NUCLEIC ACID METABOLISM IN A SLIME MOLD WITH SYNCHRONOUS MITOSIS*

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SUMMARY

In the synchronously dividing slime mold, *Physarum polycephalum*, the synthesis of DNA as shown by incorporation of ^{14}C from [6-^{14}C]orotic acid into DNA-thymine, occurred immediately after the nuclear division and lasted for 1–2 h. During the remainder of the interphase, which might last from 12–20 h, there was no significant labeling of the DNA. The incorporation of label into RNA at any time of the cell cycle suggested that RNA synthesis was continuous, although there was some indication that this process might be retarded at the time of division and DNA synthesis. The relative timing of DNA synthesis and nuclear division would tend to rule out the synthesis of DNA as a "trigger" for mitosis. Preliminary experiments indicated that the mold was capable of converting orotic acid to thymine nucleotides throughout the interphase; this suggests that the control of DNA synthesis must occur at some point very close to the final polymerization of the molecule.

INTRODUCTION

The desire to study reactions which are related to the division cycle of the cell has prompted many investigators to develop biological systems with artificially induced synchrony[2]. A few systems have been found in which synchronous division occurs spontaneously, the best known being the cleavage of the fertilized egg[3] and the formation of microspores in certain plants[4]. In the slime mold *Physarum polycephalum*, nuclear division is synchronous in relatively large plasmodia. The occurrence of spontaneously synchronous mitoses in this myxomycete was first reported by HOWARD[5]. More recently, GÜTTES *et al.*[6] have made an extensive study of the morphology of this organism and have described in detail the various stages of its life cycle.

The present report concerns the determination of the temporal relationship

Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; DNA-P and RNA-P phosphorus in deoxyribo- and in ribo-nucleic acid respectively; DNA-thymine, thymine in deoxyribonucleic acid; RNA-uracil, uracil in ribonucleic acid.

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between synthesis of DNA and mitotic nuclear division in the vegetative stage of *Physarum polycephalum*. For this purpose the conversion of [6-^{14}C]orotic acid to DNA-thymine was studied in relation to the cycle of plasmodial growth. In addition, the conversion of orotic acid to thymine derivatives of the acid-soluble fraction as well as to RNA-uracil was also determined.

METHODS

Cultures

The mold was grown on a soluble medium as described by DANIEL AND RUSCH[7]. [6-^{14}C]orotic acid was used as radioactive precursor and was added to the medium prior to autoclaving whenever radioactive medium was prepared. Unless otherwise stated, the amount of radioactive orotic acid was 0.3 μC/ml with specific activity 1 μC/μmole.

To facilitate transfer and removal of samples, the molds were grown in 9-cm Petri dishes as shown in Fig. 1. The plasmodium was supported by a piece of filter paper (Whatman No. 40), which in turn was supported by a layer of glass beads, 4 mm in diameter. The addition of approximately 15 ml of medium was sufficient to bring the liquid to the level of the filter paper; thus the upper surface of the mold

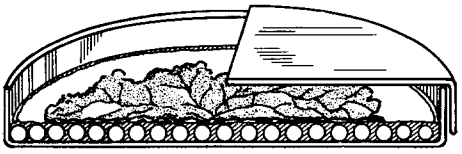

Fig. 1. Section through culture dish. Consult text for further details.

was exposed to air. Several small perforations were put in the filter paper to prevent the trapping of air bubbles below the paper, which would have reduced the contact between paper and liquid. The Petri dishes with beads and filter paper were autoclaved prior to addition of the medium and the mold. Aseptic conditions were maintained during the transfers as well as during the removal of the samples.

