A DEMONSTRATION OF SEVERAL DEOXYRIBONUCLEASE ACTIVITIES IN MAMMALIAN CELL MITOCHONDRIA

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ABSTRACT

Extracts of purified mitochondria from adult rabbit liver and kidney have been prepared by lysis with Triton X-100. Such extracts contain deoxyribonuclease activity demonstrable at alkaline pH. Studies utilizing the effects of substrate variation, differing ionic strength, nucleoside di- and triphosphates, and SH-group inhibitors reveal the existence of at least five distinguishable deoxyribonuclease activities in these extracts.

Assay of lysosomal and mitochondrial enzyme markers indicates no significant lysosomal contamination of the mitochondrial extracts. Further studies also suggest that the alkaline deoxyribonuclease activity is specifically located in or in association with mitochondria.

INTRODUCTION

Recent studies of mitochondrial DNA have revealed a number of interesting observations concerning the distribution and occurrence of varying molecular forms (1–10). Simple circular and catenated oligomeric forms have been described in a variety of situations and DNA replication intermediates containing strand displacement loops have been recognized (see reference 9 for a recent review). These observations suggest that deoxyribonuclease activity may be implicated in specific events in mitochondrial DNA metabolism, particularly in recombination and replication.

A review of the literature indicates that relatively little is known about mitochondrial deoxyribonucleases. Alkaline deoxyribonuclease was first found to be associated with mitochondrial preparations by Beaufay et al. in 1959 (11, 12). Using enzyme markers, they localized to rat liver mitochondrial fractions a nuclease active on native DNA at pH 7.5 in the presence of Mg^{++}. No purification or further characterization of this activity was reported. In the intervening years, only a few additional studies have dealt with mammalian mitochondrial deoxyribonucleases. Curtis et al. (13) partially purified from a crude preparation of rat liver mitochondria a nuclease active at pH 7.5–8.5 on RNA and, to a lesser extent, on denatured and native DNA. This activity required Mg^{++} and was stabilized by β-mercaptoethanol. Morais et al. (14) partially purified a similar enzyme, much more active on RNA than on denatured and native DNA, with a pH optimum of 7.0–7.5 and requiring Mg^{++} and β-mercaptoethanol. This enzyme was stated to have "5′-endonucleolytic" activity and to be located on the outer mitochondrial membrane and in the

intermembrane space (15). The latter conclusion has received support from the osmotic fragility studies of Baudhuin et al. (16). Grossman et al. (17) have partially purified a nuclease from crude rat liver mitochondria that requires Mg^{++} and is active on native or denatured DNA at a pH of 8.0–9.0.

Although it is difficult to make meaningful comparisons between different studies, the work previously cited (11–17) suggests the existence of only a single nuclease in extracts of mitochondrial fractions. We have investigated the possibility of there being more than one such enzyme, distinguishable by the use of different enzyme activators and inhibitors. Furthermore, we present here more rigorous evidence than has been previously reported for the localization of deoxyribonuclease activities to mitochondria specifically. A preliminary account of these results has been reported previously (18).

MATERIALS AND METHODS

Acid phosphatase, *p*-nitrophenylphosphate, and oxidized cytochrome *c* were purchased from Sigma Chemical Co., St. Louis, Mo.

Preparation of Mitochondrial Fractions

Mitochondria were prepared by a modification of the technique described by Clayton and Vinograd (3). Young adult male white rabbits were killed by occipital trauma and bled. Both kidneys were rapidly dissected free, removed, stripped of their capsules, and immersed in ice-cold mannitol-sucrose medium (MS medium) (0.21 M mannitol, 0.7 mM sucrose, 5 mM EDTA, and 10 mM Tris-HCl pH 7.5).[1] The liver was also rapidly removed and placed in MS medium at 4°C. From this point on, the organs were kept entirely separate, although the mitochondrial preparation was identical for each. Tissue was minced with razor blades in a Petri dish over ice and was then homogenized, by four strokes, with a tight-fitting motor-driven Teflon pestle. This and all following steps were done at 4°C. The homogenate was centrifuged at 350 *g* for 5 min in a refrigerated centrifuge, and the resulting supernate was centrifuged at 17,000 *g* for 20 min. The pellet from this step, representing mostly mitochondria and lysosomes, was resuspended in a convenient amount of MS medium and layered on top of a step gradient consisting of 15 ml each of 1.5 M sucrose and 1.0 M sucrose. After centrifugation at 22,000 rpm for 35 min in the Spinco SW 27 rotor at 4°C, the band at the step interface was removed with a Pasteur pipette, resuspended in MS medium, and the step gradient centrifugation repeated. The final mitochondrial band, again collected from the step interface, was diluted with an arbitrary amount of MS medium and divided into a number of portions as dictated by the needs of a particular experiment. Usually, this fraction was recentrifuged at 12,000 *g* for 15 min and the mitochondrial pellet resuspended in 50 mM Tris-HCl pH 8.0 containing 0.5% Triton X-100. After incubation for 30 min at 4°C, the fraction was spun at 12,000 *g* for 15 min to eliminate the unlysed mitochondrial debris, and the supernate was used as the crude mitochondrial extract. The mitochondrial pellet obtained as described above was examined by electron microscopy in order to evaluate morphologically the presence of other cellular organelles. These studies revealed the pellet to consist largely of recognizable mitochondria; however, a small percentage of structures had undergone degenerative changes during the experimental manipulations, resulting in a morphologic appearance not dissimilar from that of lysosomes. The Triton-lysed extract was assayed for the presence of acid DNase and alkaline phosphatase. The results of these experiments, presented in detail later, showed insignificant lysosomal contamination.

