THE ACTIVATION OF CARNOSINASE BY DIVALENT METAL IONS

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SUMMARY

1. A cell and a procedure for the electrodialysis of protein solutions has been described which is suitable for the removal of stabilizing Mn^{2+} ions from solutions of purified carnosinase. The rate of dialysis obtained is considerably higher than the rate of inactivation of the metal-ion free enzyme.

2. It has been shown that Mn^{2+} ions, besides stabilizing the enzyme, also act as activators, performing the role of a coenzyme for the enzymic reaction.

3. Of the divalent metal ions investigated Cd^{2+}, Mn^{2+}, Zn^{2+} and Co^{2+} are shown to function as activators for carnosinase. The apparent efficiency of activation is given by the order: $Cd^{2+} > Mn^{2+} \gg Zn^{2+} > Co^{2+}$.

4. The Mn^{2+} activated enzyme is inhibited both by ions functioning as activators, such as Zn^{2+} and Co^{2+}, and by non-activating ions with Be^{2+} and Fe^{2+} as the most effective inhibitors.

5. The time course of the enzymic reaction in the presence of Mn^{2+}, Cd^{2+} and Zn^{2+} is shown to be of different type for each of the ions. The factors influencing the course of the reaction are discussed.

6. The influence of the concentration of free Mn^{2+} and Cd^{2+} ions on carnosinase activity has been studied. The results are, in the case of Cd^{2+}, in accordance with the assumption that one Cd^{2+} ion interacts with each active site. The influence of Mn^{2+} ions on the measured activity is most simply explained, by assuming the necessity for two metal ions per active site. It is suggested that the second Mn^{2+} ion is necessary for stabilizing the enzyme molecule.

7. The metal-ion specificity of carnosinase and its dependence on the properties of the metal ions and the conditions of the assay have been discussed.

INTRODUCTION

Among the enzymes classified as metal-ion activated, the peptidases occupy a central position. Extensive investigations by SMITH and co-workers[1-5], who used mainly partially purified enzyme preparations, established substrate and metal-ion specificity for a large number of peptidases. These investigators found that all the peptidases examined exhibited a very high degree of specificity towards the activating metal ions. Carnosinase[1], prolidase[3], prolinase[4], glycylglycinedipeptidase[5] and leucine aminopeptidase[2] were all shown to be activated by only one or possibly two different

Abbrevations: EDTA, ethylenediamine tebracetate; THAM, tris(hydroxymethyl)aminomethane.

divalent metal ions. Such a high degree of specificity contrasts markedly with the specificity shown by other groups of metal-ion activated enzymes. Enolase, for example, thoroughly studied by a number of investigators[6,7], is activated to different degrees by Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, Cd^{2+} and Ni^{2+}. Such a broad spectrum of activation has recently been shown to be characteristic even for metallo-enzymes such as carboxypeptidase[8]. In this case, although the activity is independent of the free metal ion concentration due to extremely strong binding of the metal ion, the Zn^{2+} originally bound to the enzyme can be replaced by Co^{2+}, Cr^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+} and Mn^{2+} without the enzyme losing its catalytic properties. If the narrow metal-ion specificity of peptidases is accepted as definite, extremely selective restrictions must be imposed on the properties of the activating ion by the structure of the enzyme and the mechanism of the enzyme action. On the other hand the picture is complicated by the instability of the enzyme in solution and the observed metal-ion stabilization. For example in the case of carnosinase[1,9] Mn^{2+}, besides activating the enzyme, also functions as a stabilizer. An examination of available data on metal-ion activation of enzymes[10] reveals that in general apparent activation of an enzyme can often be adequately explained by metal-ion stabilization of the enzyme structure against thermal denaturation at the temperature of activity measurements. In this case, even if the role of the metal ion is essential and specific there is no need to postulate the existence of an enzyme–metal-substrate compound; indeed the metal ion need not be located at the active site of the enzyme or partake directly in the process of catalysis.

If the metal ion acts as a stabilizer, possible irreversible and reversible steps leading to inactivation may render the results of the activity measurements very sensitive to the conditions and handling of the enzyme sample prior to the assay. The temperature and duration of steps such as dialysis and incubation with metal ions have a considerable influence on the results. Because of this, the apparent specificity obtained when no special precautions were used to control inactivation may often be misleading. As a part of a research program on the properties and mechanism of the action of metal enzymes being carried out at this institute, it seemed advisable to re-investigate the metal-ion activation of peptidases in a more careful and systematic way, so that it may be possible to differentiate between stabilization and true activation by metal ions. Further results ought to indicate whether there should be postulated a type of mechanism for metal-ion activation essentially different from those encountered in the case of enolase and carboxypeptidase.

Carnosinase was chosen as the object of investigation because the crude preparations of carnosinase[1] showed practically all the characteristic features of peptidases, such as activation by only two metal ions, Mn^{2+} and Zn^{2+}, a lag period during the process of activation, instability of the enzyme solution, and stabilizing properties shown by Mn^{2+}. On the other hand a more refined procedure of purification[9] developed in this laboratory yields an apparently homogeneous protein material which under carefully defined conditions was stable for a period of several months.

MATERIALS AND METHODS

Purification of carnosinase

The preparation of carnosinase has been described earlier [9]. The purified enzyme

was stored in solution at pH_5 7.70* in the presence of 0.01 *M* $MnCl_2$. The specific activity of the purified product from different preparations showed a maximal variation of approximately 10%. The product from the first zone electrophoresis at pH_{20} 8.65 was used in preliminary measurements. The results were then checked with the enzyme of highest purity.

Reagents

The metal-ion solutions used were prepared from analytical grade salts, sulfates in all cases except $BaCl_2 \cdot 2H_2O$, $CaCl_2 \cdot 6H_2O$, $SrCl_2 \cdot 6H_2O$ and $HgCl_2$. Both $MnCl_2$ and $MnSO_4$ were used for the preparation of Mn^{2+} solutions, no differences being observed in the activating properties after changing the anion. The concentration of the $MnCl_2$ solutions was checked by analysis. The water used for dilution of the metal ion solutions and for preparation of buffers was distilled water that had been passed through a mixed-bed ion exchanger and stored in polyethylene bottles. The buffers used were prepared from chemicals of the highest available purity. Sigma 121 analytical grade tris(hydroxymethyl)aminomethane (THAM) was used for preparation of THAM–HCl, THAM–acetate and THAM–maleate buffers. As the pH of these buffers is strongly dependent on temperature, the pH values are stated with a suffix indicating the temperature at which the pH was measured.

The preparation and properties of carnosine (β-alanyl-L-histidine) have been described previously[9]. Glycyl-L-histidine was prepared according to the method of TURNER[11]. The melting point of the hydrochloride was 175°. Neither carnosine nor glycyl-L-histidine were free from traces of histidine. Comparison with a specially prepared and chromatographically pure carnosine sample showed no detectable difference for the kinetics of Mn^{2+}-activated hydrolysis. Both the substrate solutions and buffers within the pH region of 5–8 were purified by extraction with a 0.001% solution of dithizone in CCl_4. The procedure, the efficiency and the pH limits of the extraction have been described by MALMSTRÖM[12]. Polyethylene bottles were used for storing the different buffers. In general, both in preparing the necessary solutions and in the analytical procedures described below, contamination by metal ions was minimized by the use of techniques described by THIERS[13] for work with trace elements.

