[Reprinted from Biochemistry, (1964) 3, 1419.]

Studies on Acid Deoxyribonuclease. II. Isolation and Characterization of Spleen-Acid Deoxyribonuclease*

GIORGIO BERNARDI AND MAURICE GRIFFE†

From the Centre de Recherches sur les Macromolécules, Strasbourg, France‡

Received May 25, 1964

A method is described for the preparation of very highly purified acid deoxyribonuclease from hog spleen. The procedure involves chromatography on DEAE-cellulose, hydroxyapatite, and Amberlite IRC-50. The enzyme is free of phosphomonoesterase, splenic exonuclease, and nucleoside polyphosphatase; RNAase activity is present at a trace level. Besides its action on DNA, the enzyme shows a "phosphodiesterase" activity on a series of *p*-nitrophenylphosphodiesters. A preliminary characterization of both activities is given. Evidence is presented indicating that both activities are carried by the same protein molecule.

Since an acid deoxyribonuclease (DNAase) activity was first reported in spleen and thymus (Catcheside and Holmes, 1947; Maver and Greco, 1949a,b), several methods for the partial purification of this enzyme have been described (see Laskowski, 1961, for a review).

The isolation of the very highly purified enzyme in sizable amount was considered to be of interest, mainly on the following grounds: (a) An investigation of the kinetics of degradation of DNA by acid DNAase (Bernardi and Sadron, 1961, 1964a, 1964b; MacHattie *et al.*, 1963) showed that the enzyme degrades DNA according to two independent yet superimposed mechanisms: a "double-hit" degradation, of the type already known for pancreatic DNAase (Thomas, 1956; Schumaker *et al.*, 1956), which becomes effective in actually splitting DNA molecules only after a time lag, and a "single-hit" degradation which is effective and evident from the very start of the digestion. This latter mechanism is quite peculiar and implies, if DNA molecules are continuous double-stranded structures, simultaneous scissions of both strands at the same level. In our opinion, this phenomenon alone justifies a further inquiry into the structure of the enzyme molecule.

(b) An acid-DNAase activity appears to be widely distributed, and probably is always present in multicellular organisms (Laskowski, 1961; De Duve *et al.*, 1962; Russell, 1963). In the animal tissues investigated so far in our laboratory the activity appears to be associated with very similar protein molecules[1]; furthermore the enzyme concentration in the cells apparently is somehow related to the ability of the cells to divide.

The present article deals with the isolation and the enzymatic properties of spleen-acid DNAase. The physical and chemical characterization of the enzyme will be presented in a subsequent communication.[2] A preliminary note on both subjects has already been published (Bernardi *et al.*, 1963).

Experimental Procedure

Materials.—DNA was prepared essentially according to Kay *et al.* (1952) from calf thymus, chicken erythrocytes and *Escherichia coli*. High-molecular-weight RNA from Ehrlich ascites–tumor cells was a preparation, obtained by Drs. J. S. Colter and R. A. Brown according to their method (1956), which had already been studied in this laboratory (Bernardi and Timasheff, 1961). *E. coli* RNA was prepared as described by Littauer and Eisenberg (1959). Yeast s-RNA was a commercial product (General Biochemicals, Chagrin Falls, Ohio). RNA "core" was prepared according to Hilmoe (1960), using Pabst yeast RNA as the starting product; another RNA "core" preparation was purchased from Worthington, Freehold, N.J. Calcium [bis-(*p*-nitrophenyl)phosphate]$_2$ was prepared by Dr. A. Chersi in our laboratory as described by Privat de Garilhe and Laskowski (1955). The *p*-nitrophenyl esters of deoxyguanosine-, deoxycytidine-, and thymidine-3′-phosphates were prepared by Dr. H. E. Schaller according to Turner and Khorana (1959) and Fiers and Khorana (1963). Sodium *p*-nitrophenylphosphate was a commercial product (Calbiochem, Los Angeles, Calif.). Crystalline pancreatic DNAase (lots 932 and 10436) and RNAase (lot 593), calf spleen–acid DNAase (lots 6010 and 6011), and spleen phosphodiesterase (lot 624) were purchased from Worthington. All the reagents used were analytical grade except ammonium sulfate, which was a purified, crystalline product from Prolabo, Paris, France.

Column Chromatography.—Hydroxyapatite was prepared according to Tiselius *et al.* (1955). DEAE-cellulose was purchased from Serva, Heidelberg, Germany, or Calbiochem (Biorad Cellex D); the exchange capacities were 0.63 meq/g and 0.6 meq/g, respectively. Sephadex and CM-Sephadex were obtained from Pharmacia AB, Stockholm, Sweden. Amberlite IRC-50 was purchased from Fisher, Philadelphia, Pa. (Rexyn CG-51) or Serva (CG-50/II, 200–400 mesh); the latter product was treated according to Hirs (1955) before use. CM-cellulose was a product of Serva; its exchange capacity was 0.65 meg/g.

Chromatographic experiments were performed in a cold room (4°) using a Gilson Medical Electronics fraction collector. The transmission of the effluent at 280 mμ was continuously recorded. The flow of eluents was by gravity; 50- to 100-cm hydrostatic heads were used in most experiments. Molarity gradients were checked by refractometry using a Zeiss refractometer, or by phosphorus analysis.

Sucrose-gradient centrifugation was carried out in a Spinco Model L preparative ultracentrifuge, using a swinging-bucket rotor (SW-39) and the experimental conditions indicated by Martin and Ames (1961).

Assay of Enzymatic Activities.—The DNAase activity was routinely assayed by measuring the liberation of acid-soluble oligonucleotides from DNA. The incubation mixture contained 0.8 mg of DNA in 2 ml standard

* This work has been aided by a grant (UR-E9-[10,60]-80) from the U. S. Department of Agriculture.

† Present address: Facultés Universitaires Notre-Dame de la Paix, Namur, Belgium.

‡ Part of this work was carried out by one of the authors (G. B.) at the Department of Biophysics, Johns Hopkins University, Baltimore, Md., U.S.A.

[1] C. Cordonnier, and G. Bernardi, to be published.

[2] G. Bernardi, E. Appella, and R. Zito, to be published.

