Properties of Repressible Alkaline Phosphatase from Wild Type and a Wall-less Mutant of Neurospora crassa*

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SUMMARY

The repressible alkaline phosphatase of Neurospora crassa was purified from both the mycelium of a wild type strain and from the medium in which cultures of the slime mutant (which lacks the normal cell wall) had been grown. The enzyme preparations from the two sources had similar amino acid compositions, immunological properties, specific activities, thermal stabilities, and kinetic constants, but differed in a number of other properties. Both enzyme preparations contained carbohydrate, but the carbohydrate content of the enzyme isolated from slime medium was almost double that of the enzyme from wild type mycelium (24 and 14%, respectively). The molecular weight of the enzyme secreted by slime cells, estimated by gel filtration on Sephadex G-200 and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, was higher than that of the enzyme from wild type mycelium by an amount consistent with its increased carbohydrate content.

Electrophoresis at pH 4.7 and 9.5 indicated that the enzyme isolated from slime medium is more anionic than the enzyme from wild type mycelium. Chemical analysis revealed the presence of approximately 8 phosphate groups per enzyme molecule in the purified slime extracellular enzyme, whereas the wild type enzyme contained less than 0.5 phosphate molecule per enzyme molecule. The presence of phosphate in the slime extracellular enzyme, and the lack of significant amounts of phosphate in the wild type mycelial enzyme, was also demonstrated by determination of 32P associated with the enzymes isolated from the two sources following derepression in the presence of 32PO4.

A significant portion of the repressible alkaline phosphatase produced by derepressed wild type N. crassa was found to be secreted into the growth medium. The electrophoretic mobility of the enzyme isolated from the wild type culture medium resembled that of the enzyme isolated from the slime culture medium rather than that of the enzyme isolated from wild type mycelium.

In Neurospora crassa a number of enzymes are derepressed by phosphorus starvation or by growth on a limiting phosphorus source. These include an alkaline phosphatase (1), an acid phosphatase (2), a phosphate permease which has a high affinity for phosphate at high pH (3, 4), and one or more extracellular nucleases (5). The repressible alkaline phosphatase of N. crassa is a particularly attractive subject for the study of control of protein synthesis in a eukaryotic organism since its range of activity from full repression to full derepression is over 1000-fold (1, 4). In addition, it is easily and specifically assayed even in crude extracts containing other phosphatases (1), it is readily purified in good yield (6), and the purified enzyme has been characterized by physical and chemical methods (7).

Recent studies have concentrated on the isolation and characterization of possible structural gene mutants (8) and of regulatory mutants altered in the ability to repress or derepress (4, 8) the repressible alkaline phosphatase and the physiologically related enzymes mentioned above. Further progress in understanding such mutants requires a knowledge of the physiological factors involved in derepression of the alkaline phosphatase and, ultimately, a knowledge of the actual mechanism of derepression. With this in mind we began a study of the kinetics of derepression in the slime mutant of N. crassa. This strain lacks the normal cell wall and grows as isolated multinucleate protoplasts rather than branching hyphae (9, 10). This fact makes it much easier to manipulate in kinetic studies than the wild type strain. It quickly became apparent that, whereas the wild type strain retains most of the enzyme in a cell-bound form, slime cultures secrete nearly all of their repressible alkaline phosphatase into the growth medium. Preliminary examination of the enzyme secreted by slime cells revealed a clear difference between it and the enzyme prepared from wild type mycelia.

In the present paper we describe the purification of the repressible alkaline phosphatase secreted into the growth medium by slime cultures and compare some of its physical and chemical properties with those of the enzyme purified from wild type mycelia. A number of distinct differences were found between the two purified enzyme preparations.

EXPERIMENTAL PROCEDURES

Chemicals—Agarose, y-aminobutyric acid, barbital, 2-aminomethyl-1,3-propanediol, D-mannose, 5-bromo-4-chloro-3-indolyl phosphate, Coomassie brilliant blue, N-acetylmuraminic acid (type IV), bovine serum albumin (Fraction V), alcohol dehydrogenase (yeast, crystalline), ribonuclease A (type 1-A, bovine pancreas), ribonuclease T1 (Aspergillus oryzae), sialidase (N-acetylmuraminate glycohydrolase, type VI), Clostridium per...
fringens), alkaline phosphatase (type III, Escherichia coli), α-amylase (type I-A, hog pancreas), phosphorylase a (rabbit), and muon (type I, bovine submaxillary gland) were purchased from Sigma Chemical Co. Sephadex G-200, DEAE-Sephadex, and CM-Sephadex were obtained from Pharmacia Fine Chemicals. Reagents for acrylamide gel electrophoresis and AG 50W-X8 cation exchange resin were purchased from Bio-Rad; aniline blue-black from Canaco; p-nitrophenyl phosphate from Calbiochem; disodium EDTA from Fisher Chemical Co.; d-galactose and D-arabinose from Mannich Laboratories; sodium dodecyl sulfates from British Drug House Co. Sephadex Ltd.; glycyrri-nucleosome (bovine pancreas, crystalline) from Worthington Biochemical Co.; and carrier-free H₂¹⁷P⁴O₄ from New England Nuclear Co.

