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Henrik Tylli: A CNDO/2 Study of the Conformation of 1,2-dimethoxybenzene 16

R. U. Schenk and J. Bjorksten: The Search for Microenzymes: The Enzyme of Bacillus cereus 26

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A CNDO/2 Study of the Conformation of 1,2-dimethoxybenzene

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Abstract

The total energy of 1,2-dimethoxybenzene (veratrole) for different conformations has been calculated using the CNDO/2 approximation. If the rapid rotation of the methyl groups in the methoxy substituents is taken into account, conformations with large out-of-plane angles for the methoxy groups are predicted to be the most stable ones. The results are discussed in relation to experimental results obtained from measurements of the relaxation times, from measurements of the dipole moments and its temperature dependence and from NMR spectroscopy.

Introduction

The conformational behaviour of the methoxy group in phenol ethers has been the subject of numerous theoretical and experimental investigations. For anisole an Extended Hückel calculation [1] gave minimum in energy for an out of plane angle of 75°, whereas the more reliable CNDO/2 approximation yielded minimum in energy either for the planar conformation or for the conformation with the torsional angle 45°, depending on the orientation of the methyl group.[2] As the reorientation of the methyl group about the C–O bond is rapid compared to the torsional oscillations of the methoxy group around the $C_{Ar}-O$ bond [3] the methyl group may have any orientation when it passes by the ring hydrogen atoms *ortho* to the substituent, and instantaneous nonplanar conformations may therefore result.

Ab Initio molecular orbital calculations using a minimal STO-3G basis set [4] predicted anisole to be most stable with the torsional angle 90° if a nonflexible geometry was used. However, this result is influenced by the steric interaction with an *ortho* hydrogen atom in the planar conformation. If the $C_{Ar}OC_{Me}$ bond angle was optimized for both forms the planar conformation was found to be the more stable one. Alternatively, as

pointed out by Hehre, Radom and Pople,[4] relief of steric interactions could be achieved also by a small rotation about the $C_{Ar}-O$ bond, with less distortion of the COC angle.

Theoretical studies of veratrole have been performed by Billingsley and Bloor,[5] and by Leibovici [6] using the PPP method, and by Nagy and Hencsei [7] with the PPP and Del-Re methods. Nagy and Hencsei compared the calculated dipole moment with the experimental value for anisole and o-, m-, and p-dimethoxybenzene, and concluded that the poor agreement obtained for veratrole was a result of the hindered rotation of the methoxy groups in this compound. In fact, spectroscopic determinations of the torsional barrier for the methoxy group in anisole give a large barrier of 25.2 kJ/mole (6.02 kcal/mole) in the liquid state,[3] indicating that free rotation cannot even be assumed in anisole.

Among experimental workers it is generally agreed that no single effective conformation for veratrole can account for the experimental observations. In the present study an analysis of the conformational behaviour of the methoxy groups in veratrole is therefore undertaken using the CNDO/2 approximation in order to obtain an estimate of the energetically most favourable conformations. The parametrization of the CNDO/2 method is such that especially the calculated dipole moments frequently are in good agreement with the experimentally observed dipole moments. Bloor and Breen [8] have pointed out that this property could be useful for the calculation of molecular conformations. In the following therefore, the results of the energy calculations are combined with the calculated dipole moments and compared with the value of the dipole moment observed experimentally at different temperatures.

Method

The CNDO/2 method was applied to veratrole with the usual parametrization.[9–11] The geometry of the molecule used in the calculations is shown in *figure 1*. This geometry is based upon an electron diffraction study of anisole [12] (compare also similar data for methyl vinyl ether [13] and benzene [14]).

From the point of view of molecular dynamics the veratrole molecule is a very complicated system with torsional oscillations about the $C_{Ar}-O$ and the $O-C_{Me}$ bonds. The torsional frequencies in the liquid state of anisole are measured to be 216 cm^{-1} and 108–115 cm^{-1} for the methyl group and the methoxy group respectively.[3,15,16] Simplifying assumptions must therefore be introduced in order to reduce the problem to a manageable size. A partly flexible model of the molecule was used. The total energy of the molecule was calculated for dif-

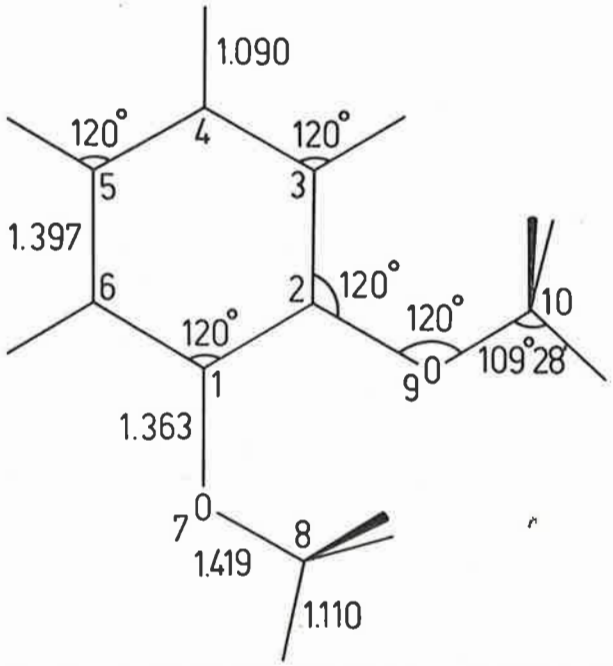

Figure 1. The atom numbering, bond lengths and bond angles in veratrole.

ferent torsional angles of the methoxy groups, and with two orientations of the methyl groups. These two orientations were chosen to give respectively minimum (orientation as in *figure 1*) and maximum (rotation of the methyl groups about the $O-C_{Me}$ bonds by 60° compared to *figure 1*) steric interaction with the hydrogen atoms *ortho* to the methoxy groups.[2] In order to obtain an estimate of the strength of the hydrogen bond in veratrole the $C_1O_7C_8$ bond angle in the planar I conformation (*figure 2*) was also varied. The various bond lengths and bond angles in the aromatic ring were kept constant.

The calculations were performed on a Burroughs 6700 computer at the University of Helsinki Computing Center. The program used was a version of the CNINDO program,[17] where the convergence criterion was slightly changed in order to obtain convergence in critical cases.

Results and Discussion

The nomenclature used for the different conformations in the present study are shown in *figure 2*. The first structural parameter to be varied is the torsional angle of methoxy group *a*. The torsional angle is defined as the angle between the ring

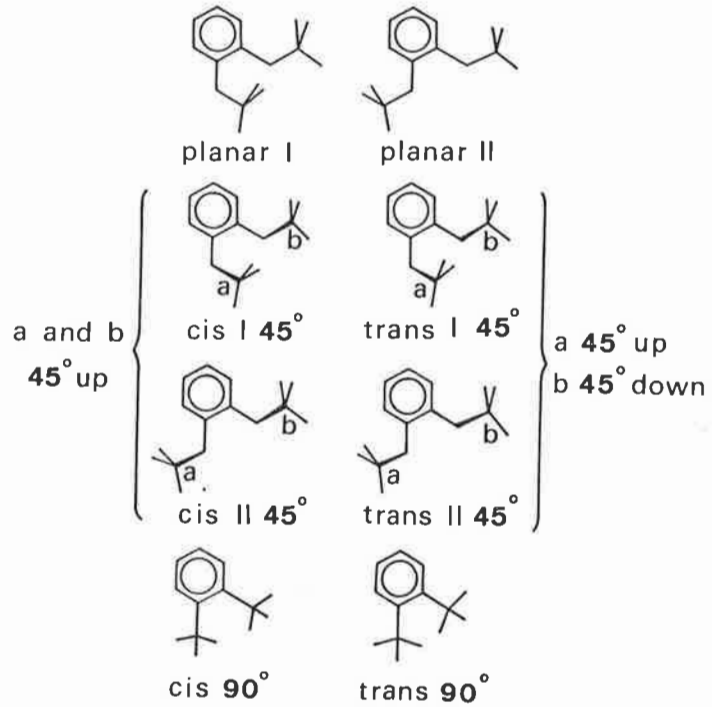

Figure 2. The nomenclature used for different conformations of veratrole.

plane and the plane containing the atoms C_1, O_7 and C_8 (or C_2, O_9 and C_{10}) (*figure 1*). The torsional angle 0° of methoxy group *a* thus corresponds to the conformation planar I, and the torsional angle 180° to the conformation planar II. These two conformations have been named *cis-trans* and *trans-trans* by Curran.[18] Model A is used as a name of the different conformations resulting if both methyl groups are oriented for minimum steric interaction with the adjacent methoxy group or with the *ortho* ring hydrogen. In model B the methyl group in the methoxy group *a* was rotated by 60° about the O_7-C_8 bond, to give maximum steric interaction.

