Purification and Physical Characterization of Stem Bromelain*

TAKASHI MURACHI, MIEKO YASUI, AND YOKO YASUDA†

From the Department of Biochemistry, Nagoya City University School of Medicine, Nagoya, Japan

Received June 27, 1963

Crude commercial bromelain from pineapple stem has been purified by successive use of ion-exchange chromatography, gel filtration, and ammonium sulfate fractionation. From 10 g of the starting material 0.87 g of purified bromelain is obtained. The purified preparation is essentially homogeneous by such criteria as sedimentation in the ultracentrifuge, free-boundary electrophoresis, diffusion studies, and chromatography. Free-boundary electrophoresis in buffers of 0.1 ionic strength over the range *p*H 2.9–11.8 gives an isoelectric point of *p*H 9.55. Slightly positive and negative dependences of sedimentation constants and diffusion constants, respectively, on protein concentration are observed in phosphate buffer at *p*H 7.4. The values computed are extrapolated to zero concentration: $s^0_{20,w}$ is 2.73 Svedberg units and $D^0_{20,w}$ is 7.77×10^{-7} cm^2 sec^{-1}. The partial specific volume, $\bar{v}$, is 0.743 ml/g, and the intrinsic viscosity, $[\eta]$, is 0.039 dl/g. The molecular weight of bromelain is computed to be 33,000. Other physical constants of bromelain obtained are: frictional ratio, $f/f_0 = 1.26$; absorbancy, $A^{1\%}_{1\,cm}$ at 280 mμ = 19.0; optical rotation, $[\alpha]_{436} = -79.1°$ and $[\alpha]_{546} = -43.1°$; optical rotatory dispersion constant, λ_c = 241 mμ; parameters, $a_0 = -190°$ and $b_0 = -78°$

Bromelain is a proteolytic enzyme found in tissues of pineapple plant and other species of family *Bromeliaceae*. Murachi and Neurath (1960) have shown that the crude bromelain preparation from pineapple stem juice can be fractionated into several components by ion-exchange chromatography. They obtained two major components by the use of cation-exchange resin Duolite CS101 at *p*H 6.05, and reported some properties of these fractions. Based on these earlier data an improvement of purification procedure has been sought in this laboratory so as to obtain homogeneous enzyme preparation at a large scale, thus enabling us to characterize further physical, chemical, and enzymatic properties of the bromelain molecule which had been only insufficiently described (Murachi and Neurath, 1960; Balls *et al.*, 1941; Ota *et al.*, 1961).

In the present communication we report a purification procedure which includes ion-exchange chromatography, gel filtration, and ammonium sulfate fractionation. The preparation obtained by this procedure is essentially homogeneous by various criteria. A molecular weight of 33,000, an isoelectric point of *p*H 9.55, and other physical constants obtained with the purified preparation are given and comparisons are made of these data with those available for papain (Smith *et al.*, 1954; Smith and Kimmel, 1960), ficin (Bernhard and Gutfreund, 1956; Cohen, 1958; Liener, 1961) and chymopapain (Ebata and Yasunobu, 1962), which have many common features with bromelain. Kinetic studies have also been made with purified bromelain and the results obtained are published elsewhere (Inagami and Murachi, 1963).

MATERIALS AND METHODS

Bromelain.—Crude commercial preparation, "Bromelain" (lots 181 and 182) from the Hawaiian Pineapple Company, Honolulu, Hawaii, was used as the starting material.[1] As compared to the crude preparation used in a previous study (Murachi and Neurath, 1960), which was also obtained from the same manufacturer, the preparation employed in the present experiments was more colored in appearance and contained more insoluble materials. These differences may reflect different manufacturing processes.

Ion-Exchange Resins and Dextran Gels.—Resins used in the present experiments were: Duolite A-2, coarse; Amberlite CG-50, Type I (100–200 mesh) and Type II (200–400 mesh); Sephadex G-25, medium; Sephadex G-50, medium; Sephadex G-75, medium; Sephadex G-100, 140–400 mesh (lot To 1291); Sephadex G-200, 140–400 mesh (lot To 39); CM-Sephadex C-25, medium (lot To 8691 M); and DEAE-Sephadex A-25, medium (lot To 7871 M). Sephadex absorbants were products obtained from Pharmacia, Uppsala, Sweden.

Substrates.—BAEE[2] was purchased from Mann Research Laboratories, Inc., New York, N. Y. Casein was a product from E. Merck, Darmstadt, Germany.

Measurement of Enzymatic Activities.—Proteinase activity of bromelain was measured by hydrolysis of casein according to the method described by Hagihara *et al.* (1958). The incubation mixture contained 5 ml of casein solution, 30 μmoles of L-cysteine, and approximately 60 μg of enzyme protein in a total volume of 6.0 ml. The incubation was carried out at 35° for 10 minutes. The absorbancy at 275 mμ of the trichloroacetic acid–soluble peptides formed was determined with a Hitachi Model EPB-U spectrophotometer. A proteinase unit is defined as the enzyme which gives an absorbancy at 275 mμ equivalent to 1 μg of tyrosine in 1 minute at 35° (Hagihara *et al.*, 1958).

Esterase activity of bromelain was assayed by determining the rate of hydrolysis of BAEE using a Radiometer Model SBR2/SBU1/TTT1 Autotitrator with 0.1 N NaOH as titrant (Inagami and Murachi, 1963). The reaction solution was 10 ml in volume containing 0.005 M L-cysteine, 0.1 M KCl, 0.05 M BAEE, and approximately 5 mg of enzyme protein. The reaction was carried out at 25° and *p*H 6.0. An esterase unit is defined as the enzyme activity which hydrolyzes 1 μmole of BAEE per minute.

Determination of Protein Concentration.—The method of Lowry *et al.* (1951) was used with crystalline bovine serum albumin (Nutritional Biochemicals Corporation, Cleveland, Ohio) as standard to determine protein concentrations of various fractions obtained in the course of purification. With purified material the

* A part of this work was carried out at the Institute for Protein Research, Osaka University, Osaka, Japan. We wish to thank Professor Toshizo Isemura for his continued interest in this work. This work was supported in part by a grant from the Ministry of Education, Japan, and by a United States Public Health Service research grant (GM 08714) from the National Institute of General Medical Sciences.

† Present address: Medizinische Fakultät, Universität des Saarlandes, Homburg/Saar, Deutschland.

[1] We are indebted to Dr. Ralph M. Heinicke for generous supply of this material.