Cultures and Growth—The wild type strain of Neurospora crassa used in these studies was 74-OL8-1a (Fungal Genetics Stock Center No. 90). Other strains included omotin-1, large culture-adapted E1120, FGSC No. 34), crisp-1 (cr-1, allele B123, FGSC No. 488), the double mutant cr-1, os-1 (alleles B122, B135, FGSC No. 280), and the multiple mutant facey, spontaneous germination, arginine-1, crisp-1, aurescent, osmic-1 (jz, sp, arg-1, cr-1, aur, os-1) known as slime. The slime was obtained as a heterocaryon (FGSC No. 321) and the slime component of the heterocaryon was removed by pig digestion.

Stock cultures, except for the slime strain, were maintained on slants of Fries' medium (11) supplemented with 1.5% sucrose and solidified with 1.5% Difco agar. The slime strain was maintained for the duration of this study by daily transfers on Fries' medium supplemented with 1.5% sucrose and 1% arginine. A 0.2-m1 aliquot of a 24-hour culture was taken each day to inoculate 100 ml of fresh medium and strains were maintained at 4.9° for Emergy's (18) et al. and the multiple mutant

Electrophoresis—Disc electrophoresis was carried out using either the alkaline buffer system (pH 9.5) of Tamura and Ui (19), or an acidic buffer system similar to that described by Reisfeld et al. (20). The latter system was modified by raising the pH of the running gel, stacking gel, and electrode buffer to 0.14 M 2-mercaptoethanol and 0.01 M sodium citrate. After electrophoresis was completed, the gel was stained with Coomassie blue R-250 and destained in methanol-acetic acid. The enzyme activity was detected by adding the buffer system and electrophoresis was carried out at 4°C using a constant current of 2.5 mA per tube for 2.5 hours.

Bands of enzyme activity were detected following electrophoresis by inactivating the gels in a 0.01 M solution of 0.1 M sodium phosphate buffer, pH 8.5, which also contained 0.01 M EDTA. Staining was then carried out with several changes of destilled water and the washed gels were stored in destilled water at 4°C.

Gels were stained for protein by immersing them for 45 min in a solution containing 0.050 M Na₂CO₃ and 0.25% (w/v) sodium dodecyl sulfate, and then heating 2 min in a boiling water bath. The rest of the electrophoretic procedure was exactly as described by Laemmli (21). The acrylamide concentration was 10%.

Estimation of Molecular Weight by SDS-Acrylamide Gel Electrophoresis—Estimation of molecular weight by SDS-acrylamide gel electrophoresis was carried out as described above. The desired N. crassa alkaline phosphatase was purified and the denatured protein was subjected to electrophoresis along with marker peptides derived from phosphorylase b (mol wt = 94,000), ovalbumin (mol wt = 45,000), bovine serum albumin (mol wt = 66,000), α-amylase (mol wt = 48,000), and E. coli alkaline phosphatase (mol wt = 40,000). The gels were stained for protein as described earlier and the mobility of each band was calculated as suggested by Weber and Osborn (22).

Estimation of Molecular Weight by Gel Filtration—Analytical gel filtration of the purified alkaline phosphatases was carried out as suggested by Andrews (23) using a column (1.5 X 82 cm) of Sephadex G-200 equilibrated with 0.05 M Tris-HCl buffer, pH 8.3, containing 0.1 M NaCl. Elution was conducted with the same buffer. Samples were applied in a volume of 1.5 ml and fractions of 3.0 ± 0.05 ml were collected at a flow rate of approximately 12 ml per hour. Bovine serum albumin (mol wt = 67,000), ovalbumin dimer (mol wt = 134,000), and yeast alcohol dehydrogenase (mol wt = 180,000) were mixed with the desired alkaline phosphatase sample prior to each run to provide internal standards for molecular weight estimation.

Immunological Methods—One milligram of purified wild type alkaline phosphatase was mixed with Freund's complete adjuvant and injected subcutaneously (subcapsular region) into an adult rabbit, 1 mg per site. Injection of the mixture was repeated three times at weekly intervals. Two weeks after the last injection the rabbit was bled and serum was prepared from the collected blood. The crude serum was fractionated with solid (NH₄)₂SO₄. The fraction precipitating between 20 and 50% saturation which contained all

The abbreviation used is: SDS, sodium dodecyl sulfate.
of the detectable antibody activity, was dissolved in one-half the original volume of 0.01 M potassium phosphate buffer, pH 7.2, and dialyzed against 20 volumes of the same buffer containing 0.1 M NaCl. The dialyzed fraction was stored frozen at -15°C until needed.

