Roy. Soc. London, Ser. B 257, 105.
Bernhard, S. A., Lee, B. F., and Tashjian, Z. H. (1966), *J. Mol. Biol. 18,* 405.
Blombäck, B. (1958), *Ark. Kemi 12,* 321.
Bradley, D. F., and Wolf, M. K. (1959), *Proc. Nat. Acad. Sci. U.S. 45,* 944.
Chase, T., and Shaw, E. (1969), *Biochemistry 8,* 2212.
Ehrenpreis, S., and Scheraga, H. A. (1959), *Arch Biochem. Biophys. 79,* 27.
Ehrlich, T., and Stivala, S. S. (1973), *J. Pharm. Sci. 62,* 517.
Fahrney, D. E., and Gold, A. M. (1963), *J. Amer. Chem. Soc. 85,* 997.
Fasco, M. J., and Fenton, J. W. (1973), *Arch. Biochem. Biophys. 159,* 802.
Feinman, R. D., Orton, C., and Li, E. H. H. (1973), Abstracts, 4th International Congress on Thrombosis and Hemostasis, Vienna, p. 438.
Glover, G., and Shaw, E. (1971), *J. Biol. Chem. 246,* 4594.
Godal, H. C. (1961), *Scand. J. Lab. Invest. 13,* 306.
Hess, G. P. (1971), *Enzymes, 3rd Ed. 3,* 213.
Hilborn, J. C., and Anastassiadis, P. A. (1971), *Anal. Biochem. 31,* 51.
Jaques, L. B. (1967), *Progr. Med. Chem. 5,* 139.
Koehler, K. A., and Magnusson, S. (1974), *Arch. Biochem. Biophys. 160,* 175.
Lundblad, R. L. (1971), *Biochemistry 10,* 2501.
Lyttleton, J. W. (1954), *Biochem. J. 58,* 15.
Magnusson, S. (1971), *Enzymes, 3rd Ed. 3,* 278.
Rosenberg, R. D., and Damus, P. S. (1973), *J. Biol. Chem. 248,* 6490.
Shinowara, G. Y. (1966), *Biochim. Biophys. Acta 113,* 359.
Stone, A. L., and Bradley, D. F. (1967), *Biochim. Biophys. Acta 148,* 172.
Wollin, A., and Jaques, L. B. (1973), *Thromb. Res. 2,* 377.

The Cooperative Binding of Two Calcium Ions to the Double Site of Apothermolysin†

Gerrit Voordouw and Rodney S. Roche*

ABSTRACT: The binding of calcium ions to apothermolysin has been studied by gel filtration at 25°, with free calcium ion concentration ranging from 10^{-3} to 10^{-6} M; pH 9.00 ± 0.05. Two calcium ions were found to dissociate simultaneously in this concentration range. Analysis of the binding data reveals a linear Hill plot with slope 2.0 and a parabolic Scatchard plot, thus demonstrating that the binding of these two calcium ions is completely cooperative. From the maximum of the Scatchard plot the overall binding constant is determined to be 2.8×10^{9} M^{-2} under these conditions. We conclude that our binding data apply to the double-site [Ca(1)-Ca(2)] wherein the calcium ions are separated by 3.8 Å as described by B. W. Matthews and L. H. Weaver [*Biochemistry 13,* 1719 (1974)].

Thermolysin is known to bind four calcium ions (Feder *et al.,* 1971). This is confirmed by the three-dimensional structure of thermolysin at 2.3-Å resolution (Colman *et al.,* 1972), which reveals the presence of four calcium binding sites: Ca(1) and Ca(2), which are only 3.8 Å apart and form a so-called double site; and Ca(3) and Ca(4), which are quite distant from each other and from the double site. The four binding sites have been confirmed crystallographically by replacing the calcium ions with strontium, barium, and trivalent lanthanide ions (Colman *et al.,* 1972). Recently, a detailed study of the site conformational changes accompanying the replacement of the calcium by lanthanide ions (Matthews and Weaver, 1974) has provided a wealth of structural detail on the four binding sites.