TABLE I
CHROMATOGRAPHIC PURIFICATION OF ACID DNAASE PREPARATION HS1 (PROCEDURE A)[a]

	Enzyme Fractions	Volume (ml)	Total OD_{280}	Total Activity	Specific Activity
	Starting product	*200*	*12.200*	*47.500*	*3.9*
I.	DEAE-cellulose				
	Phosphate, *p*H 8.0, 0.005 M	*814*	*7.090*	*51.200*[b]	*7.2*
	Idem, tail fraction	1.288	634	357	0.6
	Acetate, *p*H 5.0, 0.15 M	1.062	3110	1.074	0.3
II.	Hydroxyapatite				
	Phosphate, *p*H 6.8				
	0.02 M	1.190	5.110	3.450	0.75
	0.1 M	336	412	5.080	12.3
	0.3 M	*289*	*493*	*36.600*	*74.1*
III.	DEAE-cellulose[c]				
IV.	Hydroxyapatite				
	Phosphate, *p*H 6.8				
	Tubes 1–21 (0.2 M)	239	188	2.700	14.4
	Tubes 22–46 (0.2–0.3 M)	*285*	*157*	*19.500*	*124.2*
	Tubes 47–60 (0.5 M)	160	24	671	28.3
V.	Hydroxyapatite (Fig. 3)				
	Phosphate, *p*H 6.8				
	Tubes 48–62	47	30.2	2.820	93.4
	Tubes 63–78	*50*	*60.4*	*10.680*	*176.8*
	Tubes 79–107	90	26.0	2.680	103.5
VI.	IRC-50[d] (Fig. 4)				
	Phosphate, *p*H 6.0				
	Tubes 1–16	42	0.62	30.0	48.3
	Tubes 17–29	*34*	*5.5*	*1.650*	*302*
	Tubes 30–54	65	3.8	48.0	12.8

[a] Figures in italics refer to the fraction which was used in the following step. [b] See text for the explanation of this increase. [c] No data are available, since the fraction eluted at *p*H 8.0, $\mu = 0.005$, was immediately diluted to 0.02 M with potassium phosphate buffer, *p*H 6.8 and adsorbed on hydroxyapatite. [d] This chromatography was performed on an aliquot (18%) of the enzyme obtained from step V.

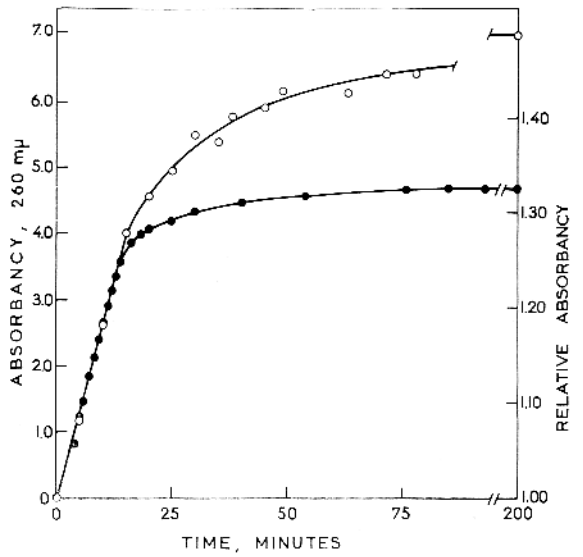

FIG. 1.—Acid-soluble oligonucleotide liberation (circles; left-hand ordinate; values not corrected for the 2:3 dilution), and hyperchromicity (points; right-hand ordinate) obtained upon digestion of calf-thymus DNA with acid DNAase. Digestion was carried out at 30°. Silica cells with a 0.1-cm optical path were used in the experiment on hyperchromicity, in order to work with the same DNA solution ($OD_{260} = 7.52$) used in the acid-soluble-oligonucleotide determination. After 200 minutes of digestion a massive dose of enzyme was added to the digestion mixtures, but no further changes were observed. The acid DNAase sample used in this experiment was obtained from step V of procedure A.

acetate buffer[3] and enzyme in 0.5 ml standard acetate buffer. Enzyme preparations of specific activity higher than 50 (*vide infra*) were diluted with standard acetate buffer containing 0.05% Armour bovine serum albumin. After 10 minutes at 37°, 0.5 ml of 12% perchloric acid was added; the mixture was chilled in an ice bath for 10 minutes and clarified by centrifugation at 4°. The extent of DNA hydrolysis was determined by measuring the absorption at 260 mμ of the supernatant in a Zeiss PMQ II spectrophotometer. Readings were corrected for dilution with the enzyme solution and the perchloric acid.[4]

Assays were performed using enzyme concentrations so as to obtain OD_{260} readings in the range 1–4. Under these conditions a linear relationship was obtained between enzyme concentration and acid-soluble-oligonucleotide formation and no time lag was evident. Figure 1 shows hyperchromicity and acid-soluble-oligonucleotide liberation as functions of digestion time. At low enzyme concentration both phenomena showed a very evident time lag.

The DNAase activity is defined as the (corrected) OD_{260} of the oligonucleotides liberated in 1 minute by 1 ml of enzyme solution. Since the molar absorption at 260 mμ of the oligonucleotide phosphorus present in the final, completely acid-soluble digest was shown to be 9100, one activity unit corresponds to the liberation of 0.22 μmole of oligonucleotide phosphorus. The total activity of an enzyme solution was obtained by multiplying its activity by its volume in ml. The specific activity was obtained by dividing the activity by the optical density at 280 mμ of the enzyme solution.

The determination of RNAase activity was essentially the same as that of DNAase activity, except that the solvents were 0.1 M acetate buffer, *p*H 5.5, for the acid activity and 0.1 M potassium phosphate buffer, *p*H

[3] Standard acetate buffer is acetate buffer prepared according to Moore and Stein (1954) diluted to 0.15 M, containing 0.01 M EDTA and 0.01 M cysteine; the final *p*H is 5.0.

[4] This was not done in our preliminary report (Bernardi *et al.*, 1963).