Double diffusion studies of enzyme and antibody in agar gels were carried out as suggested by Ouchterlony (24) using microscope slides (1 x 3 inches) covered with a 0.88% agarose gel prepared in a buffer containing 0.01 M sodium barbital (pH 8.3), 0.88% (w/v) NaCl, and 0.05% (w/v) sodium azide. Precipitin lines were allowed to develop for 72 hours at room temperature. Nonprecipitated protein was removed by washing with several changes of barosate-saline (0.85% NaCl solution) buffer and the gels were then stained for protein by immersing for 1 min in a solution of 1% (w/v) aniline blue-black in 7.5% (v/v) acetic acid. Alternatively, the gels were stained for enzyme activity using a 0.05% (w/v) solution of 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M barbital buffer, pH 8.3.

Isolation of Alkaline Phosphatase from Cells Grown in Presence of [32P]Phosphate—Cultures of the wild type or slime strains of N. crassa were labeled with 32P by growth for 36 hours in 100 ml of phosphate-free Fries' medium supplemented with 0.10 mM K13PO4 (40 μCi). At the time of harvest more than 96% of the radioactivity was present in the supernatant fluid of the cell culture. The wild type mycelia were harvested on a Millipore filter, washed with distilled water, and then homogenized in 10 volumes (4.5 ml) of 0.2 M Tris buffer (pH 7.4) by grinding with alumina powder (12). The homogenate was centrifuged 10 min at 1000 × g. The supernatant solution was centrifuged for 60 min at 105,000 × g. The pellet was discarded. The supernatant solution was pooled and concentrated from 22 to 8.6 ml by use of a Diaflo ultrafilter equipped with a UM-10 membrane (Amicon Corp., Lexington, Mass.) and used as the source of slime enzyme.

The wild type 105,000 × g supernatant and the concentrated supernatant (containing 1.5 units per ml and 17 unit per ml of repressible alkaline phosphatase, respectively) were treated as follows: 50 μg of pancreatic ribonuclease, 2.5 μg of T1 ribonuclease, and 100 μg of deoxyribonuclease were added to each, and the preparations were incubated for 30 min at 37°C. The enzyme solutions were then fractionated by the addition of solid (NH4)2SO4 as the source of wild type enzyme. The alpine type mycelia were harvested on a Millipore filter, washed with distilled water, and then homogenized in 10 volumes (4.5 ml) of 0.2 M Tris buffer (pH 7.4) by grinding with alumina powder (12). The homogenate was centrifuged 10 min at 1000 × g. The supernatant solution was centrifuged for 60 min at 105,000 × g. The pellet was discarded. The supernatant solution was pooled and concentrated from 22 to 8.6 ml by use of a Diaflo ultrafilter equipped with a UM-10 membrane (Amicon Corp., Lexington, Mass.) and used as the source of slime enzyme.

The concentrated protein solution from the medium was brought to 60% saturation by the addition of 370 mg of solid (NH4)2SO4 per ml. The mixture was stirred 30 min and then centrifuged 15 min at 19,000 × g. The precipitate was discarded and the supernatant solution was brought to 95% saturation by the addition of 257 mg of solid (NH4)2SO4 per ml. The mixture was stirred 60 min and then centrifuged as above. The supernatant solution was discarded and the precipitate was dissolved in 10 ml of 0.01 M Tris buffer, pH 7.8, and dialyzed for 24 hours against this buffer.

The dialyzed enzyme preparation was applied to a column (4.5 × 50 cm) of DEAE-Sephadex A-50 which had been equilibrated with the 0.01 M Tris buffer, pH 7.8, and the column was washed free of unbound protein with 800 ml of the same buffer. The phosphatase was then eluted by application of a linear gradient consisting of 2000 ml of 0.01 M Tris buffer, pH 7.8, in the mixing chamber and 2000 ml of the same buffer containing 0.25 M NaCl in the reservoir. Fractions (19 ml each) were collected and assayed for phosphatase activity. Fractions 83 to 125 (1590 to 2380 ml of the gradient) contained the bulk of the activity (8170); these were pooled and concentrated to 18 ml by use of a Diaflo ultrafilter equipped with a UM-10 filter and then further concentrated to 2.9 ml using a collodion bag ultrafilter (Schleicher and Schuell Co., Keene, N. H.). These concentrated fractions were subjected to the following steps.