Surprisingly, the calcium ion association constants of thermolysin in solution have not yet been determined, although an interesting calcium binding isotherm can be anticipated, especially from the structure of the double site to which an independent binding of the two calcium ions is highly unlikely. One of the problems in studying the binding of calcium ions to proteolytic enzymes is that their rate of autolytic degradation increases progressively with a decreasing amount of bound calcium ions. This problem has been recognized before in the case of thermolysin (Feder *et al.,* 1971) and columns equilibrated with 1,10-phenanthroline, as chelating agent for the active site zinc ion, have been used to suppress its autolytic degradation by formation of the apoenzyme. Unfortunately the high uv absorbance of 1,10-phenanthroline does not allow a convenient spectrophotometric determination of the resulting apothermolysin. We will describe here how the use of a non-uv absorbing zinc chelating agent, TEP,[1] allows an accurate estimation of the calcium binding isotherm for the two least firmly bound ions. The results are directly interpretable on the basis of the known X-ray structure of thermolysin.

Experimental Section

Materials. Thermolysin (crystallized, lot no. P-1512) was obtained from Sigma Chem. Co. and was used without further purification, after it was established that this material was homogeneous in gel filtration on Sephadex G-100

† From the Biopolymer Research Group, Department of Chemistry, The University of Calgary, Calgary T2N 1N4, Alberta, Canada. *Received July 9, 1974.* This work was supported by the National Research Council of Canada (Grant A 3608).

[1] Abbreviations used are: TEP, tetraethylenepentamine; Tris, tris(hydroxymethyl)aminomethane.

and in sodium dodecyl sulfate gel electrophoresis. $CaCO_3$ (chelometric standard) was purchased from Fisher and TEP from J. T. Baker Chem. Co. The TEP was used either directly or after crystallization from water–ethanol (1:1) at pH 1. No difference in the results obtained was noted between the various preparations. Sephadex G-25 Fine was from Pharmacia Fine Chemicals. All other chemicals used were reagent grade.

Equilibrium Gel Filtration. The amount of calcium ions bound to thermolysin was measured with the gel filtration technique of Hummel and Dreyer (1962). A K16/70 column (1.6 × 50 cm, Pharmacia Fine Chemicals) with flow adapters and thermostat jacket, packed with Sephadex G-25 Fine and equilibrated with 0.1 M NaCl, 0.01 M TEP (pH 9.00 ± 0.05), and a given concentration of $CaCl_2$, was used in all experiments. The temperature of the column was maintained at 25.00 ± 0.07° by a Colora circulating water bath, in which the buffer vessel also was placed. In a typical experiment 10–15 mg of thermolysin was applied to the column in a sample volume of 1–2 ml, after complete dissolution at 4°, pH 11.5 in 5 M NaCl. Thermolysin dissolves readily in high concentrations under these conditions (Matsubara, 1970; Latt *et al.*, 1969). The sample pH was adjusted to 9.0 before application to the column. The elution was then performed at a flow rate of 0.3 ml/min, fractions of 1.5 ml being collected. These were then analyzed for calcium and protein.

Determination of Thermolysin Concentration. The enzyme concentration of the collected fractions was determined from absorbance measurements at 280 nm and 22°, using a Beckman DB-G grating spectrophotometer and quartz cells with a 1-cm path length. Aliquots of 0.1–0.2 ml were withdrawn from fractions with absorbances in excess of 0.8 and measurements were made after gravimetric dilution to obtain readings between 0.2 and 0.4. An experimentally determined value of ϵ 52,400 was used as molar extinction coefficient for thermolysin (see Results). Enzyme concentrations for this determination were obtained from dry weight measurements (Schachman, 1957) using a value of 34,500 for the molecular weight of thermolysin (Titani *et al.*, 1972) and from the known stoichiometries of zinc (1:1) and calcium ion (4:1) binding to thermolysin (Colman *et al.*, 1972; Matthews and Weaver, 1974).

Atomic Absorption Spectrometry. The total calcium ion concentrations of the fractions collected in gel filtration were determined with a Heath 703 atomic absorption spectrometer at 422.1 nm, using an acetylene–air flame adjusted to stoichiometric conditions. Standard curves for calcium were evaluated over the range 2–12 ppm. The standard solutions were obtained by gravimetric dilution of a standard stock solution containing 1000 ppm of calcium. The latter was made up by dissolving the appropriate amount of $CaCO_3$ in a few milliliters of 10 N HCl and adjusting to the required concentration with double deionized water. Zinc determinations were made with a Perkin-Elmer 303 atomic absorption spectrometer at 214.0 nm, using an acetylene–air flame with standard solutions for zinc in the range 0.5–5.0 ppm.

