Spleen Exonuclease (1)

GIORGIO BERNARDI AND ALBERTO BERNARDI
CENTER FOR RESEARCH IN MACROMOLECULES
STRASBOURG, FRANCE

A method for the partial purification of spleen exonuclease (also called spleen phosphodiesterase) was described in 1960 by Hilmoe (2) and later improved by Razzell and Khorana (3). An enzymatic preparation of comparable specific activity may be obtained with the less laborious procedure described here. Some preliminary data for the further purification of this preparation are also given.

ASSAY

Reaction

3′P-oligonucleotides ⟶ 3′P-mononucleotides

The exonuclease activity is assayed by measuring the liberation of mononucleotides from 3′P-deoxyribooligonucleotides, obtained by exhaustive digestion of DNA by spleen acid deoxyribonuclease. The average size of the oligonucleotides present in these digests is 8–9, and the smallest fragments are tetranucleotides (4). The *p*-nitrophenyl ester of thymidine 3′-phosphate may also be used for the assay (3), although this substrate is also slowly hydrolyzed by spleen acid deoxyribonuclease (5).

Other substrates have serious drawbacks: (a) Bis(*p*-nitrophenyl) phosphate is a very poor substrate and is also hydrolyzed by spleen acid deoxyribonuclease (5) and spleen nucleoside polyphosphatase (6). (b) RNA "core," the water-undialyzable 3′P-ribooligonucleotides ob-

tained by exhaustive digestion of RNA with pancreatic ribonuclease, is also hydrolyzed by spleen ribonucleases (**7,8**) that are contaminants of spleen exonuclease.

Reagents

1. The reaction mixture (total volume 1.25 ml) contains
   a. 1.2 μmoles of deoxyribooligonucleotide-P (This is the product of exhaustive digestion of DNA by spleen acid deoxyribonuclease.)
      12.5 μmoles of EDTA
      187.5 μmoles of acetate buffer, pH 5.0
   b. Enzyme—if necessary this is diluted with 0.15 M acetate buffer, 0.1 M EDTA, pH 5.0, containing 0.05% beef heart cytochrome c (Sigma Chemical Company, type V)
2. 2.5% Perchloric acid containing 0.25% uranyl acetate

Procedure

After 10 min of incubation at 37 C the reaction is stopped by adding 1.25 ml of the perchloric acid–uranyl acetate reagent. The mixture is chilled for 10 min in an ice bath and clarified by centrifugation at 4 C. The optical density at 260 mμ of the supernatant fluid is measured and a suitable blank is subtracted. One activity unit is the amount of enzyme that liberates mononucleotides having at 260 mμ an optical density equal to one, under the conditions given above. The specific activity is calculated by dividing the activity by the optical density at 280 mμ of the enzyme solution. Assays are performed using enzyme concentrations to obtain OD_{260} readings not higher than 2.5. Under these conditions a linear relationship is obtained between enzyme concentration and mononucleotide liberation.

A comparison was made between the activities measured by the present method and those measured by the procedure of Razzell and Khorana (4). The initial hydrolysis rates were compared by using an enzyme fraction, purified by the chromatographic procedure described below and two additional chromatographic steps on hydroxyapatite and DEAE-Sephadex, respectively. One activity unit, as defined above, was found to liberate 1.8 μmoles of *p*-nitrophenol per hour from the *p*-nitrophenyl ester of thymidine-3′-phosphate (this was a preparation of Dr. H. Schaller). Contaminating enzymatic activities (acid deoxyribonuclease, acid and basic ribonucleases, acid phosphomonoesterase) were assayed as described elsewhere (pp. 37, 102, 236).

PREPARATION OF CRUDE SPLEEN EXONUCLEASE

Preparation Procedure

A crude spleen nuclease preparation is obtained by the following method derived from the procedures used to obtain the crude spleen nucleases (see pp. 104–106). All operations are carried out at room temperature, except where otherwise stated. Hog spleens are trimmed, ground with an electrical meat grinder, and homogenized with 0.1 N H_2SO_4 (1 liter/kg of ground spleen; 1 ml of isooctanol is added to each batch). The final pH is about 4.2. The suspension is centrifuged for 40 min at 44000 × *g* in a Spinco model L centrifuge. Solid $(NH_4)_2SO_4$ (176 g/liter; 0.3 saturation at 20 C) and EDTA (3.72 g/liter) are added to the clear supernatant fluid (*clarified extract*). The precipitate that forms is removed by filtration on a Seitz filter (p. 106). Solid $(NH_4)_2SO_4$ (198 g/liter; 0.6 saturation at 20 C) and EDTA (3.72 g/liter) are added to the clear filtrate. The precipitate that forms is collected by filtration on a Seitz filter, dissolved in a small volume of distilled water, dialyzed against distilled water at 4 C, clarified by centrifugation, and concentrated by freeze-drying.