Enzyme Purification—Tables I and II summarize the purification of the repressible alkaline phosphatase from wild type mycelia and slime culture media, respectively. The enzyme from the two sources had similar solubility properties in ammonium sulfate solutions and similar elution profiles on Sephadex G-200 columns, but differed in their behavior on ion exchangers. The enzyme from wild type mycelia is bound to CM-Sephadex at pH 8.3 but is not bound to DEAE-Sephadex at pH 7.8; the phosphatase from slime culture media behaves in the opposite manner on the two types of ion exchange media, indicating a more...
acidic nature for the enzyme from this source. The final specific activity of both enzyme preparations was essentially identical and was very similar to the value reported earlier by Kadner et al. (6).

Polycrylamide Gel Electrophoresis—The results of gel electrophoresis of the purified phosphatase at pH 4.7 and 9.5 are shown in Fig. 1. A single, somewhat broad, protein band was observed for both enzyme preparations at both pH values. The slime enzyme migrated less rapidly toward the cathode at pH 4.7 and more rapidly toward the anode at pH 9.5 than did the wild type enzyme. The differences in the electrophoretic behavior of the two enzymes are consistent with their different chromatographic behaviour on ion exchange media as noted above, with the slime enzyme behaving as the more acidic species in all cases. Duplicate gels stained for alkaline phosphatase activity (not shown) exhibited exactly the same banding pattern as the gels stained for protein (see Fig. 1).

The purified enzymes were further compared by electrophoresis at pH 8.8 in the presence of 0.1% sodium dodecyl sulfate (21), a procedure generally assumed to separate proteins on the basis of size rather than charge differences. Only a single band of protein was observed with either preparation (Fig. 2) and the mobility of the wild type enzyme was slightly greater than that of the slime enzyme.

Immunodiffusion—The immunological similarity of the repressible alkaline phosphatases purified from the two sources was tested by double diffusion in agar gels against antiserum prepared against the wild type mycelial enzyme. A sharp, fully connecting precipitin line was observed on gels stained for either protein or enzyme activity (Fig. 3). In addition a second, much weaker, precipitin line for the slime enzyme was observed on gels stained for protein. Precipitin lines in 1 were stained for protein with aniline blue-black. Precipitin lines in 2 were stained to reveal enzyme activity with 5-bromo-4-chloro-3-indolyl phosphate.

Carbohydrate Content—Quantitative determination of the neutral and amino sugars present in the purified enzymes was carried out on samples hydrolyzed under mild conditions as described under "Experimental Procedures." The observed amino acid composition of the two enzyme preparations do not appear to differ significantly; the small differences for some of the amino acids are probably close to or within the experimental error.

| Fraction | Volume | Protein | Enzyme | Specific activity | Yield |
|----------|--------|---------|--------|-------------------|-------|
| Crude extract | 880 | 4,750 | 5,980 | 1.26 | 100 |
| 60 to 96% saturation with (NH₄)₂SO₄ | 66 | 920 | 5,500 | 6.03 | 92 |
| Carboxymethyl-Sephadex eluate (concentrated) | 5.5 | 55.6 | 3,200 | 57.3 | 31 |
| Sephadex G-200 eluate | 19.5 | 39.4 | 2,420 | 61.3 | 40 |

* Based on colorimetric determination (13).

**TABLE II**

Purification of extracellular repressible alkaline phosphatase from slime mutant of N. crassa

The starting material was enzyme released into 12.8 liters of medium.

| Fraction | Volume | Protein mg | Enzyme units | Specific activity | Yield |
|----------|--------|------------|--------------|-------------------|-------|
| Cell-free medium | 12,400 | 1,550 | 16.4 | 91 |
| Concentrated medium | 303 | 86 | 1,410 | 91 |
| 60 to 90% saturation with (NH₄)₂SO₄ | 11.7 | 79 | 1,300 | 84 |
| DEAE-Sephadex eluate (concentrated) | 2.9 | 19.1 | 1,050 | 55.0 | 68 |
| Sephadex G-200 eluate | 22 | 15.4 | 842 | 62.8 | 54 |

* Based on colorimetric determination (13).
zyme differ markedly, the content of mannose and galactose being much higher in the latter enzyme. From the data in Table III the carbohydrate content of the wild type and slime phosphatases are calculated to be 13.7 and 23.8%, respectively, of the total weight of the enzymes.

**Phosphate Content**—Samples of the purified enzyme preparations were hydrolyzed in 6 N HCl and the inorganic phosphate content of the hydrolysates was measured by the method of Ames and Dubin (27) as modified by Bloch and Schlesinger (28). This method indicated less than 1 phosphate group present per approximate 8 phosphate groups per enzyme molecule.