Prevention of Contamination with Extraneous Calcium Ions. Nalgene bottles and test tubes were used routinely for the storage of buffers and the collection of column fractions. These were rinsed with double deionized water, three to five times, after standard cleaning procedures. The double deionized water was prepared by passage through a type IR-C Continental Ultra Pure deionizing unit [Biolab Equipment Ltd.]. The contaminating level of calcium in the double deionized water used was determined to be 3.0×10^{-7} M by atomic absorption spectrometry after 100-fold concentration by evaporation. Whenever necessary the calcium ion concentrations in the buffers prepared were corrected for this contaminating level. This correction amounted to less than 1–2% of the total free calcium ion concentration for almost all buffer solutions used.

Results and Discussion

The key to a successful measurement of the calcium binding isotherm of apothermolysin is the selection of the right zinc chelating agent. The chelating agent should have a high binding constant for zinc ions, should not associate with calcium ions thus enabling their concentration to be varied independently, and should have no absorbance in the 280-nm region. The zinc chelating inhibitors for thermolysin described in the literature (Vallee and Latt, 1970) do not meet the latter criterion. Because it has a high binding constant for the zinc ion ($K_1 = 2.5 \times 10^{15}$ M^{-1}; Sillen and Martell, 1964) and also meets the other two criteria outlined above, we decided to use TEP. Experiments were carried out at pH 9.00. At this pH the compromise between obtaining maximum zinc binding and the need to avoid possible denaturation is optimal. TEP also acts as a good buffer under these conditions.

When thermolysin (2 mg/ml) was dialyzed against 0.01 M $CaCl_2$–0.01 M TEP at pH 9.00 and 4° for 24 hr, 0.04 ± 0.02 mol of zinc was bound/mol of enzyme, whereas upon dialysis against 0.01 M $CaCl_2$–0.01 M Tris under similar conditions a value of 1.04 ± 0.02 mol of zinc/mol of enzyme was found. Hence TEP, by direct removal of zinc ions from the active enzyme molecule, inactivates thermolysin very similarly to 1,10-phenanthroline (Latt *et al.*, 1969). The amount of zinc bound per mole is calculated on the basis of an average molar extinction coefficient at 280 nm, 22°, and pH 9.00, ϵ = 52,400. Values for the extinction coefficient of thermolysin reported in the literature are not in close agreement and a value was determined in this study. It is seen (Table I) that the values obtained with three different methods agree within 7%, indicating our thermolysin preparation to be relatively homogeneous. We

TABLE I: Extinction Coefficient of the Thermolysin Preparation Used; Comparison with Literature Data.

Ref	Method for Determination of Enzyme Concn	E_{1cm} (1%)
This study	Zinc content of dialyzed preparations	14.6 ± 0.2
This study	Maximum calcium content of preparations after gel filtration	15.2 ± 0.2
This study	Dry weights of dialyzed preparations	15.6 ± 0.2
a		15.2
b		15.5
c		16.0
d		17.6
e	Differential refractometry	17.6

[a] Keay (1969). [b] Drucker and Borchers (1971). [c] Stauffer (1971). [d] Ohta *et al.* (1966). [e] Pangburn *et al.* (1973).

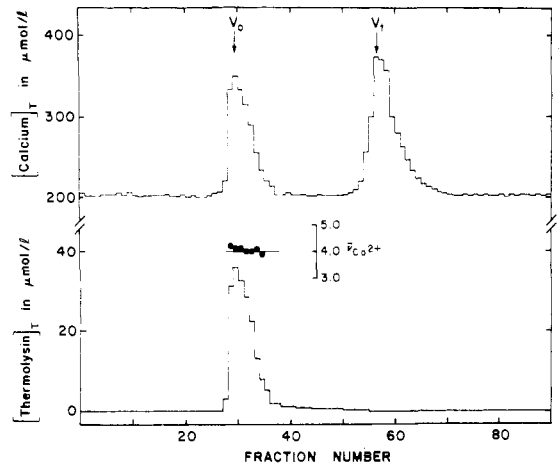

FIGURE 1: Typical elution profile for thermolysin on a column (1.6 × 50 cm) packed with Sephadex G-25 Fine and equilibrated at 25.0° with 0.1 M NaCl, 2.0 × 10^{-4} M $CaCl_2$, and 0.01 M TEP (pH 9.00). The sample (1.5 ml, *ca.* 10 mg/ml of thermolysin and 10^{-2} M Ca^{2+}) was eluted at a flow rate of 0.3 ml/min, fractions of 1.5 ml being collected. Determination of the total thermolysin and calcium concentrations, represented on the ordinate, was done as described in the Experimental Sections.

