ACTION OF MITOCHONDRIAL DNAASE I IN DESTROYING THE CAPACITY OF ISOLATED CELL NUCLEI TO FORM GELS*

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INTRODUCTION

It has been shown previously that some enzyme (or enzymes) present in isolated (and disrupted) mitochondria is capable of entering cell nuclei during the isolation procedure and destroying the capacity of the nuclei to form gels in alkali or molar saline. The same effect was observed when already isolated nuclei that were capable of gel formation were treated near pH 6.0 with broken mitochondria or aqueous extracts of the latter. It was also demonstrated that whatever enzyme caused the loss in capacity of the nuclei to form gels also caused a splitting of the DNA from the residual protein to which it normally is rather firmly bound (1, 2).

This paper is chiefly concerned with the identification of what we believe is the most important enzyme causing loss of gel-forming capacity in the case of liver nuclei, namely, DNAase I. We have already reported briefly that DNAase I is an important "antigelling" enzyme (2) and the chief results given in a recent paper claiming identification of the degelling enzyme with DNAase I (3) are confirmed by the results included in our present paper. A still more recent paper by Rotherham *et al.* (4) also confirms the ability of desoxyribonuclease to cause degradation of the desoxyribonucleoprotein of rat liver during the isolation procedure. We have previously pointed out that if the ability of isolated nuclei to form gels is dependent upon the presence of an intact desoxyribonucleoprotein structure, enzymes of any one of three types should be capable of destroying this gel-forming ability. These are desoxyribonucleases, proteases, or a type of enzyme capable of splitting whatever linkages hold the DNA to the protein. Although DNAase I action is to a large extent responsible for the destruction of the gel-forming capacity of rat liver nuclei during their isolation in the presence of disrupted mitochondria, some concomitant protease action is not excluded.

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Materials and Methods

Isolated Nuclei.—(*a*). "Gelable" nuclei containing firmly bound DNA were for the most part isolated from rat liver at pH 3.8–4.0 in dilute citric acid, using the Waring blendor for homogenization as previously described (5). In this procedure the mitochondria are disrupted, but the low pH prevents action by the mitochondrial antigelling enzyme. The reason for using this method instead of a method that permits the isolation of gelable nuclei at higher pH in sucrose solutions without mitochondrial disruption (6), was to obtain large quantities of gelable nuclei in the shortest possible time. Preliminary experiments have demonstrated that the gelable nuclei isolated at pH 4.0 in dilute citric acid behave in the same way towards disrupted mitochondria as do the gelable nuclei isolated at higher pH in the presence of sucrose.

(*b*) "Gelable" nuclei were also isolated at pH 6.0 in the presence of sucrose by the method of Dounce *et al.* (6).

(*c*) "Non-gelable" nuclei were generally isolated from rat liver in very dilute citric acid in a solution containing gum arabic, as previously described (7). These nuclei are exposed to the action of disrupted mitochondria during the isolation procedure.

(*d*) "Non-gelable" nuclei were also isolated in the presence of versene. The method used in this case was a combination of the original method used to isolate rat liver nuclei at pH 6.0 (5) with the method for isolation at pH 4.0 in less dilute citric acid (5). The initial homogenization was done in most cases with the ball-type homogenizer (6) instead of the Waring blendor, since the latter gave less satisfactory results. The homogenate was of the same composition as usual except that various amounts of 0.25 M versene solution were added to give final versene concentrations of 0.005, 0.01, 0.05, and 0.1 molar. In experiments where the two highest concentrations of versene were used, it was necessary to neutralize the versene[1] solution partially before use by adding 0.1 N NaOH.

After the first centrifugation, the nuclei were suspended in ice water containing enough 0.1 M citric acid to adjust the pH to 3.9–4.0. The subsequent steps were carried out as in the isolation of nuclei at pH 4.0 (5). Some trouble was encountered with agglutination at the higher versene concentrations, but in spite of this it was possible to isolate reasonably clean nuclei.

It was found possible to carry out the entire isolation of nuclei in versene at pH 6.0, but this was difficult owing to agglutination of the nuclei and cytoplasmic particles. There is ample time for the mitochondrial enzyme to act on the nuclei during the homogenization and first centrifugation, even though subsequent action of the enzyme is prevented by lowering the pH to 4.0. This latter step minimizes agglutination and facilitates isolation of the nuclei.