The occurrence of phosphate in the repressible alkaline phosphatase from slime medium by precipitation with antiserum; solubilization of the antibody-enzyme complex in sodium dodecyl sulfate solution, and polyacrylamide gel electrophoresis in the presence of the detergent.

**TABLE III**

| Composition of alkaline phosphatases |
|-------------------------------------|
| **Amino acid or carbohydrate** | **Wild type enzyme** | **Slime enzyme** |
| Residues | Calculated residues per 136,000 g of protein | Nearest integer | Residues | Calculated residues per 136,000 g of protein | Nearest integer |
| Cysteic acid | 0.0942 | 8.7 | 9 | 0.095 | 9.3 | 9 |
| Aspartic acid | 0.382 | 146 | 146 | 0.380 | 146 | 146 |
| Threonine | 0.323 | 103 | 103 | 0.320 | 103 | 103 |
| Serine | 0.742 | 83.2 | 83 | 0.462 | 81.3 | 81 |
| Glutamic acid | 0.479 | 98.4 | 98 | 0.554 | 97.5 | 98 |
| Proline | 0.398 | 70.1 | 70 | 0.415 | 73.1 | 73 |
| Glycine | 0.710 | 125 | 125 | 0.702 | 124 | 124 |
| Alanine | 0.577 | 102 | 102 | 0.574 | 101 | 101 |
| Valine | 0.384 | 69.4 | 69 | 0.401 | 70.6 | 71 |
| Methionine | 0.0988 | 15.6 | 16 | 0.0885 | 15.6 | 16 |
| Isoleucine | 0.274 | 48.3 | 48 | 0.281 | 49.5 | 50 |
| Leucine | 0.540 | 95.1 | 95 | 0.556 | 97.9 | 98 |
| Tyrosine | 0.274 | 48.3 | 48 | 0.270 | 47.6 | 48 |
| Phenylalanine | 0.300 | 58.2 | 58 | 0.338 | 59.7 | 60 |
| Histidine | 0.109 | 20.8 | 20 | 0.101 | 19.7 | 19 |
| Lysine | 0.488 | 86.0 | 86 | 0.496 | 82.5 | 83 |
| Arginine | 0.204 | 35.9 | 36 | 0.198 | 34.9 | 35 |
| Tryptophan | 0.110 | 19.4 | 19 | 0.108 | 19.0 | 19 |
| Glutamic acid | 0.103 | 18.1 | 18 | 0.108 | 19.0 | 19 |
| Mannose | 0.515 | 90.7 | 91 | 0.530 | 164 | 164 |
| Galactose | 0.133 | 23.4 | 23 | 0.438 | 77.1 | 77 |
| Glucose | 0.012 | 2.1 | 2 | 0.019 | 3.3 | 3 |

**TABLE IV**

| Phosphate content of purified repressible alkaline phosphatase preparations |
|---------------------------------------------------------------|
| **Enzyme** | **Amount** | **Phosphate** | **Calculated amount of enzyme** | **Ratio of phosphate to enzyme** |
| (mg) | (nmol) | (nmol/mg) | (nmol/mg) |
| Wild type | 1.33 | 4.1 | 9.8 | 0.42 |
| Slime | 0.370 | 21.9 | 2.76 | 7.9 |

* Based on colorimetric determination (13).

* Calculated assuming a molecular weight of 136,000 for the protein moiety of the repressible alkaline phosphatase, based on the data of Kadar et al. (6).

**Fig. 4.** SDS-polyacrylamide gel electrophoresis of repressible alkaline phosphatases isolated from crude extracts by precipitation with antiserum. The preparation of enzyme samples and the procedure for electrophoresis are described in detail under "Experimental Procedures." Preparations were as follows: A. 3.5 µg (0.214 unit) of purified wild type mycelial enzyme; B. 0.1-mg aliquot (equivalent to 0.210 unit) of enzyme isolated from an extract of wild type mycelia by precipitation with antiserum; C. 0.1-mg aliquot (equivalent to 0.210 unit) of enzyme isolated from slime medium by precipitation with antiserum; D. 3.5 µg (0.220 unit) of purified enzyme from slime medium. The very strong "extraneous" bands in B and C are denatured and reduced chains of immunoglobulin.
in the repressible alkaline phosphatases purified in this study was tested by incubating 15 &g of each purified phosphatase with 50 &g of sialidase (N-acetylneuraminic glycohydrolase) for 24 hours at 30° in 0.12 ml of 0.1 M potassium acetate buffer, pH 5.0 (33). This amount of sialidase released 22 &g of sialic acid from 500 &g of bovine submaxillary mucin in parallel incubations under the same conditions. The recovery of alkaline phosphatase activity was greater than 90% for both the wild type and slime enzymes at the end of the sialidase treatment. Disc gel electrophoresis of the treated alkaline phosphatases and of untreated controls was carried out at pH 4.7 and 9.5. The electrophoretic mobility of the treated samples was identical with that of the respective controls indicating the absence of terminal sialic acid residues in both forms of the enzyme.