Analytical methods

The determination of the protein content has been described earlier[9]. The total Mn content of the solutions was determined colorimetrically. The method used in the absence of chloride ions was the periodate method of SANDELL[14]. In the presence of chloride ions it was replaced by the persulfate method as described by NYDAHL[15]. In both cases a Beckman DU spectrophotometer was used for measuring the permanganate colour.

The free Mn^{2+} concentration was determined by measuring the electron spin resonance according to the method introduced by COHN[16]. The apparatus used was a Varian V-4500 EPR spectrometer with a rectangular H_{102} room temperature cavity. The temperature of the cavity was 36 ± 2° as measured by a thermocouple. The procedure for the resonance measurements and the calculations were the same

* The subscript indicates the temperature of the solution that was measured, with both the glass-electrode and the reference electrode at the temperature of the solution.

as described by MALMSTRÖM, VÄNNGÅRD AND LARSSON[17], except that the measurements were carried out in quartz tubes. Solutions of known $MnCl_2$ content were used for calibration purposes.

The concentration of free Cd^{2+} and free Zn^{2+} was determined according to the metal-indicator method described by RAAFLAUB[18]. Ammonium murexide was used as the indicator. In all cases the final murexide concentration was $1.5 \cdot 10^{-5}$ *M*. The measurements were performed at pH 7.65. A Beckman DU spectrophotometer with a thermostatically-controlled sample compartment was used for the measurements at different temperatures.

The pK values for metal–murexide complexes were determined at pH 7.65 and ionic strength 0.05. The pK of the Cd^{2+} murexide complex at 22° was 4.43. Fresh solutions of murexide were prepared for every series of measurements. A calibration curve of the extinction changes *vs.* metal ion concentration was then drawn and used for subsequent analysis of different solutions. The concentration range within which the determinations can be carried out was determined by the pK values of the metal–murexide complexes. Thus, Zn^{2+} concentrations lower than 10^{-5} *M* could not be measured. The same is valid for Cd^{2+} concentrations higher than 10^{-3} *M*.

Measurements of carnosinase activity

The amount of the substrate hydrolyzed was determined by withdrawing aliquots from the enzyme assay solution and titrating the liberated NH_2 groups in 90% ethanol according to GRASSMAN. The details of the procedure used have been described by SMITH[19]. In order to increase the reproducibility of the titration results, the visual end-point determination was replaced by a method utilizing photometric indication. The photometer assembly consisted of a filament light source and a photocell. The output from the photocell was then compared with a fixed voltage by means of a potentiometer using a light spot galvanometer as zero indicator. When thymolphtalein was used as the indicator the light source was equipped with a Hilger filter No. 606. A fixed arbitrary colour standard was used to calibrate the assembly before and between the measurements. Matched test tubes were used as titration vessels. Systematic investigations of the assay procedure showed that the relatively large error was due mainly to differences in the volume of the aliquot. The concentration of the substrate (0.05 *M*) was constant for all the measurements. The molarity of the buffers varied as they were prepared to give a constant ionic strength in the assay solutions. Detailed information on the composition of each solution is given in connection with the description of the separate experiments. The activity is expressed as moles of substrate split/min (*a*). The activity is determined from the linear part of the progress curve. No assumptions have been made as regards the order of the reaction.

Electrodialysis

The removal of the activating metal ions from the enzyme solutions was effectuated by means of electrodialysis against buffered solutions. The cell used, shown in Fig. 1, is a simple three compartment electrodialyser.

The two equal halves (A) of the cell made of Perspex (70 × 65 × 40 mm inner dimensions) with a double membrane* (C) between them, are held together by a

* A section of Visking cellulose sausage casing purified according to HUGHES AND KLOTZ[20].

metal frame (B). A suitably bent glass rod (D) (approximately 1–2 mm in diam.) is inserted between the two membranes to form a thin compartment for the protein solution to be dialyzed. The flow of fresh buffer through the electrode compartments containing Pt wire electrodes, takes place through PVC* tubes (E). The buffer in all the dialysis experiments was THAM–HCl, pH_5 7.70 and ionic strength 0.04. The

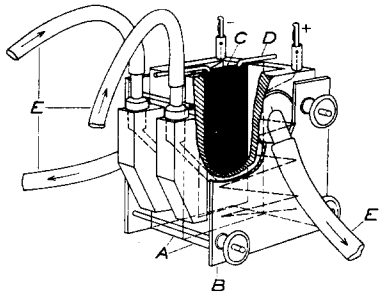

Fig. 1. Cell for electrodialysis. For description of details see text.

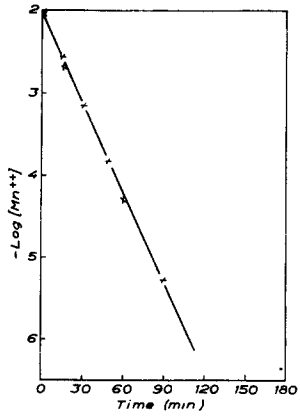

Fig. 2. Electrodialysis of 2 ml of 0.01 *M* $MnSO_4$ against THAM–HNO_3 buffer, pH_5 7.70, ionic strength 0.04. Voltage applied per cm of electrode distance, 1 V; current 35 mA.

voltage applied was 0.5–1.0 V/cm of the distance between the electrodes, which gave a current of 35–50 mA. A buffer flow of 0.5–1 l/h through each of the electrode compartments kept the pH and temperature of the protein solution steady. Fig. 2 shows a curve demonstrating the rate of dialysis achieved under the conditions specified. The concentration of the enzyme solution changes due to osmosis, but only in the case of concentrated enzyme solutions. As a rule the protein content was determined both before and after dialysis.

EXPERIMENTAL

Effect of enzyme concentration on the activity

A necessary prerequisite for using activity values as a measure of the concentration of the active enzyme, is the existence of direct proportionality between the activity and the enzyme concentration. This must be the case under conditions where the concentration of the activating metal ion as well as the concentration of all the other substances capable of influencing the activity is kept constant. Activity as a function of carnosinase concentration was measured over the range of $0.6–6.0 \cdot 10^{-3}$ mg of protein N/ml of the assay solution. The variation in concentration was obtained by diluting a concentrated enzyme solution. The concentrated enzyme solution containing 0.01 *M* Mn^{2+} was dialyzed 48 h against a THAM–HCl buffer of pH_5 7.65 having the same Mn^{2+} concentration as the enzyme solution. The buffer was then used to dilute the enzyme solution. The plot obtained was linear.

Dependence of the enzymic activity on the presence of Mn^{2+} ions

Fig. 3 represents an electrodialysis experiment where the enzyme solution

* Polyvinylchloride.

dialyzed originally contained 0.01 M Mn^{2+}. Aliquots of the dialysate were withdrawn after the indicated time intervals and one half of each aliquot was used to determine the residual activity at pH_{40} 7.65. The total Mn^{2+} concentration in the other half of each aliquot was restored to the value before electrodialysis (0.01 M) by addition of fresh Mn^{2+} and the activity determined under conditions identical with those used for determination of the residual activity. Total Mn^{2+} concentration in the assay: $2 \cdot 10^{-3}$ M.