Fibrous DNA was isolated from these nuclei by a detergent method (8), but without previous removal of globulins and histones. The nuclei often formed partial gels when treated with dilute ammonia in concentrated suspensions.

Isolation of Mitochondria.—Unless otherwise specified, mitochondria were isolated by the method of Dounce *et al.* (6), using 0.44 M sucrose with adjustment of the pH to 6.0 with citric acid. On some occasions the method was modified by leaving out the citric acid.

Isolation of DNA.—DNA was isolated from the cell nuclei by the method of Kay *et al.* (8), with two modifications. In the case of nuclei isolated at pH 4.0, the globulin fraction was extracted in cold 0.15 M NaCl at pH 7.0 and the pH was then generally lowered to 6.0–6.3, after addition of the detergent. In some cases, however, the pH was

[1] The versene was disodium ethylene diamine tetraacetate obtained from the Bersworth Chemical Company, now obtainable from Dow Chemical Company, Inc., New York, N. Y.

allowed to remain at 7.0. Incubation in the detergent solution was carried out for 3 hours or longer.

In extracting DNA from nuclei isolated at pH 6.0, the globulin fraction was first removed by extraction in cold 0.15 M NaCl at pH 7.0. The detergent solution was then added without pH adjustment and the protein was precipitated by adding solid NaCl to molar concentration immediately after mixing. After removal of the precipitated protein by centrifugation, the DNA was immediately precipitated by the addition of alcohol.

Analysis for DNA.—(*a*) *Qualitative.* The sample to be tested was dissolved or suspended in water and 2 volumes of the Dische reagent were added. The solution was then heated in a bath of boiling water for 10 minutes for color development. (*b*) *Quantitative.* The sample to be tested was extracted twice with 5 ml. portions of ice cold 10 per cent trichloracetic acid, using centrifugation to recover the sample, and then was heated at 90° C. for 15 minutes in 2.5 ml. of 5 per cent trichloracetic acid. The solution was centrifuged, and the supernatant fluid was transferred to a 5 ml. volumetric flask. The DNA was then determined according to the directions of Schneider (9), using purified calf thymus DNA as a standard.

Phosphorus Determination.—Phosphorus was determined by a slight modification (10) of the method of Sumner (11).

Absorption Spectra.—Absorption spectra in the ultraviolet region were determined with the model DU Beckman spectrophotometer.

Determination of Relative Viscosities of DNA Solutions.—Relative viscosities were determined at 30° ± 0.05° with Ostwald type viscosimeters of 5 or 10 ml. volumes. Solutions of DNA in saline were made up which always contained 0.15 gm. DNA per 100 ml. of solution, but which varied in salt concentration from zero to 5.9 per cent (1 molar).

Action of Mitochondrial Enzyme on Gelable Nuclei.—Rat liver nuclei capable of gel formation that were isolated at pH 6.0 in 0.44 M sucrose (6) or nuclei capable of gel formation that were isolated at pH 4.0 in very dilute citric acid (5) were used in these experiments. The nuclei were suspended in water and dry weights were determined from aliquots.

In these experiments, mitochondria isolated in 0.25 M sucrose (12), 0.44 M sucrose, and 0.44 M sucrose with the pH adjusted to 6.0 with citric acid (6) were used. The weight of mitochondria was approximated by taking dry weights of suspension of well centrifuged mitochondria in water, ignoring the residual sucrose.

The mitochondria were generally suspended in distilled water for use in the antigelling experiments. The total volumes of the suspensions of nuclei plus mitochondria varied from 1.5 to 3.0 ml., depending on the amount of nuclei used. Magnesium acetate when added was present in 0.002 M concentration, and versene when used was in concentrations varying from 0.001 to 0.1 M. The pH was adjusted before addition of the mitochondria to a value of approximately 6.0 with 0.01 M NaOH, using bromcresol purple as an outside indicator. The mitochondrial suspensions or lysates, previously adjusted to a pH of about 6.0, were always added last. Incubation times varied from 10 to 15 minutes, as a rule. Tenth normal NaOH was added dropwise with stirring at the end of the incubation period to test for "gelability." Addition of the NaOH was continued until a definite gel was produced or until the solution became strongly alkaline without gel formation. Suitable controls were carried out using boiled mitochondria or mitochondrial lysates.

