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Polyamine Binding to Proteins in Oat and *Petunia* Protoplasts[1]

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ABSTRACT

Previous work (A Apelbaum *et al.* [1988] Plant Physiol 88: 996–998) has demonstrated binding of labeled spermidine (Spd) to a developmentally regulated 18 kilodalton protein in tobacco tissue cultures derived from thin surface layer explants. To assess the general importance of such Spd-protein complexes, we attempted bulk isolation from protoplasts of *Petunia* and oat (*Avena sativa*). In *Petunia*, as in tobacco, fed radioactive Spd is bound to protein, but in oat, Spd is first converted to 1,3,-diaminopropane (DAP), probably by polyamine oxidase action. In oat, binding of DAP to protein depends on age of donor leaf and conditions of illumination and temperature, and the extraction of the DAP-protein complex depends upon buffer and pH. The yield of the DAP-protein complex was maximized by extraction of frozen-thawed protoplasts with a pH 8.8 carbonate buffer containing SDS. Its molecular size, based on Sephacryl column fractionation of ammonium sulfate precipitated material, exceeded 45 kilodaltons. Bound Spd or DAP can be released from their complexes by the action of Pronase, but not DNAse, RNAse, or strong salt solutions, indicating covalent attachment to protein.

Considerable evidence indicates that PAs[3] can regulate such basic processes in plants as cell division, morphogenesis, senescence, and response to stress (21). One mechanism by which such regulation might be achieved involves PA binding to specific regulatory proteins, which have been found or suspected in human lymphocytes (16), human plasma (18), chick intestine (15), sea urchin embryos (6), bovine testis (23), hamster ovary cells (4), slime mold (13), apple pollen (3), Jerusalem artichoke tubers (19), soybean cells in culture (12), and tobacco tissue culture (2). Evidence for the occurrence of transglutaminase, an enzyme capable of binding polyamines covalently to proteins, was recently found in plants (9), thus providing a possible mechanism for formation of PA-protein complexes. In our previous work with tobacco, we found it impractical to isolate large quantities of labeled protein from tissue cultures derived from thin layer explants. Thus, for large scale preparation and study of Spd-binding proteins, we have turned to oat and *Petunia* mesophyll protoplasts, whose senescence we previously found to be retarded by exogenously applied PAs (1, 20). These protoplasts can readily be isolated in large quantities, easily labeled with radioactive PAs, and conveniently solubilized for protein extraction. We reasoned that the detection of radioactive proteins after exposure of protoplasts to radioactive Spd would be an indication of Spd binding that could be substantiated by reisolation from the protein of Spd or one of its metabolites.

MATERIALS AND METHODS

Plant Material

Avena sativa cv Victory and *Petunia hybrida* cv White Joy were grown under an 18 h photoperiod with a 9:1 energy mix of fluorescent to incandescent light, at about 17.6 W m^{-2}. Oat leaves from 7 or 11 d plants were cut about 1 cm from the tip to a length of 4.5 cm and were peeled and floated on a 4 mM phosphate buffer solution at pH 5.8. Protoplasts of oats (8) were prepared by incubating peeled leaf sections at 30°C for 2 h in 0.5 M mannitol containing 0.5% Cellulysin (Calbiochem) similarly phosphate buffered at pH 5.8. They were then washed twice in the same solution minus the enzyme. Protoplasts of *Petunia* (6 weeks old) were prepared by lightly scraping the underside of the leaves with the bottom of a curved forceps and incubating them at 30°C for 3 h in 0.5 M mannitol (pH 5.8) containing 0.24% Cellulysin and 0.12% Driselase (Kyowa Hakko Kogyo-Japan). They were washed twice in a 3 mM calcium nitrate solution containing 0.5 M mannitol and 0.1 M sucrose at pH 5.8. All subsequent incubations of the oat and *Petunia* protoplasts were in their washing solutions.

