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RIBONUCLEASE FROM HUMAN GRANULOCYTES

JAN SZNAJD AND JERZY W. NASKALSKI
Clinical Chemistry Department, Institute of Internal Medicine, Kraków (Poland)

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SUMMARY

A ribonuclease has been isolated from granulocytes of healthy subjects by using NaCl fractionation, molecular filtration on Sephadex G-75 and chromatographic separation on CM-Sephadex C-50.

The isolated enzyme has optimal activity at pH 6.8, is relatively thermostable and is susceptible to inhibition by Hg^{2+}, Cu^{2+} and Zn^{2+}. The inhibitory action of metal ions can be reversed by the addition of versenate.

The isolated ribonuclease hydrolyzes polycytidylic acid to cytidylic acid but does not hydrolyze polyadenylic acid. The main products released by the enzyme from yeast RNA are the mononucleotides uridylic and cytidylic acids, numerous oligonucleotides, as well as the core fraction.

In the course of ion-exchange chromatography on CM-Sephadex C-50 the enzyme was separated into six fractions, which have been further purified and characterized. They differ with respect to molecular weight and thermostability. About 50% of the total ribonuclease activity in human normal granulocytes is represented by Fraction IV, with the amount represented by the other fractions being in the range of 6–26% each.

INTRODUCTION

It is believed that leukocytic ribonuclease liberated from the cells contributes a major part of the RNA-hydrolyzing ability of plasma[1,2]. The above hypothesis does not, however, agree with the observations that the leukocytic ribonuclease differs from plasma or serum ribonuclease with respect to the optimum pH range[3–6].

The existence of any relationship between the leukocytic and plasma ribonucleases has not yet been proved, inasmuch as there is no adequate information on the properties of ribonucleases from leukocytes and from plasma. Some attempts have been undertaken to characterize the leukocytic ribonuclease[5,6]. These studies deal, however, with leukemic leukocytes, which differ in many respects from the normal ones. The subject of the present study is the isolation of ribonuclease from normal human granulocytes and the description of its properties.

MATERIALS AND METHODS

Granulocytes were collected from "buffy coats" obtained from Blood Bank Station, Kraków Nowa Huta. Each single specimen of buffy coat was obtained from the blood of 3–6 donors (males and females). The leukocyte count of buffy coats ranged between 40 000 and 120 000 per mm^3.

Buffy coats were diluted with an equal volume of 6% dextran solution, molecular weight 80 000 (Dekstran-Polfa) and allowed to stand for about 1.5 h at 37 °C. When the sedimented erythrocytes formed a layer of about $\frac{1}{4}$ of the total volume of the suspension, the upper layer, containing the leukocytes, was collected by suction. The erythrocyte sediment was discarded. The leukocyte suspension was then centrifuged for 5 min at 1000 × *g*. The pellet was resuspended in a small volume of isotonic saline. 3 vol. of distilled water were then added and after 1 min the concentration of NaCl was adjusted to 0.84% by the addition of 2% NaCl solution. The suspension was centrifuged for 5 min at 400 × *g*. The supernatant was discarded and the cell pellet was resuspended in isotonic saline, transfered to a test tube and centrifuged again for 5 min at 400 × *g*. The leukocytic mass thus obtained contained about 85–90% polymorphonuclear leukocytes[7] and could be stored in a deep-freeze for several months.

Ribonuclease activity was determined by the method of Anfinsen *et al.*[8] modified for the estimation of ribonuclease activity in serum and urine[9]. The determinations of leukocytic ribonuclease activity were carried at two pH values: 6.6 and 5.4. Ribonuclease activity was expressed in arbitrary units calculated on the basis of the activity of standard solutions of bovine pancreatic ribonuclease. One unit of ribonuclease activity is defined as the activity of the solution of bovine pancreatic ribonuclease (Boehringer, Mannheim) containing 1 μg of the enzyme in 1 ml and was determined at pH 7.8 by the same procedure.

Non-specific nucleases were detected by estimating the ability to decompose calf thymus DNA[10] and to hydrolyze calcium(bis)-*p*-nitrophenyl phosphate (Sigma Chemical Co.)[11].