Stock cultures of the mold were grown as shake cultures, as described by DANIEL AND RUSCH[7]. To convert the mold to a surface culture, the shake suspension was centrifuged at about 250 $\times$ *g*, the old medium discarded, and a 1-ml aliquot of the mold-sediment transferred to a sterilized Petri dish containing beads and filter paper. The mold was left for 6–12 h on the moist paper before medium was added to the Petri dish. This "starvation period" was found to facilitate the fusion of the mold fragments. After about 3 days the cultures were ready for the final transfer. Pieces of filter paper, 1–2 cm^2 in size, were cut along the edge of the mold outgrowth and transferred to Petri dishes complete with filter paper and medium. After a growth period of 2 days the plasmodia were approximately 4–5 cm in diameter and were ready for the experiments.

About 30 cultures were usually prepared for an experiment. To determine the stage of the cultures within the division cycle, samples for smears were taken every hour with a pointed scalpel. The smears were fixed in alcohol and studied under a phase contrast microscope[6], at a magnification of 1000 times. The first 8–10 cultures that were found to be in mitosis when sampled were selected for the subsequent work. The frequency of sampling was increased whenever a mold was found to be in pro-

phase. By a comparison of the smears with a previously established time schedule for the various mitotic stages of this mold[6], the time of division—*i.e.*, the separation of the daughter nuclei at the end of anaphase—could be estimated with an error of less than 5 min.

For studying the incorporation of the precursor, filter paper and mold were transferred at the preselected time to a new Petri dish containing radioactive medium. Samples for chemical analyses and radioactivity measurements were obtained by cutting off and removing a part of the filter paper with adhering piece of mold, while the remainder of the mold was saved for further sampling and analyses. In this way each mold could be used for serial analyses or be made to serve as its own control.

Analytical procedures

Each piece of mold to be analyzed was homogenized in a Potter-Elvehjem homogenizer for approximately 20 sec with 4 ml of ice-cold 5 % trichloroacetic acid (TCA). The entire homogenate was transferred to a 15 ml ignition tube in which all subsequent steps were carried out. The sample was centrifuged and the sediment washed, consecutively, with 5 % TCA acid (twice), 60 % ethanol, 95 % ethanol (twice), and finally with acetone. After all samples were dried in a vacuum desiccator, their net weights were determined. Usually, the dried residues weighed 10–30 mg. With the relatively small samples involved, the isolation and separation of the nucleic acids by conventional methods proved difficult and unsatisfactory. We therefore used the method introduced by JERVELL *et al.*[8] and hydrolyzed the dried residues directly with 98 % formic acid in sealed tubes kept at 165° for 2 h.

Since [6-^{14}C]orotic acid was used as the precursor, we were interested only in the pyrimidine bases of the nucleic acids. Furthermore, since thymine and uracil are unique to DNA and RNA, respectively, the isolation of these two pyrimidine bases or their derivatives from the "acid-insoluble" material should provide a relative measure of the radioactivity as well as of the quantities of each of the two nucleic acids. With the above method for hydrolysis, DNA-thymine is liberated as the free base, whereas RNA-uracil is obtained as a mixture of uracil and uridine, with the latter component predominating. These three compounds were isolated by a combination of ion exchange and paper chromatography as previously described by JERVELL *et al.*[8]. The specific activities of the DNA-thymine and RNA-uracil were determined on the chromatographically isolated thymine and uridine, respectively. Aliquots were plated on aluminum planchets and the radioactivity measured in a gas-flow proportional counter. The amounts of the isolated compounds were determined spectrophotometrically.

In a few cases the amounts of DNA and RNA in the mold samples were determined by more direct methods. Deoxyribose was measured by a procedure described by KECK[9], and ribose by the orcinol method[10]. Assuming the ratio of purines/pyrimidines to be 1.0 in the nucleic acids of the mold, the following molar ratios were calculated for the isolated components: thymine/DNA-P = 0.26 and [uridine + uracil]/RNA-P = 0.21. A relatively good agreement among the analyses from different samples indicated that the measurement of the isolated pyrimidine derivatives could serve as a means of estimating the total amounts of nucleic acids in the samples*.

* Representative values for the content of nucleic acids in the mold were 190 μatoms RNA-P and 15–30 μatoms DNA-P/g of dried mold residue.