[2] The abbreviation used is: BAEE, benzoyl-L-arginine ethyl ester.

concentration was determined by measuring absorbancy at 280 mμ taking 19.0 as that of 1% solution at *p*H 5.2. This value was experimentally obtained by simultaneous determinations of dry weight and absorbancy of the lyophilized preparation of purified bromelain. Drying of the material for this purpose was carried out under reduced pressure at 80°, with P_2O_5 used as desiccant, for 24 hours or more.

Determination of Carbohydrates.—The carbohydrate content of the enzyme preparation was determined by orcinol-H_2SO_4 method of Winzler (1955). Heating was done at 80° for 15 minutes and the absorbancy at 540 mμ was read. Reagent-grade L-arabinose was used as standard, since a preliminary experiment with partially purified bromelain preparations indicated the presence of arabinose and xylose as well as mannose.[3]

Physical Measurements.—Sedimentation analyses were performed with a Hitachi Model UCA-1 ultracentrifuge equipped with a schlieren cylindrical lens system. The temperature was measured with a radiation thermocouple which provided a continuous indication of temperature of the base of rotor with an accuracy of ±0.10°. A Hitachi Model HTB electrophoresis apparatus was used for runs of free-boundary electrophoresis to study the purity of the preparation and also for diffusion experiments with a Neurath's type cell (Neurath, 1941). For determination of isoelectric point a Hitachi Model HTD-1 electrophoresis apparatus was used.[4] The measurement of *p*H was made with a Hitachi Model M-4 *p*H-meter. Conductivity of the solution was determined at 0° with Toa-Denpa Model CM-1D conduct-meter. A parallel stem pycnometer of approximately 3-ml capacity was employed for density measurements. Viscosity measurements were made with an Ostwald viscometer, with an outflow time of 120 seconds with 7 ml of water, in a water bath with the temperature controlled to within 0.005°. A Radiometer Model SBR2/SBU1/TTT1 Autotitrator was used for acid-base titrations in conjunction with a glass electrode, Type 202B and a calomel electrode, Type K401. Ultraviolet absorption spectrum was determined with a Cary Model 15 recording spectrophotometer. Rotations were measured with a Rudolph Model 200S photoelectric spectropolarimeter with mercury arc as a light source.

Experimental Procedure and Results

Purification

The purification procedure described below has been developed on the basis of earlier findings involving chromatography and ammonium sulfate fractionation of the crude bromelain preparation (Murachi and Neurath, 1960). All the operations were conducted at cold room temperature except for the initial extraction.

Extraction.—Commercial bromelain (10 g) was suspended in 100 ml of 0.05 M potassium phosphate buffer, *p*H 6.1, at room temperature for 30 minutes. The suspension was centrifuged at 5,000 rpm for 20 minutes and the precipitate was discarded.

Treatment with Anion-Exchange Resin.—The supernatant fluid (fraction 1) was applied to a 4.5 × 19–cm (300-ml) column with Duolite A-2 resin which had been equilibrated overnight with 0.05 M potassium phosphate buffer, *p*H 6.1. The initial 100 ml of the effluent was discarded and the following 400 ml effluent (fraction 2) was collected by a continuous flow of the same buffer. A part of the colored material was removed by this treatment.

Treatment with Cation-Exchange Resin.—Fraction 2 was then applied to a 4.5 × 31.5-cm (500-ml) column with Amberlite CG-50, Type I, which had been thoroughly equilibrated overnight with 0.2 M potassium phosphate buffer, *p*H 6.1. The column was washed with 200 ml of the same buffer and then with 5 liters of 0.05 M potassium phosphate buffer, *p*H 6.1. The effluent, except for the initial 600 ml (fraction 2a), was discarded. Fraction 2a may be saved for studying cellulase activity present in this fraction.[5] The enzyme which had been adsorbed on the cation exchanger was eluted by washing the column with 800 ml of 0.2 M potassium monohydrogen phosphate containing 1 M KCl. The initial 250 ml of the effluent was discarded and the following 250 ml eluate (fraction 3) was collected.

Gel Filtration.—To fraction 3 was added 100 g of ammonium sulfate. After 1 hour the precipitate formed was collected by centrifugation at 8,000 rpm for 20 minutes and dissolved in 50 ml of 0.05 M sodium acetate buffer, *p*H 5.2 (fraction 4). Fraction 4 was applied to a 4.5 × 31.5-cm (500-ml) column packed with Sephadex G-100 that had been thoroughly washed with 0.05 M acetate buffer, *p*H 5.2, and the column was continuously washed with the same buffer. The major part of the protein appeared in the 150-ml fraction (fraction 5) which emerged after the initial 320 ml passed through.

Ammonium Sulfate Fractionation.—To fraction 5 was added 38.5 g of ammonium sulfate to make 0.42 saturation. After 1 hour the moderately turbid mixture was centrifuged and to the supernatant fluid was added 8.8 g of ammonium sulfate to bring to 0.50 saturation. Bromelain thus precipitated was collected by centrifugation at 8,000 rpm for 20 minutes, dissolved in 30 ml of water, and dialyzed against four changes of 5 liters of water for 48 hours (fraction 6).

The whole procedure of purification up to fraction 6 can be completed within 5 days, including 2 days for final dialysis. The fractions obtained during the procedure were assayed for their proteinase and esterase activities and carbohydrate contents. A typical protocol of a purification experiment is shown in Table I. It is apparent from Table I that the procedure described gives a high yield of purified bromelain, i.e., 870 mg from 10 g of commercial product. Specific activities were increased only 1.4-fold toward casein and 1.1-fold toward BAEE as substrate, a finding consistent with the earlier observation of chromatographic purification and crystallization of bromelain (Murachi and Neurath, 1960).

As shown in Table I, most of the contaminant carbohydrates were removed by treatment with cation-exchange resin. Fraction 4, which was obtained from the eluate from the cation exchanger, still contained approximately 3% carbohydrates, although this material was found to be apparently homogeneous by ultracentrifugal and electrophoretic analyses. The carbohydrates could not be removed by either ammonium sulfate fractionation or acetone precipitation. It was found, however, that the use of gel filtration technique with Sephadex G-100 was most successful in removing a part of the carbohydrate present in fraction 4. Thus, in a small scale experiment, a 1-ml aliquot of fraction 4 was applied to a 1.12 × 20–cm (20-ml) column with Sephadex G-100 which had been washed

[3] We are indebted to Dr. Susumu Hirase for qualitative analysis on carbohydrates.

[4] We are indebted to Dr. Makoto Akutsu and Mr. Masaaki Tanizawa for their cooperation.

[5] We are indebted to Drs. Masaru Funatsu and Kiyochika Tokuyasu for assaying cellulase activities of crude bromelain and fraction 2a.

