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An Endonuclease from Mitochondria of *Neurospora crassa**

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SUMMARY

Endonuclease activity has been found to be closely associated with mitochondria of *Neurospora crassa*. The evidence for this association is that: (*a*) the activity sediments with mitochondria even after extensive washing; (*b*) purified nuclease is not bound by freshly prepared mitochondria; (*c*) nuclease and cytochrome oxidase activities cosediment through a sucrose gradient; and (*d*) the mitochondria must be disrupted in order to observe significant activity. The possibility has not been eliminated, however, that this association represents the binding to mitochondria of a nuclease of extramitochondrial origin.

The enzyme has been purified from both mitochondria and crude mycelial extracts. It degrades both deoxyribonucleic and ribonucleic acids to small oligonucleotides terminated by 5′-phosphomonoester groups, is most active at 37°, between pH values of 6.0 and 7.5, requires a divalent cation (Mg^{++}, Mn^{++}, or Co^{++}), is inhibited by ethylenediaminetetraacetic acid, and is relatively unaffected by β-mercaptoethanol. These catalytic properties distinguish it from the extramitochondrial nuclease previously described in *N. crassa*.

In the course of purification of an endonuclease from *Neurospora crassa* specific for polynucleotides lacking an ordered structure, an additional nuclease activity was observed (1, 2). Closer examination indicated that at least part of this activity was due to an enzyme closely associated with mitochondria. This enzyme has been partially purified and its properties have been examined. It is an endonuclease acting on ribonucleic acid and on native and denatured deoxyribonucleic acid and is clearly distinguishable from the extramitochondrial endonuclease described earlier (1, 2).

The presence of nuclease activity within mitochondria may have significance with regard to mitochondrial self-replication (3), particularly in view of recent observations concerning the existence of DNA (4–8), RNA (6, 9), RNA polymerase (4, 5), ribosomes (9), and peptide synthesis (10–13) within this organelle.

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EXPERIMENTAL PROCEDURE

Materials—^{32}P-Labeled *Escherichia coli* DNA was isolated according to Lehman (14). Transfer RNA, ribosomal RNA, salmon sperm DNA, and ^{32}P-dTMP were obtained as described previously (1). Concentrations of RNA and DNA are expressed as equivalents of nucleotide-phosphate.

Alkaline phosphatase from *E. coli*, free of diesterase activity, was purchased from Worthington and assayed as previously described. 5′-Nucleotidase was purified from *Crotalus adamanteus* venom (15). One unit of alkaline phosphatase and of 5′-nucleotidase is that amount of enzyme which catalyzes the formation of 1 μmole of inorganic phosphate per hour under the specified conditions. The hydrolysis of nuclease digests by these enzymes was observed by the appearance of ^{32}P, which did not adsorb to Norit (1).

Cytochrome *c* was obtained from Boehringer-Mannheim and reduced with sodium borohydride as described by Martin *et al.* (16).

Phosphocellulose, DEAE-cellulose, and hydroxylapatite were obtained and processed as before (2).

Polyethylene glycol (Carbowax 6000) was a product of Union Carbide.

Assays—The deoxyribonuclease assay measured the conversion of ^{32}P-labeled *E. coli* DNA to ^{32}P, which was soluble in perchloric acid as described previously (1). The reaction mixtures (0.3 ml) contained buffer and divalent cation as indicated for each experiment, 25 mμmoles of denatured or native ^{32}P-labeled *E. coli* DNA, and sufficient enzyme to convert 5 to 20% of the substrate to acid-soluble products. Enzyme dilutions were made in the assay buffer at 0.05 M, and incubations were for 30 min at 37°. One unit of activity is that amount catalyzing the production of 1 μmole of acid-soluble nucleotide in 30 min.

Ribonuclease activity was assayed according to Linn and Lehman (1).

Protein concentrations were determined according to the method of Waddell (17) unless otherwise indicated.

For assay of cytochrome oxidase, the reaction mixture (3 ml) contained 13.3 μM reduced cytochrome *c*, 0.11 M potassium phosphate, pH 7.4, and 6.67 μM EDTA. After equilibration to room temperature, enzyme was added (0.02 ml), and the drop in optical density at 550 mμ was measured at 30-sec intervals with a Zeiss PMQ II spectrophotometer at room temperature (18). Assays with a change of optical density of 0.01 to 0.03 per min were considered valid. One unit of activity is that amount of enzyme producing a change of absorbance of 1.0 per min.

