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Cellular Localization of the Apurinic/Apyrimidinic Endodeoxyribonucleases in Rat Liver

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A method has been developed to purify rat liver nuclei; the isolated nuclei keep both nuclear membranes and retain more than 90% of the cell apurinic/apyrimidinic (AP) endodeoxyribonuclease activity. The nuclear enzyme is located mostly in chromatin non-histones; there is also an important amount of activity in the nuclear sap and some in the nuclear membranes. The cytoplasmic AP endodeoxyribonuclease activity is shared between mitochondria, cytosol and membranes. Different cell compartments appear to contain different AP endodeoxyribonuclease species: the membrane enzyme is activated by Triton whereas the other enzymes are rather inhibited; the nuclear sap enzyme has a higher molecular weight and a higher thermal resistance than the chromatin enzyme. A hypothesis is formulated according to which: (1) the chromatin enzyme is the only species important for nuclear DNA repair; (2) the species present in the other cell compartments might be precursors of the chromatin AP endodeoxyribonuclease.

Apurinic/apyrimidinic (AP) endodeoxyribonucleases introduce breaks near apurinic or apyrimidinic sites (AP sites) in double-stranded DNA. *Escherichia coli* possesses two enzymes specific for AP sites coded by different genes: endonuclease VI [1–3] and endonuclease IV [4]. AP endodeoxyribonucleases have been found in all cells, whether bacteria or cells from plants or animals [5]. Enzymes from rat liver [6] and calf thymus [7] have been partially purified.

Mammalian cells contain several AP endodeoxyribonucleases. Linsley et al. [8] have distinguished six enzymes with different K_m values in human placenta. Kühnlein et al. [9] also found several species in human fibroblasts.

In this paper, we try to clarify the following questions. What are the cellular localizations of the AP endodeoxyribonucleases of rat liver? Are different enzymes located in different cell compartments?

MATERIALS AND METHODS

The Substrates

Radioactive DNA, prepared from *Escherichia coli* B-41 grown in the presence of [^{3}H]thymidine (10 Ci/mmol, New England Nuclear; 1 μCi/ml), had a specific radioactivity of about 50000 dis. min^{-1} μg^{-1}. Alkylation with methyl methanesulfonate and partial depurination were carried out as previously described [10]. The alkylated [^{3}H]DNA contained approximatively one alkylated site per seven nucleotides and the alkylated-depurinated [^{3}H]DNA one apurinic site per 20 nucleotides. Treatment of the alkylated-depurinated [^{3}H]DNA with 0.2 M NaOH for 15 min at 37 °C, which is known to induce a break near each AP site [10], yielded an acid-soluble fraction of 35%. For the enzyme assays, the labelled double-stranded [^{3}H]DNA (untreated, alkylated or alkylated-depurinated) was dissolved in 15 mM NaCl, 1.5 mM sodium citrate, 20 mM $MgCl_2$, pH 8.0, to a concentration of 20 μg/ml.

Enzyme Assays

To 20 μl [^{3}H]DNA solution (0.4 μg) were added 20 μl of the enzyme preparation. After a 15-min incubation at 37 °C, the tubes were cooled in ice, then 100 μl 15 mM NaCl, 1.5 mM sodium citrate, pH 7.0, containing 200 μg of calf thymus DNA, and 900 μl 5.78% perchloric acid were added successively. The tubes were left for 15 min at 0 °C, then centrifuged at 12000 × *g* for 15 min. The supernatant was used to measure the acid-soluble radioactivity. Results were corrected for controls without enzyme which always gave a very low acid-soluble fraction.

Enzyme. Apurinic/apyrimidinic (AP) endodeoxyribonucleases (EC 3.1.25.2).

