THE ISOLATION AND CHEMICAL PROPERTIES OF THE NUCLEOLI OF STARFISH OOCYTES

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The function of several of the discrete protoplasmic structures has been more completely understood where the structure itself has been isolated and studied by direct biochemical methods.[1] Progress toward the understanding of the chemical nature and of the function of the nucleolus has been hindered by the lack of a suitable technique for the isolation of nucleoli in quantities suitable for direct chemical study. During an attempt to analyze the role of the nucleolus in the growth stages of the oocytes of various forms, a technique for the isolation of nucleoli in quantities suitable for biochemical analyses was developed. This technique and a preliminary report of the chemical composition of the starfish nucleolus is given below.

The isolation procedure described has been successfully applied to the oocytic nucleoli of two starfishes: *Asterias forbesii* and *A. rubens;* the surf clam, *Mactra solidissima;* and spider crabs of the genus *Maja.* The observations and analytical data given below apply exclusively to the nucleolus of *Asterias rubens.*[2]

Isolation Procedure.—1. Preparation and Homogenization of Material: 15–20 cc. of washed ovaries or exuded eggs in the germinal vesicle stage are allowed to stand for 20 minutes in 100 ml. of water. The tissue is then squeezed through 4 layers of cheesecloth to remove connective tissue and other debris. The cheesecloth is then washed with 100 ml. of water. The combined washing and filtrate are homogenized by being forced through a No. 18-gauge hypodermic needle with a large syringe. The homogenate is filtered through 8 to 12 layers of cheesecloth, and the cheesecloth washed with 100 ml. of water.

During the development of the technique, it was found that the homogenate generally maintained a pH of 6.0 ± 0.15 without the addition of other buffering agents. This was found to be the optimum pH for isolation, as considerable agglutination of the cytoplasm took place at levels not far above or below pH 6, particularly in the presence of salts. For this reason distilled water was found to be the most suitable isolation medium. Pyrex redistilled water was used throughout, and all operations were carried out in the cold room at 2–4°.

2. Sedimentation of Nucleoli: The filtered homogenate is placed in 50-ml. centrifuge cups and centrifuged for 20 minutes at 375 g. The supernate is discarded and the surface of the pellet washed with a few milliliters

of water. The pellet is then resuspended in 20 volumes of water and centrifuged for 15 minutes at 375 g. This procedure is repeated twice, or until the proteinaceous gel is removed. The remainder of the purification is carried out in a hand centrifuge. As the time of centrifuging is quite short, the centrifuging periods are referred to in terms of the number of turns of the handle required.

The centrifuge used during the development of the technique handled 15-ml. conical centrifuge tubes and had a 14 cm. radius to the bottom of the tubes with a 15:1 gear ratio. The handle was consistently turned at the rate of two revolutions per second.

3. Removal of Large Cell Debris and Membranes: The centrifugate is resuspended in 10 ml. of water and transferred to a 15-ml. conical centrifuge tube. It is then centrifuged 10 turns in the hand centrifuge. The supernate is filtered through one or two layers of bolting silk (50 × 50 micron mesh) and the centrifugate discarded. This step is repeated until microscopic examination of the supernate shows a minimum of nuclear and membrane contamination. (The filtration of the nucleoli should be kept to a minimum, as large numbers of nucleoli adhere to the fabric and are lost.) Finally, the nucleolar suspension is concentrated by 100 turns in the hand centrifuge.

4. Removal of Small Particles and Somatic Nuclei: The concentrate is suspended in 10 ml. of water, centrifuged 60 turns and the supernate discarded. This step is repeated 2 to 4 times, or until microscopic examination reveals no nuclei.

5. Control of Preparation Purity: The following steps were taken to insure maximum purity in the final preparation. Each step of the isolation was controlled by examining a small quantity of the preparation under the microscope. A small quantity of 0.01% toluidine blue was added to the suspension on the slide to aid in identifying the nucleoli. The final preparations were examined with toluidine blue and with the phase microscope. To detect nuclear and cytoplasmic contamination the preparations were also stained with pyronin-methyl green. The low centrifugal forces used in the isolation practically precluded contamination of the preparations by the granules of the cytoplasm, and no such contamination was detected in any preparation. In cases where shed eggs were used as a source of nucleoli, the egg membranes were found to be a major contaminant. At the suggestion of Dr. Seymour Cohen, the following technique to remove this contamination was adapted from a technique for purifying virus described by Topping.[3] After step 2, the centrifugate is suspended in 1 volume of water and $1^1/_2$ volumes of ether, shaken thoroughly and centrifuged for 15 minutes at 375 g. The protein material comes to lie at the ether-water interface, trapping the membranes and other large particles with it, while the nucleoli are concentrated at the bottom of the bottle. The nucleoli

are removed with a fine pipette and transferred to a 15-ml. centrifuge tube for step 3. This procedure will also remove microsomes and other cytoplasmic granules from the preparations. The major sources of contamination found in the final preparations were nuclei which were not disrupted by the homogenization procedure and egg membrane material. The estimated contamination in no case exceeded 5% and generally was less than 1%.

