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CONTROL OF THE PRODUCTION AND PARTIAL CHARACTERIZATION OF REPRESSIBLE EXTRACELLULAR 5′-NUCLEOTIDASE AND ALKALINE PHOSPHATASE IN *NEUROSPORA CRASSA*

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Summary

A new species of orthophosphate repressible extracellular 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) was found to be released into mycelial culture media when a wild type strain of *Neurospora crassa* was grown on limiting amounts of phosphate. The production of 5′-nucleotidase and extracellular acid and alkaline phosphatase was inhibited by the addition of rifampicin when it was added at the later stage of mycelial growth, but not when it was added at a very early stage. The 5′-nucleotidase and extracellular alkaline phosphatase were partially purified and characterized. pH optimum of the former was 6.8 and that of the latter was higher than 10.0. The 5′-nucleotidase activity was inhibited by ethylenediaminetetraacetate (EDTA) and $ZnCl_2$ at pH 6.8 and stimulated by $MnCl_2$ and $CoCl_2$ at pH 4.0. Alkaline phosphatase activity was stimulated by EDTA, $MgCl_2$, $CoCl_2$ and $MnCl_2$. 5′-nucleotidase hydrolyzed various 5′-nucletides but not 3′-nucleotides or other various phosphomono- and diester compounds. Alkaline phosphatase hydrolyzed all the phosphomonoester compounds tested. Mutants, *nuc-1* and *nuc-2*, which were originally isolated by the inability to utilize RNA or DNA as a sole source of phosphate, were unable to produce 5′-nucleotidase or six other repressible enzymes reported previously. These mutants showed no or significantly reduced growth on orthophosphate-free nucleotide media depending on the number of conidia inoculated, mainly because of loss of ability to produce these repressible extracellular phosphatases.

Introduction

In *Neurospora crassa* constitutive acid and alkaline phosphatase were found in the mycelial extract [1,2]. Six orthophosphate-repressible enzymes, an

alkaline phosphatase [3—5], an acid phosphatase [6], two extracellular nucleases [7], alkaline phosphate permease [8] and ribonuclease N_1 [9] were reported to be derepressed under orthophosphate-limiting conditions. These enzymes were not derepressed in those strains having mutations in genes *nuc-1* and *nuc-2* [7—10], which were isolated on the basis of inability to utilize RNA or DNA as a sole source of phosphate [11]. Possible genetic regulation mechanism of production of these enzymes was analyzed in relation to the mutations in genes *nuc-1* and *nuc-2* [7—10] and two constitutive mutations *pcon*^{c} and *preg*^{c} [12,13]. *Nuc-1* and *nuc-2* are recessive to corresponding wild-type alleles [9,13]. *Preg*^{c} is epistatic to *nuc-2* and is recessive to the corresponding wild-type allele [12,13].

The present study describes the characterization and control mechanism of a hitherto unknown orthophosphate repressible extracellular 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) and a repressible extracellular alkaline phosphatase which has been reported to be a modified form of intracellular repressible alkaline phosphatase [5].

Materials and Methods

Strains. The following strains of *N. crassa* were used; wild-type strain (74A); *nuc-1* mutant (A1); *nuc-2* mutant (B1); and double mutant between A1 and B1 (A1B1). The isolation method and characterization of the *nuc-1* and *nuc-2* mutants have been described by Ishikawa et al. [11]. Strains B1R3, B1R4 and B1R24 were isolated as revertants from B1 [9]. Strain UW-6 (*pcon*^{c}) and UW-6 *nuc-1 inos* were kindly supplied by Dr. R.J. Metzenberg. Heterokaryon between A1 and B1 (A1 + B1) was prepared by inoculating the mixture of the conidia of the two strains.

Media. Fries minimal medium [14] was used as a high phosphate medium. A phosphate-free medium was prepared by substituting KCl (1 mg/ml) in Fries minimal medium for KH_2PO_4. A low-phosphate medium was prepared by mixing 1 part high phosphate medium and 19 parts phosphate-free medium thus reducing KH_2PO_4 concentration to 50 μg/ml. Other media containing variously reduced amounts of KH_2PO_4 were prepared in a similar way. Orthophosphate-free nucleotide media were prepared by adding nucleotide solution in sterilized water after autoclaving orthophosphate-free media. The media were stood at room temperature for two days to check for contamination. For the growth of UW-6 and UW-6 *nuc-1 inos*, 10 μg/ml of inositol was added.

Inoculation. Conidia were suspended in sterilized deionized water and filtered with double clothed gauze to remove contaminating mycelia unless otherwise mentioned. Conidial suspensions from each strain were adjusted to an absorbance of 0.1 at 630 nm (1×10^6 cells/ml). The conidial suspension (0.5 ml) was inoculated into each Erlenmeyer flask containing 20 ml of culture medium, and 1 ml of this was then inoculated into a Roux flask containg 100 ml of culture medium unless otherwise mentioned.

Culture filtrate. Mycelia were grown at 25° C without shaking. Mycelial pads were removed using filtration and the culture filtrate was then used for enzyme assay directly or after dialysis against 5 mM Tris · HCl (pH 7.4) containing 1 mM β-mercaptoethanol.

Partial purification of phosphatases. All the following experiments were carried out at 0°C and the buffer used for all partial purifications was 5 mM Tris · HCl (pH 7.4) containing 1 mM β-mercaptoethanol:

