ISOLATION AND CHARACTERIZATION OF NUCLEASE MUTANTS IN *NEUROSPORA CRASSA*

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THE nuclease activity of *Neurospora crassa* is accounted for by a number of enzymes distinguishable physically and catalytically. Of these, two nucleases specific for both DNA and RNA have been well characterized (LINN and LEHMAN 1965, 1966). More recently, an extracellular ribonuclease, RNase N_1, and two intracellular ribonucleases, RNase N_2 and N_3, have been described (TAKAI, UCHIDA and EGAMI 1966, 1967a, b). Possible functions of nucleases in cells may be the degradation of nucleic acids supplied from the surrounding environment and the turnover of nucleic acids within the cells. There arises, however, much difficulty and confusion when one attempts to understand these functions of nucleases. Furthermore, the genetics of these enzymes as well as the intracellular regulation of nucleic acid metabolism remain relatively unexplored. One approach to the elucidation of the functions of nucleases and the genetic mechanism involved in nucleic acid metabolism is to find mutants which lack one or more kinds of nuclease activity. Such mutants have been previously obtained in *Escherichia coli* (GESTELAND 1966; DÜRWALD and HOFFMANN-BERLING 1968). The present paper describes a simple screening technique for the isolation of mutants of Neurospora with the ability to digest neither DNA nor RNA and demonstrates that two genes are responsible for the activity of a particular kind of nuclease. This nuclease appears to be a complex consisting of a nuclease and an inhibitor. Preliminary reports of this work have been published (ISHIKAWA, HASUNUMA and TOH-E 1967; ISHIKAWA, TOH-E, HASUNUMA and UNO 1968).

MATERIALS AND METHODS

Materials: The wild-type strain 74A (ST. LAWRENCE) of *Neurospora crassa* was used to induce nuclease mutants and as a source of normal nucleases. Mutants 74A–Y234–M394 (*ad-1*) and 74A–T28–M4 (unknown locus), which require adenine or hypoxanthine, were also used to induce nuclease mutants. The following purine or pyrimidine-requiring mutants were used to test growth characteristics in various media: adenine-requiring mutants, *ad-4* (F2), *ad-8* (E6), *ad-3A* and *ad-6*; a guanine-requiring mutant, 74A–T51–M116 (ISHIKAWA, unpublished); pyrimidine-requiring mutants, *pyr-1* (H263), *pyr-2* (38502), *pyr-3* (KS36) and *pyr-4* (36601). The pyrimidine-requiring mutants were kindly supplied from the Fungal Genetics Stock Center, Hanover, New Hampshire.

Media: The phosphate-free medium was prepared by substituting KCl (1g/l) in Fries minimal medium for KH_2PO_4. Appropriate amounts of RNA, DNA or mononucleotides were included in the sterile phosphate-free medium as sole sources of phosphate. These media will be described in this paper as RNA medium, DNA medium or mononucleotide medium (e.g., 3′-AMP medium) depending on the supplement.

Growth measurement: To test the growth rate in various media, conidial suspensions of various strains were inoculated into 20 ml of liquid media in duplicate 100 ml Erlenmeyer flasks. Mycelial mats were harvested after a designated time, dried and weighed.

Cell-free extracts: Mycelia were grown at 25°C for designated times in 1000 ml Roux bottles containing 100 ml of Fries minimal medium. Mycelial pads were harvested on a Buchner funnel, washed with deionized water, and frozen quickly. Frozen mycelia were first ground in 0.005 M potassium phosphate buffer, pH 7.0, in a homogenizer and then disrupted through a French pressure cell at an average pressure of 400 kg/cm^2. Extracts were centrifuged at 15,000 $\times$ g for 30 min at 4°C and the supernatant solutions (crude extracts) were removed from the tubes with a syringe extending below the light lipid layer.

Fractionation of crude extracts: All the following operations were performed at 4°C. Cold ethanol (—15°C) was added with constant stirring to the crude extract to a final concentration of 75% (v/v). The precipitate formed was collected by centrifugation, dried and dissolved in 0.1 M potassium phosphate buffer, pH 7.0. The enzyme preparation was fractionated by adding an appropriate amount of solid ammonium sulfate at 0°C. The precipitate was collected by centrifugation, dissolved in 0.1 M potassium phosphate buffer, pH 7.0, and used as a sample for chromatography.

Gel filtration was performed through Sephadex G-100. A gel column of suitable size was equilibrated and eluted with 0.005 M potassium phosphate buffer, pH 7.0. Blue Dextran 2000 and NaCl were included in all runs as indicators for the void volume and the end of elution, respectively. Elution positions of these indicators (arrows) added to the samples were determined by blue color for Blue Dextran (BD) and by precipitation with $AgNO_3$ for NaCl. A diethylaminoethyl (DEAE)-cellulose column (1.5 $\times$ 35 cm) was equilibrated with 0.005 M potassium phosphate buffer, pH 7.0. Gradient elution was carried out with NaCl in a range 0–0.3 M.

Determination of RNase and DNase activities: The nuclease activities were measured by a modified method of TAKAHASHI (1961) using yeast RNA and salmon sperm DNA as substrates. The 1.0 ml reaction mixture for assaying the ribonuclease (RNase) activity contained 1.5 mg RNA, 0.1 M potassium phosphate buffer, pH 6.0, 0.005 M ethylenediamine tetraacetic acid (EDTA) and 0.2 ml of enzyme fraction. The reaction mixture for assaying the deoxyribonuclease (DNase) activity contained 0.25 mg DNA instead of RNA and no EDTA. The reaction was started by the addition of substrate to the reaction mixture. Incubation was carried out at 37°C for 18 or 60 min to assay RNase or DNase, respectively. The reaction was stopped by adding 0.25 ml of 25% perchloric acid containing 0.75% uranyl acetate. The mixture was allowed to stand in ice water at least for 10 min. and was centrifuged at 3,000 rpm for 15 min. 0.2 ml of the supernatant was diluted with 5 ml of water and the optical density at 260 mμ was measured. One unit of activity is defined as the amount of enzyme producing a change of absorbance of 1.0 under the conditions described above. The specific activity of RNase or DNase is expressed as units of activity per mg protetin.

Determination of proteolytic activity: Casein digestion was followed by incubating 1 ml of crude extract with 1 ml of a 1% solution of casein in 0.1 M phosphate buffer (pH 6.7) at 37°C for 20 min. The reaction was started by the addition of casein to the reaction mixture and stopped by the addition of 3 ml of 0.11 M trichloroacetic acid containing 0.22 M sodium acetate and 0.33 M acetic acid. After centrifugation, the absorbance at 280 mμ was determined against the blank to which the trichloroacetic acid solution described above was added at zero time. One unit of activity is defined as the amount of enzyme producing a change of absorbance of 1.0 under the conditions described above.