Preparation of Mitochondria—Mycelia were grown in 2800-ml Fernbach flasks containing 500 ml of Vogel's synthetic medium

(19) supplemented with 2% sucrose; mycelia from six flasks of 60- to 65-hour cultures (15 to 20 g, wet weight) were normally used, although mycelia which had been subsequently starved in phosphate buffer for an additional 5 days (2) gave identical mitochondrial and enzyme preparations. All subsequent operations were performed at 0–4°.

Mitochondria were purified by differential centrifugation according to Hall and Greenawalt (20). Mycelia were harvested by filtration through gauze, washed with distilled water, cut into small pieces, suspended in 250 ml of mitochondrial medium (0.25 M sucrose-0.001 M EDTA-0.15% bovine plasma albumin), and disrupted in a Gifford-Wood-Eppenbach Micro-Mill as described by Hall and Greenawalt (20). In some experiments, the mycelia were ground for 5 min with 20 g of sand by means of a mortar and pestle, and were then suspended in 250 ml of mitochondrial medium. The heavy sediment was allowed to settle out and the supernatant fluid was centrifuged for 10 min at 500 × *g;* the pellet was discarded. The 500 × *g* supernatant fluid was centrifuged for 10 min at 1500 × *g*, and the pellet was discarded. The 1500 × *g* supernatant fluid was then centrifuged for 20 min at 8000 × *g* and the pellet was resuspended in 10 ml of mitochondrial medium. This suspension was recentrifuged at 1500 × *g* and 8000 × *g* as above and the second 8000 × *g* pellet was resuspended in 5 ml of mitochondrial medium. A final removal of material at 1500 × *g* yielded the mitochondrial suspension used in all studies to be described. This fraction contained roughly 1.5% of the total cellular protein, and on sedimentation through a sucrose gradient as described in Fig. 1, 60 to 85% of its protein sedimented with the cytochrome oxidase activity.

RESULTS

Association of Nuclease Activity with Mitochondria—Mitochondria isolated from fresh mycelia were found to contain nuclease activity distinct from that found in the extramitochondrial fraction. For example, the mitochondrial fraction showed no activity when Ca^{++} was the divalent cation with either native or denatured DNA as substrate, whereas the 8000 × *g* supernatant fraction catalyzed a rapid hydrolysis of denatured DNA in the presence of Ca^{++} (Table I).

After sedimentation of the mitochondria in a sucrose gradient (Fig. 1), the ratio of cytochrome oxidase to nuclease activity was constant throughout the gradient (Fractions 1 to 8) varying from 0.16 to 0.18, compared to a value of 0.18 for the mitochondria before sedimentation.

When mitochondria containing approximately 20 units of nuclease activity were mixed with 38.9 units of purified enzyme (see below) and then reisolated, no more than 8% of the added enzyme adhered to the mitochondria (Table II).

When 0.25 M sucrose was added to an assay mixture to preserve the mitochondrial structure, only 8% of the nuclease activity of whole mitochondria assayed under the usual conditions was observed; this value is comparable to the 7% found for the 8,000 × *g* supernatant fraction of these same mitochondria (Table III). Sucrose at a concentration of 0.25 M inhibited the purified enzyme by only 8%. Furthermore, if mitochondria were disrupted by sonic irradiation, 191% of the initial level of nuclease activity was found. When the disrupted mitochondria were assayed in the presence of sucrose, the nuclease activity was reduced by about one-third, to the value found for the 144,000 × *g* supernatant fraction obtained from the disrupted mitochondria. Thus, the enzyme does not appear to be accessible when it is integrated into the mitochondrial structure, but can be "activated" either by sonic irradiation or by osmotic disruption of mitochondria during incubation in the absence of sucrose.