The calculated total energies for the two models and different angles of torsion are given in *table 1*. Plots of energy against torsional angle are shown in *figures 3a* and *3b*. It is seen that for model A two minima are obtained, corresponding to the planar I and planar II conformations, the former being the more stable one. This supports the view presented by Curran [18] who, basing his arguments on dipole moment measurements, concluded, that a considerable amount of molecules in the planar I conformation are present. The plot in *figure 3a* shows also that the con-

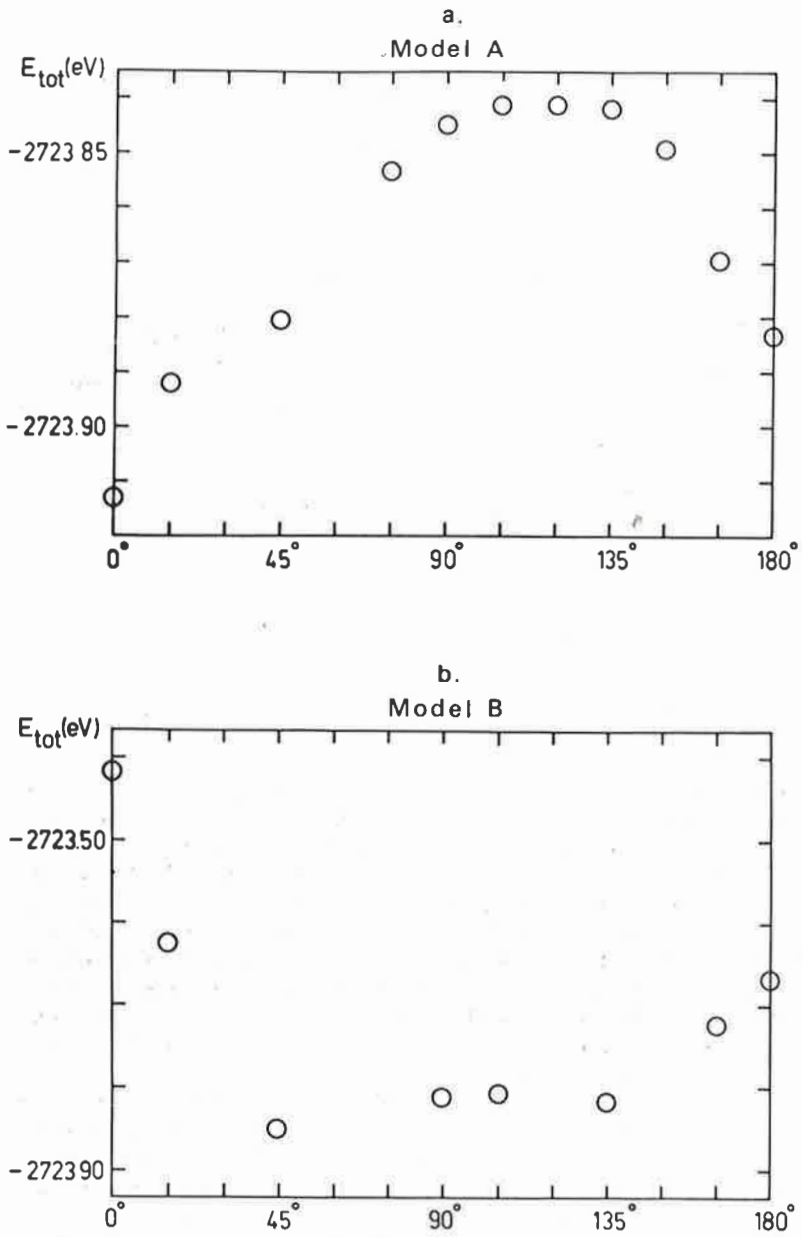

Figure 3. The total energy of veratrole as a function of the torsional angle of methoxy group *a*. In model A the methyl group of *a* was oriented for minimum steric interaction, in model B for maximum steric interaction the ortho ring hydrogen or substituent.

Table 1. The total energy and dipole moment of veratrole for different torsional angles of methoxy group *a*. In model A the methyl group of *a* was oriented as in *figure 1*, in model B rotated by 60° about the O_7-C_8 bond.

Torsional angle of methoxy group *a*	Model A		Model B	
	E_{tot}(eV)	μ(D)	E_{tot}(eV)	μ(D)
0° (planar I)	−2723.9129	2.61	−2723.4169	2.82
15°	−2723.8920	2.60	−2723.6217	2.75
45°	−2723.8807	2.64	−2723.8500	2.66
75°	−2723.8530	2.62		
90°	−2723.8447	2.56	−2723.8116	2.61
105°	−2723.8412	2.54	−2723.8065	2.56
120°	−2723.8412	2.48		
135°	−2723.8417	2.42	−2723.8173	2.39
150°	−2723.8490	2.31		
165°	−2723.8694	2.26	−2723.7234	2.30
180° (planar II)	−2723.8834	2.28	−2723.6668	2.29

formation planar I is at an energy value considerably lower than expected. Extrapolation of the energy curve to the torsional angle 0° gives the total energy −2723.8960 eV, compared to the calculated value −2723.9129 eV with the $C_1O_7C_8$ bond angle 120°. This extra stabilization is obviously due to the formation of a hydrogen bond between two of the methyl group hydrogens and the lone pair electrons on oxygen in the neighbouring methoxy group. On the other hand, the repulsion between the oxygen lone pairs makes the conformation planar II less stable than otherwise expected.

In order to obtain an estimate of the strength of the hydrogen bond, the effect of the variation of the $C_1O_7C_8$ bond angle was studied. The results of these calculations are shown in *table 2* and *figure 4*. A minimum energy of −2723.9430 eV was obtained for the bond angle 116.5°. Comparison of this energy with the extrapolated total energy −2723.8960 eV gives the rather small value 4.53 kJ/mole (1.08 kcal/mole) for the hydrogen bond.[19] It should be pointed out that the same result should be obtained if the $C_3C_2O_9$ bond angle is allowed to increase from the assumed value 120°.[12,20]

Table 2. The total energy and dipole moment of veratrole in the planat I conformation for different values of the $C_1O_7C_8$ angle.

$C_1O_7C_8$ angle	E_{tot}(eV)	μ(D)
110°	−2723.8498	2.81
115°	−2723.9352	2.70
120°	−2723.9129	2.61
125°	−2723.8261	2.50
130°	−2723.6961	2.40

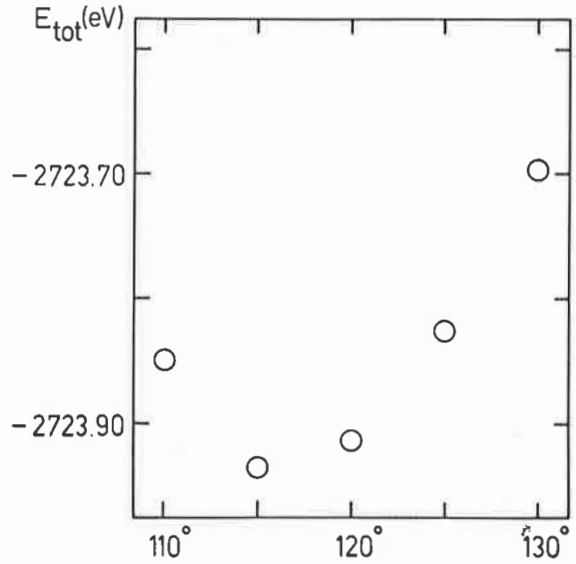

Figure 4. The total energy of the conformation planar I as a function of the $C_1O_7C_8$ bond angle.

With model B, two minima were also obtained, but now for the torsional angles 45° and 135°, the former conformation again being slightly more stable. The planar conformations are when this model is used considerably less stable than any of the non-planar conformations (*figure 3b*).

Finally the effect of variation of the torsional angles of both methoxy groups was studied. This was done with the torsional angles 45° and 90°. The calculated total energies, together with the dipole moments are given in *table 3*. All the conformations with the torsional angles 45° are of approximately the same stability, and they are less stable than the conformations planar I and planar II in model A. However, compared with the con-

Table 3. The total energy and dipole moment of veratrole for different conformations. The torsional angles of both methoxy groups were varied.

Conformation	E_{tot}(eV)	μ(D)
Cis I 45°	−2723.6875	2.90
Trans I 45°	−2723.6937	2.08
Cis II 45°	−2723.6601	2.87
Trans II 45°	−2723.6601	2.03
Cis 90°	−2723.7420	3.10
Trans 90°	−2723.7985	1.07

formation planar I model B, they are considerably more stable, and of approximately the same energy as conformation planar II model B. At still lower energies we find the conformations *cis* 90° and *trans* 90°.

If therefore the rapid reorientation of the methyl groups is taken into account, this means that the conformations with large out-of-plane angles are most readily accessible at room temperature. This conclusion is supported by the results of the measurements of the dipole moment of veratrole. As the strength of the hydrogen bond in the planar I conformation is only 4.53 kJ/mole it is concluded that the amount of molecules in that conformation is small.

A considerable amount of experimental work has been devoted to the study of the dielectric relaxation of veratrole.[21–27] According to Hase [21] the mobility of the methoxy groups calculated from the measured relaxation times is smaller in anisole than in veratrole. Later Roberti and Smyth,[23] Vaughan and Smyth [24] and Klages and Zentek [25] were able to resolve two distinct dispersion regions, one for the molecular rotation, the other caused by the torsional oscillations of the methoxy groups. In anisole the major contribution to the orientation seems to be from molecular rotation, whereas the reverse is true for veratrole. As pointed out by Hase,[21] this result can be understood if it is assumed that the methoxy groups in veratrole lie *trans* with respect to the plane of the ring, and with torsional angles near 90°.