(1) Ammonium sulfate precipitation: To the mycelial culture filtrate (12.5 l) cellulose powder (20 g/l) and $(NH_4)_2SO_4$ (660 g/l) were added with stirring. The precipitate on cellulose powder was collected, eluted with the buffer and reprecipitated with $(NH_4)_2SO_4$ (660 g/l). The precipitate was collected by centrifugation and dissolved in the buffer. It was then dialyzed against the buffer. (2) Sephadex G-100 filtration: After centrifugation the supernatant was loaded on a Sephadex G-100 column (5 × 90 cm) previously equilibrated and fractionated with the buffer. 10-ml fractions were collected. (3) Sephadex G-100 refiltration: Fractions showing enzyme activity in the first Sephadex G-100 filtration were separately pooled and the enzyme fractions were reprecipitated with $(NH_4)_2SO_4$ (0.66 g/ml). The precipitates from each fraction were collected by centrifugation and dissolved in the buffer. Each sample was loaded on a Sephadex G-100 column (5 × 90 cm) and fractionated as described above. (4) DEAE-cellulose chromatography: Enzyme samples from each Sephadex G-100 rechromatogram were loaded on a DEAE-cellulose column (2 × 12 cm) equilibrated with the buffer and eluted with a linear gradient of NaCl concentration (0—0.4 M) in the above buffer (total 1000 ml) into 10-ml fractions. Fractions showing alkaline phosphatase activity were pooled, dialyzed against the buffer, and used as partially purified alkaline phosphatase sample. (5) DEAE-cellulose rechromatography: Fractions showing 5′-nucleotidase activity in the first DEAE-cellulose chromatography were pooled, dialyzed against the buffer and rechromatographed on DEAE-cellulose column as described above (5-ml fractions) to remove contaminating acid phosphatase designated as acid phosphatase II (unpublished). Fractions showing 5′-nucleotidase activity but not acid phosphatase II activity were pooled, dialyzed against the buffer, and used as partially purified 5′-nucleotidase sample.

Determination of enzyme activity. The reaction mixture for 5′-nucleotidase (1.0 ml) contained 12.5 μmol of acetate/acetic acid (pH 4.0) or Tris/maleate (pH 6.8), 10 μmol of $MnCl_2$, 5 μmol of 5′-AMP and 0.2 ml of the enzyme fraction. The enzyme activity of mycelial culture filtrate was assayed at pH 4.0 because in this condition the activity of alkaline phosphatase was negligible, but that of the purified enzyme was assayed at pH 6.8. Reaction mixture for alkaline phosphatase (1.0 ml) contained 12.5 μmol of carbonate/bicarbonate (pH 10.4), 10 μmol of EDTA, 5 μmol of 5′-AMP or β-glycerol phosphate or 2.5 μmol of *p*-nitrophenyl phosphate and 0.2 ml of enzyme fraction. After incubation at 37°C for 1 h for 5′-nucleotidase assay and 10 min for alkaline phosphatase assay, the reaction mixture was chilled in ice water and orthophosphate or *p*-nitrophenol released was determined [6,16]. Reaction mixtures for acid phosphatase II and repressible acid phosphatase (1.0 ml) contained 12.5 μmol of acetate/acetic acid (pH 5.0), 2.5 μmol of *p*-nitrophenyl phosphate for acid phosphatase II and 10 μmol of EDTA and 2.5 μmol of bis-*p*-nitrophenyl phosphate for repressible acid phosphatase and 0.2 ml of enzyme fraction. The reaction mixture was incubated at 37°C for 10—60 min. The reaction was stopped by chilling it in ice water followed by the addition of 1 ml of 10% trichloroacetic acid. After 10 min, 2 ml of saturated sodium carbonate were

added at room temperature and absorbance was read at 405 nm. One unit of enzyme activity was defined as 1 μmol of orthophosphate or *p*-nitrophenol released per min at 37°C.

Protein and orthophosphate measurements. Protein concentration was assayed by the method of Lowry et al. [15] with bovine serum albumin as standard. Orthophosphate concentration was determined by the method of Lohman and Jendrassik [16].

Chemicals. Special chemicals used and their sources were: Sephadex G-100 (Pharmacia), DEAE-cellulose (Brown Co.), 3′- and 5′-nucleotides, rifampicin and thymidine 3′- and 5′-monophospho-*p*-nitrophenyl esters (Sigma Chemical Co.).

Results

1. Production of orthophosphate repressible extracellular phosphatases

Wild-type mycelia are able to utilize RNA as a sole source of phosphate [11], and therefore may produce orthophosphate from RNA. To confirm this assumption, the ability of dialyzed culture filtrate obtained from wild type culture grown on low- and high-phosphate media to release orthophosphate from RNA was examined. As shown in Fig. 1, a significant amount of orthophosphate was released from RNA added in low-phosphate culture filtrate, but no detectable release of orthophosphate was observed with high-phosphate culture filtrate. On the other hand, during the course of determining the

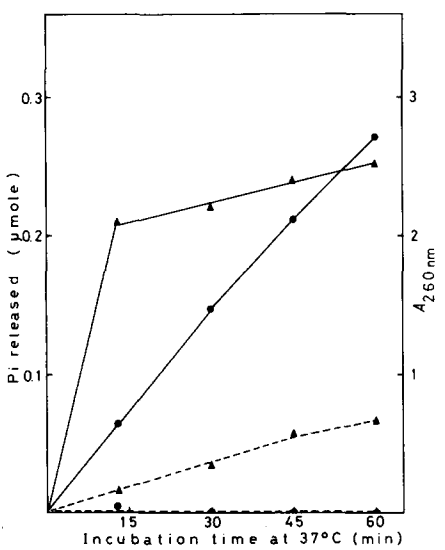

Fig. 1. Release of acid soluble materials and orthophosphate from RNA by dialyzed culture filtrate of wild-type mycelia. 0.75 ml of dialyzed culture filtrate of wild-type mycelia from low- and high-phosphate media were mixed with 0.25 ml of 6 mg/ml of yeast RNA and incubated for indicated periods at 37°C. After incubation, it was chilled in ice and 0.25 ml of 25% perchloric acid was added. After centrifugation, 0.2 ml of resulting supernatant was diluted in 5 ml of water and absorbance at 260 nm was read. Another 0.2 ml of supernatant was used for orthophosphate determination. High phosphate media (- - - - -); low phosphate media (———). Absorbance (▲); orthophosphate (●).