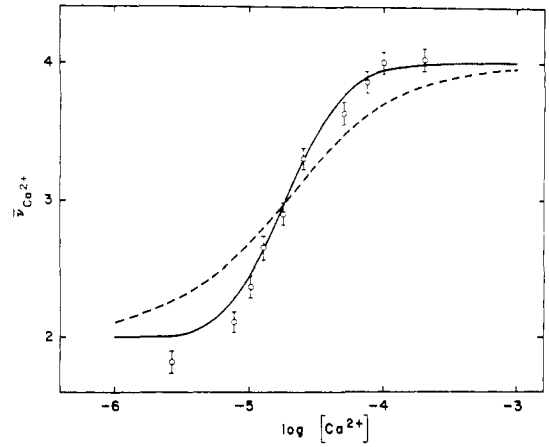

FIGURE 2: Calcium binding isotherm of apothermolysin at 25.0° in 0.1 M NaCl and 0.01 M TEP (pH 9.00). The experimentally determined number of calcium ions bound per mole of enzyme ($\bar{\nu}_{Ca^{2+}}$) is plotted as a function of the logarithm of the free calcium ion concentration (log [Ca^{2+}]). The standard deviation in $\bar{\nu}_{Ca^{2+}}$ (± 0.08) is also indicated. The solid line is calculated on the basis of cooperative binding of two calcium ions: $\bar{\nu}_{Ca^{2+}} = 2 + [2Kc^2/(1 + Kc^2)]$ with $K = 2.8 \times 10^9$ M^{-2} and $c = [Ca^{2+}]$. The dotted line is for two identical independent sites: $\bar{\nu}_{Ca^{2+}} = 2 + [2Kc/(1 \bullet Kc)]$, $K = 5.4 \times 10^4$ M^{-1}.

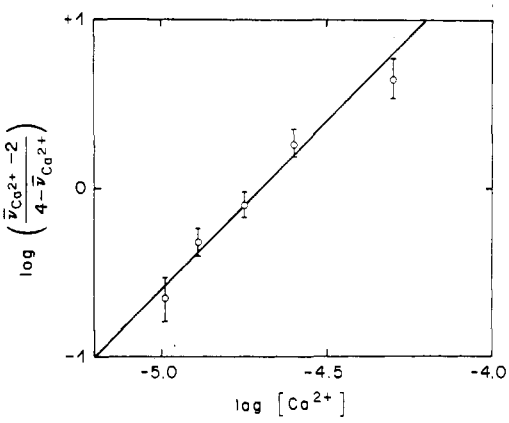

FIGURE 3: Hill plot using the data from Figure 2, for which $0.2 < \bar{\nu}_{Ca^{2+}} - 2 < 1.8$. The line drawn has a slope 2.0. The error bars have been calculated from the measured standard deviation in $\bar{\nu}_{Ca^{2+}}$ (± 0.08).

therefore used an average value $E_{1\,cm}(1\%)$ 15.2 (ε 52,400) in all our calculations. This value agrees well with some literature values but differs greatly from others. The reason for this discrepancy is not known.

A typical chromatogram for apothermolysin at 25.00° and an equilibrating buffer containing 0.1 M NaCl, 2.0 × 10^{-4} M $CaCl_2$, and 0.01 M TEP (pH 9.00) is shown in Figure 1. The enzyme elutes at the void volume (V_0) of the column, where the changes in total enzyme concentration are completely paralleled by changes in the total calcium concentration. It is found that the number of calcium ions bound per mole of apothermolysin ($\bar{\nu}_{Ca^{2+}}$) is 4.02 ± 0.08 under these conditions. A second calcium peak is eluted at the total volume (V_t) of the column and represents the excess of calcium ions present in the sample. We did not attempt to estimate $\bar{\nu}_{Ca^{2+}}$ from a trough in calcium ion concentration, that would be observed upon chromatography of a calcium free thermolysin sample (Hummel and Dreyer, 1962), since this approach did not prove to be feasible experimentally due to solubility problems with the calcium-free protein.