A partial fractionation of the acid-soluble material to yield thymine of nucleotide and non-nucleotide origin was carried out in one experiment. The molds in this case were homogenized with 5 ml of ice-cold 0.3 *N* perchloric acid and the insoluble material washed with a second 5-ml portion of the acid. One volume of ethanol was added to the combined extracts to precipitate mucous material. Following the addition of 0.2 ml of 0.25 *M* Na_2SO_4 to each sample, the solutions were neutralized with KOH and back-titrated with HCl to give a reaction slightly acid to phenol red. The addition of Na_2SO_4 served to remove calcium ions which had been carried along from the medium and which had been found to form precipitates with organic phosphate esters under the prevailing conditions. Calcium sulfate and potassium perchlorate were removed by centrifugation in the cold, and the neutral supernatant solution was added through a 3-ml column of Dowex 50-X8 (hydrogen form) onto a 3-ml column of Dowex 1-X10 (formate form). The columns were washed with 20 ml of 0.01 *M* acetic acid, and the effluent and wash were collected as "fraction A." This fraction should contain, among other compounds, any thymidine and free thymine from the mold. Subsequent elution of the Dowex 1 column with 30 ml of 1.0 *N* HCl yielded a "fraction B". It had previously been established that this latter fraction would contain orotic acid as well as any phosphorylated thymidine derivatives present in the original perchloric acid extract.

All fractions "A" and "B" were evaporated to dryness *in vacuo*, and the residues were hydrolyzed with 98 % formic acid and carried through the subsequent steps for isolation of thymine, following the procedure given above for the acid-insoluble materials. Since in this particular experiment we were interested only in determining the total ^{14}C present in the thymine derivatives of the two fractions, carrier thymine was added to the samples before the paper chromatography to facilitate location of the compound. The isolated thymine spots were eluted and their content of radioactivity determined as described above.

RESULTS

DNA synthesis

To obtain a preliminary estimate of the period of DNA synthesis, individual plasmodia were transferred to radioactive medium at different times following nuclear division. After 3 hours' exposure to the radioactive medium, one half of each mold was harvested for analysis and the other half sampled periodically to determine the time of the next division. In the upper frame of Fig. 2 the heights of the columns show the specific activity of the DNA-thymine obtained from the various molds. The width of each column indicates the period during which the particular mold was exposed to the precursor. The time axis has been adjusted to an interdivision time of 18.6 h, which was the average found in this group. It can be seen that the most active incorporation of ^{14}C from orotic acid into DNA-thymine occurred very close to the time of division. The columns in the lower frame of Fig. 2 represent the specific activities of RNA-uracil obtained from the same molds. The incorporation of ^{14}C into the RNA-uracil in all of the samples demonstrated that the virtual absence of labeling of the DNA-thymine during most of interphase was *not* caused by failure of the mold to take up the precursor from the medium.

It was clearly desirable to time the period of the labeling of DNA more precisely.

Since a shortening of the period of exposure to radioactive medium would lead to less efficient incorporation, a different approach was tried. As already indicated from the previous experiment, essentially no labeling of DNA occurred during the greater part of the interphase. Leaving the mold exposed to radioactive medium at a time