TABLE I
PURIFICATION OF BROMELAIN

Step	Fraction	Volume (ml)	Total Protein (g)	Carbohydrates (g/g protein)	Proteinase Activity: *PU*/g Protein (10^3)	Proteinase Activity: Total *PU* (10^3)	Esterase Activity: *EU*/g Protein	Esterase Activity: Total *EU*
Commercial product			(Total solid, 10)					
Extraction	1	101	5.12	0.657	1.15	5.90	0.224	1.15
Duolite A-2	2	400	4.04	0.758	1.23	4.98	0.226	0.913
Amberlite CG-50	2a	600	0.400	4.47	0.054	0.022	0.0174[a]	0.069[a]
(Elution)	3	250	2.72	0.0255	1.42	3.86	0.232	0.631
$(NH_4)_2SO_4$ pptn	4	50	1.75	0.0283	1.50	2.62	0.252	0.441
Gel filtration	5	150	1.18	0.0210	1.62	1.92	0.250	0.295
$(NH_4)_2SO_4$ fractionation	6	30	0.871	0.0205	1.64	1.44	0.252	0.219
Further treatment of fraction 6:								
$(NH_4)_2SO_4$ fractionation				0.0207	1.63		0.250	
Acetone precipitation				—	1.28		0.225	
Lyophilization				—	1.20		—	

[a] Assay for esterase activity was made with the material obtained by precipitation from fraction 2a with ammonium sulfate (0.8 saturation).

with 0.05 M sodium acetate buffer, *p*H 5.2. The column was washed with the same buffer and 1-ml fractions were collected. The absorbancy at 280 mμ of each fraction was read and the carbohydrate content was determined. The results obtained are shown in Figure 1. It is apparent from Figure 1 that the contaminant carbohydrates present in fraction 4 have a molecular size larger than that of bromelain. The use of Sephadex G-200 in place of Sephadex G-100 did not result in better separation and Sephadex G-75 gave a poorer separation, whereas no separation was achieved with either Sephadex G-50 or Sephadex G-25 under the conditions employed. Even after the gel filtration, the enzyme contained some 2% carbohydrates.

Reprecipitation with Salts.—From the aqueous solution of the purified bromelain (fraction 6) the enzyme could be reprecipitated by 0.42–0.50 saturation with ammonium sulfate. The reprecipitation neither increased specific activity of the enzyme nor decreased carbohydrate content (Table I). The fully active enzyme could also be precipitated by 80% saturation with NaCl.

Acetone Precipitation.—When an equal volume of acetone was added to fraction 6, a faint turbidity developed. The mixture was centrifuged at 8,000 rpm for 20 minutes, and the supernatant fluid was brought to −20°. A distinct turbidity immediately appeared and increased with time, yielding approximately 80% of the protein as a precipitate after 2 hours. The precipitate, which was not crystalline, was collected by centrifugation and enzymatic activities were assayed. The results are also shown in Table I. The acetone treatment caused a marked decrease in activity, although this method seemed to promise the most toward possible crystallization of the enzyme. Nevertheless, the acetone-reprecipitated material was found to be indistinguishable from fraction 6 by various physical methods employed.

Lyophilization.—Lyophilized bromelain showed a marked decrease in specific activity (Table I).

Homogeneity of Purified Bromelain.—Fraction 6 was subjected to various analyses for purity determina-

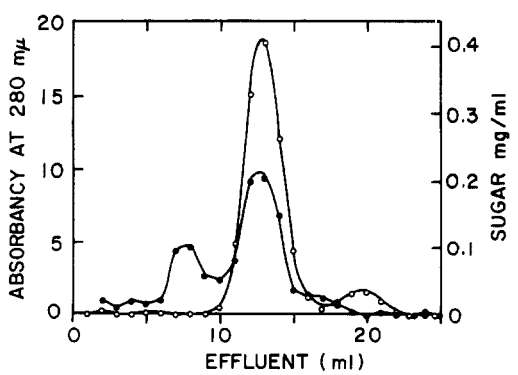

FIG. 1.—Gel filtration of partially purified bromelain (fraction 4) with Sephadex G-100. ○, absorbancy at 280 mμ; ●, carbohydrate content. One ml of fraction 4 corresponding to 35 mg of protein was filtered through a 1.12 × 20-cm column with 0.05 M acetate buffer, *p*H 5.2, at 4°. One-ml fractions were collected.

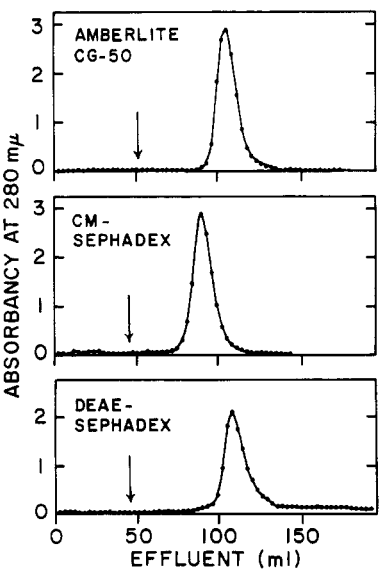

FIG. 2.—Chromatography of purified bromelain (fraction 6) with ion-exchange resins. Two ml of 1.3% bromelain solution was applied to a 1.12 × 20-cm column. Three-ml fractions were collected at 4°. A logarithmic concentration gradient was started from the fraction indicated by an arrow (↓) in the figure. Phosphate buffers were used for Amberlite CG-50 and CM-Sephadex, and Tris buffer for DEAE-Sephadex. For other experimental details, see text.