Gel filtration was accomplished on Sephadex G-75 (Pharmacia, Uppsala) prepared in 0.01 M sodium phosphate buffer pH 7.8 in a solution of 0.5 M NaCl and 0.1% human blood albumin (B.D.H., England). For preparative purposes, 40–60-ml aliquots were applied to a 4.6 cm × 68.8 cm column (containing about 760 ml of the gel suspension) and the filtrate flowing at the rate of 60 ml/h was collected. For better resolution and for the determination of the molecular weights of partly purified ribonuclease fractions, 4-ml aliquots of ribonuclease solutions were applied to a 3.4 cm × 68.8 cm column (containing about 590 ml of the gel suspension), at a flow rate of 12 ml/h. In the latter procedure the effluent volume was measured with an accuracy of about 1% and the procedure was repeated 3 or 4 times.

Molecular weights were estimated using the procedure of Whitteaker *et al.*[12]. Egg albumin (ovalbumin Koch-Light), pepsin (*ex* hog, Koch-Light Lab.), bovine pancreatic ribonuclease (Boehringer, Mannheim) and cytochrome *c* (Biomed, Poland) were used as molecular weight standards.

Ion-exchange chromatography was carried out on CM-Sephadex C-50 equilibrated with 0.01 M sodium phosphate buffer (pH 6.0) in 0.14 M NaCl. The slurry of the equilibrated exchanger was packed into 1.2 cm × 25 cm glass columns. The

columns were then washed with 250 ml of the above buffer. After each run the columns were repacked and the exchanger regenerated by washing with water, 0.5 M NaOH, 0.2 M HCl, water, and then equilibrated with 0.01 M sodium phosphate buffer pH 6.0 in 0.14 M NaCl.

The RNA digestion products were separated with the aid of chromatography on Sephadex G-10 fine columns (1.2 cm × 160 cm) prepared in 0.03 M ammonium carbonate buffer[13] pH 7.8, with gaseous CO_2.

PROCEDURE AND RESULTS

A leukocytic mass (collected during a 3-month period and stored in the deep freeze) was suspended in 10 vol. of water and homogenized for 6 min at 12 000 rev./min in a Waring-Blendor type homogenizer. Solid NaCl was then added to the homogenate to a concentration of 6% (w/v). The mixture was left overnight at 4 °C and the precipitate formed was separated by centrifugation (10 min at 12 000 × *g*) and discarded.

The supernatant containing 80–90% of the total ribonuclease activity was treated with protamine sulphate (Koch-Light Lab.) (about 100 mg of protamine sulphate in 100 ml of supernatant) and applied to a column of Sephadex G-75 (4.6 cm × 68.8 cm). Ribonuclease emerged from the column following a large protein peak (containing acid phosphatase, β-glucuronidase and myeloperoxidase) (Fig. 1).

Part of the effluent exhibiting ribonuclease activity was collected, treated with protamine sulphate (20 mg per 100 ml of effluent) and dialyzed for 20 h against 0.01 M sodium phosphate buffer (pH 6.0) in 0.14 M NaCl. The sediment formed on dialysis was centrifuged and rejected, and the clear supernatant was adjusted precisely to pH 6.0 and to 0.14 M NaCl. Then the dialysate was applied to the column of CM-Sephadex C-50 (1.2 cm × 25 cm).

The column effluent, exhibiting only a trace of ribonuclease activity, was rejected. The adsorbed enzyme was then eluted with 0.01 M sodium phosphate buffer (pH 6.0), with the NaCl concentration gradient rising sigmoidally.

The NaCl concentration gradient was developed by the continuous mixing of

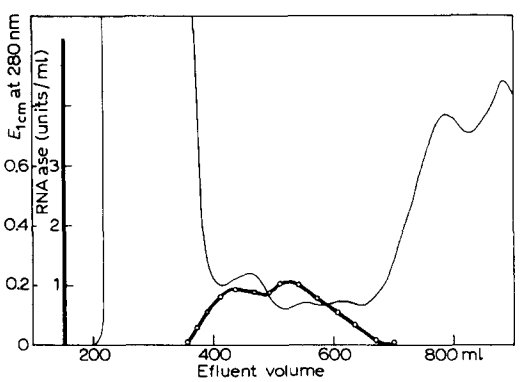

Fig. 1. The results of molecular filtration of the 6% NaCl extract on a Sephadex G-75 column. ○—○, ribonuclease activity; ———, protein content.