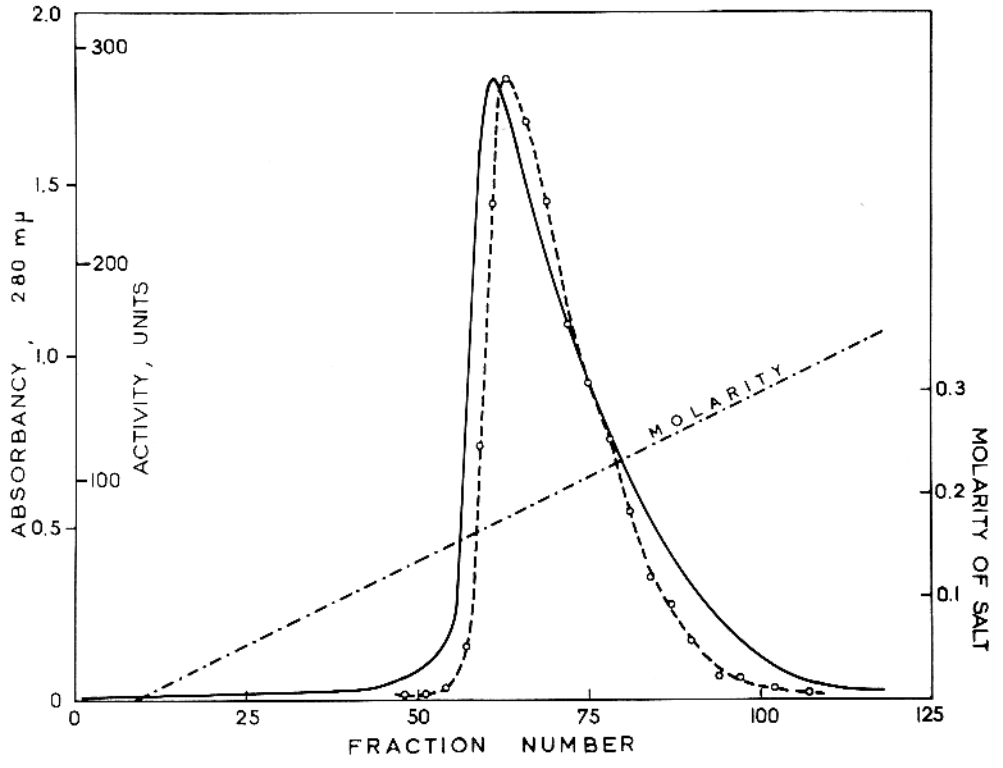

FIG. 2.—Chromatography of acid DNAase on hydroxyapatite (step V of procedure A; see Table I). Absorbancy of the fractions at 280 mμ (left-hand ordinate, outer scale), ——; DNAase activity of the fractions (left-hand ordinate, inner scale), —O—O—. A linear molarity gradient of potassium phosphate buffer, *p*H 6.8 was used (right-hand ordinate).

7.3, containing 0.01 $MgCl_2$, for the basic activity (Maver and Greco, 1962). The phosphodiesterase and phosphomonoesterase activities were determined according to Burton and Petersen (1960). Phosphodiesterase activity was also measured according to Hilmoe (1960), using RNA "core" as the substrate; the volume was increased ten times as suggested by a Worthington leaflet.

The nucleoside polyphosphatase activity was measured using adenosine triphosphate as the substrate and the experimental conditions of Laskowski and Filipowicz (1958). Inorganic phosphorus was determined according to Martin and Doty (1949).

RESULTS

Isolation of the Enzyme

Preparation of the Crude Enzyme.—A method already described elsewhere (Bernardi, 1961) was slightly modified in order to handle much larger amounts of tissue. A description of a standard preparation follows. All operations were done at 4°; the precipitates were allowed to stand overnight; the centrifugations were carried out in a Lourdes centrifuge at 8,000 *g* for 1 hour, except where otherwise stated.

Spleens were obtained at the abattoirs from freshly slaughtered pigs; 5 kg was trimmed, minced, and homogenized for 3 minutes in a Waring Blendor with 7 liters of 0.15 M NaCl–0.02 M $CaCl_2$. Batches of 250 g were treated with 300 ml of solvent to which 1 ml of isooctanol had been added. The suspension was shaken for 18 hours and then centrifuged for 30 minutes at 3000 *g*. The sediment was again homogenized with 4 liters of fresh solvent and treated as before. The turbid combined supernatants were centrifuged. The supernatant now obtained was filtered through cheesecloth, brought to 0.34 saturation with solid $(NH_4)_2SO_4$, and centrifuged. The supernatant was filtered through cheesecloth and paper and brought to saturation with solid $(NH_4)_2SO_4$. The precipitate that formed was collected by centrifugation or filtration, dissolved in 3 liters of water, adjusted to *p*H 2.5, by dropwise addition of 0.3 N HCl, then centrifuged. The sediment was discarded and the supernatant was treated with $(NH_4)_2SO_4$. The precipitate obtained between 0.4 and 0.8 saturation was dissolved in 500–750 ml of water, dialyzed against 10 liters of water with several changes, clarified by centrifugation and freeze-dried. No loss of enzymatic activity was found upon freeze-drying at this stage of purification. Some loss of activity was found during storage at −15° of the freeze-dried preparations for periods of several months. The crude enzyme was obtained in a yield of about 4.5 g, about 1 g/kg of trimmed tissue. It contained about 55% of the acid-DNAase activity present in the clarified extract and its specific activity was 3–4; the total activity was about 15,000. The specific activity of the crude enzyme was about the same as that of the two commercial acid-DNAase preparations, which were prepared according to the procedure of Shimomura and Laskowski (1957) through dialysis against acetate buffer (step 5).

Phosphomonoesterase, phosphodiesterase, and nucleoside-polyphosphatase activities were detected in the crude enzyme preparation; both basic and acid RNAase were present, the ratios DNAase/RNAase being 3.6 and 1.8, respectively. These contaminating activities were also found in the two commercial preparations.

Chromatographic Purification.—After many small-scale trials of chromatographic purification on substances already used by previous authors, namely, Amberlite IRC-50 (Koszalka *et al.*, 1957), CM- and DEAE-cellulose (Shimomura and Laskowski, 1957; Maver *et al.*, 1959; Maver and Greco, 1962; Hodes and Swenson, 1962), hydroxyapatite (Fredericq and Oth, 1958), and combinations of those methods, two procedures (A and B, *vide infra*) were worked out.

Three large-scale preparations were carried out: HS1, HS2, and HS3; amounts of 17 g, 9 g, and 37 g,