Assay of Deoxyribonuclease Activity

Deoxyribonuclease activity was measured by the degradation of high molecular weight DNA to acid-soluble nucleotide. Incubation mixtures (1.0 ml) contained 15 nmol (as nucleotide) ^{3}H-labeled *Escherichia coli* DNA. In some instances, the DNA was denatured by heating in a boiling water bath for 10 min followed by quenching in ice. The concentration of divalent and monovalent cations, specific enzyme inhibitors, and other enzyme cofactors are presented with the data on individual experiments. For measuring alkaline deoxyribonuclease activity, 50 mM Tris-HCl buffer at pH 8.2 was included in the incubation. For acid deoxyribonuclease activity, 50 mM citrate buffer pH 4.8 was used. Mitochondrial extract was added to a final concentration of 0.25–1.5 mg protein/ml. All experiments included a control containing all the components of the incubation mixture except the active fraction being assayed. Incubation was carried out at 37°C for 30 min, at which point 0.5 ml each of ice-cold 1% bovine serum albumen and 20% trichloroacetic acid (TCA) were added. Tubes were kept cold for 15 min and then centrifuged at low speed. 1.0 ml of the supernate was mixed with 1 drop of NH_4OH in 10 ml liquid scintillation fluid and then counted in a Beckman LS-50 spectrometer. One unit of enzyme activity is defined as the amount that will liberate 1 nmol of nucleotide in 30 min at a pH of 8.2. Using either heat-denatured or native DNA with either 1 mM $MgCl_2$ or $MnCl_2$, this assay is linear for a protein concentration

[1] *Abbreviations used in this paper:* MS medium, mannitol-sucrose medium (0.21 M mannitol, 0.7 mM sucrose, 5 mM EDTA, and 10 mM Tris-HCl pH 7.5); PCMPSA, *p*-chloromercuriphenylsulfonic acid; POPOP, 1,4-bis[(5-phenyloxazolyl)]benzene; PPO, 5,5-diphenyloxazole; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

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range between 50 and 500 μg/ml over a period of 30 min at 37°C.

Assay of Cytochrome Oxidase

Cytochrome oxidase was assayed by the procedure of Smith (19), using the following modification of her equation to calculate the rate constant:

$$K = \frac{2.3 \log \dfrac{ODt_1 - ODox}{ODt_2 - ODox}}{t_2 - t_1}.$$

Assay of Acid Phosphatase

Acid phosphatase was assayed according to the procedure described in Sigma Technical Bulletin no. 104 (revised August 1971), Sigma Chemical Co.

^{3}H-Labeled DNA

^{3}H-labeled DNA from *E. coli* B-3 was prepared by the method of Thomas et al. (20).

Protein Determination

Protein concentrations were measured by the procedure of Lowry et al. (21), using bovine serum albumen as a standard.

Sedimentation Velocity Studies

Reaction mixtures included 50 μl crude mitochondrial extract, 15 nmol native [^{3}H]*E. coli* DNA, either 0.1 mM β-mercaptoethanol with 1.0 mM EDTA, or 0.1 mM *p*-chloromercuriphenylsulfonic acid (PCMPSA) with 1.0 mM $MgCl_2$, and 50 mM Tris-HCl buffer pH 8.5 to a final reaction volume of 1.0 ml. After incubation for 20 min at 37°C, reactions were stopped by the addition of 0.1 ml each of 0.1 M EDTA and 1% sodium dodecyl sulfate (SDS). 0.1 ml of the entire mixture, representing usually about 10,000 cpm from the experimental and control incubation mixtures, was placed on 5–20% sucrose density gradients containing 0.8 N NaCl and 0.2 N NaOH. Gradients were centrifuged in a Spinco SWT-56 rotor at 35,000 rpm for 180 min. Gradients were collected as 8-drop fractions to which 1 ml H_2O and 10 ml scintillation fluid were added. The scintillation fluid consisted of a mixture of toluene containing 0.5% 5,5-diphenyloxazole (PPO) and 0.003% 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) plus Triton X-100 in a ratio of 2:1 (vol/vol). Radioactivity was measured in a Beckman LS-250 spectrometer.

RESULTS

Demonstration of Deoxyribonuclease Activity in Mitochondrial Extracts

The results shown in Fig. 1 provide evidence that the purified mitochondrial fraction isolated as described in the previous section contains deoxyribonuclease activity that degrades both single- and double-stranded DNA in the presence of 1.0 mM $MnCl_2$. In the absence of added mitochondrial extract, less than 0.05 nmol of DNA was rendered acid soluble. An identical result was obtained when incubations contained extract previously heated to 60°C for 10 min. We have consistently observed that under these conditions the rate of degradation of single-stranded DNA is slightly greater than that of double-stranded DNA. Qualitatively similar results are obtained in the presence of $MgCl_2$ except that the rate of single-stranded DNA degradation is much greater than that of double-stranded DNA. Comparison of the specific activity and total nuclease activity present in the cytoplasmic, nuclear, and mitochondrial fractions of a single preparation of rabbit liver showed that the specific activity of deoxyribonuclease assayed by the degradation of both double-stranded and single-stranded *E. coli* DNA was about 16-fold higher in mitochondrial than in nuclear fractions and about 2,000-fold higher than in cytoplasmic