Optimization of Labeling

Typically, protoplasts from about 22 peeled oat leaves, 7 or 11 d old, or seven scraped *Petunia* leaves, 6 weeks old, each in a Petri dish with 10 mL of their washing solution, were labeled with 40 μL of [^{3}H]Spd trihydrochloride (New England Nuclear), specific activity >30 Ci/mmol, 1 mCi/mL. To determine optimal conditions for labeling, protoplasts from 7 or 11 d oat leaves were incubated in the dark or light for 2, 4, or 6 h and for 4 h at temperatures ranging from 4 to 35°C. To estimate injury to protoplasts during isolation, 7 d old peeled oat leaves were labeled for 4 h in the dark and then compared to similarly labeled protoplasts isolated from the same age and number of peeled leaves. To determine if there was an artifact in labeling due to the replacement of [^{3}H] by

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[3] Abbreviations: PA, polyamine; DAP, 1,3-diaminopropane; βHEH, hydroxyethylhydrazine; Put, putrescine; Spd, spermidine; Spm, spermine.

[^{1}H] and to compare binding of ^{14}C-labeled and ^{3}H-labeled Spd, oat protoplasts (11 d leaves) were also labeled for 4 h in the dark with 120 μL of [^{14}C]Spd trihydrochloride (Amersham), specific activity >120 mCi/mmol, 50 μCi/mL. In subsequent experiments, protoplasts were labeled for 4 h at 24°C in the dark unless otherwise indicated.

Protein Extraction and Measurement

The proteins of oat protoplasts (11 d leaves) were extracted by either 0.1 M Tris, phosphate, or sodium carbonate buffers at pHs ranging from 4.8 to 10.6, and containing either 1% SDS or 1% Triton X-100 in addition to 0.7% DTT (to inhibit phenol oxidation), 0.001% leupeptin (to inhibit proteases), and 0.04% protamine sulfate (to precipitate nucleic acids). Typically, the protoplasts from 1 Petri dish of oats or *Petunia* were ground in an ice-chilled glass-on-glass homogenizer in 0.7 mL of a suitable protein extraction buffer. The homogenate was centrifuged at 13,000 *g* for 10 min, and the remaining pellet was reextracted in 0.3 mL of buffer and recentrifuged. Each supernatant fraction was therefore combined with the previous one and the pellet discarded. To test for the best means of mechanical disruption, oat protoplasts (11 d leaves) in a protein extraction buffer were either (a) sonicated for 90 s, then frozen at −70°C and thawed, (b) ground in a glass homogenizer, then frozen and thawed, or (c) frozen, thawed, and then ground in a glass homogenizer. All three treatments were followed by centrifugation as above. When oat leaves were used in experiments, they were first cut into small segments with a razor blade and ground with pieces of dry ice in a mortar and pestle. Extraction buffer was added, followed by glass-on-glass homogenization over ice.

Radioactive Spd bound to protein was determined by a filter paper disc method involving TCA precipitation of protein, followed by ethanol and acetone washes in place of the last ether washes called for in the original method (14). We applied 100 μL of extract per disc, then scintillation-counted the dried discs in 12 mL of OptiFluor. Blank discs with 100 μL of an appropriate solution as a control were also subjected to the same treatment and their counts subtracted from the other discs. Total protein was measured by the Bradford method (5), on aliquots taken either from the protoplast homogenates or from dried discs treated overnight with 750 μL per disc of 80% formic acid (14) to elute all proteins. For specific activity calculations, 100 μL of this aliquot was added to 12 mL of OptiFluor.

In subsequent experiments, all protein extractions were performed after glass-on-glass homogenization of frozen-thawed material.