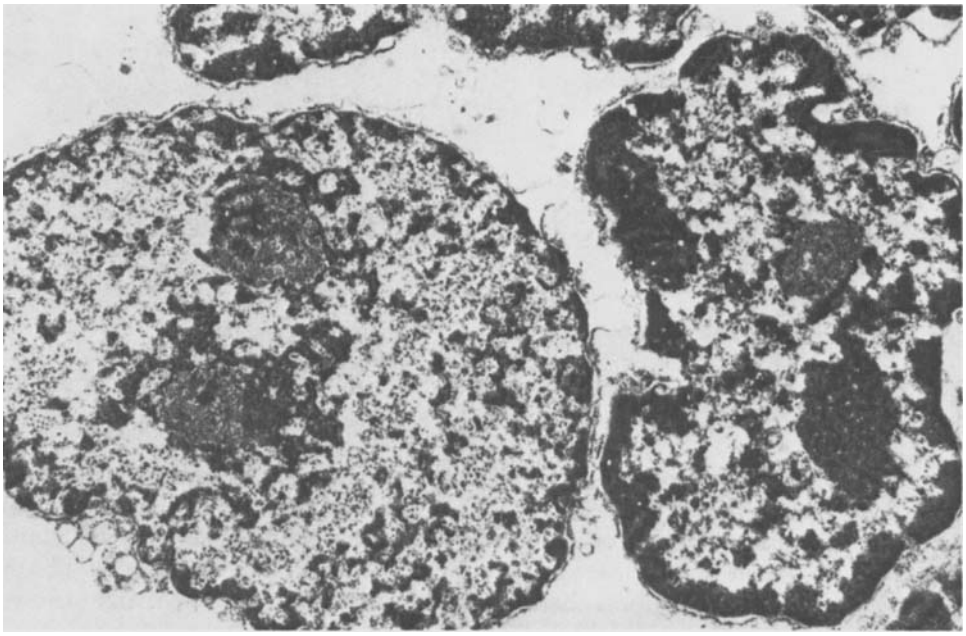

Fig. 1. *Electron micrography of isolated rat liver nuclei*

With the alkylated-depurinated [^{3}H]DNA, the acid-soluble fraction was nearly proportional to the AP endodeoxyribonuclease activity as long as it is between 1% and 12%. A unit of AP endodeoxyribonuclease activity is that amount which releases 10% of the total alkylated-depurinated [^{3}H]DNA radioactivity in the acid-soluble fraction in the conditions of the described assay.

Preparation of Nuclei

Young male rats, weighing 80–100 g and fasted for 24 h, were anesthetized with ether/chloroform (3:1, v/v) and, after a quick laparotomy, the liver was perfused through the portal vein with an ice-cold Locke's solution. The sus-hepatic veins were cut, the liver excised and immediately placed in ice-cold buffer A (2.5% Ficoll 400, 5% dextran T2000, 0.1% bovine serum albumin, 0.25 M sucrose, 4 mM 2-mercaptoethanol, 4 mM $CaCl_2$, 2.4 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.0). All subsequent manipulations were carried out between 0° and 4 °C. After removing the big vessels, the liver was cut in pieces and homogenized in buffer A (10 ml/g liver) with 15 strokes (700 rev./min) using a Potter-Elvejhem apparatus of 90–120-μm clearance. The homogenate was filtered through 12 layers of cheesecloth, then centrifuged at 20000 × *g* for 25 min. The sediment of crude nuclei (which also contained unbroken cells and part of the mitochondria) was resuspended in buffer A (5 ml/g tissue) with the Potter-Elvejhem apparatus (10 strokes, 400 rev./min) and layered on top of discontinuous sucrose gradients. Each gradient was constituted of 10 ml 1.7 M, 9 ml 1.4 M, 9 ml 1.0 M sucrose in buffer B (1% Ficoll, 2.5% dextran, 0.1% bovine serum albumin, 4 mM 2-mercaptoethanol, 4 mM $CaCl_2$, 2.4 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.0) and 10 ml of the crude nuclei suspension. The tubes were centrifuged during 120 min in an SW-27 rotor in a Beckman centrifuge at 131000 × *g*. The purified nuclei were found at the bottom of the tubes.

Electron microscopic examination showed that the preparation of purified nuclei did not contain other cell organelles and that the nuclei had kept both nuclear membranes with very little adhering cytoplasmic contamination. The nucleoli had a normal structure; the euchromatin and heterochromatin distribution seen in resting hepatocytes was preserved in most nuclei (Fig. 1).

Chromatin Preparation and Dissociation

The purified nuclei were suspended in water containing 0.5 mM phenylmethylsulfonyl fluoride and kept 30 min at 0 °C. The swollen nuclei were disrupted with a Potter-Elvejhem apparatus of 10–30-μm clearance and the suspension centrifuged 5 min at 5000 × *g*; a second treatment of the sediment with the Potter-Elvejhem apparatus was followed by another centrifugation and the procedure repeated until no more intact nuclei could be detected by phase microscopy. The chromatin gel forming the last sediment was washed twice with 0.5 mM phenylmethylsulfonyl fluoride.

The method used to dissociate the chromatin was a modification of those described by Gilmour and

Paul [11] and Stein et al. [12]. The gel was dissolved in urea/NaCl buffer (5 M urea, 3 M NaCl, 10 mM Tris · HCl, pH 8.3) to 700 μg DNA/ml and the solution dialyzed five times against 200 vol. urea/NaCl buffer. DNA was sedimented by a 12-h centrifugation at $420000 \times g$ or a 48-h centrifugation at $130000 \times g$. The protein solution, after an exhaustive dialysis against urea buffer (5 M urea, 10 mM Tris · HCl, pH 8.3), was mixed with a suspension of QAE-Sephadex A-25 (about 1 g/2 mg protein) in the same buffer. After 1 h, the suspension was filtered under reduced pressure on a Büchner funnel using a Whatman 1 paper and the Sephadex washed with small portions of urea buffer. The filtrate contained the histone fraction. The Sephadex was then eluted with urea/NaCl buffer (3 ml/g QAE-Sephadex) to obtain the non-histone protein fraction.

