Preparation and Purity of Chymotrypsinogen B*

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The merits of various available methods for the purification of chymotrypsinogen B have been investigated. A procedure involving gradient elution from carboxymethyl-cellulose columns at *p*H 4.37 has been developed and shown to yield a product of high potential and low free activity as judged by the hydrolysis of *N*-acetyl-L-tyrosine ethyl ester and the stoichiometric reaction with *N-trans*-cinnamoylimidazole. The homogeneity of the preparations has been demonstrated by a number of physical methods and a low level of neochymotrypsinogen contamination shown by carboxyl-terminal analyses.

Relative to the extensive studies on chymotrypsinogen α in many laboratories over the years, the properties of chymotrypsinogen B have received little attention. Undoubtedly this lack of interest has arisen in part from the lack of satisfactory methods for the isolation and purification of the zymogen in good yield. Furthermore, it was not appreciated until recently that the B zymogen comprised 16% of the proteins of the pancreatic juice of cattle (Keller *et al.*, 1958). The procedures available for the preparation of the zymogen when this work was begun may be summarized as follows: first, the isolation of chymotrypsinogen B by ammonium sulfate fractionation and crystallization according to the method of Kassell and Laskowski (1961); second, the separation of freeze-dried pancreatic extract on DEAE-cellulose,[1] *p*H 8.0, (Keller *et al.*, 1958); third, the elution of chymotrypsinogen B from CM-cellulose columns by a stepwise increase of the *p*H of 0.05 M sodium citrate buffer from 4.2 to 4.6 (Rovery *et. al.*, 1960).

The original procedures of Laskowski (1955) for the isolation of chymotrypsinogen B gave preparations contaminated either by active enzyme or by neochymotrypsinogens (Laskowski and Kassell, 1960). Improvements in the preparations were obtained by carrying out the procedures in the presence of diisopropylfluorophosphate (Kassell and Laskowski, 1961) but only a single crystallization was attempted. The presence of neochymotrypsinogens was indicated by the presence of 0.05–0.07 mole of carboxyl-terminal tyrosine per mole of chymotrypsinogen B. In our hands this method has proved unsatisfactory and for this reason our major efforts have been directed toward chromatographic procedures at low *p*H on columns of CM-cellulose.

EXPERIMENTAL

Assay of Chymotrypsin.—The progress of the purification of chymotrypsinogen B was followed by assaying the activated zymogen against 2.5 ml of 0.01 M ATEE in 0.01 M Tris-HCl buffer, *p*H 8.0, containing 0.02 M $CaCl_2$ and 0.1 M KCl, in the *p*H-Stat at 25° (Radiometer Titrator TTT-1a and Ole Dich recorder). From the initial slope of the hydrolysis, activities have been calculated in terms of the apparent specific zero-order reaction constant (k' = meq substrate hydrolyzed per ml per minute per mg of enzyme nitrogen per ml). Calcium has been included in the assay system because of its activation of both chymotrypsin α and B (Green *et al.*, 1952; Wu *et al.*, 1956). Concentrations of enzyme were estimated from the absorbancy at 280 mμ and the $E^{1\%}_{1cm}$ = 18.7 (Enenkel, *et al.*, in preparation). For activation, a 1% solution of chymotrypsinogen B in 0.1 M Tris-HCl buffer, *p*H 8.0, was incubated with trypsin (Worthington Biochemical Corporation, lot TR 836-38; ratio of trypsin to zymogen 1:40) at 0° for 10 minutes. Under these conditions maximum activity was attained in less than 10 minutes as evaluated by the zero-order rate constant (k') of hydrolysis of ATEE. Preparations with high potential activities were also reacted with *N-trans*-cinnamoylimidazole[2] after activation, according to Method A of Schonbaum *et al.* (1961). The reaction was followed in a Beckman DK-2 recording spectrophotometer at a chart speed of 1 inch per minute. A suitable sample (usually 0.1 ml) of a 1–4% chymotrypsin B solution was introduced into a 1-cm silica cuvet previously filled with 3 ml of 0.1 M sodium acetate buffer, *p*H 5.0, and 0.01 ml of a suitable stock solution of *N-trans*-cinnamoylimidazole in acetonitrile. From the drop in absorbancy at 335 mμ due to acylation, the normality of chymotrypsin B could be calculated. The molar extinction coefficient of *N-trans*-cinnamoylimidazole was taken as 9.37×10^3 (Schonbaum *et al.*, 1961) and the $\Delta\epsilon_{335}$ for cinnamoyl-chymotrypsin B versus chymotrypsin B as 0.42×10^3. The latter value was measured independently for the chymotrypsin B system and found to be the same ($\pm$ 5%) as for the α enzyme. Occasionally some opalescence appeared upon the addition of the enzyme to the acylating reagent but this did not occur with the better preparations of chymotrypsin B (k' versus ATEE > 3.0). In all other respects, the behavior of chymotrypsin B was identical to that of chymotrypsin α under these conditions. Assuming a molecular weight of 25,000 and with an independent measurement of concentration of the stock solution of enzyme from the absorbancy at 280 mμ, the purity of the chymotrypsin B could be calculated.

