EVIDENCE FOR SYNTHESIS OF DEOXYRIBONUCLEIC ACID IN ENUCLEATED *RANA PIPIENS* EGGS

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The large quantity of DNA found in the cytoplasm of some eggs raises the question of whether DNA can be synthesized outside the nucleus. Data on the *Rana pipiens* egg were obtained some time ago and were first mentioned in a paper by Schultz in 1956 (1). In the enucleated egg of this species, DNA, or a DNA-like molecule, doubles in amount in the first 30 hours after activation. Since the amount present in the whole egg before activation is at least 2000 times the haploid value (2, 3), an amount comparable to this may be synthesized by the cytoplasm.

METHODS

Rana pipiens eggs were activated with a clean glass needle and were enucleated by flicking the nucleus out of the egg according to the Porter technique (4). The jelly was removed from the two to fifteen eggs needed for a single determination. The eggs were put intact into a glass tube and dried in an oven at 90°C to inactivate DNase which might break down DNA before determinations were made. No measurable amounts of DNA were digested when this procedure was tested in a control experiment in which known amounts of DNA and egg homogenate were subjected to 90°C for 24 hours.

For the determination of DNA there were available two microbiological methods, both dependent on the utilization of deoxyribosides by the test organism (5 to 7). These exist in the egg as low molecular weight compounds, in which case they can be directly determined by the microbiological assay, or as part of the DNA molecule which must then be broken down into its constituent deoxyribosides. Enzyme hydrolysis with a combination of DNase and snake venom phosphatase was found satisfactory. A typical protocol for two eggs is as follows: the dried eggs were heated at 100°C in 0.1 ml of 0.5 N NaOH while being homogenized with a stirring rod. They were then brought to pH 7 with about 0.05 ml of 0.06 N maleic acid. Then 0.01 ml of freshly prepared DNase (1 mg/ml), in 0.01 M $MgSO_4$, and 0.1 ml snake venom phosphatase (12 μg/ml) were added to the homogenate. If more than two eggs were to be assayed correspondingly larger proportions of the solutions were used. After being shaken, the tubes were incubated at 37°C for 18 hours and then autoclaved for 3 minutes. Non-soluble material was centrifuged down at 20,000 *g* and the supernatant decanted and saved; the precipitate was washed with distilled water and this supernatant added to the first and brought to a final volume of 1 milliliter with distilled water. The amount of unbound deoxyribosidic material was also determined for the various stages by assaying eggs that had not been previously treated with enzymes. Thus, by the comparison of values obtained for eggs subjected to enzymatic digestion and for untreated ones, both the DNA and the free deoxyribosides could be evaluated.

MICROBIOLOGICAL ASSAY

In order to double-check the results, two independent assays for DNA were used. One, devised by Hoff-Jørgensen (6), uses *Thermobacterium acidophilus* R-26 and is specific for deoxyribosides and deoxyribotides; the other uses *E. coli* and is specific for thymine or thymidine. Travaglini's modifications of these methods for *Drosophila* eggs were followed (see Miller (7)). The inoculum in each case was 0.05 ml of the bacteria in their log phase of growth obtained from bacteria maintained in their respective basal media. After 18 hours at 37°C the inoculated tubes were read in a Model DU Beckman spectrophotometer at 650 mμ.

For each experiment a new standard curve was established using thymine deoxyriboside in a medium containing all the solutions except the egg homogenate. For the calculation of DNA values, the amount of deoxyribosides in the homogenate before hydrolysis was subtracted from the total value after hydrolysis. Calculations were made on the basis that 1 g mole of deoxyribosides is equivalent to 327 grams of DNA.

E. coli assays indicate the effect of homogenate to be even less than for *Thermobacterium acidophilus.*

Bacterial contamination as a cause of the increase in DNA is unlikely since after 30 hours there is a decrease in the amount of DNA found in the eggs, instead of the increase expected from a postulated bacterial growth.

Another factor to be considered is the completeness of DNA hydrolysis. Occasionally a whole

TABLE I

Thermobacterium acidophilus Assay

Deoxyriboside Content of Enucleated R. pipiens Eggs from a Single Female at 0 and 27 Hours, Determined in the Presence of Varying Levels of Added Deoxyriboside

Each entry, with its standard deviation, is the mean of six separate determinations. Each amount is the amount determined minus what was added.

Age of eggs	Amount deoxyriboside added		
hrs.	*μg* 0	*μg* 0.023	*μg* 0.040
0	0.072 ± 0.016	0.077 ± 0.014	0.080 ± 0.010
27	0.111 ± 0.010	0.112 ± 0.014	0.103 ± 0.008

TABLE II

Content of Bound Deoxyribosides of Enucleated Eggs of R. Pipiens, Compared with that of Eggs from the Same Clutch Prior to Activation (0 hours)

Before activation	After activation	Time after activation	Method	No. of determinations
μg	*μg*	*hrs.*		
0.13	0.44	21	*T. acidophilus*	1
0.21	0.47	20	*E. coli*	1
*0.06 ± 0.01	0.09 ± 0.02	20	*T. acidophilus*	2
0.16 ± 0.03	0.35 ± 0.01	27	*T. acidophilus* and *E. coli*	4

* The low values in this experiment may be due to a failure of enzyme digestion.