Nature of the Binding Reaction

To determine whether the label could be easily removed from the protein, aliquots of oat protoplast homogenates (11 d leaves) on the discs were washed with 1 and 5 M NaCl for 30 min before and after TCA treatment. To establish whether the label was merely adsorbed to the TCA insoluble material or required some metabolic reaction for binding, similar extracts of freshly prepared unlabeled oat protoplasts (11 d leaves) were exposed to [^{3}H]Spd either before or after TCA treatment. To test further the necessity of protoplast viability for the binding reaction, oat protoplasts (11 d leaves) were either boiled (see below) or were aged at 5°C for 19 h after isolation. This latter procedure has been shown to diminish macromolecular synthetic activity in protoplasts (8). The aged protoplasts were then brought to room temperature, labeled with Spd for 4 h, and counted as usual for radioactivity in the TCA precipitate.

To determine the nature of the TCA insoluble material bound to the radioactive label, discs containing 100 μL aliquots of the homogenized labeled oat protoplasts (11 d leaves) were treated at 37°C with either 50 μg/mL Pronase (100 mM Tris, pH 8.0) for 1 or 18 h, 50 μg/mL RNAse (100 mM acetate, pH 5.5) for 1 h, or 50 μg/mL DNAse (25 mM Tris plus 5 mM $MgCl_2$, pH 7.0) for 1 h (10). The discs containing the enzyme-treated aliquots were then processed with TCA as before.

Metabolism of Spermidine

Once labeled proteins were found, we sought to determine whether the bound moiety was Spd or one of its metabolites. Using a TLC method for PA separation and identification (7), the [^{3}H]Spd used was first analyzed to verify that only Spd was present. Then the extracted labeled proteins from the oat (11 d leaves) and *Petunia* (6 week leaves) protoplasts were similarly analyzed. The protein extract was treated with ice-cold, 20% TCA for 1 h and centrifuged at 2000 *g* for 10 min, the TCA soluble supernatant fraction being kept for analysis later. The pellet, called the TCA-insoluble fraction, was washed several times with 5% TCA and then left overnight in 5% TCA. More TCA washes followed until fewer than 50 cpm/mL were recorded in the washing volume. Ethanol and acetone washes followed until counts reached background levels. The washed pellet was solubilized overnight in 1 N NaOH, hydrolyzed at 110°C for 24 h in sealed ampoules containing an equal volume of 12 N HCl to release bound PAs, then analyzed by TLC (7). In addition, the supernatant incubation media left after the labeled protoplasts were first spun down in the washing procedure were also analyzed. For analysis, dansylated samples from each fraction and treatment were separated on TLC plates (Whatman LK6D silica gel) with a cyclohexane:ethylacetate (5:4, v/v) solvent system (system 1). For the better separation of DAP from putrescine, a chloroform:triethylamine (25:2, v/v) solvent system (system 2) was used (7). After development and comparison with standards, each plate was divided into about 20 fluorescent bands which were scraped off and counted in 12 mL of OptiFluor.

If the labeled Spd were converted to another polyamine, an enzymatic process would probably be involved. Heat killing the protoplasts should prevent such enzymatic conversion and could also establish whether living cells are necessary for Spd binding. With this in mind, oat protoplasts (11 d leaves) were placed in a boiling water bath (reaching 97°C) for 10 min (which congealed them into small individual spheres), then exposed to labeled Spd and subsequently treated as though they were still viable. They were extracted in SDS carbonate buffer at pH 8.8, with or without 100 mM Spd in the TCA treatment through the ethanol washes. A viable,

unboiled group of protoplasts obtained from the same age leaves was also extracted for protein in the presence or absence of 100 mM DAP during the same treatments. Finally, to block the conversion of Spd to DAP (22), viable oat protoplasts (11 d leaves) were labeled in the presence of 1 mM βHEH, an inhibitor of polyamine oxidase (11), the only enzyme in plants known to convert Spd to DAP.

To determine the specificity of Spd uptake and binding in the presence of related compounds, appropriate competition experiments were performed. A variety of unlabeled PAs and amino acids, all at 0.01 mM, were added one at a time to the oat protoplasts (11 d leaves) at the time of labeling; these included spermidine trihydrochloride, spermine tetrahydrochloride, putrescine dihydrochloride, DAP, lysine monohydrochloride, and histidine monohydrochloride.