Fig. 4 represents an experiment which was designed to show the effect of chelating agents on the residual activity and to determine whether the losses during prolonged dialysis were due to denaturation of protein or contamination by low-molecular weight substances. Two ml of the same enzyme solution as used in the previous experiment were electrodialyzed under identical conditions. After 2 h of electrodialysis,

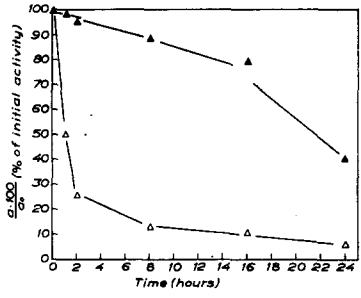

Fig. 3. Effect of removal of ions by electrodialysis on the activity of carnosinase. Two ml of a solution of carnosinase containing 0.01 M Mn^{2+} dialyzed against THAM–HCl buffer pH_5 7.70, ionic strength 0.04. Voltage 0.9 V/cm of electrode distance, current 35 mA. △—△, residual activity at pH_{40} 7.65; ▲—▲, activity after reactivation of the assay with $2 \cdot 10^{-3}$ M Mn^{2+} at pH_{40} 7.65.

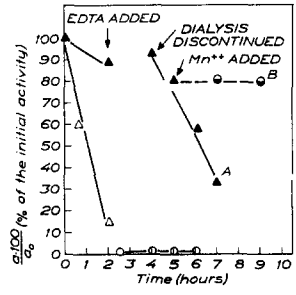

Fig. 4. The influence of EDTA on the electrodialysis of 2 ml of a solution of carnosinase, containing 0.01 M Mn^{2+}, against THAM–HCl buffer of pH_5 7.70, ionic strength 0.04. Voltage 0.9 V/cm of electrode distance, current 35 mA. △—△, residual activity measured at pH_{40} 7.65 before the addition of EDTA; ○—○, after the addition of EDTA; ▲—▲, activity after reactivation of the assay with $2 \cdot 10^{-3}$ M Mn^{2+}; ◒—◒, activity in a part of the dialysate stored after addition of Mn^{2+} (0.01 M Mn^{2+} in the solution).

0.2 ml of EDTA was added as a 0.1 M solution, the pH_5 value of which was adjusted with NaOH to 7.70*. The electrodialysis was then continued for an additional 2 h after which the dialysate was removed and stored at the temperature of dialysis, while the activity measurements were continued after intervals indicated in the figure. At point A in Fig. 4, one-tenth volume of 0.1 M Mn^{2+} was added to one part of the dialysate. The activity in this new solution was then measured simultaneously with the measurements on the unaltered dialysate. The experiment represented by Fig. 4 was repeated with the activity measurements carried out at 10° instead of 40°. A six-fold higher enzyme concentration was used, with THAM–acetate pH_{10} 7.65 buffer instead of THAM–HCl. The activity changes during dialysis showed the same pattern as shown in Fig. 4.

* The activity after the addition of EDTA was zero. Under the conditions employed this means that any possible activity higher than 0.5% of the initial value would have been measurable. The activity after the removal of EDTA varied between 2–6% of the value for the reactivated enzyme.

The following two experiments illustrate the influence of changing Mn^{2+} concentration on the time course of the hydrolytic reaction.

The experiment represented by Fig. 5 represents the case where the enzymic hydrolysis of carnosine in the presence of Mn^{2+} was suddenly interrupted by addition of EDTA (0.02 ml of 0.2 *M* EDTA to 2.5 ml of assay solution). Fifteen minutes later an excess of Mn^{2+} (0.05 ml of 0.2 *M* solution) was added in order to bind the EDTA and restore the free Mn^{2+} concentration. Curve B represents a control experiment performed with an unchanged part of the original assay solution. The three lines are drawn separately as the addition of EDTA and Mn^{2+} alters the titration values of the aliquots. The activity is not zero in the presence of EDTA because the amount of EDTA added had to be limited so that it could later easily be removed by addition of reasonable amounts of Mn^{2+}. High Mn^{2+} concentrations result in extensive formation of MnO_2 during the titration which prevents the detection of the end-point.

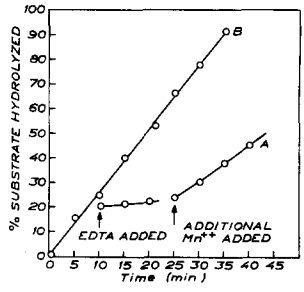

Fig. 5. Hydrolysis of carnosine as a function of time at pH_{40} 7.65. Initial total concentration of Mn^{2+}, $2 \cdot 10^{-3}$ *M*. Curve A represents the activity after the successive addition of EDTA ($1.6 \cdot 10^{-3}$ *M*) and Mn^{2+} ($4 \cdot 10^{-3}$ *M*), curve B the activity with no addition of any kind.

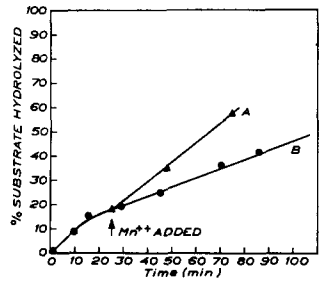

Fig. 6. Hydrolysis of carnosine as a function of time at pH_{40} 7.65. Curve A in presence of $3 \cdot 10^{-3}$ *M* Mn^{2+}; curve B in presence of approx. $5 \cdot 10^{-5}$ *M* Mn^{2+}.

The purpose of the experiment illustrated by Fig. 6 was to show that the results of the previous experiments were not due to reversible inhibition of the enzyme by EDTA. An incompletely dialyzed solution of the enzyme, with a residual activity of approximately 20 % of the initial value and a free Mn^{2+} concentration of approximately $5 \cdot 10^{-5}$ *M* was used for the assay (no EDTA was used during the dialysis). After the hydrolysis had proceeded for 10 min the assay solution was divided into two equal parts and an additional amount of Mn^{2+} was added to one of them (the final total concentration in this case being $2 \cdot 10^{-3}$ *M*). The velocity of hydrolysis in both halves of the solution is represented by curves A and B in Fig. 6.

Activation by different divalent metal ions

The effect of Ba^{2+}, Ca^{2+}, Mg^{2+}, Co^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+} and Mn^{2+} on the rate of the enzymic hydrolysis of carnosine was investigated under the conditions described below. The activity measurements were in all cases conducted at 40°. THAM–HCl and THAM–maleate buffers were used to vary the pH between 5 and 9. As a rule the stable enzyme solution containing 0.01 *M* Mn^{2+} was dialyzed by the addition of EDTA as described in the preceding section. The dialyzed enzyme solution with a residual activity of approximately 5 % of the initial value was mixed with the

buffer to be used. A suitable amount of a metal ion was added and the assay performed immediately. In order to ascertain the losses of activity during the dialysis* a control with Mn^{2+} as the activator was run for every dialysis. Table I summarizes the experimental conditions and the qualitative results of the activity measurements.