Treatment of Nuclei or Nucleoprotein with DNAases I and II.—140 to 250 mg. samples of "gelable" nuclei isolated at pH 4.0 were suspended in about 40 ml. of distilled water and treated with 0.01 to 1.0 ml. volumes of aqueous solutions of crystallized DNAase I containing from 0.002 to 2.0 mg. of enzyme. Magnesium acetate was present in 0.002 M concentration and the pH was adjusted to about 6.0 by the addition of 0.1 N NaOH. Twenty to 30 mg. samples of gelable nucleoprotein, made by removing the globulins and histones

from above mentioned type of nuclei by extraction at 0° C. with 0.9 per cent NaCl and then 0.2 N HCl, were treated in a similar manner with the enzyme. In all of the experiments in which the amounts of DNAase used were not very small, there was a tendency for the pH to fall during the digestion with the enzyme, since no buffer was used, and hence it was often necessary to add small amounts of 0.1 N NaOH from time to time to keep the pH from falling appreciably below 6.0. Periods of incubation varied from 15 minutes to 1 hour. Distinct changes in the physical appearances of the nuclei or nucleoprotein mixtures were noted as incubation with DNAase I proceeded.

The gel-forming capabilities of the nuclei or nucleoprotein were tested after the incubation with enzyme by adding dilute NH_4OH dropwise to small aliquots of the incubation mixtures until gel formation occurred or until the solutions became very alkaline without gel formation.

In order to find out how much DNA remained firmly bound to protein after treatment with DNAase I, the nuclei were extracted twice after the incubation period with cold 0.9 per cent NaCl and then were stirred for a few minutes in 0.5 per cent duponol solution at pH 6. Enough solid NaCl was then added to make the concentration of the latter 1 molar, and the precipitated protein was removed by centrifugation. The treatment with detergent was repeated once. The amount of DNA left in the residual protein was determined by the Schneider-Dische technique, using hot 5 per cent trichloracetic acid to extract the residual DNA from the protein.

Treatment of suspensions of whole nuclei with partially purified DNAase II,[2] was carried out in a manner similar to treatment with DNAase I, using about 10 to 20 mg. of nuclei, except that a rather concentrated stock solution of enzyme was used whose enzyme content was not known. Magnesium acetate was not required for activation and it was found that neither 0.002 M $Mg(Ac)_2$ nor 0.002 M citric acid inhibited the antigelling action of the DNAase II.

RESULTS

Using the experimental procedures reported in the section on materials and methods, we have obtained the following results.

1. The DNA obtained from nuclei incapable of gel formation, isolated at pH 6.0 in very dilute citric acid, is definitely depolymerized. This is shown by its decreased tendency to form long fibers when precipitated from aqueous solution with alcohol, and by the very pronounced decrease in its viscosity in water or saline solutions as compared with the viscosities of corresponding solutions of known high-polymer DNA. The latter finding is illustrated in Fig. 1. Hence DNAase action must have occurred during the isolation procedure. The properties of DNA isolated from rat liver nuclei obtained at pH 4 and pH 6 under various conditions are summarized in Table I. Some of the DNA samples are obviously contaminated with protein, judging from the analyses for phosphorus. This is because insufficient material was available for more complete purification.

2. The mitochondrial antigelling enzyme requires an activator, such as Mg^{++} ions. This fact appears to preclude appreciable DNAase II action. The

[2] We are indebted to Mr. T. Kosalka of the Atomic Energy Division of this University for the DNAase II preparation, which was made from calf spleen.