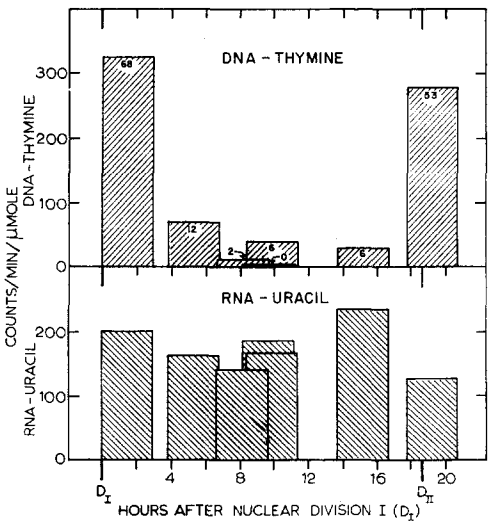

Fig. 2. Incorporation of ^{14}C into pyrimidines of nucleic acids after 3 hours' exposure of culture to [6-^{14}C]orotic acid. The columns represent in the upper frame the DNA-thymine and in the lower frame the RNA-uracil. The numbers on the columns for the DNA-thymine indicate the total radioactivity in counts/min, recovered in this compound from the entire sample. The radioactivity of the medium in this particular experiment was 0.2 μC of ^{14}C/ml. The time axis in this graph was adjusted to an average inter-division time of 18.6 h. In this and in the subsequent graphs the time of nuclear division is defined as the time of the separation of the daughter nuclei at the end of anaphase.

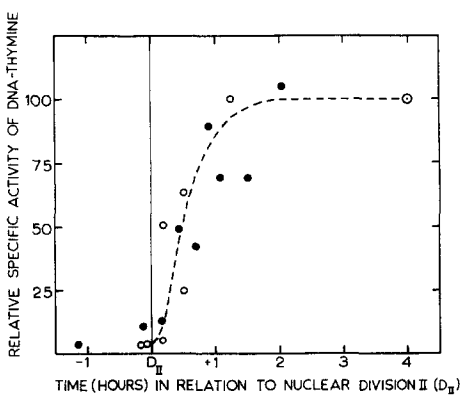

Fig. 3. Incorporation of ^{14}C from [6-^{14}C]orotic acid into DNA-thymine in relation to the time of nuclear division. The cultures were transferred to radioactive medium approximately 4 h after the previous division and left in this medium until the time of sampling. The data have been corrected for individual differences among the cultures by expressing the specific activity of each sample as % of the specific activity of the second half of the same plasmodium, which was left in the medium until 4 h after division. The specific activity of the DNA-thymine in these latter samples averaged about 1600 counts/min/μmole. The open and closed circles indicate the values obtained with two different groups of mold cultures.

when there is no synthesis of DNA would nevertheless permit the uptake of radioactive orotic acid by the mold and might also lead to the production of some more immediate radioactive precursor of the DNA molecule. In this way the efficiency of the incorporation of radioactivity into DNA, when synthesis does occur, might be greatly increased. The analysis of molds harvested during the period when DNA was presumed to be synthesized should then yield information as to the exact onset and duration of this process.

Fig. 3 shows the data obtained from two groups of molds which were transferred to radioactive medium approximately 4 h after nuclear division (division I), and allowed to grow in this medium until the final sampling, One half of each mold was sampled at a preselected time close to the next division (division II); the other half in all cases was left in the radioactive medium until 4 h after division II so that it would complete its synthesis of DNA. In each case the specific activity of the DNA-thymine from the first half of the mold was expressed as a percentage of the value obtained from the second half; this served as a means of correcting for individual differences among the cultures. The graph (Fig. 3) clearly shows that there was no significant incorporation of ^{14}C into DNA prior to the nuclear division II. Shortly after the division there was a rapid uptake of radioactivity, and a maximum level of labeling appeared to be reached between 1 and 2 h after mitosis*.

Obtaining a pure compound rather than a quantitative recovery was emphasized in the isolation of DNA-thymine. It was found, however, that the DNA-thymine per unit dry wt. increased greatly during the period of ^{14}C incorporation. The maximum value observed was 172 % of the premitotic value. If we assume that the dry wt. of the mold increased at a normal rate during the period of DNA synthesis, the reported value would suggest that the DNA content was indeed doubled during the period of ^{14}C-incorporation, indicating further that the labeling was a result of *net* DNA synthesis.