The activating effect of Cd^{2+}, Mn^{2+}, Zn^{2+} and Co^{2+} was studied as a function of the pH of the assay solution. Fig. 7 represents the activity curves obtained. THAM–HCl buffers were used between pH_{40} 7.5 and 8.6, THAM–maleate between 7.5 and 6. acetic acid–Na acetate below 6 and boric acid–Na borate above 8.6. The pH_{40} of the reaction mixture was determined with a pH meter in the absence of the enzyme.

TABLE I

METAL IONS TESTED AS ACTIVATORS FOR CARNOSINASE AT 40° AND A SUBSTRATE CONCENTRATION OF 0.05 *M*

Metal ion	*The concentration* range of the metal ion investigated* ($\times 10^{-3}$)	*Range of pH tested*	*Activity*
Ca^{2+}	2	7.5–9.0	—
Mg^{2+}	2–5	7.5–9.0	—
Ba^{2+}	2	7.5–8.65	—
Mn^{2+}	2	6.0–9.0	+++
Ni^{2+}	2–4	7.0–7.5	—
Co^{2+}	2	5.5–8.0	+
Fe^{2+}	1–2	5.0–7.0**	—
Zn^{2+}	2	5.0–8.0	++
Cd^{2+}	2	5.0–8.0	+++

* Refers to the total metal ion concentration.
** The concentration at higher pH values is nominal due to oxidation.

The ionic strength of the assay due to the buffer and metal ion was kept constant and equal to 0.056. The contribution from the 0.05 *M* substrate is not included but as the substrate concentration is constant the only variation of the ionic strength is due to the shift of the equilibrium between the different ionized forms of the substrate resulting from the changing pH. Due to the method used for activity determinations it is not possible to use buffer concentrations high enough to keep the pH of the reaction mixture constant. The pH changes after 50 % hydrolysis do not, however, exceed 0.1 unit. The total metal-ion concentration was in all the different measurements equal to $2 \cdot 10^{-3}$ *M*. The free metal-ion concentration varies from case to case depending on the stability constants of the metal complexes with the different molecules and ions of the solution. The concentrations of the enzyme solutions used for the different measurements were all identical before the electrodialysis. Due to variations in activity losses during the dialysis, the specific activity values of the dialyzed solutions vary from 5 to 25 %. In order to utilize data from several experiments for the same group of curves the activity of every dialysate is determined at pH_{40} 7.65 in the presence of $2 \cdot 10^{-3}$ *M* Mn^{2+}. The activity at this arbitrarily chosen point is represented by 100 and the results of the other activity determinations expressed relative to it.

* The losses during the dialysis varied between 10–25 %. In the case of the losses exceeding 30 % the enzyme solution was discarded.

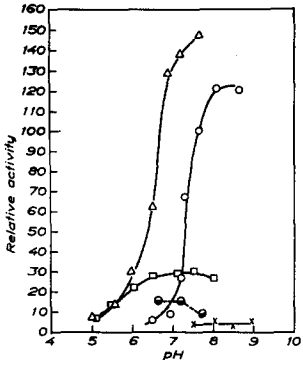

Fig. 7. Effect of pH on the hydrolysis of carnosine at 40° in presence of different metal ions: ○—○, $2 \cdot 10^{-3} M$ Mn^{2+}; △—△, $2 \cdot 10^{-3} M$ Cd^{2+}; □—□, $2 \cdot 10^{-3} M$ Zn^{2+}; ◒—◒, $2 \cdot 10^{-3} M$ Co^{2+}; ×—×, residual activity after electrodialysis of the Mn^{2+}–carnosinase.

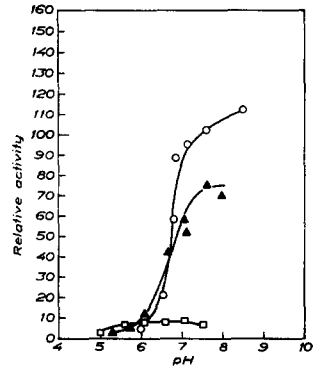

Fig. 8. Effect of pH on the hydrolysis of glycyl-L-histidine at 40° in the presence of different metal ions: ○—○, $2 \cdot 10^{-3} M$ Mn^{2+}; ▲—▲, $2 \cdot 10^{-3} M$ Cd^{2+}; □—□, $2 \cdot 10^{-3} M$ Zn^{2+}.

Fig. 8 represents analogous curves of pH dependence with the same metal ions and under the same conditions, but with carnosine replaced by glycyl-L-histidine as the substrate. The activity curves in this case, as in the case of carnosine, are discontinued at the point of metal hydroxide formation, as the activity at higher pH values is affected not only by the diminishing metal-ion concentration but also by inactivation of the enzyme due to co-precipitation with metal hydroxide. It is interesting to note that Mn^{2+} activation is only slightly influenced by the change of the substrate. The maximum activity obtained with the same total concentration of Cd^{2+} and Zn^{2+} diminishes considerably. This is not unexpected as the replacement of an β-amino group by an α-amino group increases the binding of Cd^{2+} and Zn^{2+} to the substrate and as a result, among other possible changes influencing the activity, the free metal-ion concentration diminishes as compared to the assay with carnosine. Some relative values are given in Table II.

TABLE II

THE EFFECT OF DIFFERENT SUBSTRATES ON THE CONCENTRATION OF FREE METAL ION AT 20°, pH 7.65

Metal ion	*Total conc.* ($\times 10^{-4}$) *M*	*Buffer* (0.05 *M*)	*Free Me^{2+} conc. in presence of*	
			0.05 M carnosine	*0.05 M glycyl-L-histidine*
Cd^{2+}	2	THAM–maleate	$5 \cdot 10^{-5}$	$2.8 \cdot 10^{-5}$
Zn^{2+}	2	THAM–HCl	$9.5 \cdot 10^{-4}$	$4.5 \cdot 10^{-4}$*

* The error in determinations of free Zn^{2+} can be rather large due to possible interference by $Zn(OH)_2$.

Progress curves of the enzyme reactions in the presence of different metal ions

Before a further comparison can be made between the effects of different metal ions on the enzymic reaction it is necessary to study the time course of the enzymic reaction in the presence of different metal ions. It is by no means necessary that the progress of the reaction is of the same type for all the activating ions. SMITH AND

SPACKMAN[2] have for example found both zero and first-order reactions with leucine aminopeptidase depending on the activating ion. Although progress curves of enzyme reactions often do not fit the standard equations of homogeneous chemical reactions, it is necessary to know the form of the progress curves, as relative differences between the progress curves in the presence of different ions may depend on a different mechanism of the reaction or some factor selectively influencing the rate in the presence of one metal ion. These differences must be accounted for if the activation by different ions is to be compared.