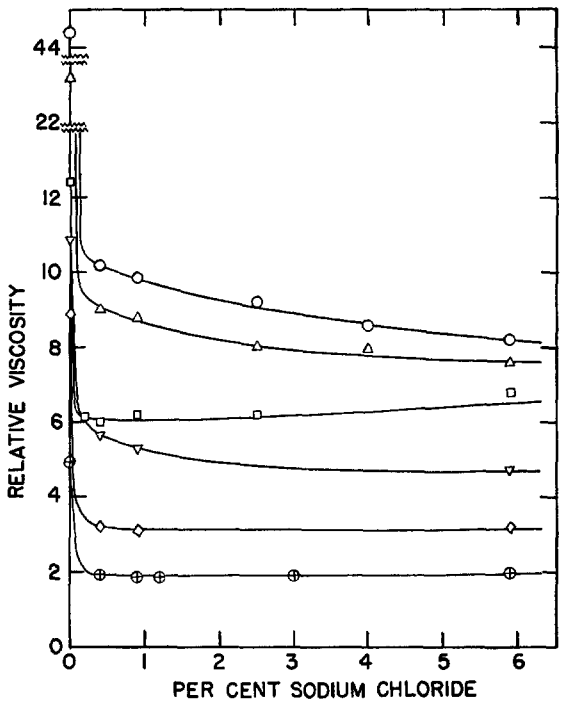

FIG. 1. Relative viscosities of various Na-DNA samples, plotted against NaCl concentration. All DNA solutions contained 0.15 per cent NaDNA.

O Fibrous DNA prepared from rat liver cell nuclei isolated at pH 4.0. Exhaustively purified by four treatments with sodium dodecyl sulfate and washed in graded concentrations of alcohol to remove NaCl. See Table I, Row 3.

△ Same as **O** except that graded concentrations of ethanol not used in washing. See Table I, Row 2.

□ DNA prepared from rat liver nuclei isolated at pH 6 → 4, using 0.05 M versene solution. See Table I, Row 8.

▽ DNA prepared from rat liver nuclei isolated at pH 4.0 and then treated with DNA-ase I. See Table I, Row 9.

◇ DNA prepared from rat liver nuclei isolated at pH 6 → 4 using 0.0053 M versene. See Table I, Row 6.

⊕ DNA prepared from rat liver nuclei isolated at pH 6.0 in gum arabic solution (without versene). See Table I, Row 4.

results of what we consider to be the most reliable experiments showing the necessity of magnesium ions for the mitochondrial antigelling enzyme are shown in Table II. The variability of all experiments is shown in Table III.

Certain of the results in these tables may require special comment. The mitochondria isolated in the presence of citric acid *uniformly* fail to prevent gelation in the absence of magnesium ions. Using the same mitochondria, there

are only two out of 26 experiments where the addition of magnesium very definitely had little or no tendency to cause the prevention of gelation, and only three other experiments where one could reasonably question whether there was any effect. On the other hand, mitochondria isolated in the absence of added citrate will sometimes prevent the gelation phenomenon. (This has also been found in a number of preliminary experiments not listed in the tables.) We interpret this to mean that enough divalent metal may remain in the mitochondria isolated in the absence of citrate to activate the DNAase I.

TABLE I

DNA sample	Per cent phosphorus	$D^{1}_{1\ cm.}$ per cent 260 mμ in H_2O	Relative viscosity in H_2O (0.15 per cent solution)	No. of treatments with detergent
pH 4 (fibrous)	9.45	205	21.57	4
pH 4 (fibrous)	9.38	—	23.17	4
pH 4 (fibrous)	—	—	44.40	4*
pH 6 (fibrous)	9.74	180	4.93	4
pH 6 (semi-fibrous)	9.74	209	4.18	4
pH 6 → 4 (fibrous) isolated in 0.0053 M versene	7.62	169	8.87	2
pH 6 → 4 (fibrous) isolated in 0.0112 M versene	6.63	—	8.92	2
pH6 → 4 (fibrous) isolted in 0.05 M versene	—	—	12.46	4*
pH 4 (fibrous) treated with DNAase I (1.1×10^{-5} mg. enzyme per mg. nuclei)	8.71	238 204‡	10.86	2
pH 4 (fibrous) treated with DNAase I (1.4×10^{-4} mg. per mg. nuclei)	—	334	—	2
Calf thymus DNA§	9.3	192	18.5	2

* Treated with graded concentrations of ethanol to remove salt.

‡ Value in 2 per cent saline.

§ Isolated according to Kay, Simmons, and Dounce (8).

The fact that whole homogenate made in the presence of citrate prevents gelation is not surprising, since the concentration of added citrate is in the neighborhood of only 0.001 M, and is apparently not sufficient to keep all of the naturally occurring metallic activator in the homogenate in a complexed state. However, when mitochondria are isolated from such an homogenate, the metallic activator is effectively removed from them, apparently because there is enough citrate in the original homogenate to "loosen" the activator so that it can be effectively removed by the subsequent washing procedure.

