NUCLEASE INHIBITOR FROM THE NUCLEAR SAP OF LIVER AND HEPATOMA CELLS

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1. Introduction

Several nuclease inhibitors of a protein nature have been found in animal cells [1–4] in the soluble fraction of the cytoplasm. There has been no direct indication of the presence of such inhibitors in the nucleus.

While studying the RNase activity of purified histones isolated from rat liver and hepatoma cells (to be reported in due course) we sometimes observed partial or even complete inhibition of this activity which could be released by treatment of histone preparations with *p*-chlormercuribenzoate (p-CMB). It was possible to avoid inhibition of RNase activity only when an exhaustive extraction of nuclear sap from the nuclei was carried out before isolation of histones. The above observation suggested that a potent RNase inhibitor of a protein nature, sensitive to p-CMB, might be present in the nuclear sap. This paper describes the purification and some properties of the nuclease inhibitor from the nuclear sap.

2. Materials and methods

Nuclei were isolated from the liver of rats (140–200 g) by the modified method of Chauveau [5]. The liver was washed to remove blood, minced in the cold, forced through a French press and homogenized in a glass homogenizer with a teflon pestle (500–800 rpm, 4–5 strokes) in 0.88 M sucrose–1.5 mM $CaCl_2$–0.05 M tris-HCl, pH 7.4. The filtrate was layered on a sucrose solution (d = 1.268) containing 0.5 mM $CaCl_2$ and centrifuged at 60,000 *g* for 90 min (Spinco L-2, $\neq$ 30). For additional purification of the nuclei, the procedure was repeated twice. The nuclear preparations were examined microscopically (stained with toluidine blue) and were characterized by their content of RNA and DNA (P-DNA, 7.0 pg; P-RNA, 2.0 pg).

For isolation of nuclei from Zajdela hepatoma, the cell suspension was centrifuged at 200 rpm for 10 min; to remove erythrocytes, the cells were suspended in 0.14 M NaCl (pH 7.2) and recovered by centrifugation at 600 *g* for 3 min. The washed cells were subjected to an osmotic shock and homogenized in 0.25 M sucrose–0.025 M KCl–0.0015 M $CaCl_2$–0.05 M tris-HCl buffer pH 7.4. The homogenate was layered on a 0.88 M sucrose–0.05 M tris buffer pH 7.4 and the nuclei sedimented at 20,000 *g* for 15 min. The procedure was repeated once more.

The nuclear sap from the liver and hepatoma nuclei was removed repeatedly with 0.14 M NaCl–0.001 M $MgCl_2$ pH 7.2 at 3–4° until no protein was detectable in the extract.

Nuclear ribosomes were removed from the sap by centrifugation at 190,000 *g* ($\neq$ 50) for 2 hr.

3. Results

In the ribosome-free nuclear sap only proteins with solubility properties characteristic of globulins (precipitating with ammonium sulfate at 0.35–0.7 saturation) were found to possess inhibitory activity as revealed in experiments with various RNases (for experimental conditions see legend to tables 1 and 3). The albumins from the nuclear sap showed no activity (table 1).

Table 1
Inhibitory activity of protein fractions from the nuclear sap.

Additions	Pancreatic RNase activity*	Inhibitor units**	Pancreatic RNase activity in the presence p-CMB
–	0.80	0	–
a) Nuclear sap	0.40	1.3	0.85
b) Globulins	0.03	29	0.89
c) Albumins	1.035	0	–
d) Residue***	0.84	0	–

* Expressed as an increase in the absorbance at 260 nm of the acid soluble fraction of the incubation mixture.
** Inhibitory unit: 50% inhibition of the activity of 0.05 μg pancreatic RNase.
*** Left after the extraction of globulins and the bulk of albumins from the total protein precipitated by ammonium sulfate 1.0 saturation.

Incubation conditions: total volume of the sample 3 ml; RNase 0.15 mg; rat liver ribosomal RNA 1.5 mg; a) 746 μg; b) 62 μg; c) 600 μg; d) 130 μg; incubation time 1 hr at 37°. The assay was performed in 0.05 M tris-HCl containing 0.1 M NaCl.

Table 2
Inactivation of the inhibitors by different factors.