Additional structural information about the conformational behaviour of the methoxy groups in veratrole is provided by measurements of the electric dipole moment and especially by its temperature dependence.[18,23,26,28–31]

At room temperature the dipole moment of veratrole is found to be 1.31 D. The calculated dipole moment of the *trans* 90° conformation is 1.07 D, and for the conformations *trans* I 45° and *trans* II 45° 2.08 and 2.03 D respectively. The rather low experimental value means that the contribution of the less polar conformations should be large at room temperature.

The dipole moment of veratrole increases with temperature.[23,26,28,30,31] DiBello, McDevitt and Roberti [31] measured the dipole moment of liquid veratrole over the temperature range 25 to 160°C, and over a total range of −20 to 165°C in benzene, decalin and paraffine oil. In these measurements the dipole moment increased with temperature with nearly the same slope, and reached above 90°C the constant values 1.58 D in paraffine oil, and 1.64 D in decalin. The dipole moment of the pure liquid showed no levelling even at the highest temperature 160°C, where its value was 1.63 D. However, above 60°C the increase of the dipole moment with temperature was not linear. The fact

that the dipole moment increases with the temperature can be understood if the energies of the different conformations is considered. The observed dipole moment is a weighted average, where the contribution from each possible conformation of the molecule is weighted by a Boltzmann factor according to its energy. At higher temperature therefore, the contribution from the more polar conformations with a higher total energy increases. The maximum observed dipole moment in paraffine oil and decalin in which levelling was observed, was still considerably lower than the value 1.93 D, calculated for a hypothetical state of free rotation of both methoxy groups about the $C_{Ar}-O$ bonds.[32] This means that certain more polar conformations are so severely restricted that their contribution is practically constant, and shows no appreciable increase with temperature.

From measurements of both dipole moments and molar Kerr constants Aroney, LeFèvre and Chang [33] concluded that no single conformation of veratrole could account for the experimental results. The hypothesis was advanced that the oxygen atoms in veratrole, by analogy with o-dichlorobenzene [34] perhaps were not coplanar with the aromatic ring. However, a recent X-ray crystallographic investigation of the structure of 2-hydroxy-1,3,4,7-tetramethoxyanthrone [20] shows that the deviation from the least squares plane for the oxygen atoms staying *ortho* to each other does not exceed 0.06 Å. On the other hand the methoxy groups in the positions 3 and 4 are rotated out of the least squares plane by 58.1° and 83.0° respectively about the $C_{Ar}-O$ bonds. In contrast, the angle of rotation is only 5.1° for the methoxy group in position 7 which is not hindered by adjacent substituents. This again strongly suggests large out-of-plane angles for the methoxy groups in veratrole.

Anomalous behaviour of veratrole can also be demonstrated by NMR spectroscopy. The ring proton chemical shifts for protons in the *ortho* position to the methoxy groups cannot be calculated as simple sums of the substituent shielding constants.[35] For a number of benzene derivatives, Lauterbur has demonstrated an additive relationship for the effect of substituents on aryl ^{13}C shieldings.[36–38] Dhami and Stothers [39] found that this additive relation does not hold in *ortho* substituted anisoles, and the two methoxy groups in veratrole seem to have a larger effect on the ring carbons than they would have separately. From the data obtained one may conclude that the preferred conformation is one with the methoxy groups turned away from each other. Similar results were also obtained by Crecely, McCracken and Goldstein [40] from the study of long-range spin-spin couplings in substituted anisoles.

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The Search for Microenzymes: The Enzyme of Bacillus cereus

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Abstract

Microenzymes are defined as enzymes, including subunit forms with molecular weight less than 10,000. Microenzymes were initially detected on the basis of their ability to penetrate thru an Amicon UM-10 membrane which effectively excludes substances of molecular weight greater than 10,000, and which did in practice exclude over 99 % of Cytochrome C (MW 12,400). A single exogenous microenzyme was found to exist in equilibrium with oligomers, with oligomers predominating, in a strain of Bacillus cereus (BRL-70).

Calcium ion was found to stabilize and to activate the enzyme. Lead ion also served this role. Other divalent ions were less effective stabilizers of the enzyme. Monovalent and aluminum ions had little or no stabilizing effect. The enzyme was extremely unstable below pH 5.0, had peak activity about pH 6.5 and retained considerable activity even after prolonged storage at pH 10.0.

Gel filtration gave an enzyme activity peak at a molecular weight somewhat greater than Cytochrome C (MW 12,400) but with activity tailing out to the region corresponding to molecular weight 2,500. Treatment with anhydrides caused the enzyme activity to peak at a higher molecular weight. Sodium dodecyl sulfate treated enzyme in acrylamide gel electrophoresis gave bands around 5,000—5,500, 8,000—8,200, 10,700 and 16,000. These relate to each other in the ratio of 2:3:4:6, implying a monomeric weight of about 2,700 for this microenzyme.

Introduction

Microenzymes are defined as enzymes, including monomer or subunit forms, with molecular weights below 10,000.[1] The search for microenzymes was initiated at this laboratory as a result of aging research which indicated a possible therapeutic utility for such enzymes.[2]

In the aging process, amorphous "hyalin" materials accumulate in and around cells and gradually impede the functionality of the cells and consequently of the body. These hyalin materials are mostly proteinaceous, and appear to be highly cross-linked, dense, insoluble, and resistant to dissolution by the body's enzymes.[3] The insolubility and high density of the materials suggests that inability to penetrate the hyalin matrix

may explain why normal body enzymes do not eliminate this material.

Since these materials *are* broken down naturally after death, enzymes for achieving such dissolution clearly do exist. If the high density of these hyalin materials prevents their dissolution by the body's enzymes, then a very low molecular weight proteolytic enzyme — a microenzyme — seems called for. The search for such enzymes in our laboratory has lately centered around spore-forming microorganisms whose spores also have extremely dense walls, which they obviously must at least partially dissolve in order to germinate.

Evidence for the existence of low molecular weight enzymes was reported earlier.[2] One such enzyme was found to dissolve hyalin materials in kidney in vitro.[4] Subsequent evidence has indicated that the enzyme most intensely studied — that from a strain of Bacillus cereus (BRL-70) — exists in equilibrium with oligomers of molecular weights between 10,000 and 25,000. The effects of pH and ionic composition on this enzyme are reported here, as are ultrafiltration and electrophoresis studies, plus other efforts to establish the molecular weight of the monomeric enzyme.

Methods

Production of enzyme solution

A strain of Bacillus cereus (BRL-70) was inoculated into a 500 ml shaker flask with 100 ml of broth containing 0.5 g of bacteriological peptone (Nutritional Biochemicals Co., Cleveland, Ohio), 0.3 g of yeast extract (also Nutritional Biochemicals Co.), and 0.5 g of glucose, pH 7.0 ± 0.2, 34°C, (SAR Broth). Sixteen hours later the culture was inoculated into a 14 liter New Brunswick fermentor containing 10 L of broth at 34°C with 150 g of bacteriological peptone, 90 g of yeast extract, and 50 g of glucose in unsoftened tap water, adjusted to pH 7.0 ± 0.2. Growth was monitored by turbidity, pH measurements, and finally for proteolytic activity by Congocoll assay.[5] A 20 % glucose solution was added periodically until the total glucose added was 450 g.

After 74 hours, the broth, at pH 7.8, contained proteolytic activity of over 1,000 Congocoll units per ml. A sample of cell-free broth was filtered thru an Amicon UM-10 membrane designed to retain materials of molecular weight greater than 10,000. Approximately 6 units of proteolytic activity per ml passed thru the membrane. The microbial cells were removed from the rest of the broth by passage thru a Sharples supercentrifuge and the broth was then frozen until used. Over 300

units or 30 % of the activity passed thru the UM-10 membrane when the broth was subsequently thawed and filtered. High percentages of activity have passed thru intact UM-10 membranes on several occasions but we have not as yet been able to achieve this consistently.

Assays for proteolytic activity

Proteolytic activity was routinely determined by the Congocoll method of Nelson et al.[5] This method was found reliable in this work, except where variability was sometimes observed at very high enzyme concentrations. For extremely low enzyme concentrations, the caseinolysis method of Williams and Chase was used.[6]

After it became apparent that calcium ion protected enzyme activity, 1×10^{-4} M $CaCl_2$ was incorporated in the pH 7.2 Tris buffer in which the enzyme and Congocoll were incubated. A Congocoll unit, as used in this study, is defined as an absorbance of 1.0 at 495 nm produced by 1.0 ml of enzyme solution reacting with 20 mg. of Congocoll for 30 minutes at 30°C.

Ultrafiltration

Enzyme samples were filtered thru Amicon UM-10 membranes (Amicon Co., Lexington, Mass.) with nitrogen at 40 – 50 psi pressure at 4°C. These membranes are designed to retain substances with molecular weights greater than 10,000, and did routinely retain over 99 % (generally over 99.5 %) of an aqueous solution of Cytochrome C (M. W. 12,400; Calbiochem, A grade, equine heart).