Protein Characterization

Proteins extracted from labeled oat protoplasts (11 d leaves) were fractionally precipitated by the stepwise addition of saturated ammonium sulfate to produce cuts of 25 and 62% saturation, and the specific activity of all fractions was determined. After centrifugation and washing, each precipitate was resolubilized in extraction buffer. The combined protein fractions were applied to a Sephacryl 200 HR column (2.5 cm i.d., 50 cm length), eluted with the SDS extraction buffer, with fractions collected at 5 min intervals for radioactivity counting and protein measurement by the Bradford method (5). An identical column containing only colored mol wt markers (turkey albumin 18 kD and horse myoglobin 45 kD) was run for protein sizing, as the green color of the protoplast protein fraction obscured their visualization when run together with the plant samples.

Each experiment was repeated at least once, and the results given are the average of the multiple experiments. Large differences in cpm/disc between essentially similar treatments reflect either more protoplasts used, more label added, or both. In addition, we noted that extraction of protein on discs with formic acid yields 6 to 7 times higher cpm/disc than the discs processed through the TCA treatment alone because, unlike the original report (14), disc quenching is eliminated.

RESULTS

Effect of Source of Cells, Extraction Buffer, and Incubation Conditions on Labeling.

Oat protoplasts incubated with ^{3}H-labeled Spd and extracted with Triton-phosphate buffer of pH 5.8 showed a linear labeling rate for the TCA precipitate over a 6 h period. In this respect, oat protoplasts were superior to peeled oat leaf segments. When roughly identical numbers of cells were extracted with Triton-phosphate buffer (pH 5.8), protoplasts yielded 487 cpm/disc compared with 148 cpm/disc for leaf segments. Part or all of this difference could be due to the more rapid penetration of labeled Spd into protoplasts, both because of the greater surface area exposed and the increased permeability of protoplast membranes. *Petunia* protoplasts were in turn superior to oat protoplasts, yielding 14,442 cpm/disc compared with 9,521 cpm/disc when extracted with SDS-carbonate buffer of pH 8.8.

Protoplasts from 11 d old oat leaves were about 50% better than 7 d old leaves with respect to binding of label from Spd. Also, dark incubation was superior to incubation in white light of the growth room for both ages. Light inhibited the uptake of label by 45% and binding to TCA-precipitable material by 22% in the 11 d old leaves. In the 7 d old leaves, the figures were 31 and 15%, respectively.

Binding of label was markedly temperature dependent, rising between 4 and 23°C, then declining between 23 and 35°C. The Q_{10} for binding (pH 8.8 Triton-carbonate extraction) is 1.6 between 4 and 11 degrees and 1.4 between 11 and 23°C.

Extraction

Freezing, then grinding oat protoplasts yielded somewhat higher TCA-insoluble cpm/disc than either sonication or grinding before freezing when extractions were by a pH 5.8 Triton phosphate buffer.

In a systematic study of the effect of pH and chemical nature of the buffer, oat protoplasts were labeled, then homogenized with phosphate, Tris, or carbonate buffers containing Triton over the pH range 4.8 to 10.6. The carbonate buffers at pH 8.8 and 9.7 yielded the highest counts; we settled on 8.8 as our standard since pH 9.7 could interfere with subsequent column separation and future electrophoretic procedures. The use of these alkaline buffers lessened the danger that binding of label would result merely from the attachment of highly protonated PAs to negative groups on protein.

Many more counts (10910 cpm/disc) were obtained from the oat protoplasts with the SDS protein extraction buffer than with the Triton buffer (1610 cpm/disc).

Release of Label from the TCA-Insoluble Material by Various Agents

Using the Triton-Tris extraction buffer at pH 8.8 on oat protoplasts, we found that 1 and 5 M NaCl, applied either before or after TCA treatment, did not dislodge the label from the discs, indicating that the retention of counts was not due to simple cation adsorption. The counts are higher with more salt as the high salt concentration decreases sample elution during the extraction. Of the enzymes used, only Pronase was effective in reducing radioactivity bound to the TCA insoluble material; it caused a reduction of 38% in cpm/disc, while DNAse produced no change and RNAse a paradoxical 25% gain, possibly due to its binding of the TCA precipitable material to the filter-paper disc matrix. These results legitimized the use of the term 'protein' to designate the macromolecule to which counts were attached.

