High Molecular Weight Phosphorus Compound in Nucleic Acid Extracts of the Slime Mold

Physarum polycephalum

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Orthophosphate labeled with $^{32}$P was added to the growth medium of the plasmodium of Physarum polycephalum. The nucleic acid extracts of such plasmodia contained $^{32}$P that was not removed by nuclease, protease, or amylase. This labeled material was shown to be separable from nucleic acids, could be eluted from a methylated albumin-kieselguhr column at 0.5 M NaCl, was of high molecular weight, and had several characteristics in common with polyphosphate. A fraction of this polyphosphate-like material was also found in extracts of isolated nuclei.

During the course of an experiment in which nucleotides labeled with $^{32}$P were isolated from the ribonucleic acid (RNA) extracted from the plasmodium of Physarum polycephalum, a highly labeled fraction was found (3). This material was clearly distinguishable from deoxyribonucleic acid (DNA) and RNA. Further study of this fraction suggested that it was a polyphosphate, a substance previously reported to be present in the plasmodium of Physarum (7, 10) and in other organisms (8). This paper reports the isolation of the $^{32}$P-labeled substance and presents data that are consistent with the suggestion that it is a polyphosphate.

MATERIALS AND METHODS

Chemicals. The various chemicals used, together with their sources, were as follows: carrier-free $^{32}$P, inorganic orthophosphate (E. R. Squibb, Franklin Park, Ill.); $\alpha$-amylase and $\beta$-amylase (Schwarz Bio-Research Inc., Orangeburg, N.J.); purified deoxyribonuclease and acid phosphatase (Worthington Biochemical Corp., Freehold, N.J.); ribonuclease (from bovine pancreas), ribonuclease-T1, and Pronase (Calbiochem, Los Angeles, Calif.). Methylated albumin was a gift from H. Kubinski of the McArdle Laboratory. Other chemicals were reagent grade. Solutions used were as follows: (i) buffer I [0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.2), 1 M MgCl$_2$, 0.1 M NaCl]; (ii) poly-some medium [0.1 M Tris-hydrochloride (pH 7.2), 0.2 M sucrose, 0.05 M KCl, 0.0015 M MgCl$_2$]; (iii) wash buffer (0.25 M sucrose, 0.01 M ethylenediaminetetraacetate, pH 7.5); (iv) nuclear isolation medium [0.01 M Tris-hydrochloride (pH 7.2), 0.25 M sucrose, 0.01 M CaCl$_2$, 0.1% Triton X]; (v) 1 M sucrose medium [1 M sucrose, 0.002 M CaCl$_2$, 0.01 M Tris-hydrochloride (pH 7.5), 0.1% Triton X]; (vi) methylated albumin-kieselguhr (MAK)-buffers [0.1 M, 0.4 M, or 1.6 M NaCl in 0.01 M phosphate buffer (pH 6.7)].

Culture of plasmodia. Axenic cultures were maintained as shaken suspensions of microplasmodia in 20 ml of semidefined, citrate-hematin medium in 500-ml Erlenmeyer flasks, as described earlier (4). Every 2 or 3 days, 2 ml or 0.6 ml, respectively, of a culture was inoculated into fresh growth medium.

Extractions. $^{32}$P was added directly to the culture medium, and at the end of the specified period of time cells were harvested by centrifugation at 200 X g for 3 min and washed briefly by resuspension in an equal volume of unlabeled medium before centrifugation. The pellet was frozen in liquid nitrogen and homogenized in 10 volumes of buffer I in a Potter-Elvehjem homogenizer for total extracts. Subcellular fractionations were made as described previously (13). Frozen microplasmodia were homogenized in polysome medium and centrifuged at 30,000 X g for 7 min, yielding a pellet and a supernatant fraction. Nuclei were isolated as described by Mohberg and Rusch (J. Cell Biol. 23:61A, 1964). Unfrozen cells were suspended in wash buffer, collected by centrifugation, and then broken in the nuclear isolation medium in a Waring Blender. Debris was retained on a milk filter pad (Rapid-Flo, Johnson & Johnson, Chicago, Ill.), and cytoplasmic contaminants could be eliminated by two centrifugations of the nuclear suspension through 1 M sucrose medium (J. Mohberg, personal communication). Nucleic acids were isolated by a modified phenol extraction (15). Sodium dodecyl sulfate (SDS) was added to buffer I to a final concentration of 2%, and the protein was removed.

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by two extractions with equal volumes of water-saturated redistilled phenol, followed by one extraction with chloroform-amyl alcohol (15:1). Two volumes of ethyl alcohol were added to the aqueous phase, and the nucleic acids were precipitated overnight at -20°C. The precipitate was dissolved in 0.4 M NaCl buffer for direct application to a MAK column or in buffer I for enzyme treatment.