Assay for nuclease inhibitor activity: The activity of nuclease inhibitor was measured according to the method of ROTH (1958). An inhibitor preparation was added in the RNase-assay mixture containing nuclease N_3' described later before the addition of substrate. After leaving at 0°C for 60 min, the RNase activity was measured as described above. The unit of inhibitor is defined as the unit of RNase activity that is 50% inhibited by the particular inhibitor preparation being tested.

Protein measurement: Protein concentration was determined by the method described by LOWRY, ROSEBROUGH, FARR and RANDALL (1951).

Chemicals: RNA was kindly supplied by PROF. F. EGAMI. Salmon sperm DNA was a product of Calbiochem. (highly polymerized, A grade). Other special chemicals used were: casein (Merck); Sephadex G-100 and Blue Dextran (Pharmacia); DEAE-cellulose (Brown Co.).

RESULTS

Isolation of nuclease mutants: The Neurospora wild type shows considerable growth in RNA or DNA media. Some adenine requiring mutants can also utilize both RNA and DNA as a source of adenine. Nuclease mutants which can not grow in the RNA medium were obtained from wild-type 74A or adenine-requiring mutants by the filtration-concentration technique of WOODWARD, DE ZEEUW and SRB (1954) as revised by CASE (1963). Macroconidia, untreated or exposed to ultraviolet light (UV) or X rays, were utilized to obtain mutants. Conidia suspended in the RNA medium were incubated at 34°C in a low speed reciprocating shaker. Following filtration, platings were made on minimal agar (in case of an adenine-requiring mutant, adenine was added), and single colonies were isolated for testing. Nuclease mutants were identified by testing growth ability in liquid minimal medium supplemented with 100 μg RNA in place of KH_2PO_4. 168 nuclease mutants which do not utilize RNA have been thus isolated to date (Table 1).

Classification of nuclease mutants: Preliminary classification of nuclease mutants was made by means of the heterocaryon tests described by ISHIKAWA (1962). Utilizing several arbitrarily chosen mutant strains as standards, all other nuclease mutants were tested for heterocaryon complementation with these strains by mixing conidial suspensions of the two mutants on the RNA agar medium. On the basis of these heterocaryon tests, two distinct groups of mutants were detected: group A (58 mutants) and group B (110 mutants) as described in Table 1. Combinations between group A mutants and group B mutants were found to show a positive heterocaryon response on the RNA medium after two days at 25°C.

Further evidence for two distinct groups of mutants has been obtained from genetic studies. Opposite mating types were obtained through backcrosses with 3.1a wild type. Five independent crosses between mutants in groups A and B gave approximately 25% wild-type segregants, indicating that groups A and B are

TABLE 1

Origin of nuclease mutants

Group	Mutants derived from conidia exposed to: UV	X rays	No treatment	Total
A	24	30	4	58
B	39	71	0	110
Total	63	101	4	168

not linked. Crossing analyses have shown that group A mutants are located at one locus in linkage group I which is designated as nuclease-1 (*nuc-1*). Group B mutants are located at a second locus in linkage group II which is designated as nuclease-2 (*nuc-2*). In the preliminary report (ISHIKAWA, HASUNUMA and TOH-E 1967) these two loci were designated as *RNase-1* and *RNase-2*, respectively. In subsequent work, A1 (74A-T28-M1) and B1 (74A-T28-M2) were usually used as the two representative mutant strains for groups A and B, respectively. A double mutant of these two loci, A1 B1 was isolated after tetrad analysis of a cross between A1 and B1.

Growth characteristics of nuclease mutants: The wild-type 74A and nuclease mutants isolated were examined for their response to various concentrations of RNA, DNA and nucleotides as shown in Figure 1. The wild type utilizes these substances for growth on a minimal medium without phosphate. Nuclease mutants exhibit negative or very much reduced growth responses on RNA or DNA media. Group B mutants show considerable leaky growth in the RNA medium. Mutants of both groups could utilize adenosine 3′-monophosphate (3′-AMP) as a source of phosphate as shown in Figure 1. Other nucleotides, adenosine 5′-mono-

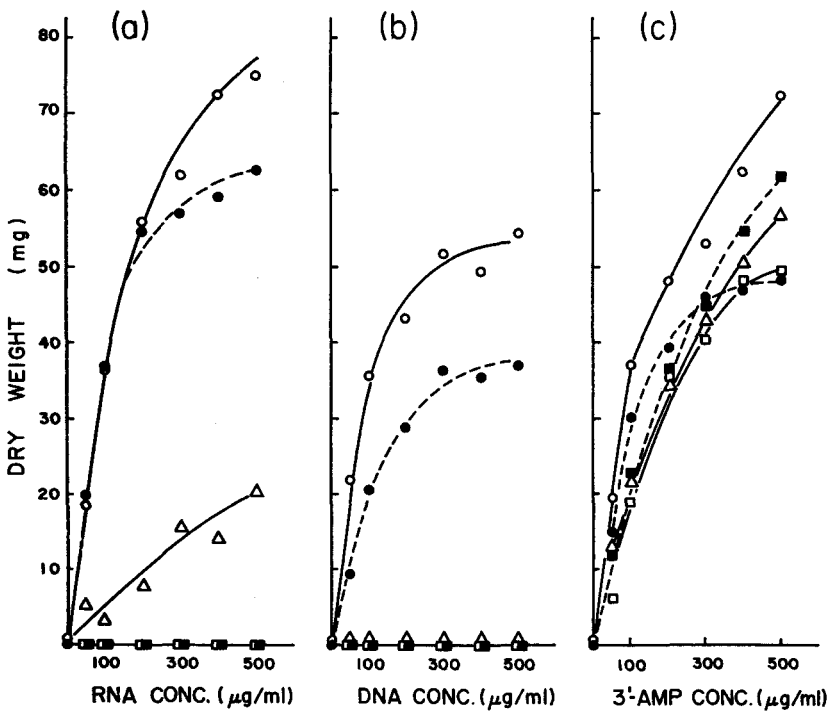

FIGURE 1.—Growth characteristics of the wild type and nuclease mutants in phosphate-free media supplemented with various amounts of RNA, DNA or 3′-AMP. Growth is expressed as mg dry weight of mycelia in 100 ml Erlenmeyer flasks containing 20 ml of media at 25°C for 72 hrs. (a) RNA media. (b) DNA media. (c) 3′-AMP media. Strains used: 74A (○); A1 (□); B1 (△); A1B1, double mutant (■); A1 + B1, heterocaryon (●).

phosphate (5′-AMP), guanosine 3′-monophosphate, (3′-GMP), guanosine 5′-monophosphate (5′-GMP), uridine 5′-monophosphate (5′-UMP) and cytidine 5′-monophosphate (5′-CMP), were equally utilized as sources of phosphate. However, the growth of nuclease mutants on nucleotide media was slightly less than that of the wild type. Heterocaryons formed between A1 and B1 utilized nucleic acids although the amount of growth in nucleic acid media was less than that of the wild type. Nuclease mutants show no significant difference from the wild type in either growth rate in minimal medium (Table 2) or in morphological characteristics.