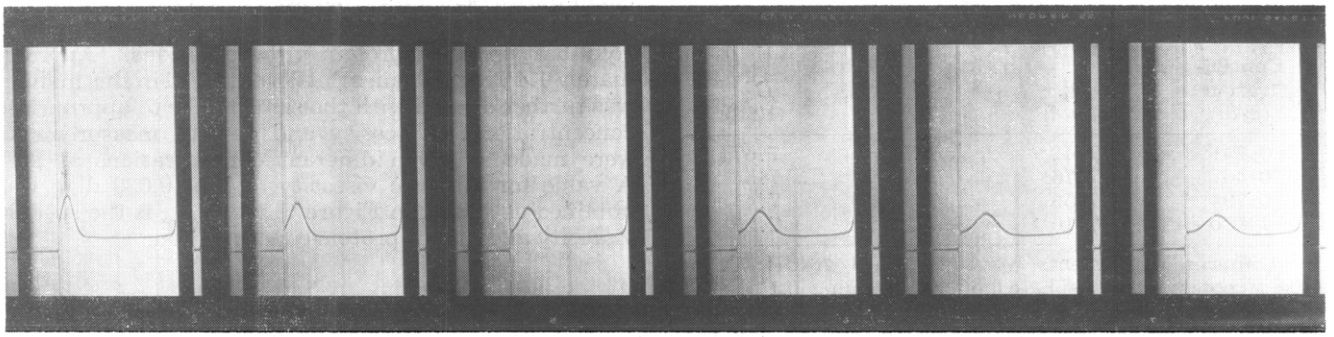

FIG. 3.—Ultracentrifugal pattern of purified bromelain (fraction 6) at a concentration of 1.19% at 60,000 rpm in 0.04 M phosphate buffer, pH 7.4, containing 0.1 M KCl at 20.2°. The pictures shown were taken at a phase plate angle of 75° at 16, 26, 36, 46, 56, and 66 minutes after the top speed was reached. The direction of the sedimentation is toward the right. The computed $s_{20,w}$ = 2.90 S.

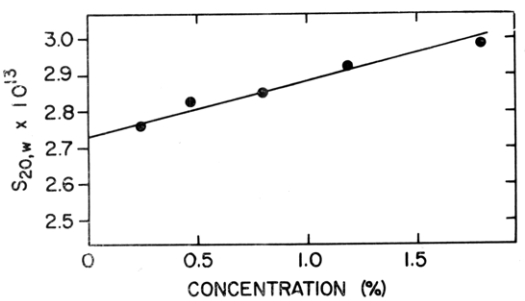

FIG. 4.—Sedimentation constants ($s_{20,w}$) of purified bromelain (fraction 6) as a function of protein concentration. The individual $s_{20,w}$ values were obtained from the runs in 0.04 M phosphate buffer, pH 7.4, containing 0.1 M KCl at room temperature. The extrapolated value, $s^0_{20,w}$ = 2.73 S.

tions such as sedimentation by ultracentrifuge, free-boundary electrophoresis, diffusion experiments, and chromatography. The results of the chromatographic experiments are described below, while those of other physical measurements will be described in the following section, under Physical Characteristics.

For chromatography of purified bromelain three different ion-exchange resins were used at different pH values. Amberlite CG-50, Type II, at pH 6.1; CM-Sephadex C-25 at pH 6.3; and DEAE-Sephadex A-25 at pH 9.6. In each of the experiments a 1.12 × 20-cm (20-ml) column was used, all at 4°. Amberlite CG-50 was equilibrated with 0.1 M potassium phosphate buffer, pH 6.1. Two ml of 1.3% bromelain solution (fraction 6, diluted), which had been dialyzed vs. 500 ml of the same buffer at 4° overnight, was applied to the column. A continuous flow of 0.1 M potassium phosphate buffer, pH 6.1, was begun and 17 fractions of 3 ml each were collected. A logarithmic concentration gradient was then started with 100 ml of 0.1 M potassium phosphate buffer, pH 6.1, in a mixing flask, and 200 ml of the same buffer containing 2 M KCl in a reservoir. Concentration of protein in the fractions was determined by measuring the absorbancy at 280 mμ. The results are shown in the top section of Figure 2. The middle section of Figure 2 represents a similar experiment with CM-Sephadex with 0.05 M potassium phosphate buffer, pH 6.3, with a gradient up to 2 M KCl, while the bottom section of the figure shows the results with DEAE-Sephadex with 0.05 M Tris buffer, pH 9.6, and a gradient up to 2 M KCl. Recoveries of the protein in chromatography were 95% for both Amberlite CG-50 and CM-Sephadex and 85% for DEAE-Sephadex. All these chromatograms indicate the presence of one single major component in fraction 6.

Physical Characteristics

All the physical measurements were made with fraction 6. Protein concentrations in these experiments were determined spectrophotometrically at 280 mμ.

Sedimentation Analyses.—All runs were made at room temperature. The rotor temperature was maintained at the initial value with an accuracy of ±0.5° by manually controlling the refrigerator. The data have been corrected to sedimentation values corresponding to water at 20°, $s_{20,w}$, in the usual manner.

For purity determination a run was made at 60,000 rpm with 1.19% bromelain solution in 0.04 M potassium phosphate buffer, pH 7.4, containing 0.1 M KCl. The sedimentation pattern obtained is shown in Figure 3. The preparation appeared to be monodisperse. In this case $s_{20,w}$ was calculated to be 2.90 S.

For determination of sedimentation constant at zero concentration, runs were made at 44,000 rpm with 0.241%, 0.467%, 0.794%, 1.18%, and 1.79% solution of bromelain in 0.04 M potassium phosphate buffer, pH 7.4, containing 0.1 M KCl. Six photographs were taken at 12-minute intervals. The observed dependence of $s_{20,w}$ on the concentration of bromelain is shown in Figure 4. The extrapolation has led to a value of 2.73 S at zero concentration. The value is very close to that obtained in a previous investigation (Murachi and Neurath, 1960), i.e., 2.73 S and 2.76 S for two fractions obtained by chromatography.

Diffusion Studies.—A 5-ml aliquot of bromelain solution (fraction 6) of appropriate concentration was dialyzed vs. two changes of 2 liters of 0.04 M potassium phosphate buffer, pH 7.4, containing 0.1 M KCl at 4° overnight. The dialyzed material was used for the diffusion run vs. the same buffer at 20°. The protein concentrations employed were 0.241%, 0.467%, 0.794%, and 1.18%. Eight photographs were taken at intervals between 5 and 160 minutes after the boundaries were established. Tracings of projected enlargements were made for the individual schlieren curves. Diffusion constants were calculated by three different methods (Neurath, 1942); $D_\mu = \mu^2/2t$ by the maximum ordinate method, where μ is the abscissa of inflection point and t is the time in seconds; $D_A = A^2/4\pi tH^2$ by the height-area method, where A is the area under the curve and H is the maximal height; $D_M = m^2/2t$ by the statistical or moment method, where m^2 is the second moment of the curve. The values calculated were corrected to the diffusion constants corresponding to water at 20°. The values obtained are compared in Table II. A satisfactory agreement of D_A value with D_M at each of the four different concentrations studied may indicate monodispersity of the material. The monodispersity was also evidenced by

TABLE II
DIFFUSION CONSTANT OF BROMELAIN[a]

Concentration (g/dl)	D_μ (10^{-7})	D_A (10^{-7})	D_M (10^{-7})
0.241	7.42	7.76	7.67
0.467	7.76	7.62	7.55
0.794	7.55	7.50	7.45
1.180	7.10	7.21	7.25

[a] Diffusion experiments were performed at *p*H 7.4 in 0.04 M potassium phosphate buffer containing 0.1 M KCl at 20°. D_μ was calculated by the maximum ordinate method, D_A by the height-area method, and D_M by the moment method. All constants were corrected to $D_{20,w}$ in $cm^2\ sec^{-1}$.

the fact that the individual diffusion curves appeared to closely represent Gaussian distribution.