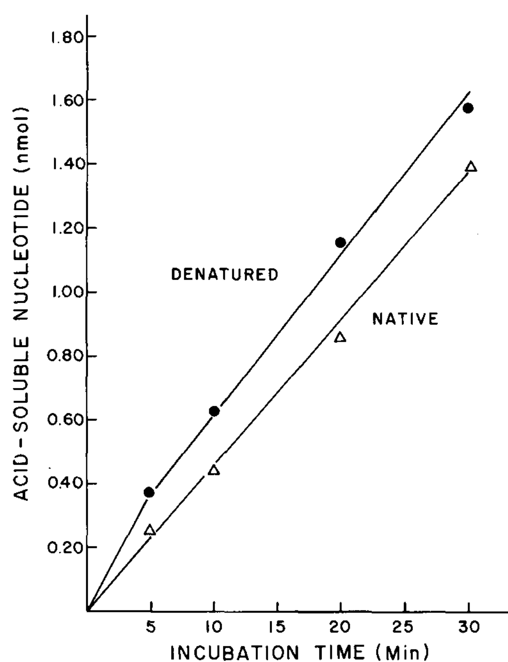

FIGURE 1 Kinetics of degradation of native and heat-denatured DNA by lysed liver mitochondria. Incubation mixtures (1.0 ml) contained *E. coli* DNA (15 nmol) either native or heat denatured; $MnCl_2$, 1.0 mM; Tris-HCl buffer, pH 8.2, 50.0 mM; and mitochondrial extract, 150 μg. Incubation was carried out at 37°C for the times indicated. Measurement of acid-soluble nucleotide product is described in the text.

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fractions (data not shown). Less than 1% of the total deoxyribonuclease units was present in the cytoplasm, the remainder being about equally divided between mitochondrial and nuclear fractions. These results clearly indicate that deoxyribonuclease activity is present in significant amounts in mitochondria relative to other subcellular fractions.

Parameters Affecting Mitochondrial Deoxyribonuclease Activity

The results obtained with extracts of mitochondria obtained from either rabbit liver or rabbit kidney were qualitatively identical. There was some quantitative variation from one enzyme preparation to another.

THE SH-GROUP INHIBITOR PCMPSA: When undialyzed liver mitochondrial extract is assayed in the presence of increasing concentrations of the SH-inhibitor PCMPSA, there is a variable inhibition of nuclease activity depending on the substrate and divalent cation present. As is shown in Fig. 2, the activity on native DNA in the presence of $MgCl_2$ is virtually completely inhibited at about 5×10^{-5} M PCMPSA. However, in the presence of $MnCl_2$ about 10% residual activity remains at 10^{-3} M PCMPSA. When denatured DNA is used as the substrate, there is considerable residual activity insensitive to PCMPSA inhibition in the presence of either divalent cation. This experiment clearly defines two classes of mitochondrial nuclease activities; those insensitive to PCMPSA inhibition and those sensitive to PCMPSA inhibition.

Although less than 1% acid-soluble nucleotide is detected when double-stranded DNA is incubated in the presence of $MgCl_2$ and 1.0 mM PCMPSA,

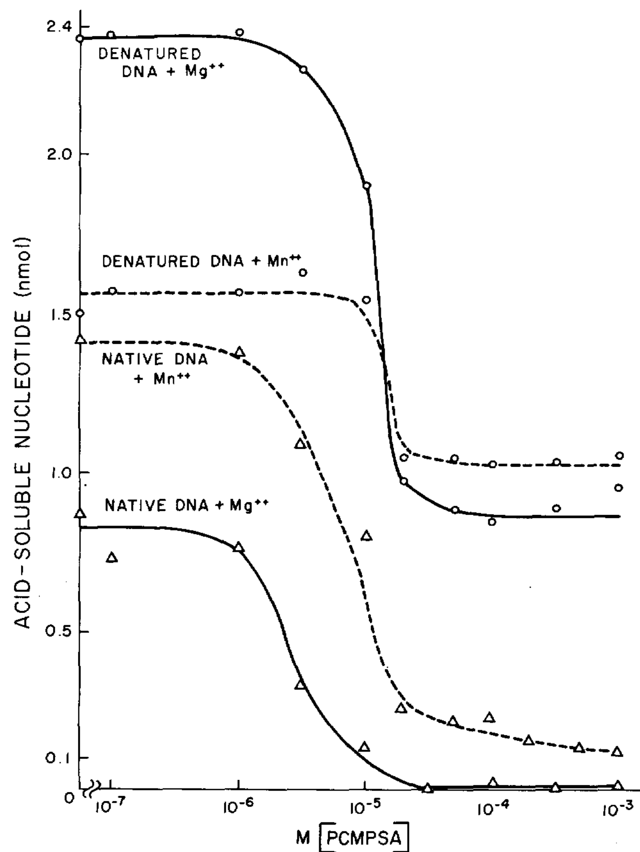

FIGURE 2 The effect of the SH-inhibitor PCMPSA on mitochondrial nuclease activity. Assay conditions were identical to those described in Fig. 1 except for the use of $MgCl_2$ (1.0 mM) and PCMPSA as indicated. The mitochondrial extract was derived from rabbit kidney, and 200 μg of protein were added to each reaction. Incubations were carried out for 20 min at 37°C.