Purification of Mitochondrial Nuclease—The nuclease activity was partially purified from a preparation of mitochondria (Table IV, Fig. 2). The catalytic properties of the nuclease of mitochondria and its behavior during this purification (particularly in the DEAE-cellulose chromatography) were identical with those of several side fractions obtained during purification of the

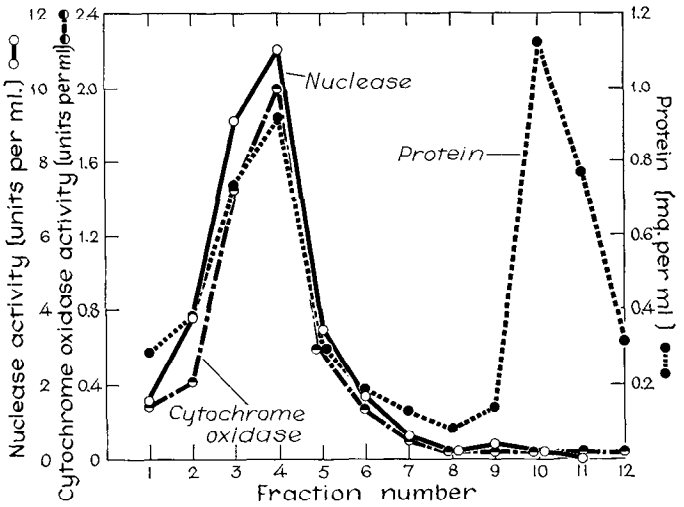

FIG. 1. Sedimentation of mitochondria in a sucrose gradient. A suspension of mitochondria (0.5 ml) prepared as described in "Methods" was layered onto a constant sucrose gradient (4.4 ml, 0.58 through 1.9 M sucrose) and centrifuged for 4 hours at 39,000 × *g* in an SW 39 rotor in a Spinco model L centrifuge. Fractions (0.4 ml) were then collected from a puncture in the bottom of the tube. Cytochrome oxidase was measured as described in "Methods," nuclease activity was estimated with denatured ^{32}P-labeled *E. coli* DNA in 0.1 M Tris, pH 7.5-0.01 M $MgCl_2$, and protein was determined by the method of Lowry *et al.* (21). The recovery of cytochrome oxidase was 91% and that of nuclease was 104%. Some slowly sedimenting protein is normally found, although this particular preparation of mitochondria contained an unusually large amount of this material.

TABLE I

DNase activity in mitochondrial and extramitochondrial fractions of mycelial extract

Mitochondria were prepared from an Eppenbach Micro-Mill extract of 62-hour mycelia as described in "Methods." Assays were performed in the standard manner with 0.1 M buffer and 0.01 M divalent cation. These two fractions accounted for all of the nuclease activity of the extract.

Conditions of assay				Total units	
DNA	Buffer	Buffer pH	Divalent cation	Mitochondrial fraction	8000 × *g* supernatant fraction
Native	Sodium acetate	5.5	$MnCl_2$	31	7.1
Native	Tris	7.5	$MnCl_2$	110	98
Native	Tris	7.5	$CaCl_2$	<2.5	<1.3
Denatured	Tris	8.2	$MgCl_2$	98	247
Denatured	Tris	7.5	$CaCl_2$	<4.3	485

TABLE II

Absence of binding of purified nuclease by mitochondria

Fresh mitochondria were obtained from mycelia ground with sand. Mitochondrial suspension, 1 ml containing approximately 20 units of nuclease activity, was mixed with 1 ml of mitochondrial medium and either 0.15 ml of purified enzyme containing 38.9 units of nuclease activity, or 0.15 ml of 0.05 M potassium phosphate, pH 6.5 (mitochondria alone). After 5 min at 0°, the supernatant fluids were obtained by centrifugation for 20 min at 8000 × *g*. The pellets were resuspended in 2 ml of mitochondrial medium and recentrifuged as above. The wash fluid was removed, and the mitochondrial pellet was suspended in 1 ml of mitochondrial medium. Nuclease activity was assayed with native ^{32}P-labeled *E. coli* DNA in 0.1 M Tris, pH 7.5-0.01 M $MnCl_2$. All assay mixtures contained the same amount of mitochondrial medium, because the components of the medium stimulate nuclease activity.