Studies on the effect of calcium ion concentration and pH on penetration of the enzyme thru the membrane were carried out. Salts of lithium, sodium, magnesium, calcium, barium, strontium, and zinc were added at 0.05 M concentration to calcium-free enzyme solutions in 0.01 M N-ethyl morpholine (NEMO) at pH 7.0 (zinc at pH 5.7), and left to equilibrate for four days at 4°C. The solutions were then diluted 50× with 0.01 M NEMO, pH 7.0, and were immediately passed thru a UM-10 membrane. The total activities of these solutions and the activities which passed thru the UM-10 membrane were then determined.

Enzyme samples were frequently concentrated over UM-2 membranes which are designed to retain substances with molecular weights over 1,000. Solutions in which the enzyme was dissolved were also changed by washing in the new solution over the UM-2 membrane.

Gel Filtration

Most gel filtration studies were run with Biogel P-10. A number of runs were also made with Sephadex G-50 as the fractionating material. Initially, filtration was with a pH 7.65 solution of 0.02 M N-ethyl morpholine (NEMO) containing 0.1 M NaCl and 0.001 M $CaCl_2$, but in most experiments the solutions were adjusted to pH 7.0. Other filtrations were carried out between pH 5.5 and 9.5 using 0.02 M Tris buffer, usually with maleate or glycine. Unless specifically excluded, $CaCl_2$ was routinely incorporated as a stabilizer in these solutions at 0.0001 M to 0.001 M.

Enzyme previously passed thru a UM-10 membrane was washed free of calcium ion with 0.02 M NEMO and 0.1 M NaCl. The solution was mixed with 0.3 M salt solutions to achieve a 0.1 M concentration of LiCl, NaCl (only), KCl, $MgCl_2$, $BaCl_2$, $Sr(NO_3)_2$, or $ZnCl_2$. Solutions containing 0.01 M lead acetate or aluminum nitrate were also prepared. The samples were equilibrated for at least one week at 4°C, and were then fractionated on a Biogel P-10 column with solutions at pH 7.0 containing 0.1 M NaCl, 0.02 M NEMO and 0.001 M concentrations of the particular salt tested. Since the Zn and Al equilibrated enzyme samples had essentially no activity when they came off the Biogel column, a $CaCl_2$ equilibrated enzyme of comparable potency was later fractionated thru the $ZnCl_2$ or $Al(NO_3)_3$ equilibrated columns.

Standards for molecular weight determination included Cytochrome C (M.W. 12,400), Vitamin B-12 (M.W. 1,355), a blue dextran of M.W. 10^6 and sucrose (M.W. 342). Pancreatic trypsin inhibitor (Worthington Biochem Co., Freehold, N.J.), one of the few compounds available with molecular weight about 6,000, appeared to have a contaminant (trypsin?) which made it unusable as a standard. A yellow dextran of M.W. 20,000 supplied by Pharmacia (Uppsala, Sweden) was used in several instances.

Electrophoresis

The preparative electrophoresis method of Whitehead et al[7] was used initially as a technique for purification of the microenzyme. Electrophoretic mobility and enzyme purity were evaluated with this technique using Sephadex G-25 as the support medium. The enzyme moved toward the cathode at pH 5.5 and 7.0 and toward the anode at pH 9.0, so these poles were placed at the bottom of the column. After electrophoresis the samples were eluted from the column with the same buffer.

Gel electrophoresis using the method of Weber and Osborn[8] was utilized to determine the molecular weight of the micro-

enzyme. A 4X concentration of their cross-linker (methylene bisacrylamide) was used to achieve density high enough to fractionate the low molecular weight components.

Dispersement Studies

Treatment of oligomeric proteins with succinic and other anhydrides has been reported to disperse these proteins to monomeric form.[9,10] Several anhydrides were therefore used in attempts to disperse and stabilize the monomeric or subunit form of the microenzyme. Molecular weight distributions of the resulting substances were evaluated by gel filtration.

Treatment of proteins with sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (2ME) was also reported to disperse proteins into their subunits.[11] Enzyme solutions treated with these materials were fractionated by gel filtration as well as electrophoretically.

Results

Initial Studies

The initial criterion for the detection of microenzymes was ultrafiltration thru an Amicon UM-10 membrane, which reputedly retains substances of molecular weight greater than 10,000; and which did in practice routinely retain 99+ % of Cytochrome C (M.W. 12,400).

It gradually became clear, however, that the enzyme is not a minor component in a solution containing a large amount of high molecular weight enzyme; but rather that the enzyme exists in a monomer-oligomer relationship with an equilibrium normally greatly favoring the oligomer. Thus a Biogel P-10 fractionation of an enzyme which had previously passed thru a UM-10 membrane, showed the enzyme activity centered at a molecular weight above Cytochrome C, but its activity tailed out to the low molecular weight side as shown in Figure 1. This characteristic pattern occurred routinely in these studies. Unfiltered enzyme was found to give an identical activity curve, indicating that we were dealing with a single enzyme system, so subsequently unfiltered systems were normally used.

Fractionation on Sephadex G-50 also gave a distribution curve where the enzyme eluted substantially before Cytochrome C (Figure 2), but again the enzyme exhibited the characteristic tailing.

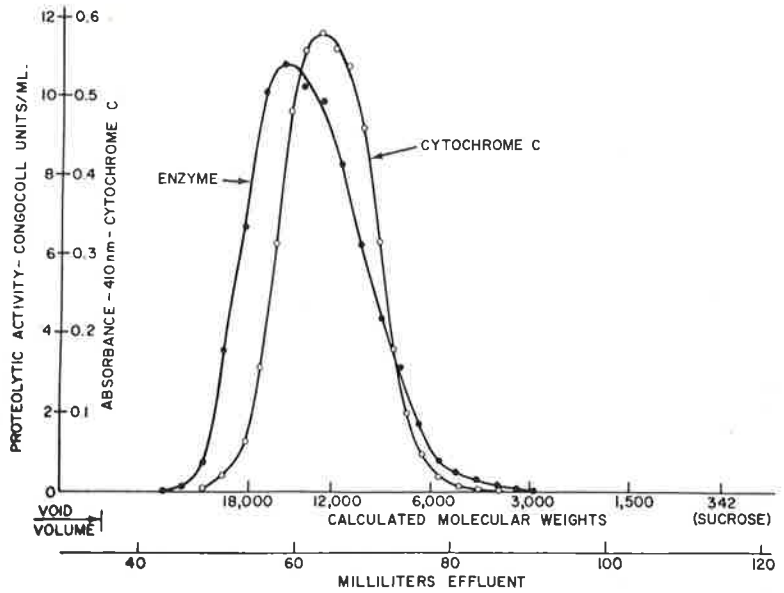

Fig. 1. Activity distribution of enzyme after fractionation on Biogel P-10 column. Cytochrome C distribution curve was added to show the tailing of enzyme activity to the lower molecular weight region.

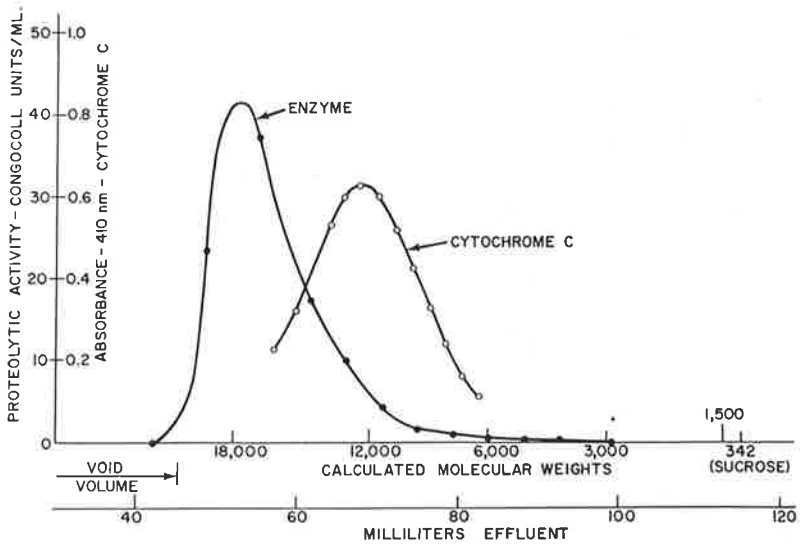

Fig. 2. Activity distribution of enzyme after fractionation on Sephadex G-50 column. Cytochrome C distribution curve was again added to show the tailing of enzyme activity.

Evidence for a single enzyme

Electrophoretic studies at pH 5.5, 7.0, and 9.5 showed that the enzyme migrated toward the cathode slowly at pH 5.5 and very slowly at pH 7.0; and moved slowly toward the anode at pH 9.5 (Figure 3). As in the gel filtration, only one enzyme peak was observed, plus the usual tailing which occurred also when a sample was passed thru the Sephadex G-25 supporting medium without prior electrophoresis. Since Sephadex G-25 fractionates only up to about M.W. 5,000, the tailing suggests that the monomeric enzyme may have a molecular weight less than 5,000. In conjunction with the gel filtration data the presence of only one electrophoretic peak at three differing pH's makes it highly unlikely that there is more than one proteolytic enzyme system present.