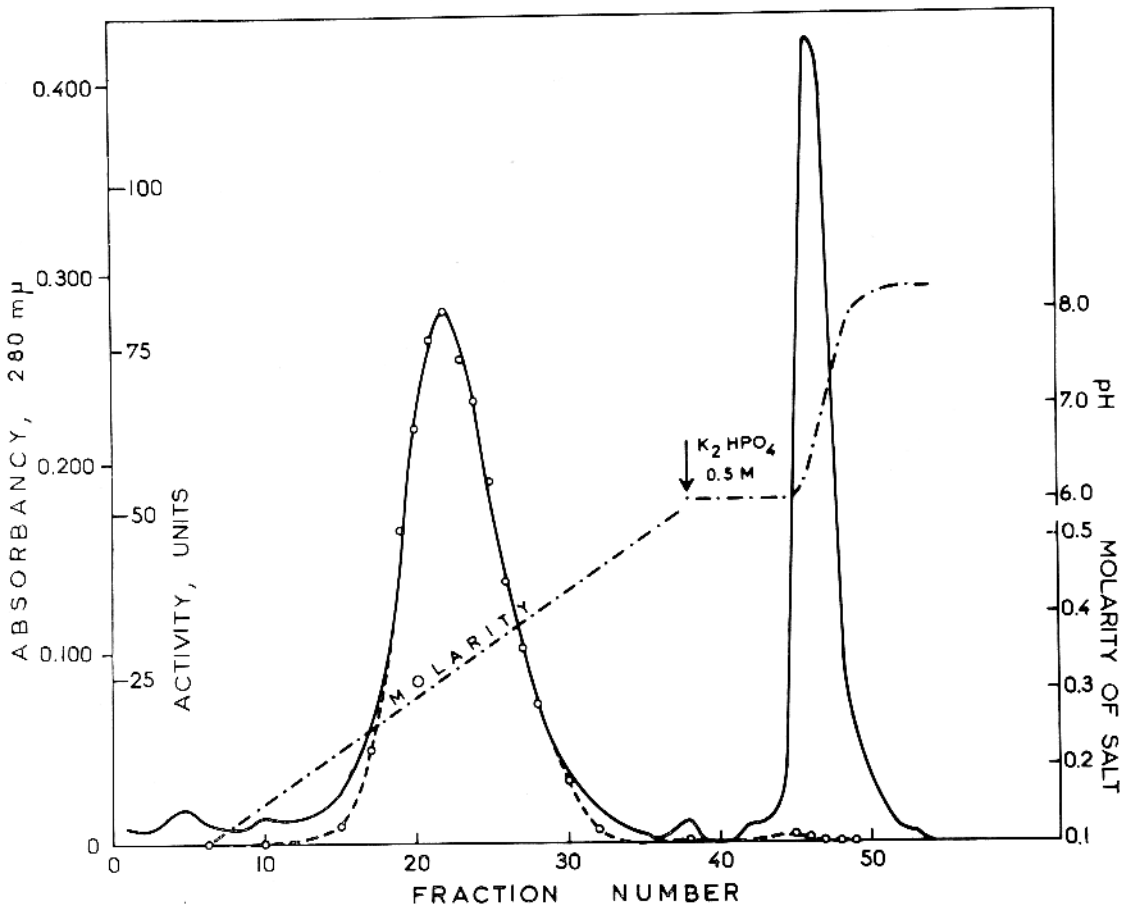

FIG. 3.—Chromatography of acid DNAase on Amberlite IRC-50 (step VI of procedure A; see Table I). Indications as in Fig. 2. A molarity gradient of phosphate buffer, *p*H 6.0 (right-hand ordinate lower scale), was followed by 0.5 M K_2HPO_4 (the *p*H of effluent is given in the right-hand ordinate, upper scale).

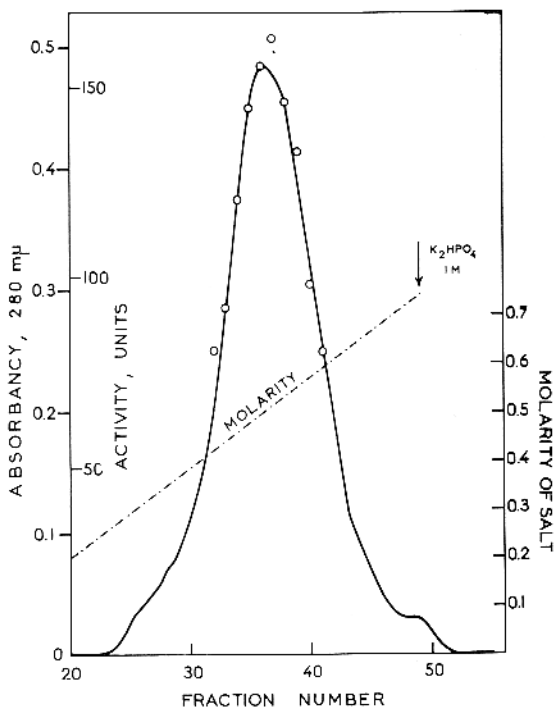

FIG. 4.—Rechromatography on IRC-50 (2 × 12.5 cm) of the first peak of the chromatographic run of acid DNAase on IRC-50 (step IV of procedure B; preparation HS2). Elution was carried out with a molarity gradient of phosphate buffer, *p*H 6.0. No optical density was eluted with a subsequent *p*H gradient. Fractions of 5 ml were collected. Circles indicate the DNAase activity.

respectively, of crude enzyme were treated. Preparation HS1 was obtained using procedure A; HS2 and HS3 were obtained using the simpler procedure B. The emphasis in the three preparations was quite different. In HS1 yields of optical density and DNAase activity were studied in detail; HS2 was intended to obtain a higher final yield; and in HS3 the chromatographic behavior of some accompanying enzymatic activities was studied as well.

Procedure A.—Results are shown in Table I. Step I. DEAE-Cellulose: This step was patterned after Maver *et al.* (1959). The crude enzyme was dissolved in water, diluted to 0.005 M in phosphate buffer, *p*H 8.0, and adsorbed on four DEAE-cellulose columns (3 × 30 cm). Under these conditions DNAase was not retained. The total DNAase activity eluted was about 10% higher than that of the starting material, because of the adsorption of an inhibitor on the column. This inhibitor was later identified as sulfate ion. The adsorbed proteins could be eluted with 0.15 M acetate buffer, *p*H 5.0; this buffer also eluted sulfate. A brown substance, unidentified as yet, was removed from the column with 0.1 N HCl.

Step II. Hydroxyapatite: The activity-rich fraction from Step I was diluted to 0.02 M with potassium phosphate buffer, *p*H 6.8, and adsorbed on four hydroxyapatite columns (2 × 30 cm). Elution was carried out stepwise, using 0.02 M, 0.1 M 0.3 M, and 0.5 M potassium phosphate buffer, *p*H 6.8.

Step III. DEAE-Cellulose: The 0.3 M fraction from previous step was freeze-dried, dissolved in water, and dialyzed against 0.005 M phosphate buffer, *p*H 8.0, and then adsorbed on DEAE-cellulose (3 × 30 cm). The fraction which was not retained was immediately

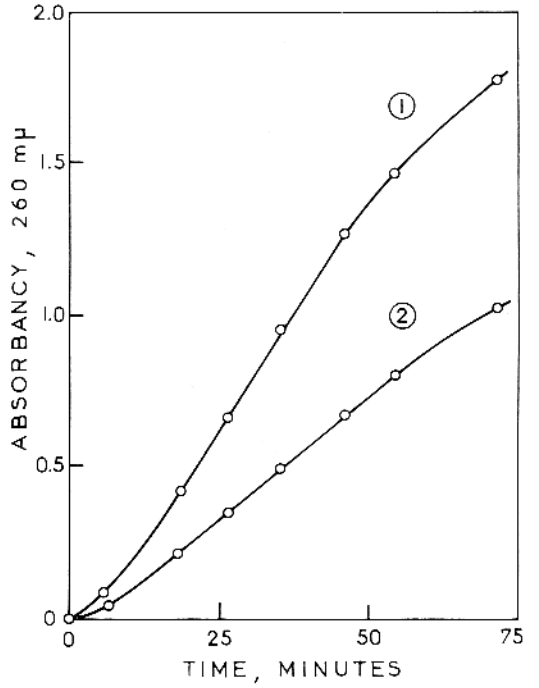

FIG. 5.—Acid-soluble-nucleotide liberation during the digestion of (1) native and (2) heat-denatured calf-thymus DNA by acid DNAase. The OD_{260} of native DNA was 2.24; that of denatured DNA, 2.45. Values not corrected for the 2:3 dilution.

diluted to 0.02 M with potassium phosphate buffer, *p*H 6.8 and adsorbed on hydroxyapatite.