Chromatin Reconstitution

DNA, isolated from chromatin using an adapted Marmur's method [13], was dissolved in 10 mM Tris · HCl, pH 8.3, to 2.5 mg/ml. The DNA, histone and non-histone protein solutions were separately dialyzed five times against 300 vol. urea/NaCl buffer, then mixed at a weight ratio of DNA : histones : non-histones of 1:2:1. The urea was dialyzed away first [14] using 3 M NaCl, 10 mM Tris · HCl, pH 8.3, then the NaCl was removed by steps using 10 mM Tris · HCl, pH 8.3, buffers containing successively 2.5 M, 2.0 M, 1.5 M, 1.0 M, 0.5 M and 0 M NaCl. The reconstituted chromatin was centrifuged 40 min at $40000 \times g$; the sediment was suspended with the Turrax in 10 mM Tris · HCl, pH 8.0, to about 700 μg DNA/ml and dialyzed against the same buffer.

EXPERIMENTS AND RESULTS

Factors Affecting the Retention of the AP Endodeoxyribonuclease Activity in the Isolated Nuclei

We have studied the influence of the medium used for homogenizing the liver on the activity of AP endodeoxyribonuclease measured in the supernatants obtained at $20000 \times g$ (or $5000 \times g$, when the medium did not contain high-molecular-weight polymers) and also on the appearance, in these supernatants, of DNA resulting from the disruption of nuclei.

The enzyme activity was measured in the supernatant and in the nuclei. The crude nuclei were suspended in distilled water and homogenized with the Potter-Elvejhem apparatus of low clearance whereas the supernatant was used directly. The enzyme activities of these preparations, suitably diluted with water, were assayed on alkylated-depurinated [^{3}H]-DNA as described in Materials and Methods.

Except in one set of experiments, 10 ml homogenization medium/g liver tissue was used. Table 1 shows that the AP endodeoxyribonuclease activity in the supernatants varied from 5% to 72% and the DNA content from 0 to 22% of the total (supernatant + sheared sediment) depending on the homogenization medium. A minimum of enzyme activity and no DNA was found with buffer A, an isotonic medium of low ionic strength rich in high-molecular-weight hydrophilic polymers (2.5% Ficoll, 5% dextran, 0.1% bovine serum albumin, 0.25 M sucrose, 4 mM 2-mercaptoethanol, 4 mM $CaCl_2$, 2.4 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.0).

We tried to change the homogenization procedure with buffer A in several ways: adding Tris · HCl increases the AP endodeoxyribonuclease activity found in the supernatant and also the amount of DNA; increasing the pH above 7 leads to the same results as does a reduction of the volume of buffer used per weight of liver tissue.

In another experiment, nuclei isolated in buffer A were resuspended, with a Potter-Elvejhem apparatus of large clearance, either in water or in buffer A. The nuclei swollen in water were disrupted with the Potter-Elvejhem apparatus of low clearance. The nuclei in buffer A were divided into two parts and one of these was submitted to eight 15-s pulses of a MSE ultrasonic disintegrator (20 kHz) at the lowest output (1.5 W). To these three preparations, alkylated-depurinated [^{3}H]DNA was added and, after a 15-min incubation at 37 °C, the acid-soluble radioactivity was measured. Table 2 gives the results which are nearly the same for the nuclei broken in distilled water and the nuclei sonicated in buffer A. However intact nuclei in buffer A yielded a radically different result; the apparent enzyme activity was very low which indicates that the nuclear membranes are nearly impermeable to the enzyme as well as to the substrate.

Cellular Localization of the AP Endodeoxyribonuclease Activity

We distinguish four cellular compartments: nuclei, mitochondria, membranes and cytosol. The homogenization of liver in buffer A was done as described in Materials and Methods, but the first centrifugation was at $40000 \times g$ for 1 h in order to have all the mitochondria in the sediment. This sediment was fractionated on a discontinuous sucrose gradient as described in Materials and Methods: the purified nuclei were found at the bottom of the tube, the mitochondria rested between the 1.4 M and 1.0 M sucrose layers. The $40000 \times g$ supernatant was diluted twice with water and further centrifuged at $100000 \times g$ during 2 h to sediment the membranes; the last supernatant represents the cytosol.

Table 1. *Influence of the homogenization medium on the AP endodeoxyribonuclease activity and DNA content of the nuclei supernatants*
The liver was excised and homogenized as indicated in Materials and Methods except that various homogenization media were used. DNA in whole homogenates and in supernatants was measured with the diphenylamine method following the method of Schneider [18]. Determinations of AP endodeoxyribonuclease activity in crude nuclei and supernatants are described in the text. The table gives the supernatant enzyme activity and DNA content as a percentage of the total (nuclei + supernatant)

Homogenization medium	pH	Volume	Enzyme activity	DNA
		ml/g liver	% total	
0.15 M NaCl, 2.4 mM EDTA, 5 mM Tris · HCl	8.0	10	60–72	20–22
0.3 M sucrose, 4 mM $CaCl_2$	8.0	10	49	1.5–2.5
Buffer A	7.0	10	5– 7	0–1
Buffer A + 5 mM Tris · HCl	7.0	10	11	4
Buffer A + 50 mM Tris · HCl	7.0	10	32	8
Buffer A + 100 mM Tris · HCl	7.0	10	45	9
Buffer A	8.0	10	8–10	2–3
Buffer A	9.0	10	21	8
Buffer A	7.0	8	10–12	3
Buffer A	7.0	5	19	4