Mutants requiring purines or pyrimidines were tested for their ability to utilize nucleic acids as a source of purine or pyrimidine which these mutants require (Table 2). Adenine-nonspecific mutants such as *ad-1*, *ad-3* and *ad-6* mutants which grow on either adenine or hypoxanthine utilized RNA or DNA as a source of adenine. Phosphate in the culture media repressed significantly the utilization of nucleic acids as indicated in Table 2. Double mutants between *ad-6* and A1 or B1 failed to utilize nucleic acids as a source of adenine, indicating that A1 and B1 mutations are really related to the supply of purine from nucleic acids. Adenine-specific mutants such as *ad-4* and *ad-8* mutants which do not grow on hypoxanthine did not utilize nucleic acids as a source of adenine. A guanine-requiring mutant utilized nucleic acids slowly as a source of guanine. It is well known that the growth of adenine-specific mutants is stimulated by the presence of histidine (MAGASANIK 1958; ISHIKAWA 1962). However, the growth response of adenine- and guanine-requiring mutants to nucleic acids was not affected by the addition of histidine. Pyrimidine-requiring mutants at the four known loci, *pyr-1*, *pyr-2*, *pyr-3* and *pyr-4*, showed no significant growth on nucleic acids as a source of pyrimidine.

Nuclease activity in wild type and nuclease mutants: The wild type and some of the nuclease mutants were grown on minimal medium and the RNase and DNase activities of culture filtrates and crude mycelial extracts prepared from them were assayed (Table 3). No significant difference in the RNase activity was observed between culture filtrates of the wild type and of nuclease mutants, whereas no detectable DNase activity was found in culture filtrates of all strains tested. On the other hand a significant difference in the RNase and DNase activities of crude mycelial extracts was observed between the wild type and nuclease mutants.

Since crude mycelial extract of the wild type contains at least three species of RNase and of DNase (HASUNUMA and UNO, unpublished), the nuclease species responsible for the reduced nuclease activities found in crude extracts of nuclease mutants should be identified after fractionation. Therefore, crude extracts prepared from 7-day old mycelia of the wild type and nuclease mutants were subjected to filtration through Sephadex G-100. Two major peaks were found in the RNase assay in potassium phosphate buffer, pH 6.0, in the presence of EDTA (Figure 2a). The second peak corresponds in chromatographic mobility and in pH optimum to RNase N_3 described by TAKAI, UCHIDA and EGAMI (1967b) and designated here as nuclease N_3. The same chromatogram was analyzed for DNase

TABLE 2

*Comparative growth characteristics**

Strains	None +p†	RNA (μg/ml) +p† 100	+p† 500	—p† 100	—p† 500	DNA (μg/ml) +p 100	+p 500	—p 100	—p 500	Species used	Mononucleotide (μg/ml) +p 100	+p 500	—p 100	—p 500
74A	84.1	77.9	83.8	29.9	72.8	81.1	90.5	27.5	63.7	3′-GMP	80.1	84.5	31.2	74.2
A1	85.1	84.4	87.0	0	0.4	85.2	86.5	0	0	3′-GMP	81.0	83.5	7.4	57.8
B1	83.2	82.2	80.0	2.9	5.6	88.3	87.3	0.1	0.2	3′-GMP	78.3	85.9	14.6	60.8
ad-3A	0	4.5	31.0	21.0	69.4	1.1	2.0	17.4	52.0	3′-AMP	42.0	72.6	35.7	65.6
ad-6	0	2.5	24.8	26.4	64.3	1.3	2.4	20.6	54.2	3′-AMP	57.1	78.1	42.8	68.9
ad-6 A1	0	0.8	1.7	0	0	0.2	0.7	0	0	3′-AMP	53.6	77.3	17.3	48.0
ad-6 B1	0	1.7	17.1	0.8	5.7	0	0.6	0	0	3′-AMP	48.2	68.9	17.3	48.7
ad-4	0	0.3	2.0	0.4	4.2	0	0	0	0	3′-AMP	15.0	44.9	23.6	55.7
ad-8	0	0.2	0.7	0.1	1.5	0	0	0	0	3′-AMP	33.8	68.5	33.5	61.9
gua⁻‡	0	1.7	25.1	0.7	19.1	0	2.8	0	4.8	3′-GMP	44.6	62.4	16.2	55.3
pyr-1	0	0	0	0	0	0	0	0	0	5′-UMP	42.4	64.2	31.7	57.2
pyr-2	2.6	6.2	6.2	9.1	9.9	2.2	2.2	0.2	0.4	5′-UMP	49.7	69.0	28.3	74.3
pyr-3	0	0	0	0	0	0	0	0	0	5′-UMP	40.0	73.5	31.2	62.7
pyr-4	0	0	0	0	0	0	0	0	0	5′-UMP	36.1	60.9	17.9	58.1

* Growth is expressed in mg dry weight of mycelia grown in 100 ml Erlenmeyer flasks containing 20 ml of various kinds of media at 25°C for 72 hrs.

† +p and —p indicate minimal medium and phosphate-free minimal medium, respectively.

‡ Guanine requiring mutant, 74A-T51-M116.

TABLE 3

Nuclease activity in culture filtrates and crude extracts of wild type and mutant cultures of various ages

Culture age (days)	RNase activity				DNase activity			
	74A	A1	B1	A1 B1	74A	A1	B1	A1 B1
Culture filtrate*								
3	0.090	0.066	0.073	0.091	0.003	0.000	0.002	0.001
5	0.356	0.331	0.344	0.471	0.005	0.000	0.000	0.001
7	0.837	0.883	0.887	0.904	0.005	0.001	0.000	0.001
Crude extract†								
3	0.031	0.013	0.017	0.011	0.083	0.015	0.026	0.016
5	0.078	0.028	0.044	0.028	0.087	0.055	0.044	0.052
7	0.076	0.028	0.040	0.036	0.096	0.041	0.045	0.044

* Units per ml culture filtrate.
† Specific activity (units per mg protein).

activity. Two major peaks were found under the present assay conditions and the second one appeared to be identical in mobility with peak N_3 in the RNase assay (Figure 2a). Crude extracts of the two nuclease mutants, A1 and B1, were fractionated likewise through Sephadex G-100 (Figure 2b, c). In analyses for both RNase and DNase activities, the activity of peak N_3 was significantly lower in both mutants than in wild type.