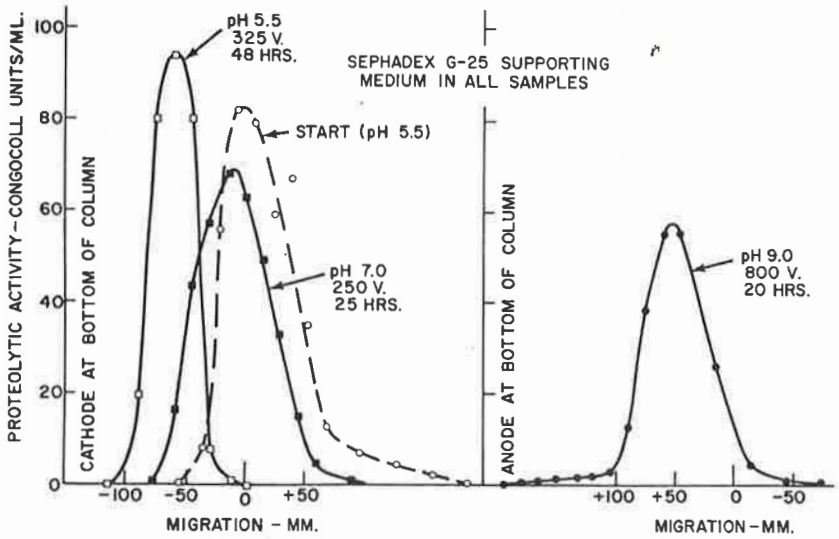

Fig. 3. Electrophoretic mobility of enzyme at pH 5.5, 7.0, and 9.5. Samples were placed at the top of a Sephadex G-25 column at the start of electrophoresis; and were eluted from the column after electrophoresis was completed.

Evidence for the presence of only one enzyme contrasts with the results of Furukawa et al [12] who reported three exogenous proteolytic enzymes produced by their strain of Bacillus cereus. However these were alkaline proteases, whereas ours is a neutral protease. Feder et al [13] reported only one "neutral protease" in their strain of Bacillus cereus. Their enzyme was also stabilized by calcium and had a maximum activity around pH 7.0 – 7.2. These properties resemble those of our enzyme. They reported

the presence of 1 mole of zinc per 63,000 g of enzyme, thus indicating a minimum molecular weight of 63,000.

Aronson et al [14] also found only one principal protease in broth from their Bacillus strains. This was an extracellular enzyme activated by calcium ion.

Tests of inhibitors and preservatives

Crude enzyme solutions were mixed with a number of enzyme inhibitors and potential preservatives. The activities of the solutions were determined after 20 hours at 4°C. Table I shows that many of the solutions had activities greater than the control. Only sodium fluoride, ferric chloride and cobaltous chloride appeared to inhibit the enzyme activity. In another series stored for 78 days at room temperature, cobaltous chloride treated enzyme gave the lowest activity. Thimerosal appeared to be most effective for protecting the enzyme activity, so it was incorporated at 0.001 – 0.01 % in our solutions.

Table I. Effects of inhibitors and preservatives on proteolytic activity of enzyme solutions.

Substance	Proteolytic Activity, Congocoll Units/ml	
	20 Hours	78 Days
Cadmium chloride, 0.001 M	14.2	
Cupric chloride, 0.001 M	19.2	11.2
Cobaltous chloride, 0.001 M	10.8	3.0
Cysteine, 0.001 M	15.6	
Ferric chloride, 0.001 M	10.8	
Plumbous nitrate, 0.001 M	13.6	
Manganous chloride, 0.001 M	16.0	15.1
Mercuric chloride, 0.001 M	14.6	
Sodium azide, 0.001 M	13.2	
Sodium cyanide, 0.01 M	16.6	13.3
Sodium fluoride, 0.001 M	8.4	
Sodium salicylate, 0.001 M	17.2	14.7
Sodium benzoate, 0.1 %	16.6	11.2
Thymerosal, 0.01 %	16.8	19.6
Thymol, 0.05 %	17.4	13.7
Control	13.0	

Calcium ion and pH effects

Calcium concentration – Enzyme containing solutions were stored in 0.01 M Tris solutions containing several levels of $CaCl_2$ for 6 days. Total enzyme activity was then assayed. Table II shows that a concentration of 3×10^{-5} M $CaCl_2$ or higher substantially stabilized the enzyme.

Table II. Effects of calcium ion concentration on enzyme stability in 0.01 M Tris solutions, pH 7.2, after storage for 6 days at 4°C

$[Ca^{+2}]$	Proteolytic Activity, Congocoll Units/ml
0	9.0
1×10^{-5}	11.0
3×10^{-5}	21.1
1×10^{-4}	16.5
3×10^{-4}	22.2
1×10^{-3}	22.5

A further study at several pH's indicated that high calcium ion either activated the enzyme or deactivated an enzyme inhibitor, since the activity at 0.01 M $CaCl_2$ was substantially higher than the original enzyme activity (Table III). The enzyme was found to be less stable in the absence of calcium ion, particularly at higher pH.

Table III. Proteolytic activity of enzyme solutions after storage for three weeks at 4°C.

Sample	Proteolytic Activity, Congocoll Units/ml		
Original solution	150		
	0 Ca^{+2}	10^{-3} Ca^{+2}	10^{-2} Ca^{+2}
pH 7.1	117	157	216
pH 7.6	67	157	229
pH 8.2	52	150	183

However, at very high calcium concentrations (i.e. 0.1 M or higher) enzyme activity decreased, though this may be an effect of the calcium on the substrates collagen and casein.

Enzyme Dilution Effects – Ultrafilterability, or passage thru a UM-10 membrane, was reduced by calcium ion; but dilution of the enzyme greatly enhanced its ultrafilterability, even with calcium ion present (Tavle IV). Such a shift toward the monomeric form is expected for monomer-oligomer equilibria upon dilution. Therefore, this behavior suggests a monomer-oligomer equilibrium.

Table IV. Effects of Calcium ion and dilution on ultrafilterability of microenzyme.

Enzyme Solution	Proteolytic Activity, Congocoll Units/ml		
	Original Enzyme	Ultrafiltrate	
		Orig. Conc.	1/100 Dilution
0.02 M NaCl	150	0.27	11.1
0.001 M $CaCl_2$ in 0.1 M NaCl and 0.02 M NEMO	150	0.13	2.1

pH and Temperature Effects – Enzyme samples buffered at pH 3.5 thru 10.0 were assayed after storage for one week at 4°C. Table V – A shows that samples stored below pH 5.0 had lost all activity. Most of the activity was retained in the rest of the samples. Optimum storage pH was at pH 6.5 or 7.0. This is also the optimum pH for enzyme activity (Table V – B).

Table V. Effects of pH on enzyme activity.

A. Identical enzyme samples stored at different pH's. Assayed at pH 7.2

pH (Storage)	3.5–4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	9.0	10.0
Proteolytic Activity, Congocoll Units/ml	0	139	159	162	184	177	161	159	148	119

B. Identical enzyme samples assayed at different pH's.

pH (Assay)	4.5	5.0	6.0	6.5	7.0	8.0	9.0	10.0
Proteolytic Activity, Congocoll Units/ml	75	83	145	160	135	90	24	7

Congocoll assays of the enzyme, run at 5°C intervals, showed that the enzyme activity increased to a temperature of 50°C (Figure 4) in solution containing 10^{-4} M $CaCl_2$.

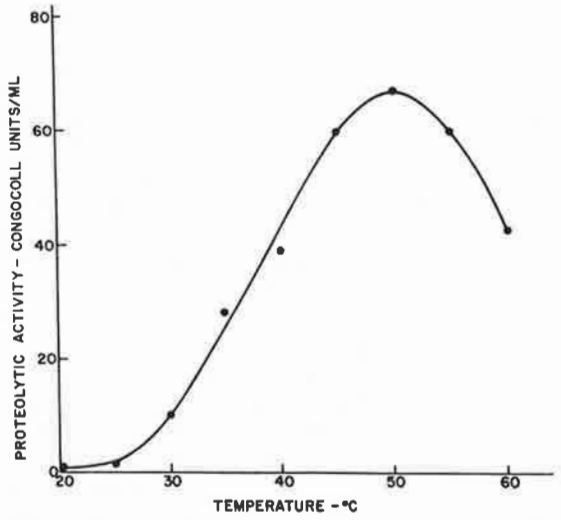

Fig. 4. Activity of enzyme at different temperatures as determined by a Congocoll assay requiring a 30-minute incubation.

EDTA – A 100 ml enzyme sample, washed free of calcium ion with 0.1 M NaCl and 0.002 M NEMO solution at pH 7.0, was placed in an ultrafiltration cell over a UM-10 membrane. Ninety mls of the solution was filtered thru the membrane under 40 psi nitrogen pressure and the sample was rediluted with the same solution except that 0.0001 M EDTA was added. Ninety mls was filtered thru again. Ninety ml samples with increasing concentrations of EDTA were subsequently added and filtered thru the membrane. The proteolytic activity passing thru the UM-10 membrane increased substantially and then decreased as the EDTA concentration was increased (Table VI). The

Table VI. Effects of increasing concentrations of EDTA on penetration of microenzyme thru UM-10 ultrafilter.