Table 2. *AP endodeoxyribonuclease activity of intact and disrupted nuclei on an added alkylated-depurinated DNA*

Nuclei	AP endodeoxyribonuclease
	units/g liver
Disrupted in distilled water	2830
Sonicated in buffer A	2750
Intact in buffer A	175

Table 3. *Cellular localization of the AP endodeoxyribonuclease activity*

Cell compartment	Activity
	% total
Nucleus	91
Mitochondria	2
Membranes	0.1
Cytosol	6.9

The purified nuclei were taken up in distilled water and disrupted with the Potter-Elvejhem apparatus of low clearance. The mitochondria were also taken up in distilled water, resuspended with the Potter-Elvehjem apparatus of low clearance, then sonicated with eight 15-s pulses of the MSE ultrasonic disintegrator (20 kHz) at the lowest output (1.5 W). The membranes were suspended in water with the Potter-Elvehjem apparatus of low clearance. The $100000 \times g$ supernatant was used directly. All these preparations were suitably diluted with water for the enzyme assay on alkylated-depurinated [^{3}H]DNA. The results are expressed in Table 3 as percentages of the sums of the activities. Most of the apparent AP endodeoxyribonuclease activity (about 90%) was in the nucleus. The value for the cytosol is probably a maximum since it is possible that some of it might have leaked from the nucleus. There was some activity in mitochondria and a very low, although significant, activity in cytoplasmic membranes.

Nuclear Localization of the AP Endodeoxyribonuclease Activity

We distinguish three nuclear compartments: chromatin, nuclear sap and nuclear membranes. The chromatin was dissociated and the histone and non-histone protein fractions separated.

Purified nuclei and chromatin were prepared as described in Materials and Methods. The pooled supernatants in the chromatin preparation contained a mixture of nuclear sap and nuclear membranes; absence of DNA in this fraction was checked. These supernatants were centrifuged at $100000 \times g$ for 2 h to separate a sediment of nuclear membranes from the nuclear sap.

The chromatin gel was sheared in water adjusted at pH 8.0 with a Turrax to obtain a homogeneous suspension; the membranes were suspended in water adjusted at pH 8.0 also with the help of the Turrax, whereas the fraction containing the nuclear sap was used directly. After suitable dilution of these preparations with water, their enzyme activity was measured on alkylated-depurinated [^{3}H]DNA. Table 4 shows that the apparent AP endodeoxyribonuclease activity of chromatin was lower than that of the nuclear sap; there was also some activity in the nuclear membranes.

The chromatin was dissociated in DNA, histones and non-histones as described in Materials and Methods. Little AP endodeoxyribonuclease activity was found in the histone fraction, but the activity in the non-histone protein fraction was higher than in the starting chromatin in spite of the drastic treatment (NaCl/urea) used to dissociate the chromatin. To try

Table 4. *Nuclear localization of the AP endodeoxyribonuclease activity*
The figures in parentheses are corrected values trying to account for the inactivation of the enzyme during the manipulations to dissociate the chromatin. The results on reconstituted chromatin are not corrected and the corrected values for the non-histones from the reconstituted chromatin take account only of the inactivation during the second cycle of dissociation. Results from three experiments

Fraction	Enzyme activity		
	units/g liver		
Nuclear membranes	250		
Nuclear sap	4100	3700	8400
Chromatin	2800	800	1800
Histones	300	150	190
	(860)	(430)	(540)
Non-histones	3500	2000	4200
	(10000)	(5700)	(12000)
Reconstituted chromatin	500	170	340
Non-histones from reconstituted chromatin	1530 (4400)		1130 (3200)

to correct for the loss of enzyme activity resulting from this treatment, the nuclear sap AP endodeoxyribonuclease was submitted to the manipulations used to isolate the non-histone fraction; the enzyme activity, measured before and after, indicated an inactivation of 65%. Table 4 gives between parentheses the results corrected as if the percentage survival were the same for the chromatin enzyme.

DNA isolated from chromatin using an adapted Marmur's method [13], histone and non-histone protein fractions were used to reconstitute chromatin as described in Materials and Methods and the reconstituted chromatin was redissociated to separate again the non-histones. Table 4 indicates that the reconstitution of chromatin masks most of the enzyme activity which reappears in the non-histone protein fraction after redissociation.

In every case, a DNA determination was made on the chromatin suspension and the results are given per weight of liver.