Ammonium Sulfate Fractionation.—Two preparations of extracted and freeze-dried pancreatic proteins were employed for the separation on the substituted cellulose columns; protein powder 1 (PP 1) was pre-

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[1] The following abbreviations are used: DEAE-cellulose, diethylaminoethyl-cellulose; CM-cellulose, carboxymethyl-cellulose; ATEE, *N*-acetyl-L-tyrosine ethyl ester; Tris, tris(hydroxymethyl)aminomethane; PP, protein powder (with digit to designate which type, e.g., PP 1, PP 2).

[2] *N-trans*-cinnamoylimidazole was kindly supplied by Dr. G. R. Schonbaum.

TABLE I
SUMMARY OF YIELDS AND ACTIVITIES FOR CHROMATOGRAPHY OF CHYMOTRYPSINOGEN B

Method	Yield of Protein (%)	Yield of Activity (%)	Potential Activity (k')	Free Activity (k')
DEAE-cellulose[a]	25	75	2.4	0.05
CM-cellulose				
stepwise elution[a]	20	56	2.55	0.003
gradient elution[a]	15	58	3.1	0.0009
gradient elution[b]	36	77	3.2	0.0009

[a] Starting material PP 1. [b] Starting material PP 3.

pared according to Rovery *et al.* (1960).[3] In the method of preparation finally adopted by this laboratory, an additional series of fractionations with ammonium sulfate was introduced. The protein, precipitated between 0.2 and 0.4 saturation, was dissolved in a minimum amount of 0.005 M HCl, dialyzed exhaustively against the dilute acid, and centrifuged at 9000 rpm for 60 minutes in a Servall high-speed centrifuge to remove insoluble material. The protein concentration of the supernatant was adjusted to 2% (using $E^{1\%}_{1cm}$ = 18.7 at 280 mμ) and the *p*H to 3.0. A saturated solution of ammonium sulfate, brought to *p*H 3.0 by the addition of concd. HCl, was then added slowly to obtain 0.25 saturation (25 ml for every 75 ml of enzyme solution). The resulting precipitate was collected by centrifugation (30 minutes at 2500 rpm) and discarded. The supernatant was brought to 0.30 saturation (7 ml of saturated ammonium sulfate solution for every 100 ml of enzyme solution) and centrifuged. The precipitate was dissolved in a small volume of 0.005 M HCl, dialyzed, and freeze-dried (protein powder 2 [PP 2]). To every 100 ml of the supernatant 17 ml of saturated ammonium sulfate solution was added to attain 0.40 saturation. The suspension was centrifuged and the precipitate was processed as before to yield protein powder 3 (PP 3). Since PP 2 had considerable potential chymotryptic activity (k' = 0.95), it was saved and fractionated as before whenever enough material had accumulated.

Chromatography on DEAE-Cellulose.—DEAE-cellulose (0.9 meq/g) was purchased from Schleicher and Schuell Co. and purified according to Peterson and Sober (1956). It was equilibrated with 0.005 M sodium phosphate buffer, *p*H 8.0, and packed into a 1-cm × 60-cm column for analytical runs and into a 5-cm × 60-cm column for preparative experiments. One hundred mg of PP 1 and 2.5 mg of soybean trypsin inhibitor were applied to the analytical column in 0.005 M sodium phosphate buffer, *p*H 8.0, containing 0.001 M diisopropylfluorophosphate, and eluted as previously described by Keller *et al.* (1958). Several fractions along the eluted peak containing chymotrypsinogen B were collected, brought to *p*H 2.5 by the addition of 5 N HCl, dialyzed, and freeze-dried. The free and potential chymotryptic activities of each fraction were determined.