The fact that boiling in the presence of magnesium is more effective than boiling in the absence of magnesium in destroying the antigelling action of the

mitochondrial enzyme probably indicates a partly reversible heat denaturation in the absence of magnesium.

TABLE II

Action of Mitochondria on Gelable Nuclei at pH 6–6.2

Test No.	Approximate dry weight of mitochondria or other material used	Presence of absence of citrate in isolating mitochondria	$Mg(Ac)_2$ concentration	Dry weight of nuclei used*	pH at which nuclei were prepared	Approximate* ratio of mitochondria to nuclei	Volume of 0.1 N NaOH used to produce gelation	Gel‡ formation
1	10.5 mg.§	Present	Not added	2.3 mg.	4	4.6/1	0.15 ml.	+++
2	10.5 mg.§	Present	0.002 M	2.3 mg.	4	4.6/1	0.1 ml.	±
3‖	6.3 mg.§ boiled	Present	0.002 M	1.4 mg.	4	4.6/1	0.1 ml.	+++
4	—	—	0.002 M	2.3 mg.	4	—	0.15 to 0.2 ml.	+++
5	10 to 11 mg.¶	Present	0.002 M	4.3 mg.	4	2.5/1	0.1 to 0.2 ml.	+
6	5 to 5.5 mg.¶	Present	0.002 M	17.3 mg.	4	1/3	0.15 to 0.2 ml.	++
7	4.0 mg.¶	Absent	Not added	3.2 mg.¶	4	1.25/1	0.05 ml.	±
8	4.0 mg. boiled¶	Absent	Not added	3.2 mg.¶	4	1.25/1	0.05 ml.	+++
9	30 mg. water homogenate¶	Present	Not added	4 mg.¶	6	—	0.05 to 0.1 ml.	±
10	30 mg. water homogenate boiled¶	Present	Not added	4 mg.¶	6	—	0.05 to 0.1 ml.	+++

* The nuclear weights are based on actual dry weights taken on aliquot suspensions. These values do not take into account the 50 per cent weight loss of the nuclei during the isolation procedure. If the 50 per cent loss were taken into account the ratio of mitochondria to nuclei would be half as great in all cases.

‡ Gels graded from ± (no gel or very slight recoil) thru +++ (strong gel).

§ Water lysate of mitochondria.

‖ Mitochondria boiled in 0.002 M $Mg(Ac)_2$ do not prevent gelation of nuclei; mitochondria boiled previous to the addition of 0.002 M $Mg(Ac)_2$ do prevent gelation, apparently because of reactivation of DNAase.

¶ Approximate dry weight.

3. When gelable nuclei are treated with crystallized DNAase I in the presence of Mg^{++}, subsequent gelation is prevented and a rapid cleavage of DNA from the residual protein is caused. If sufficient quantities of DNAase are used, there is also considerable depolymerization of the DNA. When low concentrations of the enzyme are employed, gelation can be prevented and the DNA is still cleaved from the protein, but only slight depolymerization of the DNA

occurs. Apparently bonds in the DNA molecule close to points of attachment of the latter to protein are preferentially attacked at pH 6.0 by the DNAase. It should be emphasized, however, that there is always a *slight* depolymeriza-

TABLE III

*Variability of Experiments**

Test No.	Incubation materials	Approximate mitochondria/ nuclei ratio	Number of experiments with a given degree of gelation‡			
			±	+	++	+++
1	Nuclei plus H_2O-citrate homogenate	—	3	—	—	—
2	Same as 1 but boiled	—	—	—	—	3
3	Nuclei plus non-citrated mitochondria	<2.5/1	2	—	—	—
		2.5/1	—	—	1	2
4	Same as 3 but boiled	<2.5/1	—	—	—	2
5	Nuclei plus citrated§ mitochondria	<2.5/1	—	1	—	1
		2.5/1	—	—	—	1
		>2.5/1	—	—	1	1
		≫2.5/1	—	—	1	—
6	Nuclei plus citrated mitochondria plus Mg^{++}	<2.5/1	5	2	2	1
		2.5/1	1	2	—	2
		>2.5/1	8	3	—	—
7	Same as 6 but boiled	<2.5/1	—	—	—	2
8	Nuclei plus mitochondria boiled in Mg^{++}	—	—	—	—	3
9	Nuclei plus mitochondria boiled previous to Mg^{++} addition	—	3	1	—	—

* Nuclei isolated at pH 4 in dilute citric acid have not been distinguished in this table from those isolated at pH 6.0 in 0.44 M sucrose, since both are gelable and both behave identically on being treated with mitochondria under various conditions.