The nuclease peak N_3 was partially purified by the following procedure. The crude extract prepared from 7-day old wild-type mycelia was subjected to ammonium sulfate fractionation. The nuclease activity precipitated between 40 and 60% saturation of ammonium sulfate (231 g/l and 369 g/l) at 0°C proved to be mostly peak N_3 in succeeding Sephadex G-100 filtration (Figure 3a). Peak N_3 thus obtained was further chromatographed on a DEAE-cellulose column (Figure 3b). Peak N_3 always behaved as a single peak on chromatography, showing coinciding RNase and DNase activities. These results may indicate that peak N_3 exhibits specificity toward both RNA and DNA.

Nuclease-inhibitor complex: The latency of the nuclease activity was first suggested by the fact that the RNase activity of a mycelial extract increased considerably after incubation at 37°C. To analyze the activation process, the crude extract from 7-day old mycelia was prepared in potassium phosphate buffer (0.4 M, pH 6.0) containing $MgCl_2$ (0.001 M) and incubated at 37°C for several hours. 100 μg per ml of streptomycin and penicillin were added in the incubation mixture to prevent bacterial contamination. The RNase activity was assayed without $MgCl_2$ at pH 6.0. The maximum activation has been attained after 3 hours' incubation under the same conditions (Figure 4). A similar time and amount of maximum activation was observed for both mutants (A1, B1, A1B1) and wild type.

Crude extract obtained from wild-type mycelia was incubated as described above for 3 hrs, fractionated with ammonium sulfate, and subjected to filtration through Sephadex G-100. A new peak (fraction number 60–80) which is desig-

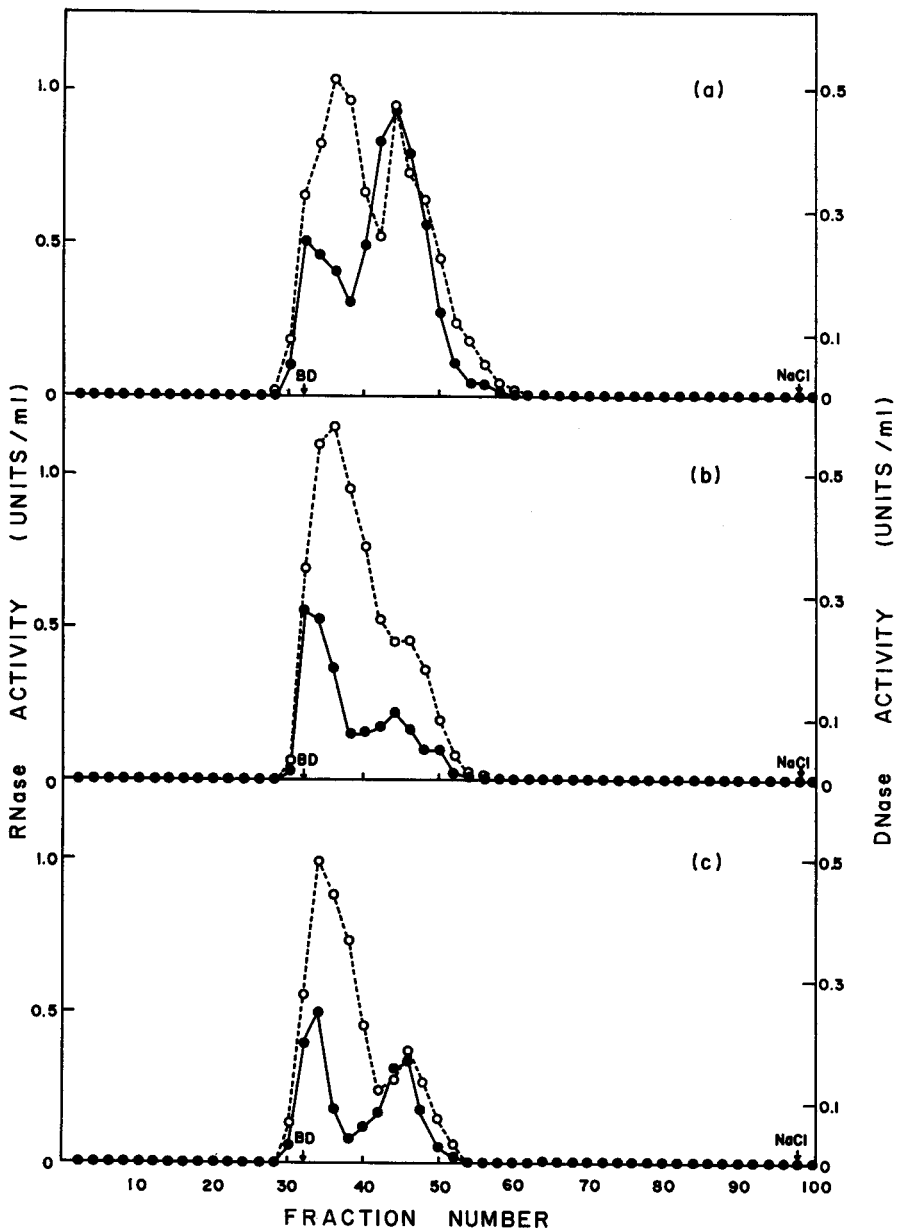

FIGURE 2.—Filtration of crude extracts through Sephadex G-100. Crude extract obtained from 7-day old mycelia was precipitated with ethanol and subsequently with 100% ammonium sulfate. The enzyme preparation thus prepared was put onto a gel column (1.5 × 106 cm) and eluted into 2 ml fractions with 0.005 M potassium phosphate buffer (pH 7.0). Each fraction was assayed for the RNase and DNase activities. Elution positions of Blue Dextran (BD) and NaCl are indicated by arrows. (a) 74A. (b) A1. (c) B1. RNase activity (○); DNase activity (●).