Step IV. Hydroxyapatite (3 × 30 cm): Elution was carried out in four steps: 0.02, 0.2, 0.3, and 0.5 M potassium phosphate buffer, *p*H 6.8. Activity was found in both the 0.2 and 0.3 M fractions. Active fractions were pooled together and adsorbed on another hydroxyapatite column.

Step V. Hydroxyapatite (2 × 15 cm): Elution was carried out with a linear molarity gradient of potassium phosphate buffer, *p*H 6.8, from 0.05 to 0.5 M. A single peak was eluted (Fig. 2), but the activity curve did not coincide with the optical-density curve. The central fractions of the peak were pooled, concentrated by being rolled in Visking cellulose tubes over dry Sephadex, frozen at an optical density at 280 mμ of about 3.0, and stored at −60°. An aliquot of the frozen enzyme was thawed and rerun on hydroxyapatite; no improvement whatsoever over step V was obtained.

Step VI. Amberlite IRC-50 (Fig. 3): Other aliquots of enzyme from the previous step were dialyzed against 0.1 M phosphate buffer, *p*H 6.0, and adsorbed on Amberlite IRC-50 (0.7 × 12 cm) equilibrated with the same buffer. Some inactive material was not retained. A molarity gradient of phosphate buffer, *p*H 6.0, from 0.1 to 0.5 eluted the enzyme at a constant specific activity of 300,[5] the eluting molarity being extremely dependent upon the *p*H of the buffer. Additional inactive material was eluted with 0.5 M K_2HPO_4. Upon rechromatography the first fraction was again eluted at a constant specific activity of 300[5] with the molarity gradient, whereas the inactive fraction still required the *p*H gradient for elution.

Procedure B.—A scheme of this procedure has been outlined elsewhere (Bernardi *et al.*, 1963). Step I.

[5] The enzyme fractions were diluted with standard acetate buffer, without cysteine, containing 0.1% bovine serum albumin. This was seen later to give lower activity values than when diluting with standard acetate buffer containing 0.05% bovine serum albumin.

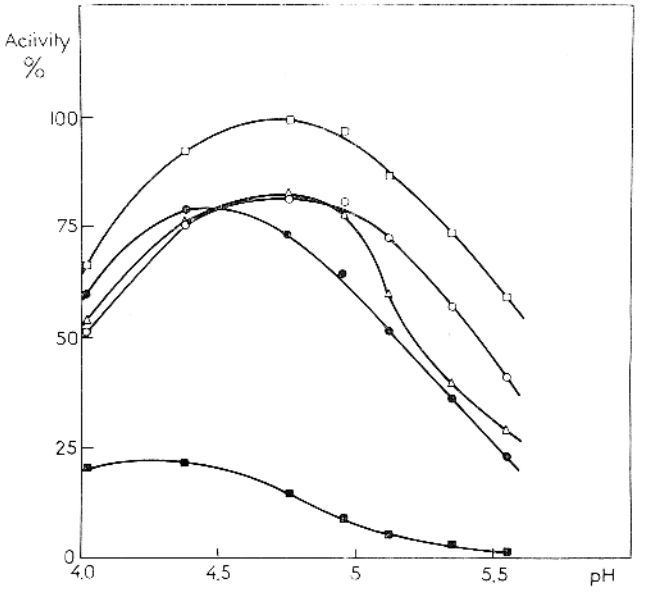

FIG. 6.—DNAase activity of acid DNAase, as assayed by the acid-soluble-oligonucleotide formation at μ 0.15. Acetate buffer, O—O; acetate buffer + 0.01 M $MgCl_2$, ●—●; acetate buffer + 0.01 M EDTA, □—□; acetate buffer + 0.01 M Na_2SO_4, ■—■; acetate buffer + 0.01 M KH_2PO_4, △—△.

DEAE-Cellulose. This was used as in Procedure A. The fraction not retained by the column contained DNAase, phosphodiesterase, and acid phosphomonoesterase in that order; basic RNAase and some acid RNAase were also presented. Adsorbed proteins were eluted with a gradient of molarity and/or *p*H, with 0.15 M acetate buffer *p*H 5.0 as the final eluent; this resulted in a partial separation of a second acid phosphatase and the bulk of acid RNAase.

Step II. Hydroxyapatite: Elution was carried out as in step V of Procedure A. DNAase was eluted immediately after a bright-red fraction showing absorption bands at 280, 414, 520, and 550 mμ (tentatively identified with cytochrome c). Phosphodiesterase and acid phosphomonoesterase were eluted before DNAase, in that order.

Step III. Hydroxyapatite: Elution was performed as before, the enzyme being desorbed at about 0.2 M potassium phosphate buffer, *p*H 6.8. The active fractions were concentrated and frozen.

Step IV. Amberlite IRC-50: Elution was performed as in Step VI of Procedure A, with the same results. The rechromatography of the active fractions from the previous step is shown in Figure 4. The central fractions (tubes 32–41) showed a constant specific activity and were pooled, dialyzed against 0.01 M phosphate buffer, *p*H 6.0–0.02 M NaCl, concentrated to an OD_{280} = 2.5, and frozen. DNAase activity was recovered in a final yield of 25%, taking as 100% the activity measured after chromatography I. The specific activity was 350.

Properties of the Enzyme

Spleen acid DNAase, as obtained by the foregoing methods, appears to be a homogeneous protein as judged by physical techniques (sedimentation velocity and electrophoresis on cellulose acetate at several different *p*H values; see Bernardi *et al.*, 1963), amino acid analysis,[1] sucrose gradient centrifugation, and enzymological methods (see below).

Properties.—Acid DNAase was found to be active on both DNA and a series of *p*-nitrophenylphosphodiesters.