In the presence of Mn^{2+} ions in the pH region between 7 and 9, the substrate is hydrolyzed in accordance with zero order kinetics. With the Mn^{2+} ion concentration lower than necessary for maximum activity or with the pH below 7, the time curve diverges from that of zero order (see Fig. 6). As these divergences are strongly influenced by the temperature of the assay mixture, they can be explained by the fact that the stability of the enzyme is a function of the Mn^{2+} concentration and the pH of the assay. Figs. 9 and 10 show the progress curves of the reaction in the presence

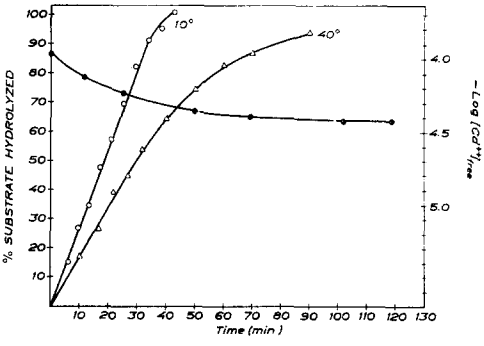

Fig. 9. Hydrolysis of carnosine as a function of time in presence of $4 \cdot 10^{-3}$ *M* Cd^{2+}. Buffer THAM–maleate, pH of the assay 7.65: O—O, per cent hydrolysis at 10°; △—△, per cent hydrolysis at 40°; ●—●, concentration of free Cd^{2+} during the reaction at 40°.

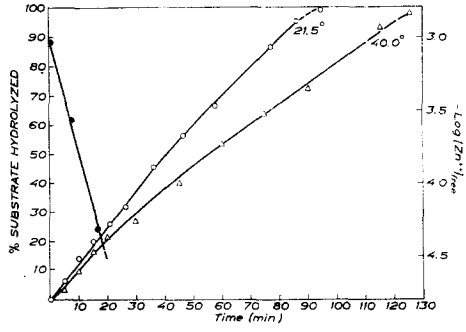

Fig. 10. Hydrolysis of carnosine as a function of time in presence of $2 \cdot 10^{-3}$ *M* Zn^{2+} at pH 7.65. The buffer used was THAM–HCl: △—△, per cent hydrolysis at 40°; O—O, per cent hydrolysis at 21.5°; ●—●, concentration of free Zn^{2+} at 40°.

of Cd^{2+} and Zn^{2+} at an initial concentration giving maximal activity. In both cases the curves diverge considerably from the zero order curve. Two of the possible factors responsible for the deviation can easily be derived from the experimental data. The first factor reveals itself in the sharp change of the free metal-ion concentration during the progress of the reaction, especially striking in the case of Zn^{2+} activation. The downward slope of the curve representing the free metal-ion concentration is determined by the difference in metal binding capacity between the substrate and the products of the reaction.

The presence of a second factor is clearly indicated by the temperature dependence of the shape of the progress curves. In both cases, though more drastically in the case of Cd^{2+}, the downward curvature of the reaction curve diminishes when the reaction is carried out at lower temperatures.

The time course of the activation process

As the enzyme was stored in the presence of Mn^{2+}, the lag period observed by

HANSON AND SMITH[1] showing the time dependence of the activation process would naturally not show up. In the case of activation by Cd^{2+} and Zn^{2+} of the dialyzed solutions, no lag period was observed. A more detailed investigation of this question will be published in connection with investigations of metal-ion stabilization of carnosinase, which are now in progress.

Activity as a function of metal ion concentration

The enzyme used for all the measurements described in this section originated from the same concentrated solution of enzyme in THAM–HCl buffer of pH_5 7.70 containing 0.01 *M* Mn^{2+}. The measurements in the presence of Mn^{2+} were carried out at a constant enzyme concentration obtained by dilution of the stock solution. The activity measurements were carried out immediately after dilution. For measurements in the presence of Cd^{2+}, suitable amounts of the stock solution were dialyzed as described before. The dialyzed solution was immediately used for activity measurements. Separate dialyses were carried out for each series of activity measurements. The activating metal ions were added to the assay immediately before the addition of the enzyme. The buffer used for measurements in the presence of Mn^{2+} was THAM–HCl. When the temperature was lowered, the content of THAM had to be reduced considerably in order to keep the pH at 7.65 and to retain the same ionic strength. At lower temperatures the buffer capacity of THAM–HCl is very small in the region of pH 7.65. Thus already at 10° the pH is buffered by the substrate–HCl system instead.

THAM–maleate buffers of the same ionic strength were used for measurements in the presence of Cd^{2+} ions added in the form of $CdSO_4$. The Cl^- ions were deliberately omitted in order to avoid complex formation between Cd^{2+} and the excess of chloride ions. The free metal-ion concentration was in the case of Mn^{2+} determined as described in the section devoted to methods. As the measurements could be carried out at 36° only, the values given at other temperatures are subject to an error, the magnitude of which depends on the temperature dependence of the metal binding capacity of the substrate. From the available data on the metal binding capacity of imidazole[21,22], it can be estimated that the temperature ought not to influence the order of magnitude of the free Mn^{2+} concentration.

Direct studies of the chelate formation between the substrate and the activating metals now in progress will furnish the necessary corrections. The influence of temperature on the Mn^{2+} binding by THAM molecules has been neglected as the pK value of the THAM–Mn^{2+} complex cannot be higher than 1.1[23]. In the case of Cd^{2+}, the concentration is determined at the different temperatures used for activity measurements.

Fig. 11 shows the influence of Mn^{2+} concentration on the activity of carnosinase. The range of the metal-ion concentration was in this case, and in the case of Cd^{2+} limited by the method of activity determinations. At higher metal-ion concentrations, the formation of metal hydroxide precipitates disturbs the titration. Fig. 12 shows the same type of curves in the presence of Cd^{2+}.

An attempt can be made to plot the data in terms of the simple empirical rate equation of WARBURG AND CHRISTIAN[24],

$$a = \frac{a_{max} M}{K_a + M} \qquad (1)$$

where a represents activity and M the concentration of the free metal ion, conveniently used in the form introduced by LINEWEAVER AND BURK[25]. K_a represents a hypothetical dissociation constant.

$$\frac{1}{a} = \frac{K_a}{a_{max}} \frac{1}{M} + \frac{1}{a_{max}} \qquad (2)$$

The experimental points from the experiments with Mn^{2+} at 20°, 30° and 40° do not yield straight lines. Fig. 13 shows the form of the LINEWEAVER–BURK function at 30°. The results from measurements at 10.5° fit reasonably well on a straight line.

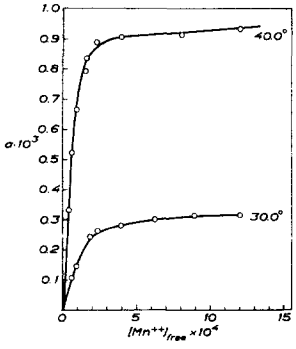

Fig. 11.

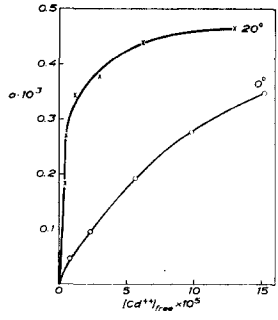

Fig. 12.

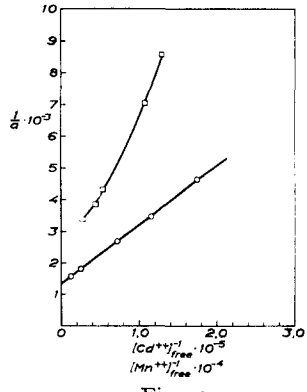

Fig. 13.