In order to determine diffusion constant at zero concentration, $D^0_{20,w}$, D_M values in Table II were plotted against the concentration of bromelain as shown in Figure 5. By extrapolation a value of $7.77 \times 10^{-7}\ cm^2\ sec^{-1}$ was obtained.

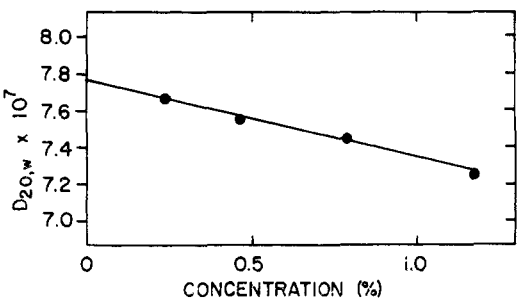

FIG. 5.—Diffusion constants ($D_{20,w}$) of purified bromelain (fraction 6). The individual runs were made in 0.04 M phosphate buffer, *p*H 7.4, containing 0.1 M KCl at 20°. The diffusion constants computed by the moment method (see text) are shown in the figure as a function of protein concentration. The extrapolated value, $D^0_{20,w} = 7.77 \times 10^{-7}\ cm^2\ sec^{-1}$.

Partial Specific Volume.—The apparent partial specific volume, $\bar{v}$, was calculated from measurements of density of bromelain solutions in 0.04 M potassium phosphate buffer, *p*H 7.4, containing 0.1 M KCl at three different concentrations and at 20°. The results obtained are shown in Table III with an average value of 0.743 ml/g.

TABLE III
PARTIAL SPECIFIC VOLUME OF BROMELAIN[a]

Concentration (g/dl)	Density (g/ml)	$\bar{v}$ (ml/g)
0	1.00845	—
0.290	1.00918	0.742
0.579	1.00990	0.743
0.842	1.01055	0.744
		Av, 0.743

[a] The measurements were made at *p*H 7.4 in 0.04 M potassium phosphate buffer containing 0.1 M KCl at 20°.

Viscosity.—The buffer used for viscosity measurements contained 0.02 M sodium acetate, 0.005 M acetic acid, and 0.08 M KCl, the ionic strength being 0.1. The *p*H was 5.2. The sample of bromelain (fraction 6, 25 ml) was passed through a 3.2 × 30-cm (300-ml) column with Sephadex G-50, which had been thoroughly washed with the same buffer, in order to attain quick equilibration of the protein with buffer ions. Approximately 1.4% bromelain solution obtained in this manner was further diluted with the same buffer to appropriate concentrations. Viscosity and density measurements were made at seven different concentrations at 20°. A value for intrinsic viscosity, $[\eta]$, of 0.039 dl/g was obtained as shown in Figure 6, where η_{sp} is the specific viscosity and *c* is the protein concentration.

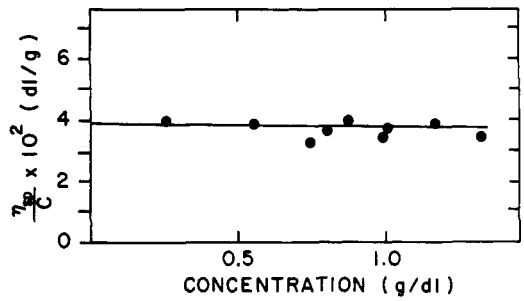

FIG. 6.—Plot of reduced viscosity (η_{sp}/c) of purified bromelain (fraction 6) vs. protein concentration (c). Viscosity and density measurements were made in acetate buffer, *p*H 5.2, 0.1 ionic strength with KCl making up 80% of the ionic strength, at 20°. The intrinsic viscosity obtained $[\eta] = 0.039$ dl/g.

Molecular Weight.—The molecular weight, *M*, of bromelain as determined from sedimentation and diffusion measurements, was computed from the formula $M = RTs/D\ (1 - \bar{v}\rho)$, where *T* is the absolute temperature, *R* is the gas constant, and ρ is the density of water. As described above, $s = 2.73 \times 10^{-13}$, $D = 7.77 \times 10^{-7}$, and $\bar{v} = 0.743$, hence $M = 33{,}200$.

The molecular weight was also computed from the formula (Scheraga and Mandelkern, 1953),

$$M = \left\{\frac{Ns[\eta]^{1/3}\eta_0}{\beta(1 - \bar{v}\rho)}\right\}^{3/2} \quad (1)$$

where *N* is the Avogadro number, η_0 is the coefficient of viscosity of the pure solvent, and β is a constant which is assumed to be 2.16×10^6 (Schachman, 1957). With $s = 2.73 \times 10^{-13}$ and $[\eta] = 0.039$, the molecular weight is 32,100, which is in fairly good agreement with the *M* value determined from sedimentation-diffusion measurements.

The third method employed for determination of the molecular weight of bromelain was the Archibald procedure of sedimentation in the ultracentrifuge (Archibald, 1947; Klainer and Kegeles, 1956). A 5-ml sample of approximately 1% protein concentration was dialyzed vs. 2 liters of 0.04 M potassium phosphate buffer, *p*H 7.4, containing 0.1 M KCl at 4° for 18 hours. The dialyzed solution was subjected to an ultracentrifuge run at 21,300 rpm at room temperature. Six photographs were taken at 10-minute intervals. Tracings of projected enlargements of the individual patterns were used for the determination of molecular weight according to the method by Klainer and Kegeles (1956). Only the pattern at the meniscus was used. The protein concentration of the sample which should be known for the foregoing computation was determined by a separate run of the ultracentrifuge with a synthetic-boundary cell, under otherwise identical conditions. The concentration was determined as being represented by the area under the schlieren curve in the projected enlargement (Klainer and Kegeles, 1956). The molecular weights computed for the individual patterns at different time intervals were found to decrease slightly

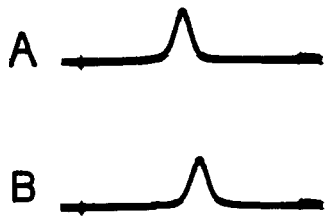

FIG. 7.—Electrophoretic patterns of purified bromelain (fraction 6) at a concentration of 0.788% in 0.04 M phosphate buffer, *p*H 7.4, containing 0.1 M KCl at 1.0°. A, ascending pattern after 201 minutes of migration at 4 ma. The direction of migration is toward the right. B, descending pattern after 203 minutes at 4 ma. The direction is toward the left. The electrophoretic mobility computed for the descending peak, $u = +2.58 \times 10^{-5}$ cm^2 volt^{-1} sec^{-1}.