Fraction	Activity			
	Mitochondria + purified enzyme (38.9 units)	Mitochondria alone	Recovery of added enzyme	Net change in indicated fractions
	units			
Supernatant	33.6	0.1	33.5	
			37.7	−1.2
Wash	4.5	0.3	4.2	
Pellet	22.8	19.4	3.4	+3.4

TABLE III

Increase in nuclease activity on disruption of mitochondria

Mitochondria were prepared from an Eppenbach Micro-Mill extract and purified enzyme was Fraction A (Table V). The disrupted mitochondrial sample was prepared by treatment of a mixture of 1 ml of mitochondrial suspension and 1 ml of 0.05 M Tris, pH 7.5, for two 1-min intervals with an MSE-Mullard ultrasonic disintegrator. The 8,000 × *g* mitochondrial supernatant fraction was prepared by centrifugation of the mitochondria for 20 min, whereas the 144,000 × *g* supernatant fraction was prepared by centrifugation of a mixture of 1 ml of the disrupted mitochondria and 2 ml of 0.05 M Tris, pH 7.5, for 30 min. Assays were performed with denatured ^{32}P-labeled *E. coli* DNA in 0.1 M Tris, pH 7.5-0.01 M $MgCl_2$ in the presence or absence of sucrose as indicated.

Sample	Sucrose (0.25 M)	Activity relative to untreated sample
		%
Untreated mitochondria	−	(100)[a]
Untreated mitochondria	+	7.6
8,000 × *g* supernatant fluid of untreated mitochondria	−	7.0
Disrupted mitochondria	−	191
Disrupted mitochondria	+	126
144,000 × *g* supernatant fluid of disrupted mitochondria	−	123
Purified enzyme	−	(100)
Purified enzyme	+	91.5

[a] This value was equivalent to 24.2 units per ml of suspension.

extramitochondrial nuclease from a crude mycelial extract (2). Because these side fractions were available in large amounts, they were used for further purification and characterization of the mitochondrial enzyme (Table V).

TABLE IV

Isolation of nuclease from mitochondria

The pellet from 3.5 ml of a mitochondrial suspension was suspended in 3 ml of 0.05 M glycylglycine, pH 7.0, treated for five 1-min intervals in the Mullard sonicator, and centrifuged for 30 min at 144,000 × *g*. To 2.5 ml of the supernatant solution were added 0.26 ml of 1 M Tris, pH 7.5, and 1.69 g of finely powdered ammonium sulfate. The ammonium sulfate was dissolved by stirring at 0° for 30 min, after which the suspension was kept at 0° for 180 min. The precipitate was removed by centrifugation at 20,000 × *g* and suspended in 2 ml of 0.02 M potassium phosphate, pH 6.5. Insoluble material was removed by centrifugation and the extract was dialyzed against two changes, 3 liters each, of 0.02 M potassium phosphate, pH 6.5. Neither the ammonium sulfate supernatant fraction nor the material insoluble in the phosphate buffer had significant nuclease activity.

The dialyzed material was then applied to a phosphocellulose column (1 × 12 cm) which had previously been equilibrated with 0.02 M potassium phosphate, pH 6.5. The column was washed with two 20-ml portions of 0.02 M potassium phosphate, pH 6.5, and one 12.5-ml aliquot of 0.05 M potassium phosphate, pH 6.5. Finally, 0.50 M potassium phosphate, pH 6.5, was passed through the column and 5-ml fractions were collected. Elution with all buffers was at a rate of approximately 0.5 ml per min. The second and third 0.5 M potassium phosphate fractions, containing essentially all of the activity recovered, were pooled and dialyzed against 6 liters of 0.004 M potassium phosphate, pH 7.5. The total recovery of activity was 79%.

The dialyzed material was applied to a DEAE-cellulose column (1 × 3 cm) which had been equilibrated with 0.004 M potassium phosphate, pH 7.5. The column was then eluted with potassium phosphate, pH 7.5, at 0.3 ml per min as follows: two 6-ml aliquots at 0.004 M; two 7.5-ml aliquots at 0.013 M; two 7.5-ml aliquots at 0.02 M; and two 7.5-ml aliquots at 0.07 M. Enzyme activity eluted at 0.004 M and 0.013 M (Fig. 2), with little material appearing at higher salt concentrations at which elution of the extramitochondrial nuclease would have been expected (Fig. 3). The total recovery of activity was 81%.