‡ Gels graded from ± (no gel or very slight recoil) thru +++ (strong gel).

§ Many more experiments were carried out where the dry weight ratio of mitochondria to nuclei must have been high but was not measured, in which no degelation at all could be observed.

tion of the DNA and an appreciable nucleotide residue that remains firmly bound to the residual protein after action of DNAase I at pH 6.0, so that cleavage of the DNA from the residual protein is undoubtedly caused by the splitting of internucleotide phosphodiester bonds, *not* by the splitting of DNA-protein bonds.

That cleavage of DNA from the residual protein occurs as the result of DNAase action on gelable isolated nuclei is demonstrated by the fact that DNA can be isolated from these nuclei by the use of detergent without the lowering of pH and without the necessity for an incubation period in the detergent solution.

The results of the experiments in question are summarized in Table IV. DNAase I action can be seen to imitate very closely the action of the mitochondrial antigelling enzyme system, but there is a slight difference, since the mitochondrial enzyme system does not leave any detectable nucleotide residue in the residual protein.

4. Pretreatment with partially purified DNAase II at pH 5.7–6.0 completely prevents gel formation with nuclei that are otherwise capable of gel formation.

TABLE IV

Action of Crystalline DNAase I on Rat Liver Nuclei Isolated at pH 4.0

Experiment	Dry weight nuclei	Dry weight DNAase I	Enzyme digest time	Dry weight DNA recovered	Per cent DNA in residue based on DNA as 12 per cent of nuclei	Condition of DNA isolated
	mg.	*mg.*		*mg.*		
A	169.6	2.2	1 hr.	22.5	0.51	N.F.*
B	141.5	0.5	½ hr.	18.2	1.13	N.F.
C	161.1	0.1	¼ hr.	25.1	0.98	F.‡
D	170.0	0.024	¼ hr.	29.4	1.85	F.
E	252.0§	0.0024	10 min.	57.0	0.59	F.

* N.F. = non-fibrous.

‡ F. = fibrous.

§ These nuclei contained a fair proportion of non-nuclear fragments and there was obvious contamination of the isolated DNA with protein.

5. Versene will not block the antigelling action of disrupted mitochondria completely, even if it is added in concentrations as high as 0.05–0.1 molar to the original homogenizing medium used for isolating nuclei. Its addition does, however, permit the subsequent isolation from the nuclei of completely fibrous DNA which has a considerably higher viscosity than does DNA subjected to the action of disrupted mitochondria during the isolation procedure in the absence of versene. The viscosities of two samples of DNA of this type are included in Fig. 1. Since the phosphorus content of both these sampls is low, it is possible that the lowered viscosities are due to the presence of impurity rather than to partial depolymerization of the DNA. Further work would be required to establish this point with certainty.

6. It was found that versene can activate the antigelling action of disrupted isolated mitochondria in the absence of added magnesium ions, although

versene alone at pH 6.0 does not appear to cause the degelation of gelable nuclei (previously isolated at pH 4.0).

7. At a pH of 7.0, sodium dodecyl sulfate will not cleave the DNA from the residual protein of gelable nuclei isolated at pH 4.0, even during the course of stirring the nuclei in detergent, after removal of globulins, for a period of 96 hours at room temperature. However, at a pH of 6.0–6.3, cleavage proceeds smoothly in the detergent solution during the course of 2 to 3 hours, as usual. By way of contrast, no incubation period in detergent is required to separate DNA by the detergent method from the residual protein of nuclei isolated in very dilute citric acid at pH 6.0, as already indicated.

8. If an attempt is made to isolate DNA at pH 7–7.5 from rat liver nuclei previously isolated in dilute citric acid at pH 4.0, DNA containing some firmly bound protein is obtained which gives a somewhat low phosphorus analysis and forms a stiff gel in detergent.