Test enzyme	Treatment of the inhibitor	Residual activity of the inhibitor in %	
		Cytoplasmic	Globulin fraction of the nuclear sap
Pancreatic RNase 0.05 μg	Heating (60°, 5 min) pH 6.8	82	100
	0.25 N H_2SO_4 (60 min)	0	0
	Cu^{2+} (10 μM)	71	91
Pancreatic DNase 0.15 μg	Heating (60°, 5 min) pH 6.8	0	94
	0.25 N H_2SO_4 (60 min)	0	0

Some properties of the nuclear inhibitor and cytoplasmic RNase inhibitor (the latter was isolated by the method of Roth [1]) were compared. As can be seen from table 2, both nuclear and cytoplasmic inhibitors can act not only on RNases but also on DNases (their inhibitory activities were tested by the effect on pancreatic RNase and DNase, separately).

The nuclear inhibitor is more resistant than the cytoplasmic one to heating and action of the heavy bivalent cations (table 2). There is also dissimilarity in their effects on different nuclease (table 3). Of the two inhibitors studied, the nuclear one proved to be more efficient especially towards histone and chromatin acidic protein RNase activities.

Preparations of nuclear and cytoplasmic inhibitors were then subjected to successive chromatography on the hydroxyapatite [4] and DEAE-Sephadex columns, which allowed us to purify the nuclear inhibitor

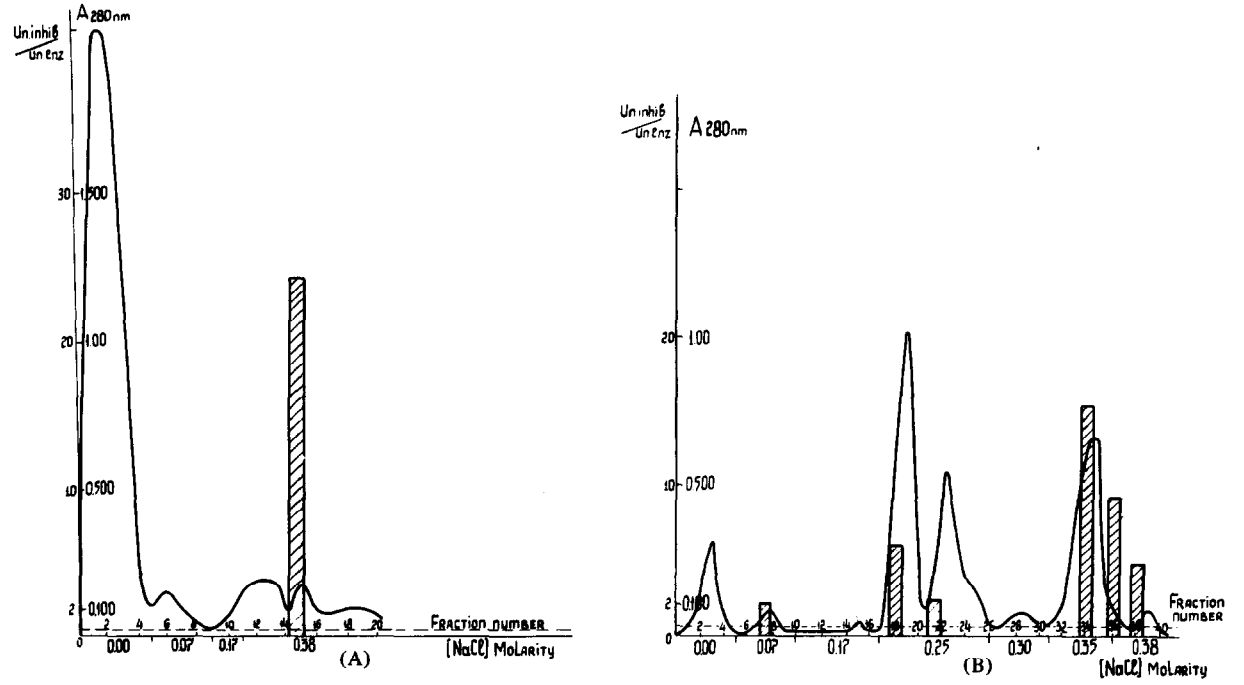

Fig. 1. DEAE-Sephadex A-50 chromatography. Column 1 X 15 cm. Flow rate 20 ml per hr. Stepwise elution with NaCl solution containing 0.02 M tris-HCl buffer pH 7.4. Fractions (3 ml each) were collected by the automatic collector (LKB). Amount of protein in the fractions was determined (after dialysis) by the Lowry's method [10]. Activity of the inhibitor was measured as its effect on the pancreatic RNase in 0.05 M tris-HCl buffer pH 7.6 containing 0.1 M NaCl (incubation at 37° for 1 hr) and expressed in inhibitory units (see the legend to table 1), striped columns. - - - - - - original level of the inhibitor effect. (A) Cytoplasmic inhibitor; (B) Nuclear sap.