The Chromatin AP Endodeoxyribonuclease Activity

The non-histone protein fraction of chromatin was tested on untreated [^{3}H]DNA, alkylated [^{3}H]-DNA and alkylated-depurinated [^{3}H]DNA. Fig. 2 shows that the enzymic activities on untreated and on alkylated DNA were low whereas the activity on alkylated-depurinated DNA was high. The conclusion is that the chromatin non-histones contain an AP endodeoxyribonuclease activity. Fig. 2 also shows that the acid-soluble radioactivity obtained after a long exposure of the alkylated-depurinated [^{3}H]DNA to the enzyme preparation was higher than the acid-soluble radioactivity given by a treatment with 0.2 M

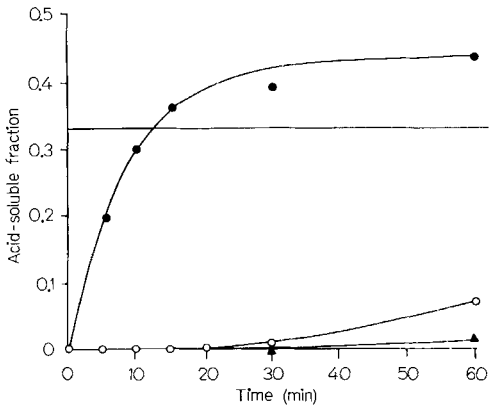

Fig. 2. *Action of non-histone proteins of rat liver chromatin on untreated, alkylated and alkylated-depurinated DNA.* The same amount of chromatin non-histone proteins (5 µg) was incubated with 10 µg of untreated [^{3}H]DNA (▲), alkylated [^{3}H]DNA (one alkylated site per seven nucleotides) (○), or alkylated-depurinated [^{3}H]DNA (one AP site per 20 nucleotides) (●) at 37 °C in a total volume of 1 ml and aliquots were taken at various times to measure the acid-soluble radioactivity. The horizontal line indicates the acid-soluble radioactivity given by a 15-min treatment in 0.2 M NaOH of the alkylated-depurinated [^{3}H]DNA

NaOH; this indicates that the chromatin non-histones contain nuclease activities other than the one acting at AP sites. Comparison of the kinetics given on untreated DNA and alkylated-depurinated DNA suggests that these additional activities are mostly exonucleolytic since they are more evident when an AP endodeoxyribonuclease has introduced breaks near the AP sites.

The purification of the chromatin AP endodeoxyribonuclease was approached in two different ways. In a first trial, the mixture of chromatin proteins (130 mg) in 210 ml urea buffer (pH 8.3) was poured onto a column (2.6 × 50 cm) of QAE-Sephadex A-25 at a rate of 28 ml/h and 7-ml fractions immediately collected. The column was subsequently eluted, at the same rate, with 700 ml of a linear 0–3 M NaCl gradient in urea buffer. Absorbance at 280 nm was measured on each fraction which was subsequently dialyzed against 10 mM Tris · HCl, pH 8.0, before measuring the AP endodeoxyribonuclease activity. Fig. 3 shows that some AP endodeoxyribonuclease activity comes out with the histones although most of it appears with the non-histones as a single peak associated with a small amount of protein eluted around 2 M NaCl.

To avoid the denaturation in a high concentration of urea, another approach was investigated. The chromatin gel was dispersed with a Turrax in 50 mM Tris · HCl, pH 8.0 (1 ml buffer/1.5 mg chromatin DNA) and the suspension divided into two equal parts. To one half, 8% streptomycin sulfate in the same buffer was added slowly at 4 °C to a final concen-

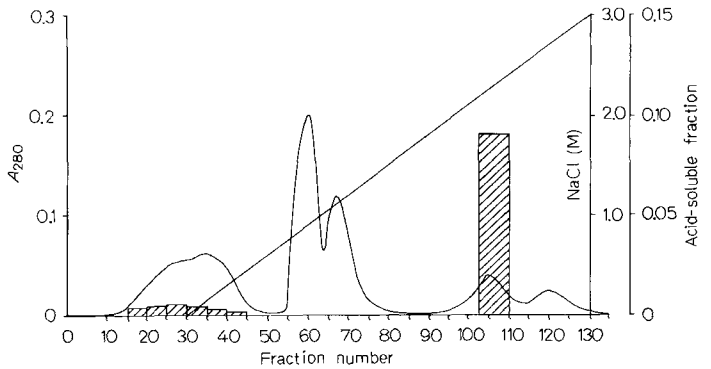

Fig. 3. *Chromatography on QAE-Sephadex of the chromatin proteins of rat liver.* The mixture of chromatin proteins (130 mg) in 5 M urea, 10 mM Tris · HCl, pH 8.3, was poured on the QAE-Sephadex column which was subsequently eluted with a linear 0–3 M NaCl gradient in the same buffer. The absorbance (A) at 280 nm was measured (——) and, after dialysis of each collected fraction against 10 mM Tris · HCl, pH 8.0, the enzyme activity of 20-µl aliquots was determined on alkylated-depurinated [^{3}H]DNA; it is expressed as acid-soluble fractions (striped rectangles)