**MAK chromatography.** MAK columns (12) were prepared in a modified form as described elsewhere (11). The basal layer was made up from 3 g of kieselguhr supplemented with 0.5 ml of a 1% solution of methylated albumin; the middle layer contained 2 g of kieselguhr and 1.5 g of protein-coated kieselguhr from a stock suspension. The protective upper layer of 1 g of kieselguhr was added from a suspension in 0.4 M NaCl buffer. After the column was washed with 150 ml of 0.4 M NaCl buffer at 35°C, the sample was adsorbed onto the column and washed with 250 ml of 0.4 M NaCl buffer. Elution was achieved by a linear gradient that was generated by adding 1.6 M NaCl dropwise to a mixing vessel which contained 200 ml of 0.4 M NaCl buffer. The effluent flow was regulated by a peristaltic pump and was led through a flow cell (Beckman 97290), where the presence of ultraviolet (UV)-absorbing material was monitored by a spectrophotometer (Beckman DB) and recorded by a Sargent recorder. Radioactivity was measured in samples taken from 500-drop fractions, which were either dried on planchets for gas-flow counting or precipitated, together with 500 μg of carrier albumin, by 5% trichloroacetic acid on glass fiber filters (Gelman, type E) for liquid scintillation counting.

**Gel filtration.** Sephadex columns (1 by 20 cm, Sephadex G25 or G50) were equilibrated with 0.01 M Tris-hydrochloride buffer, pH 7; fractions of 100 drops were collected and the radioactivity of samples was determined.

**Chemical analyses.** Phosphorus analysis was done as described earlier (1). Samples were ashed at 150°C in 4.5 N HCl for 45 min and in H₂O₂ for 30 min. Acid-labile phosphate was determined after the samples were heated at 100°C in 1 N H₂SO₄ for up to 30 min. The anthrone test was done as described (17), with glucose used as a standard.

**RESULTS**

³²P as orthophosphate was added for a 4-hr period to the medium of microplasmodia grown in shaken culture. Nucleic acid extracts of these plasmodia were applied to a MAK column. Extensive washing with 0.4 M NaCl buffer at 35°C removed soluble RNA, degraded RNA, and all unincorporated ³²P from the column. Elution of the adsorbed material and fractionation were done as described in Materials and Methods. The presence of UV-absorbing material was continuously monitored and was expressed as optical density (OD) at 260 nm (Fig. 1). No OD was measured in the first half of the elution profile. A small peak (later identified as DNA by labeling with ³H-thymidine) appeared at fraction 19, and there was some UV-absorbing material before the two major components were eluted at fractions 29 and 32. These latter fractions were shown to contain the two ribosomal RNA fractions, since they were found in RNA extracts from ³H-unicellular labeled ribosomes. The ³²P label was spread over the nucleic acid region of the profile designated by the OD readings and also over that part of the profile devoid of measurable UV absorption.

To investigate the nature of the highly labeled material which had no UV absorption, nucleic acid extracts were treated with deoxyribonuclease and ribonuclease prior to application to the MAK column. In the elution profile, no OD₃₂₅ could be detected, whereas the unidentified compound was still present and eluted at 0.5 M NaCl (Fig. 2). Pretreatment of the extracts with Pronase (200 μg/ml) or with α- or β-amylase (250 μg/ml) for 4 hr at room temperature did not remove the unknown compound.

To characterize the ³²P-labeled material, appropriate fractions from the MAK eluate were pooled and found to have the following properties: 85 to 95% of the label remained in the dialysis bag after dialysis against a 1,000-fold volume of Tris buffer (1 mm, pH 7), and the label was not retained in gel filtration (Sephadex G25 or G50), thus indicating a high molecular weight. It was precipitated by 70% ethyl alcohol, 5%
trichloroacetic acid, barium acetate, or streptomycin sulfate (5 mg/ml). At least partial degradation was assumed when $^{32}$P could no longer be precipitated by 5% trichloroacetic acid, and degradation could be observed after treatment with acid phosphatase (300 $\mu$g/ml, 2 hr at room temperature) or with 1 N NaOH (3 hr at room temperature), or after hydrolysis in 1 N H$_2$SO$_4$ at 100 C. After hydrolysis in H$_2$SO$_4$, the release of inorganic phosphate was determined directly, and the amount of phosphate released reached a plateau after 15 min at 100 C (Fig. 3). The amount of acid-labile phosphate at this time had reached 75% of the total phosphorus, as determined by ashing the sample. Less than 2 $\mu$g of glucose equivalents were present, as concluded from a negative anthrone test; thus, polysaccharide composed of neutral sugars could not be a major component of the sample. Charcoal did not adsorb the $^{32}$P label.