6. *Yield:* The yield from a typical preparation was usually 5 to 10 mg. (dry weight) and 30 to 70% of the total nucleoli.

Physical Properties of the Nucleolus.—The nucleolus of the starfish oocyte is a spherical, highly refractile body containing one to many vacuoles of varying sizes.

The nucleoli demonstrate a definite change in appearance upon contact with the isolation medium. This change is simliar to that seen in nuclei which are isolated by the citric acid technique. The change appears as an increase in the refractive index of the nucleolus. Upon prolonged contact with distilled water, the surface of the nucleolus changes from a clear, hyaline state to a granular, less refractile condition. This change becomes accentuated in the presence of calcium binding agents such as oxalate or citrate. No other change in the size or internal structure of the nucleolus has been observed during the isolation procedure. The nucleolus does not flatten appreciably under pressure, but rather fragments into unequal pieces when excess pressure is applied. There is no evidence that it possesses an osmotic membrane. The nucleolus swells very greatly in alkaline media. This swelling begins at about pH 7.6, and is reversible with the addition of acid. There is no evidence of structural change (such as alteration in size or number of vacuoles, etc.), even after two or three alternate exposures to alkali and acid. The normal size of the nucleolus is little affected by acid conditions. The nucleolus is rapidly broken down by dilute solutions of trypsin. It does not dissolve in salt solutions or strong acid, and dissolves slowly in *N* NaOH. The density of isolated nucleoli, as measured in aqueous sucrose solutions, is between 1.35 and 1.4.

From this evidence and visual observation, it is concluded that the nucleolus, at least after isolation, is a solid or semisolid structure.

Chemical Properties of the Nucleolus.—1. Nucleic Acid: The nucleic acid of the nucleolus is entirely of the ribose type. No desoxyribose or thymine could be detected by colorimetric or chromatographic means. The content of ribonucleic acid (RNA) in five different nucleolar preparations ranged between 2.2 and 4.6% of the dry weight of the nucleolus. A series of determinations was made on the relative base content of the RNA of the nucleolus and on the RNA of the cytoplasmic granules, using the chromatographic technique of Markham and Smith.[4] A marked difference in the ratio of guanine and uracil between the two nucleic acids was

found. The results on a molar ratio basis are given below (the mean of all the molar values = 1).

MOLAR RATIOS

BASE	NUCLEOLUS	CYTOPLASM
Guanine	1.45 ± 0.021	1.23 ± 0.016
Adenine	0.87 ± 0.015	0.80 ± 0.020
Cytosine	1.09 ± 0.014	1.15 ± 0.012
Uracil	0.59 ± 0.025	0.88 ± 0.014

Seven determinations were made on each base on material taken from three different nucleolar preparations. The deviations listed are the standard errors. The differences between the amounts of adenine and cytosine in the two nucleic acids are probably not significant (t test).

2. *Nucleolar Protein:* The nucleolus (dry weight) contains 16 to 20% nitrogen. Acid extraction removes 25 to 35% of this amount. The nature of the protein has been examined by solubility and by chromatographic means. Using the technique for extraction of basic proteins described by Daly, Mirsky, and Ris,[5] no histone could be found in the intact nucleolus. Some material with the characteristics of protamines was extracted at 60°. This extraction did not alter the size or appearance of the nucleolus. The extracted residue was hydrolyzed in 6 N HCl for 24 hours at 105° in a sealed tube, and the hydrolysate chromatographed in butanol-ammonia to separate the amino acids. The following amino acids were identified: glutamic acid, proline, arginine, lysine, glycine, tyrosine, phenylalanine, valine, leucine and isoleucine. The presence of tryptophan was established by a positive Hopkins-Cole reaction. Histidine could not be detected on the chromatogram. The amounts of glutamic acid, proline, arginine, lysine, histidine and phenylalanine differed markedly from that found in thymus histone. This protein residue, which makes up the bulk as well as the structural framework of the nucleolus, contains 0.5 to 1% phosphorus.

3. *Nucleolar Enzymes:* Tests were made to determine the presence of certain enzymes in the nucleolus. The results are tabulated below for frozen-dried nucleolar preparations and for a similar quantity of identically treated whole egg powder.

ENZYMATIC ACTIVITY

ENZYME	NUCLEOLUS	EGG POWDER
DPN reductase	−	+
Dipeptidase (alanylglycine)	−	+
Alkaline glycerophosphatase	−	+
Acid glycerophosphatase	+	+

The egg powder was also able to reduce methylene blue, both in the ab-

sence and the presence of succinate. The nucleolus did not reduce methylene blue under these circumstances.

The techniques used were sensitive to at least one-tenth of the activity found in the whole egg material, so that any significant concentration of enzyme in the nucleolus would have been detected. The activity of nucleolar acid phosphatase was approximately one-fifth that of the whole egg powder on a dry weight basis. On a nitrogen basis, the activity would be less than one-tenth that of the whole egg material.