TABLE I

EFFECT OF PHOSPHATE CONCENTRATION IN CULTURE MEDIA ON THE PRODUCTION OF EXTRACELLULAR 5′-NUCLEOTIDASE AND ALKALINE PHOSPHATASE

Concentration of orthophosphate in culture media (μg/ml)	Enzyme activity *			Dry weight of mycelia (mg)
	5′-nucleotidase	Alkaline phosphatase		
		5′-AMP substrate	β-glycerol phosphate substrate	
1000	0.036	0.000	0.000	76.4
100	0.147	0.636	0.683	64.0
50	0.257	1.94	2.58	52.7
20	0.460	2.56	3.17	34.6
10	0.588	3.60	4.43	18.4

* Culture filtrate was dialyzed against 5 l of 5 mM Tris · HCl (pH 7.4) containing 1 mM β-mercaptoethanol. Enzyme activity was expressed in units/ml per g dry mycelia.

specificity of partially purified repressible extracellular nucleases using RNA as substrate [7], production of large amounts of nucleosides was observed by thin layer chromatography. This suggested the existence of unknown phosphatase(s) having nucleotidase activity which seem to be derepressed in orthophosphate-limited mycelial culture medium. To confirm these results, the relationship between phosphatase activity and the concentration of orthophosphate in the culture medium was analyzed by assaying with 5′-AMP, β-glycerol phosphate and bis-*p*-nitrophenyl phosphate as substrates (Table I). The repressible extracellular acid phosphatase reported by Nyc [6] has no ability to hydrolyze 5′-AMP [6,10], and repressible alkaline phosphatase shows almost no activity at pH 4.0 in acetate buffer in the presence of $MnCl_2$ (see Fig. 5). Therefore the apparent increase in the 5′-AMP-hydrolyzing activity at pH 4.0 in the presence of $MnCl_2$ indicates the presence of new species of phosphatase(s) which are derepressed under orthophosphate limitation. Later this enzyme was found to be repressible extracellular 5′-nucleotidase. The increases in 5′-nucleotidase, acid phosphatase and alkaline phosphatase activities in low-phosphate media were respectively 16, 175 and more than 1000 times those in high-phosphate media. To examine whether 5′-nucleotidase is repressible or not, rifampicin was added to the low-phosphate culture media to the final concentration of 1 μg/ml at the different times after inoculation of wild-type conidia. The 5′-nucleotidase activity as well as activities of repressible extracellular acid and alkaline phosphatases increased immediately after the disappearance of orthophosphate in culture media (Fig. 2). Growth of mycelia and production of the extracellular 5′-nucleotidase as well as acid and alkaline phosphatases were inhibited by the addition of rifampicin. The degree of inhibition was dependent on the growth stage of mycelia (Fig. 2). When rifampicin was added at a very early stage of mycelial growth (24 h after inoculation of conidia), it showed a slight effect on mycelial growth as well as the production of these phosphatases. Whereas, when it was added at the later growing stage of mycelia

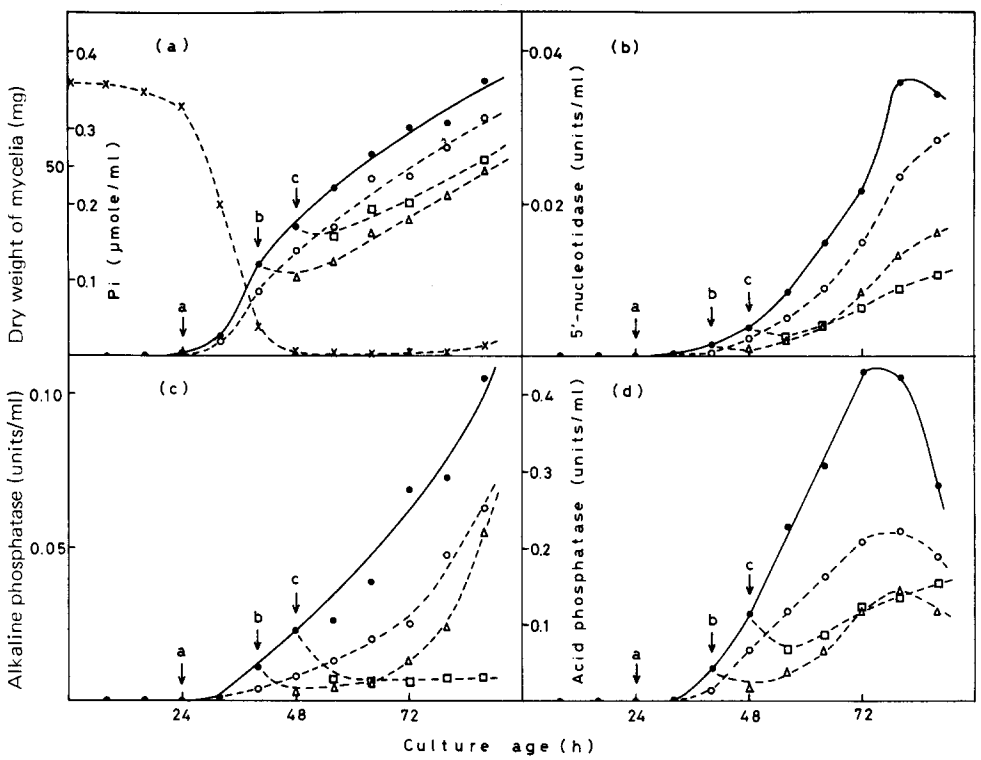

Fig. 2. Effect of rifampicin on growth and production of extracellular phosphatases in wild-type mycelia. Conidial suspension of wild-type strain was inoculated in 100-ml Erlenmeyer flasks containing 20 ml of low-phosphate media and incubated at 25°C. At appropriate intervals mycelia were harvested, dried and weighed, and culture filtrates were used for enzyme and phosphate assays. Arrows a, b, and c indicate the times when 20 μg of rifampicin was added to several flasks. (a) Dry weight of mycelia of the control cultures (—●—). Dry weight of mycelia of the cultures added 20 μg of rifampicin at 'a' point (- - -○- - -), at 'b' point (- - -△- - -) and at 'c' point (- - -□- - -). Concentration of phosphate (P_i) (- - -X- - -). (b) 5'-nucleotidase activity of control cultures (—●—), the enzyme activity of the cultures with 20 μg of rifampicin added at 'a' point (- - -○- - -), at 'b' point (- - -△- - -) and at 'c' point (- - -□- - -). (c) Extracellular alkaline phosphatase activity and (d) extracellular acid phosphatase activity. Symbols are the same as in (b).

(48 h after inoculation of conidia), it inhibited mycelial growth and enzyme production more efficiently. The results were essentially the same as those found in the production of ribonuclease N_1 [9]. The activities of these phosphatases were not affected in the presence of 0.2 μg/ml of rifampicin. Thus these phosphatases were found to be derepressed under orthophosphate-limited conditions.

