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The Phosphorylation of Ribosomal Protein in *Lemna minor*

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ABSTRACT

Sterile cultures of *Lemna minor* have been labeled with $^{32}P_i$, and the ribosomal proteins have been examined for radioactivity. In relatively short term labeling a radioactive protein was found which ran as a single component in both urea/acetic acid and sodium lauryl sulfate gel electrophoresis. Acid hydrolysis of the labeled protein permitted the isolation of serine phosphate. After labeling to equilibrium with $^{32}P_i$, calculation indicated only 0.6 to 0.75 atom of this protein phosphorus per ribosome.

The phosphorylated protein is found in both polysomes and "derived" monomers and appears to be located in the ribosomal small subunit. Its apparent molecular weight is 42,000. Addition of growth-inhibiting concentrations of abscisic acid does not alter the apparent degree of labeling of this protein in 5 hours, but after 24 hours of treatment the total protein phosphorus was reduced from 0.75 atom of phosphorus per ribosome to 0.36 atom of phosphorus per ribosome.

Many of the cellular events mediated by cyclic AMP in animal tissues appear to be related to the control of protein kinase activity (7). The groups of proteins which have been demonstrated to be phosphorylated in animal systems, and therefore may have their activity modulated by protein kinases, include nuclear acidic proteins (15) and histones (1), plasma membrane proteins (14), various enzymes (13), and microsomal proteins (4). This last category includes ribosomal proteins themselves, and the phosphorylation of this group of proteins was first definitively demonstrated by Kabat (4). He was able to show that phosphorylation of at least one ribosomal protein in reticulocytes was associated with the production of inactive monomers. It has recently been shown that phosphorylation of ribosomes *in vitro* with a protein kinase reduces their amino acid-incorporating ability (9).

The possible role and presence of cyclic AMP in plants are still under investigation (3, 5, 10, 12, 18). The presence of phosphorylated proteins and protein kinases in plants does not appear to have been seriously examined except by Kuo and Greengard (7), who reported the apparent absence of histone kinases in some half-dozen plant tissues they examined. This paper reports a first step in an examination of the possible role of this regulatory system in plants with the detection of a phosphorylated ribosomal protein in a sterile plant system. Preliminary experiments indicate that abscisic acid may control the degree of phosphorylation of this protein.

MATERIALS

Carrier-free $^{32}P_i$ was obtained from the Radiochemical Centre, Amersham, and neutralized before use.

The same strain of *Lemna minor* was used as previously described (16). Growth conditions were identical except for the use of a 12-hr day instead of continuous light. This reduced growth rates slightly, the average fresh weight rate constant of increase being 0.30 day^{-1} (16). All experiments were carried out under sterile conditions, and nonsterile experiments were discarded. Assays for sterility were carried out as previously described (16).

Three media have been used for labeling *Lemna* with $^{32}P_i$: (*a*) for 5 hr labeling, 50 ml of sucrose-mineral salts but with KH_2PO_4 reduced to 4 μM and with $^{32}P_i$ at 50 μc/ml; (*b*) for 24 hr labeling, 50 ml of sucrose-mineral salts but with KH_2PO_4 reduced to 40 μM and containing $^{32}P_i$ at 50 μc/ml; (*c*) for 12 days labeling, 100 ml of sucrose-mineral salts containing 4 μc/ml $^{32}P_i$ (2).

As far as can be determined, growth (measured as frond number) continues normally on these media for the duration of the labeling period.

METHODS

Isolation and Separation of Ribosomes and Subunits. Plants were ground in 5 volumes of ice-cold 14% sucrose; 0.05 M KCl; 0.05 M tris, pH 8.5; 10 mM $MgCl_2$. After filtering through Mira-cloth, the homogenate was spun at 15,000*g* for 15 min, and the supernatant was collected and made 2% in Triton X-100. Ribosomes were then layered over 2 ml of 35% sucrose containing 10 mM tris, pH 8.5; 10 mM $MgCl_2$; and either 20 mM KCl (for the experiments shown in Figs. 2, 7, 8, and 10) or 0.25 M KCl (for the experiments shown in Figs. 3–6 and 9). The latter concentration of KCl gave cleaner ribosome preparations, containing about 39% protein, while 20 mM KCl ribosomes were about 45% protein. The ribosomes were spun through the 35% sucrose at 125,000*g* for 2 hr.

The pellet was resuspended in 0.7 ml of 0.05 M KCl, 10 mM tris (pH 7.4), and 10 mM $MgCl_2$; layered onto a 20-ml 10 to 35% exponential sucrose gradient containing 0.05 M KCl, 10 mM $MgCl_2$, and 10 mM tris (pH 7.4); and spun for 2.5 hr at 80,000*g*.

Fractions of either 15 or 20 drops were collected after the tube was punctured and counted by Cerenkov radiation in a Packard scintillation counter. The desired fractions were collected, and the ribosomes were precipitated with 0.7 volume of ethanol. In some experiments (shown in Figs. 2, 7, 8, and 10) and in particular when derived monomeric ribosomes were being prepared, the pH of the initial extraction medium and the 1 M sucrose was 7.9 and 7.4, respectively.

Monomeric ribosomes were prepared from *Lemna* which was gassed in the dark with nitrogen for 1 or 2 hr (8). Subunits were prepared from these derived monomers by suspending the ribosomal pellet in 0.7 ml of 0.05 M KCl, 0.05 mM $MgCl_2$, and 10 mM tris (pH 7.4) and centrifuging on a 20-ml 10 to 35% sucrose gradient containing the above concentrations of KCl, tris, and $MgCl_2$ at 50,000*g* for 16 hr.

Preparation of Labeled Ribosomal Protein. Ethanol-precipitated ribosomes were resuspended in small volumes of 0.01 M tris (pH 7.4), 0.05 M KCl, 1 mM EDTA, Na_2, and an equal volume of 4 M LiCl, 2% mercapto-ethanol added. After standing at 0 C overnight, the precipitate was removed, and the supernatant was made to 0.1% with sodium sarcosyl-L (pH 8) (Geigy U.K. Ltd.). One microgram of ribonuclease (previously heated to 60 C for 10 min) was added. The solution was dialyzed against two changes of 0.5% sodium sarcosyl-L, 10 mM tris (pH 7.4), and 1% mercaptoethanol. Protein was stored in this condition at −15 C. In the experiments shown in Figures 2 and 3 the labeled protein was diluted with unlabeled carrier protein before storage.