Properties of the Clarified Extract and the Crude Spleen Exonuclease

The *clarified extract* has a total activity of 13,000 units/kg of spleen, a specific activity of 0.4, and an OD_{280}/OD_{260} ratio of 1.55. The crude spleen exonuclease has a total activity of 3500 units/kg of spleen, a specific activity of about 4, and an OD_{280}/OD_{260} ratio of 1.75. The properties of crude spleen exonuclease are summarized in Table 1. The loss in activity occurring when going from the *clarified extract* to the crude spleen exonuclease seems to be mainly due to the dialysis against distilled water; it may be greatly reduced by dialyzing the enzyme preparation against 0.1 M acetate buffer, pH 5.0.

TABLE 1. *Properties of Crude Spleen Exonuclease*[a]

	Total Activity	Specific Activity
Exonuclease	15,000	3.9
Acid ribonuclease	200,000	17.5
Acid deoxyribonuclease	7,500	0.65
Acid phosphomonoesterase	6,000	0.48

[a] This preparation was obtained from 13 kg of spleen.

CHROMATOGRAPHIC PURIFICATION

The chromatographic purification of spleen exonuclease is derived from the purification procedures developed to prepare spleen acid deoxyribonuclease. Column chromatography is performed as already described (see p. 107). A summary of the purification is given in Table 2.

TABLE 2. *Chromatographic Purification of Spleen Exonuclease*

	Volume	Total OD_{280}	Total Units	Specific Activity
Crude spleen nuclease	80	9,130	36,000	3.9
Step 1. CM-Sephadex[a]	300	157	9,000	57
Step 2. Hydroxyapatite (fractions 35–38)	60	11.1	5,650	500

[a] The values reported refer to the fractions loaded on hydroxyapatite as assayed after the dialysis step. The recovery of activity from CM-Sephadex, as assayed before dialysis, was essentially complete.

Step 1. CM-Sephadex C-50 (Fig. 1)

Crude spleen exonuclease, dialyzed against 0.1 M acetate buffer, pH 5.6, is loaded on a CM-Sephadex C-50 column equilibrated with the same buffer. A molarity gradient (0.1 to 1.0) of acetate buffer, pH 5.6, was used to elute the proteins. Two acid phosphomonoesterase activity peaks are eluted at the beginning of the gradient and at 0.4 M acetate, respectively. The exonuclease activity is eluted at a molarity of 0.7, between two acid ribonuclease activity peaks.

Step 2. Hydroxyapatite (Fig. 2)

The exonuclease-rich fractions are pooled, dialyzed against 0.05 M phosphate buffer, pH 6.8, and loaded on a hydroxyapatite column. Elution is carried out with a molarity gradient (0.05 to 0.5) of phosphate buffer, pH 6.8. Exonuclease activity very closely follows an ribonuclease activity peak eluted at 0.1 M.