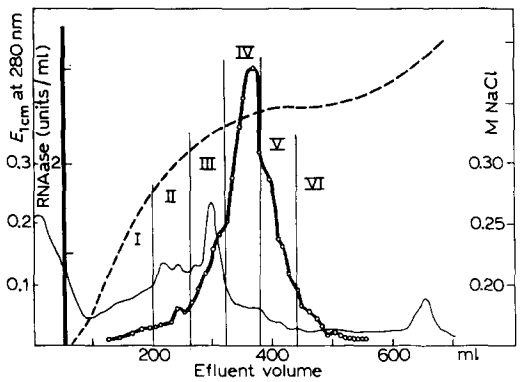

Fig. 2. Chromatographic separation of granulocytic ribonuclease on a CM-Sephadex C-50 column. ○—○, ribonuclease activity (at pH 6.6); ———, protein content; — — —, NaCl concentration gradient. A predominant part of granulocytic ribonuclease emerged from the column when the NaCl concentration reached plateau level = 0.380 M.

four 200-ml portions of the mentioned sodium phosphate buffer in the four-chamber "Varigrad" gradient mixer of Petterson and Sober[14], containing: 0.14 M NaCl, 0.60 M NaCl, 0.00 M NaCl (no NaCl) and 1.00 M NaCl in Chambers I, II, III and IV, respectively. The effluent was collected in 4-ml fractions (at the rate of 16 ml/h) and tested for absorbance at 280 nm, NaCl concentration and ribonuclease activity.

As a result of this procedure granulocytic ribonuclease was separated into six fractions differing with respect to their elution conditions (Fig. 2). A predominant

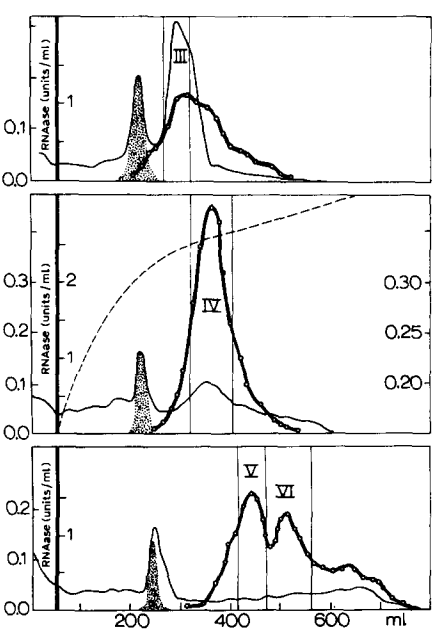

Fig. 3. Rechromatography of the separated ribonuclease Fractions III–VI on CM-Sephadex C-50 columns. The dotted peak at about 230 ml represents bovine heart cytochrome *c* used as an elution standard. The other markings are the same as in Fig. 2.

fraction was ribonuclease Peak IV which contributed about 50% of the total ribonuclease activity.

The fractions were collected separately, and were each separately rechromatographed on CM-Sephadex C-50 columns using the same procedure as above. As a result of the rechromatography, all six fractions appeared as single peaks emerging under their own characteristic elution conditions. In particular, it was found that ribonuclease Fraction III, which does not form a separate peak during first chromatography, shows a small ribonuclease peak at about 300 ml of the effluent. The results of the rechromatography of ribonuclease III–VI are shown on Fig. 3. A schematic pattern of the locations of ribonuclease Fractions I–VI on the CM-Sephadex C-50 chromatogram is shown in Fig. 5.

The separated fractions were collected and concentrated about 15 times with Aquacide I (Calbiochem). Then a 3-ml portion of the concentrated fraction was treated with 1 ml of a solution of 50 mg cytochrome *c* per 100 ml, and the mixture was applied to a Sephadex G-75 column (3.4 cm × 68.8 cm).

As a result of molecular filtration on Sephadex G-75, the ribonuclease fractions emerged from the column in different effluent volumes. Ribonuclease Fractions IV

TABLE I

SUMMARY OF RIBONUCLEASE ISOLATION PROCEDURE FROM HUMAN GRANULOCYTES

Procedure step	*Total ribonuclease activity*	*Purification ratio*	*Recovery (%)*
1. Homogenate	3300	1	100
2. NaCl fractionation	3200	1.2	97
3. 1st chromatography on Sephadex G-75	2960	2.8	89
4. CM-Sephadex C-50 chromatography Combine:	1780	—	54
Fraction I	95	2.5	2.9
Fraction II	148	2.5	4.5
Fraction III	198	11	6
Fraction IV	979	85	29
Fraction V	207	57	8
Fraction VI	122	14	3
5. CM-Sephadex C-50 rechromatography			
Fraction I	40	4.3	1.2
Fraction II	73	4.0	2.2
Fraction III	80	7.0	2.4
Fraction IV	450	101	13.6
Fraction V	107	30	3.2
Fraction VI	36	4	1.1
6. 2nd chromatography on Sephadex G-75*			
Fraction I	31	—	—
Fraction II	43	—	—
Fraction III	41	—	—
Fraction IV	330	—	—
Fraction V	72	—	—
Fraction VI	20	—	—

* From peak region only.