EDTA Concentration	Proteolytic Activity, Congocoll Units/ml Thru Membrane	Above Membrane at start
None	0.2	975
0.0001 M	2.0	870
0.0002 M	5.9	700
0.0003 M	0.6	–
0.00036 M	0.1	–
10^{-6} M $CaCl_2$	0.1	660

proteolytic activity above the membrane also decreased somewhat, but not proportionally. This behavior appears to corroborate other evidence which indicates the presence of inactive higher molecular weight oligomers under conditions where calcium concentration is suboptimal. Thus, enzyme samples stored in solutions devoid of calcium ion for several weeks and then fractionated on a Biogel P-10 column with an effluent containing $CaCl_2$ consistently showed an increase in enzyme activity two weeks after fractionation (Figure 5). The increased activity usually peaked around the 15,000 and 20,000 molecular weight regions. This indicates a molecular weight around 5,000 or a fraction of this for the monomeric enzyme.

Figure 6 shows that the enzymatic activity was greatly reduced merely by fractionating the enzyme on a Biogel column in the absence of calcium ion. Addition of 5×10^{-4} M EDTA to chelate ions released by the enzyme almost totally eliminated any residual enzyme activity. This indicates that calcium ion is essential for the stability and the activity of the enzyme. Also it suggests that the enzyme has a strong chelating effect on calcium ion.

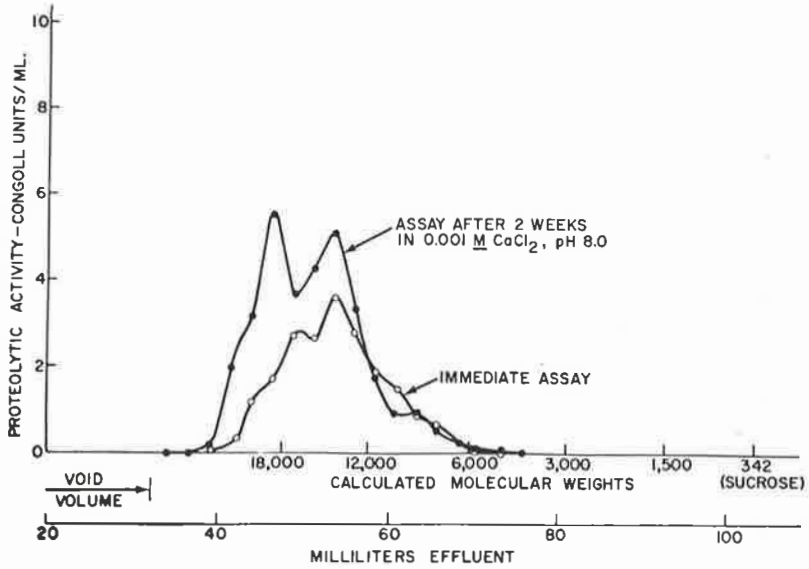

Fig. 5. Activity distribution of enzyme previously stored without calcium ion, immediately after fractionation on Biogel P-10 column with solution containing 10^{-3} M $CaCl_2$; and increased activity of fractionated samples after storage for two weeks in the calcium containing solution.

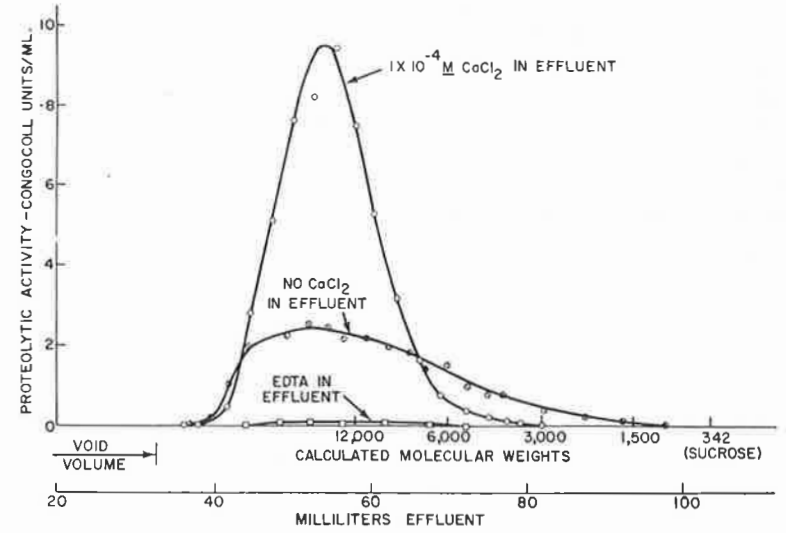

Fig. 6. Distribution and total activities of enzyme samples fractionated on Biogel P-10 in the presence of calcium ion, in the absence of calcium ion, and with EDTA to chelate any calcium ion present.

Effects of other metallic ions

The proteolytic activities of enzyme solutions stored in the presence of a number of different cations are shown in Table VII. A comparison with the percentage activity which passed thru the UM-10 membrane, also shown in the table, indicates an inverse correlation between total activity and amount of enzyme passing thru the membrane. This implies that the monomeric form of the enzyme is less stable than the oligomer. Sodium and calcium stand out as exceptional in that they permitted a substantial amount of activity to pass thru the membrane but still retained most of their total activity. Barium and strontium had this effect to a lesser degree.

Table VII. Proteolytic activity and ultrafilterability of enzyme solutions after storage for 4 days at 4°C in 0.05 molar solutions of the specified salt. The solutions were buffered with 0.01 M N-ethyl morpholine at pH 7.0 (zinc at pH 5.7)

	Proteolytic Activity, Congocoll Units/ml						
Salt							
Total Proteolytic Activity	LiCl 6.5	NaCl 11.5	$MgCl_2$ 5.5	$CaCl_2$ 12.8	$BaCl_2$ 8.7	$Sr(NO_3)_2$ 11.9	$ZnCl_2$ 15.8
Activity Thru UM-10 Membrane	0.22	0.71	0.34	0.33	0.53	0.08	0.03
% Thru Membrane	3.4	6.2	6.2	2.6	6.1	0.7	0.2

The total proteolytic activities of enzyme solutions equilibrated with salts of several cations for two weeks are shown in Table VIII. Again, as was seen in Table VII, sodium ion preserved the activity better than did the other monovalent cations. Aluminum and zinc containing solutions had lost almost all of their activity. These two samples contained rather large precipitates. Calcium and strontium had retained most of their activity. Activity retained and molecular weight of the equilibrating ion did not appear to be correlated.

Table VIII. Proteolytic activities of enzyme solutions after storage for 2 weeks at 4°C in 0.1 M salt solutions also containing 0.07 M NaCl.

Salt	Congocoll Units/ml	Salt	Congocoll Units/ml
$AlCl_3$	9		
$MgCl_2$	540	$ZnCl_2$	21
$CaCl_2$	1,380	LiCl	150
$BaCl_2$	405	NaCl (only), 0.1 M	534
$Sr(NO_3)_2$	1,200	KCl	195

The proteolytic activities and activity distributions of the above enzyme solutions and also of a sample equilibrated with lead acetate are shown in Figure 7 after fractionation on Biogel

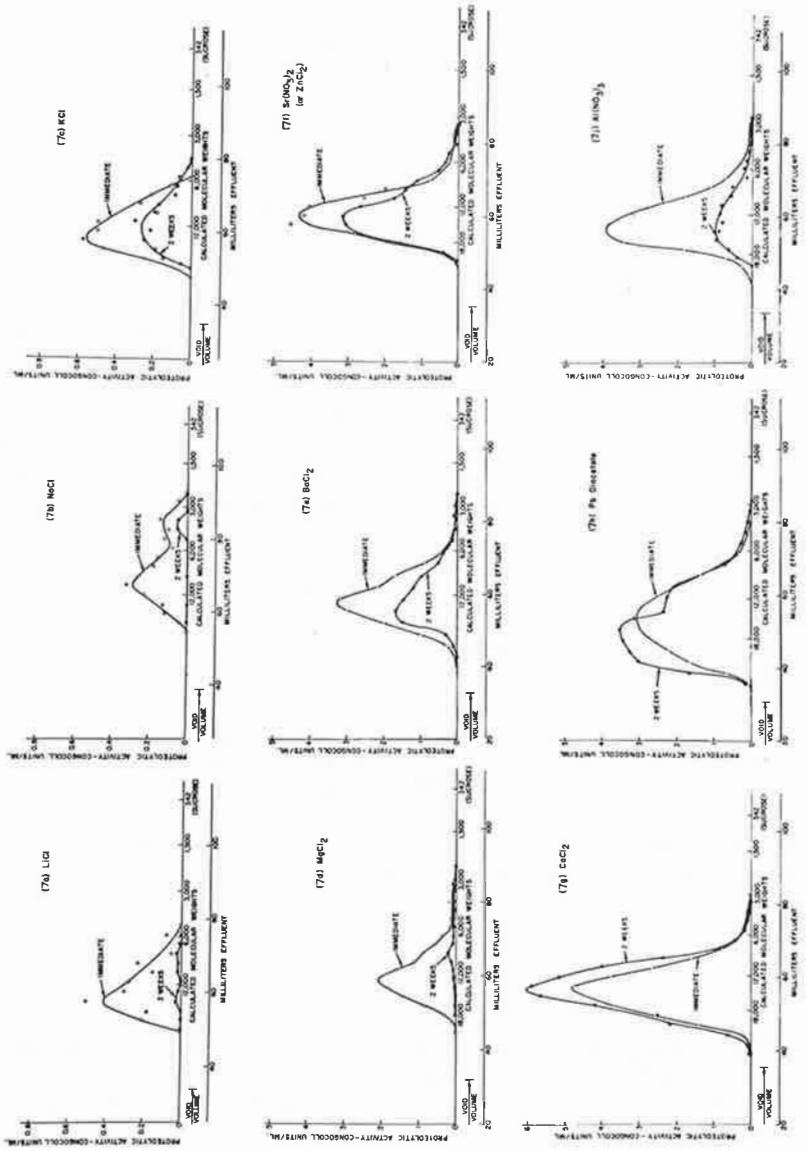

Fig. 7. Activity distribution of enzyme fractionated in the presence of a number of differing monovalent, divalent and trivalent ions. Activities were assayed immediately and then two weeks after fractionation.