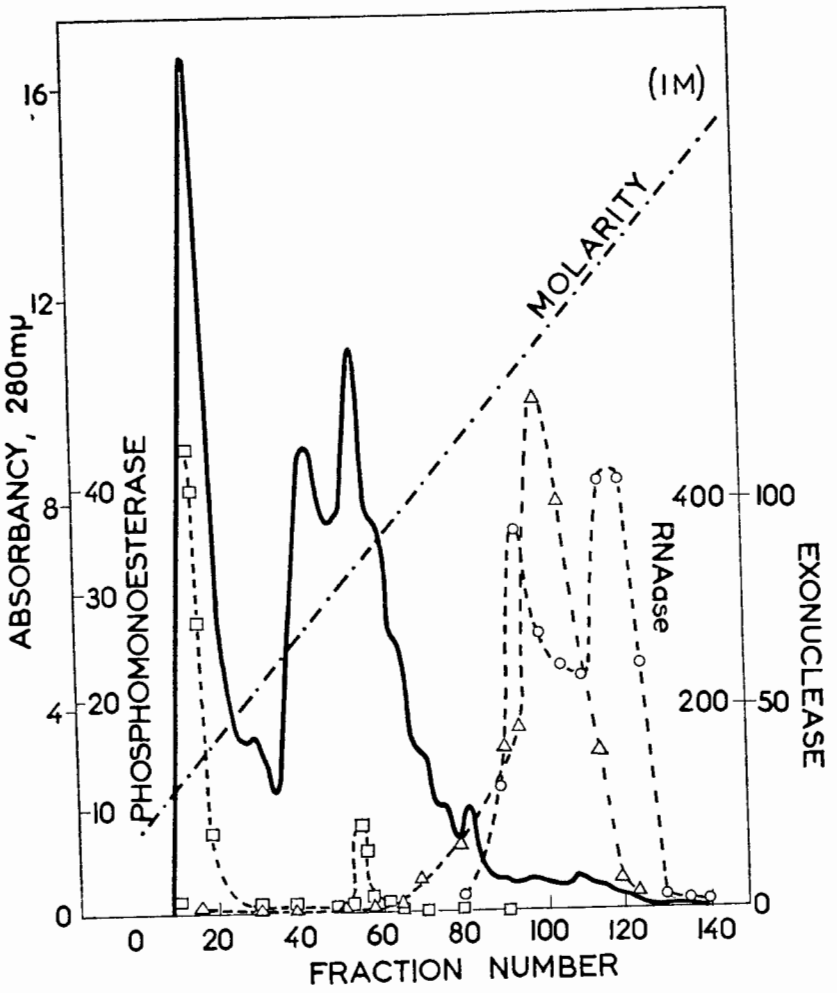

Fig. 1. Chromatography on CM-Sephadex C-50 of crude spleen exonuclease (Step 1). 80 ml of crude spleen exonuclease ($OD_{250} = 114$; $OD_{280} = 65$) were loaded on a 3- × 90-cm column of CM-Sephadex C-50 equilibrated with 0.1 M acetate buffer, pH 5.6. A molarity gradient (0.1–1 M) was used for the elution. 20 ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Squares indicate the acid phosphomonoesterase activity (left-hand inner scale); circles, the acid ribonuclease activity (right-hand inner scale); triangles, the exonuclease activity (right-hand outer scale).