DNAASE ACTIVITY.—The enzyme is able to hydrolyze

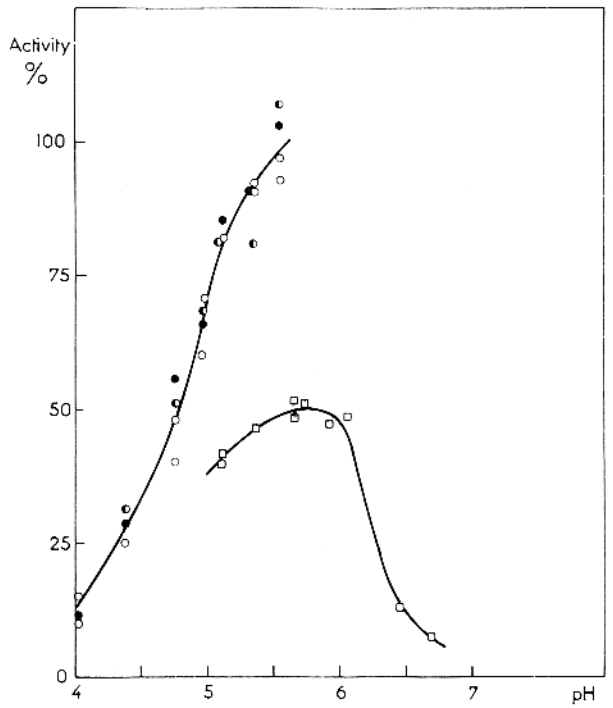

FIG. 7.—"Phosphodiesterase" activity of acid DNAase, as assayed on Ca [bis(*p*-nitrophenyl)phosphate]$_2$ at μ 0.15. Acetate buffer, O—O; acetate buffer + 0.01 M $MgCl_2$, ●—●. acetate buffer + 0.01 M EDTA, ◐—◐; succinate buffer, □—□.

both native and heat-denatured DNA. The acid-soluble nucleotide formation, however, is slower in the latter case (Fig. 5). Furthermore the initial degradation kinetics is different in the two cases (Bernardi and Sadron, 1964a,b). The *p*H-activity curves at μ = 0.15 are shown in Figure 6. No bovine serum albumin was added when the enzyme was diluted in these experiments. The *p*H optimum appears to be close to 4.8. Magnesium is slightly inhibitory above *p*H 4.5 and shifts the *p*H optimum to the lower value of 4.4. EDTA enhances the enzymatic activity without shifting the *p*H optimum. Phosphate ion seems to be slightly inhibitory only above *p*H 5.0. Sulfate ion is a very strong inhibitor, the inhibition being less pronounced below *p*H 4.5. In succinate buffer, μ = 0.15, *p*H = 6.7, the activity is less than 3% of that in acetate buffer, *p*H = 5.0, μ = 0.15. Cysteine has a very evident activating effect on preparations having a specific activity higher than 50.

"PHOSPHODIESTERASE" ACTIVITY.—The enzyme shows an activity on Ca [bis-(*p*-nitrophenyl)phosphate]$_2$ and the *p*-nitrophenyl esters of thymidine-, deoxyguanosine-, and deoxycytidine-3′-phosphates (the deoxyadenosine derivative was not assayed). The results obtained on this latter series of compounds will be reported in detail elsewhere.[6]

Using as a substrate Ca [bis-(*p*-nitrophenyl)phosphate]$_2$ the *p*H optimum was found to be between 5.6 and 5.9. In the 5.1–5.6 *p*H range the activity in acetate is about twice as large as in succinate buffer. In the *p*H range 4.0–5.6 no significant changes occur upon addition of Mg^{2+} or Versene (Fig. 7); in the *p*H range 4.0–7.0 SO_4^{2-} and HPO_4^{2-} give a very strong inhibition at a 0.01 M level.

In order to show that the DNAase and the "phosphodiesterase" activities are carried out by the same protein molecule the following experiments have been carried out. (A) CHROMATOGRAPHY ON AMBERLITE

[6] G. Bernardi and H. E. Schaller, to be published.

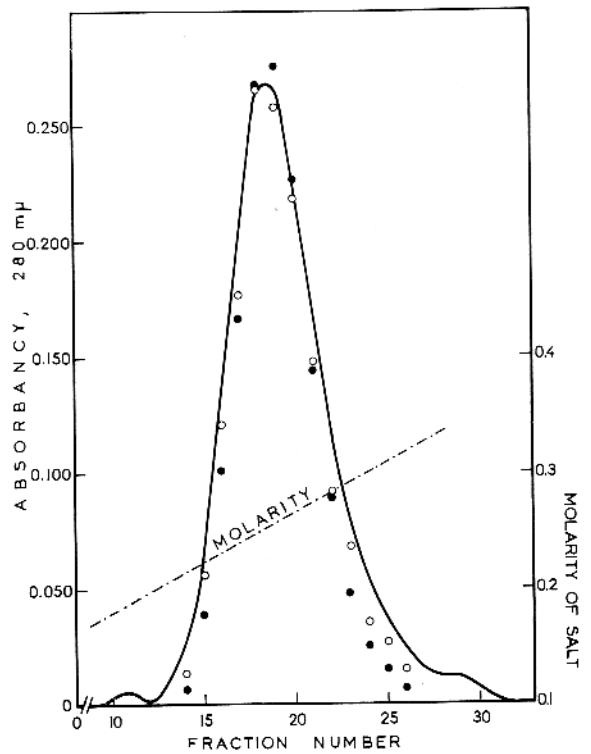

FIG. 8.—Chromatography of acid DNAase on IRC-50. The enzyme was eluted by a molarity gradient of phosphate buffer, *p*H 6.0. The solid line gives the absorbancy at 280 mμ (left ordinate); the broken line indicates the molarity (right-hand ordinate). Circles indicate the DNAase activity, points the "phosphodiesterase" activity; both activities are given in arbitrary units.

IRC-50, CM-SEPHADEX, AND SEPHADEX G-50, AND G-100.—When running pure acid DNAase on these columns only one symmetrical peak was obtained and the ratio of the two activities was constant through the peak. The results obtained on Amerlite IRC-50, when using the same experimental conditions as in the isolation of the enzyme, are shown in Figure 8. On CM-Sephadex the enzyme was eluted by a molarity gradient (0.1–0.3 M) of phosphate buffer, *p*H 6.8. The runs on Sephadex G-50 and G-100 were performed using 0.01 M acetate buffer, *p*H 5.0, and 0.001 M potassium phosphate buffer, *p*H 6.8, respectively, as the solvents.

Splenic exonuclease (Hilmoe, 1960), when present, shows up as a separate peak in the chromatographies on Amberlite IRC-50 and CM-Sephadex, or as a partially separated component on Sephadex G-50 and G-100.

(B) THERMAL INACTIVATION (Fig. 9).—Enzyme samples (0.4 ml; OD_{280} = 0.148) were kept for 20 minutes at several temperatures ranging from 25 to 75° in standard acetate buffer, chilled in an ice bath, and used in digestion experiments at 37°. The inactivation curves of both DNAase and "phosphodiesterase" activities were identical. The thermal stability of the enzyme was lower at higher *p*H values.