Instead of calculating $\bar{\nu}_{Ca^{2+}}$ for each fraction (Figure 1) the same quantity can also be obtained by plotting the total calcium concentration *vs.* the total enzyme concentration for the various fractions. Straight lines were obtained with $\bar{\nu}_{Ca^{2+}}$ as slope and the free calcium ion concentration as intercept. These plots indicate the analogy between gel chromatography and other transport processes like sedimentation and electrophoresis (Cann, 1970) in studying small molecule–macromolecule interactions.

The results of a number of gel chromatography experiments, with various free-calcium ion concentrations ([Ca^{2+}]) are shown in Figure 2. It is seen that apothermolysin binds a maximal number of 4.0 calcium ions, as has been found before by a number of workers (see Introduction). When the free calcium ion concentration is reduced to values between 10^{-4} and 10^{-6} M two calcium ions dissociate simultaneously from the enzyme molecule.

We could not fit the observed calcium binding isotherm by models involving independent sites with different or similar intrinsic affinities and tested therefore the binding data for cooperativity (Hill, 1913). Since only two of the total of four calcium ions are dissociating we have plotted log $[x/(2 - x)]$ *vs.* log [Ca^{2+}], where $x = (\bar{\nu}_{Ca^{2+}} - 2)$ (Figure 3). We find a Hill coefficient of 2.0 ± 0.2. This value indicates that complete positive cooperativity exists between the two calcium binding sites, since only in this case does the Hill coefficient equal the total number of dissociating ligands (*e.g.*, Levitzki and Koshland, 1969).

Further analysis of the binding data according to the method of Scatchard (1949) shows a parabolic plot (Figure 4). From the data in the vicinity of the Scatchard plot maximum at $x = (\bar{\nu}_{Ca^{+2}} - 2) = 1$, we estimate that $K^{1/2} = 5.3 \times 10^4$ M^{-1}, hence the overall association constant $K = 2.8 \times 10^9$ M^{-2} under these conditions. The solid line in Figure 4 is calculated using this value for K (see Appendix) and fits the experimental data well. The shape of the Scatchard plot is a further diagnostic of positively cooperative ligand binding and has been described before (Cook, 1972).

When the above value for the overall association constant K is used to calculate the binding isotherm of apothermolysin the solid line in Figure 1 is obtained. This line gives a

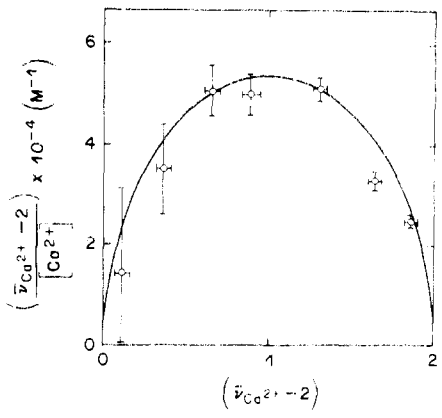

FIGURE 4: Scatchard plot using the data from Figure 2 for which $0.1 < \bar{\nu}_{Ca^{2+}} - 2 < 1.9$. The error bars have been calculated as in Figure 3. The curve drawn is the theoretical Scatchard plot for completely positive cooperative binding of the two calcium ions: $y = [Kx(2 - x)]^{1/2}$, with $y = x/c$, $x = \bar{\nu}_{Ca^{2+}} - 2$, and $K = 2.8 \times 10^9\ M^{-2}$ (see Appendix)

good fit to the experimental data, except for the region $[Ca^{2+}] < 10^{-5}$ M. In the latter region, the deviation from the calculated isotherm, beyond the error limits, has to be ascribed, presumably, to the dissociation of a third calcium ion. Since the dissociation of more than two calcium ions was accompanied by the precipitation of the enzyme during the course of a gel filtration experiment, no measurements could be done below $[Ca^{2+}] = 5 \times 10^{-6}$ M and the magnitudes of the remaining association constants could not be determined.