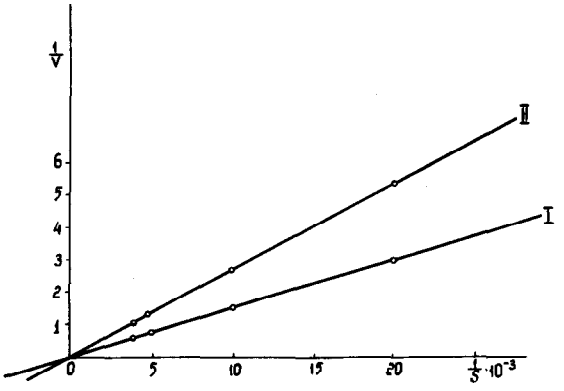

Fig. 2. Reciprocals of the substrate concentration in μg ($1/S$) plotted against the reciprocal of the reaction rate expressed as A_{260} ($1/V$) of the acid soluble fraction of the incubation mixture. Composition of the incubation mixture: calf thymus DNA, ribosomal DNase, globulin inhibitor from the nuclear sap. Time of incubation 30 min at 37°. (I) Ribosomal DNase; (II) ribosomal DNase + inhibitor.

2,000-fold compared with the original sap. These procedures revealed one more substantial difference between the two inhibitors: the cytoplasmic inhibitor was eluted from the DEAE-Sephadex column as a single peak whereas the nuclear one appeared in several protein fractions on the DEAE-Sephadex chromatography (fig. 1), all of which possess inhibitory activity towards both RNases and DNases.

The results of special experiments expressed according to the equation of Lineweaver and Burk provided evidence that the relationships between both pancreatic DNase and RNase and the nuclear inhibitor are of a competitive character (fig. 2).

The data presented in this paper reveal the presence in the liver and hepatoma nuclear sap of a potent RNase and DNase inhibitor. Its activity is confined to the globulin fraction only. This nuclear inhibitor differs in some properties from the previously known

Table 3
Effect of the inhibitors on different enzymes (% of inhibition).

Nuclease	Inhibitors			
	Cytoplasmic		Globulin fraction of nuclear cell sap	
	Liver	Hepatoma	Liver	Hepatoma
RNase				
1) Bovine pancreatic	70	90	48	94
2) Rat liver acidic [6]	0	–	0	0
3) Guanylic (actinomycetes) [7]	0	–	0	0
4) Acidic protein of the nuclear nucleoprotein (rat liver) [8]	50	–	82	98
5) Ribosomal (rat liver)	66	30	50	50
6) Histones (rat liver)	52	65	100	100
DNase				
Bovine pancreatic	98	94	100	100
Ribosomal (rat liver)	80	50	90	100
Ribosomal (rat hepatoma)	72	80	94	96

Incubation mixture (3 ml) for all of the RNases contained rat liver ribosomal RNA (1.5 mg) as substrate and a) tris-HCl buffer 0.05 M pH 8–NaCl 0.1 M (for the RNases with alkaline pH optimum) or b) acetic buffer 0.02 M pH 5.6 (for acidic RNase). Incubation 1 hr at 37°.

The amounts of the given enzymic activity (in E units) were equal in the experiments in which the action of different inhibitors on the same enzyme were compared for a) acidic RNase 3, b) guanylic 10, c) acidic protein of chromatin 0.82, d) ribosomal 8, e) histones 1.0. The enzyme unit: amount of the protein bringing about an increase in the absorbance (A_{260}) of the acid soluble fraction by 1 A within 60 min incubation. Pancreatic RNase 0.02 μg.

Content of inhibitors in μg protein: a) rat liver nuclei 246, b) hepatoma nuclei 306, c) rat liver cytoplasm 264.

Incubation mixture (3 ml) for all of the DNases contained 600 μg rat liver DNA. Time of incubation 2 hr at 37°. a) Pancreatic DNase: enzyme 0.15 μg, $MgCl_2$ 5 mM, tris-HCl buffer 0.05 M pH 7.6, NaCl 0.1 M, b) rat liver ribosomal DNase: enzyme 2.5 units, $MgCl_2$ 5 mM, tris-HCl 0.05 M pH 8.1, NaCl 0.1 M, c) hepatoma ribosomal DNase: enzyme 2.27 units, $MgCl_2$ 5 mM, tris-HCl buffer pH 8.1. Content of inhibitors in μg protein from: a) rat liver nuclei 560, b) hepatoma nuclei 600, c) rat liver cytoplasma 650.

cytoplasmic RNase inhibitor. It is possible that our inhibitor is the one whose presence in the "bound" form of nuclear RNase might be suggested from the experiments of Busch et al. [9].

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