tration of 0.8%; after 30 min, the suspension was centrifuged for 15 min at 10000 × g. To the supernatant cooled at 0 °C, solid ammonium sulfate was added to 85% saturation. After 30 min of slow mixing, the suspension was centrifuged for 15 min at 10000 × g; the sediment was dissolved in a volume of 50 mM Tris · HCl, pH 8.0 equal to that in which the chromatin was initially suspended and dialyzed exhaustively against the same buffer. To the other half of the chromatin suspension at 0 °C, solid ammonium sulfate was directly added up to 85% saturation. A clog which formed was removed with tweezers and the protein precipitate treated as already described. The method using streptomycin sulfate gives from 1000–5000 enzyme units/g liver because a variable amount of AP endodeoxyribonuclease activity binds to the streptomycin precipitate. Without the streptomycin pretreatment, the yield is 4000–6000 enzyme units/g liver.

The Mitochondrial AP Endodeoxyribonuclease Activity

The mitochondrial fraction contained 2% of the overall apparent AP endodeoxyribonuclease activity of the broken cells (Table 3). It seems that the enzyme active on alkylated-depurinated [^{3}H]DNA truly belongs to the mitochondria. Phase-contrast microscopy failed to show nuclei in the mitochondrial preparation. On the other hand, the small amount of cytosol or membranes, which themselves have a very low activity, that might contaminate the mitochondria, can contribute only an insignificant amount of enzyme.

A preparation of mitochondria, suspended in water with the Potter-Elvejhem apparatus of low clearance and sonicated, was tested on untreated [^{3}H]DNA, alkylated [^{3}H]DNA and alkylated-depurinated [^{3}H]-DNA. Fig. 4 indicates no action on untreated DNA, a very small one on alkylated DNA (alkylated DNA always has a few AP sites) and a high activity on alkylated-depurinated DNA. The mitochondria thus

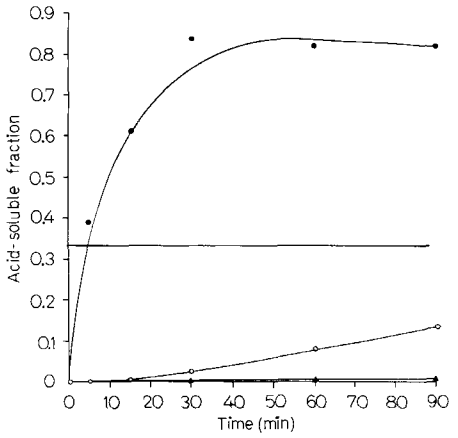

Fig. 4. *Action of sonicated mitochondria from rat liver on untreated, alkylated and alkylated-depurinated DNA.* The same amount of sonicated mitochondria was incubated with 10 µg of untreated [^{3}H]DNA (▲), alkylated [^{3}H]DNA (one alkylated site per seven nucleotides) (○) or alkylated-depurinated [^{3}H]DNA (one AP site per 20 nucleotides) (●) at 37 °C in a total volume of 1 ml; aliquots were taken at various times to measure the acid-soluble radioactivity. The horizontal line indicates the acid-soluble radioactivity given by a 15-min treatment in 0.2 M NaOH of the alkylated-depurinated [^{3}H]DNA

possess an AP endodeoxyribonuclease activity. But here again (and it is more important than with the non-histone proteins of chromatin), a long exposure of the alkylated-depurinated [^{3}H]DNA to the preparation yielded an acid-soluble radioactivity larger than that which was given by a treatment with 0.2 M NaOH; in fact, nearly all the [^{3}H]DNA became acid-soluble. This observation suggests the presence of an important exonuclease activity in the mitochondria.

The AP Endodeoxyribonuclease Activity of the Membranes

The AP endodeoxyribonuclease activity of a 40000 × g supernatant (buffer A) of crude nuclei

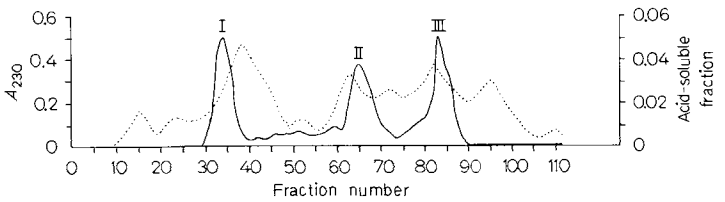

Fig. 5. *Chromatography on Sephadex G-75 of a mixture of dissociated membranes, nuclear sap and chromatin non-histones.* The mixture (total volume = 2.5 ml) was poured onto a column (1.6 × 65 cm) of Sephadex G-75 and eluted at a rate of 7 ml/h; 4-ml fractions were collected. (·····) The absorbance (*A*) at 230 nm; (— · —) the enzyme activity of 20-µl aliquots determined on alkylated-depurinated [^{3}H]DNA and expressed as acid-soluble fractions. I = membrane enzyme; II = nuclear sap enzyme; III = chromatin enzyme

(= cytoplasmic membranes + cytosol) was increased fourfold on addition of 0.5% Triton X-100. This 40000 × *g* supernatant, after a twofold dilution, was further centrifuged at 100000 × *g* for 4 h. The AP endodeoxyribonuclease activity of the 100000 × *g* supernatant (= cytosol) was slightly inhibited by 0.5% Triton X-100. The 100000 × *g* sediment (= cytoplasmic membranes) was resuspended in twofold diluted buffer A; a fivefold increase of the enzyme activity was observed on addition of 0.5% Triton X-100.

