THYMINE IN THE ACID-SOLUBLE FRACTION OF ARBACIA EGGS*

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INTRODUCTION

To our knowledge, thymine, the characteristic pyrimidine of desoxyribonucleic acid (DNA), has not been found hitherto in that fraction of tissues or cells which may be extracted with cold dilute acid; *i.e.*, in the extract usually designated as the acid-soluble fraction. Results of previous experiments led us to believe that thymine would be found in such an extract obtained from mature echinoderm eggs. The observations leading to this expectation were (*a*) the finding of an abundance of DNA in the nuclei of immature eggs, and (*b*) the complete absence of evidence for its presence in mature eggs, which suggested that the DNA originally present was degraded (1, 2). Analysis of extracts of mature *Arbacia* eggs has shown that thymine is indeed present in this fraction and the amount found is approximately equal to the total thymine of these eggs as determined in previous experiments (1). In addition guanine, cytosine, and a mixture of adenine and uracil have been identified in the digest of the acid-soluble fraction but the quantities present have not been measured.

Procedure

Eggs.—Shedding of eggs was induced by injection of 0.53 M KCl and the eggs washed 5 times in filtered sea water. A total of $11.88 \pm 0.10 \times 10^7$ eggs were collected for analysis. In an aliquot taken for microscopic examination one immature egg and 9 masses of syncytial cells were found in 1,000 eggs counted. With respect to these elements, this egg preparation was quite similar to the one used in the previous analyses (1). After the bulk of the sea water was removed by centrifugation, the eggs were immediately washed once with cold 70 per cent ethanol and twice with cold 95 per cent ethanol which removed most of the salt and a considerable amount of echinochrome. The eggs were then extracted twice with acetone at room temperature, twice with boiling ethanol-ether (2:1), methanol-chloroform (2:1), and petroleum ether, the extraction period being about 20 minutes in each case. After drying by evaporation of the last solvent at room temperature, the dried residue was extracted twice with 120 ml. portions of ice cold 6 per cent $HClO_4$ with continuous stirring, allowing 20 minutes for each extraction (3, 4). To remove most of the echinochrome in the acid extract it was shaken with ether (3 extractions, each with 300 ml. portions of ether), the ether extract being washed with 6 per cent $HClO_4$ and the aqueous fractions combined. The volume of the solution was reduced by vacuum distillation and perchlorate precipitated in the cold as the potassium salt, the precipitates being washed with 0.6 per cent $HClO_4$ and the washings

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combined with the concentrated solution. The perchlorate precipitation was carried out in 5 steps as the volume of solution was successively reduced to 140, 50, 10, 1, and 0.6 ml., the pH being kept below 5. The final precipitate wash was with cold water. At volumes of 10 ml. and less further concentration was obtained by evaporation in a stream of dry nitrogen. It was noted that colored matter, presumably the small amount of echinochrome left in the solution, behaved as an indicator, being yellow at pH less than 4, green at about pH 5–7, and brown when made alkaline. After reducing the volume to 0.3 ml., 0.5 ml. of 70 per cent $HClO_4$ was added and the mixture heated in a steam bath for 1 hour (5). After removal of the carbonized residue perchlorate was again precipitated keeping the pH below 1. The volume of the digest was then brought to 6 ml. and the pH adjusted to about 3. On standing, a black flocculent precipitate appeared which was removed by centrifugation, after which the volume was again reduced. When the volume reached 0.5 ml. an unidentified crystalline precipitate which was soluble in the cold, but not in the hot solution was removed. The concentrated digest was then deposited as streaks on two strips of filter paper and subjected to chromatography in butanol-ammonia using a "standard" mixture of adenine, guanine, cytosine, and thymine in another lane on each strip for locating the corresponding bases in the digest (1, 5). Bands of ultraviolet-absorbing substances were found at the levels corresponding to guanine, cytosine, a mixture of adenine and uracil, and thymine. The "thymine" bands were eluted with 20 ml. of water, the eluates combined, reduced to 0.3 ml., and deposited as a drop 7 mm. in diameter on filter paper, and subjected to chromatography in butanol-hydrochloric acid (1, 5). A strongly ultraviolet-absorbing spot was found at the level of the "standard" thymine. The absorption spectrum of the eluate of this spot agreed with that of pure thymine in the region 240 to 290 mμ within 2 per cent. The amount of thymine recovered as determined by the absorption spectrum was 47.0 μg. which was equivalent to 3.96×10^{-7} μg. per egg. *Arbacia* sperm had previously been found to contain 8.2×10^{-8} μg. per cell (1). The amount of thymine per cell recovered from the acid-soluble fraction was, therefore, 4.83 times the amount in the sperm.