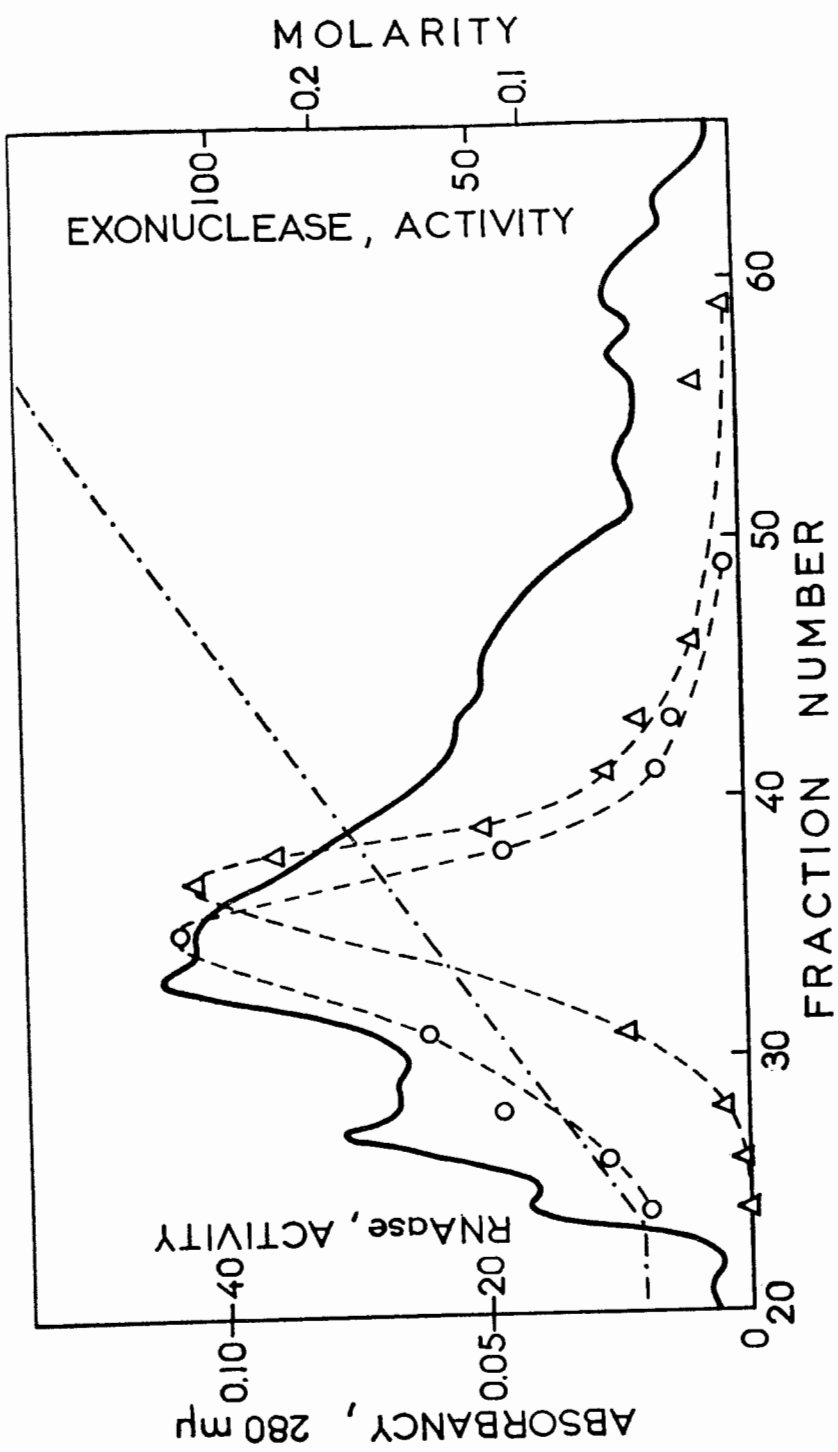

Fig. 2. Chromatography on hydroxyapatite of fractions 95–110 from Step 1 (Step 2). 300 ml ($OD_{250} = 0.525$) were loaded on a 2- × 38-cm column of hydroxyapatite equilibrated with 0.05 M phosphate buffer, pH 6.8. A molarity gradient (0.05–0.5) of phosphate buffer was used for the elution (right-hand outer scale). 15-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Circles indicate the acid ribonuclease activity (left-hand inner scale); triangles, the exonuclease activity (right-hand inner scale).