Protein extracted from unlabeled oat protoplasts with Triton-Tris buffer (pH 8.8) failed to bind counts when exposed to radioactive Spd while on the discs, either before or after TCA treatment. This shows that attachment of label to protein requires the intervention of some active metabolic step.

Aged oat protoplasts, exposed to [^{3}H]Spd 19 h after initial separation from the leaf, were extracted with Triton carbonate buffer (pH 8.8). They produced a lesser binding of polyamine to protein than did fresh protoplasts (1315 cpm/disc for aged protoplasts *versus* 2718 cpm/disc for fresh protoplasts). This

mirrors the decreased rate of macromolecular synthesis previously found in aged oat protoplasts (8).

Spermidine Metabolism

An analysis by TLC of the tritiated Spd used in the experiments showed that it was not contaminated with any other polyamine. A similar analysis of labeled oat protoplasts revealed that the [^{3}H]Spd had been converted to DAP in the soluble and insoluble fractions as well as the incubation medium (Fig. 1). By contrast, in *Petunia* protoplasts the Spd remained essentially unaltered in all three fractions (Fig. 2). It is well known that cereals such as oat contain polyamine oxidase, which yields DAP, but that dicotyledonous families such as the *Solanaceae* lack this enzyme (22). The PAO inhibitor βHEH reduces the labeling of proteins extracted from oat protoplasts with pH 8.8 SDS carbonate buffer by about 75%. As Figure 3 indicates, only a portion of the added Spd is converted into DAP and then bound to proteins.

After being labeled, the boiled oat protoplasts were extracted for protein in the SDS carbonate buffer (pH 8.8) in the presence or absence of cold Spd. These extracts were then compared to those from unboiled oat protoplasts extracted in the presence or absence of cold DAP. This was done with the knowledge that boiled protoplasts did not convert the labeled Spd to DAP (Fig. 4). Cold Spd almost totally eliminated the label from the boiled protoplasts, whereas cold DAP did not totally eliminate the label from the unboiled ones (Table I).

The results of competitive inhibition binding experiments with oat protoplasts demonstrated that DAP added to the incubation medium was the most effective inhibitor of the binding of labeled polyamines to protein, followed in order by Spd, spermine, putrescine, histidine, and lysine.

Protein Characterization

Specific activity measurements of the ammonium sulfate cuts of proteins extracted in the SDS carbonate buffer indicated that the bulk of the labeled protein was in the 25% cut (3484 cpm/mg protein) compared to the 62% cut (666 cpm/mg protein). No measurable amount remained in the supernatant after the 62% cut.

The results of applying the combined 25 and 62% cuts to the Sephacryl column can be seen in Figure 5. Marker M2 was run with the column, and the protein measurement in the figure under it represents that marker in addition to protein from the protoplasts. Running the marker M1 under identical conditions on another column indicated a mol wt of the Spd binding protein in excess of 45000.