Chromatography on CM-Cellulose.—Several batches of CM-cellulose of varying capacity (0.9, 0.67, 0.59, 0.50 meq/g) were purchased from Bio-Rad Laboratories Inc. and purified according to Peterson and Sober (1956). All chromatographic runs using stepwise elution were performed in a manner similar to that described by Rovery *et al.* (1960). Small changes had to be introduced pertaining to the molarities of the eluting buffers as will become evident under Results.

For the purification of chymotrypsinogen B by gradient elution, CM-cellulose (0.7 meq/g) was purified as above and equilibrated with 0.03 M sodium citrate buffer, *p*H 4.47, in the cold room. It was packed into a 3-cm × 50-cm (for analytical purposes) or 6.5-cm × 50 cm (preparative) column. Protein powder 1 and PP 3 were used as the starting material. For analytical runs, the linear gradient consisted of 1 liter of 0.03 M citrate buffer and 1 liter of 0.15 M citrate buffer, *p*H 4.47. A 2% solution of PP 1 or PP 3 in 0.03 M citrate buffer, containing 0.005 M diisopropylfluorophosphate, was applied to the column and washed in with a few ml of the same buffer. Four hundred mg was charged onto the small column and 2 g onto the preparative one. Gradient elution was begun immediately and the flow rate was adjusted to 0.3 ml/minute per cm² of column surface. Eighteen-ml fractions were collected in an automatic fraction collector and the absorbancy of each tube was read at 280 mμ. Various fractions were tested for free and potential chymotryptic and tryptic activity in the *p*H-stat. To determine the potential activities, the *p*H of 1-ml aliquots was adjusted to 8.0 by the addition of 1 M Tris, and sufficient of a 0.5% solution of trypsin was added to give a ratio of protein to trypsin of approximately 40:1. After incubation overnight at 0° the esterolytic activity was estimated in the *p*H-stat. Control experiments had shown that essentially full activation was achieved under these conditions, even with the more dilute fractions. Deoxyribonucleolytic activity was determined by the method of Kurnick (1950) after dialysis of various fractions against 0.1 M sodium acetate buffer, *p*H 5.0. For standardization purposes, deoxyribonucleic acid–methyl green and deoxyribonuclease I were purchased from Worthington Biochemical Corporation. In order to investigate the effect of *p*H on the elution patterns, chromatographic runs were also performed at *p*H 4.37 and 4.27 in a manner identical to that outlined above.

Carboxypeptidase-A was purchased from Worthington Biochemical Corporation and was treated with diiospropylfluorophosphate before use. The method employed for the determination of carboxyl-terminal amino acids was similar to that described by Gladner and Neurath (1953). Amino acids were determined by the method of Richmond and Hartley (1959), which in our hands gave recoveries of control samples of ± 15% of the theoretical values.

Electrophoresis experiments were performed in a Perkin-Elmer apparatus (Model 38a) equipped with a modified Philpot-Svenson cylindrical lens system. The runs were carried out in a 2-ml Tiselius cell at 0°, and photographs were taken on Kodak sheet film using the film adaptor contained in the viewing hood.

RESULTS

The extraction of 20 beef pancreas glands with dilute sulfuric acid yielded approximately 24 g of PP 1 (potential k' = 0.7–0.9) or 8 g of PP 3 (potential k' = 1.4–1.6). Since 2 g of either PP 1 or PP 3 could be applied to a preparative CM-cellulose column, the additional fractionation with ammonium sulfate significantly reduced the number of chromatographic runs necessary for the preparation of the same amount of chymotrypsinogen B. Table I presents a summary of the results of different approaches to the purification of the zymogen.

Chromatography on DEAE-Cellulose.—As was the case with whole pancreatic juice (Keller *et al.*, 1958),

[3] Beef pancreas glands were donated by Swift Canadian Co. Ltd., Edmonton.

a large breakthrough peak occurred on chromatographing PP 1. On application of the gradient, however, only one peak emerged. The chymotrypsinogen B contained therein exhibited a potential k' of 2.40 and 1.5% of free chymotryptic activity. It was found to be electrophoretically heterogenous at *p*H 7.5 in 0.1 M Tris-HCl buffer.