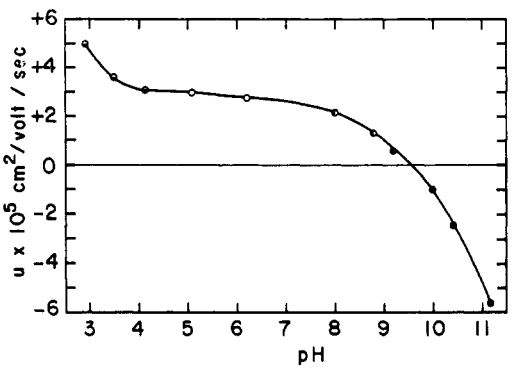

FIG. 8.—Electrophoretic mobility (u) of purified bromelain (fraction 6) as a function of *p*H. ◒, glycine-HCl; ○, acetate; ◐, Veronal-HCl; and ●, glycine-NaOH. The individual u values were obtained from the runs in univalent buffers of 0.1 ionic strength, at 5 ± 0.5°. The isoelectric point found, $pI = 9.55$.

with time. An extrapolation to time zero has led to $M = 33{,}500$ with $\bar{v} = 0.743$.

From values of s, D, and $\bar{v}$, the frictional ratio, f/f_0, was computed to be 1.26.

Electrophoretic Analyses.—The material used for free-boundary electrophoresis was dialyzed vs. respective buffer solutions at 4° for at least 18 hours before the runs. The *p*H of the buffer was determined at 25°.

For purity determination a 0.788% solution of bromelain in 0.04 M potassium phosphate buffer, *p*H 7.4, containing 0.1 M KCl was used. The run was made at 1.0° at 4 ma for 4 hours. In Figure 7 are shown electrophoretic patterns obtained after 201 and 203 minutes of migration, with a calculated mobility for descending peak at $+2.58 \times 10^{-5}$ cm^2 · volt^{-1} · sec^{-1}. The patterns shown in Figure 7 indicate apparent homogeneity of the material at this *p*H. Although in the descending pattern a minor peak can be seen which moves faster toward the cathode, this represents less than 1% of the total protein. Apparent monodispersity was also demonstrated in similar runs at *p*H 4.18 in 0.02 M sodium acetate buffer containing 0.08 M KCl and at *p*H 8.10 in 0.04 M Tris buffer containing 0.1 M KCl.

For determination of the isoelectric point, runs were made with approximately 1% solution of bromelain over the range *p*H 2.90–11.80. All the buffers used were univalent with an ionic strength of 0.1, containing 0.08 M KCl in every case. Glycine-HCl buffer was used for a *p*H range between 2.9 and 3.5, sodium acetate–acetic acid for *p*H 4.1–6.1, sodium Veronal–HCl for *p*H 7.9–8.9, and glycine–NaOH for *p*H 9.4–11.8. The temperature was maintained at 5 ± 0.5°. Photographs were taken at 10–15-minute intervals and the

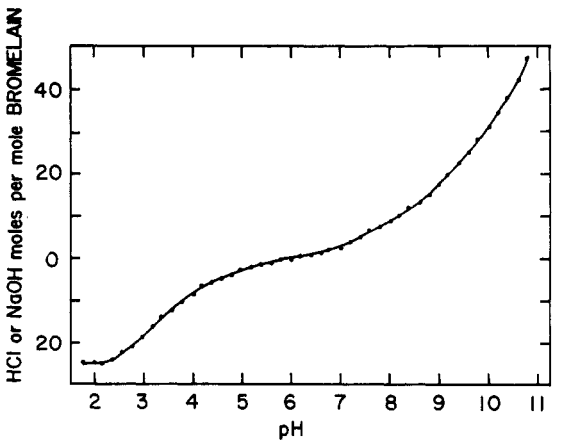

FIG. 9.—Titration curve for purified bromelain (fraction 6) at 20°. Ten ml of 1.81 mM bromelain in 0.1 M KCl was titrated with 1.00 N HCl or 1.00 N NaOH. The data were collected for titration of 0.1 M KCl with standard acid or alkali.

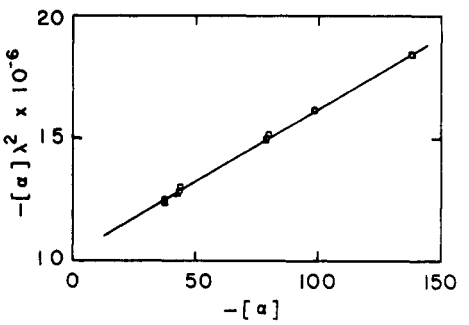

FIG. 10.—Yang's plot for purified bromelain (fraction 6) in 0.04 M phosphate buffer, *p*H 7.4, containing 0.1 M KCl at 20°. For symbols used, see equation (2) in text. The concentrations of bromelain were: ○, 2.37%; △, 1.42%; and □, 0.71%. The optical rotatory dispersion constant obtained, $\lambda_c = 241$ mμ.

run was continued for 70–130 minutes. From the tracings of projected enlargements of the individual schlieren curves the mobility, u, was determined using the descending pattern only. The computed mobilities are shown in Figure 8 as a plot of u vs. *p*H in order to determine the isoelectric point, *pI*. A value of *p*H 9.55 was thus obtained, which is considerably higher than the *pI* value of 8.75 for crystalline papain (Smith *et al.*, 1954) but lower than the *pI* 10.0 for chymopapain (Ebata and Yasunobu, 1962).

Titration Studies.—Ten ml of approximately 0.6% solution of bromelain in 0.1 M KCl was used for *p*H titrations. Standard 1.00 N HCl or 1.00 N NaOH used as titrant was delivered from a syringe buret with vigorous stirring under a stream of CO_2-free nitrogen. All the runs were made at 20°. The data were corrected for titration of 0.1 M KCl with standard acid or alkali under identical conditions. Figure 9 shows the results in terms of moles acid or alkali per mole of bromelain as a function of *p*H. The molar concentration of bromelain was computed from the absorbancy at 280 mμ with $\epsilon = 6.33 \times 10^4$ (see below). It seems from Figure 9 that approximately 25 acidic groups per mole are present which can be dissociated over the range *p*H 1.6–6.0.