2. Partial purification of repressible extracellular phosphatases

The repressible extracellular phosphatases were partially purified by the method described in Materials and Methods from mycelial culture filtrate obtained from wild-type culture grown in low-phosphate media. The results of Sephadex G-100 gel-filtration of $(NH_4)_2SO_4$ precipitate of culture filtrate was shown in Fig. 3 (a). The phosphatase eluted immediately after Dextran Blue (fraction numbers 60—72) was repressible extracellular alkaline phosphatase

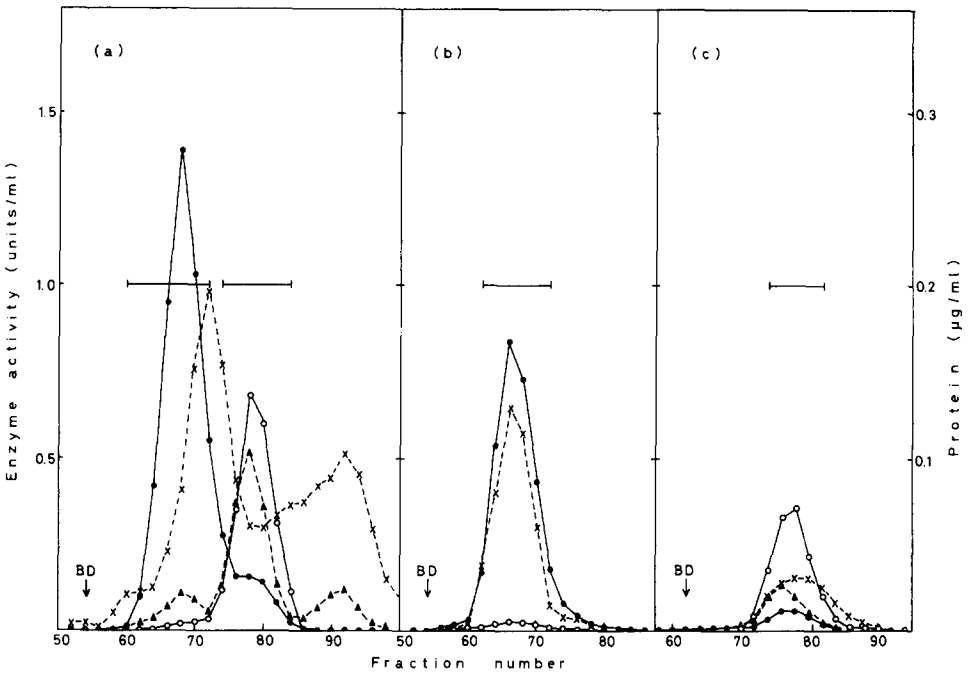

Fig. 3. Gel-filtration of $(NH_4)_2SO_4$ precipitate of culture filtrate and refiltration through Sephadex G-100 column. Precise experimental conditions were as described in Materials and Methods. (a) Gel-filtration of $(NH_4)_2SO_4$ precipitate of culture filtrate obtained from the wild-type cultures in low-phosphate media. (b) Repressible extracellular alkaline phosphatase fractions indicated with a left-side bar in (a) were collected, precipitated with $(NH_4)_2SO_4$ and rechromatographed. (c) 5′-nucleotidase acid phosphatase II and phosphodiesterase fractions indicated with a right-side bar in (a) were collected, precipitated with $(NH_4)_2SO_4$ and rechromatographed. Phosphatase activities assayed with a reaction mixture for 5′-nucleotidase (——○——), acid phosphatase II (- - -▲- - -) and alkaline phosphatase (5′-AMP as substrate) (——●——). Protein concentration (- - -X- - -). Arrows with BD indicate elution position of Dextran Blue.

[5] and the second peak was found to contain two newly found phosphatases, 5′-nucleotidase and acid phosphatase II. The precise character of the latter will be described elsewhere. The last peak corresponded to repressible acid phosphatase reported by Nyc [6]. In alkaline pH, repressible acid phosphatase is unstable [6]. Therefore the apparent relative amount of acid phosphatase in Fig. 3 (a) is very small. To remove the two newly found phosphatases, alkaline phosphatase fractions were refiltered through Sephadex G-100 (Fig. 3 (b)) and then chromatographed with a DEAE-cellulose column (Fig. 4 (a)). The fractions showing alkaline phosphatase activity were dialyzed and used as a repressible extracellular alkaline phosphatase fraction which appeared to be free of other phosphatase activity. Fractions containing 5′-nucleotidase and acid phosphatase II in the first Sephadex G-100 chromatogram (fraction numbers 74—84) were refiltered through Sephadex G-100 (Fig. 3 (c)) to remove repressible extracellular acid and alkaline phosphatases and then chromatographed with a DEAE-cellulose column (Fig. 4 (b)). Fractions containing 5′-nucleotidase (Fraction numbers 34—37) were pooled, dialyzed and rechromatographed with a DEAE-cellulose column to remove acid phosphatase II. 5′-nucleotidase frac-