Addition of 0.5% Triton X-100 also increased sixfold the AP endodeoxyribonuclease activity of nuclear membranes prepared as described in Materials and Methods, whereas the enzyme activities of the nuclear sap and the chromatin non-histones were untouched or decreased.

Properties of the Enzymes of the Different Cell Compartments

Molecular Weights. The enzyme preparation in 2.5 ml of 50 mM NaCl, 50 mM Tris · HCl, 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol, pH 8.0, was layered on top of a column (1.6 × 65 cm) of Sephadex G-75 equilibrated with the same buffer and the elution was performed, always with the same buffer, at a rate of 7 ml/h; 4-ml fractions were collected. The preparations were first analyzed separately, then together. Fig. 5 shows the chromatography of a mixture of membranes dissociated by addition of 0.5% Triton X-100, nuclear sap and chromatin non-histones; there are three peaks of AP endodeoxyribonuclease activity which have the same position as when the preparations are run separately. The membrane activity (I) emerges at the void volume of the column; then comes the nuclear sap enzyme (II) and finally the chromatin non-histone enzyme (III).

Thermostability. The enzyme preparation (1 ml) in 50 mM NaCl, 50 mM Tris · HCl, 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol, 1% bovine serum albumin, pH 8.0, was heated at 45 °C in polypropylene tubes; 20-µl aliquots were taken at various times to measure the AP endodeoxyribonuclease activity on alkylated-depurinated [^{3}H]DNA during 10 min at 30 °C. In each case, the logarithm of the residual enzyme activity was a linear function of time. Cytoplasmic and nuclear membrane enzymes both had a half-life of 25 min at 45 °C. The half-life of the chromatin non-histone enzyme was 2.5 min. The nuclear sap enzyme was stable at 45 °C.

DISCUSSION

Most of the AP Endodeoxyribonuclease Activity is in the Nucleus and the Nuclear Membrane has a Low Permeability to the Enzyme

In this section, we discuss apparent activities demonstrated on an added alkylated-depurinated [^{3}H]DNA, leaving for later discussion of the question of a masked activity in chromatin as well as in membranes. In a first approach, we have distinguished the sediments and supernatants of 20000 × *g* centrifugations (or 5000 × *g* when there is no high-molecular-weight polymer). The sediments contain the nuclei and a part of the mitochondria; although the mitochondria raise an interesting point that will be dealt with later, their contribution to the activity of the sediment is negligible compared to that of the nuclei. The enzyme assay on the sediments was done after dispersing them in water in the presence of a protease inhibitor and disrupting the swollen nuclei in a Potter-Elvejhem apparatus of low clearance. The supernatants were used as such.

The fraction of the total AP endodeoxyribonuclease activity found in the supernatant depends on the conditions used for the homogenization of the liver tissue. A minimum, between 5% and 7% of the total apparent activity, is observed when using 10 vol. buffer A, an isotonic medium of low ionic strength rich in high-molecular-weight hydrophilic polymers. This minimum is probably the maximum existing in the cell cytoplasm and, when more activity is found in the supernatant, this is probably because the homogenization has released some nuclear enzyme. This release appears to have two possible causes: the rupture of the nuclear membranes and the supernatant then contains DNA; permeation of the nuclear membranes to the nuclear enzyme. The results pre-

sented in Table 1 indicate that a high ionic strength helps to break open the nuclei which releases DNA in the supernatant and moreover permeates the membranes of the remaining nuclei to the enzyme. A low ionic strength seems to preserve the anatomical integrity of the nuclei, but is not sufficient to prevent the leakage of the nuclear enzyme through the membranes unless high-molecular-weight hydrophilic polymers are added.

We also showed (Table 2) that very little AP endodeoxyribonuclease activity can be demonstrated on alkylated-depurinated [^{3}H]DNA added to a suspension of intact nuclei in buffer A. Rupture of the nuclear membranes either by sonication or by shearing the nuclei swollen in water reveals the enzyme activity. This experiment indicates that, in buffer A, the nuclear membranes have only a low permeability to DNA as well as to the AP endodeoxyribonuclease.

Cellular Localization of the AP Endodeoxyribonuclease Activities

AP endodeoxyribonuclease activities have been found in nucleus and cytoplasm.