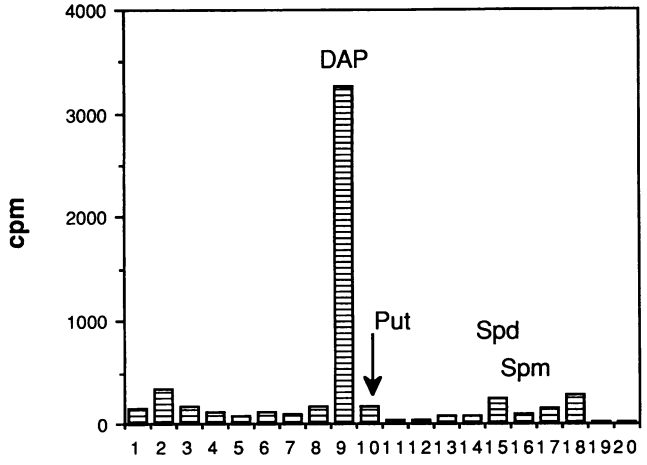

Figure 1. Fate of labeled Spd fed to oat protoplasts. TLC analysis was done after 4 h of labeling with [^{3}H]Spd. The labeled polyamine bound to proteins was extracted and determined for the TCA-insoluble, -soluble and incubation medium fractions. Only the insoluble fraction is shown, as the other two fractions are similar. The detected PAs are DAP, Put, Spd, and Spm.

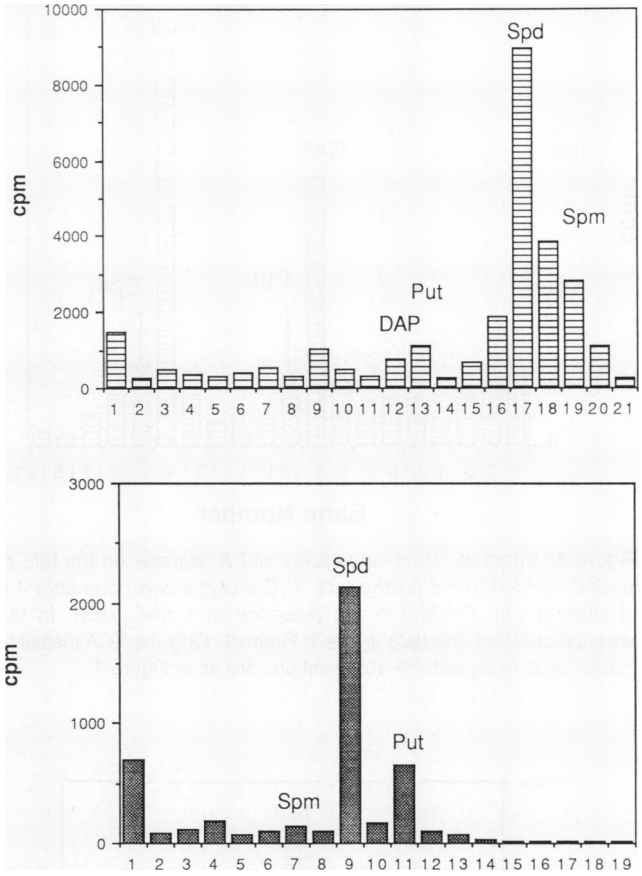

Figure 2. Fate of labeled Spd fed to *Petunia* protoplasts. TLC analysis was done after 4 h of labeling with [^{3}H]Spd. The labeled PA bound to proteins was determined for the TCA-insoluble (top graph), -soluble (bottom graph), and incubation medium fractions. The latter fraction is not shown, as it is similar to the soluble fraction. Use of a different solvent system accounts for the different baseline for the top graph. PA abbreviations are as in Figure 1.

DISCUSSION

Because of the highly protonated and thus charged condition of Spd at cellular pHs, it would not be surprising if it bound to a variety of cellular components, especially following cellular disruption. It is therefore important to determine whether any binding is related to a biological role of Spd or whether it is an artifact. Since the binding described here depends upon the physiological state of the protoplasts, vary-