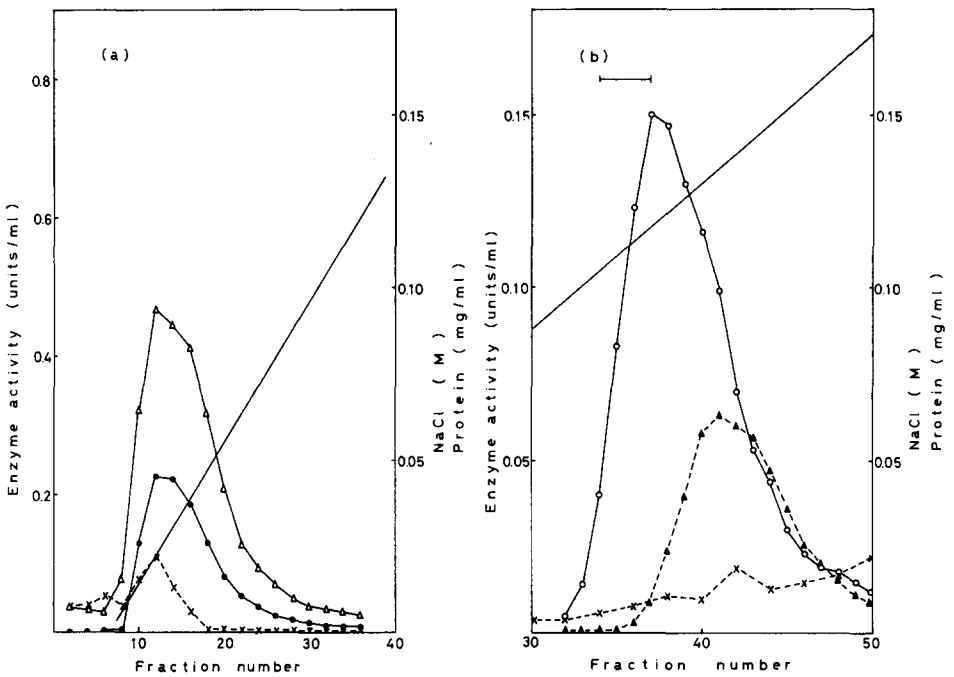

Fig. 4. DEAE-cellulose column chromatography on repressible extracellular alkaline phosphatase, 5'-nucleotidase and acid phosphatase II fractions. (a) Fractions obtained by gel-filtration through Sephadex G-100 (Fig. 3 (b) indicated with a bar) were loaded on a DEAE-cellulose column and eluted with linear gradient of NaCl concentration (0-0.4 M). (b) Fractions obtained by gel filtration through Sephadex G-100 (Fig. 3 (c) indicated with a bar) were chromatographed with a DEAE-cellulose column as in (a). 5'-nucleotidase fractions indicated with a bar were collected and used for further purification. Precise purification procedures were described in Materials and Methods. Enzyme activity assayed in the reaction mixture fo · alkaline phosphatase with *p*-nitrophenyl phosphate as substrate (—△—). NaCl concentration (———). The other symbols are the same as in Fig. 3.

tions which do not show acid phosphatase II activity were pooled, dialyzed and used as a partially purified 5'-nucleotidase sample. In the partially purified 5'-nucleotidase fraction a small amount of phosphodiesterase is detected as a contaminant, the character of which will be described elsewhere. The results of partial purification of 5'-nucleotidase and repressible extracellular alkaline phosphatase are summarized in Table II.

3. Characteristics of repressible extracellular 5'-nucleotidase and alkaline phosphatase

From the elution profile through Sephadex G-100, the molecular weights of 5'-nucleotidase and repressible extracellular acid phosphatase were calculated to be around 80 000 and 52 000, respectively. The molecular weights of these enzymes clearly differ from those of repressible alkaline phosphatase (mol. wt. 165 000 [5]) and peak II (mol. wt. 19 400) and III (mol. wt. 10 500) enzymes of ribonuclease N_1 [9].

Using partially purified enzyme samples, enzyme characteristics such as pH optimum, ion requirement and substrate specificity were determined. pH

TABLE II

PARTIAL PURIFICATION OF REPRESSIBLE EXTRACELLULAR 5′-NUCLEOTIDASE AND ALKALINE PHOSPHATASE

Purification step	5′-nucleotidase					Alkaline phosphatase				
	Total units	Total protein	Specific activity (units/mg protein)	Recovery (%)	Purification	Total units	Total protein	Specific activity (units/mg protein)	Recovery (%)	Purification
Culture filtrate *	118	336	0.351	100	1	492	336	1.46	100	1
$(NH_4)_2SO_4$ precipitate	58.1	85.7	0.678	49.2	1.93	228	85.7	2.66	46.3	1.82
$(NH_4)_2SO_4$ re-precipitate	65.1	80.3	0.817	55.2	2.33	159	80.3	1.98	32.1	1.36
Sephadex G-100 filtration	49.4	22.8	2.17	41.9	6.18	69	26.3	2.64	14.1	1.81
Sephadex G-100 refiltration	30.7	3.63	8.47	26.0	24.1	64	15.6	5.30	12.9	3.63
DEAE-cellulose chromatography	6.68	0.561	11.9	5.7	31.1	47	1.34	35.3	9.4	24.2
DEAE-cellulose re-chromatography	0.55	—	—	0.5	—					

* Culture filtrate dialyzed against 10 mM Tris · HCl (pH 7.4) containing 1 mM β-mercaptoethanol was used for enzyme assays.

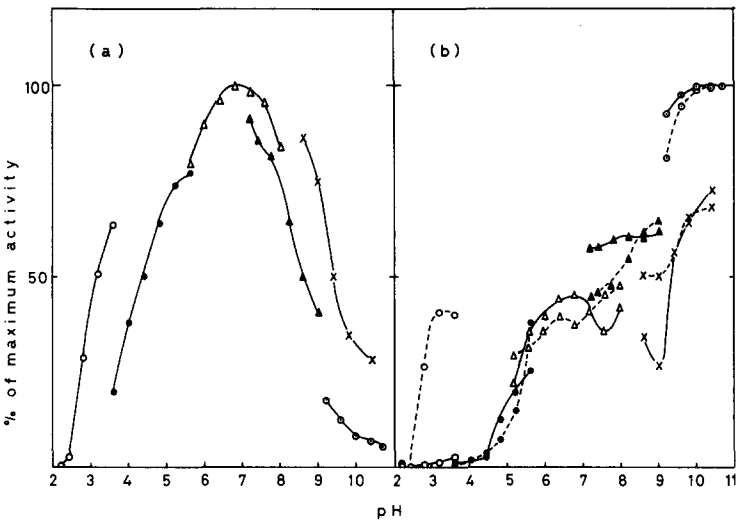

Fig. 5. pH optima of 5′-nucleotidase and extracellular alkaline phosphatase. Each reaction mixture contained 12.5 μmol of indicated buffers having a series of pH values from 2.2 to 10.7. The other part of reaction mixture was the same as described in Materials and Methods. (a) 5′-nucleotidase. (b) Extracellular alkaline phosphatase with 5′-AMP (———) and *p*-nitrophenyl phosphate (- - - - -) as substrates. Glycine/HCl (○); sodium acetate/acetic acid (●); potassium phosphate (△); Tris · HCl (▲); glycine/NaOH (X); carbonate-bicarbonate (○).

optima of 5′-nucleotidase and alkaline phosphatase were determined to be 6.8 and higher than 10.0, respectively, using various phosphomonoester compounds as substrates (Fig. 5). 5′-nucleotidase and alkaline phosphatase showed activity in the very wide pH ranges. In the case of repressible extracellular alkaline phosphatase, no apparent optimum was observed at pH 9.0—9.5 which is the pH optimum of repressible intracellular alkaline phosphatase [3]. The enzyme is more active in Tris · HCl than in glycine/NaOH (Fig. 5 (b)).