Assays were performed with denatured ^{32}P-labeled *E. coli* DNA in 0.1 M Tris, pH 7.5-0.01 M $MgCl_2$.

Fraction	Volume	Total units
Mitochondrial suspension	2.6	63.0
144,000 × *g* supernatant	2.6	58.5[a]
Dialyzed ammonium sulfate	2.4	30.7[b]
Phosphocellulose	11.4	18.8[c]
Dialyzed phosphocellulose	14.4	18.9
DEAE-cellulose		
Potassium phosphate, 0.004 M	12.4	8.5
Potassium phosphate, 0.013 M	15.6	5.0
Potassium phosphate, 0.02 M	15.4	0.8
Potassium phosphate, 0.07 M	14.9	1.1

[a] The pellet contained an additional 18.9 units which were discarded.

[b] An additional 0.6 unit was found in the ammonium sulfate supernatant fraction after dialysis.

[c] An additional 2 units were found in the first 0.02 M potassium phosphate wash, and an additional 3 units were found in later 0.50 M potassium phosphate fractions.

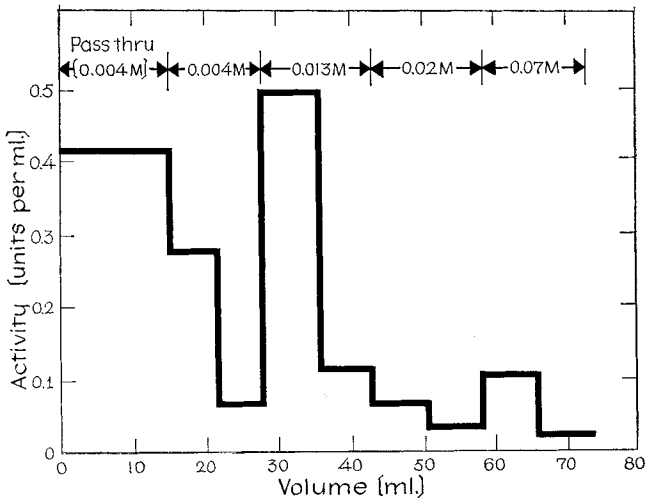

FIG. 2. DEAE-cellulose chromatogram of nuclease activity isolated from mitochondria. The chromatogram was developed as described in Table IV.

A crude mycelial extract was prepared, and its nuclease activity was partially purified by Mg^{++} precipitation, ammonium sulfate fractionation, phosphocellulose chromatography, and polyethylene glycol concentration, as described previously (2). This material was applied to a DEAE-cellulose column and eluted as described in Fig. 3. The fractions eluting with 0.004 M and 0.013 M potassium phosphate (designated as Fractions A and B, respectively) corresponded to the analogous fractions obtained when purified mitochondria were used as starting material (Table IV). Peak C contained a small amount of nuclease of undetermined origin, and Peak D represented the extramitochondrial nuclease (2).

Peaks A and B were each concentrated to approximately 15 ml by dialysis against 30% polyethylene glycol (w/v), then dialyzed against two 3-liter portions of 0.02 M potassium phosphate, pH 6.5, and applied to hydroxylapatite columns (1 × 3 cm) which had been equilibrated with 0.02 M potassium phosphate, pH 6.5. The columns were washed with 75 ml of 0.02 M potassium phosphate, pH 6.5, and 35 ml of 0.05 M potassium phosphate, pH 6.5, then eluted with a constant gradient (total volume, 330 ml) from 0.05 M to 0.35 M potassium phosphate, pH 6.5; 10-ml fractions were collected at 0.2 to 0.3 ml per min. Both columns had identical elution patterns, enzyme activity appearing in symmetrical peaks between 0.16 and 0.25 M potassium phosphate. The activity determined with denatured DNA as substrate (measured at pH 7.5 in the presence of Mg^{++}) cochromatographed with the activity on native DNA (measured at pH 5.5 in the presence of Mn^{++}) and total yields were approximately 88%. The fractions from each chromatogram with significant levels of activity were pooled and concentrated by pressure dialysis (22) for 23 hours against 2 liters of 0.05 M potassium phosphate, pH 6.5. The concentrated fractions had specific activities comparable to crystalline pancreatic DNase when measured under conditions optimal for each enzyme (Table V).