Having established experimentally that thermolysin binds two calcium ions with complete positive cooperativity, we now turn to a discussion of their most probable location, using the three-dimensional structure of the molecule (Colman *et al.*, 1972) and the structures of the calcium binding sites, as given in detail by Matthews and Weaver (1974). Colman *et al.* (1972) denoted the four calcium binding sites as follows: Ca(1), the inner of the double site; Ca(2), the outer of the double site; Ca(3), the single site at Asp-57; and Ca(4), the single site at Asp-200. Inspection of the location of these sites in the thermolysin molecule (Colman *et al.*, 1972, Figure 7) reveals that the distances between Ca(3) and Ca(4) and the double site are large compared to the interatomic distance of 3.8 Å between the two calcium ions in the double site. The transmittance of site–site interactions over large distances is, of course, well known for subunit enzymes. Such interactions are, however, never completely cooperative as indicated by the fact that the Hill coefficient is always less than the maximum number of ligands bound for such positively cooperative systems. The fact that the two calcium ions which bind to the apothermolysin molecule in the concentration range 10^{-6}–10^{-3} M do so with complete cooperativity, as shown by the Hill coefficient of 2, indicates therefore that long range site–site interactions are not likely to be involved. Assuming then that the observed site–site interactions are due neither to Ca(3) and Ca(4) interacting with each other nor with the double site, Ca(1)–Ca(2), we focus our attention on the latter.

Since it is situated rather close to the active site cleft, Colman *et al.* suggested that the calcium double site might be particularly important for the stability of the enzyme since it links together the two halves of the molecule. The inner calcium ion, Ca(1), is shielded from the solvent by the outer calcium ion, Ca(2), and by the carboxyl groups acting as complexing ligands. Of the carboxyl groups coordinated to the double site (Asp-138, Glu-177, Asp-185, Glu-190) three are particularly interesting, namely Glu-177, Asp-185, and Glu-190, which coordinate *both* Ca(1) and Ca(2) (Matthews and Weaver, 1974, Figure 4a). The behavior of the double site when trivalent lanthanide ions bind to thermolysin provides an experimental basis for suggesting the nature of the molecular mechanism underlying the observed cooperativity in the binding of Ca(1) and Ca(2). Matthews and Weaver (*loc. cit.*) have shown that Ca(2) is displaced without substitution and that Ca(1) is substituted by trivalent lanthanide ions at a locus displaced from the Ca(1) site by 0.33 Å. Within the resolution of the X-ray results, there is no indication of a substantial rearrangement of the bridging side chains of Glu-177, Asp-185, and Glu-190 indicating that the geometry of the Ca(1) site is preserved. Thus, in the absence of Ca(2), the integrity of the Ca(1) site requires a 3+ ion with a higher coordination number than Ca^{2+}. A single Ca^{2+} ion could not provide such stabilization. The reason for the observed cooperativity is thus revealed. When Ca(2) dissociates the resulting destabilization of the Ca(1) site leads to its simultaneous disruption and the dissociation of Ca(1).

It is interesting to note that Pauling (1935) postulated, in the first theory describing the oxygen binding to hemoglobin, that direct site–site interactions between the four hemes, located close together on the corners of a square, accounted best for the observed cooperativity in the binding of oxygen to hemoglobin. Although Pauling's model proved wrong for hemoglobin, thermolysin can be said to exhibit "Pauling cooperativity," since one can regard the double site as two closely spaced single sites capable of direct site–site interactions.

Having attributed our data to the double site, we can conclude that the two single binding sites have a higher affinity for calcium ions than the double site. Although the experimental situations are not directly comparable, this agrees with results obtained by Matthews and Weaver (1974) from their competitive europium–calcium substitution experiments in the crystalline state.

The biological importance of the double site is apparent from Figure 2. If we assume that the enzyme with an occupied double site is considerably more stable against autolytic degradation or various denaturation processes, than the enzyme with an unoccupied double site, it is seen from Figure 2 that in a cooperative model the stable enzyme form predominates in the concentration range 10^{-4} M $< [Ca^{2+}] < 10^{-3}$ M, compared to the case of noncooperative binding. Calcium concentrations of this order may be considered to be typical of natural conditions.

It is an interesting question whether other proteases, homologous to thermolysin, like the neutral protease of *Bacillus subtilis* (Pangburn *et al.*, 1973) have double sites. The experimental criteria for the recognition of such a site have been clearly delineated in the present study.