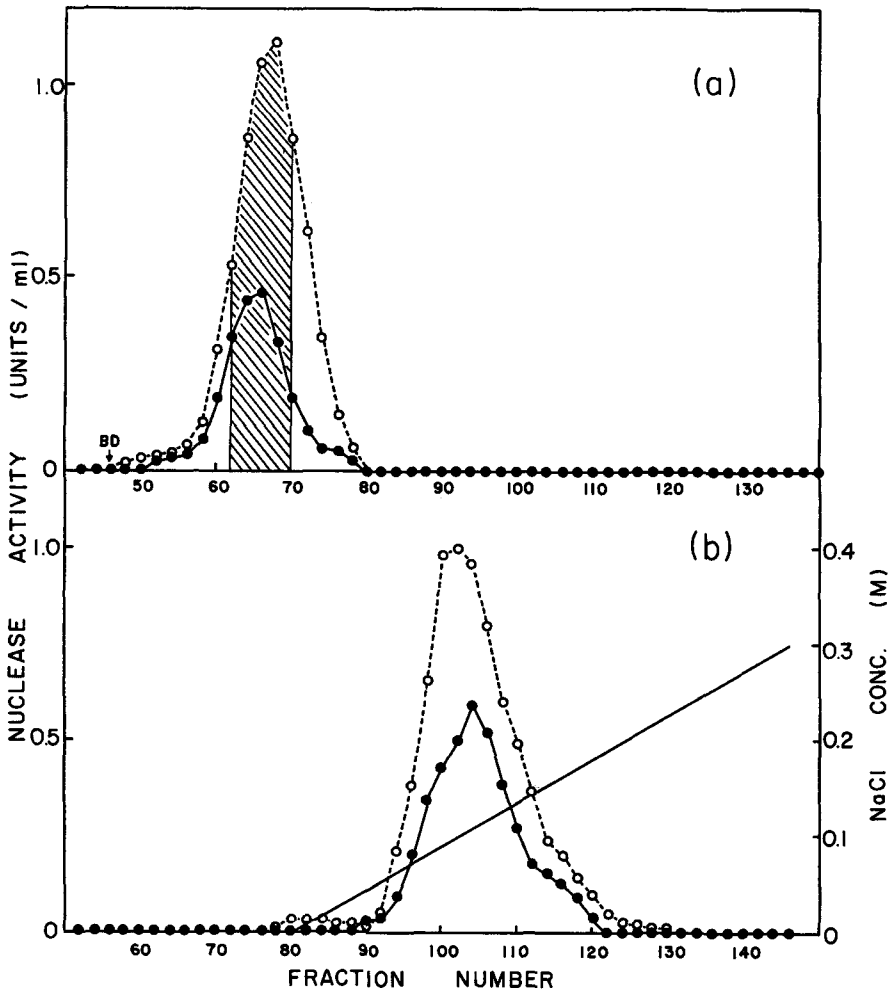

FIGURE 3.—DEAE-cellulose chromatography of peak N_3 of the wild type obtained from Sephadex G-100 filtration. Crude extract obtained from 7-day old mycelia was precipitated with ethanol. The enzyme preparation subsequently precipitated between 40 and 60% ammonium sulfate saturation was filtered through a Sephadex G-100 column (3 × 75 cm). N_3 fraction shown by hatched region in the gel filtration was collected and chromatographed on a DEAE-cellulose column. Fractions (4.0 ml) were eluted with a linear gradient of NaCl from 0 to 0.3 M. (a) Sephadex G-100 filtration. (b) DEAE-cellulose chromatograph. RNase activity (○); DNase activity (●); gradient of NaCl concentration (—).

nated here as nuclease N_3' (designated previously as nuclease N_5 by ISHIKAWA *et al.* 1968) was observed after 3 hours' incubation at 37°C (Figure 5). The ratios of the DNase activity to the RNase activity at peak N_3 and N_3' appeared to be different from preparation to preparation. The shift in these ratios from N_3 to N_3' observed after 3 hours' incubation at 37°C was not reproducible. The DNase activity found in fractions 30–40 was increased but the RNase activity found in

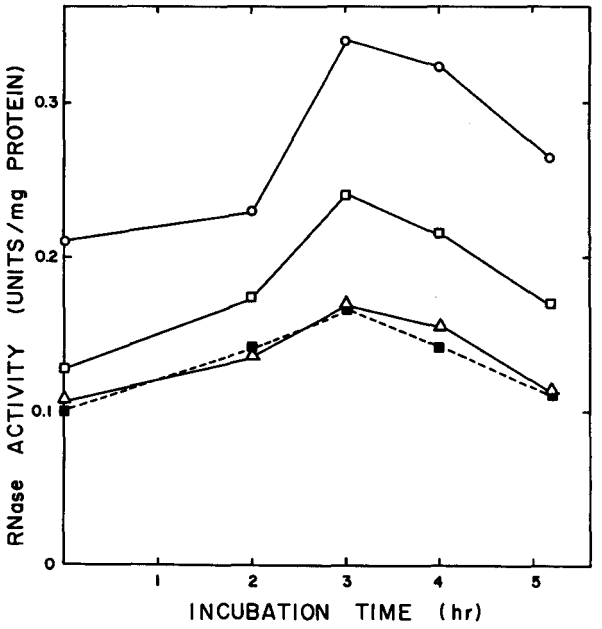

FIGURE 4.—Effect of preincubation at 37°C on the RNase activity of crude extracts prepared from 7-day old wild-type and mutant mycelia. Preincubation was made in 0.4 M potassium phosphate buffer, pH 6.0 containing 0.001 M $MgCl_2$. 100 μg per ml of streptomycin and penicillin were added in the preincubation mixture to prevent bacterial contamination. See Figure 1 for symbols.

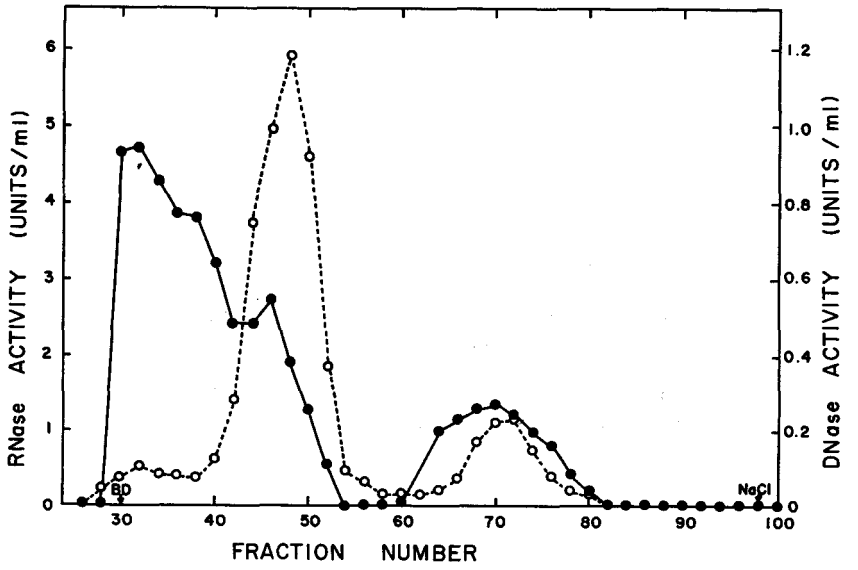

FIGURE 5.—Filtration of nucleases through Sephadex G-100 column (1.5 × 106 cm) after incubating crude extract of wild-type mycelia at 37°C for 3 hrs and precipitated with 60% ammonium sulfate. RNase activity (O); DNase activity (●).

the same fractions was significantly reduced after incubation and subsequent ammonium sulfate fractionation. To confirm that peak N_3' had been derived from peak N_3 during the incubation, the N_3 fraction in the first gel filtration was collected, incubated at 37°C for another hour with 0.13 M potassium phosphate buffer, pH 6.0, containing 0.0003 M $MgCl_2$, and rechromatographed through a Sephadex G-100 column. On the chromatogram thus obtained, only peak N_3' was observed instead of peak N_3 (Figure 6). This led to the conclusion that peak N_3' was actually derived from peak N_3. The mobility ratio of N_3' to N_3 indicates a reduction of molecular weight of *ca.* 60%.

