experiments indicate that the nucleolar RNA to a large extent contributes to the RNA in the cytoplasm (22–26). The present results as well as earlier base analyses on the spider oocyte (5) are in agreement with this view, since the cytoplasmic RNA is much more similar to the nucleolar RNA than to the nucleoplasmic RNA. The fact that the former two types of RNA do differ significantly in spite of their similarity may have several alternative or combined causes, without excluding the possibility that the cytoplasmic RNA present at a given moment is practically completely derived from the nucleolar RNA. Only one of these possible causes will be taken up in discussion here. Vincent (4) using mature starfish oocytes showed that the guanine/uracil quotient is considerably higher in the nucleolar RNA than in the cytoplasmic RNA. In the younger, growing oocytes in our material, a difference in the same quotient is present, although it is considerably smaller, primarily on account of a less elevated guanine/uracil quotient in the nucleolus. This suggests that an increase in this ratio may occur in the nucleolus during development, and work in progress in this laboratory supports this idea. In such a case the change of the quotient in the cytoplasmic RNA will lag behind and always remain smaller than that of the nucleolus, provided that there is retention of the nucleolar RNA in the cytoplasm.

The present results add support to those of earlier reports which ascribe to the nucleolus a quantitatively dominating role in the production of cytoplasmic RNA, but only in the sense that the major part of the cytoplasmic RNA present at a given moment is of nucleolar type. They say nothing about how high the relative contributions of nucleolar and nucleoplasmic RNA may be per time unit. The base analyses indicate, however, that in case there is a high relative contribution of nucleoplasmic RNA it is necessary that this RNA should have a considerably shorter life than that of the nucleolus. The nucleoplasmic RNA could thus very well fit into the role of the messenger RNA described in experiments on normal and phage-infected E. coli. Here an unstable RNA fraction has been found which is related to bacterial or phage DNA in base composition and which becomes associated with the ribosomes and shows a great speed of turnover (27, 28). It forms at most a few per cent of the RNA present in the cells although it may be produced at a significant rate. It is of interest in this connection to point out that the nucleoplasmic RNA of the starfish oocyte shows some points of similarity with the general type of animal DNA (29) in the respect that adenine predominates over guanine and uracil over cytosine (in contrast to nucleolar and cytoplasmic RNA). (The ratio of the sum of purines to the sum of pyrimidines deviates, however, far from unity.) The great variations in the amounts of nucleoplasmic RNA from cell to cell are also of some interest, in light of the rhythmic gene activities known from giant chromosomes (30, 31), as an indication of the possibility that similar phenomena may be present also in other kinds of nuclei.

To sum up: Our data are compatible with the concept that the stable and unstable parts of the ribosomal RNA are furnished by the nucleolus and nucleoplasm respectively.
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and the Zoological Station of Kristineberg, Sweden,
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