Fig. 11. The influence of the concentration of Mn^{2+} on the activity, a, of carnosinase in THAM-HCl buffer at 30° and 40°, pH 7.65.

Fig. 12. The influence of the concentration of Cd^{2+} on the activity, a, of carnosinase in THAM-maleate buffer at 20° and 0°, pH 7.65.

Fig. 13. LINEWEAVER–BURK plots, eq. (2), for the activation of carnosinase by: □—□, Mn^{2+} at 30° in THAM–HCl buffer; ○—○, Cd^{2+} at 12° in THAM–maleate buffer.

The measurements in the presence of Cd^{2+} at 2°, 12° and 20° all yield straight lines when plotted according to LINEWEAVER AND BURK. Fig. 13 shows the plot at 12°. Due to the well-known insensitivity of the plotting at high metal-ion concentrations the apparent linearity does not prevent the K_a values from varying within approximately 20 % when calculated from the experimental points. The K_a values for Cd^{2+} measurements at different temperatures are given in Table III. The K_a values for Mn^{2+} activation at 20°, 30° and 40° are replaced by limits of variation of the constant for the unaltered Warburg equation.

Inhibition of Mn^{2+} carnosinase by metal ions

The determination of the inhibitory properties of metal ions on the Mn^{2+}-activated

TABLE III

THE VALUES FOR CONSTANT K_a (EQ. 1) FOR Mn^{2+} AND Cd^{2+} ACTIVATION AT pH 7.65

Metal ion	K_a *values at different temperatures*				
	10.5°	*21°*	*30°*	*40°*	
Mn^{2+}	$8.0 \cdot 10^{-4}$	$3-1 \cdot 10^{-4}$	$1.5-0.4 \cdot 10^{-4}$	$0.6-0.3 \cdot 10^{-4}$	
	2°	*12°*	*21°*		
Cd^{2+}	$1.2 \cdot 10^{-4}$	$1.5 \cdot 10^{-5}$	$5.0 \cdot 10^{-6}$		

carnosinase furnishes us with an additional experimental parameter, namely the classification of metal ions with respect to their relative abilities to inhibit the enzyme. The information obtained from such studies has been used to draw conclusions about the groups participating as ligands in the binding of the activating ion to the assumed site of catalytic action.

A more strict investigation on the quantitative aspects of inhibition characterized by determination of the competitive or non-competitive character of the inhibition has not been carried out in the case of carnosinase. This is due to the fact that an investigation of a system with two different metal ions both interacting with the substrate, necessitates the determination of substrate–metal association constants for all the metal ions involved. A qualitative survey of the inhibiting effects of some divalent metal ions has been carried out prior to a more thorough investigation.

The measurements were carried out at pH_{40} 7.65 with THAM–HCl as the buffer. The concentration of Mn^{2+} and of the metal studied was in all cases 0.002 *M*.

These concentrations were for some of the ions investigated (Fe^{2+}, Hg^{2+}) only nominal, representing the total amount of the metal ion added. The results obtained are presented in Table IV.

TABLE IV

INHIBITION (IN %) OF CARNOSINASE ACTIVITY (100% = TOTAL INHIBITION) IN THAM–HCl BUFFER AT pH 7.65 AND 40° IN THE PRESENCE OF 2×10^{-3} *M* Mn^{2+} AND 2×10^{-3} *M* OF THE INHIBITING ION

Metal ion	*Inhibition (%)*	*Metal ion*	*Inhibition (%)*	*Metal ion*	*Inhibition (%)*
Be^{2+}	86	Sr^{2+}	0	Co^{2+}	80
Mg^{2+}	0[a]	Ba^{2+}	0	Fe^{2+}	64
Ca^{2+}	0[b]	Zn^{2+}	85	Ni^{2+}	65
		Cu^{2+}	0	Hg^{2+}	40

[a, b] In addition measurements were carried out at pH_{40} 8.65–9.10 which showed no inhibition.

DISCUSSION

Activation and stabilization

In order to show that an observed dependence of activity on the presence of metal ions is not due to several possible artifacts[10] but to the fact that the metal ion functions as a coenzyme, it is necessary, as a first step, if the enzyme solution contains

metal ions, to show that their removal is followed by loss of activity which can be restored by adding suitable amounts of the activating ion.

If the stability of the enzyme molecule allows the successive removal of the metal ions by prolonged dialysis against chelating agents, the experiments can easily be performed. If there is reason to suspect that the metal-free enzyme is unstable, it is essential that the rate of dialysis should considerably exceed the rate of the subsequent denaturation. For carnosinase preliminary experiments showed that dialysis, even in its most effective form, always resulted in considerable activity losses. Electrodialysis described in the section for methods was, however, found to satisfy the requirements for the rate of dialysis. The pH used for the dialysis is very near the iso-electric point of the enzyme as determined by qualitative electrophoretic experiments. Experiments with pH values lower or higher than this invariably led to precipitation of the protein on the membranes.

The first part of the curve, Fig. 3, representing activity changes during dialysis shows a rapid decrease in the residual activity. This process is reversible, as reactivation restores about 90 % of the original activity. After 3 h, the rate of the dialysis has decreased so far that further decrease of the residual activity takes place only very slowly and is accompanied by a gradually increasing amount of irreversible inactivation indicated by the downward slope of the reactivation curve. The drop of activity might perhaps be caused by accumulation of strongly inhibiting ions from the buffer used for dialysis. It is clear that the residual activity after 2 h of dialysis can scarcely be cut down further by prolonged dialysis. Even if the inactivation could be avoided the dialysis is bound to lead to an equilibrium where the removal of ions is compensated by ions from the continuously changing buffer. The proportions of the dialysate (2 ml) and the buffer (5.1 l) make this feasible even if the buffer has been prepared with the utmost care.

Experiments with the use of chelating agent show that EDTA completely inhibits the activity. The residual activity after the removal of EDTA by electrodialysis is about 2 % of the initial value. The continuous decrease of activity, even after the dialysis has been discontinued, shows that the losses of activity during dialysis are due to irreversible inactivation which proceeds at a considerable rate* even at 5°. The addition of Mn^{2+} to a part of the dialysate (A in Fig. 4) immediately stopped the inactivation. The most important consequence of this surprisingly rapid inactivation is that no definite conclusion can be drawn about the role of the Mn^{2+} ion. The observed activation can be explained as stabilization in view of the fact that the dialysis takes place at 5° and the activity measurements at 40°. The metal-free enzyme is probably denatured so fast at 40° that the activity is entirely determined by the amount of enzyme stabilized by the remaining Mn^{2+} ions. In order to prove that, beside its role as a stabilizer, the Mn^{2+} does function as a true activator it will be necessary to show that the metal-free enzyme cannot function as a catalyst during the short period before it is irreversibly inactivated.

A repetition of the electrodialysis with the activating measurements performed at 10° instead of 40°, however, confirmed the observations of the preceding experiment.

As the validity of this kind of experiment depends on the relative rates of the

* The rate of denaturation varied considerably in different electrodialysis runs although the same enzyme solution was used. Before the process of denaturation has been studied systematically, the data given should be considered only as a qualitative indication of the rate of denaturation.

supposed catalytic action of the metal-free enzyme and the inactivation process, two additional experiments were designed to give more experimental material before drawing any definite conclusion.