4. *Other Substances:* A small amount of material which contained about 5% of the total phosphorus of the nucleolus was extracted by alcohol-ether at 60°. This treatment did not alter the appearance of the nucleolus. Also a small amount of material was found which could be extracted in cold dilute acid. This material, from absorption and chromatographic data, appeared to be a nucleotide or mixture of nucleotides, but has not yet been positively identified.

Discussion.—From these data, it is evident that the structural basis of the nucleolus of the starfish is phosphoprotein. This protein is present in high concentrations, and constitutes the bulk of the nucleolus. The failure to find significant amounts of basic protein is not in accord with Caspersson's concept that the nucleolus consists primarily of protein rich in basic amino acids.[6] The failure to find histone in the starfish nucleolus agrees with the results of Pollister and Ris, who failed to find histone in the nucleolus of maize using microspectrophotometric techniques.[7]

Subsequent to the studies of Caspersson and Schultz,[8] and Brachet,[9] the nucleic acid of the nucleolus has been considered to be of the ribose type. The chromatographic analyses made during the present study have confirmed this view. The concentration of nucleotides in the starfish nucleolus cannot be considered as being very high. The granular fraction of the starfish cytoplasm (containing some mitochondria and the microsomes) contained approximately 10% RNA as compared with the value of 3 to 4% for the nucleolus. The only other value for the nucleic acid content of the nucleolus which could be found in the literature is that given by Caspersson and Schultz[8] for the nucleolus of the salivary gland of a female Drosophila. They estimated that the absorption curve which they obtained microspectrophotometrically could be approximated by a nucleoprotein solution containing 3.5% nucleic acid and a protein of the globulin type. The male Drosophila salivary gland nucleolus was said to have an absorption curve with about twice this value of nucleic acid, but with another type of protein. In the same paper, these authors gave absorption curves for sea urchin oocyte nucleoli and spinach nucleoli. They stated that the nucleotide concentration was much higher in these forms, but no values were given.

The demonstration that the nucleolus contains a different RNA from that

of the cytoplasmic granules complements the work of Marshak,[10] who found that comparable differences existed between the nuclear and cytoplasmic RNA of several tissues. In contrast to the results of Marshak, no significant difference with respect to adenine was found between the nucleolus and cytoplasm of *Asterias rubens*.

Except for the failure to find alkaline phosphatase, the lack of enzymatic activity of the nucleolus is not surprising, in view of the low level of enzymatic activity of the nucleus as a whole. In the case of alkaline phosphatase, the histochemical technique for *in situ* localization of this enzyme on tissue sections has generally shown the nucleolus to be the site of intense activity. It is possible that the enzyme may have been lost in the aqueous media during the course of isolation, as has been found in the case of nuclei.[11] However, the detailed study of Cleland[12] on the mechanism of the histochemical demonstration of alkaline phosphatase has shown that considerable caution must be exercised in interpreting the distribution patterns of the enzyme, particularly in the nucleus. He has found that alkaline phosphatase is localized in the large granule, and in the "soluble" fractions of cell homogenates depending upon the organ, with little or no activity in the nucleus. The results of the present study support this contention.

The weak activity of acid phosphatase in the nucleolus may be due to contamination or adsorption from the cytoplasm. Such contamination in isolated nuclei has been found by Pallade.[13] The very high activity of the enzyme in the whole egg material would make such an artifact quite possible.

Summary.—A technique for the isolation of nucleoli from the oocytes of marine eggs has been described.

The nucleolus of *Asterias rubens* has been shown to be composed largely of phosphoprotein, which makes up its main structural component. Associated with the protein component is a significant amount of RNA, small amounts of lipid and soluble nucleotide.

No histone could be demonstrated in the nucleolus.

The ribonucleic acid of the nucleolus has been shown to differ from that of the cytoplasm in its content of guanine and uracil.

The only enzymatic activity detected in the nucleolus was that of acid phosphatase.

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POTENTIAL THEORY IN SPACE OF n DIMENSIONS (PART I)

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1. In order to develop Potential Theory in a Euclidean space of n dimensions, the following extension of Newton's Law of Gravity is adopted.

Newton's Law of Universal Gravitation. In a Euclidean Universe of n dimensions, any two particles of masses m_1, and m_2 situated at the positions P_1 and P_2 attract each other with a force directed along the line joining P_1 and P_2. The magnitude of the force F is directly proportional to the product of the masses and inversely proportional to the $(n-1)$ power of the distance r between P_1 and P_2. Thus

$$|F| = \frac{km_1m_2}{r^{n-1}}. \tag{1}$$

For $n = 3$, this reduces to Newton's well-known inverse square law, and for $n = 2$, this becomes the law of gravity used in the theory of the Logarithmic Potential. Thus by our work, both theories can be developed from a uniform point of view.

2. Let $x = x^i = (x^1, \ldots, x^n)$ denote cartesian coordinates in a Euclidean space of n dimensions so that the square of the differential of arc length is $ds^2 = \delta_{ij}\,dx^i\,dx^j$, where the δ_{ij} are the Kronecker deltas. An N dimensional hypersurface or Riemannian manifold V_N where $1 \leqq N \leqq n$, immersed in this Euclidean space is the locus of points defined by the system of n equations