RNA synthesis

In all of the cultures there was extensive incorporation of ^{14}C from orotic acid into the uracil moiety of the RNA. To investigate the course of the RNA labeling, a group of molds was transferred to radioactive medium approximately 4 h after division, and 4–5 consecutive samples were taken for determination of specific activity of the RNA-uracil. Multiple samplings were possible in this case, since the greater RNA content of the mold permitted the use of smaller samples for analysis. The results from this experiment are plotted in Fig. 4. In agreement with the preliminary observations, recorded in Fig. 2, there was a continuous labeling of RNA during interphase, at a time when no DNA synthesis occurred. The apparent break in the curves at the time of division and of DNA synthesis is another interesting feature of the data.

Acid-soluble thymine derivatives

The conversion of orotic acid to DNA-thymine involves a series of intermediate steps[11] and it became of interest to test whether some of the more immediate pre-

* Similar data (unpublished) have been obtained by the use of [8-^{14}C]adenine or [2-^{14}C]-thymidine as precursor and by the separation of the DNA from RNA after the addition of carrier DNA.

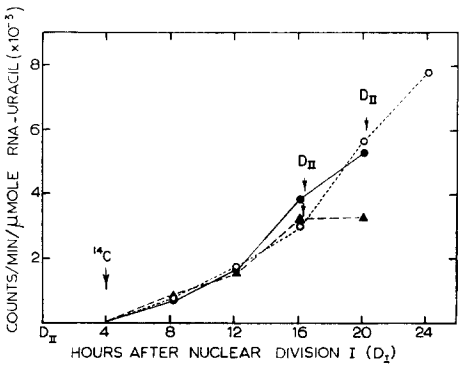

Fig. 4. Incorporation of ^{14}C from [6-^{14}C]orotic acid into RNA-uracil in relation to the time of nuclear division. The cultures were transferred to radioactive medium approximately 4 h after the first division (D_I) as indicated by the arrow (^{14}C). Each curve indicates the results obtained in a single plasmodium. Since the inter-division periods were different in these cultures the second division (D_{II}) has in each case been indicated by an arrow.

cursors of the DNA would be formed only at the time of DNA synthesis, or whether their production would occur in other periods of the cell cycle as well. Three cultures were transferred to radioactive medium 4 h after division. One half of each plasmodium was harvested 4.5 h later, at which time the molds were in mid-interphase. The second half was collected in late prophase, which for these particular cultures occurred 10–12 h after the transfer to radioactive medium. As it was believed that the "pool size" of the individual thymine derivatives would be quite small, isolation of the individual compounds was not attempted. Instead, an estimate was made of the amount of radioactivity in the nucleotide and non-nucleotide thymine derivatives in the acid-soluble fraction as described above. The results of this fractionation are shown in Table I. It should be noted that thymine nucleotides were formed from orotic acid in mid-interphase, as judged from the radioactivity recovered in the nucleotide-thymine fraction (fraction "B"). There was very little difference between the values found at mid-interphase and in late prophase when the data were expressed

TABLE I

CONVERSION OF [6-^{14}C]OROTIC ACID TO THYMINE DERIVATIVES

Time of sampling	*Exposure to [6-^{14}C]orotic acid* (h)*	*Mold No.*	*Acid-soluble*		*Acid-insoluble*	
			*Fraction A non-nucleotide thymine counts/min***	*Fraction B nucleotide thymine (counts/min)*	*DNA-thymine (counts/min)*	*Estimated final DNA-thymine (counts/min)*
Mid-interphase	4.5	I	47	376	14	(800)
	4.5	II	131	687	62	
	4.5	III	99	527	26	
Late prophase	11	I	164	418	40	
	10	II	74	762	37	
	12	III	114	535	67	

* 0.5 μC/ml of medium (1.9 μC/μmole).

** All of the radioactivity data represent total activity calculated for a sample of mold containing 1.0 μmole RNA-uracil or approximately 25 mg of dry wt. material.

as total radioactivity in a sample of standardized size. In the period between the two samplings, however, the plasmodia were increasing in mass, so that on a "per nucleus" basis there was an increase in the thymine nucleotide level, estimated to be about 35 %*.