DISCUSSION AND CONCLUSIONS

The results presented in this paper show that DNAase I is importantly concerned in the antigelling activity of disrupted mitochondria. Granted that this be so, it would seem logical to suppose that versene should block the mitochondrial antigelling activity, by removing metal activators necessary for DNAase I action. Versene in fact can be used to block DNAase I action in assaying for DNAase II in the presence of DNAase I (13). The fact that at pH 6.0 versene failed to block the antigelling action of disrupted mitochondria completely, and indeed seems to have caused some activation of the antigelation action of the mitochondria, could possibly be explained on the basis of an activation of DNAase II by the versene, concomitant with the inhibition of DNAase I. However, it is not yet known for certain whether proteases and possibly a more specific enzyme capable of cleaving whatever bonds hold the DNA to the residual protein also operate in the mitochondrial antigelling process; and hence it is equally possible, on the basis of the present data, that protease action might account for the decrease in gel-forming ability that occurs in the presence of versene. The degree of depolymerization of the DNA in the presence of versene, if any, cannot be stated with certainty, as already explained.

There is certainly more than enough DNAase I action to account for the observed antigelling action when nuclei are isolated at pH 6.0 in the presence of disrupted mitochondria, and for the concomitant cleavage of the DNA from the residual protein. However, the fact that crystallized DNAase I fails to cleave 100 per cent of the DNA from the residual protein of gelable nuclei, taken together with the fact that no detectable DNA is found in the residual protein obtained from nuclei isolated at pH 6.0 in the presence of disrupted mitochondria, indicates that DNAase I is probably not the sole mitochondrial enzyme responsible for the degelation of gelable nuclei.

The discovery that DNA normally seems to be firmly bound to the residual protein of cell nuclei and must be cleaved from this protein before it can be isolated in protein-free form (1), raises the question of how such cleavage is accomplished in the various methods for isolating DNA. In methods using alkali, or heat, the alkali or the heating could doubtless bring about the cleavage, judging from our previous work (1). In milder methods involving prolonged extraction periods, it is very likely that mitochondrial enzyme action could play a role. The fact that poor yields were generally obtained in these methods indicates that cleavage was generally incomplete. In methods involving the use of sodium dodecyl sulfate, it may be the detergent itself that causes the cleavage; but the pH apparently must be in the right range if such cleavage is to occur, as shown in this paper. Why the dodecyl sulfate should cleave the DNA from the protein at pH 6–6.3 but not at pH 7.0–7.5 is not known, but it has been suggested by Dr. M. Schoenberg of the Department of Pathology of this medical school that excessive micelle formation in the detergent at the higher pH range may interfere with its action.

As further evidence that it is the action of the detergent itself which liberates DNA from the residual protein of isolated cell nuclei at pH 6.0–6.3, it may be added that we have been unable to observe any activity of crystalline DNAase I on purified DNA at pH 6.0–6.3 in the presence of 0.41 per cent sodium dodecyl sulfate, although a slight action was observed at pH 7.0–7.5.

We do not yet know for certain whether lowering the pH to 4.0 during the isolation of nuclei might cause the DNA to become more difficult to separate from the residual protein by means of detergent than would be the case if the nuclei were isolated at pH 6.0 by a method that did not involve mitochondrial disruption. However, such a possibility seems very unlikely, since work on the isolation of DNA from calf thymus shows that very stiff gels are formed when the crude thymus chromosomes obtained in 0.9 per cent NaCl solution are placed in the detergent solution, and that a period of about 3 hours of stirring in detergent at room temperature is required to break this gel and at the same time liberate the DNA from the residual protein.

Although we do not believe that histone is importantly involved in gel formation by isolated nuclei (1), our work does not conflict with the possibility that many of the DNA phosphate groups are ionically bound to positively ionized groups of histones as suggested by Davidson and Butler (15), from studies of isolated nucleohistones. It is clear, however, that histone may be extracted from cell nuclei with 0.2 N HCl at zero°C. without disturbing the firm binding of the DNA to the residual protein of the nuclei.