Acrylamide Gel Electrophoretic Methods. The urea-acetic method was substantially the method described by Panyim and Chalkley (11), and electrophoresis was carried out for 4.5 hr at 2 ma/gel. Proteins in 0.5% sarcosyl-L were precipitated with 2 volumes of ethanol and dissolved in a small volume of 6 M urea-0.9 N acetic acid containing 10% sucrose and 1% mercaptoethanol before electrophoresis.

Sodium Lauryl Sulfate Method. Gels were prepared 7.5 cm in length with the acrylamide and bis-acrylamide being 15% and 0.4% respectively. The gel and electrophoresis buffer were 6 M urea, 0.1% SLS,[1] and 25 mM tris brought to a pH of 7.8 with glacial acetic acid. Samples for separation were precipitated from 0.5% sodium sarcosyl-L with 2 volumes of ethanol and dissolved in small volumes of 1% SLS, 6 M urea, 25 mM tris (pH 7.8), 10% sucrose, and 1% mercaptoethanol. Gels were pre-electrophoreted for about 15 min before protein samples were loaded. Electrophoresis was for 6 to 6.5 hr at 2.5 ma/gel.

Protein standards used for molecular weight determinations (17) were bovine serum albumin, ovalbumin, myoglobin, and cytochrome *c;* and these were dissolved in the 1% SLS buffer described above. Gels have been loaded with protein samples between 70 and 200 μg in different experiments except for the experiment shown in Figure 10, where the loadings were 275 μg protein/gel.

Gel Staining and Slicing. After electrophoresis the gels were removed from the tubes and stained in 0.025% Coomassie Blue in 50% methanol-7% glacial acetic acid (17). Gels were destained by washing in 5% methanol-5% glacial acetic acid, scanned at 575 nm, frozen, and sliced into 1-mm slices. After drying on paper strips, the slices were cut out and counted in a scintillation counter.

RESULTS

Figure 1 shows a typical sucrose density gradient profile of *Lemna* polysomes isolated at pH 8.5 and using 0.05 M KCl. The yield is about 65% polysomes, and this yield is reduced to about 50% if the extractions are carried out at pH 7.4 and the ribosomes pelleted through 20 mM KCl. For the purposes of preparing ribosomal proteins the fractions denoted as P (representing dimer to pentamer) and M (monomer) have been bulked. Other fractions have always been discarded.

RNA has been prepared from these polysome and monosome fractions and analyzed by gel electrophoresis in order to fully identify and determine the degree of purity of these ribosomes (results not published here). The major components were clearly from 80S ribosomes and some degradation of the 0.7×10^6 component was noted. Estimations of purity indicated that the polysomes contained about 3% 70S and the monosomes 5% 70S ribosomes, the remainder being 80S.

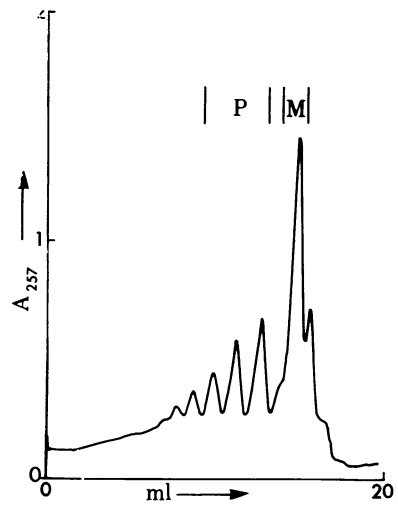

FIG. 1. Sucrose density gradient profile of polysomes isolated from *Lemna minor*. Polysomes were isolated from *Lemna minor* at pH 8.5 and 0.05 M KCl. They were carefully layered on a 20-ml 10 to 35% sucrose gradient and centrifuged at 10^5 *g* for 2.5 hr. Gradients were removed by pumping heavy sucrose into the bottom of the tube, and the A_{257} of the gradient was measured through a constant recording apparatus. Fractions between P and M were collected and precipitated with ethanol, and the RNA was prepared from the precipitate with phenol.

Gel Electrophoresis of Labeled Protein. In the experiment shown in Figure 2 *Lemna* was labeled for 24 hr with $^{32}P_i$, and the proteins were prepared from polysomes isolated at pH 7.4 and a salt concentration of 20 mM KCl. The labeled proteins have been separated by urea-acetic acid (Fig. 2a) and SLS gel methods (Fig. 2b). Chromoscan traces have been included for reference. In Figure 2c the labeled protein sample in 0.5% sarcosyl-L was treated for 1 hr with 1 μg of pronase at 25 C before separation as for Figure 2b.

The results show that in both separations (Fig. 2, a and b) 50% of the radioactivity is located in one component, the remaining radioactivity being distributed over a number of fractions. The pronase treatment in Figure 2c indicates the likelihood that the radioactivity detected in all fractions is attached to proteins.

In order to test the possibility that the labeled proteins shown in Figure 2, a and b, may not be ribosomal in origin but loosely associated contaminants, we have employed the method described by Kabat (4), which involves centrifuging ribosomes through 0.25 M KCl instead of 20 mM KCl. This procedure reduced the protein content of the ribosomes from about 45% protein to 39% protein. Figure 3, a and b, shows urea-acetic acid and SLS gel separations of labeled proteins from polysomes isolated in this way. Figure 3c shows SLS gel separation of four standard proteins: serum albumin, ovalbumin, myoglobin, and cytochrome *c*. The results of Figure 3, a and b, indicate, firstly, that the single major component seen in Figure 2 is still present under these more stringent conditions of isolation and, secondly, that the other labeled proteins seen in Figure 2 are now almost totally eliminated, the main component occupying over 80% of the total radioactivity on the gel. This suggests that these other labeled proteins may be loosely associated contaminants. Comparison of the data in Figures 3b and 3c gives an estimate of the molecular weight for the major component of 42,000. Four separate determinations of the molecular weight have given a similar value.

[1] Abbreviation: SLS: sodium lauryl sulfate.