Attempts were made to locate the $^{32}$P-labeled material within the various particulates of the plasmodium by differential cell fractionation and MAK chromatography of nucleic acid extracts. After being labeled for 2 hr, one culture was homogenized and centrifuged at 30,000 $\times$ g for 7 min to remove nuclei, pigment granules, and mitochondria, with most of the ribosomes left in the supernatant fluid. Nuclei were isolated from a second culture. Nucleic acids from these three cell fractions were eluted from MAK columns, and the $^{32}$P profiles were determined.

![FIG. 3. Acid-labile hydrolysis. Fractions with $^{32}$P-labeled material from an experiment like that shown in Fig. 2 were pooled and dialyzed. One sample was ashed to determine total phosphorus (26 $\mu$g). Other samples were heated at 100 C for the times indicated on the curve, and inorganic phosphate was determined.](image)

![FIG. 4. Localization of polyphosphate. Two plasmodial cultures were labeled with $^{32}$P (5 $\mu$g/ml) and harvested after 2 hr. One culture was homogenized in polysome medium and centrifuged for 7 min at 30,000 $\times$ g. Nuclei were isolated from the other culture. Phenol extracts were made from the pellet, the supernatant fraction, and the nuclei, and were adsorbed onto and eluted from MAK columns as described in the legend of Fig. 1. The three elution profiles are combined in this graph: $\bigcirc$, 30,000 $\times$ g pellet; $\times$, 30,000 $\times$ g supernatant fraction; $\bigcirc$, nuclei. The DNA eluted in fractions 19 to 21, as detected by the OD tracing. The position of the RNA fractions showed slight variations, probably due to different loads per column; whenever variations occurred in the radioactivity profile, a corresponding change was noted in the OD profile.](image)
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total cell extracts. The 32P label from isolated nuclei was found in the region of nucleic acids as well as in the unknown material. On comparison with the profiles derived from the 30,000 x g pellet or from total extracts (Fig. 1), the 32P-labeled material from nuclear extracts seemed to correspond to that subfraction which eluted at a slightly higher NaCl concentration. As in the profiles discussed above, the 32P-labeled fraction was distinct from DNA, which was marked by the OD260 tracing in fractions 19 to 21 of the experiments shown in Fig. 4.

DISCUSSION

The results of these experiments clearly demonstrate that the fraction in nucleic acid extracts which is rapidly labeled with 32P is a separate entity and is not DNA, RNA, protein, glycosgen, or polysaccharide. It was: not degraded by deoxyribonuclease or ribonuclease, extractable by phenol and not affected by Pronase, not changed by amylose, and precipitated with streptomycin sulfate under conditions in which polysaccharides remained in the supernatant fluid (5). Thus, although this substance is not one of the more common macromolecules, it appears to be a distinct molecule of appreciable size.

The evidence from the present experiments indicates that the 32P-labeled compound is polyphosphate. 32P Incorporation, phenol extraction, and MAK column chromatography have also revealed the presence of polyphosphate in frog embryos in early developmental stages; this material eluted at a higher salt concentration (0.6 M NaCl) than in our system and was eluted shortly after the DNA but before the bulk of RNA (16). Polyphosphates have been found in many organisms (8) and have been reported to be present in the plasmodium of Physarum (7, 10). The findings that the compound is of high molecular weight, precipitated by barium acetate, labile to acid hydrolysis, and not adsorbed on charcoal indicate that it is polyphosphate, but its precipitation by trichloroacetic acid and lability to alkali suggest that it is not pure polyphosphate. It is of interest, however, that a fraction of all polyphosphates of biological origin are insoluble in trichloroacetic acid, and lability to alkali may have been caused by the Mg2+ ions present in the extraction buffer (8). Furthermore, degradation in KOH of an RNA-polyphosphate complex has been reported in Chlorella; as in the present experiments, acid hydrolysis accounted for only 75% of the total phosphorus as polyphosphate (2). It appears, therefore, that the "acid-insoluble polyphosphate" in the plasmodium of Physarum is not a pure chain of inorganic phosphate.

Polyphosphate granules showing metachromatic staining with Toluidine Blue were reported in the cytoplasm of Physarum (10). In addition to the polyphosphate in the cytoplasmic particles, the material was shown in this report to be associated with the nuclei as well. Excessive phosphorus in DNA extracts obtained from isolated nuclei could be isolated after CsCl centrifugation and was demonstrated to be polyphosphate (Goodman et al., submitted for publication). Polyphosphates were also reported in the nuclei of mammalian cells (6).

The function of polyphosphate has not been clarified, but an inverse relationship of RNA content to polyphosphate was described by several investigators (9, 14, 18). A study of the possible relationship of the polyphosphate to the synthesis of nucleic acids during the various stages of growth and differentiation is now in progress.

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