**Molecular Weight**—The molecular weights of the wild type and slime enzymes were estimated by gel filtration, along with protein standards of known molecular weight, on a Sephadex G-200 column as described under "Experimental Procedures." The peak elution volume of the slime alkaline phosphatase (Fig. 6) was less than that of the wild type enzyme, in agreement with the larger size expected of the former enzyme due to its increased carbohydrate content (Table III). The apparent molecular weights, estimated from the line formed by plotting elution volume against log molecular weight of the standards, were 165,000 for the wild type enzyme and 178,000 for the slime enzyme.

The native form of the N. crassa repressible alkaline phosphatase is a dimer (8). The molecular weight of the subunits was estimated by gel electrophoresis of the N. crassa alkaline phosphatase and standards of known molecular weight in the presence of sodium dodecyl sulfate as described under "Experimental Procedures." The results are shown in Fig. 7. The molecular weights of the subunits, estimated from the standard curve, were 85,000 for the wild type enzyme and 90,000 for the slime enzyme.

**Thermal Stability**—The rate of loss of activity during heat denaturation of the two enzyme preparations was measured at three pH values. The enzymes were prepared in the indicated buffer by dialysis against a large excess of the buffer, followed by dilution to a concentration of 2 &g per ml in the same buffer. Aliquots of the enzyme solutions were heated at the indicated temperature for appropriate lengths of time, cooled rapidly, and surviving enzyme activity was determined in the standard enzyme assay. The half-life of each enzyme under the indicated conditions of temperature and pH were derived from the linear plots of incubation time against the logarithm of the surviving enzyme activity. As shown in Table V, the half-lives of the wild type and slime enzymes did not differ significantly under the conditions tested. It is interesting to note however that, whereas a precipitate formed in solutions of the wild type enzyme heated
alkaline phosphatase activity in slime culture was found almost exclusively in the culture medium. However, even in wild type a significant fraction of this enzyme appeared to be liberated into the media from each culture were carried out and the results are summarized in Table VI. As already mentioned, the repressible alkaline phosphatase of N. crassa has been considered to be primarily, if not exclusively, cell-bound (1,2) rather than excreted into the media as is the repressible acid phosphatase of the same organism (2, 35). The finding that in cultures from wild type mycelium as shown in Fig. 9. The structure of this enzyme molecules would liberate phosphate from each other was tested by prolonged incubation of highly concentrated enzyme solutions. Samples of enzyme, 670 \( \mu \)g each, were prepared by dialysis against distilled water and then dried by lyophilization in conical tubes. The dried material was dissolved by the addition of 5 \( \mu \)l of Fries' salts (minus phosphate). The concentrated enzyme solutions were incubated 24 hours at 37\(^\circ\)C in a closed container over moistened filter paper and then diluted to a concentration of 2 mg per ml with 0.2 M Tris-HCl buffer, pH 7.4. Enzyme assays indicated a recovery of greater than 95% of the enzyme activity. Disc electrophoresis at pH 4.7 revealed no observable change in the electrophoretic mobility of either enzyme.

**Kinetic Properties**—The possibility that the phosphatase secreted by slime cells might be capable of a self-modification process in which neighboring enzyme molecules would liberate phosphate from each other was tested by prolonged incubation of highly concentrated enzyme solutions. Samples of enzyme, 670 \( \mu \)g each, were prepared by dialysis against distilled water and then dried by lyophilization in conical tubes. The dried material was dissolved by the addition of 5 \( \mu \)l of Fries' salts (minus phosphate). The concentrated enzyme solutions were incubated 24 hours at 37\(^\circ\)C in a closed container over moistened filter paper and then diluted to a concentration of 2 mg per ml with 0.2 M Tris-HCl buffer, pH 7.4. Enzyme assays indicated a recovery of greater than 95% of the enzyme activity. Disc electrophoresis at pH 4.7 revealed no observable change in the electrophoretic mobility of either enzyme.