The effect of divalent cations and EDTA were examined for these enzyme samples (Table III). Both of the enzymes showed their activities in the absence of divalent cations. In the presence of EDTA, 5′-nucleotidase showed reduced activity at pH 6.8 and 4.0, whereas alkaline phosphatase was activated. 5′-nucleotidase was inhibited by $ZnCl_2$ but relatively unaffected by $MgCl_2$, $MnCl_2$, $CoCl_2$ and $CaCl_2$ at pH 6.8 but at pH 4.0 the enzyme was stimulated by $CoCl_2$ and $MnCl_2$. Repressible extracellular alkaline phosphatase was stimulated by $MgCl_2$, $CoCl_2$, $ZnCl_2$ and $MnCl_2$. This is different from the characteristics of intracellular repressible alkaline phosphatase which is unaffected by these divalent cations [3].

Substrate specificities of these enzymes were analyzed with use of eighteen kinds of phosphomono- and diester compounds (Table IV). 5′-nucleotidase was found to be strictly specific to 5′-nucleotides. This enzyme hydrolyzed a small amount of fructose 6-phosphate, but this may be within fluctuation of assays because fructose 6-phosphate shows a relatively high background value in this assay system. Hydrolysis of a small amount of bis-*p*-nitrophenyl phosphate may be due to a remaining activity of phosphodiesterase contaminating in the

TABLE III

EFFECT OF EDTA AND DIVALENT CATIONS ON THE ACTIVITY OF EXTRACELLULAR 5′-NUCLEOTIDASE AND ALKALINE PHOSPHATASE

Partially purified enzyme samples prepared from culture filtrate of wild type were assayed with indicated amount of additives and the relative activity (% of control) was calculated.

Additive	Concentration (mM)	5′-nucleotidase				Alkaline phosphatase	
		pH 6.8		pH 4.0			
		nmol P_i released/min	Per cent of control	nmol P_i released/min	Per cent of control	nmol P_i released/min	Per cent of control
none		16.5	100	7.3	100	19.1	100
EDTA	10	12.0	72.2	4.5	61.6	23.0	120
EDTA	1	15.5	94.0	4.8	65.7	31.6	165
$MgCl_2$	1	16.7	101	8.4	115	33.4	175
$MnCl_2$	1	16.4	99.4	14.9	202	24.3	127
$CoCl_2$	1	16.9	102	14.5	199	27.2	142
$CaCl_2$	1	14.6	88.5	8.7	119	19.0	99.5
$ZnCl_2$	1	6.2	37.6	8.9	122	26.8	140

* Alkaline phosphatase was assayed with 5′-AMP as substrate.
** Acid phosphatase activity was assayed in the absence of EDTA.
*** Low and high P_i denote low-phosphate and high-phosphate media, respectively.

enzyme sample. The relative rates of hydrolysis of phosphomonoester compounds by extracellular repressible alkaline phosphatase were found to be very similar to those of intracellular enzyme [3].

4. Ability of nuc-1 and nuc-2 mutants to derepress repressible extracellular 5′-nucleotidase and acid and alkaline phosphatases

Since failure of *nuc-1* and *nuc-2* mutants to derepress at least six repressible enzymes has been reported, the ability of *nuc-1* mutant (A1), *nuc-2* mutant (B1), double mutant between A1 and B1 (A1B1) and heterokaryon between A1 and B1 (A1 + B1) to derepress the repressible 5′-nucleotidase and acid and alkaline phosphatases were examined by growing mycelia in low-phosphate media (Table V). A1, B1 and A1B1 had no ability to derepress these repressible extracellular phosphatases, but heterokaryon A1 + B1 restored the ability partially. The derepression of 5′-nucleotidase and alkaline phosphatase by B1R3, B1R4 and B1R24 which were isolated as revertants from B1 was found to be very low compared to acid phosphatase and in the case of B1R24 no derepression of alkaline phosphatase was observed. Strain UW-6 which was isolated as a constitutive mutant for repressible alkaline phosphate permease was found to derepress these enzymes at very low levels. But as UW-6 *nuc-1 inos* shows almost no derepression of the enzymes *nuc1* is epistatic for this derepression.

Since all these phosphatases are excreted into culture media, these enzymes are expected to participate in hydrolyzing nucleotides added in culture media. Though in the previous paper *nuc-1* and *nuc-2* mutants were reported to grow on orthophosphate-free nucleotide media [11], the failure of *nuc* mutants to derepress the repressible extracellular alkaline phosphatase and 5′-nucleotidase

TABLE IV

SUBSTRATE SPECIFICIES OF REPRESSIBLE EXTRACELLULAR 5′-NUCLEOTIDASE AND ALKALINE PHOSPHATASE

Partially purified enzyme samples prepared from culture filtrate of wild type were assayed with indicated substrates and the relative rate of hydrolysis was calculated.

Substrates	5′-nucleotidase		Alkaline phosphatase	
	nmol P_i released/min *	Relative rate of hydrolysis (%)	nmol P_i released/min *	Relative rate of hydrolysis (%)
β-Glycerol phosphate	0.00	0.0	25.10	100
p-nitrophenyl phosphate	0.00	0.0	29.58	118
Sodium pyrophosphate	0.00	0.0	17.42	34.8
5′-AMP	6.29	100	17.32	69.0
5′-GMP	5.60	89.0	13.43	53.5
5′-IMP	5.87	93.3	13.14	52.4
5′-CMP	3.73	59.3	10.51	41.9
5′-UMP	4.71	74.9	10.99	43.8
3′-AMP	0.00	0.0	15.67	62.4
3′-GMP	0.00	0.0	14.60	58.1
3′-CMP	0.00	0.0	16.15	64.4
3′-UMP	0.00	0.0	15.19	60.5
Glucose 6-phosphate	0.00	0.0	6.81	27.1
Glucose 1-phosphate	0.00	0.0	10.41	41.5
Fructose 6-phosphate	0.59	9.4	2.04	8.1
Bis-*p*-nitrophenyl phosphate	0.25	4.0	0.43	1.7
Thymidine 3′-monophospho-*p*-nitrophenyl phosphate	0.18	2.9	0.61	2.4
Thymidine 5′-monophospho-*p*-nitrophenyl phosphate	0.00	0.0	0.40	1.7