In both cases the time course of the reaction is studied when the Mn^{2+} concentration in the assay is rapidly changed during the reaction.

In the case of experiments with EDTA (Fig. 5), straight lines representing the time course of the reaction are found and would be expected if Mn^{2+} functions as a true activator independently of the stabilization phenomenon. In the next experiment the addition of Mn^{2+} to a reaction mixture of low Mn^{2+} content results in a considerable increase of activity clearly distinguished from a possible stabilization effect. That the first part of the time course of this reaction is non-linear is explained by the rapid inactivation of the metal-free enzyme which directly influences the equilibrium: $Mn^{2+} + E^n \rightleftharpoons MnE^{n+2}$. MnE^{n+2} represents here the stable enzyme and not the assumed active enzyme–coenzyme compound, the site for the metal ion in the stabilization process not necessarily being identical with the enzymically active site. The results of the series of experiments described above lead to the conclusion that the Mn^{2+} ions in the reaction medium have two functions to fulfil. In the first place, they are indispensable for the catalytic activity of the enzyme, as is characteristic of metal-ion-activated enzymic reactions. These data alone naturally do not allow one to conclude whether the metal ion participates directly in the catalytic reaction or not. The second function of the metal ion is the stabilization of the enzyme molecule. This aspect of the Mn^{2+}–enzyme interaction can scarcely be compared to the frequently observed, rather unspecific, influence of metal ions on the thermal stability of proteins. In these cases the proteins are as a rule stable without the metal ion under normal conditions, and the stabilization reveals itself as a decrease in the rate of denaturation at higher temperatures[26–28]. The rapid inactivation of metal-free carnosinase at 5° and full stability in the presence of Mn^{2+} at temperatures as high as 40° make it plausible that the stabilization is due to specific interaction between the enzyme and one or more metal ions; a similar situation is found in the case of Ca^{2+} ion stabilization of amylase[29].

The metal-ion specificity of the activation and inhibition

If we compare the metal ions that have been shown to activate carnosinase with respect to the physical properties generally used to characterize ions, it becomes obvious that the electro-negativities of the activating ions lie very close to each other and consequently, when the well-known order of complex stability[30] is considered the activating ions all fall together in the middle of a series $Hg^{2+} > Cu^{2+} > Ni^{2+} > Pb^{2+} > Zn^{2+} > Co^{2+} > Cd^{2+} > Mn^{2+} > Mg^{2+} > Ca^{2+} > Ba^{2+}$ listed according to decreasing complex stability with amino acids.

TABLE V

SOME PROPERTIES OF THE ACTIVATING METAL IONS[10]

Metal ion	*Electronegativity*	*Ionic radius*	*Covalent radius*
Mn^{2+}	1.4	0.78–0.80	—
Cd^{2+}	1.5	0.96	1.48 tetrahedral
Zn^{2+}	1.5	0.72–0.74	1.31 tetrahedral
Co^{2+}	1.7	0.73	1.23 octahedral

The comparison of the maximal activities obtained with different ions is rather difficult as the time course of the hydrolytic reaction shows considerable differences in the presence of the different activating ions. The product formation curves in the presence of Cd^{2+} and Zn^{2+} indicate the possibilities of product inhibition and enzyme denaturation. Due to the insensitivity of the activity measurements, the real initial velocities may differ considerably from the values estimated from the curves since the first experimental point often represents as much as 10 % hydrolysis.

If we consider the pH region of activation for different ions, it is easy to realize that the activity obtained for example in the presence of Co^{2+} need not represent the maximal possible activity in the presence of free metal ions in concentrations equal to the total amount of metal ion added. Oxidation of the Co^{2+}–substrate compound manifested by strong colour formation in the assay solution can, by altering the free metal-ion concentration, also produce chelated forms that act as inhibitors. The decisive influence of the interaction of metal ions with the substrate molecules is further illustrated by Fig. 8 which shows the pH dependence of the metal-ion activation with carnosine replaced by glycyl-L-histidine. The relative activities obtained with the same nominal metal-ion concentration as was used in the case of experiments with carnosine show quite a different pattern.

The specific inhibition of Mn^{2+} activated carnosinase by metal ions can advantageously be discussed in this connection as both the specificity of activation and inhibition are largely determined by the same factors. The most interesting feature of the results from inhibition studies is the strong inhibitory effect shown by Be^{2+}, especially when compared to the other alkaline-earth ions which show no inhibitory tendencies at all. Pronounced inhibition by Be^{2+} has in the case of the phosphatases[31] been interpreted as indicating the participation of a phenolic –OH in the formation of the binding site for the metal ion. Before any such suggestion can finally be accepted it should be remembered that Be^{2+} differs radically from all other divalent ions with respect to its size. The small size of Be^{2+} may enable the ion to block some necessary negatively charged groups (not necessarily phenolic –OH) in the interior of the protein structure which are not available for other larger divalent ions.

The strong inhibiting properties of Zn^{2+} and Co^{2+} are expected since both ions can function as activators. The apparent non-inhibiting properties of Cu^{2+}, which are peculiar when compared to the inhibitory effect shown by Ni^{2+}, are explained easily enough in terms of the chelate-forming ability of the substrate. The pK values of Cu–carnosine[32] indicate that in this case the substrate acts as a chelating agent comparable to EDTA so that the concentration of metal ions free to react with the protein is negligible.

When correlating the physical properties of the metal ions with the results of the activity and inhibition measurements it must be remembered that the observed specificity need not only reflect the spatial and electronic requirements of the active site for catalytic action but can be determined by a number of factors influencing the free metal-ion concentration of the reaction medium.

Generally speaking the factors determining the metal ion specificity can be divided into two groups:

A. Factors influencing the equilibrium in the assay solution between the metal ions and the active site.

B. Factors directly connected with the physical properties of the metal ion and of the

group or groups that combine with the metal ion in the course of the catalytic reaction.

The first group of factors do not influence the intrinsic binding constant of the metal ion to the active site but rather influence the concentrations of the different molecular species participating in the enzyme-catalyzed reaction (*e.g.*, chelate formation between the substrate or the product and the metal ions, formation of metal hydroxides, enzyme denaturation and instability of the oxidation state of the metal ion.) The free metal-ion concentration is not only diminished by direct removal of ions but in addition the formation of mixed compounds of the type enzyme-Me-$(OH)_n$ or substrate-Me-$(OH)_n$ can inhibit the reaction.

For the second group it should be possible to find a correlation between the physical properties of the metal ion and the degree of activation, as the factors of this group can directly be studied by physical measurements of different, separated and well-defined molecules acting as models for the enzyme reaction.

It is in principle not impossible that the observed specificity should be entirely determined by the secondary factors listed in the first group. In the case of carnosinase practically all the factors of the first group seem to influence the results; thus it is very doubtful if a significant pattern can emerge from the correlation of activity data with the properties of the metal ion. The one definite conclusion that can be drawn is that the previously assumed high specificity of carnosinase is misleading and is due to difficulties in obtaining a stable metal-free enzyme and to not taking necessary precautions to control the secondary factors indirectly influencing the activity (for example enzyme denaturation).