The small amount of radioactivity recovered from the acid-insoluble thymine fraction (DNA-thymine) was probably a result of contamination by the acid-soluble thymine derivatives. The data clearly demonstrate that the radioactivity of the acid-soluble thymine could not be the result of a partial hydrolysis of radioactive DNA during the fractionation of the samples. The last value in the table represents an estimate of the radioactivity to be found in the DNA if the cultures had been permitted to go through with the division and subsequent DNA synthesis. This value, which is an average from a series of other cultures grown in the same medium, is of the same magnitude as the total radioactivity found in the nucleotide-thymine fraction. It appears, therefore, that in this organism the precursors of DNA-thymine, at least up to the thymidylic acid stage, are available much earlier than the onset of the DNA synthesis.

DISCUSSION

From the data reported it seems clear that in the synchronously dividing slime mold, *Physarum polycephalum*, synthesis of DNA takes place during a very short part of the cell cycle. The duration of the synthesis in a single nucleus may possibly be shorter than indicated in Fig. 3, since a less rigid synchrony of DNA synthesis than that of division would tend to increase the over-all period of incorporation. The time of the period of synthesis in relation to mitosis is somewhat unexpected since, for most materials studied, the synthesis of DNA occurs during the latter part of interphase. DNA synthesis in telophase or in early interphase is, however, not unique and has been reported earlier[12]. In the present case we are dealing with an organism which has an extremely short period between mitosis and synthesis of DNA and a long period between synthesis of DNA and the next division—*i.e.*, a short G_1 and a long G_2 in the terminology of HOWARD AND PELC[13].

The low level of radioactivity found in the DNA isolated during the greater part of interphase was possibly a result of contamination by the acid-soluble fraction (*cf.* Table I). Although this explanation still needs to be proved, the present data can at least rule out the possibility of any extensive exchange of DNA material in the absence of DNA synthesis.

The timing of the synthesis of the DNA in this case renders it unlikely that synthesis of DNA *per se* should be a "trigger" for nuclear division, however necessary the duplication of DNA is for further mitotic division of the mold nuclei. The extremely close synchrony in the division of the nuclei further suggests that the "triggering principle", if such exists, either originates in the cytoplasm or can be transmitted to the other nuclei through the cytoplasm.

As to what "triggers" the DNA synthesis, we have very little information. It is known that several enzymic steps, not required for other functions of the cell, are involved in the synthesis of DNA[11]. Among these are the conversion of riboside

* In a parallel experiment (unpublished) the conversion of [2-^{14}C]thymidine to thymine nucleotides was also shown to occur during interphase. In this case the total radioactivity of the thymine nucleotides in late prophase was on the average 30 % higher than that at mid-interphase.

derivatives to the corresponding deoxyriboside derivatives, the methylation of deoxyuridylic acid to thymidylic acid, the formation of deoxyriboside triphosphates, and the final condensation of these to DNA polymer. Any one of these steps could *a priori* be considered as a possible point of control for DNA synthesis.

Our data on the production of thymidine nucleotides in the absence of DNA synthesis bear directly on this question. Although our present fractionation procedure does not permit any conclusion as to the original phosphorylation level of the thymine nucleotides, the data indicate that the mold retained its ability to convert orotic acid to at least thymidylic acid throughout the cell cycle. Thus, the synthesis of DNA is probably controlled at a point subsequent to the formation of deoxymononucleotides. The question of whether the ultimate control is at the point of the deoxynucleotide kinase or the DNA-polymerase[14], or whether it depends instead on the physical state of the pre-existing DNA (*e.g.*, the availability of a template surface), should now be open for experimental attack.

The apparent break in the incorporation of ^{14}C from orotic acid into the RNA, as indicated by the curves in Fig. 4, may imply depression of RNA synthesis at the time of division and DNA synthesis. It is, at present, impossible to say whether this phenomenon is related to the division or whether a competitive situation exists between the synthesis of RNA and the synthesis of DNA. Results obtained by other investigators[15,16] indicate that the latter explanation is the more likely one.

ACKNOWLEDGEMENT

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