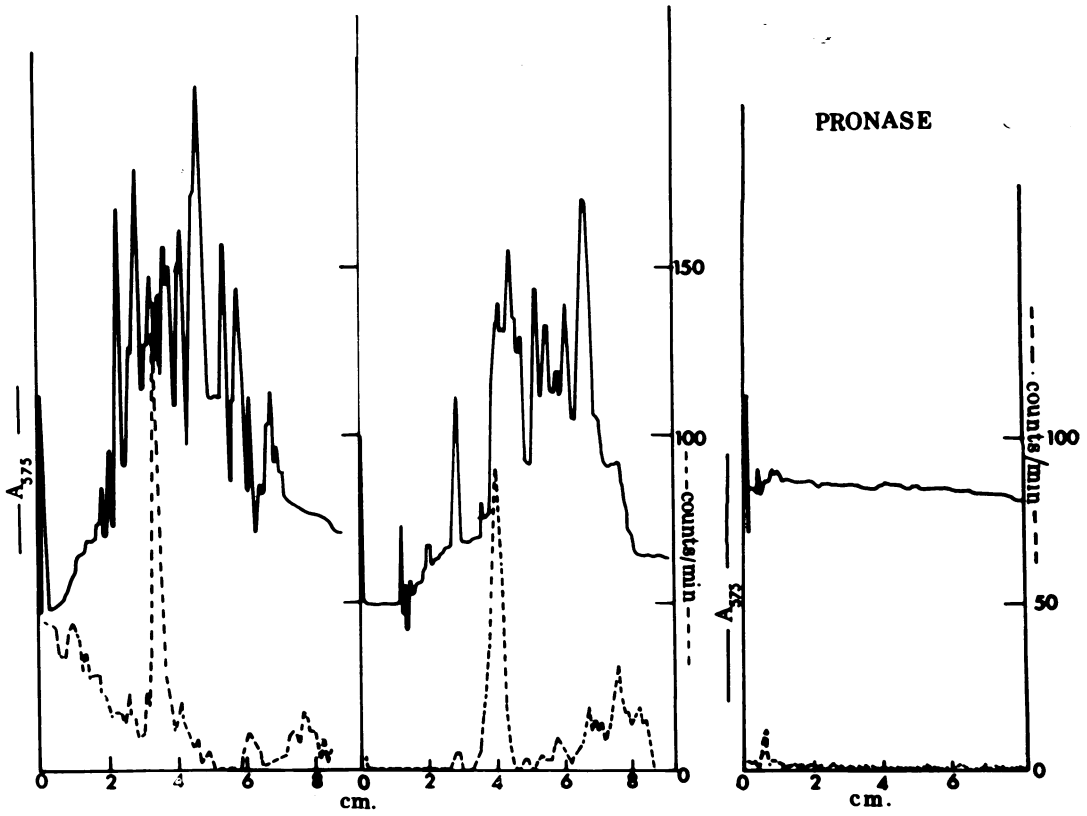

FIG. 2. Gel electrophoretic profiles of phosphorylated ribosomal proteins isolated from polysomes prepared at neutral pH and low salt. Plants were labeled for 24 hr on sucrose-mineral salts containing 50 μc/ml $^{32}P_i$. Polysomes were prepared at pH 7.4 and using 20 mM KCl. After separation on sucrose density gradients the fractions containing the monomer to the pentamer were collected, and the ribosomal proteins were prepared. After gel electrophoresis the gels were stained with Coomassie blue, destained, scanned at 575 nm, cut into 1 mm slices, and counted. a (left): Urea-acetic acid gel electrophoresis; b (center): SLS gel electrophoresis; c (right): SLS gel electrophoresis of protein treated with 1μg of pronase for 1 hr at 25 C before electrophoresis. —: A_{575}; - -: radioactivity.

Evidence for the Chemical Nature of the Major Phosphorylated Component. The data in Figures 2 and 3 would indicate that the detected $^{32}P_i$ may be attached to a macromolecule. There are three pieces of evidence which suggest that this macromolecule is a protein. Firstly, the molecule behaves as a cation at neutral pH levels in SLS and as an anion in dilute acetic acid and is attached to polysomes. Secondly, the molecule is stable to the action of RNase but unstable to the action of pronase. During the preparation of ribosomal protein, ribonuclease is added for a period of at least 8 hr under conditions in which it is still active. The evidence shown in Figure 2c indicates that pronase, however, is able to digest the radioactivity. Thirdly, the data in Figure 4 indicate that the $^{32}P_i$ is attached to a serine residue. Phosphorylated ribosomal protein was prepared from *Lemna* incubated for 24 hr in $^{32}P_i$. The labeled protein (~1 mg) was hydrolyzed in 6 N HCl, and the components were separated by paper electrophoresis (4). Standards containing P_i, serine phosphate, and threonine phosphate were run at the same time, and their position was revealed using phosphomolybdate spray. The electrophoresis strip was then cut into 0.5-cm sections and counted. The results are shown in Figure 4.

They clearly reveal the presence of labeled serine phosphate in the ribosomal protein despite the fact that serine phosphate is relatively unstable under the hydrolysis conditions used and considerable losses would have occurred (4). A trace of labeled threonine phosphate can also be seen to be present. The radioactivity present in the P_i peak has three probable sources: firstly, hydrolyzed serine phosphate; secondly, contaminating RNA in the preparation which under these conditions will be hydrolyzed to the component bases, ribose, and inorganic phosphate; and thirdly, contaminating $^{32}P_i$ itself.

Number of Labeled Phosphate Groups per Ribosome. The amount of any phosphate-containing compound in *Lemna* may be determined by growing *Lemna* for 4 to 5 generations on $^{32}P_i$, counting the required compound, and relating this radioactivity to the original specific radioactivity of the medium (2). To obtain continuous growth, however, on labeled media, low specific radioactivities have to be used, and this consequently lowers the radioactivity detectable in labeled compounds.

Accordingly, *Lemna* was grown for 12 days on sucrose-mineral salts containing 4 μc/ml $^{32}P_i$, the increase in fresh weight under these conditions being about 16-fold. The labeled ribosomal protein was prepared and electrophoreted by both urea-acetic acid and SLS methods. The results are shown in Figure 5.