Sperm.—Sperm were collected after stimulating shedding with 0.53 M KCl, suspended in filtered sea water, passed through bolting cloth, and centrifuged for 5 minutes at 900 *g* to remove a red sediment. The volume of the suspension was then made to 630 ml. and 1.0 ml. taken for counting. Counts were made in a hemocytometer on aliquots in which the sperm were immobilized with 1 per cent formalin. The number of sperm taken for analysis was $68.56 \pm 1.85 \times 10^{10}$. They were subjected to the same extraction procedure described for the eggs. Since there was no echinochrome in the acid extract it was not shaken with ether. Perchlorate was removed and the volume reduced as described for the eggs. At a volume of 1.0 ml., an amorphous white precipitate was removed and dried. The volume of the solution was then reduced to 0.2 ml. and digested with 0.5 ml. of 70 per cent $HClO_4$. The dried white precipitate was similarly digested, the digests combined and subjected to chromatography in butanol-ammonia. Although strongly ultraviolet-absorbing bands were found at the levels for guanine, and adenine plus uracil, there were none at the thymine level. Nevertheless, this region together with 2.0 cm. of the paper on either side of it was eluted and the eluate deposited as a drop 7.0 mm. in diameter on paper and subjected to chromatography in butanol–hydrochloric acid. No ultraviolet-absorbing material was found at or near the level for thymine. The only ultraviolet-absorbing material detected was a blue fluorescent spot near the solvent front identical in position with a similar spot observed in the chromatograms of the egg extract. In contrast to the eggs, therefore, the acid extract of the sperm yielded no detectable thymine.

DISCUSSION

The most significant result of these experiments is the recovery of thymine in the acid-soluble fraction of mature *Arbacia* eggs as predicted from the

theory we previously presented that the DNA of the egg is degraded during maturation. We have shown that isolated nuclei of *Asterias* oocytes in the germinal vesicle stage contain an amount of DNA equal to at least six times the amount in sperm (2). We have also found that in mature *Tripneustes* eggs after thorough extraction with cold dilute citric acid, all of the thymine found could be accounted for by the DNA of contaminating somatic nuclei and polar bodies in the preparation, leaving none that could be assigned to the egg cells themselves (2). Analyses of *Arbacia* eggs not previously acid-extracted gave the result that of the amount of thymine recovered from the insoluble fraction after KOH digestion and acid precipitation, a considerable portion was attributable to the egg after allowance was made for somatic contamination and for polar bodies. It was also found that in these three species, the pronuclei of the mature eggs were Feulgen-negative and that this condition could not be explained away as a dilution of DNA in an exceptionally large nuclear volume nor by the assumption of a diffuse or lampbrush structure for the chromonemata (1). Chromatin of the usual type has been described by Wilson and by Mathews for the pronuclei of *Asterias*, *Arbacia*, and *Toxopneustes* (10, 11). Examination in phase-contrast of the pronuclei of living eggs of *Arbacia* and *Tripneustes* has revealed chromonemata comparable in dimensions with those of the fixed and stained preparations illustrated by the above mentioned authors (12). From these observations, we were led to the deduction that the DNA of the oocyte nucleus together with that which may have been synthesized during the maturation divisions was degraded to the level of small molecules which were extractable with cold dilute acid. The experiments we have here reported show that *Arbacia* eggs do indeed contain thymine derivatives of the sort predicted.