**Distribution of Repressible Alkaline Phosphatase between Cells and Media of Wild Type and Several Mutant Strains of N. crassa**—The repressible alkaline phosphatase of N. crassa has been considered to be primarily, if not exclusively, cell-bound (1,2) rather than excreted into the media as is the repressible acid phosphatase of the same organism (2, 35). The finding that in cultures of the slime strain the repressible alkaline phosphatase is primarily free in the medium, prompted us to do a quantitative examination of the distribution of this enzyme in cultures of wild type and of several mutant strains affected in cell wall synthesis (36). Cultures were grown for 24 hours in 100 ml of phosphate-free Fries' medium supplemented with either 0.05 mM KH\(_2\)PO\(_4\), or 2 mM \( O \)-phosphorylethanolamine; this latter phosphorus source allows essentially maximal rates of growth of the organism, but at the same time gives quite a high degree of derepression of alkaline phosphatase (37). Cells were harvested and extracts were prepared by homogenization with alumina powder (12) in 0.05 M sodium acetate buffer, pH 5.0 (2). Alkaline phosphatase determinations on both the cell extracts and the cell-free culture media from each culture were carried out and the results are summarized in Table VI. As already mentioned, the repressible alkaline phosphatase activity in slime culture was found almost exclusively in the culture medium. However, even in wild type a significant fraction of this enzyme appeared to be liberated into the medium (20 to 30% under the conditions tested). The proportion of the phosphatase released into the medium by the cell wall mutants (os-I; os- \( \sigma \); cr; and os, \( \sigma \), cr, aur) was intermediate between the value observed for the wild type and slime cultures.

**Electrophoretic Mobility of Repressible Alkaline Phosphatase Isolated from Wild Type Culture Medium**—Medium from a derepressed culture of wild type N. crassa (grown for 20 hours on 0.05 mM KH\(_2\)PO\(_4\) medium) was concentrated approximately 30-fold with a Diaflo ultrafilter (UM-10 membrane). A further 10-fold concentration was achieved by the addition of Lyphogel (Gelman Instrument Co., Ann Arbor, Mich.). The final concentrated solution (0.3 ml) contained 0.10 unit of repressible alkaline phosphatase activity (an extract of the mycelium harvested from this medium contained 0.90 unit of enzyme). This solution was dialyzed overnight against 0.01 M Tris-HCl buffer, pH 7.4, and was then subjected to electrophoresis in the pH 9.5 buffer system. Standards consisting of the purified wild type mycelial and slime medium enzymes were run at the same time. The bands of enzyme activity observed following electrophoresis are shown in Fig. 8. The repressible alkaline phosphatase isolated from wild type culture medium migrates at a rate more similar to that of the slime enzyme than that of the enzyme purified from wild type mycelium.

**Properties of the Residual Repressible Alkaline Phosphatase in Washed Slime Cells**—Although the bulk of the repressible alkaline phosphatase in slime cultures is found free in the medium (Table VI), approximately 5% of the total activity is found still associated with the cells even after they have been washed with fresh medium. Attempts to purify the enzyme from extracts of washed slime cells by ammonium sulfate fractionation revealed that a considerable portion (15 to 25% depending on how the cells were washed) of the activity was precipitated in 65% saturated solutions of the salt. Less than 5% of the enzyme isolated from slime medium or wild type mycelium is precipitated under the same conditions. The electrophoretic mobility at pH 0.5 of this "65% ammonium sulfate precipitable" alkaline phosphatase is less than that of the enzyme isolated from either slime medium or wild type mycelium as shown in Fig. 9. The structure of this more soluble, more basic form of the enzyme was shown to be immunologically similar to the other repressible alkaline phosphatase species by precipitation of the expected number of enzyme units with graded amounts of the antisem prepared against the wild type mycelial enzyme (Table VII).
The repressible alkaline phosphatase secreted by the slime mutant was purified to apparent homogeneity as judged by polyacrylamide gel electrophoresis at pH 8.8 in the presence of sodium dodecyl sulfate, and at pH 4.7 and pH 9.5 in the absence of the detergent. The enzyme was also purified from the mycelium of a wild type strain by the procedure of Kadner et al. (6). The two purified enzyme preparations exhibited essentially identical glucosamine, mannose, and galactose found in the wild type purified wild type enzyme was found to contain a carbohydrate content of the purified enzyme was found to be approximately 11.5% of the total weight. In the present study the carbohydrate content of the purified wild type enzyme was found to be approximately 13.7%. The relative amounts of glucosamine, mannose, and galactose found in the wild type enzyme are very similar to those found by Kadner et al. (6).

The somewhat higher total amounts found in this study may be due to differences in the wild type strains used in the two studies. The slime enzyme purified in this study was found to contain a
much larger per cent of carbohydrate than the wild type enzyme, and the relative amounts of glucosamine, mannose, and galac-
tose were also quite different. The glucosamine content of the slime enzyme was almost identical with that of the wild type enzyme, but the mannose content was approximately 80% higher and the galactose content was over 3-fold higher. No other carbohydrate, other than probably insignificant traces of glucose, was found in either enzyme preparation.