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analysis of sedimentation velocity of the DNA in alkaline sucrose density gradients reveals significant endonucleolytic cleavage of the DNA (Fig. 3). An identical result is obtained in the absence of $MgCl_2$ and in the presence of 1.0 mM EDTA. Thus, at least one of the mitochondrial nucleases insensitive to PCMPSA inhibition is an endonuclease that attacks double-stranded DNA and which has no specific requirement for added divalent cation.

IONIC STRENGTH: In the presence of increasing concentrations of monovalent cation, other differential effects are observed that further serve to distinguish the various deoxyribonuclease activities present in rabbit mitochondria. Fig. 4 demonstrates that when native DNA is used as substrate, deoxyribonuclease activity is inhibited by KCl under all conditions tested, i.e. in the presence or absence of PCMPSA with either $MgCl_2$ or $MnCl_2$. With denatured DNA as the substrate, the PCMPSA-sensitive activity is stimulated maximally at 24 mM KCl and inhibited at higher concentrations. These results suggest that among the mitochondrial nucleases affected by SH-group inhibitors there are two distinct activities: a nuclease that is stimulated by KCl to degrade denatured DNA, and a nuclease whose ability to degrade native DNA is inhibited by KCl.

ATP AND OTHER NUCLEOSIDE DI- AND TRIPHOSPHATES: As indicated earlier, in the presence of 1.0 mM PCMPSA, nuclease activity can be readily detected that degrades denatured DNA in the presence of either $MgCl_2$ or $MnCl_2$. This activity is stimulated by the addition of ATP (Fig. 5). The results of numerous experiments indicate that ATP causes a two to fourfold stimulation that is maximal at concentrations between 0.75 and 1.125 mM. When this experiment is repeated without PCMPSA addition, and the value obtained in the presence of PCMPSA is subtracted, the results show that the PCMPSA-inhibitable nuclease fraction is *not* stimulated by ATP. Thus, the effect of ATP further serves to differentiate nucleases inhibited by PCMPSA and those not inhibited by this reagent. In addition, the stimulation observed in the presence of ATP and $MnCl_2$ is seen only with use of denatured DNA as the substrate. When native DNA is used as substrate, ATP is inhibitory (Fig. 5). The endonuclease activity detected by sedimentation velocity studies on DNA incubated with extract in the presence of PCMPSA and $MgCl_2$ is unaffected by ATP.

In order to evaluate the specificity of the ATP

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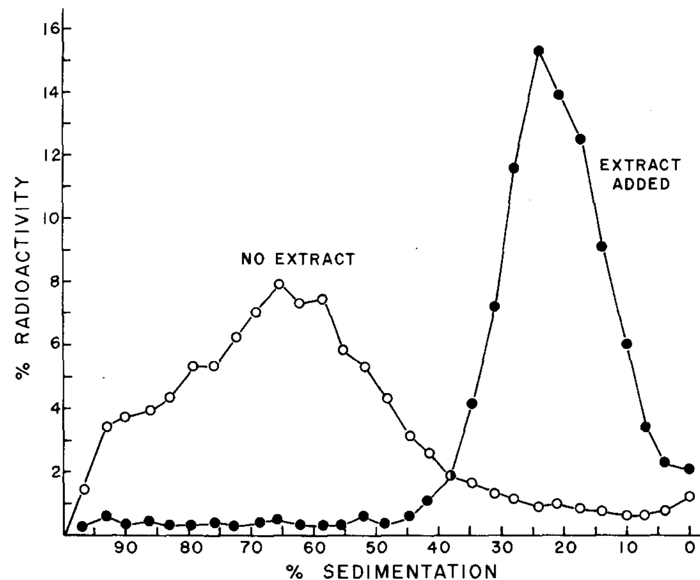

FIGURE 3 Endonucleolytic degradation of *E. coli* DNA by extracts of liver mitochondria. Incubations (1.0 ml) contained *E. coli* DNA, 15.0 nmol; PCMPSA, 0.1 mM; $MgCl_2$, 1.0 mM; Tris-HCl buffer, pH 8.5, 50 mM; liver mitochondrial extract, 250 μg. Incubations were carried out at 37°C for 20 min at which time 0.1 ml 0.1 M EDTA and 0.1 ml 1% SDS were added. 0.05 ml of the mixture was layered on a 5–20% alkaline sucrose density gradient. Preparation of the gradients and conditions of sedimentation are described in the text.