It is possible to obtain gelable nuclei by means of the Behrens method (1), and this indicates that lipide probably does not play an important role in gel formation, since the repeated use of the organic solvents in the Behrens procedure must cause a very complete removal of lipide from the nuclei. Although lipide as well as DNA is associated with the residual protein of nuclei, as shown

by Engebring and Laskowski (16) and by Thomas *et al.* (17), there appears to be no connection between gelability and lipide content of the nuclei or the nucleoprotein.

In a recent publication, Doty and Zubay (18) claim to have overcome the gelation of thymus nucleoprotein and to have isolated undegraded nucleoprotein of the nuclei. However, repetition of their procedure led to the isolation of what appeared to be essentially bundles of chromosomes which were found to contain histone and residual protein as well as DNA and lipide. This material when first dissolved in distilled water initially forms a gel if the blendor is not run too rapidly during the mixing process. During the course of stirring for one hour, the gel disappears completely and becomes a viscous fluid. A fall in pH occurs during this time, which we take to indicate enzyme action, but owing to the high concentration of versene used in the preparation as well as the relatively high pH (8.0), DNAase action seems excluded. It is possible, however, that protease may be involved in such degelation, as has also been suggested by Bernstein (14).

The work reported in this paper shows what mitochondrial DNAase I can do to the nucleoprotein of cell nuclei upon breakdown of compartmentalization of this enzyme within the mitochondria. The firm binding of DNA to chromosomal protein is broken by the DNAase and some depolymerization of the DNA occurs. In view of the probable genetic role of DNA, such effects could be expected to cause irreversible damage to the cell. Anything that tended to cause mitochondrial disruption within the living cell therefore could be expected to produce genetic effects or even to cause eventual death of the cell. We thus become aware of a possible basis for cytopathology that seems not to have been emphasized previously.

Finally, the relationship of our work to the recently reported lysosomes of de Duve *et al.* (19) should be briefly mentioned. We have not yet seen evidence of lysosomes in our mitochondrial preparations, but if it turns out that lysosomes do exist and that the intracellular DNAase I of liver cells is located in these bodies rather than in the mitochondria, it will only be necessary to substitute the word *lysosome* for the word *mitochondria* to reinterpret the results outlined in this paper. The general reasoning and conclusions would remain unaltered.

SUMMARY

1. DNA prepared from non-gelable rat liver nuclei isolated in the presence of disrupted mitochondria at pH 6.0, has been compared with DNA obtained from gelable nuclei isolated at pH 4.0. The DNA of the non-gelable nuclei is partially depolymerized relative to the DNA of the gelable nuclei.

2. It has been found that sufficiently small quantities of crystallized DNAase I can cleave a very large part of the DNA of gelable nuclei isolated at pH

4 from the residual protein of these nuclei without causing extensive depolymerization of the DNA. At the same time the gelable nuclei are rendered non-gelable.

3. Partially purified DNAase II can also render gelable nuclei isolated at pH 4 non-gelable, and in so doing presumably also cleaves the DNA from the residual protein of the nuclei.

4. Mitochondrial DNAase I appears to be the enzyme responsible to a large extent for the cleavage of DNA from the residual protein of gelable rat liver cell nuclei with concomitant destruction of the gel-forming capability of these nuclei, when the nuclei are subjected to the action of disrupted mitochondria at pH 6.0 during the isolation procedure.

5. Mitochondrial DNAase II does not appear to exert appreciable action on nuclei during the course of isolation of the nuclei at pH 6.0 in the presence of disrupted mitochondria.

6. It is probable that DNAase I is not the sole enzyme responsible for destroying the gelability of nuclei isolated at pH 6.0 in the presence of disrupted mitochondria. Protease may be involved.

7. Sodium dodecyl sulfate at pH 6.0–6.3 cleaves the DNA of isolated gelable nuclei from the residual protein of these nuclei over a period of 2 to 3 hours. At pH 7.0–7.5, however, there is negligible cleavage over a period of 96 hours.

8. If non-gelable nuclei are isolated at pH 6.0 in the presence of disrupted mitochondria, DNA subsequently can be removed from them by the use of detergent at pH values ranging from 6.0–7.5 without the necessity of incubation in the detergent solution, since the DNA had already been detached from the residual protein by the action of the mitochondrial enzyme system during isolation of the nuclei.

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