The electrophoretic mobility of a protein in polyacrylamide gel is determined by both the size and the net charge on the pro-
tein. The fact that the slime phosphatase is the faster species when migration is toward the anode (pH 9.5) and is the slower species when migration is toward the cathode (pH 4.7) indicates that it is separated from the wild type enzyme primarily due to a difference in charge. The behavior of the two enzyme prepara-
tions on DEAE- and CM-Sephadex during purification supports this conclusion. The possibility that the difference be-
tween the slime and wild type enzymes could be at least partially due to a difference in amino acid composition cannot be com-
pletely ruled out at this time. As pointed out above, amino acid analysis is not sensitive enough to show conclusively a dif-
ference of only one, or a few, residues in such a large molecule.

However, wild type N. crassa also secretes a significant amount of repressible alkaline phosphatase into the growth medium (Table VI), and the electrophoretic mobility of this secreted wild type enzyme more nearly resembles that of the secreted slime enzyme than that of the enzyme retained by wild type mycelia (Fig. 8). This secreted wild type enzyme has not been obtained in sufficient quantity to allow its purification and chemical anal-
ysis, but its similarity to the secreted slime phosphatase strongly suggests that the secreted wild type enzyme and the mycelial wild type enzyme differ in degree of post-translational modifica-
tion rather than differing in their primary amino acid structure.

There is precedent for such a phenomenon: the exo-1 (38) and T9 (39) mutants of N. crassa appear to be simultaneously affected in cell wall synthesis, and they synthesize glucoamylase with altered gel filtration (39) or isoelectric focusing (39) properties.

Sialic acid, which is known to account for the electrophoretic heterogeneity of a number of enzymes (29-32), is probably not present in either the wild type or slime repressible alkaline phospha-
tases. Treatment of the purified enzymes with a large excess of sialidase failed to alter their respective electrophoretic mobili-
ties, a result which would have been expected if terminal sialic acid residues had been present.

The purified slime phosphatase was found to contain approxi-
mately 8 phosphate groups per enzyme molecule (Table IV) whereas the wild type enzyme contained less than 0.5 phosphate group per enzyme molecule. This difference in phosphate content would seem to provide a sufficient explanation for the electrophoretic difference between the two forms of the enzyme. Bloch and Schlesinger (28) have shown that the purified, native E. coli alkaline phosphatase contains 1.6 to 2.1 moles of tightly
bound inorganic phosphate per mole of enzyme. This phosphate is removed by dialysis against nitriolotrisetic acid, a procedure which removes tightly bound zine ions from the enzyme. The alkaline phosphatase isolated from slime cells labeled with $P^{32}$ retained most of its bound $P^{32}$ during an isolation procedure which included dialysis against phosphate buffer, heating at 100$^\circ$ in phosphate buffer containing 0.1% sodium dodecyl sulfate, and SDS-polyacrylamide gel electrophoresis (Fig. 4). Retention of the radioactive phosphate under these conditions indicates that in this case it is probably present as covalently bound organic phosphate rather than tightly bound inorganic phosphate.

The subcellular localization of the repressible alkaline phospha-
tase of N. crassa has not been studied, although the enzyme has been considered to be primarily cell-bound rather than secreted into the medium (1, 2). The analogous enzyme in several bacte-
ria is known to be located in the periplasmic space between the cell membrane and cell wall (40-43). The fact that the slime mutant, which lacks the normal cell wall, secretes approximately 95% of the repressible alkaline phosphatase into the medium strongly suggests that this enzyme also is normally located in the periplasmic space. A similar distribution of enzyme between cells and growth medium in slime cultures has recently been observed for invertase (10), an enzyme previously shown to be primarily located in a position external to the plasma membrane (44).

If the similar electrophoretic mobility of the phosphatase se-
creted into the medium by both wild type mycelia and slime cells (Fig. 8) is a reflection of similar structures, then the slime enzyme may represent a natural stage in maturation rather than an "abnormal" form resulting from the mutant phenotype. It is even possible that this is the form of the enzyme as it initially enters the periplasmic space. According to this hypothesis the bulk of this enzyme, which is retained in the periplasmic space, would then be further modified, perhaps by alkaline phosphatase itself, to produce the "wild type" form of the enzyme. The enzyme secreted by slime would escape this modification due to its rapid dilution into the surrounding medium. Incubation of the purified purified slime phosphatase at a concentration (approximately 50%, w/v) which might occur if all of the enzyme were localized in the periplasmic space did not result in "self-modification" as judged by lack of change in electrophoretic mobility. Further studies will be required to determine whether enzyme(s) capable of converting either the cell-bound or secreted form of the enzyme to the alternative form can be isolated from N. crassa.

A small amount of repressible alkaline phosphatase activity remains associated with the slime cells even after they have been washed with fresh medium. Much of this activity is indistin-
guishable from the enzyme found secreted into the medium. However, a significant proportion (15 to 75% depending on the thoro,

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