Properties of DNase Activity—Except for minor differences in pH optima and stability at 0° (see "Discussion"), the properties of the enzyme preparations derived from DEAE-cellulose Fractions A and B were identical.

The rate of digestion of either native or denatured DNA was roughly proportional to enzyme added until about 20% of the substrate became acid-soluble. The rate of hydrolysis proceeded linearly for about 30 min and then dropped off, although some degradation continued for at least 150 min.

Dilute fractions of enzyme were unstable when stored, particularly in low salt or at alkaline pH, losing as much as 50% of their activity per day. The concentrated, purified fractions were stable indefinitely when stored above liquid nitrogen and were

TABLE V

Further purification of mitochondrial nuclease

Purification was as described in the text, and assays were performed with denatured ^{32}P-labeled *E. coli* DNA in 0.1 M Tris, pH 7.5-0.01 M $MgCl_2$. All individual and pooled column fractions were also assayed with native ^{32}P-labeled *E. coli* DNA in 0.1 M sodium acetate, pH 5.5-0.01 M $MnCl_2$ and were found to be 33 to 40% as active when assayed under these conditions. Protein estimates were not made for the polyethylene glycol fraction because of interfering ultraviolet absorption or for the hydroxylapatite fraction because of its extremely low protein content.

Fraction	Volume	Total units	Total protein	Specific activity
	ml		*mg*	*units/mg*
Enzyme Fraction A				
DEAE-cellulose peak, eluted with 0.004 M phosphate	139	1810	3.7	490
Polyethylene glycol concentrate	17	890		
Hydroxylapatite	120	762		
Concentrated hydroxylapatite	3.1	588	0.385	2070
Enzyme Fraction B				
DEAE-cellulose peak, eluted with 0.013 M phosphate	53	566	0.400	1410
Polyethylene glycol concentrate	11	249		
Hydroxylapatite	120	220		
Concentrated hydroxylapatite	2.3	131	0.039	3350

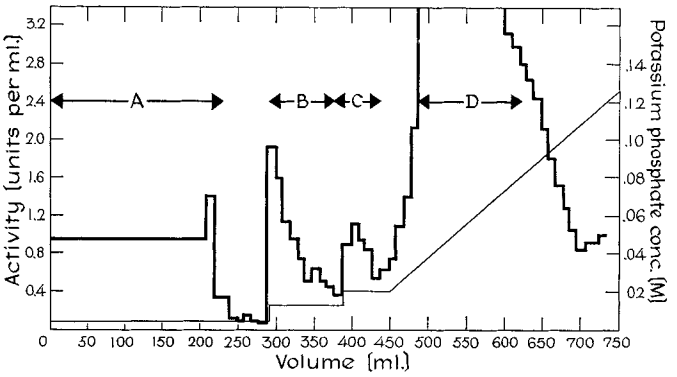

FIG. 3. DEAE-cellulose chromatogram of partially purified mycelial extract. A column (1 x 20 cm) containing 16 ml of packed DEAE-cellulose was equilibrated with 0.004 M potassium phosphate, pH 7.5. Concentrated phosphocellulose fraction (2), 195 ml containing 6300 units of activity and 10.7 mg of protein, was passed through the column at 0.4 ml per min. The column was then washed successively with 70 ml of 0.004 M, 100 ml of 0.013 M, and 50 ml of 0.02 M potassium phosphate, pH 7.5, at a rate of 0.7 ml per min. Fractions of 10 ml each were collected; all collection tubes contained an amount of 1 M potassium phosphate, pH 6.5, sufficient to bring the phosphate concentration to 0.05 M to stabilize the enzyme. Because of the low protein concentration of these fractions, elution and concentration were performed as quickly as possible. Peak D was eluted as described previously (2).

stable for several months at −20°. However, they were unstable at 0° unless 20% glycerol was present.