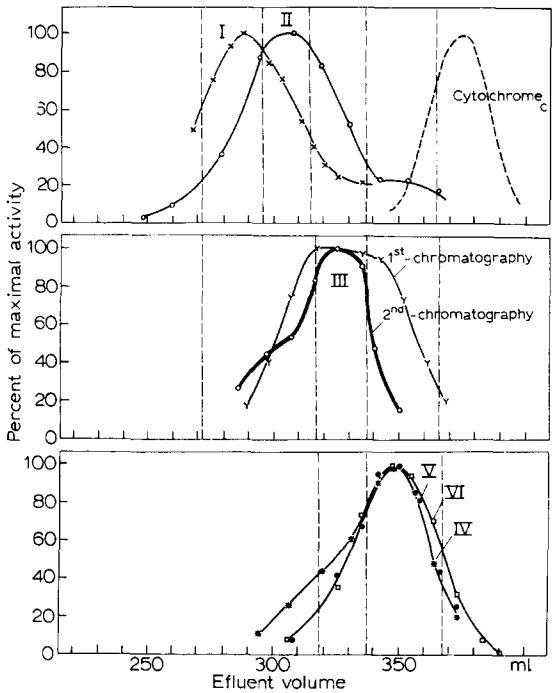

Fig. 4. The results of molecular filtration on Sephadex G-75 columns (3.4 cm × 68.8 cm) of the separated ribonuclease Fractions I–VI. — — —, (upper graph), elution pattern of cytochrome c used as an elution standard; the broken vertical lines mark elution regions characteristic for the fractions studied.

V and VI emerged in the same effluent volume (Fig. 4). The effluent volumes obtained were the basis for the calculations of the molecular weights present in Table II

Characterization of the granulocytic ribonuclease

10 mg of RNA substrate was digested with about 50 activity units of ribo-

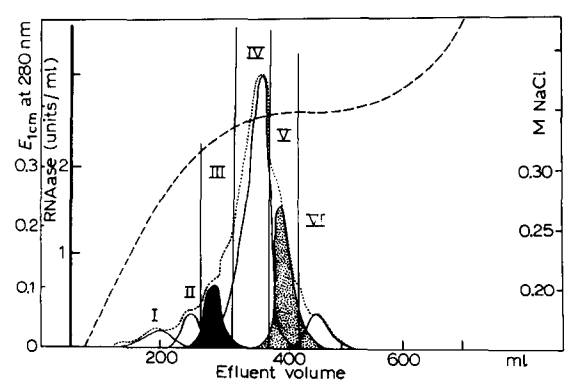

Fig. 5. Schematic pattern of ribonuclease I–VI locations no the CM-Sephadex C-50 chromato gram. ·····, ribonuclease activity graph from Fig. 2.

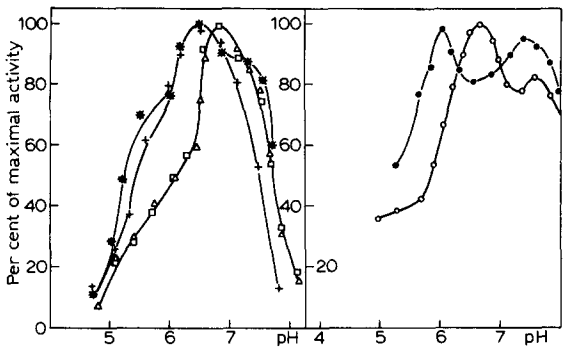

Fig. 6. The effect of pH on the activity of granulocytic ribonuclease fractions. +—+, Fraction I; *—*, Fraction II; △—△, Fraction III; □—□, Fraction IV; ●—●, Fraction V; ○—○, Fraction VI.

nuclease IV for 20 h. The obtained digestion products were applied to a Sephadex G-10 fine column (1.2 cm × 160 cm) in ammonium carbonate buffer (pH 7.8) and eluted until the complete removal of substances adsorbing light at 260 nm. The same procedure was applied to 3 mg of undigested RNA substrate and to the RNA hydrolyzed with 0.3 M KOH for 24 h at 20 °C. The results of the chromatographic separation of the investigated reaction products are shown in Fig. 7.