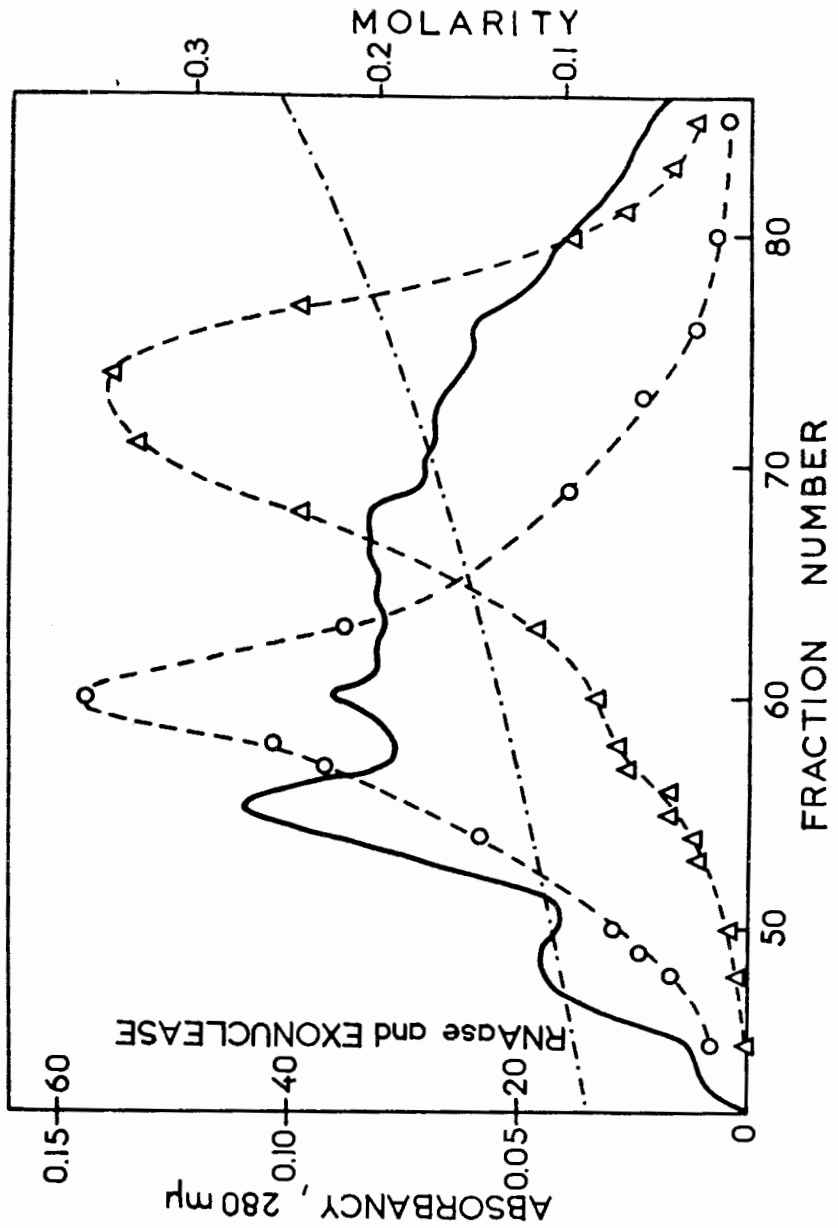

Fig. 3. Chromatography on hydroxyapatite of exonuclease-rich fractions from an hydroxyapatite chromatography. 180 ml ($OD_{280} = 0.215$) were loaded on a 2- × 20-cm column of hydroxyapatite equilibrated with 0.05 M phosphate buffer, pH 6.8. A molarity gradient (0.05–0.4) was used for the elution (right-hand scale). 6-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Circles indicate the acid ribonuclease activity; triangles, the exonuclease activity (both activities, left-hand inner scale).

Further Purification

Two different types of chromatography were tried on exonuclease fractions derived from Step 2, in order to purify the enzyme from the contaminating ribonuclease activity.

The first one is a rechromatography run on hydroxyapatite using a shallower molarity gradient (Fig. 3). An improved separation of the ribonuclease and the exonuclease activities was obtained, and the exonuclease fractions showed a higher specific activity than those obtained from the first hydroxyapatite chromatography. The second type of chromatography tried was on DEAE-Sephadex A 50, using a molarity gradient (0.01–0.3 M) of phosphate buffer, pH 6.8, to elute the proteins (Fig. 4). A very satisfactory separation of the two enzymatic activities was obtained.

Properties of the Enzyme

The specific activity of the exonuclease fraction from Step 2 is 500; that of the peak fraction of the hydroxyapatite rechromatography is 770. By assuming, on the basis of the biuret reaction, an $E^{1\%}_{280} = 10$ for spleen exonuclease, these specific activities are equivalent to 900 and 1400 units of Razzell and Khorana (3), respectively. These values are well above that of Hilmoe's preparation [estimated to 470 by Razzell (9)], and comparable to those (995–2040) of the preparation by Razzell and Khorana (3).

A higher specific activity is likely to be associated with the DEAE-Sephadex exonuclease fraction (Fig. 4); however, the optical density at 280 mμ was so low that the specific activity could not be assessed.

Several possible contaminating activities were checked on the DEAE-Sephadex exonuclease fraction. No endonucleolytic attack on DNA was detected by viscometry, using exonuclease concentrations and digestion times such that acid-soluble nucleotides could be demonstrated. Acid phosphomonoesterase activity could be detected in trace amount by using large enzyme concentrations and very long digestion times (17 hr). Yeast-soluble RNA was hydrolyzed at only about half the rate shown by high molecular weight DNA. The hydrolysis rate was essentially the same in 0.15 M acetate buffer–0.01 M EDTA, pH 5.0, and in 0.1 M phosphate buffer–0.01 M $MgCl_2$, pH 7.2. This is in contrast with the results obtained when digesting soluble RNA with the ribonuclease fraction eluted from the DEAE-Sephadex column (Fig. 4). In this case, the ratio of acid to basic ribonuclease activity (using the buffers indicated above) equals 50. This finding may be considered as a preliminary evidence supporting the idea that the very slow digestion