Figure 5a shows that the label in the ribosomal protein is now distributed among five bands, the largest corresponding to that previously seen and shown in Figure 3. This main band now occupies only 50% of the radioactivity on the gel. Figure 5b, illustrating SLS gel electrophoresis, shows these other proteins to be of lower molecular weight than the main band.

From the total radioactivity in the main band and from the measured radioactivity of the RNA of the ribosomes from which the protein was prepared it can be calculated that there are about 100 pmoles of phosphate attached to this protein per μmole of RNA phosphate. Assuming that there are about 6000 nucleotides of RNA per ribosome, this indicates a value of 0.6

molecule of phosphate attached to this main band protein per ribosome. A second determination (see Fig. 10) has indicated a value of 0.75 molecule of protein phosphate per ribosome.

Ribosomal Location of Phosphorylated Protein. Kabat (4) was able to demonstrate that in reticulocytes one phosphorylated protein was associated with the production of inactive monomeric ribosomes. We have examined the ribosomal location of the phosphorylated protein in *Lemna*, firstly, to check the possibility of association with monomers, and secondly, in order to obtain some possible clue as to the function of this phosphorylation.

The data shown in Figure 6 are electrophoretic profiles of

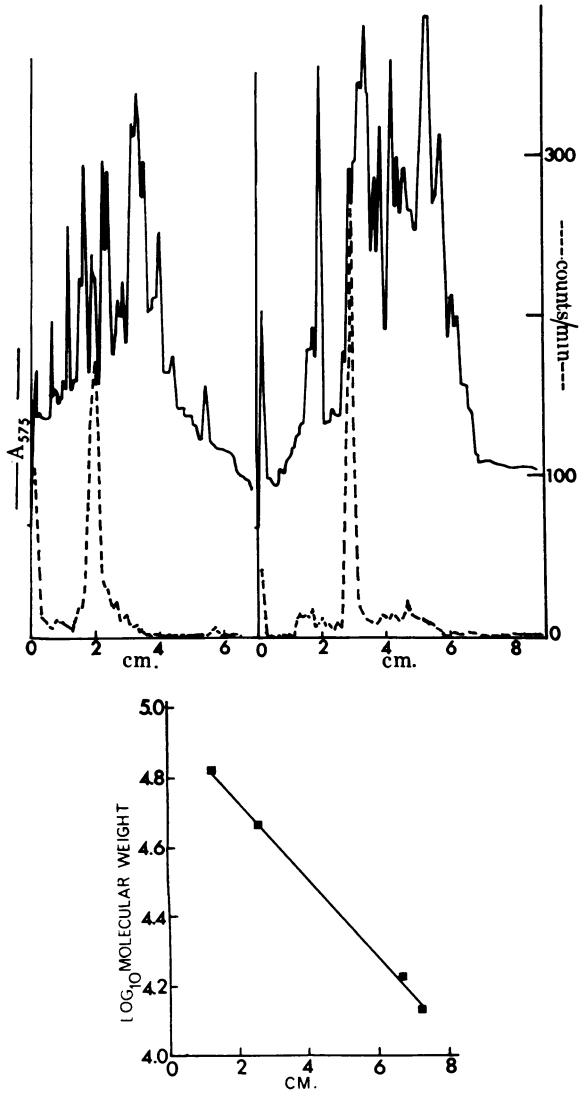

FIG. 3. Gel electrophoretic profiles of phosphorylated ribosomal proteins isolated from polysomes prepared at pH 8.5 and using high salt. Plants were labeled for 24 hr on sucrose-mineral salts containing 50 μc/ml $^{32}P_i$. Polysomes were prepared at pH 8.5 and using 0.25 M KCl. After separation on sucrose density gradients, fractions corresponding to the monomer to the pentamer were bulked, and the ribosomal proteins were prepared from these. After gel electrophoresis the gels were stained with Coomassie blue, destained, scanned at 575 nm, cut into 1-mm slices, and counted. a: Urea-acetic acid gel electrophoresis; b: SLS gel electrophoresis; —: A_{575}; – –: radioactivity; c: graph of $\log_{10}$ mol wt against mobility for serum albumin, ovalbumin, myoglobin, and cytochrome *c*. Electrophoresis conditions identical to that for part b.

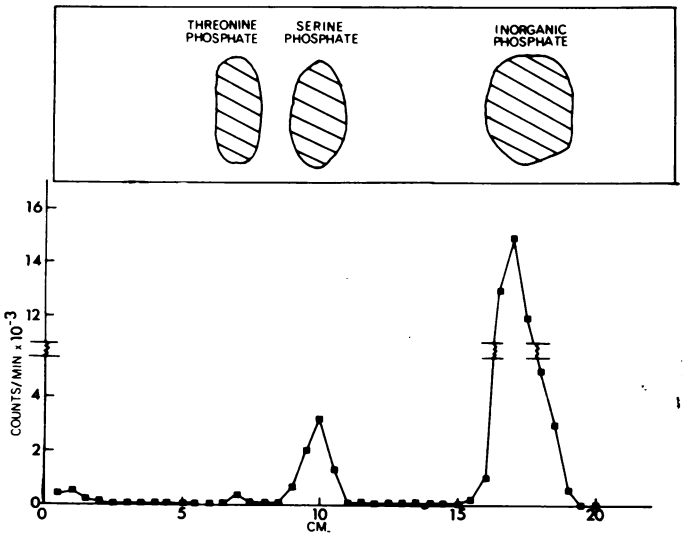

FIG. 4. Paper electrophoretic separation of acid hydrolysate of phosphorylated ribosomal protein. Phosphorylated ribosomal protein was prepared from *Lemna* labeled for 24 hr with 50 μc of $^{32}P_i$ sucrose-mineral salts. After ethanol precipitation the pellet (about 1 mg of protein) was hydrolyzed for 7 hr at 112 C in 6 N HCl. Paper electrophoresis was carried out at pH 1.8 at 40 v/cm for 2 hr (4). Markers of P_i, serine phosphate, and threonine phosphate were electrophoresed at the same time, and their mobilities were revealed with phosphomolybdate reagent. The sample strip was cut into 0.5-cm sections and counted.