It was found that the RNA substrate forms a single peak at 58 ml of eluate, whereas the alkaline-hydrolysis products separate into two peaks: the first at about 110 ml, containing uridylic and cytidylic acids and the second at 130 ml, containing guanylic and adenylic acids (Fig. 7).

The products of RNA substrate digestion with ribonuclease IV were separated into four fractions: Fraction I, corresponding to undegraded RNA substrate; Fractions II and III corresponding to numerous oligonucleotides, and Fraction IV corresponding to uridylic and cytidylic acids (Fig. 7).

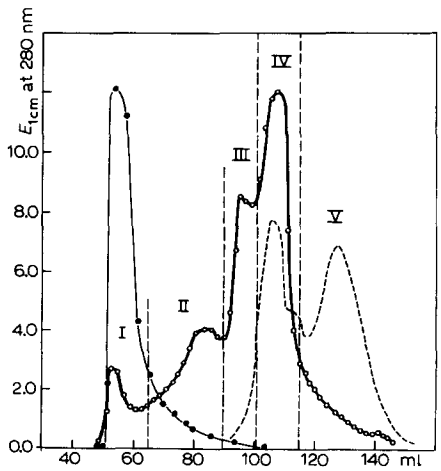

Fig. 7. Comparison of the results of molecular filtration on a Sephadex G-10 fine column: ———, RNA substrate; ○—○, ribonuclease digestion products; — — —, alkaline hydrolysate of RNA.

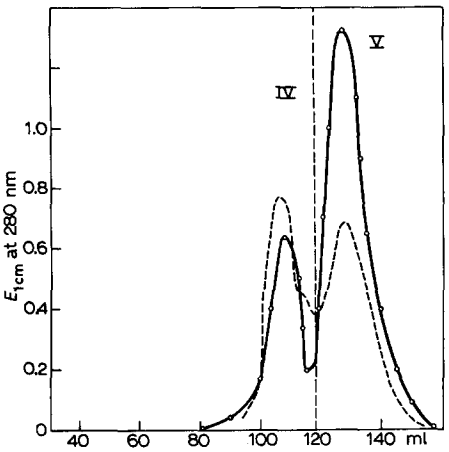

Fig. 8. Comparison of the results of molecular filtration on Sephadex G-10 fine column: — — —, alkaline hydrolysate of RNA substrate; ○—○, alkaline hydrolysate of RNA from Fraction I ribonuclease digestion products (core fraction).

Fraction I was then hydrolyzed for 24 h with 0.3 M KOH and chromatographed again on a Sephadex G-10 fine column. It was found that this fraction has about a 3-fold lower content of pyrimidine nucleotides and about a 4-fold higher content of purine nucleotides as compared to the standard RNA substrate (Fig. 8).

A specimen of Fraction IV was separated by electrophoresis on Whatman No. 1 filter paper (0.3 M acetate buffer (pH 3.5); 6 V/cm) in the presence of 3'-uridylic and 3'-cytidylic acids (Koch-Light Lab.) used as standards. It was found that Fraction IV was separated into two spots exactly corresponding to the used standards. The absorption spectra of the eluted spots proved that the uridylic and cytidylic acids are the main components of the RNA digestion products Fraction IV.

The used procedure leaves, however, unresolved the question of whether 3'- or

TABLE II

SOME PROPERTIES OF THE ISOLATED GRANULOCYTIC RIBONUCLEASE FRACTIONS

Fraction	*Optimal activity at pH*	*Molecular weight*	*Time for 50% inactivation at pH 2.5 and 95 °C (min)*
Ribonuclease I	6.9	23 000 ± 1 100**	3
Ribonuclease II	6.5	21 000 ± 1 200**	6
Ribonuclease III	6.6	18 500 ± 700**	9
Ribonuclease IV	6.9	15 500 ± 700***	12
Ribonuclease V	6.0† 7.4	15 500 ± 700**	10
Ribonuc lease VI	6.6† 7.3	15 500 ± 700*	12

* From two measurements.
** From three measurements.
*** From four measurements.
† Two pH optima.