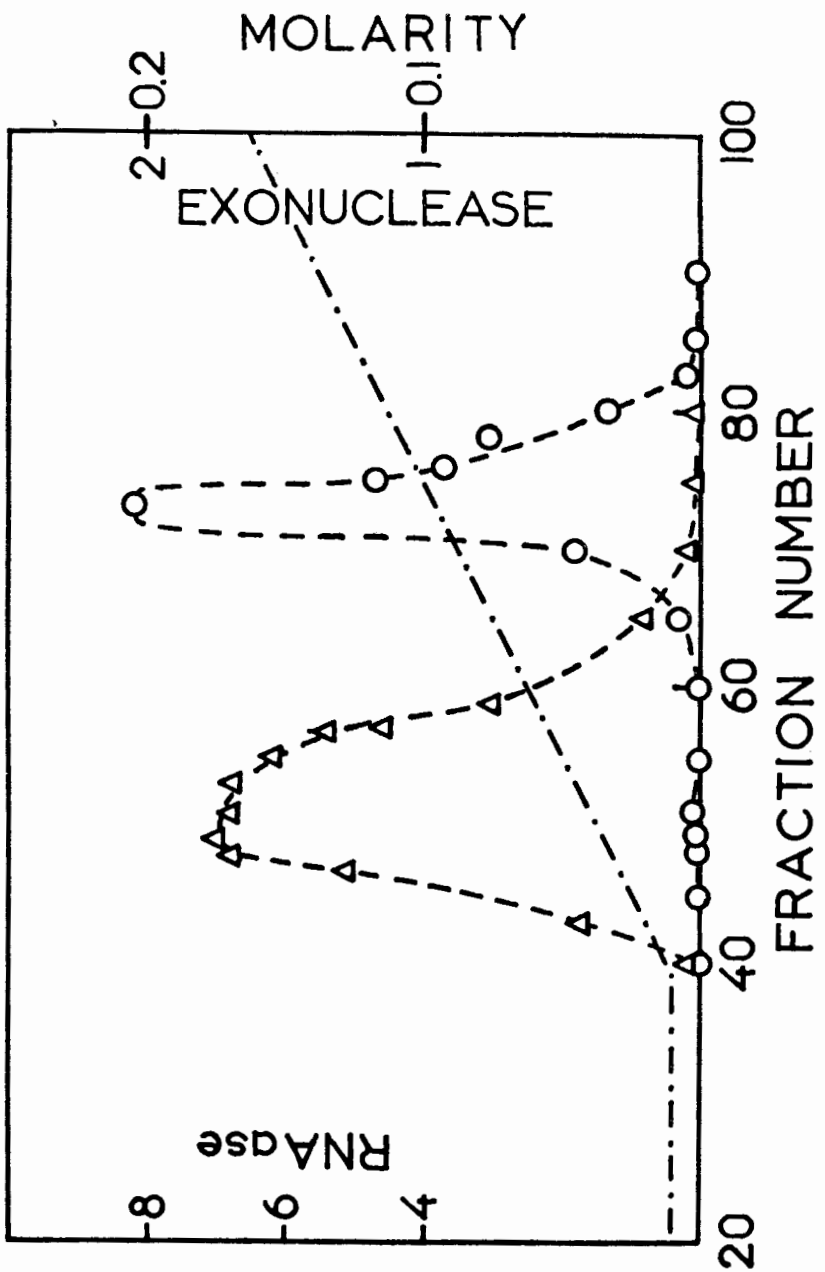

Fig. 4. Chromatography on DEAE-Sephadex A-50 of exonuclease-rich fractions from an hydroxyapatite chromatography. 10.8 ml ($OD_{280} = 0.120$) were loaded on a 1- × 100-cm column of DEAE-Sephadex A-50 equilibrated with 0.01 M phosphate buffer, pH 6.8. A molarity gradient (0.01–0.3 M) was used for the elution (right-hand outer scale). 2.7-ml fractions were collected. Circles indicate the ribonuclease activity (left-hand inner scale); triangles, the exonuclease activity (right-hand inner scale).

of transfer RNA by the exonuclease fraction is basically due to a true exonucleolytic attack on contaminating ribosomal RNA fragments present in the transfer RNA preparation or on pre-existing, ribonuclease provoked, breaks in the transfer RNA.

REFERENCES

1. BERNARDI, A., and G. BERNARDI. In preparation.
2. HILMOE, R. J. 1960. J. Biol. Chem. *235:* 2117.
3. RAZZELL, W. E., and H. G. KHORANA. 1961. J. Biol. Chem. *236:* 1144.
4. CARRARA, M., and G. BERNARDI. In preparation.
5. BERNARDI, G., and M. GRIFFÉ. 1964. Biochemistry. *3:* 1419.
6. LASKOWSKI, M., and B. FILIPOWICZ. 1958. Bull. Soc. Chim. Biol. *40:* 1865.
7. MAVER, M. E., and A. E. GRECO. 1962. J. Biol. Chem. *211:* 907.
8. BERNARDI, A., and G. BERNARDI. In preparation.
9. RAZZELL, W. E. 1963. *In* S. P. COLOWICK and N. O. KAPLAN, editors, Methods in enzymology. Academic Press, New York. Vol. VI, p. 247.