The quantitative aspects of these observations are not without significance. In the previous experiments, the amount of thymine per egg, when determined as DNA, was found to be equivalent to $10X$ in which X was the amount per sperm. From experiments on other materials which indicated that the polar bodies each had the X amount of DNA (13, 14), it was assumed that $3X$ of the total could be reasonably assigned to the polar bodies. In addition it was found that in spite of repeated washings there was a considerable number of somatic cells in the preparation (1). The extent of contamination with somatic cells was found to be the same in the preparation used in the present experiments as in those previously reported. Making the assumption that these cells contained the diploid quantity of DNA, it was estimated from the frequency of the masses of these cells and the number of cells per mass that the minimum amount of DNA that might be assigned to these cells was $2X$ and the maximum, $4X$. We find in the present experiments that the amount of thymine recovered in the acid-soluble fraction was equivalent to $4.8X$ calculated as DNA. We may then safely assume that at least this much thymine does not exist in the cell as part of DNA. This would leave $5.2X$

as non-acid-soluble or DNA thymine. There is thus good agreement with the data of the previous experiments when the minimum value there given is used. If, alternatively, the estimate for the maximum possible contamination is used, we could expect to recover only about three-fifths of the amount of acid-soluble thymine actually found. We believe therefore that the minimum estimate is the best approximation to the true value. The experiments with the eggs of the sea urchin *Tripneustes* are of interest in this connection. After thorough extraction with cold 5 per cent citric acid, the amount of thymine found was just enough to account for the DNA of the polar bodies and of the contaminating somatic nuclei whose frequency in this material could be obtained with considerable accuracy. In other words after acid extraction, we could find no thymine which could be assigned to the egg itself (2). These three sets of experiments are taken as evidence not only that the DNA of the egg is degraded during maturation, but that all or almost all of it is so degraded.

The failure to obtain any thymine in the acid extract of the sperm makes it impossible to argue that the thymine in the egg extract may have been split from DNA by the procedures employed. The amount of sperm extracted represented more than 5,000 times as many cells as the eggs used. Whatever amount of DNA might be reasonably assumed to be present in the eggs, the amount in the sperm extracted was many times greater, so that if thymine were split from DNA, a readily detectable amount should have been obtained in the sperm extract. Other investigators have found that cold 6 per cent $HClO_4$ will not extract DNA from plant or mammalian tissues in the brief time intervals used (3, 4). An assumption that the polar bodies are the source of the acid-soluble DNA would not be justified because of the observation that they remain Feulgen-positive and also because they could account for only three-fifths of the thymine found. Thus the only justifiable deduction that can be made from the observations is that the thymine extracted with acid came from the egg cells but not from any DNA that they contained.

Although the thymine recovered in these experiments was found in that fraction which usually contains nucleotides, it cannot be assumed that the source of the thymine was necessarily free thymidylic acid. The previous experiments cited suggest that it is not. Further work is necessary to determine the form in which the thymine exists in these cells, but it is reasonable to assume that it must be contained in molecules which are small in comparison to DNA.

All the arguments for the theory that DNA is the sole or the major genic material (6, 7) are based on the assumption that it must be present at all times in the polymerized form in cells which have the capacity for reproduction, for otherwise it would be difficult to obtain both the marked specificity and the wide diversity of activity that seem to be characteristic of genes.

The eggs we have studied may be induced to proliferate and to develop into normal embryos parthenogenetically; *i. e.*, without the introduction of DNA by sperm. One may conclude therefore that DNA cannot be the sole genic material. It has been proposed that DNA may have a regulatory function in the cell by acting as a competitive inhibitor of ribonucleic acid. This hypothesis has been generalized to include similar antagonism of ribonucleotides and ribonucleosides by the corresponding desoxyribose compounds (8, 9). The finding that relatively large quantities of desoxyribose compounds are released by the nucleus during maturation of *Arbacia* eggs is consistent with that theory.

SUMMARY

Mature *Arbacia* eggs were extracted with cold dilute perchloric acid, the extract concentrated, and the concentrate digested in hot perchloric acid. Thymine was recovered from the digest by paper chromatography, and the amount per egg found to be about 5 times the amount per sperm. This was the amount expected from previous experiments and is believed to represent all or almost all of the thymine in the egg. The result supports previous observations that DNA is absent from the mature egg although present in the nucleus of the egg in the germinal vesicle stage. No thymine could be recovered from a similar extract of 5,000 times as many sperm of the same species. The observations are consistent with the theory that DNA and its derivatives act as metabolic antagonists of the corresponding ribose compounds.

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