Chromatography on CM-Cellulose, Stepwise Elution.— The successful application of the method of Rovery *et al.* (1960) was found to depend on the capacity of the cellulose exchanger. For example, with CM-cellulose of 0.9 meq/g capacity, essentially no chymotrypsinogen B could be eluted even with citrate buffer as concentrated as 0.2 M. On celluloses with lower capacities, the protein was bound less strongly and was eluted in good yields by sodium citrate buffers having molarities identical or close to that reported previously. CM-cellulose with a capacity of 0.59 meq/g and 0.06 M citrate buffer yielded the best preparation (potential k' = 2.55) and an elution diagram essentially identical to that reported by Rovery *et al.* (1960).

Chromatography on CM-Cellulose, Gradient Elution.— Since gradient elution of chymotrypsinogen B from ion exchangers might be expected to give a more homogeneous product than a stepwise method, a procedure was developed employing a linear gradient of sodium citrate buffers at a *p*H close to 4.4. Figure 1A presents the elution diagram of a 3 × 50-cm column charged with 400 mg of PP 1 in 20 ml of 0.03 M citrate buffer, *p*H 4.47, containing 0.005 M diisopropylfluorophosphate. Several fractions between tube numbers 48 and 114 were tested for nucleolytic activity and for chymotryptic activity after activation with trypsin (Fig. 1B). It is evident that protein possessing chymotryptic activity is eluted from the column in three peaks. The first peak, which has been identified as chymotrypsinogen B, showed maximal activity in the center fractions with a potential k' toward ATEE of 3.1 after tryptic activation. All gradient runs analyzed in the same way showed a sharp increase in the specific activity in the leading edge of the peak. The trailing edge, which showed lower specific activity, was not included in pooled fractions for preparative purposes. The second peak of chymotryptic activity is not normally present and in this particular experiment may represent partially activated B zymogen. This conclusion is supported by the experiment described later. The third peak may represent neochymotrypsinogen B or chymotrypsinogen α. Since Laskowski and Kassell (1960) were unable to resolve neochymotrypsinogen B and the native zymogen on CM-cellulose, we favor the latter possibility. Maroux *et al.* (1962) have shown that the α zymogen can be eluted from CM-cellulose with 0.08 M ammonium acetate at *p*H 6.0.

In order to determine the elution characteristics of chymotrypsin B on the gradient system the following experiment was done. Ten mg of PP 1 and 10 mg of trypsin-activated PP 1 were mixed and chromatographed on a 0.9 × 43-cm column with gradient elution. Protein concentration of the fractions was measured by optical density at 280 mμ and activity measurements toward ATEE were estimated before and after activation with trypsin. The results of Figure 2, in agreement with Laskowski and Kassell (1960), show that active forms of chymotrypsin B are eluted after the zymogen and do not seriously contaminate the fractions ordinarily pooled for preparative purposes.

In 1950, Kunitz showed that deoxyribonuclease I was present in the 0.2–0.4 saturated $(NH_4)_2SO_4$ fraction of beef pancreas extracts (Kunitz, 1950). As early as 1946, Laskowski observed that his preparations of chymotrypsinogen B were contaminated with nucleolytic activity (Laskowski, 1946). Protein powder 1 when tested showed 3% of deoxyribonuclease activity. It was of interest to investigate the distribution of deoxyribonuclease activity in the fractions from the column of Figure 1. The results shown in Figure 1B indicate that deoxyribonuclease is eluted shortly after the proteolytic zymogen. Assuming the specific activity of pure deoxyribonuclease to be 1.0 (Kurnick, 1950), the contamination of fractions 62 and 66 amounts to 13% and of fraction 55 containing the B-zymogen to 2%. When a number of lyophilized preparations of chymotrypsinogen B were tested, 1–2% of nucleolytic activity was detected.