Since proteolytic activity was observed in mycelial extracts of Neurospora, a possible participation of proteolytic enzymes in the activation of nuclease N_3 was considered. Ten μg per ml of subtilopeptidase A was enough to activate nucleases in the crude extract in 1 hr incubation at 37°C as shown in Figure 7. A rapid inactivation of nucleases was observed, however, at higher concentrations of subtilopeptidase A. The proteolytic activity of crude extracts obtained from the wild type and nuclease mutants, A1 and B1, was assayed, and no essential differences were found among these strains as indicated in Table 4.

To analyze the process of production of nuclease N_3' from N_3, the following chemical reagents have been tested for their ability to produce peak N_3'; monoiodoacetate, *p*-chloromercuribenzoate (*p*-CMB), sodium dodecyl sulfate and β-mercaptoethanol. N_3 fraction was obtained from wild-type mycelia and treated

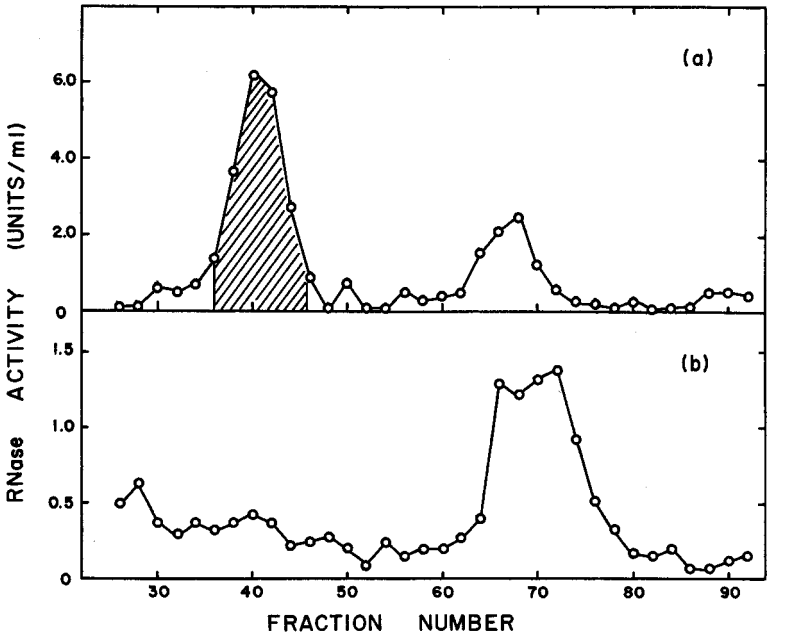

FIGURE 6.—Production of peak N_3' from peak N_3. N_3 fraction obtained from the first Sephadex G-100 filtration (1.5 × 106 cm column) as shown by hatched region was incubated at 37°C for 1 hr and then applied on another column. The RNase activity was measured. (a) The first chromatograph. (b) The second chromatograph (after treatment).

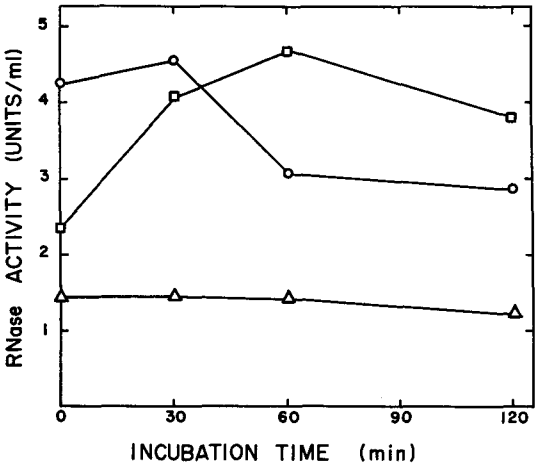

FIGURE 7.—Treatment of crude extract with subtilopeptidase A. Crude extract from 74A mycelia was incubated at 37°C in 0.4 M potassium phosphate buffer, pH 6.0 containing 0.001 M $MgCl_2$. Aliquots of crude extract thus incubated were removed at the designated time and assayed for the RNase activity. Concentrations of subtilopeptidase A: 0 (△); 10 μg/ml; (□); 100 μg/ml (○).

with these reagents for 10 min at 37°C. 5 ml of N_3 fraction thus treated (25 units of RNase activity) were filtered through a Sephadex G-100 column (2 × 38 cm), and the RNase activity was measured for fractions obtained after the gel filtration. As shown in Figure 8, *p*-CMB (0.001 M) and sodium dodecyl sulfate (2%) were effective in producing peak N_3', whereas β-mercaptoethanol (0.001 M) had no apparent effect on the N_3 complex. The treatment with monoiodoacetate (0.01 M) resulted in the production of peak N_3' with simultaneous disappearance of peak N_3.

In a crude mycelial extract some part of nuclease N_3 appears to exist in an active state, while another part remains in an inactive state. The concentration of inactive nuclease N_3 involved in a mycelial extract was compared among the wild type and nuclease mutants. N_3 fractions were prepared from 14-day-old mycelia and incubated at 37°C for 2 hrs to activate nuclease N_3. N_3 fractions containing the same amount of the RNase activity (27 units) were further treated with monoiodoacetate (0.01 M) at 37°C for 40 min, and fractionated through a

TABLE 4

*Proteolytic activity in crude extracts of wild type and mutant mycelia of various ages**

Culture age (days)	74A	A1	B1	A1 B1
3	0.308	0.253	0.304	0.292
5	0.320	0.272	0.270	0.280
7	0.248	0.282	0.283	0.299

* Specific activity (units per mg protein).