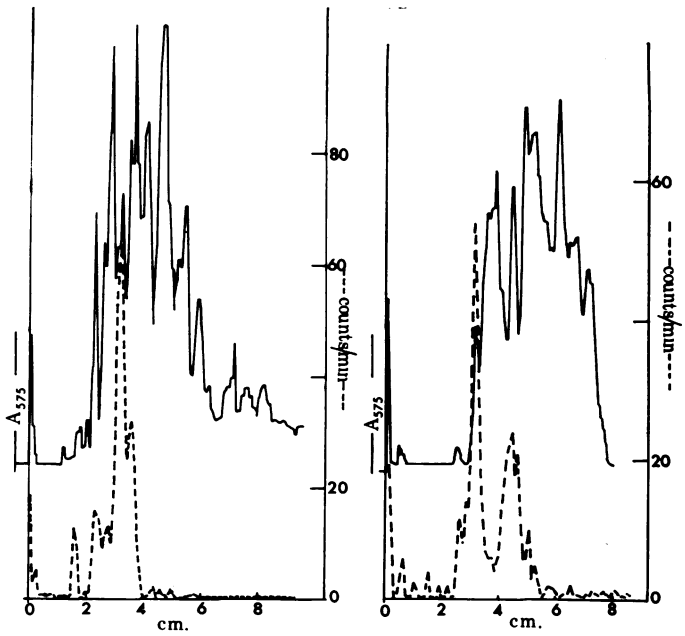

FIG. 5. The effect of labeling to equilibrium on the distribution of phosphate among radioactive proteins. *Lemna* was grown for 12 days on sucrose-mineral salts containing 4 μc/ml $^{32}P_i$. Labeled ribosomal protein was prepared and electrophoresed. Other conditions as under Figure 3. a (left): Urea-acetic acid separation of labeled protein; b (right): SLS gel electrophoresis of labeled protein; —: A_{575}; – –: radioactivity.

labeled protein obtained from the type of sucrose gradient shown in Figure 1. Polysomes (Fig. 6a) represent protein isolated from the dimer to pentamer region, and monosomes represent protein isolated from the monosome region of the same

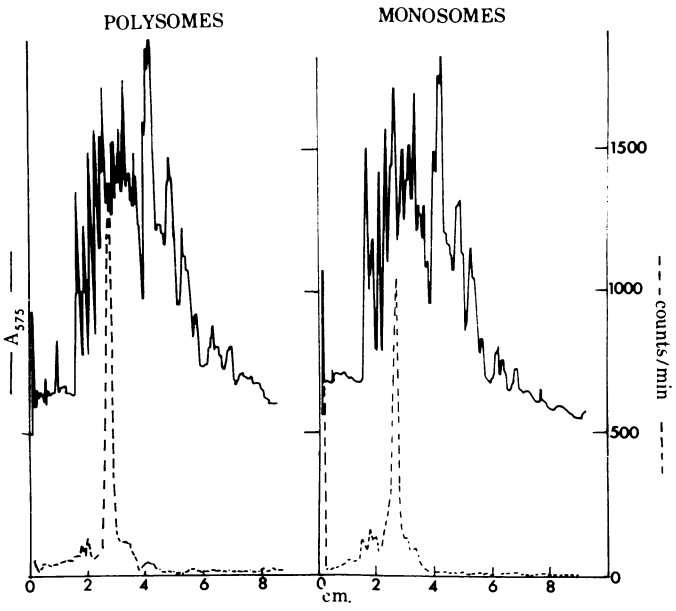

FIG. 6. Separation of labeled ribosomal protein isolated from polysomes and monosomes. Labeled polysomes were prepared and separated on sucrose gradients as shown in Figure 1. Fractions from the dimer to the pentamer and the monomer were collected separately as polysomes and monosomes, and the ribosomal proteins were isolated and separated by gel electrophoresis. a (left): Urea-acetic acid gel electrophoresis of polysomal protein; b (right): urea-acetic acid gel electrophoresis of monosomal protein; —: A_{575}; – –: radioactivity.

gradient. The phosphorylated protein is clearly detectable in both fractions and cannot therefore be associated with the production of inactive monomers.

The monosome fraction analyzed in Figure 6 will probably contain both inactive monomeric ribosomes and degraded polysomes. In an attempt to see if the phosphorylated protein is located only in polysomes, inactive monomers have been prepared from *Lemna*, ribosomal proteins have then been prepared, and the amount of label in the phosphorylated protein has been compared with that in ordinary polysomes. In the experiment shown in Figure 7, ribosomes were prepared from labeled *Lemna* gassed with nitrogen in the dark for 0, 1, and 2 hr. After separation on sucrose gradients the monomer region in *each* gradient was collected, and the monomer ribosomal proteins were prepared. In agreement with Lin and Key (8) the nitrogen treatment substantially increased monosome levels from 47% total ribosomes at zero time to 84% after 1 hr and to 85.5% after 2 hr. Figure 7, a to c, shows the electrophoretic profiles of the protein from the monomer fractions. Although the nitrogen treatment resulted in substantial increases in monosomes, this was not accompanied by a comparable change in the phosphate attached to protein. There is a reduction of about 30% in the phosphorus attached to protein after 2 hr of nitrogen treatment but some diminution would be expected because of the probable reduction in phosphorylation rates in the dark and the possible continuation of turnover of this phosphate. For further information we have also attempted to locate in which of the two ribosomal subunits the protein is located. This proved to be somewhat more difficult than anticipated. Attempts to dissociate ribosomes in EDTA resulted in complete breakdown of the subunits. Treatment of inactive monomeric ribosomes with 0.5 M KCl resulted in the detection of two subunits on sucrose gradients (8), but considerable quantities of labeled material were left on the gradient origin. Subsequent analysis of these salt-produced subunits indicated total loss of the phosphorylated protein. The method finally adopted was to treat inactive monomeric ribosomes with low salt concentrations (0.05 M KCl) in the presence of very low magnesium levels (0.05 mM $MgCl_2$) (8). Figure 8, a to d, shows urea-acetic acid and SLS gel electrophoretic profiles of phosphorylated protein from small and large subunits, respectively. These data clearly locate the main phosphorylated protein as being present in the small subunit and thus indicate its probable functions with known functions of this subunit. The profiles of proteins from the large subunit show that in the SLS gel separations a small peak of radioactivity runs in the same position as the main phosphorylated component in the small subunit. In urea-acetic acid, however, this small peak runs 0.4 cm faster than the main peak of radioactivity seen in the small subunit profiles, and thus the origin of this large subunit component is unlikely to be cross contamination between the two subunits.