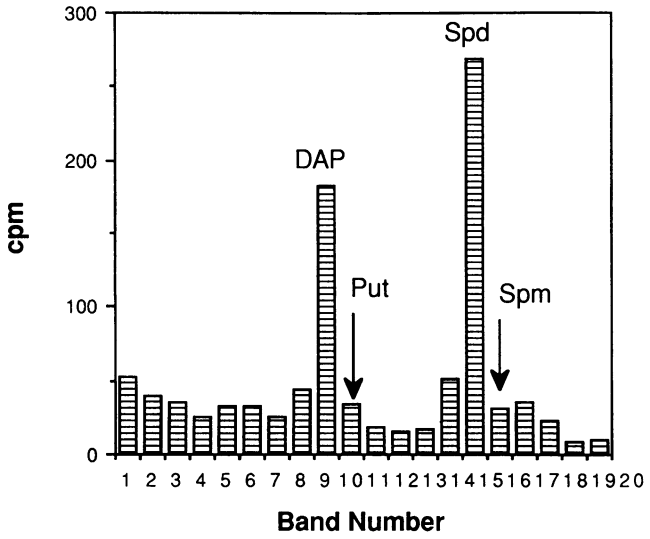

Figure 3. Effect of βHEH, an inhibitor of PA oxidase, on the fate of labeled Spd fed to oat protoplasts. TLC analysis was done after 4 h of labeling with [^{3}H]Spd in the presence of 1 mM βHEH. In the absence of βHEH, the data are as in Figure 1. Only the TCA insoluble fraction was analyzed. PA abbreviations are as in Figure 1.

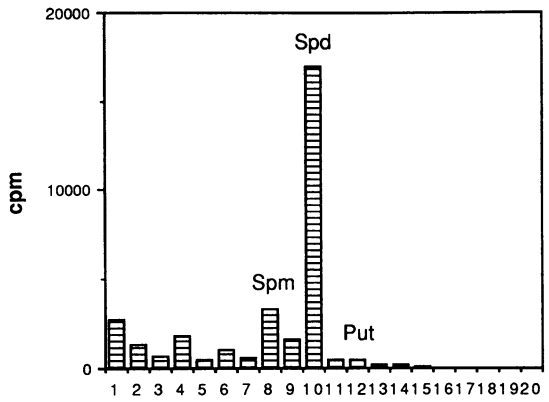

Figure 4. Fate of labeled Spd fed to boiled oat protoplasts. TLC analysis was done after 4 h of labeling with [^{3}H]Spd. The labeled PA bound to proteins in protoplasts that had been boiled prior to labeling was determined for the TCA-insoluble, soluble, and incubation medium fractions. Only the insoluble fraction is shown, as the other two fractions are similar. PA abbreviations are as in Figure 1.

Table I. *Effect of DAP and Spd on the Binding of [^{3}H]Spd to TCA Precipitable Protein in Unboiled and Boiled Oat Protoplasts*

Since the unboiled protoplasts converted Spd to DAP, and the boiled did not, the unboiled was tested only against DAP.

Treatment	Soluble Fraction	Pellet Fraction
	cpm/disc	
Unboiled	4445	127
Unboiled + DAP	206	236
Boiled	3884	2894
Boiled + Spd	0	57

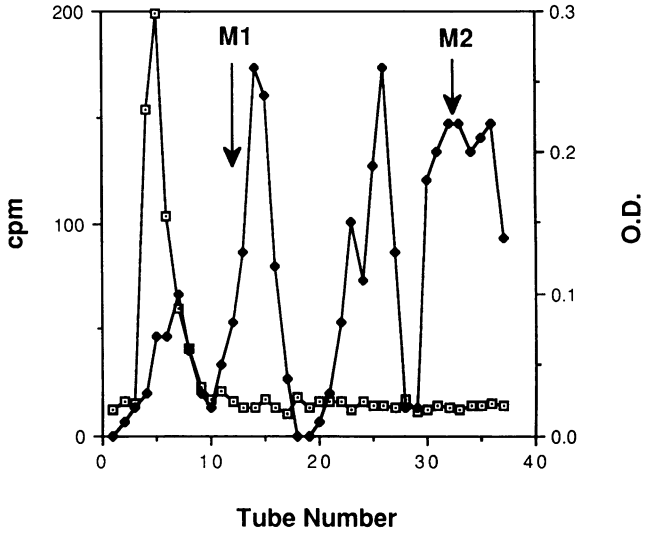

Figure 5. Fractionation of protein from Spd labeled oat protoplasts on a Sephacryl 200HR column. After labeling for 4 h with [^{3}H]Spd, the protoplasts were homogenized in a protein extraction buffer and precipitated with 25%, then 62% ammonium sulfate. The precipitates were combined, resolubilized in buffer and applied to the column. Fractions were collected every 5 min. Aliquots of 200 μL were applied to filter paper discs treated with TCA and counted (⊡ for cpm). Aliquots of 10 μL were measured for protein with the Bradford method (● for o.d.). M2 represents the location of 18 kD marker run with the column, and M1 represents a 45 kD marker run on a separate but identical column.