The enzyme showed maximum activity in 0.05 to 0.1 M Tris or imidazole buffer, pH 6.0 to 7.5, and required either Mg^{++} or Mn^{++} at 0.001 to 0.01 M. There was some activity with Co^{++} (20% of maximum), but none with Ca^{++} or Zn^{++}. EDTA inhibited the enzyme only when added in excess of the divalent cation.

Glutathione in either the oxidized or reduced form had no significant effect on enzyme activity, although β-mercaptoethanol (3 to 7 mM) stimulated activity by 50% at acid, but not at neutral or alkaline, pH values.

The enzyme was maximally active from 37° to 45°.

Identification of Products of Digestion of Denatured DNA—The purified enzyme had no detectable 5′-nucleotidase activity (less than 0.03% of its DNase activity) at pH 5.5, 7.5, or 9.5, as measured by the liberation from 5′-^{32}P-dTMP of ^{32}P that did not adsorb to Norit (15).

The products obtained from limited and extensive digestion of DNA were examined for their susceptibility to alkaline phosphatase and 5′-nucleotidase. Early in digestion (30 to 50% of the substrate made acid-soluble), approximately 10% of the products were sensitive to alkaline phosphatase, but none (<0.2%) were susceptible to 5′-nucleotidase. After extensive digestion (beyond the point at which the substrate was 100% acid-soluble), approximately one-fourth of the ^{32}P was susceptible to bacterial alkaline phosphatase, and slightly over 1% was sensitive to 5′-nucleotidase. These results imply that the mitochondrial nuclease is an endonuclease, producing 5′-mononucleotides only after extensive digestion has taken place.

The products of an extensive digestion of DNA were separated and identified by paper chromatography and electrophoresis (Table VI). 5′-Mononucleotides and a large number of di-, tri-, tetra-, and pentanucleotides were found. An examination of the residues at the 5′-terminals of the oligonucleotide fraction showed dGMP, dCMP, dAMP, and dTMP to be present in nearly equivalent amounts, indicating that despite the unequal distribution of mononucleotides in the digest (Table VI), the enzyme has no obvious base specificity.

TABLE VI

Identification of products of digestion of DNA by mitochondrial nuclease

The reaction mixture contained (in 0.11 ml): 0.1 M Tris, pH 7.5-0.01 M $MgCl_2$, 100 mμmoles of denatured ^{32}P-labeled *E. coli* DNA (specific activity, 3×10^6 cpm per μmole), and 0.88 unit of enzyme. After incubation for 120 min at 37°, the digest was applied to Whatman No. 3MM paper and resolved by chromatography followed by electrophoresis as described previously (1).

Product	Total ^{32}P
	%
dAMP	0.3
dTMP	0.06
dCMP	1.0
dGMP	1.7
Dinucleotides	15.9[a]
Trinucleotides	26.1[b]
Tetranucleotides	22.5
Pentanucleotides	24.7
> Pentanucleotides	7.8

[a] This fraction was further resolved by electrophoresis into peaks containing 1.7, 3.7, 2.4, 2.3, 3.1, 2.4, and 0.3% of the total ^{32}P.

[b] This fraction contained at least 11 components.

Properties of RNase Activity—The mitochondrial nuclease was approximately as active on ribosomal and transfer RNA as on DNA. As was found with DNA, maximal activity was observed in Tris or imidazole buffer, pH 6.5 to 7.5. Above pH 6, in the presence of Mg^{++}, transfer RNA was attacked at a lower rate than ribosomal RNA, whereas at lower pH values, the reverse was found. The RNase activity had the same requirement for divalent cations as did the DNase activity.

DISCUSSION

Several lines of evidence support the idea that the nuclease activity described here is closely associated with mitochondria. (*a*) The mitochondrial preparation contained a large proportion of the total nuclease activity of the cell despite the fact that it contained only 1.5% of the total cellular protein. (*b*) The nuclease and cytochrome oxidase activities had identical distributions after sedimentation of the mitochondrial preparation in a sucrose gradient. (*c*) Purified enzyme failed to bind mitochondria. (*d*) Disruption of the mitochondria greatly stimulated enzyme activity, suggesting that the nuclease activity is integrated into the mitochondrial structure.