(C) SUCROSE-GRADIENT CENTRIFUGATION.—A constant ratio of the two activities was found through the peak in several experiments (Fig. 10). When spleen exonuclease (Hilmoe, 1960) was added to acid DNAase, it showed up as a heavier component. This behavior suggests that splenic exonuclease has a higher molecular weight than acid DNAase; this is also indicated by the fact that both on Sephadex G-50 and G-100 splenic exonuclease is eluted before acid DNAase.

Enzymological Purity.—Several possible contaminating activities were assayed in acid DNAase. Phospho-

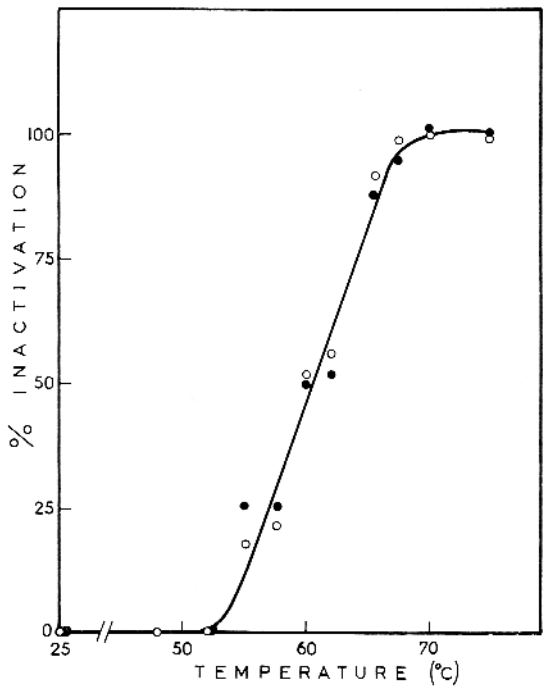

FIG. 9.—Thermal inactivation curve. Acid DNAase samples in standard acetate buffer were kept 20 minutes at the temperatures indicated in the abscissa; they were chilled in ice bath and assayed at 37° for both DNAase (open circles) and "phosphodiesterase" activity (solid circles).

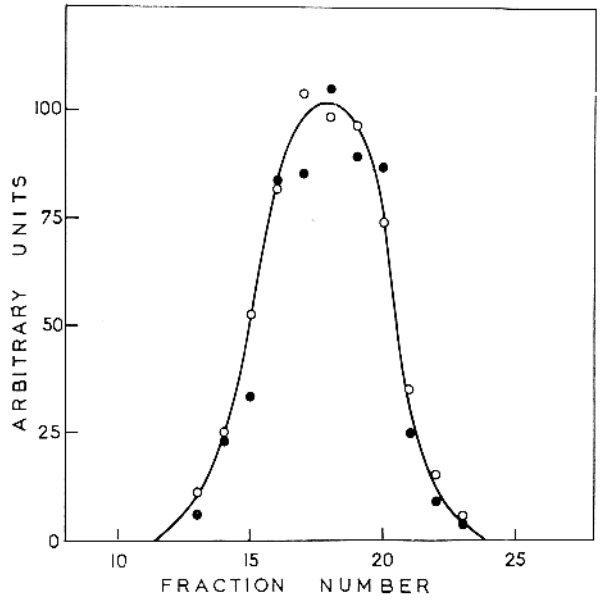

FIG. 10.—Sucrose-gradient centrifugation of acid DNAase. A total of 32 fractions was collected. Circles indicate the DNAase activity; points indicate the "phosphodiesterase" activity. Bottom of the cell to the left. A linear molarity gradient was obtained using 5% and 20% sucrose solutions in standard acetate buffer. Centrifugation was carried out for 16 hours at 4° at 38,000 rpm using a SW-39 rotor and a Spinco Model L ultracentrifuge.

monoesterase activity was found to be zero after 3.5 hours of digestion at *p*H 5.0 and 6.7, using 0.1 ml of acid DNAase with an OD_{280} = 0.259; under the same conditions and the same OD_{280} the crude enzyme gives off-scale readings after 3 minutes. Nucleoside polyphosphatase activity (Laskowski and Filipowicz, 1958) was absent; no activity on ATP could be detected with digestion times up to 24 hours, when using amounts of DNAase capable of producing an extensive hydrolysis of Ca[bis-(*p*-nitrophenyl)phosphate]$_2$.

RNAase activity was present at about the same level as in Worthington crystalline pancreatic DNAase (lot 10436), the ratio DNAase/RNAase being of the order of 5.10^3. A much lower ratio was found in the older pancreatic DNAase sample (lot 932). In spleen DNAase the RNAase activity at *p*H 5.5 was higher than at *p*H 7.3, whereas the reverse was true for the pancreatic DNAase. Ehrlich ascites-tumor-cell RNA was hydrolyzed at a slower rate than soluble RNA; *E. coli* RNA was hydrolyzed at an intermediate rate; RNA "core" was also attacked (*vide infra*). Splenic exonuclease activity (Hilmoe, 1960) was absent, judged by the complete lack of activity at *p*H 6.7 (0.25 M succinate buffer) on RNA "core" by acid DNAase samples which caused an evident degradation of Ca-[bis-(*p*-nitrophenyl)phosphate]$_2$ in the same buffer (see Table II), and from the absence of mononucleotides in the final DNA digest.[6]

TABLE II
RATES OF HYDROLYSIS OF RNA "CORE" AND Ca[BIS-(*p*-NITROPHENYL)PHOSPHATE]$_2$ BY SPLENIC PHOSPHODIESTERASE AND ACID DNAASE[a]

Enzyme	Rate on RNA "core" (A_{260}/hr)[a]	Rate on RNA "core" (units/ml[20])	Rate on Ca[bis-(*p*-nitrophenyl)phosphate]$_2$ (A_{400}/hr[a])	Rate on Ca[bis-(*p*-nitrophenyl)phosphate]$_2$ (mμmoles/ml/hr[b])
Phosphodiesterase	0.900	21.2	0.007	17
Acid DNAase	0	0	0.022	62

[a] In 0.25 M succinate buffer, *p*H 6.6; using the conditions described under Experimental Procedure with 0.1 ml of enzyme. [b] Assuming an E_{400} for *p*-nitrophenol of 12,000 (Razzell and Khorana, 1961).