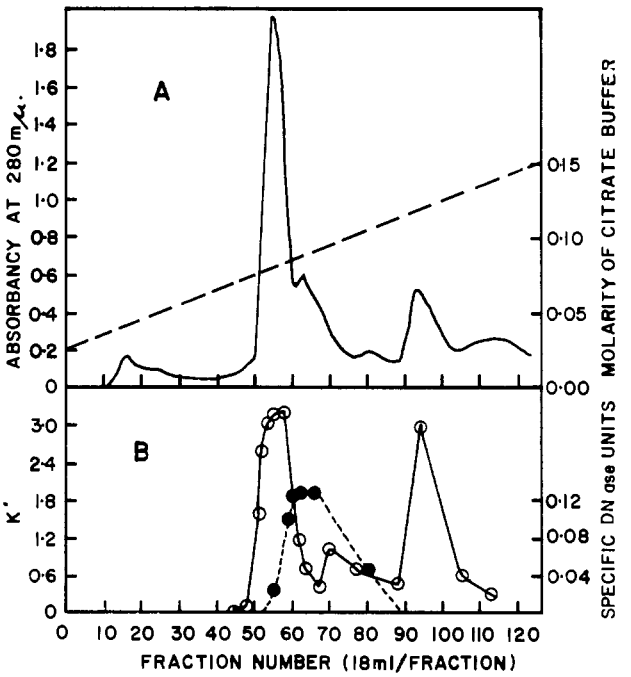

FIG. 1.—(A) Gradient elution diagram of PP 1 on CM-cellulose at *p*H 4.47 in sodium citrate buffer. (B) Potential chymotryptic (O——O) and deoxyribonuclease (●——●) activities of effluent fractions.

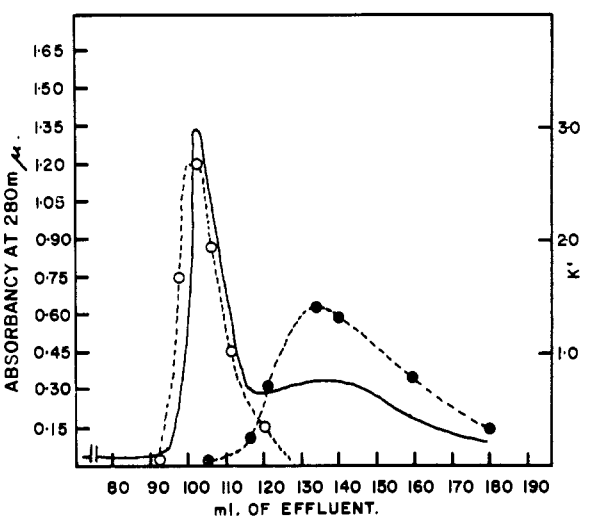

FIG. 2.—Gradient elution of PP 1 and trypsin-activated PP 1; absorbancy at 280 mμ, ——; potential chymotryptic activity O——O; free chymotryptic activity ●——●.

When gradient elutions were performed at *p*H 4.37 and *p*H 4.27, the trailing shoulder of the main peak of Figure 1 developed into a second peak further removed from the fractions containing chymotrypsinogen B. At the same time, the yield of the zymogen decreased by 15% at *p*H 4.37 and by 50% at *p*H 4.27. Nucleolytic contamination was less than 1% in pooled chymotrypsinogen B fractions from *p*H 4.37 columns, but no less in *p*H 4.27 columns.

Homogeneity of Chymotrypsinogen B.—The physical parameters of chymotrypsinogen B prepared by gradient elution on CM-cellulose have been examined and will be reported in a subsequent paper (Enenkel *et al.*, in preparation). In sedimentation-velocity runs at 59,788 rpm in a Spinco Model E ultracentrifuge, a single symmetrical peak was observed. The molecular weights determined at the meniscus and cell bottom of Archibald runs were similar and showed no tendency to change with time. Electrophoresis runs in buffers of ionic strength 0.1 over the *p*H range 3.0–7.5 showed a single boundary.

Carboxyl-terminal analyses have been used by Kassell and Laskowski (1961) as a measure of contamination of their preparations by neochymotrypsinogens. With native chymotrypsinogen B no carboxyl-terminal asparagine was liberated by carboxypeptidase A (Kassell and Laskowski, 1962), but detectable amounts of other amino acids were produced when the B-zymogen was treated with high molar ratios (1:10) of carboxypeptidase A. The liberation of tryosine was taken as an indication of the presence of neochymotrypsinogens. However, in our experience, carboxyl-terminal leucine has always been found in higher molar concentration than tyrosine. We have used these analyses as a criterion of the extent of chymotryptic autolysis during our purification procedures. When several preparations of chymotrypsinogen B prepared by the gradient elution technique were treated with carboxypeptidase A under conditions identical to those of Kassell and Laskowski (1961) only small amounts of amino acids were found. Only traces of tyrosine, 0.07–0.09 mole of leucine, 0.06–0.08 mole of valine, 0.03–0.06 mole of alanine, and 0–0.06 mole of phenylalanine could be identified.