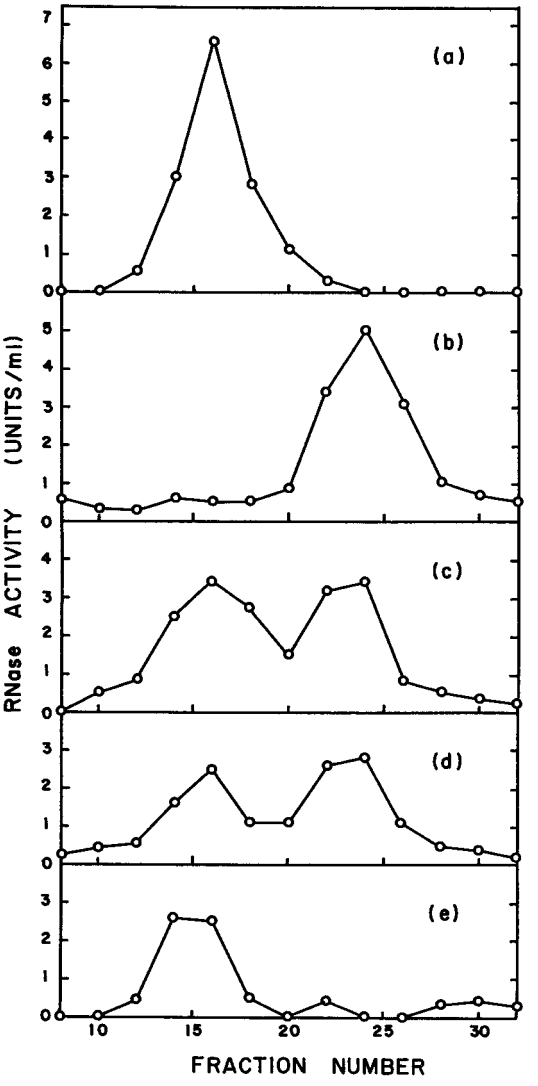

FIGURE 8.—Gel filtration of nuclease N_3 complex treated with various reagents through Sephadex G-100. Nuclease N_3 was obtained from 7-day old wild-type mycelia after Sephadex G-100 filtration, and treated with monoiodoacetate (0.01 M), p-CMB (0.001 M), sodium dodecyl sulfate (2%) and β-mercaptoethanol (0.001 M) at 37°C for 10 min. 5 ml of N_3 fraction (25 units of RNase activity) thus treated were filtered through a Sephadex G-100 column (2 × 38 cm). 4 ml fractions were collected and assayed for the RNase activity. (a) No treatment. (b) Monoiodoacetate. (c) *p*-CMB. (d) Sodium dodecyl sulfate. (e) β-mercaptoethanol.

Sephadex G-100 column (2 × 38 cm). As shown in Figure 9, N_3 activities were reduced and peak N_3' was observed. The RNase activity of peak N_3' produced from the N_3 fraction of the mutant strain B1 was three times as high as that produced from N_3 fraction of 74A or A1. The result indicates that group B mu-

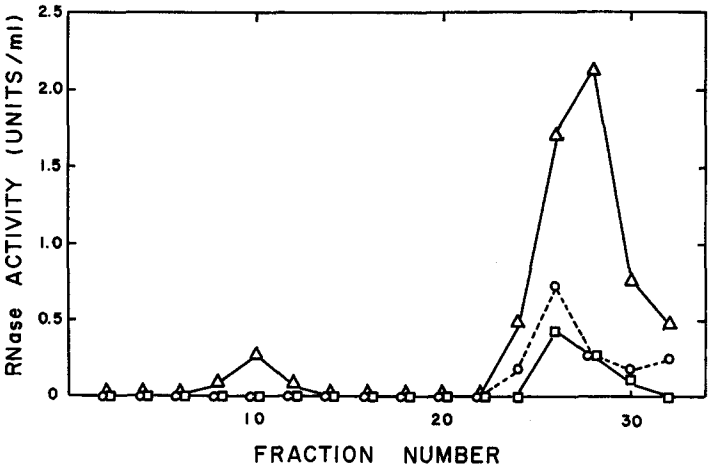

FIGURE 9.—Amount of peak N_3' appearing after the treatment of peak N_3 with monoiodoacetate. Peak N_3 fractions were prepared from 14-day old mycelia of the wild type and nuclease mutants after gel filtration and incubated at 37°C for 2 hr. 5.4 ml of N_3 fraction (27 units of RNase activity) thus prepared were treated further with 0.01 M monoiodoacetate at 37°C for 40 min. The RNase activity was assayed for fractions obtained through a Sephadex G-100 column (2 × 38 cm). See Figure 1 for symbols.

tants have an excess of latent nuclease N_3 *in vivo* which is dissociated and activated by monoiodoacetate, and also suggests that the *nuc-2* mutation results in the alteration of the binding properties or of the dissociation process of nuclease N_3' and a natural inhibitor.

Characteristics of the inhibitor: It was found that the crude mycelial extract inhibits the RNase activity of N_3' fraction when added to the reaction mixture in the RNase assay. This may reflect the presence of free inhibitor molecules in crude extracts. The free inhibitor could be assayed against the N_3' fraction prepared from an extract of 14-day old wild-type 74A mycelia by incubation at 37°C for 4 hr. The inhibitor activity of crude extracts from 5-day old and 10-day old mycelia of the wild type and nuclease mutants was assayed. As described in Table 5, 5-day old mycelia appear to contain a larger amount of inhibitor activity than 10-day old mycelia in all strains tested. Crude extracts from B1 and the double mutant, A1 B1, exhibited a tendency to have higher inhibitor activity than those from the wild type and A1. The result may indicate that B1 mycelia contain a more efficient inhibitor or a larger amount of inhibitor.

The inhibitor activity found in wild-type mycelial extract was stable at 37°C for at least 1 hr in 0.50 M potassium phosphate buffer, pH 6.0, containing 0.0005 M $MgCl_2$, but it was completely inactivated at 60°C within 15 min. No significant difference has been observed in the heat stability among inhibitors obtained from the wild type and nuclease mutants.

DISCUSSION

A large number of nuclease mutants in *Neurospora crassa* has been isolated by a simple selection procedure for their inability to digest nucleic acids added

TABLE 5

Inhibitor activity found in crude extracts of wild type and nuclease mutants

Strain	Culture age (days)	Amount of protein (mg/0.1ml extract)	Inhibitor activity* (units/0.1ml extract)	Specific activity (units/mg protein)
74A	5	0.341	0.189	0.560
	10	0.218	0.089	0.411
A1	5	0.302	0.149	0.495
	10	0.200	0.064	0.322
B1	5	0.315	0.228	0.722
	10	0.204	0.103	0.504
A1 B1	5	0.311	0.246	0.791
	10	0.223	0.142	0.637

* Unit of inhibitor activity is defined as the unit of nuclease N_3' activity that is 50% inhibited by the crude extract being tested, when added in the RNase-assay procedure before the addition of substrate and left at 0°C for 60 min.

to the culture medium. A single mutation resulted in a loss of ability to digest either RNA or DNA. Sephadex G-100 filtration experiments revealed that the mutation resulted in reduced nuclease activity of a particular nuclease fraction, N_3 present in cell extracts. The N_3 fraction has been previously reported by TAKAI *et al.* (1967b) to be a kind of ribonuclease which digests RNA, producing guanylate and oligonucleotides with a terminal guanylate. In the present work, it was shown that exactly the same elution profiles of the N_3 fraction on Sephadex G-100 filtration and subsequent DEAE-cellulose chromatography were obtained for both RNase and DNase activities. Furthermore, nuclease N_3' derived from the N_3 fraction by certain treatments also exhibited specificity toward both RNA and DNA. This evidence indicates that peaks N_3 and N_3' are two kinds of nuclease which have specificity toward both RNA and DNA. However, more work is required to explain why the ratios of RNase activity to DNase activity were different from preparation to preparation. RNase N_1 found in culture filtrate of the wild type (TAKAI *et al.* 1966) appears to have a similar molecular weight and the same specificity to produce guanylate as compared with nuclease N_3'. However, no DNase activity was observed in culture filtrates of both wild type and nuclease mutants, and the activity of RNase N_1 of the mutants was of the same level as that found in the wild type. Thus, it is concluded that RNase N_1 and nuclease N_3' are different molecular species and that the nuclease mutants described have normal RNase N_1.