When using enormous amounts of acid DNAase (a sample particularly rich in contaminating RNAase was used in this experiment), an activity on RNA "core" could be shown; the *p*H-activity curve, however, was similar to that of contaminating RNAase rather than to that of splenic phosphodiesterase. In agreement with the interpretation that the activity on RNA "core" was due to RNAase is the report that spleen RNAases hydrolyzed RNA "core" (Maver *et al.*, 1959) and the finding that a pure preparation of acid RNAase which showed no activity at all on Ca[bis-(*p*-nitrophenyl)phosphate]$_2$ was very active on RNA "core".[7]

DISCUSSION

A comparison, in terms of specific activity, of our DNAase preparation with those by previous authors is very difficult, owing to the scarcity of published data and to differences in the assay procedures. According to Laskowski (1961), the three best available methods for the partial purification of acid DNAase, those of Koerner and Sinsheimer (1957), Shimomura and Laskowski (1957), and Fredericq and Oth (1958), lead to preparations of similar potency. A very rough comparison of the last two preparations and ours may be attempted, as follows. Shimomura and Laskowski (1957) obtained a 30-fold purification from their step IV to the final product. Assuming that the Worthington preparations (whose specific activity was found to be about 4) had the same specific activity as Shimomura and Laskowski's (1957) product at step IV, we should estimate the specific activity of the final product of those authors at about 120, in our units.

[7] A. Bernardi, G. Rialdi, and G. Bernardi, to be published.

The preparations of Fredericq and Oth (1958) were purified by two or three chromatography runs on hydroxyapatite. Taking into account our finding that it is impossible to obtain a specific activity higher than 175 by repeating purifications on hydroxyapatite, we may estimate that the preparations by Fredericq and Oth (1958) had specific activities of the order of 100–150. Therefore the purity of the best preparations obtained by previous authors may be estimated at about 30–40% of our preparation. The spleen-acid DNAase obtained by the methods described here appears to be free of phosphomonoesterase, phosphodiesterase (Hilmoe, 1960), and nucleoside polyphosphatase activity; the only contaminating impurity detected in the preparation is RNAase activity, which is present, however, at about the same trace level as in Worthington crystalline pancreatic DNAase.

A surprising and unexpected finding is that acid DNAase, besides its activity on DNA, also is endowed with a "phosphodiesterase" activity on a series of *p*-nitrophenylphosphodiesters like Ca[bis-(*p*-nitrophenyl)phosphate]$_2$ and the *p*-nitrophenyl esters of deoxyriboside-3′-phosphates. Comments on this finding and its possible relationship with the degradation kinetics of DNA must await further work.

Acknowledgments

The authors wish to thank Prof. Charles Sadron and Dr. C. A. Thomas, Jr., for their interest; Miss B. Nubel and Mr. H. Stebler for their excellent technical assistance; Mrs. C. Cordonnier, Dr. G. Rialdi, and Dr. A. Chersi for their help at various moments of the present work; and Dr. H. E. Schaller, Max Planck-Institut für Virusforschung, Tübingen, for the preparation of the *p*-nitrophenyl esters of deoxyriboside-3′-phosphates.

References

Bernardi, G. (1961), *Biochim. Biophys. Acta 53*, 216.
Bernardi, G., Griffé, M., and Appella, E. (1963), *Nature 198*, 186.
Bernardi, G., and Sadron, C. (1961), *Nature 191*, 809.
Bernardi, G., and Sadron, C. (1964a), Baselli Conference on Nucleic Acids and Their Role in Biology, Milan (Italy), September, 1963 (in press).
Bernardi, G., and Sadron, C. (1964b), *Biochemistry 3*, 1411 (this issue, preceding paper).
Bernardi, G., and Timasheff, S. N. (1961), *Biochem. Biophys. Res. Comm. 6*, 58.
Burton, K., and Petersen, G. B. (1960), *Biochem. J. 75*, 17.
Catcheside, D. G., and Holmes, B. (1947), *Symp. Soc. Exptl. Biol. 1*, 225.
Colter, J. S., and Brown, R. A. (1956), *Science 124*, 1077.
De Duve, C., Wattiaux, R., and Baudhuin, P. (1962), *Advan. Enzymol. 24*, 291.
Fiers, W., and Khorana, H. G. (1963), *J. Biol. Chem. 238*, 2780.
Fredericq, E., and Oth, A. (1958), *Biochim. Biophys. Acta 29*, 281.
Hilmoe, R. J. (1960), *J. Biol. Chem. 235*, 2117.
Hirs, C. H. W. (1955), *Methods Enzymol. 1*, 113.
Hodes, M. E., and Swenson, M. K. (1962), *Biochim. Biophys. Acta 61*, 612.
Kay, E. R. M., Simmons, N., and Dounce, A. L. (1952), *J. Am. Chem. Soc. 74*, 1724.
Koerner, J. F., and Sinsheimer, R. L. (1957), *J. Biol. Chem. 228*, 1039.
Koszalka, T. R., Falkenheim, R., and Altman, K. I. (1957), *Biochim. Biophys. Acta 23*, 647.
Laskowski, M. (1961), *Enzymes 5*, 123.
Laskowski, M., and Filipowicz, B. (1958), *Bull. Soc. Chim. Biol. 40*, 1865.
Littauer, U. Z., and Eisenberg, H. (1959), *Biochim. Biophys. Acta 32*, 320.
MacHattie, L., Bernardi, G., and Thomas, C. A., Jr. (1963), *Science 141*, 59.
Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236*, 1372.
Martin, J. B., and Doty, D. M. (1949), *Anal. Chem. 21*, 965.
Maver, M. E., and Greco, A. E. (1949a), *J. Biol. Chem. 181*, 853.
Maver, M. E., and Greco, A. E. (1949b), *J. Biol. Chem. 181*, 861.
Maver, M. E., and Greco, A. E. (1962), *J. Biol. Chem. 237*, 736.
Maver, M. E., Peterson, E. A., Sober, H. A., and Greco, A. E. (1959), *Ann. N.Y. Acad. Sci. 81*, 599.
Moore, S., and Stein, W. H. (1954), *J. Biol. Chem. 211*, 907.
Privat de Garilhe, M., and Laskowski, M. (1955), *Biochim. Biophys. Acta 18*, 370.
Razzell, W. E., and Khorana, H. G. (1961), *J. Biol. Chem. 236*, 1144.
Russell, A. P. (1963), Ph.D. dissertation, Boston University.
Schumaker, V. N., Richards, E. G., and Schachman, H. K. (1956), *J. Am. Chem. Soc. 78*, 4230.
Shimomura, M., and Laskowski, M. (1957), *Biochim. Biophys. Acta 26*, 198.
Thomas, C. A., Jr. (1956), *J. Am. Chem. Soc. 78*, 1861.
Tiselius, A., Hjertén, S., and Levin, Ö. (1955), *Arch. Biochem. Biophys. 65*, 132.
Turner, A. F., and Khorana, H. G. (1959), *J. Am. Chem. Soc. 81*, 4651.