The Effect of Hormones on Phosphorylation of Ribosomal Protein. The growth of *Lemna* is subject to the action of two plant growth hormones, cytokinins and ABA (16). We have tested the possibility that either of these two hormones may alter the degree of phosphorylation of ribosomal protein.

In the experiment shown in Figure 9 *Lemna* was grown for 5 hr in the presence of $^{32}P_i$ and in the presence and absence of ABA (5 μM). The ribosomal protein was isolated and electrophoreted, and the radioactivity profiles are shown in Figure 9. The results show that no new phosphorylated proteins appear as a result of abscisic acid treatment. They do also indicate that there is a reduction of about 30% in the label attached to protein as a result of the ABA treatment. Under the same conditions there is a reduction of about 12% in the specific activity of the RNA although the ribosomal profiles indicated no apparent change in the level of the polysomes.

This has been further developed in two ways. Firstly, the experiment cited above has been repeated but with the difference that *Lemna* was labeled for 20 hr and ABA added for the last 4 hr. The results were very similar to those shown above with a slight reduction (about 25%) in labeling of the phosphorylated protein. To analyze longer term effects of

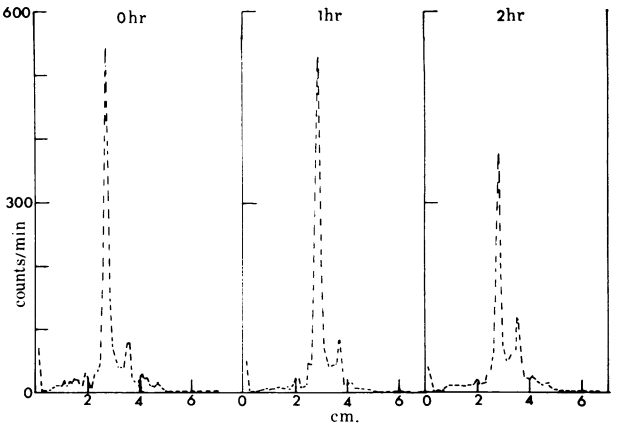

FIG. 7. Separation of labeled ribosomal protein isolated from derived monomers. *Lemna* was labeled for 24 hr with $^{32}P_i$ and divided into three. One part was gassed with nitrogen in the dark for 2 hr, one part for 1 hr, and the remainder used as a zero time control. Ribosomes were isolated at pH 7.4 in 20 mM KCl on sucrose gradients. The monomer fractions only in all gradients were collected, and the proteins were prepared and separated by urea-acetic acid gel electrophoresis.

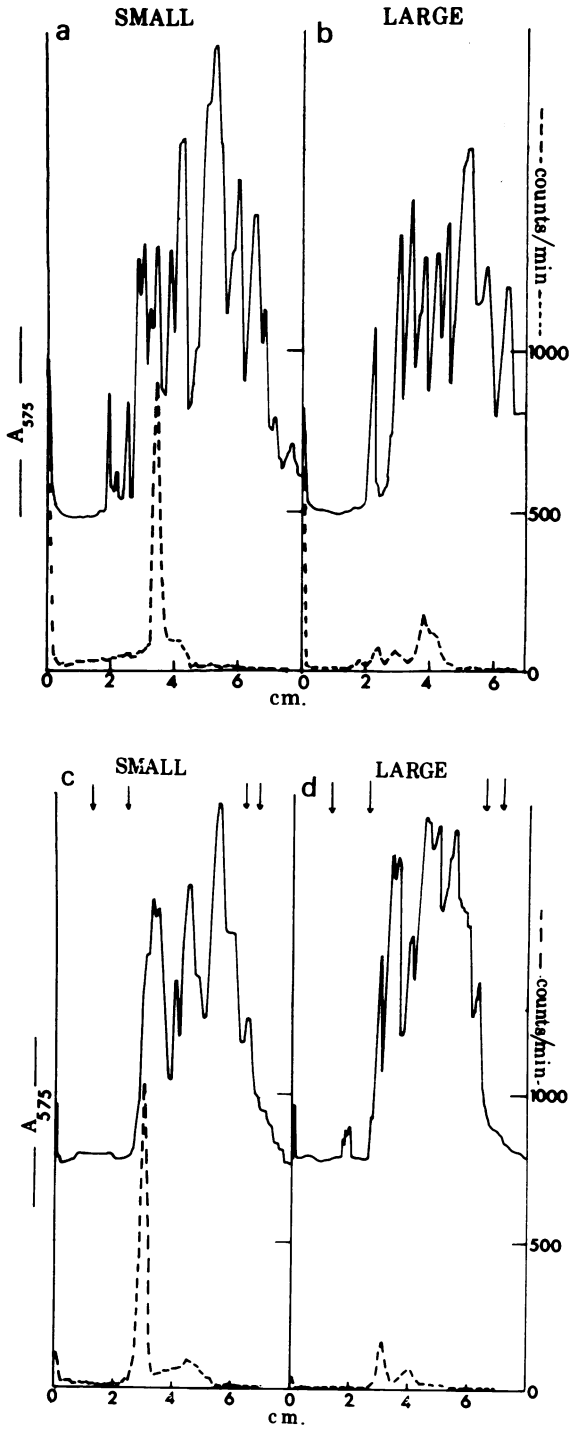

FIG. 8. Gel electrophoretic separations of proteins isolated from ribosomal subunits. *Lemna* was labeled for 24 hr with $^{32}P_i$ and then treated for 1 hr in the dark with nitrogen. Monomeric ribosomes were isolated at pH 7.4 in 20 mM KCl and suspended in 0.05 M KCl, 0.05 mM $MgCl_2$, and 10 mM tris, pH 7.4. The resulting subunits were isolated on sucrose density gradients. Fractions containing small and large subunits were collected. Proteins were prepared from these fractions and separated by SLS and urea-acetic acid gel electrophoresis. a. and b: Urea-acetic acid gel electrophoresis of small and large subunit proteins, respectively; c. and d: SLS gel electrophoresis of small and large subunit proteins, respectively. Arrows indicate the positions of bovine serum albumin, ovalbumin, myoglobin, and cytochrome *c* run under identical conditions. —: A_{575}; – –: radioactivity.