The nuclear activity is divided between nuclear sap, chromatin and nuclear membranes. The AP endodeoxyribonuclease activity of chromatin is for its greater part not demonstrable on an added alkylated-depurinated [^{3}H]DNA unless the chromatin has been dissociated into DNA and proteins; most of the AP endodeoxyribonuclease activity is then found in the non-histone proteins. It is even possible that the low apparent activity observed on chromatin sheared with a Turrax apparatus is due to partial denaturation and that true native chromatin has no activity on a foreign DNA containing AP sites. The results suggest that the AP endodeoxyribonuclease in chromatin is oriented to work on chromatin DNA. We previously made a similar observation on the chromatin from *Phaseolus multiflorus* embryos [15,16]. The AP endodeoxyribonuclease of the nuclear membranes is also partially masked and can be revealed on addition of Triton. Thus, if the apparent AP endodeoxyribonuclease activity of the broken nucleus is about 90% of what is found in the cell (Table 3), the above remarks indicate that the enzyme content of the nucleus is even proportionally greater.

In the cytoplasm, three different localizations have been found: cytosol, mitochondria and cytoplasmic membranes. The membrane AP endodeoxyribonuclease is activated by Triton. The enzyme found in mitochondria seems to be actually in these organelles since the preparation was not contaminated with nuclei and the contribution from cytosol or cytoplasmic membranes can be only negligible.

In each cell compartment, the nuclease activity is greatly increased by the presence of AP sites in DNA

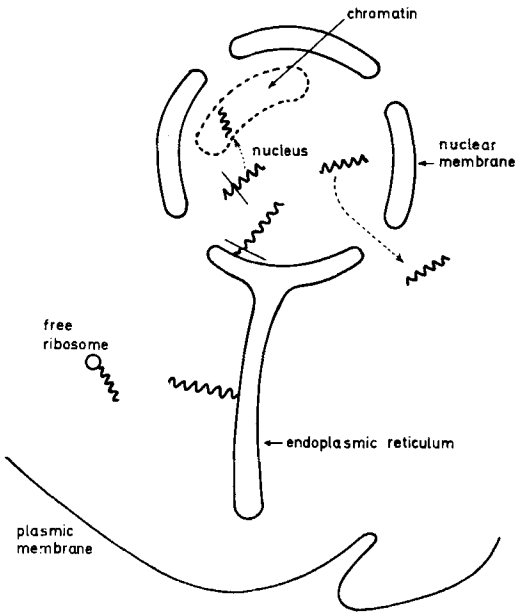

Fig. 6. *Hypothetical scheme of the protein maturation leading to the chromatin AP endodeoxyribonuclease.* See text for explanations

so that we are truly dealing with AP endodeoxyribonucleases. Nevertheless, alkylated-depurinated DNA activated by the AP endodeoxyribonuclease is easily broken down; the preparations thus contain active exonucleases able to degrade double-stranded DNA from single-stranded breaks. Purification of the enzymes will be necessary to learn whether some of the AP endodeoxyribonucleases possess an exonuclease activity as is the case for *Escherichia coli* endonuclease VI which is the same enzyme as exonuclease III [3,17].

Plurality of the AP Endodeoxyribonucleases in the Rat Liver

We have seen the presence of AP endodeoxyribonuclease activities in different cell compartments; we should like to know whether or not they are different enzymes.

The mitochondria might have a particular enzyme perhaps synthesized in this organelle. We previously found that the chloroplastic AP endodeoxyribonuclease of *P. multiflorus* was different from the enzyme present in the nuclear sap and cytosol [15].

The AP endodeoxyribonucleases of the membranes appear to be different from the enzymes found in the other cell compartments since they are activated by Triton whereas the other ones are inhibited. On the other hand, the cytoplasmic and the nuclear membrane enzymes might be identical since they are both activated 5–6-fold by 0.5% Triton and have the same thermal resistance (25-min half-life at 45 °C).

The nuclear sap enzyme and the chromatin enzyme, both different from the membrane enzyme, cannot be the same protein; they differ by their molecular weights and thermal resistances.

These results show that different AP endodeoxyribonucleases are located in different cell compartments. One may ask whether these enzymes are coded by different genes or whether they correspond to steps in the maturation of the same protein. Sephadex G-75 chromatography has shown that the nuclear sap enzyme has a higher molecular weight than the chromatin enzyme; the membrane enzyme emerging at the void volume of the column, although it is likely to form a complex with Triton, is probably bigger than the nuclear sap enzyme. One may thus suggest the hypothesis depicted in Fig. 6. Free ribosomes synthesize a protein which attaches to the inner side of the endoplasmic reticulum. This protein, which might be the membrane enzyme activated by Triton, is carried into the nucleus, as on a conveyor belt, by the membrane system. The hydrophobic part of the molecule is split off by a protease and the enzyme released in the nuclear sap. Some of the nuclear sap enzyme leaks into the cytosol, the direction of the flow and the poor permeability of the nuclear membranes to the enzyme explaining the much lower concentration found in cytosol as compared to nuclear sap. The nuclear sap enzyme is finally cut by a second protease to yield a species which is immediately taken up by chromatin. The chromatin enzyme is most likely the only one involved in the repair of nuclear DNA.

We plan to isolate the different AP endodeoxyribonucleases of rat liver and compare their primary structures.

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