* When *p*-nitrophenyl esters were used, *p*-nitrophenol released was determined.

made the authors recheck the growth of *nuc* mutants on orthophosphate-free nucleotide media. The results shown in Table VI indicate that the growth of *nuc* mutants on orthophosphate-free nucleotide media depended on the number of conidia inoculated. When a small number of conidia (5×10^5) were inoculated into 20 ml of nucleotide media, A1 showed no growth on any 3′- or 5′-nucleotides except 5′-GMP, and B1 was found to grow slightly on any 3′- or 5′-nucleotides except 3′- and 5′-CMP, but when a large number of conidia (2.5×10^6 or 5×10^6) were inoculated, A1 showed growth to the level of 1/3—1/2 of that of wild type, and B1 showed more growth than A1. When the initial growth of a small number of conidia of both mutants was supported by the addition of a small amount of orthophosphate, they showed substantial growth on nucleotide media. These results suggested that constitutive acid and alkaline phosphatase may be involved in the hydrolysis of nucleotides.

TABLE V

PRODUCTION OF REPRESSIBLE EXTRACELLULAR 5′-NUCLEOTIDASE AND ACID AND ALKALINE PHOSPHATASES IN HIGH AND LOW PHOSPHATE MEDIA BY WILD TYPE STRAIN AND *NUC* MUTANTS

Strains	5′-nucleotidase *		Acid phosphatase **		Alkaline phosphatase		Dry weight of mycelia (mg)	
	High P_i ***	Low P_i	High P_i	Low P_i	High P_i	Low P_i	High P_i	Low P_i
74A	0.057	0.567	0.02	28.4	0.006	4.40	57.0	38.1
A1	0.008	0.027	0.05	0.00	0.005	0.003	59.6	34.8
B1	0.004	0.032	0.18	0.00	0.004	0.004	56.8	33.7
A1B1	0.010	0.034	0.05	0.16	0.006	0.012	60.0	31.2
A1 + B1	0.004	0.079	0.03	8.45	0.005	0.114	63.3	38.1
B1R3	—	0.031	0.23	0.19	—	0.043	68.3	32.1
B1R4	—	0.058	2.34	4.23	—	0.103	63.5	45.6
B1R24	—	0.025	0.27	0.06	—	0.000	59.6	31.4
UW-6	—	0.088	2.10	17.5	—	0.142	50.1	46.9
UW-6 *nuc-1 inos*	—	0.034	0.01	0.06	—	0.012	59.3	32.1

* Enzyme activity was expressed in units/ml per g dry mycelia and culture filtrates from high phosphate media were dialyzed against 5 mM Tris · HCl (pH 7.4).

TABLE VI

GROWTH CHARACTERISTICS OF WILD TYPE, *NUC-1* MUTANT (A1) AND *NUC-2* MUTANT (B1) ON NUCLEOTIDES

Number of conidia inoculated	Nucleotides	Dry weight of mycelia (mg)		
		Strain 74A	Strain A1	Strain B1
$5 \cdot 10^5$	5′-AMP	36.2	0	2.8
$5 \cdot 10^5$	5′-GMP	28.8	2.3	8.1
$5 \cdot 10^5$	5′-CMP	29.1	0	0.2
$5 \cdot 10^5$	5′-UMP	29.0	0	4.2
$5 \cdot 10^5$	3′-AMP	31.4	0	4.5
$5 \cdot 10^5$ *	3′-AMP	30.8	0	3.0
$2.5 \cdot 10^6$ *	3′-AMP	32.9	11.3	16.7
$5 \cdot 10^6$ *	3′-AMP	29.5	16.5	19.2
$5 \cdot 10^5$	3′-GMP	33.1	0	5.7
$5 \cdot 10^5$	3′-CMP	34.1	0	0
$5 \cdot 10^5$	3′-UMP	35.3	0	5.9

* Conidia used were not filtered through gauze.

Discussion

A 5′-nucleotidase, strictly specific to 5′-nucleotides, was newly found in mycelial culture media of a wild type strain of *N. crassa* when it was grown on limiting amounts of orthophosphate. The release of the enzyme into culture media in the growing phase of mycelia may not be the result of non-specific release of intracellular enzymes by the lysis of mycelia. Since the activity of the enzyme is apparently increased under orthophosphate limitation, the enzyme was thought to be repressible. Since rifampicin inhibited the production of 5′-nucleotidase and acid and alkaline phosphatases, these enzymes are synthesized de novo in low phosphate medium, as has been reported in the case of ribonuclease N_1 [9]. A lower inhibition was observed when rifampicin was added at the very early phases of mycelial growth. The mechanism of inhibition by rifampicin and the differential effect of rifampicin on the production of these enzymes may be essentially the same as those discussed for the production of ribonuclease N_1 [9].

Characteristics of 5′-nucleotidase, such as pH optimum, substrate specificity and sensitivity to EDTA are enough to differentiate the enzyme from repressible extracellular acid and alkaline phosphatases described previously [5,6], as well as from the constitutive acid and alkaline phosphatases [1,2]. Since the partially purified 5′-nucleotidase sample showed strict specificity to 5′-nucleotides, this enzyme will provide a useful biochemical tool. Repressible extracellular alkaline phosphatase has been reported to be modified form of repressible intracellular alkaline phosphatase [5]. The present report provides further information that both enzymes are different in their pH optima and sensitivity to divalent cations. It is noticeable that modification of the intracellular enzyme molecules, probably by increased carbohydrate content [5], resulted in the alteration of these enzymic characteristics.