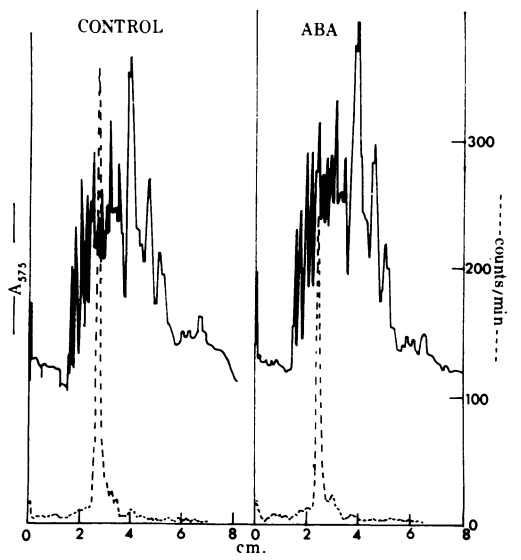

FIG. 9. Short term effect of ABA on the accumulation of $^{32}P_i$ in ribosomal protein. *Lemna* was labeled for 5 hr with 50 μc/ml $^{32}P_i$ in the presence and absence of 5 μM ABA. Ribosomes were prepared and separated on sucrose density gradients. Fractions containing the monomer to pentamer were isolated, and the proteins were prepared from these and separated by gel electrophoresis using the urea-acetic acid method. —: A_{575}; – –: radioactivity.

ABA, *Lemna* was labeled for 12 days with $^{32}P_i$, and ABA was added for the last 24 hr to one half. Sucrose density gradient profiles of polysomes (results not shown here) indicated that this treatment reduced polysome levels 2.5-fold with a consequent increase in the monomer. Proteins were isolated from the monomeric region of both gradients and separated using urea-acetic acid. The results are shown in Figure 10, a and b.

Under these conditions *Lemna* has been labeled to equilibrium, and the radioactivity on the protein represents levels of phosphate attached to the protein. From the control values (Fig. 10a) it can be calculated (as described for Fig. 5) that in this experiment there are 0.75 atom of protein phosphorus in the main band per ribosome. Other minor phosphorylated components can again be seen, as shown in Figure 5, and again occupying about 50% of the total activity on the gel. The effect of ABA is to reduce strongly the total phosphorus attached to ribosomal proteins. For example, there is 0.36 atom of protein phosphorus in the main band per ribosome instead of 0.75 atom for the control. Other differences can be seen in the minor components; again, for example, one component (running just ahead of the main band) appears to have been eliminated altogether. Despite these differences in the protein phosphorus, the total ribosomes extracted and the specific activity of the RNA were unaltered by the treatment with ABA.

Two experiments have been carried out in which *Lemna* has been treated for 24 hr with $^{32}P_i$ in the presence and absence of 5 μM benzyladenine. Gel electrophoresis of the ribosomal protein failed to reveal any differences in the profile or in the degree of labeling.

DISCUSSION

The evidence in this paper may be construed as supporting the notion that a sterile higher plant, *Lemna minor*, has at least one ribosomal protein which is phosphorylated. The term ribosomal in this context is purely operational because of the difficulties which attend the demonstration that a protein is

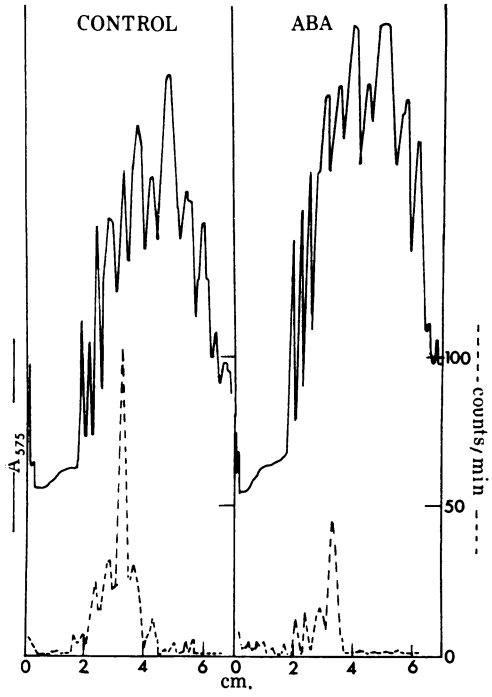

FIG. 10. Long term effect of ABA on the level of phosphorus attached to ribosomal protein. *Lemna* was labeled for 11 days on sucrose-mineral salts containing 4 μc/ml $^{32}P_i$ and then divided into two lots. These lots were then grown for a further day on $^{31}P_i$ in the presence and absence of 5 μM ABA. Polysomes were isolated on sucrose density gradients, and ribosomal proteins were prepared from the monomeric region of each gradient only. Proteins were separated by urea-acetic acid gel electrophoresis, stained, scanned at 575 nm, and sliced. Total RNA extracted as ribosomes in control sample = 297 μg and in ABA sample = 280 μg and the specific radioactivity of the ribosomal RNA for control = 7.8×10^{-3} counts/min·μg RNA and for ABA sample = 7.7×10^{-3} counts/min·μg RNA. (left): Gel electrophoresis of control sample; b (right): gel electrophoresis of ABA-treated sample; —: A_{575}; – –: radioactivity.

ribosomal from the functional standpoint. This one phosphorylated protein (which accounts for over 80% of the protein phosphorus after 24 hr of labeling) has a molecular weight of about 42,000 and is almost the largest protein in *Lemna* ribosomes.

Our results do not give any immediate indication of the possible function of this protein-attached phosphate. Its continued presence in both polysomes and derived inactive monomers would seem to argue against notions of a direct role in the mechanism of protein synthesis or attachment of messenger to ribosomes. On the other hand, the fact that the phosphorylated protein is located in the small ribosomal subunit would ally the function of this protein with the known functions of this subunit.