Ultraviolet Absorption Spectrum.—The spectrum was recorded with a 0.51% solution of bromelain in 0.05 M sodium acetate buffer at *p*H 5.2 and at 20°. At 280 mμ the absorbancy was found to be 0.984, which corresponds to a molar extinction coefficient, ϵ, of 6.33×10^4 by taking a molecular weight of 33,200

(see above). The absorption maximum was found to be at 2788 *A* and the minimum at 2503 *A*. The curve represents a typical absorption pattern of a protein solution, the ratio of the absorbancy at 260 mμ to that at 280 mμ being 0.524. A small rise at around 290 mμ was noted which is possibly due to contribution from tryptophan residues.

Optical Rotation Studies.—The optical rotatory dispersion was measured between 400 and 600 mμ with bromelain solutions of 0.71%, 1.42%, and 2.37% in 0.04 M potassium phosphate buffer, *p*H 7.4, containing 0.1 M KCl, at 20°. A 1-dm polarimeter tube was used. The data obtained were plotted according to the Drude's equation,

$$[\alpha] = \frac{K}{\lambda^2 - \lambda_c^2} \qquad (2)$$

to compute λ_c, the optical rotatory dispersion constant. In equation (2) $[\alpha]$ is the specific rotation at wavelength λ and K is the constant. Plots were also made according to the Moffitt-Yang equation (Moffitt and Yang, 1956),

$$[m'] = \frac{3}{n^2 + 2}\frac{M_0}{100}[\alpha] = \frac{a_0\lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \qquad (3)$$

to compute a_0 and b_0, constants relating to the conformation of the protein molecule studied. In equation (3) $[m']$ is the mean residue rotation, n is the refractive index of the solvent, M_0 is the mean residue molecular weight, and λ_0 is a constant.

As shown in Figure 10, dispersion data obtained with bromelain conform well to equation (2). A λ_c value of 241 mμ was thus obtained. By averaging data at three different concentrations of the sample the following constants were also obtained: $[\alpha]_{436} = -79.1°$ and $[\alpha]_{546} = -43.1°$. For equation (3), $[m']$ was calculated from $n = 1.33$ for the solvent used and with M_0 being assumed to be 110. The λ_0 is assumed to be 212 mμ (Urnes and Doty, 1961). From a Moffitt-Yang plot a_0 was computed to be −190° and b_0 to be −78°. These characteristics of bromelain are listed in Table IV in comparison with some of the available data in literature for several different proteolytic enzymes (Jirgensons, 1959; Imahori *et al.*, 1960).

TABLE IV

CHARACTERISTICS OF OPTICAL ROTATORY DISPERSION OF SEVERAL PROTEOLYTIC ENZYMES[a]

Enzymes	λ_c (mμ)	$-[\alpha]_{546}$ (deg)	$-a_0$ (deg)	$-b_0$ (deg)
Bromelain	241	43.1	190	78
Papain	231	77.0		
Ficin	261	48.7		
Trypsin	235	48.0		
Pepsin	227	75.0		
α-Chymotrypsin	241	76.6		
π-Chymotrypsin		74.3[b]	403	138

[a] The data for bromelain were obtained in the present study. Others are from Jirgensons (1959) except for π-chymotrypsin (Imahori *et al.*, 1960). For the definitions of constants, see text. [b] $-[\alpha]_D$ value.

By assuming $b_0 = -600°$ for a completely helical molecule, one may compute from the observed b_0 value of −78° an excess right-handed helical content of bromelain to be (78/600) × 100, i.e., 13%.

DISCUSSION

The purification procedure described above is an improvement over the earlier method of Murachi and Neurath (1960) in that effective removal of the colored material and of the contaminant carbohydrates was achieved by the use of Duolite A-2 resin and Sephadex G-100, respectively, and that an elution of the enzyme from the cation-exchange resin was performed by a single, stepwise increase in ionic strength of the buffer instead of a gradient elution. A stepwise elution offers a striking simplicity of the procedure which was aimed at in developing a method suitable for a large-scale preparation. In contrast to this, however, the present method seems to represent no practical improvement over the earlier method in obtaining an enzyme with higher specific activity. Thus, with the present method an increase in specific proteinase activity of 1.43 was achieved, whereas fraction 5 of the previous method (Murachi and Neurath, 1960) showed a specific activity 1.48 times as high as that of the crude material. In view of the differences in methods of determining proteinase activity and protein concentration between the present and the earlier experiments, it is reasonable to state that both methods achieved an increase in specific activity to almost the same extent. Amberlite CG-50 was chosen in place of Duolite CS101 used in the earlier experiment because of the availability of the resin in this country.

The small increase in specific activity during purification (Table I) is explicable in view of the earlier findings (Murachi and Neurath, 1960) that all the components obtained from the crude stem bromelain by ion-exchange chromatography were enzymatically active and that two major components, amounting together to more than 60% of the total protein of the crude material, had comparable specific activities. The major achievement of the present method is therefore the effective removal of nonprotein constituents from the enzyme protein rather than a preferential loss of inert protein.

Although the bromelain preparation obtained by the purification procedure described above was not crystalline, fraction 6 was found to be essentially homogeneous by various physical criteria. However, the material still contained approximately 2% carbohydrates which could not be removed by various methods including ion-exchange chromatography and gel filtration. Whether bromelain is a true glycoprotein like ovalbumin remains to be determined. Papain (Smith and Kimmel, 1960), chymopapain (Ebata and Yasunobu, 1962), and ficin (Bernhard and Gutfreund, 1956) contain no carbohydrate, while among proteolytic enzymes of animal origin plasmin (Shulman *et al.*, 1958;) and enterokinase (Yamashina, 1958) have been reported to contain 1.5% hexose and 41.1% carbohydrate, respectively.

The sedimentation constants of bromelain computed from the results of runs at *p*H 7.4 were shown to increase with protein concentration. Also in a previous study with either one of the two fractions of bromelain a positive dependence of $s_{20,w}$ values on concentration had been noted, although numerical data were not included in the paper published (Murachi and Neurath, 1960). A much more marked increase in sedimentation values with protein concentration was reported to occur with crystalline papain at *p*H 4 in the absence of cysteine or at *p*H 5.4 in the presence of cysteine (Smith *et al.*, 1954; Smith and Kimmel, 1960). Smith *et al.* (1954) explained such behavior as increased tendency of papain monomer to aggregate as the protein concentration increases. One might be able to apply the same explanation to the observed dependence of *s* values of bromelain on protein concentration. Similar reasoning could be also applicable to a negative dependence of diffusion constants on concentration (Fig.