The *nuc-1* and *nuc-2* mutants were unable to produce repressible extra-

cellular 5′-nucleotidase and acid and alkaline phosphatases, but heterokaryon between them restored the ability partially. Thus, these mutants are regulatory mutants which failed to derepress at least 7 repressible enzymes, acid and alkaline phosphatases [3—6], two extracellular nucleases [7], ribonuclease N_1 [9], alkaline phosphate permease [8] and 5′-nucleotidase. Although the production of extracellular alkaline phosphatase and 5′-nucleotidase seems to be regulated by essentially the same mechanism as found in the other repressible enzymes [7—10], the relative levels of derepression of extracellular alkaline phosphatase and 5′-nucleotidase in heterokaryon (A1 + B1), B1R4 and UW-6 were less than one tenth of those of repressible acid phosphatase and ribonuclease N_1 [9]. Since the *nuc-1* and *nuc-2* genes are thought to be related to the production of inducer for these repressible enzymes [10] and the level of inducer in heterokaryon between A1 and B1 (A1 + B1) is thought to be lower than that in wild type, a higher level of inducer may be required for the production of extracellular alkaline phosphatase and 5′-nucleotidase as compared with those of extracellular repressible acid phosphatase and ribonuclease N_1. The possibility that differential production of these enzymes to be mere reflection of differential excretion of these enzymes can not however be excluded.

Metzenberg and his co-workers [13] presented an elaborate model of an hierarchic regulation mechanism of these repressible enzymes which is based on the following facts. *Nuc-1* and *nuc-2* are null mutants. *Pcon*^{c} synthesize acid and alkaline phosphatase and alkaline phosphatase permease constitutively, and it is allelic to *nuc-2*, dominant to wild type allele, and hypostatic to *nuc-1*. *Preg*^{c} synthesizes the above enzymes constitutively, and it is epistatic to *nuc-2*, hypostatic to *nuc-1*, and recessive to the corresponding wild-type allele. They also reported the isolation of a constitutive mutant of *nuc-1*. From this evidence they explained the regulation mechanism of these enzymes as follows. Orthophosphate inactivates *nuc-2* product, *nuc-2* product inactivates *preg*^{c} product, *preg*^{c}-product inactivates *nuc-1* product and *nuc-1* product induces enzyme synthesis. There will be a possibility that *nuc-2* and *preg*^{c} are concerned with different metabolic pathways for the production of precursors of inducers for these repressible enzymes. *Nuc-2* may be responsible for the production of a precursor of normal inducer for these repressible enzymes. *Preg*^{c} appears to be a type of mutant which lacks an enzyme activity and therefore accumulates some metabolite which may become a precursor of the second inducer for these repressible enzymes. This explains that *preg*^{c} is recessive to the corresponding wild-type allele and epistatic to *nuc-2*. These precursors of inducers will be converted into inducers by the action of *nuc-1* gene product. The *nuc-2* gene product is thought to be sensitivie to orthophosphate, and *pcon*^{c} may be a mutation causing lack of sensitivity to orthophosphate. It may be postulated that the *nuc-1* gene product is partially sensitive to orthophosphate, and therefore mutants partially constitutive in the production of the repressible enzymes have been found.

Since at the early stage of this work we reported that the *nuc* mutants can grow on nucleotide media though with reduced rate in both mutants, we must further report that the growth of *nuc* mutants on orthophosphate-free nucleotide media is conditional. The effect of inoculation size on the growth of *nuc*

mutants on nucleotide media may be the result of some growth factor in conidia [20]. Since *nuc* mutants are known to have no ability to derepress repressible enzymes in low-phosphate media containing nucleotide (Hasunuma, K., unpublished) the growth of mycelia with large inoculation size on orthophosphate-free nucleotide media may depend on an unknown mechanism of utilizing nucleotides, such as the participation of constitutive acid and alkaline phosphatases. It is probable that once conidia of *nuc* mutants germinate either by abundant inoculation of them or addition of a small amount of orthophosphate, the resulting mycelia utilize nucleotides as a sole source of phosphate. The result that the *nuc-2* mutant showed almost no growth on 3′- and 5′-CMP may suggest that leakiness of this mutant depends on some phosphatases exhibiting a particular base specificity.

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References

1 Kuo, M.-H. and Blumenthal, H.J. (1961) Biochim. Biophys. Acta 52, 13—29
2 Kuo, M.-H. and Blumenthal, H.J. (1961) Biochim. Biophys. Acta 54, 101—109
3 Nyc, J.F., Kadner, R.J. and Crocken, B.J. (1966) J. Biol. Chem. 241, 1468—1472
4 Kadner, R.J., Nyc, J.F. and Brown, D.M. (1968) J. Biol. Chem. 243, 3076—3082
5 Burton, E.G. and Metzenberg, R.L. (1974) J. Biol. Chem. 249, 4679—4688
6 Nyc, J.F. (1967) Biochem. Biophys. Res. Commun. 27, 183—188
7 Hasunuma, K. (1973) Biochim. Biophys. Acta 319, 288—293
8 Lehman, J.F., Gleason, M.K., Ahlgren, S.K. and Metzenberg, R.L. (1973) Genetics 75, 61—73
9 Hasunuma, K., Toh-e, A. and Ishikawa, T. (1976) Biochim. Biophys. Acta 432, 223—236
10 Toh-e, A. and Ishikawa, T. (1971) Genetics 69, 339—351
11 Ishikawa, T., Toh-e, A., Uno, I. and Hasunuma, K. (1969) Genetics 63, 75—92
12 Metzenberg, R.L., Gleason, M.K. and Littlewood, B.S. (1974) Genetics 77, 25—43
13 Littlewood, B.S., Chia, W. and Metzenberg, R.L. (1975) Genetics 79, 419—434
14 Beadle, G.W. and Tatum, E.L. (1945) Am. J. Bot. 32, 678—686
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 265—275
16 Lohman, K. and Jendrassik, L. (1926) Biochem. Z. 178, 419—426
17 Kuntzel, H. and Schafer, K.P. (1971) Nat. New Biol. 231, 265—269
18 Barath, Z. and Kuntzel, H. (1972) Nat. New Biol. 240, 195—197
19 Jacob, S.T., Sajdel, E.M. and Munro, H.M. (1968) Biochem. Biophys. Res. Commun. 32, 831—838
20 Charlang, G.W. and Horowitz, N.H. (1971) Proc. Natl. Acad. Sci. U.S., 68, 260—262