Estimates of the amount of phosphorus attached to this protein indicate values substantially less than 1 atom of protein phosphorus per ribosome. There are a number of possible explanations for this, including (*a*) there are losses of either protein or phosphorus during the isolation procedure, (*b*) only 60 to 70% of the ribosomes at any one time have this protein phosphorylated, or (*c*) the phosphorylated protein is only attached to the ribosome some 60 to 70% of the time. The evidence we have at the present time does not distinguish among all these possibilities, but we do have some preliminary evidence that the protein phosphorus is relatively stable in crude homogenates, indicating the two latter explanations as better possibilities.

One interesting feature appears when the results of Figures 5, 6, and 10 are compared, representing, respectively, 12, 1 and 0.21 days of labeling with $^{32}P_i$. The proportions of label in the main band of protein phosphorus to the total activity on the gel are, for three time periods, 50, 80, and 90%. One possible interpretation of this evidence is that the phosphorus in the major labeled protein shows a more rapid rate of turnover than the other residual proteins on the gel. The results in Figure 7 showed that after 2 hr of nitrogen treatment there was approximately a drop of 30% in the protein phosphate which may be representative of turnover of this phosphate.

The main reasons for the work described in this paper are the data shown in Figures 9 and 10. These results do show that ABA does alter the amount of phosphate attached to ribosomal protein (a 2-fold reduction after 24 hr of treatment), but this effect is slow to operate, being only just detectable after 5 hr of treatment with ABA. The effects of animal hormones on protein kinases are usually very quick and extremely large (1), and it would certainly be difficult to construe the evidence shown in Figures 9 and 10 as indicating an effect of ABA on a protein kinase in a similar manner. Since the effect of ABA on *Lemna* is to inhibit growth, it can also be argued that rates of phosphorylation are likely to be reduced substantially, and thus continuing turnover would lower the level of phosphate attached to the ribosomal protein. One measurement which is important in this argument is the lag period before ABA causes growth inhibition. One difficulty in determining this parameter is that it is very difficult to determine the growth of *Lemna* over very short time periods. We have carried out a number of experiments in recent years in which the effects of ABA on growth (measured as frond number and fresh weight) have been determined over 7 to 8 days (16). If the growth curves are plotted as $\log_{10}$ fresh weight or frond number, a satisfactory straight line is obtained which may then be extrapolated back until it hits the control curve (drawn in the same way). From this an apparent lag time before growth inhibition commences may be determined. Various experiments have given values between 12 and 16 hr for this lag time. The results of Figure 9 indicate that the reduction in protein phosphate is detectable after 5 hr and increases until at least a 2-fold reduction occurs as measured at 24 hr. This event may thus start in the lag period of growth inhibition and may have some significance in understanding the growth inhibition of *Lemna* induced by ABA.

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LITERATURE CITED

1. BALHORN, R., W. O. RIEKE, AND R. CHALKLEY. 1971. A reinvestigation of phosphorylation of lysine-rich histones during rat liver regeneration. Biochemistry 10: 3952–3958.
2. BIELESKI, R. L. 1968. Levels of phosphate esters in *Spirodela*. Plant Physiol. 43: 1297–1308.
3. GALSKY, A. G. AND J. A. LIPPINCOTT. 1969. Promotion and inhibition of amylase production in barley endosperm by cyclic AMP and ADP. Plant Cell Physiol. 10: 607–620.
4. KABAT, D. 1970. Phosphorylation of ribosomal proteins in rabbit reticulocytes. Characterization and regulatory aspects. Biochemistry 9: 4160–4174.
5. KAMISAKA, S., H. SANO, M. KATSUMI, AND Y. MASUDA. 1972. Effects of cyclic AMP and gibberellic acid on lettuce hypocotyl elongation and mechanical properties of its cell wall. Plant Cell Physiol. 13: 167–173.
6. KAMIYAMA, M. AND B. DASTUGUE. 1972. Rat liver non-histone proteins: correlation between protein kinase activity and activation of protein synthesis. Biochem. Biophys. Res. Commun. 44: 29–36.

7. Kuo, J. F. and P. Greengard. 1969. Cyclic nucleotide-dependent protein kinases. Proc. Nat. Acad. Sci. U.S.A. 64: 1349–1355.
8. Lin, C. Y. and J. L. Key. 1971. Dissociation of N_2 gas-induced monomeric ribosomes and functioning of derived sub-units in protein synthesis in pea. Plant Physiol. 48: 547–552.
9. Monier, D., K. Santhanam, and S. R. Wagle. 1972. Studies on the inhibition of amino acid incorporation into protein by isolated ribosomes by protein kinases. Biochem. Biophys. Res. Cmmun. 46: 1881–1886.
10. Narayanan, A., J. Vermerson, and A. Pradet. 1970. Dosage enzymatique de l'acide adenosine 3′5′ monophosphate cyclique dans les semences de laitue. C. R. Acad. Sci. Paris 271: 2404–2407.
11. Panyim, S. and R. Chalkley. 1969. High resolution acrylamide gel electrophoresis of histones. Arch. Biochem. Biophys. 130: 337–346.
12. Pollard, C. J. 1970. Influence of gibberellic acid on the incorporation of 8-^{14}C-adenine into cyclic AMP in barley aleurone layers. Biochim. Biophys. Acta 201: 511–512.
13. Robinson, G. A., R. W. Butcher, and E. W. Sutherland. 1968. Cyclic AMP. Annu. Rev. Biochem. 37: 149–174.
14. Shlatz, L. and G. V. Marinetti. 1971. Protein kinase mediated phosphorylation of the rat liver plasma membrane. Biochem. Biophys. Res. Commun. 45: 51–56.
15. Teng, C. S., C. T. Teng, and V. G. Allfrey. 1971. Studies on nuclear acidic proteins. J. Biol. Chem. 246: 3597–3609.
16. Trewavas, A. 1970. The turnover of nucleic acids in *Lemna minor*. Plant Physiol. 45: 742–751.
17. Weber, K. and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulphate polyacrylamide gel electrophoresis. J. Biol. Chem. 244: 4406–4412.
18. Wood, H. M., M. C. Lin, and A. C. Braun. 1972. The inhibition of plant and animal 3′5′ cyclic monophosphate phosphodiesterases by a cell division promoting substance from tissues of higher plant species. Proc. Nat. Acad. Sci. U.S.A. 69: 403–406.