On the other hand, the observed association of nuclease activity with mitochondria could represent the binding of a nuclease of extramitochondrial origin at some site within the mitochondrion.

The mitochondrial nuclease activity described here is clearly distinct from the extramitochondrial endonuclease of *N. crassa* (Table VII). The two enzymes are well separated by chromatography on DEAE-cellulose and are partially resolved on columns of hydroxylapatite. Furthermore, inasmuch as the mitochondrial enzyme is inactive in the absence of added divalent cation, in the presence of Ca^{++}, or at 55°, it is presumably free of the extramitochondrial nuclease.

It is not known why the mitochondrial nuclease is eluted from DEAE-cellulose in two peaks. The presence of two peaks is modified neither by starvation of the cells before harvesting, nor by isolation of the enzyme from purified mitochondria rather than from a whole cell extract (compare Figs. 2 and 3). After further purification on hydroxylapatite, the two fractions are indistinguishable in their properties with two exceptions. (*a*) The enzyme derived from Peak A is considerably less stable than that from Peak B when stored at 0° in the absence of glycerol. (*b*) The enzyme from Peak A has a pH optimum ranging from pH 6.8 to 7.5, whereas the enzyme derived from Peak B is maximally active from pH 6.0 to 7.0. Although definite conclusions as to the origin of the two fractions are clearly unwarranted, the two fractions might be tentatively regarded as slightly modified forms of the same protein. In practice, they may be pooled and fractionated together on hydroxylapatite.

Beufay *et al.* (23, 24) have observed an association of the alkaline DNase of rat liver with mitochondria by methods similar to those which we have used. Although these authors did not isolate the DNase, they described optimal reaction conditions for it (25) which were similar to the enzyme described here.

Beard and Razzell (26) have reported the purification of RNase activity from liver mitochondria, but their mitochondrial fraction contained microsomes and lysosomes, both of which are

TABLE VII
Comparison of properties of mitochondrial and extramitochondrial nucleases

	Mitochondrial	Extramitochondrial
pH optimum	pH 6.0 to 7.5	pH 7.0 to 8.5
Divalent cation requirements	Absolute requirement for Mg^{++}, Mn^{++}, or Co^{++}; inactive with Ca^{++}	No requirement, but stimulated by Mg^{++}, Ca^{++}, and Fe^{++}
Inhibition by EDTA	Reversed by stoichiometric levels of Mg^{++}, Mn^{++} or Co^{++}	Reversed only by Co^{++}
Temperature optimum	37° to 45°; inactive above 50°	47° to 62°
Effect of thiols	Stimulation at pH $\leqslant$ 6.5; no effect at pH > 6.5	Stimulation at pH $\leqslant$ 6.5; strong inhibition at pH > 6.5
Substrate specificity	Attacks polyribo- or polydeoxyribonucleotides with ordered or nonordered structure	Specific for polyribo- or polydeoxyribonucleotides with nonordered structure
Products of digestion	Mononucleotides and small oligonucleotides terminated by 5′-phosphate. Guanylate and cytidylate are predominant mononucleotides.	Mononucleotides and small oligonucleotides terminated by 5′-phosphate. Guanylate is predominant mononucleotide; cytidylate present in lowest concentration.

known to be rich in nuclease activity (27). It is possible that the mitochondrial preparation used here was similarly contaminated with particles the sedimentation properties of which are identical with those of the mitochondria. However, electron microscopic examinations of mitochondrial preparations obtained from *N. crassa* by the same procedure used here (4, 20) suggest that contamination by nonmitochondrial particles is minor.

Considerations of the function of the nuclease must remain speculative, particularly as it is not clear whether the natural substrate for the enzyme is RNA, DNA, or both. DNA (4–8), ribosomes (9), RNA of low molecular weight (9), and RNA of high molecular weight (6, 9) have all been observed in mitochondria. Furthermore, with the demonstration in mitochondria of peptide synthesis (10, 11) and RNA polymerase activity (4, 5) it seems that, with the exception of DNA polymerase, all the factors required for self-duplication of the mitochondria (3) have been found within this organelle. The identification of a nuclease with mitochondria provides an additional instance of the close association noted earlier (28) between nuclease activity and nucleic acid replication.

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