As for the validity of the method, it is possible that a substance other than DNA may arise in the egg which would affect the growth of the bacteria and so invalidate the assays. However, it was found that if various combinations of homogenate and standard deoxyriboside solution were made, the results of these combinations were usually within 10 per cent of the results when homogenate and standard solution were incubated separately as is shown in Table I. Similar tests made with series of experiments gave extremely low results, and this is thought to be due to some unexplained interference with the enzyme reaction. In the hydrolysis of standard DNA solutions under the conditions used, an average close to 80 per cent of expected values was obtained.

RESULTS

Table II demonstrates the synthesis of DNA by a comparison of bound deoxyriboside content of

eggs before activation and at about 20 to 30 hours after activation, the time at which the maximum amount of DNA is usually present. After this time, there is a decrease in bound deoxyriboside and the eggs degenerate.

In Table III there are more detailed results for one clutch of eggs determined by both assay methods and showing values for bound and unbound deoxyriboside. Note that the latter represent less than 15 per cent of the bound plus free compounds in each case. The difference in the amount of bound deoxyriboside at 0 hours and at 27 hours is significant ($P < 0.01$ if the 0.04 reading is omitted); DNA or a DNA-like molecule has been synthesized.

TABLE III

Content of DNA, Deoxyribosides, and Thymine-Containing Compounds in Enucleated Eggs from a Single Female, at Varying Times after Activation

Values given are for micrograms of the compound per egg; average values are shown with the standard deviation.

Time after activation	μg DNA			μg Free deoxyribosides		μg Free thymine compounds	
	T. acid. method	*E. coli* method	Average	Average	Per cent of total deoxyribosides	Average	Per cent of total thymine compounds
hrs.							
0	0.19 0.13	0.16 0.18	0.16 ± 0.03	0.004	2	0.002	6
6	0.33* 0.14	0.18 0.18	0.17 ± 0.02	0.010	5	0.005	12
12	0.19 0.25	0.25 0.25	0.24 ± 0.03	0.013	6	0.008	14
20	0.06* 0.39	0.28 0.36	0.34 ± 0.05	0.016	5	0.006	10
27	0.34 0.04*	0.35 0.36	0.35 ± 0.01	0.002	0.5	0	0

* Values not used in computing averages shown.

If DNA synthesis in normal development is compared with that in enucleated eggs (Table IV), it is clear that the latter synthesize DNA at about the normal rate until they reach a time corresponding to normal early mid-cleavage (12 hours at 19°C). It is of interest that haploids, which have nuclei, maintain a normal rate for a somewhat longer time than enucleated eggs (8).

DISCUSSION

Before consideration of the significance of cytoplasmic DNA synthesis in *Rana pipiens* eggs, two questions must be answered: first, is the compound synthesized DNA, or a DNA-like molecule, or both of these?; and second, is the process really independent of the nucleus? It is now known that di- and triphosphates of pyrimidines are present in at least some cells (9, 10), including sea urchin eggs (10); such compounds support the growth of *Thermobacterium acidophilus* R-26 after only phosphatase digestion (in the absence of DNase action). Since both enzymes were always used together, the possible contributions of such compounds to the results is unknown. The *Thermobacterium* assay method itself has been subjected to some criticism (11, 12). However, experiments such as those shown in Table I, and the use of snake venom phosphatase which removes nucleotides found to have an inhibiting effect (12), and the fact that both types of assays show a similar result, all would make these objections less serious.

The question of nuclear participation in this synthesis is complicated by the fact that the exovate containing the nucleus remains attached by a thin stalk to some of the enucleated eggs. However, if it is assumed that the exovate contains the diploid amount of DNA, since it has passed the second meiotic metaphase, the nuclear DNA would either have to replicate over 1000 times or produce at least ten generations of nuclear divisions in order to synthesize the DNA formed in the enucleated eggs. Neither of these alternatives is likely to occur consistently enough to give the reported results.

TABLE IV

Content of Bound Deoxyribosides per Egg, at Various Times after Fertilization or Activation, in Normally Developing and Enucleated Eggs

Determinations by *E. coli* method

Normal			Enucleated	
Time after fertilization	Stage	DNA	Time after activation	DNA
hrs.		*μg*	*hrs.*	*μg*
0	1	0.16	0	0.16
		0.21		0.18
3	2	0.21	6	0.18
		0.11		0.18
12	7	0.26	12	0.25
		0.26		0.25
24	8	0.68	20	0.28
		0.62		0.36
36	10−	1.19	27	0.35
		0.62		0.36
60	12−	1.98		
		2.03		
120	17	4.46		
		4.72		

Although the work of Kornberg and his group on *in vitro* synthesis of DNA (13), as well as that of several authors on thymidine incorporation (14, 15) have made the idea of cytoplasmic DNA synthesis less startling than it was at the time of the experiments reported here, little or no progress has been made since then in explaining how the large amounts of DNA present in the cytoplasm of some cells are either synthesized or utilized.

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