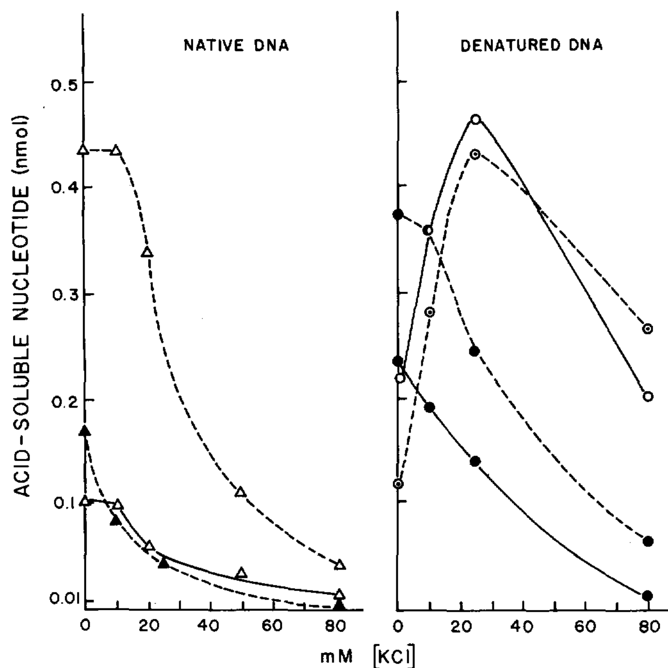

FIGURE 4 Effect of KCl on mitochondrial nuclease activity. Incubations (1.0 ml) contained: *E. coli* DNA (15 nmol), native or heat denatured; $MgCl_2$ or $MnCl_2$, 1.0 mM; PCMPSA when added, 1.0 mM; Tris-HCl buffer, pH 8.2, 50 mM; KCl at the concentrations indicated; and kidney mitochondrial extract, 275 μg. Incubations were carried out at 37°C for 25 min. Acid-soluble nucleotide product was measured as described in the text. The data for nuclease activity inhibited by PCMPSA were calculated by subtraction of results obtained with PCMPSA from those obtained in its absence. The data points shown in the figure for activity inhibitable by PCMPSA are the derived values. Open triangles and open circles, PCMPSA inhibitable. Closed triangles and closed circles, PCMPSA noninhibitable. Solid lines, Mg^{++} present. Dashed lines, Mn^{++} present.

stimulation observed with denatured DNA in the presence of PCMPSA and $MnCl_2$, a number of further studies were carried out. Fig. 6 shows the results of addition of either inorganic phosphate, pyrophosphate, or ATP. In the presence of $MnCl_2$, only ATP is stimulatory; both inorganic phosphate and pyrophosphate cause inhibition. Table I indicates that a variety of other nucleoside di- and triphosphates and deoxynucleoside triphosphates produce effects that are qualitatively identical with those of ATP.

Localization of Nuclease Activity to Mitochondria

We were more concerned, in these studies, with an analysis of the nucleases present in isolated mitochondria than in demonstrating the exclusivity of their association with this cellular organelle. Indeed, some or all of the nuclease activities described may be present in other cellular fractions. For this reason, the results of detailed comparative studies on the specific activity of individual nucleases during stages of the mitochondrial isolation are not presented. We show here the results of a series of experiments which were carried out in an attempt to demonstrate that alkaline deoxyribonuclease activity present in lysed mitochondrial preparations did in fact originate in the mitochondria rather than by contamination of the mitochondrial fraction with extramitochondrial enzymes. Despite the fact that the mitochondria were purified from other cellular constituents, possible sources of contamination to be considered are cytoplasmic enzyme and lysosomal enzyme. In addition, it is conceivable that nucleases could become nonspecifically bound to the exterior of mitochondria during cell lysis and remain bound during the entire purification procedure.

Mitochondria purified by step-gradient centrifugation were incubated in MS medium at 4°C for

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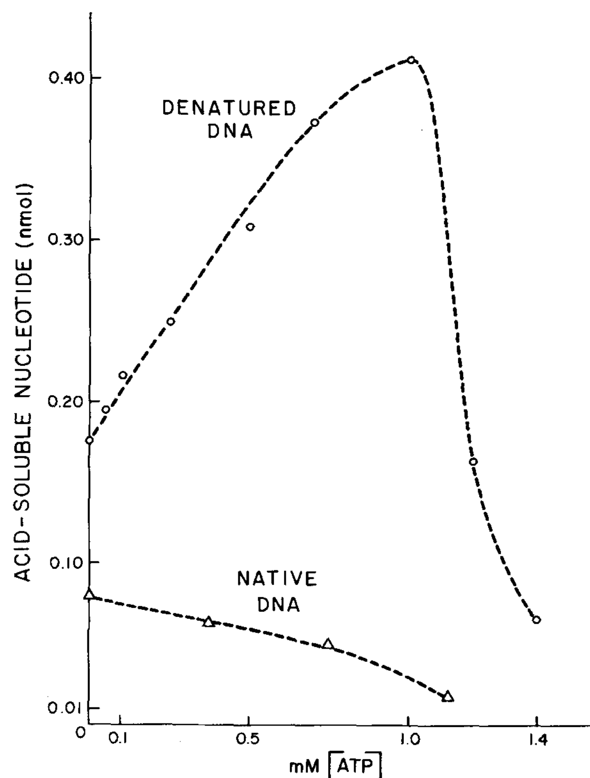

FIGURE 5 Effect of ATP on the degradation of native and denatured DNA, in the presence of PCMPSA and $MnCl_2$. Incubations (1.0 ml) were carried out as described in earlier figure legends. ATP was present at the concentrations shown in the figure. Incubations were carried out at 37°C for 25 min, using liver mitochondrial extract (250 μg).