7A	LiCl	7F	$Sr(NO_3)_2$ or $(ZnCl_2)$
7B	NaCl	7G	$CaCl_2$
7C	KCl	7H	$Pb(OAC)_2$
7D	$MgCl_2$	7J	$Al(NO_3)_3$
7E	$BaCl_2$		

P-10. A second curve in each graph shows the activities of the same sample following a subsequent two week storage at 4°C. The activities of the samples equilibrated with monovalent ions was so weak that an expanded scale was required to effectively show the activities. The maximum lithium (7A) and potassium (7C) enzyme activities eluted at around molecular weight 15,000, but the activity tailed out to the region at which substances of 5,000 molecular weight elute; sodium (7B) enzyme activity eluted at about molecular weight 10,000 and extended to the region corresponding to molecular weight 2,500.

The divalent cations gave greater activity, and the activity increased with molecular weight of the cation (7D to 7F). The decrease in activity two weeks after fractionation was also proportionately less with the higher molecular weight divalent ions. Zinc gave results essentially identical to strontium (7F) and so was not shown. Calcium was a clear exception (7G). It not only gave exceptionally high initial activity, but it also usually produced an increase in activity after two weeks. Lead also gave an increase in activity two weeks after fractionation (7H). The lead-enzyme distribution curve was much broader than for the other cations, due to activity beginning at a higher MW. This may indicate a greater tendency for higher molecular weight oligomers.

These results indicate that calcium and probably lead have a protective as well as an activating effect on the enzyme. On the other hand, the trivalent aluminum ion (7J) had less protective effect than did the higher molecular weight divalent ions. The results support the earlier ultrafiltration studies (Table VII) which showed an increased total activity with increasing mass for divalent ions. Divalent ions again tended to give greater stability than did monovalent ions.

Molecular weight studies

Double Biogel run – The variable rates at which the enzyme moved thru the UM-10 membrane as well as the regular tailing out of the enzyme activity – often to the 2,500 molecular weight region – indicated that we were dealing with a microenzyme in equilibrium with its oligomers, with the oligomers predominating.

Early evidence had suggested a molecular weight of 5,700 ± 20 % for the enzyme.[3] To check this a sample was passed thru Biogel P-10 and a second sample at the 5,000 – 6,000 molecular weight region was promptly repassed thru the same column. Figure 8 shows that the profiles of both samples were very similar. This indicates that the equilibration rate of the enzyme

was rather rapid. The absence of any second peak in the second pass also further confirms the presence of only one enzyme system.

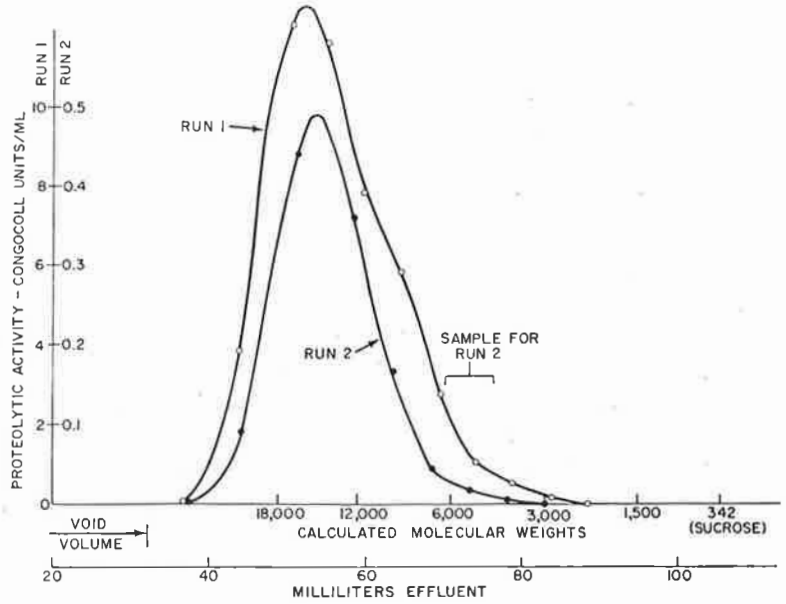

Fig. 8. Activity distribution of enzyme fractionated on Biogel P-10 column and activity distribution of the 5,000 – 6,000 molecular weight region from above sample after it was again fractionated on the Biogel P-10 column (Run 2).

Treatment with anhydrides – Reaction of aggregated proteins with succinic and other anhydrides, reported to favor dispersal of protein subunits, instead appeared to favor aggregation. Figure 9 shows the effects of treatment with citraconic anhydride. The enzyme activity peaked at a higher than usual molecular weight, 20,000 or higher, and had much less intense tailing toward lower molecular weights. The activity increased substantially after incubation for two weeks at pH 6.0 and 40°C. Similar high molecular weight fractions occurred after treatment with other anhydrides, but the activity was not increased by subsequent incubation.

Treatment with sodium dodecyl sulfate – An enzyme sample treated with 1 % sodium dodecyl sulfate (SDS) in the presence of 2-mercaptoethanol (2 ME) was fractionated on a Biogel P-10 column with pH 7.0 buffer containing 0.1 % SDS, 0.1 % 2 ME, 0.02 M NEMO and 0.1 M NaCl. A comparable run was made with Cytochrome C previously treated with SDS and 2 ME.

The SDS-Cytochrome C peaked almost at the void volume

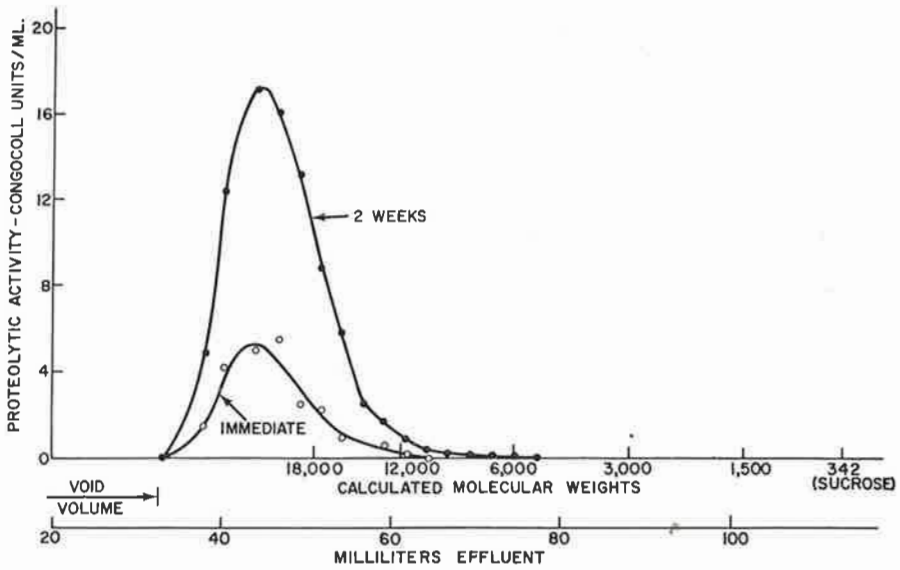

Fig. 9. Activity distribution of enzyme fractionated on Biogel P-10 column following reaction with citraconic anhydride. Sample fractions were assayed immediately after reaction and fractionation, and again after two weeks incubation at 40°C and pH 6.0.

on Biogel P-10, whereas normally it peaks midway in the effluent (Figure 10). The SDS-enzyme peaked with the SDS-Cytochrome C, but showed a quite substantial tailing. These results indicate that the effective molecular volumes of proteins are greatly increased by SDS treatment, probably due to attachment of large numbers of SDS molecules. The tailing of the enzyme also supports the evidence for a low molecular weight monomer in equilibrium with oligomer.

SDS-enzyme on acrylamide gel — An acrylamide gel with 4X the concentration of cross-linker used by Weber and Osborn [8] finally succeeded in fractionating a concentrated microenzyme solution.

The bands for insulin and for the microenzyme were quite diffuse, whereas the bands for Cytochrome C and other markers were quite sharp. This is interpreted by us as resulting from a tendency for the insulin and microenzyme to oligomerize even in the presence of SDS. The front of the diffuse band was therefore considered to correspond to the distance migrated by that particular fraction. On this basis, in comparison with Cytochrome C (MW 12,400) and insulin (MW 5,700, dimer MW 11,400), the enzyme gave three bands corresponding to MW 5,500, 8,200 and 16,000 (Figure 11).