The digestion products of nucleic acids by Neurospora cells *in vivo* may not include pyrimidine except as oligonucleotides, since pyrimidine-requiring mutants were unable to utilize nucleic acids. The fact that adenine-non-specific mutants were able to utilize nucleic acids but adenine-specific mutants were not may indicate that the digestion products of nucleic acids may not be adenylate. Since adenine-non-specific mutants are unable to grow on guanylate and a guanine-requiring mutant utilizes nucleic acids at significantly low rates, it is

suggested that the digestion products of nucleic acids *in vivo* may not include a significant amount of guanylate. Therefore, it is postulated from these results that the digestion products of nucleic acids are immediately transformed to inosinate or its derivatives as a precursor of adenylate and guanylate.

Evidence has been presented that nuclease N_3 goes to N_3' and that possibly IN_3' goes to N_3' and I, where I is an inhibitor molecule. The mobility change of N_3 to N_3' on the Sephadex G-100 chromatogram indicates either that N_3 is a polymer enzyme or that N_3 releases I which has *ca.* 60% of the molecular weight of N_3. The fact that the crude mycelial extract inhibits the RNase activity of N_3' may indicate the existence of free inhibitor molecules in crude mycelial extracts. The existence of a complex of nuclease and its inhibitor is well known in other organisms (ROTH 1956, 1958; LINDBERG 1967; SMEATON and ELLIOTT 1967). Fraction N_3 may be heterogeneous, containing both active and inactive forms of nuclease, or it may contain only one, partially active species. The nuclease activity found in the crude mycelial extract and identified as the N_3 complex in the first gel filtration was further activated after certain treatments. Free nuclease N_3' molecules may not be produced *in vivo*, since no N_3' fraction was observed without particular treatments such as incubation of extract at 37°C or its treatment with particular chemical reagents. The treatment of N_3 complex with monoiodoacetate, *p*-chloromercuribenzoate or sodium dodecyl sulfate led to the formation of nuclease N_3'. β-mercaptoethanol showed no such effect on N_3 complex. These results may suggest that cysteine residue(s) participate in transforming the complex from an inactive state to an active state and in binding between nuclease N_3' and the natural inhibitor molecule. The inhibitor (if it is a distinct species) may be a protein since proteolysis led to dissociation, but its exact nature has not yet been elucidated.

Two genes, nuclease-1 (*nuc-1*) and nuclease-2 (*nuc-2*), have been identified in genetic analyses of nuclease mutants isolated. The mutations in either gene resulted in a similar phenotype, except that mutants at the *nuc-2* locus were more leaky in the RNA medium than those at the *nuc-1* locus. Enzymatic analyses indicated that the activity of nuclease N_3 was significantly reduced in both groups of mutants. *nuc-1* mutants have decreased activity of nuclease N_3' as well as nuclease N_3. *nuc-2* mutants possess supernormal latent nuclease N_3 activity as revealed by the treatment with monoiodoacetate (Figure 9). A possible participation of proteolytic enzymes is proposed in the process of activation of nuclease N_3 *in vivo*. However, the existence of more latent nuclease activity in mutant strain B1 may not be due to the lower activity of proteolytic enzymes in the cell, since no difference has been observed in the activity of proteolytic enzymes of the mycelia among the wild type and nuclease mutants (Table 4). Although no direct evidence has been obtained in this study for an altered inhibitor in these mutants, it was shown that crude extracts obtained from *nuc-2* mutants exhibited an elevated level of inhibition. From these data it is suggested that the mutation at the *nuc-2* locus does not affect the nuclease molecule itself, but increases the binding affinity of the inhibitor for free nuclease, N_3', resulting in a more completely inhibited complex.

Thus the evidence obtained here suggests that the *nuc-1* gene controls directly the activity of nuclease N_3' and that the *nuc-2* gene affects the nuclease activity by controlling the activity of an inhibitor in some way. An alternative possibility may be that one of two genes controls a factor which has an effect on the dissociation of nuclease N_3 to N_3' and I, or on the depolymerization of nuclease N_3 to N_3'. To obtain a final conclusion, characterization of nuclease N_3, N_3' and inhibitor should be performed and direct effects of mutation on these molecules should be studied. Isolation and characterization of temperature-sensitive nuclease mutants may help to elucidate the roles of these genes.

Nuclease mutants isolated to date have no obvious defects that make them morphologically different from the wild-type strain. The same result has been obtained for RNase and DNase mutants isolated in *Escherichia coli* (DÜRWALD and HOFFMANN-BERLING 1968). However, Neurospora nuclease mutants show slightly reduced growth in mononucleotide media. This may be due to a secondary physiological alteration in phosphate metabolism and further work on this point is now underway. Furthermore, our preliminary data indicate that *nuc-2* mutants are more sensitive to ultraviolet light irradiation than the wild type (UNO and ISHIKAWA 1969). It seems most likely that these mutant strains will be useful for studies on the physiological roles and regulation mechanisms of nucleases in Neurospora.

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SUMMARY

One hundred and sixty eight mutants which utilize neither RNA nor DNA as a sole source of phosphorus have been isolated in *Neurospora crassa*. A single mutation results in a loss of ability to digest either RNA or DNA. Two genes, nuclease-1 (*nuc-1*) and nuclease-2 (*nuc-2*), were found to be responsible for such nucleic acid digestion. These mutants showed a reduced activity of a nuclease fraction, N_3, which was found in the wild-type cell extract and digested both RNA and DNA. After treatment with reagents such as monoiodoacetate or after incubation at 37°C, the N_3 fraction was transformed into another nuclease fraction N_3'. It was suggested that nuclease N_3 is a complex consisting of nuclease N_3' and an inhibitor molecule. Comparative studies of mutants indicated that the *nuc-1* locus may be responsible for nuclease N_3' and the *nuc-2* locus for the inhibitor.

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