Acknowledgment

Gerrit Voordouw acknowledges the award of a N.R.C. Postgraduate Scholarship and an Izaak Walton Killam Memorial Scholarship. The authors are indebted to Dr. G. M. Gaucher for the loan of column equipment and to Dr. H. L. Yeager for making the atomic absorption spectrometer available to us.

Appendix

The overall association constant for the binding of two

calcium ions to the double site of thermolysin was calculated from the theoretical maximum in the Scatchard plot, occurring at $x = \bar{\nu}_{Ca^{2+}} - 2 = 1$, in the following way. For a completely cooperative binding of the two calcium ions the binding isotherm is given by

$$\bar{\nu}_{Ca^{2+}} = 2 + [2Kc^2/(1 + Kc^2)] \quad (1)$$

where c is the free calcium ion concentration and K the association constant in the reaction: $ECa_2 + 2Ca^{2+} \rightleftharpoons ECa_4$. We have assumed the two remaining calcium ions of ECa_2 not to be involved in association–dissociation reactions at $[Ca^{2+}]$ in the range studied. Combination of $x = \bar{\nu}_{Ca^{2+}} - 2$ with (1) gives

$$c = \sqrt{x/K(2 - x)} \quad (2)$$

A Scatchard plot is defined as a plot of $y = (x/c)$ *vs.* x. Combination with (2) yields

$$y = \sqrt{Kx(2 - x)} \quad (3)$$

hence y is the square root of a true parabola; $y = 0$ for $x = 0$ and $x = 2$. The maximum in the Scatchard plot occurs at $x = 1$: $y_{max} = K^{1/2}$. After estimation of $K^{1/2}$ from data points in the vicinity of $x = 1$ we can calculate y, using (3) and see whether the calculated curve fits the data points, distant from $x = 1$. The isotherm (eq 1) can then be calculated and compared with the actual experimental data.

References

Cann, J. R. (1970), *in* Interacting Macromolecules, Molecular Biology Series, Horecker, B., Kaplan, N. O., Marmur, J., and Scheraga, H. A., Ed., New York, N.Y., Academic Press, p 47.

Colman, P. M., Jansonius, J. N., and Matthews, B. W. (1972), *J. Mol. Biol. 70,* 701.

Cook, R. A. (1972), *Biochemistry 11,* 3792.

Drucker, H., and Borchers, S. L. (1971), *Arch. Biochem. Biophys. 147,* 242.

Feder, J., Garrett, L. R., and Wildi, B. S. (1971), *Biochemistry 10,* 4552.

Hill, A. V. (1913), *Biochem. J. 7,* 471.

Hummel, J. P., and Dreyer, W. J. (1962), *Biochim. Biophys. Acta 63,* 530.

Keay, L. (1969), *Biochem. Biophys. Res. Commun. 36,* 257.

Latt, S. A., Homquist, B., and Vallee, B. L. (1969), *Biochem. Biophys. Res. Commun. 37,* 333.

Levitzki, A., and Koshland, D. E. (1969), *Proc. Nat. Acad. Sci. U. S. 62,* 1121.

Matsubara, H. (1970), *Methods Enzymol. 19,* 642.

Matthews, B. W., and Weaver, L. H. (1974), *Biochemistry 13,* 1719.

Ohta, Y., Ogura, Y., and Wada, A. (1966), *J. Biol. Chem. 241,* 5919.

Pangburn, M. K., Burstein, Y., Morgan, P. H., Walsh, K. A., and Neurath, H. (1973), *Biochem. Biophys. Res. Commun. 54,* 371.

Pauling. L. (1935), *Proc. Nat. Acad. Sci. U. S. 21,* 186.

Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51,* 660.

Schachman, H. K. (1957), *Methods Enzymol. 4,* 32.

Sillen, L. G., and Martell, A. E. (1964), *Chem. Soc., Spec. Publ. No. 17,* 576.

Stauffer, C. E. (1971), *Arch. Biochem. Biophys. 147,* 568.

Titani, K., Hermodson, M. A., Ericsson, L. H., Walsh, K. A., and Neurath, H. (1972), *Nature (London), New Biol. 238,* 35.

Vallee, B. L., and Latt, S. A. (1969) *in* Structure-Function Relationships of Proteolytic Enzymes, Desnuelle, P., Neurath, H., and Ottesen, M., Ed., New York, N. Y., Academic Press, p 155.