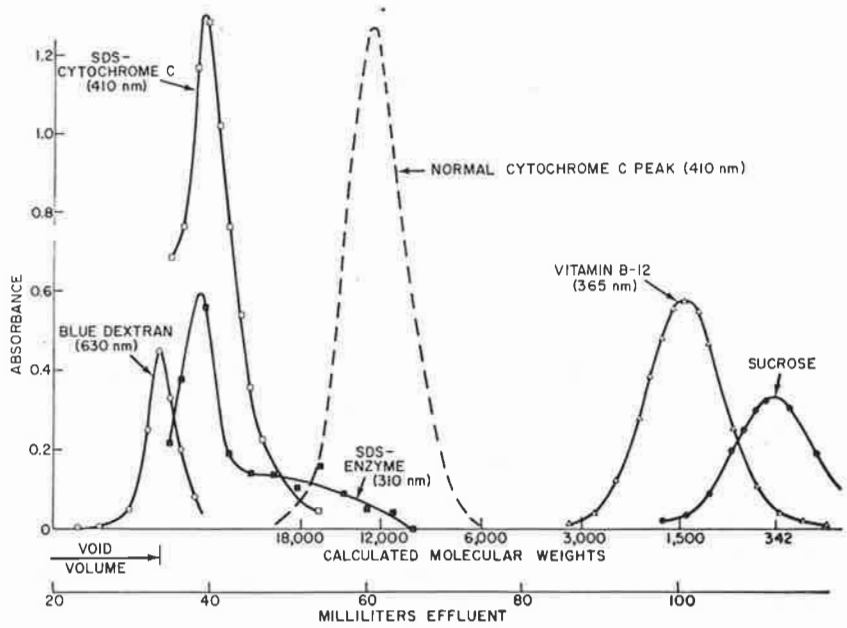

Fig. 10. Distribution of SDS-treated enzyme and of SDS-treated Cytochrome C after fractionation on Biogel P-10. The normal curve for Cytochrome C is also shown as are the curves for the blue dextran, Vitamin B-12 and sucrose.

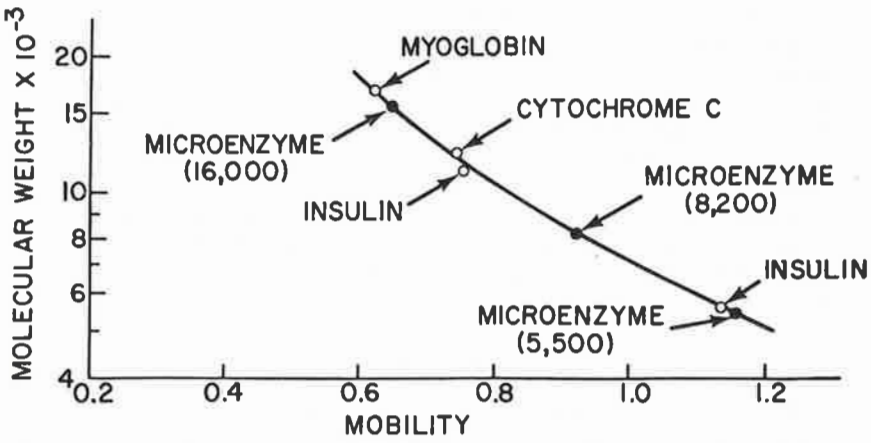

Fig. 11. Determination of molecular weights of *Bacillus cereus* microenzyme by plotting of mobility of enzyme fractions in comparison with substances of known molecular weight.

Other acrylamide gel runs gave values of 5,400 and 5,000 for the smallest component; and 8,000 and 10,700 for the next larger component. The 16,000 MW band did not stand out in these latter samples. The values obtained in these runs approximate ratios of 2:3:4:6; suggesting that the smallest component is a dimer and thus that the monomer probably has a molecular weight around 2,700.

This method is the only one of a multitude of methods used by us that has yielded reasonably convincing evidence for the molecular weight of the monomer of this enzyme. A number of methods have given results which could best be interpreted as revealing an enzyme with molecular weight near 5,000 or multiples thereof. If the molecule was actually a dimer, then these results seem in accord with the present observations.

Further Biogel studies – Alcohol was incorporated in the effluent of the Biogel P-10 column to lower the polarity of the solution in the hope that this would slow the equilibration rate and permit detection of the microenzyme monomer. When 10 % alcohol was incorporated into the effluent on the Biogel column, two rather unclear peaks at 3,000 and 6,000 molecular weights were observed (Figure 12). However, the enzyme activity also extended out to beyond the sucrose peak. At 33 % alcohol, the enzyme was evidently adsorbed, since none of the activity passed thru the column. This suggests that adsorption may have been responsible for much of the retardation in the 10 % alcohol fraction. However, the peaks around 6,000 and 3,000 have occurred consistently in several runs, and so may be real. Certainly they are consistent with the acrylamide gel values.

DEAE purification – A massive brown-colored impurity in the enzyme solution was observed to move during electrophoresis toward the anode even at pH 5.5. Removal of this impurity with DEAE cellulose or DEAE Sephadex was therefore tried and found to be successful. Subsequent to this purification step, UV absorption peaks at 220 nm and 278 nm were obtained with the enzyme solution, indicating that this enzyme may well be a polypeptide.

Passage of the enzyme thru these ion-exchangers also increased the activity of the enzyme to about 140 % of its prepassage level; but there are some indications that stability has been adversely affected.

Discussion

Viewing the molecular weight determinations in detail, ultracentrifugation is not accurate below Mol, wt. 10,000. We could only conclude that we had active substances below this value. In the last decade gel filtration has been developed as a promising method. Adsorption does occur with some proteins, however, in which case too low molecular weight values result. For this reason we used two different gels, the dextran derived Sephadex G-50 and the polyacrylamide, Biogel P-10, both of a degree of crosslinkage appropriate for the 2,000 – 20,000 mol. wt. determinations.

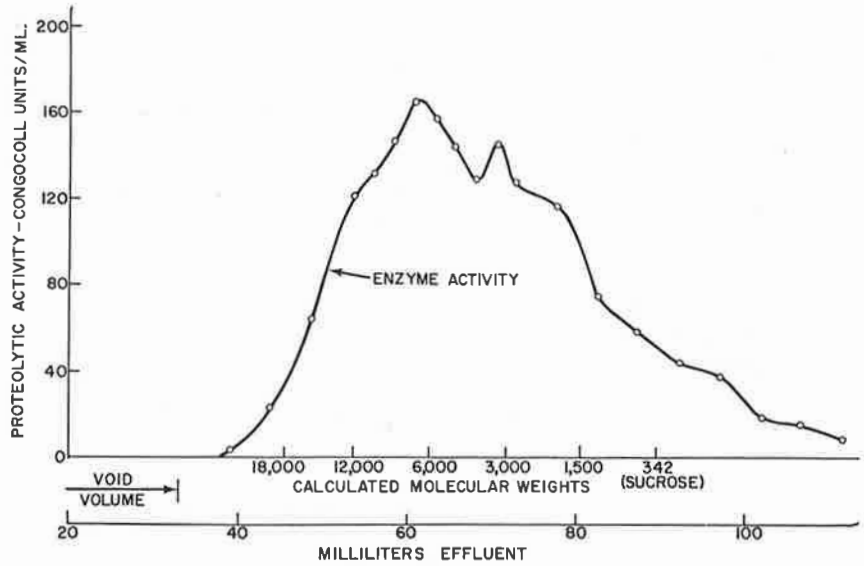

Fig. 12. Comparison of mobilities of *Bacillus cereus* microenzyme of insulin and of Cytochrome C on polyacrylamide gel with 4X concentration of cross-linker. Disc electrophoretic columns are shown following development, with marks beside the columns to show the extent of the diffuse bands.

In both gels enzyme activity tailed out into low molecular weight regions. While this tailing out does not define the molecular weight, it does indicate that material of low molecular weight was present.

Proof of the existence of a low molecular weight moiety was clearly provided by the results of extensive ultrafiltration studies. These showed that significant amounts of the enzyme passed through a membrane that effectively excludes materials with molecular weight above 10,000, and that penetration of the enzyme through the membrane could be substantially influenced by changes in concentration, metallic ions, and EDTA; while these did not similarly affect Cytochrome C which was used to confirm the integrity of the membrane.

Penetration of a substantial part of the proteolytic activity thru these membranes is clear proof that the enzyme monomer has a molecular weight less than 10,000.

Continually there have been indications that the monomer of this enzyme has a molecular weight of 5,000 to 6,000 or some fraction of this. There are yet to be confirmed indications of values as low as 1,500. Our results show that we are dealing with an enzyme system involving several oligomers.

In the SDS-2 ME polyacrylamide gel electrophoresis (Figure 11) both the enzyme and the insulin bands showed diffuseness, which is a characteristic of systems subject to rapid shifts between monomer and oligomers. Thus, it appears that the SDS-2 ME treatment did not completely disperse either of these two systems. The diffuseness reduces the precision of the molecular weight determinations, but still leaves no doubt about their exceptionally low values.

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