TABLE V

COMPARISON OF PHYSICAL PROPERTIES OF PLANT SULFHYDRYL PROTEINASES

Physical Property[a]	Bromelain	Papain[a]	Chymo-papain[b]	Ficin[c]
Sedimentation constant, s (in S)	2.73[d]	2.42[e]	2.71[f]	2.56[g] 2.61[h]
Diffusion constant, D (in 10^{-7} cm^2 sec^{-1})	7.77[i]	10.23[j]		
Partial specific volume, $\bar{v}$ (in ml/g)	0.743	0.724		
Intrinsic viscosity, $[\eta]$ (in dl/g)	0.039			
Frictional ratio, f/f_0	1.26	1.16		
Isoelectric point, pI	9.55	8.75	10.0	9.0[k]
Absorbancy, $A_{1\text{ cm}}^{1\%}$ at 280 mμ	19.0	25[l]	18.7	
Molecular weight	33,200[m]	21,000[m]	27,000[r]	26,000[s]
	32,100[n]	20,700[n]		
	33,500[p]	20,900[q]		

[a] Smith and Kimmel (1960). [b] Ebata and Yasunobu (1962). [c] Bernhard and Gutfreund (1956). [d] $s^0_{20,w}$. [e] $s_{20,w}$ at pH 3.9–4.0, independent of protein concentration. [f] $s_{20,w}$ at 1.26% in 0.01 M phosphate buffer at pH 7.2. [g] s_{20} at 1% in 0.1 M phosphate buffer at pH 6.7. [h] $s_{20,w}$ at 1% in 0.1 M phosphate buffer at pH 6.9 (Liener, 1961). [i] $D^0_{20,w}$. [j] $D_{20,w}$ at pH 3.9, independent of protein concentration. [k] Cohen (1958). [l] At 278 mμ (Glazer and Smith, 1961). [m] By sedimentation-diffusion. [n] From sedimentation constant and intrinsic viscosity. [p] By Archibald method. [q] From amino acid composition. [r] A molecular weight of 35,500 for chymopapain was obtained by a more recent experiment (Ebata, M., personal communication). [s] By osmotic pressure. [t] Optical rotatory properties are shown in Table IV.

5). However, since the sedimentation patterns of bromelain at concentrations as high as 1.2% (Fig. 3) and 1.8% were obviously symmetric, and since no indication of apparent polydispersity was obtained by diffusion experiment even at 1.2%, the validity of the above cited explanation must await further studies. Nevertheless, it has been possible to calculate a molecular weight of bromelain from the present results by using s and D values as extrapolated to zero concentration. In view of the magnitude of errors involved, it may be safe to state at present that bromelain has a molecular weight of approximately 33,000.

Table V summarizes the results of physical measurements conducted with fraction 6 in comparison with the data for three other sulfhydryl proteinases of plant origin. It is interesting to note that all four enzymes listed in Table V are basic proteins of comparable molecular size. These apparent similarities in physical properties are contrasted to rather different specificities and mechanisms of action among these proteinases (Murachi and Neurath, 1960; Inagami and Murachi, 1963).

After the completion of the present study El-Gharbawi and Whitaker (1963) have reported that crude stem bromelain was fractionated into five proteolytically active components by ion-exchange chromatography with Bio-Rex 70 at pH 6.10. Since their report contains only limited information on the physical properties of these components, a comparison of any one of the five components to the purified bromelain obtained by our procedure seems practically impossible.

ACKNOWLEDGMENTS

We wish to express our sincere gratitude to Dr. Kozo Hamaguchi for many helpful discussions, to Dr. Kinji Kakiuchi for the ultracentrifugal examinations, and to Dr. Toshio Takagi for the diffusion and titration experiments.

REFERENCES

Archibald, W. J. (1947), *J. Phys. & Colloid Chem. 51*, 1204.
Balls, A. K., Thompson, R. R., and Kies, M. W. (1941), *Ind. Eng. Chem. 33*, 950.
Bernhard, S. A., and Gutfreund, H. (1956), *Biochem. J. 63*, 61.
Cohen, W. (1958), *Nature 182*, 659.
Ebata, M., and Yasunobu, K. T. (1962), *J. Biol. Chem. 237*, 1086.
El-Gharbawi, M., and Whitaker, J. R. (1963), *Biochemistry 2*, 476.
Glazer, A. N., and Smith, E. L. (1961), *J. Biol. Chem. 236*, 2948.
Hagihara, B., Matsubara, H., and Nakai, M., and Okunuki, K. (1958), *J. Biochem. (Tokyo) 45*, 185.
Imahori, K., Yoshida, A., and Hashizume, H. (1960), *Biochim. Biophys. Acta 45*, 380.
Inagami, T., and Murachi, T. (1963), *Biochemistry 2*, 1439.
Jirgensons, B. (1959), *Arch. Biochem. Biophys. 85*, 532.
Klainer, S. M., and Kegeles, G. (1956), *Arch. Biochem. Biophys. 63*, 247.
Liener, I. E. (1961), *Biochim. Biophys. Acta 53*, 332.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Moffitt, W., and Yang, J. T. (1956), *Proc. Nat. Acad. Sci. U. S. 42*, 596.
Murachi, T., and Neurath, H. (1960), *J. Biol. Chem. 235*, 99.
Neurath, H. (1941), *Science 93*, 431.
Neurath, H. (1942), *Chem. Rev. 30*, 357.
Ota, S., Fu, T., and Hirohata, R. (1961), *J. Biochem. (Tokyo) 49*, 532.
Schachman, H. K. (1957), *in* Methods in Enzymology, Vol. IV, Colowick, S. P., and Kaplan, N. O., eds., New York, Academic, p. 32.
Scheraga, H. A., and Mandelkern, L. (1953), *J. Am. Chem. Soc. 75*, 179.
Shulman, S., Alkjaersig, N., and Sherry, S. (1958), *J. Biol. Chem. 233*, 91.
Smith, E. L., and Kimmel, J. R. (1960), *Enzymes 4*, 133.
Smith, E. L., Kimmel, J. R., and Brown, D. M. (1954), *J. Biol. Chem. 207*, 533.
Urnes, P., and Doty, P. (1961), *Advan. Protein Chem. 16*, 401.
Yamashina, I. (1958), *Koso Kagaku Shimpoziumu 13*, 120; *Chem. Abstr. 53*, 11464.
Winzler, R. J. (1955), *Methods Biochem. Analy. 2*, 279.