20 min, after which the mitochondria were centrifuged, and the supernate was saved. The pellet was resuspended in MS medium and the procedure repeated. In the presence of 1.0 mM $MgCl_2$, no deoxyribonuclease activity was detected in either supernate. In the presence of 1.0 mM $MnCl_2$, deoxyribonuclease activity in both supernates was only about 5% of that present in the extract from detergent-lysed pellets. This experiment demonstrates that after extensive washing of the purified mitochondrial preparation, nuclease activity is still mainly associated with a sedimentable fraction. The requirement for lysis with detergent in order to release nuclease in a soluble form indicates that the activity is located in or on a membrane-associated organelle. Both of these results are consistent with a mitochondrial localization of the deoxyribonuclease activities assayed.

Table II shows the results of a comparison between lysed and unlysed preparations of mitochondria in terms of lysosomal and mitochondrial enzyme markers. Acid phosphatase and acid deoxyribonuclease were only two to eight times more active in lysed compared with unlysed preparations. On the other hand, alkaline deoxyribonuclease and cytochrome oxidase activity increased about 100-fold after lysis with detergent.

In an attempt to demonstrate that deoxyribonucleases are not bound nonspecifically to the external surface of mitochondria, deoxyribonuclease activity of intact mitochondria was compared to that of lysed organelles. To control for leakage of deoxyribonucleases from mitochondria during incubation at 37°C, the supernatant fraction of an intact suspension of mitochondria incubated at 37°C for 30 min was assayed for deoxyribonuclease activity. The results, shown in Table III, indicated that, under all assay conditions used, the nuclease activity associated with intact mitochondria is low relative to that released by mitochondrial lysis, and that all of the former can be accounted for by leakage of nuclease during the incubation. We conclude that nucleases are not bound nonspecifically to the outside of mitochon-

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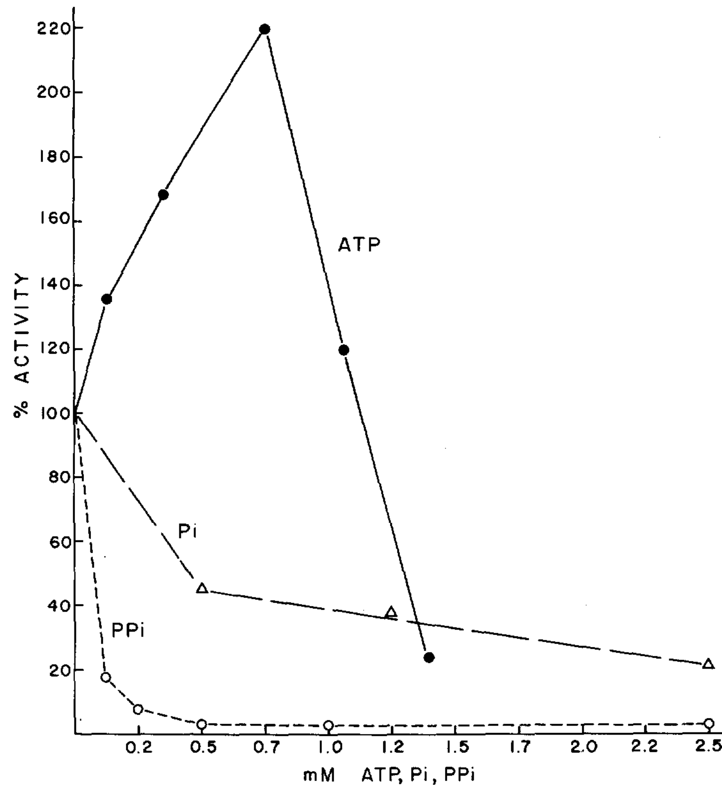

FIGURE 6 Effect of ATP, Pi, and PPi on the degradation of denatured DNA in the presence of PCMPSA. Incubation conditions were those previously described. The concentrations of ATP, Pi, and PPi are indicated in the figure. Incubations were carried out at 37°C for 25 min, using liver mitochondrial extract (250 μg). Nuclease activity without phosphate or ATP addition is normalized to 100%, and changes in activity in the presence of phosphate are expressed relative to that value.

dria, at least not in an active form. A consideration that is extremely difficult to eliminate is that nucleases are bound to the outside of mitochondria and are inactive until the mitochondria are exposed to detergent.

DISCUSSION

Those aspects of DNA metabolism in which specific deoxyribonuclease activity is most frequently considered are replication, recombination, and repair. Studies on mitochondrial nucleic acid metabolism carried out in recent years have strongly implicated, and in some instances convincingly demonstrated, the existence of at least some of these metabolic activities in mitochondria (1-10, 22). Improved electron microscope techniques have facilitated detailed studies on the topology of mitochondrial DNA and have indicated the existence of molecular forms highly consistent with replicative intermediates. Experiments with mouse L cells growing in tissue culture have shown a novel type of branched circle which consists of a closed circular duplex to which a 7S single-stranded segment is hydrogen bonded, displacing one of the strands to form a displacement loop (D-loop). More advanced replicative forms have been identified as expanded D-loop molecules and circular molecules containing gaps. It has been suggested that nicking of the D-loop molecule in vivo might permit branch migration with release of the newly synthesized 7S DNA intact. Closure of the molecule would permit this process to be repeated. Robberson and Clayton (10) have shown in vitro that the loss of 7S DNA by the process of branch migration is dependent upon nicking of the covalently closed circular D-loop molecules by X irradiation.