All preparations of chymotrypsinogen B were routinely assayed by the titration procedure with cinnamoylimidazole (Schonbaum *et al.*, 1961). Preparations prepared by gradient elution on CM-cellulose were found to be 90–95% activatable. On the assumption that all the potential chymotryptic activity in these preparations is present as a single molecular species, it was calculated that the potential k' toward ATEE of pure native chymotrypsinogen B is 3.35.

Discussion

When the three methods of preparation of chymotrypsinogen B are compared, the superiority of the gradient-elution technique over the other procedures becomes apparent. Chromatography on DEAE-cellulose according to Keller *et al.* (1958) yields not only zymogen of relatively low potential activity (k' = 2.4) but also a preparation with exceptionally high free chymotryptic activity (1.8%).

Under well-defined conditions of chromatography, chymotrypsinogen B can be purified by the stepwise elution technique of Rovery *et al.* (1960). Zymogen prepared in this laboratory by this method contained up to 0.1% free enzyme. Judged by the cinnamoylimidazole assay, such preparations are activatable to the extent of 80%. In view of the results obtained by gradient elution it may be assumed that the contaminating proteins eluted after chymotrypsinogen B by the citrate gradient emerge together with the zymogen when the *p*H of the eluting buffer is increased stepwise.

Highly homogenous chymotrypsinogen B can be prepared by gradient elution of carboxymethyl-cellulose columns. Careful control of the *p*H of the eluting buffer is essential, since deoxyribonuclease I tends to emerge from the column shortly after the proteolytic zymogen. Citrate buffer, *p*H 4.37, represents a compromise between good yield of chymotrypsinogen B and minimal contamination with deoxyribonuclease I. Such preparations are 90–95% activatable and show a single peak in the ultracentrifuge and the electrophoresis apparatus. Only small quantities of carboxyl-terminal amino acids are liberated by carboxypeptidase A, a criterion which demonstrates that little autolysis occurs during the preparative procedure. The use of diiospropylfluorophosphate has been found advantageous during the gradient elution procedure to maintain the free activity at a low level. Protein prepared in its absence had free activities 50–100 times higher and potential activities about 20% lower than the values quoted in Table I.

In Laskowski's laboratory, chymotryptic activity is measured with casein as substrate. Results from this assay cannot be directly compared to *p*H-stat measurements. Because of possible variations in the nature and quality of the casein preparations used in this assay, experiments which were designed to arrive at a conversion factor gave inconclusive results.

References

Enenkel, A. G., Smillie, L. B., and Kay, C. M. (in preparation).

Gladner, J. A., and Neurath, H. (1953), *J. Biol. Chem. 205*, 345.

Green, M. M., Gladner, J. A., Cunningham, L. W., Jr., and Neurath, H. (1952), *J. Am. Chem. Soc. 74*, 2122.

Kassell, B., and Laskowski, M. (1961), *J. Biol. Chem. 236*, 1996.

Kassell, B., and Laskowski, M. (1962), *J. Biol. Chem. 237*, 443.

Keller, P. J., Cohen, E., and Neurath, H. (1958), *J. Biol. Chem. 233*, 344.

Kunitz, M. (1950), *J. Gen. Physiol. 33*, 349.

Kurnick, N. B. (1950), *Arch. Biochem. 29*, 41.

Laskowski, M. (1946), *J. Biol. Chem. 166*, 555.

Laskowski, M. (1955), *in* Methods in Enzymology, Vol. II, Colowick, S. P., and Kaplan, N. O., eds., New York, Academic, p. 16.

Laskowski, M., and Kassell, B. (1960), *Acta Biochim. Polon. 7*, 253.

Maroux, S., Rovery, M., and Desnuelle, P. (1962), *Biochim. Biophys. Acta 56*, 202.

Peterson, E. A., and Sober, K. A. (1956), *J. Am. Chem. Soc. 78*, 751.

Richmond, V., and Hartley, B. S. (1959), *Nature 184*, 1869.

Rovery, M., Guy, O., and Desnuelle, P. (1960), *Biochim. Biophys. Acta 42*, 554.

Schonbaum, G. R., Zerner, B., and Bender, M. L. (1961), *J. Biol. Chem. 236*, 2930.

Wu, F. C., and Laskowski, M. (1956), *Biochim. Biophys. Acta 19, 110.*