5'-nucleotides are formed. This, and some other questions concerning the specificity of the isolated granulocytic ribonuclease, need further study.

The above described procedure for RNA substrate digestion and for the investigation of the obtained digestion products was applied to the mixture of the isolated ribonuclease Fractions I, II, III, V and VI. The results obtained were very similar to those obtained for the products of RNA digestion with ribonuclease Fraction IV.

The isolated ribonucleases hydrolyzed polycytidylic acid to cytidylic acid but did not hydrolyze polyadenylic acid. They also did not cause depolymerisation of DNA, nor were they able to hydrolyze calcium (bis)-*p*-nitrophenyl phosphate, nor to split off inorganic phosphate from 3'-uridylic and 5'-adenylic acids.

Optimal activity, molecular weights and the effects of heating of the isolated ribonuclease fractions are presented in Table II, and in Fig. 6.

The data concerning inhibition of the isolated ribonuclease fractions in the presence of some metal ions are presented in Table III.

TABLE III

INHIBITION OF GRANULOCYTIC RIBONUCLEASE FRACTIONS BY METAL IONS

Data represent percentage of standard test activity in the absence of ion. Concentration of ions $3 \cdot 10^{-3}$ M.

Fraction	Mg^{2+}	Ca^{2+}	Zn^{2+}	Cu^{2+}	Hg^{2+}
Ribonuclease I	86	107	46	11	9
Ribonuclease II	72	96	54	13	18
Ribonuclease III	68	70	54	26	13
Ribonuclease IV	82	62	60	29	22
Ribonuclease V	92	83	54	25	14
Ribonuclease VI	73	73	45	10	19

DISCUSSION

The presented investigations demonstrated that normal human granulocytes contain ribonuclease with optimal activity near pH 6.8. This enzyme contributes a major part of the RNA-degrading ability of the studied granulocytes. The optimal pH value found for the granulocytic ribonuclease justifies an assessment that the enzyme is an acid ribonuclease[15]. On the other hand, human granulocytes seem to be devoid of an alkaline ribonuclease with an optimal activity at pH 7.8–8.0, similar to the pancreatic alkaline ribonuclease or to alkaline ribonuclease of normal and leukemic human urine[16,17]. We also failed to demonstrate the presence of an inhibitor directed towards granulocytic ribonuclease or towards bovine pancreatic ribonuclease[18].

The obtained enzyme is different in all properties from the acid thermolabile ribonuclease isolated from rat liver with optimal activity at pH 5.4 and which required Mg^{2+}. The enzyme isolated in this study is rather similar to the acid ribonuclease of human spleen[20] or to acid ribonuclease of normal[16] and leukemic human urine[13].

There is some evidence that in polymorphonuclear leukocytes the ribonuclease is bound to the granule fraction[21,22]. Leukocytic granules are usually identified with

lysosomes[21,22,23], and thus it can be assumed that the isolated enzyme is predominantly derived from the granulocytic lysosome fraction. This suggestion implies that leukocytic acid ribonuclease is different from the lysosomal acid ribonuclease of rat liver.

The heterogeneity of granulocytic ribonuclease causes some difficulties in the interpretation of the obtained results. It is unknown whether all the ribonuclease fractions are of lysosomic origin, or if some of them are derived from different subcellular structures. It cannot be excluded without further broad studies that some of the smaller ribonuclease fractions are derived from some other blood cells, *e.g.* lymphocytes, which always contaminate the initial granulocyte preparation.

Some other studies on ribonuclease in human leukemic granulocytes have been published[5,6,24]. Silber *et al.*[6] observed in human leukemic granulocytes an acid ribonuclease with optimal activity at pH 6.5. In the previous study[5] we isolated from human leukemic granulocytes three ribonuclease fractions displaying optimal activity at pH 5.8, 6.8 and 7.4, respectively. The general properties of the enzyme studied by us[5] and by Silber *et al.*[6] are very similar to the enzyme isolated now from normal granulocytes. The only important difference concerns the homogeneity of the enzymatic preparations. This difference may be, however, due to different separation procedures rather than to the difference between the normal and leukemic cells. It is likely that ribonuclease Fractions IV, V and VI, showing a considerable degree of similarity, were unresolved during our previous experiments[5,25]. On the other hand the improved separation procedure based on the "Varigrad" chromatographic system elaborated in the present study opens up the possibility for a comparison of the ribonucleases in normal and leukemic granulocytes.

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