In addition, all normal animal cells contain catenated oligomers of mitochondrial DNA (two or more molecules interlocked as rings in a chain)

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TABLE I

Effect of Ribonucleoside Di- and Triphosphate and Deoxyribonucleoside Triphosphate on Deoxyribonuclease Activity

Addition	Concentration	Stimulation
	mM	%
ADP	1.125	335
ATP	1.125	395
GTP	0.75	343
CTP	0.75	317
UTP	0.75	343
dATP	0.75	364
dGTP	0.75	339
dCTP	0.75	336
TTP	0.75	324

Incubation mixtures (0.3 ml) contained heat-denatured *E. coli* DNA, 15 nmol; PCMPSA, 0.1 mM; Tris-HCl buffer, pH 8.2, 25 mM. Nucleoside di- and triphosphates were added at concentrations between 0.375 and 1.125 mM. Incubations were carried out at 37°C for 30 min. Acid-soluble nucleotide product was measured as described in the text. The table shows the concentration of phosphorylated nucleoside that gave maximal nuclease stimulation expressed as a percentage of the activity measured in its absence.

the number of which varies from 4 to 25% by weight, depending on the origin of the cells. It is not known at present what conditions promote their origin or what the mechanism of catenane formation is. However, it is possible that recombination reactions involving DNA breakage and rejoining may be important in their genesis. Such processes might also require the action of one or more specific deoxyribonucleases. Westergaard et al. (22) found that damage to *Tetrahymena* mitochondrial DNA by UV light, electron irradiation, ethidium bromide, or thymine starvation leads to an increase in the mitochondrial DNA polymerase activity up to 40-fold. No studies have yet shown active DNA repair in mammalian mitochondria; however, recent experiments in our laboratory (23) have aimed at investigating possible mechanisms of DNA repair in mitochondria by using a phage-induced endonuclease that can specifically detect pyrimidine dimers in DNA. The detailed results of the studies to be published elsewhere (23) indicate no evidence for an excision repair mechanism in mitochondria, but do not rule out the possibility of a postreplication repair mechanism which might involve deoxyribonuclease activity.

TABLE II

Comparison of Deoxyribonuclease Activity with Lysosomal and Mitochondrial Enzyme Markers

Experiment no.	Sample	Acid phosphatase	Acid DNase (acid-soluble nucleotide cpm)	Alkaline DNase (acid-soluble nucleotide cpm)	Cytochrome oxidase
		OD units			$K \times 10^{4}$
1	Control	0.4	340	190	6.9
	Lysed mitochondria from kidney	1.60	600	23,200	711.0
2	Control	0.09	—	1,752	7.7
	Lysed mitochondria from liver	0.8	—	148,400	1,870.0

In this table the activities of acid phosphatase, acid deoxyribonuclease, alkaline deoxyribonuclease, and cytochrome oxidase are compared in lysed and unlysed preparations of purified mitochondria. Mitochondria were prepared from rabbit liver and kidney as described in the text. The mitochondrial band obtained from the second-step gradient was resuspended in 10.0 ml MS medium and divided into two equal aliquots. Each was centrifuged at 5,000 *g* for 5 min, and the pellets were resuspended in 50 mM Tris-HCl buffer pH 8.0 with or without added 0.5% Triton X-100. The two samples were incubated for 30 min at 4°C and then centrifuged at 12,000 *g* for 15 min. The supernates were collected and identical volumes of each used for enzyme assays. In each case the control refers to the mitochondrial preparation that was not exposed to Triton X-100. Enzyme assays were performed as described in the text. For measurement of acid and alkaline deoxyribonuclease, incubation mixtures (0.3 ml) contained *E. coli* DNA, 5 nmol; $MgCl_2$, 1.0 mM; β-mercaptoethanol, 1.0 mM; 50 mM citrate buffer pH 4.8, or 25 mM Tris-HCl pH 8.2; and mitochondrial extract, 50 μg. The same amount of protein was also used for the acid phosphatase and cytochrome oxidase incubations. The protein concentration of the control fractions was too low to measure, but in each case the volume of supernate used was the same as that from the lysed mitochondrial preparation.

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TABLE III

Comparison of Deoxyribonuclease Activity between Lysed and Unlysed Mitochondria

Fraction	Relative deoxyribonuclease activity			
	No PCMPSA		PCMPSA present	
	Native DNA	Denatured DNA	Native DNA	Denatured DNA + ATP
	%	%	%	%
Supernate from mitochondria incubated without substrate	2.12	1.0	18.2	3.5
Mitochondria incubated with substrate	0.61	0.5	7.12	4.7
Mitochondrial extract	100.0	100.0	100.0	100.0