ing with the age of the leaves and illumination, it appears to have some physiological relevance.

We have shown that polyamines bind to TCA-precipitable material in oat leaves and in protoplasts of oat and *Petunia*. Such binding is linear over time, which could mean that the uptake and nonspecific binding of Spd are slow. However, experiments with carrot protoplasts demonstrate that uptake of polyamines is rapid (17). If this is also true of oat protoplasts, then the binding of polyamines to proteins is rate limiting.

The Q_{10} for the different labeling temperatures is somewhat lower than that usually found for such processes as active uptake or enzymatically mediated attachment to protein. However, since 'aged' protoplasts do not bind Spd as well as controls, and since protein extracted from protoplasts fails to bind Spd *in vitro*, we conclude that an enzymatic process is probably responsible for binding the polyamine to protein, while uptake may be nonenzymatic. The unequal extraction of TCA precipitable material at different pHs also indicates specificity in the binding of polyamines. Why a pH 8.8 carbonate buffer and freeze grinding yield the best extraction procedure is not clear, but SDS probably increases the detectable cpm by unfolding proteins and exposing more labeled sites.

The effectiveness of Pronase, and the ineffectiveness of RNAse and DNAse, in releasing counts from the TCA-precipitable material argues that polyamines are indeed bound to proteins. The fact that a high concentration of NaCl cannot elute the counts from the complex suggests a strong nonionic

binding of the polyamine to the protein. Counts remain even after the labeled protoplasts are extracted in the presence of a high concentration of DAP, to which they convert Spd, while a high Spd concentration removes the counts only from the Spd-labeled boiled protoplasts, which cannot metabolize Spd. Thus, the boiled protoplasts would appear to be binding Spd loosely and in a nonspecific manner, in contrast with the living protoplasts.

The *Petunia* results are consistent with our previous results with tobacco (2), also a member of the *Solanaceae.* In oats, PAO is known to convert Spd to DAP; thus when the enzyme is inactivated by boiling, the oats are unable to make the Spd to DAP conversion. Using the PAO inhibitor βHEH, we can also reduce the conversion of Spd to DAP. Since PAO was found in the cell walls of oats (11) it is not surprising that we find some residual activity in our protoplast preparations, thus accounting for the DAP in the incubation medium. PAO has not been found in plants other than the cereals (22), and this probably explains why *Petunia* protoplasts and tobacco cells do not convert Spd into DAP.

Cold DAP in the presence of radioactive Spd reduces the counts associated with protein by competing for DAP binding sites on oat protoplast proteins. Added cold Spd also reduces the relative amount of radioactive Spd that can be converted to DAP for protein binding. Other charged PAs and amino acids are less specific and less effective as inhibitors of the binding of label from fed Spd.

The 25% ammonium sulfate cut precipitates the largest proteins and aggregates of macromolecules. As this cut yields the highest specific activity proteins, the labeled proteins are probably of high mol wt. The results of the molecular sieve column, suggesting a mol wt in excess of 45 kD, confirm this. This is far higher than the 18 kD previously found in tobacco (2), a plant that lacks PAO and thus does not convert Spd to DAP. Fluorography of SDS-PAGE gels will be necessary to determine a more accurate mol wt and to ascertain whether specific proteins are involved in binding Spd and DAP. It should be recalled that even though DAP is a degradation product of Spd, it is active in delaying senescence in oat leaves (20). Thus, its binding to protein might have a physiological function.

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