Kinetics of metal-ion activation

The Cd^{2+} activation of carnosinase can adequately be described by the simple mass-action equation of WARBURG AND CHRISTIAN[24]. The experimental values plotted according to LINEWEAVER AND BURK yield straight lines. The values of the constant K_a in eq. (2) are listed in Table IV. The conclusion from these data is that one metal ion is necessary for every active site. In the concentration range investigated no other function can be discovered that influences the activity and is dependent on metal-ion concentration. Inhibition at higher metal-ion concentrations cannot be discovered from the activity curves. The results naturally give us no information as to whether the enzyme is stable or whether an independent process of inactivation is taking place. The temperature dependence of the time course of the reaction indicates that although Cd^{2+} activates the enzymic reaction it does not necessarily stabilize the enzyme structure in the way that Mn^{2+} ions do.

The Mn^{2+} activation curves at room temperature (20°) and higher do not fit the WARBURG–CHRISTIAN equation, and as can be seen from Fig. 13 a LINEWEAVER–BURK plot does not yield a straight line.

The WARBURG–CHRISTIAN mass action equation can naturally be modified by the introduction of an additional parameter to give an equation that fits the experimental points of the Mn^{2+} activation curves better. Eq. (3) for example adequately describes the functional dependence of the activity (a) on the free Mn^{2+} concentration (M).

$$a = \frac{a_{max}M}{B + \frac{A}{M} + M} \qquad (3)$$

The need for a modification in the empirical rate equation can be deduced from the assumption that an inactivation process dependent on Mn^{2+} concentration takes place. Observations on the instability of the metal-ion free enzyme and the stabilizing role of Mn^{2+} make it apparent that inactivation at low metal-ion concentration must be included in the deduction of a rate equation.

Let us assume that after the lowering of the metal-ion concentration the process of inactivation, leading to different forms of the enzyme, arrives at a state of apparent equilibrium. This means that further changes in the relative amounts of the different forms of the enzyme are slow when compared to the hydrolysis of the substrate. Further, if we assume that the equilibrium with the metal ion determining the rate of hydrolysis is identical with the stabilization equilibrium (the same metal ion functions in both capacities), it is easy to see that the rate equations for the reaction are of the same type as eq. (1), regardless of the number of steps postulated for the inactivation process. It is assumed that activation and stabilization, both influencing the total activity measured, are dependent on the equilibrium (4) where ME designates the stable

$$K = \frac{ME}{M_{free} \times (E_{tot.} - ME)} \tag{4}$$

active form of the enzyme. The introduction of different forms of inactive enzyme E' combined or uncombined with the metal ion leads only to a lowering of the free enzyme concentration represented by the additional terms in eq. (5). Consequently, when

$$K = \frac{ME}{M_{free} \times (E_{tot.} - ME - E'M - E')} \tag{5}$$

the two assumptions concerning equilibrium conditions are fulfilled, the LINEWEAVER–BURK plots should yield straight lines independent of the number of forms of the inactivated enzyme present.

The concave curve representing the LINEWEAVER–BURK plot for Mn^{2+} activation is of the type expected for a second order reaction. If we assume that activation and stabilization are represented by two different and independent equilibria with different dissociation constants, K_1 and K_2, the rate eq. (6) arrived at is analogous to the

$$\frac{V_m}{V} = 1 + \frac{K_1}{M} + \frac{K_2}{M^2} \tag{6}$$

equation derived by FRIEDENWALD AND MAENGWYN-DAVIES[35] for cases where the same substance acts as both activator and substrate. Eq. (6) is identical with the empirical rate eq. (3). In deducing eq. (6) we assume the existence of an activated enzyme species $EM_{act.}$ that is not stabilized but can combine with the stabilizing atom to form $EM_{2stab.act.}$. On the contrary, if we assume that the enzyme must be stabilized before it can be activated, $EM_{act.}$ can be assumed to be non-existent, and the rate equation will be further simplified. Which of the two equations represents the experimental curves better cannot be decided as differences between the two theoretical curves lie within the limits of error for the activation measurements. The theoretical curve for Mn^{2+} activation at 30° according to eq. (7) coincides with

$$\frac{V_m}{V} = A + \frac{K_1 K_2}{M^2} \tag{7}$$

the curve drawn in Fig. 11. In the case of activation at 40°, the experimental curve

differs very slightly at the two points representing the lowest Mn^{2+} concentration. In this case eq. (6) gives a somewhat better result.

There may be a second explanation for deviations from the linearity for the LINEWEAVER–BURK plot. The condition assumed for deducing the rate expressions, namely the existence of an equilibrium between the different forms of the enzyme, may not be fulfilled. Experimental evidence on this point is not sufficient yet. We know that the inactivation is not totally reversible, which should naturally exclude any possibility of equilibrium. On the other hand the progress curve of the reaction (Fig. 6) which represents the activity at low metal-ion concentrations, shows that, after a period of decreasing activity, the system seems to arrive at a sort of equilibrium where the rate of further denaturation of the enzyme is so low that for calculations during a reasonable time interval it can be considered as being in equilibrium. A more detailed study of inactivation is necessary before the kinetic data can definitely be interpreted.

The conclusion that can definitely be drawn is that Mn^{2+} activation cannot be represented by a simple mass law treatment and that the simplest way to explain it, is to assume two different equilibria with the Mn^{2+} ion, the first for stabilization and the second for activation. This adequately explains the concave curve of the simple LINEWEAVER–BURK plot. It does, in addition, account for the observed effect of inactivation during the course of the other experiments described. The data are not sufficient yet to say whether the number of active sites and the number of ions per enzyme molecule necessary for stabilization is equal as has been tacitly assumed for calculating the rate equations.

The strong temperature dependence of the K_a values is rather interesting as the metal-ion binding to proteins is reported to be very little influenced by temperature changes[34]. As the constant K_a represents an algebraic combination of a number of equilibrium constants, including the acid dissociation constants of the groups responsible for binding the metal ion to the enzyme or to the substrate, the thermodynamic quantities calculated cannot be directly correlated to any possible activation mechanism. However, the apparent values can possibly be used to differentiate between different possible rate equations when the temperature dependence of the constants included in K_a can be determined separately. In the case of the substrate, this certainly is feasible and studies on this question are being planned. Thus, the findings from the kinetics of metal-ion activation confirm the results from the other experiments and emphasize the dual role of metal ions played in the case of carnosinase. It is by no means evident that the metal-ion specificity for activation is the same as for stabilization; if this is not the case, the total or apparent specificity is a combination of the influences imposed by a pair of metal ions.

As regards the nature of the activation process less can be said. The equilibrium seems to be established fast enough, in the case of Cd^{2+} and Zn^{2+}, to prevent any type of lag period being observed with the rather insensitive methods used for activity measurements. It is possible that the lag period in the activation by Mn^{2+} ions observed by previous workers[1] is connected with the stabilizing properties of this ion. The investigation of this problem is in progress and will be published separately. A more general discussion of the data presented, in the light of current concepts of the mechanism of peptidases, will be given in a subsequent communication describing the stabilization of enzymes by metal ions.

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