This table shows a comparison of the deoxyribonuclease activities present in extracts of lysed mitochondria and those present in intact mitochondria incubated under the identical conditions. Mitochondria were prepared as described in the text. The purified mitochondrial pellet was resuspended in 20.0 ml MS buffer and contained 28.0 mg/ml of protein. An aliquot of this material was lysed by the addition of 0.5% Triton X-100 (final concentration) at 4°C. Equivalent sized aliquots of mitochondrial suspension were supplemented with either $MgCl_2$ (1.0 mM), or $MnCl_2$ (1.0 mM) plus ATP (1.125 mM). A final aliquot was maintained without any additions. Deoxyribonuclease activity was measured by the acid-soluble nucleotide method as described in the text. Incubation mixtures (1.0 ml) contained 15 nmol native or heat-denatured *E. coli* DNA. Assays without PCMPSA contained $MgCl_2$ (1.0 mM); those with PCMUA (1.0 mM) contained $McCl_2$ (1.0 mM), and, in some cases, ATP (1.125 mM). All incubation mixtures contained 0.21 M mannitol, 0.7 mM sucrose, and 10.0 mM Tris-HCl buffer pH 7.5. Nuclease was added either as 56 μg protein from lysed mitochondria or as an equivalent amount of protein as intact mitochondrial suspension in MS buffer. To serve as controls for leakage of nuclease during the incubation of intact mitochondria, incubations of equivalent amounts of intact mitochondria with either added $MgCl_2$, or $MnCl_2$ and ATP, but without added substrate, were carried out at the same time. Incubations were carried out at 37°C for 30 min. Samples incubated with DNA were precipitated with TCA (5% final concentration), and acid-soluble nucleotide released was measured as described in the text. Samples incubated without DNA were centrifuged at 5,000 *g* for 10 min at 4°C. The supernates were saved and aliquots assayed for nuclease activity as described in the text. The deoxyribonuclease activity present in extracts of lysed mitochondria was normalized to 100% in each case, and the activities measured in the supernate of incubated mitochondria, or in intact mitochondrial suspensions, are expressed relative to these values. The data obtained from incubations in the presence and absence of PCMPSA are not directly comparable because of the normalization.

In the light of the observations summarized in the preceding paragraphs, and particularly since our review of the literature indicates an emphasis on only a single deoxyribonuclease activity in mitochondria, we have been prompted to examine the deoxyribonuclease content of mitochondria in greater detail. These studies confirm the existence of deoxyribonuclease activity in extracts of mitochondrial fractions purified from mammalian cells. This is most convincingly demonstrated by showing that when this fraction is treated with detergent, there is a large increase in nuclease activity, paralleling a rise in the activity of cytochrome oxidase, a known mitochondrial enzyme marker. .

In evaluating the significance of this observation, however, we have made an effort to eliminate, insofar as possible, the presence of extramitochondrial sources of contamination. The most obvious source of contaminating nuclease would be cytoplasmic protein carried over into the soluble phase of the mitochondrial fraction. Such contamination should be removed by repetitive washing of the mitochondrial fraction, and therefore the supernates from successive washes should contain progressively less nuclease activity. Our results indicate, instead, that the supernates of successive washes contain a constant amount of deoxyribonuclease activity, which we interpret as a consequence of leakage from damaged mitochondria.

A second possible source of contamination would be other cellular organelles present in the mitochondrial fraction. Electron microscope examination of our mitochondrial pellets showed largely mitochondria; however, a small percentage of them had undergone degenerative changes during the experimental manipulations that resulted in a morpholigic appearance not dissimilar from that of lysosomes. Assay of lysosomal enzyme markers such as acid phosphatase and acid deoxyribonuclease, however, revealed very little lysosomal contamination. Finally, we have considered the possibility that nucleases from any extramitochondrial source could bind nonspecifically to the mitochondrial surface. Under these conditions, one would expect to have demonstrable nuclease activity with

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TABLE IV

Summary of Mitochondrial Nuclease Activities

I. Inhibited by PCMPSA	A. Stimulated by KCl with *denatured* DNA
	B. Inhibited by KCl with *native* DNA
II. Not Inhibited by PCMPSA	A. Stimulated by ATP with *denatured* DNA
	B. Inhibited by ATP with *native* DNA
	C. Endonucleolytic activity with *native* DNA in the presence of EDTA

intact mitochondria. Our results indicate that when intact mitochondria are incubated with exogenous DNA in a slightly hypertonic medium, only a small degree of nonmitochondrial DNA degradation occurs. Even this low level of activity can be accounted for by leakage of nuclease from the mitochondria during the incubation. Although this result indicates that there is probably no deoxyribonuclease bound in an active form external to the mitochondria, it leaves unresolved the possibility that enzyme is bound in an inactive form and only becomes active when the mitochondria are lysed. Unfortunately, we know of no way of adequately excluding that possibility. However, we consider it highly unlikely.

Of particular interest in these studies is the observation that the use of different DNA substrates, enzyme inhibitors, ionic strength, and nucleoside di- and triphosphates have delineated more than a single deoxyribonuclease activity in mitochondria. The data presented are consistent with the existence of at least five distinguishable activities, summarized in Table IV. It remains to be seen, when purification is undertaken, whether or not all of these activities are associated with separable proteins, and until such time we prefer not to designate these nucleases with specific names. At present, we wish to draw attention to the observation that ATP stimulates the degradation of denatured DNA by a nuclease that is present in extracts of mitochondria and, in this respect, resembles a nuclease present in a number of microorganisms which is believed to be involved in genetic recombination (24–29). In addition, we are intrigued with the observation that mitochondrial extracts contain an endonuclease that nicks native DNA in the absence of added divalent cation. Further studies on these enzyme activities are in progress.

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