Effect of Metal Ions on Brain Peptidase Activity.* (26836)

S. VAN DEN NOORT AND L. LAHUT UZMAN
Harvard Neurological Unit, Boston City Hospital, and Dept. of Neurology, Harvard Medical School, Boston, Mass.

The substrate specificity of mouse brain peptidase activity toward a wide variety of peptides representing different structural arrangements of constituent amino acids has been demonstrated(1,2). This specificity was present without addition of any co-factors or activators. The present study concerns itself with the effect of some biologically significant metal ions on cerebral peptidase activity towards 2 representative peptides. The first of these, glycyl-L-leucine, represents the prototype of N-glycyl dipeptides with an aliphatic (or aromatic) lipophilic side-chain adjacent to the terminal carboxyl group which is maximally split by the cerebral peptidase system(2). The second, triglycine, represents a class of N-glycyl tripeptides in which the N-glycyl residue is split independently of the other residues(2).

Materials and methods. The mice used were Swiss albino mice from the colony maintained at the Harvard Medical School. Extensive chemical and histological data on the brain of this strain were already available (3). Fresh peptidase preparations in 30% glycerol in 4% phosphate buffer (0.067 M) at pH 7.0 were prepared for each experiment in the manner described in detail earlier(2), to yield 1.0 mg of dry tissue equivalent enzyme per 50 μl of extract. The metal ions Cu^{++}, Zn^{++}, Cd^{++}, Mg^{++}, Ca^{++}, Mn^{++}, $MoO_4^{=}$, Fe^{+++}, Co^{++}, Al^{+++}, Ni^{++} and Hg^{++} were added to the enzyme extract in the form of $CuSO_4$, $ZnSO_4$, $CdCl_2$, $MgSO_4$, $CaCl_2$, $MnCl_2$, Na_2MoO_4, $FeCl_3$, $CoCl_2$, $AlCl_3$, $NiCl_2$, and mercuric acetate respectively. All metal salts were analytical reagent grade. Concentration of metal ion was varied (1-5 μg metal/mg dry tissue equivalent enzyme), while the volume added was kept constant at 4 μl. Thorough mixing of the metal ions with the peptidase preparation was achieved by briefly "buzzing" the microtubes on the shaft of a micromotor. The peptide substrates (5 μM peptide in 50 μl volume) were then added. Incubation and formol titration were performed as described in detail previously (2). The purity of the peptides used was rigidly controlled chromatographically(2), and as an added precaution the triglycine was recrystallized twice from boiling water and ascertained to be chromatographically pure in 2 different solvent systems. Every experiment had parallel duplicate blanks for enzyme alone, enzyme plus metal, and metal plus peptide which were titrated in the same experiment. Blank values were subtracted from the test values. In no case did the presence of the metal ion, within the concentration range used, interfere with titration of liberated carboxyl groups.

Results. The effect of the different metal ions on hydrolysis of glycyl-L-leucine by cerebral peptidases is presented in Fig. 1. The results are expressed as percent of the hydrolysis of peptide without added metal ions, this value being 100%. These values are plotted against the different amounts of metal ions present (in moles). It is immediately apparent that the inhibitory effect of metal ions on the enzymatic hydrolysis of glycyl-L-leucine by brain is different for most divalent metals tested. Mercury, cadmium and copper ions, in that order, showed highest inhibition of glycyl-L-leucinase activity. The effect of Zn^{++} was intermediate between this group and Mn^{++} and Co^{++} whose inhibition never was more than 40%. Fe^{+++}, Al^{+++}, Ca^{++}, Mg^{++}, Ni^{++} and $MoO_4^{=}$ were without effect on this system except at very high concentrations (10-20 μM $\times$ 10^{-2} Metal/mg dry tissue equivalent enzyme) in which range they produced non-specific precipitation of protein in the incubation mixtures. The effect of metal ions on the hydrolysis of triglycine (Fig. 2) was more complex in view of the fact that the hydrolysis of 2 peptide

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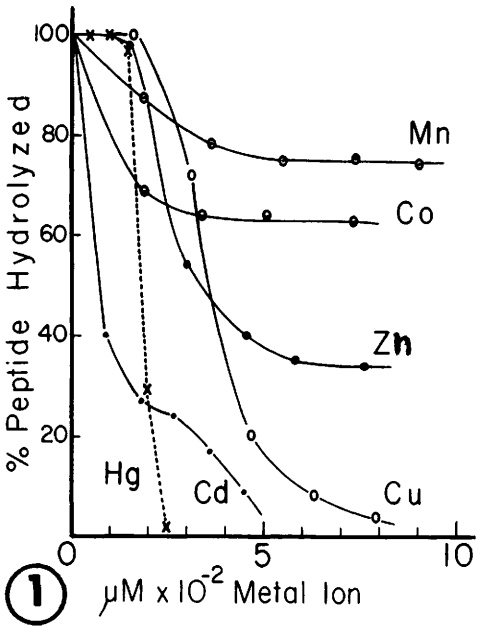

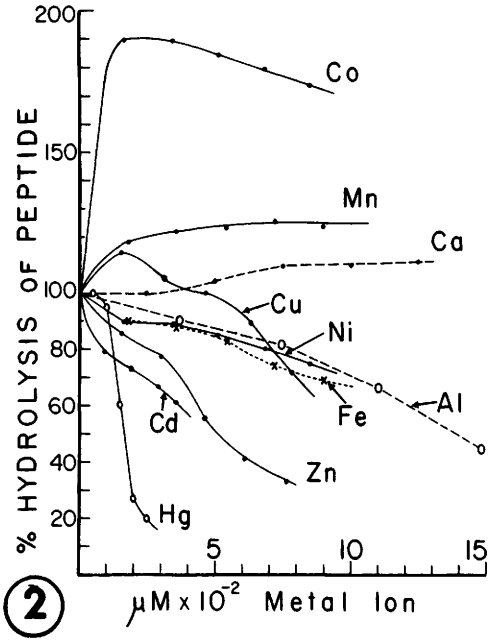

FIG. 1. Inhibitory effect of Hg^{++}, Cd^{++}, Cu^{++}, Zn^{++}, Co^{++} and Mn^{++} on hydrolysis of glycyl-L-leucine by cerebral peptidases. Ni^{++}, $MoO_4^{=}$, Fe^{+++}, Al^{+++}, Ca^{++} and Mg^{++} were without effect.

FIG. 2. Effect of metal ions on the enzymatic hydrolysis of glycyl-diglycine. Co^{++} ions activate hydrolysis of the diglycine residue.

bonds was involved. The hydrolysis of the N-terminal glycine residue which is hydrolyzed without metal ions(2), was inhibited maximally by mercury, cadmium and zinc ions, in that order. Copper, nickel, trivalent iron, and aluminum ions exhibited less pronounced inhibition (less than 50% at 10 μM $\times$ 10^{-2} Metal ion/mg dry tissue equivalent). Molybdate and magnesium ions were without any effect. At low concentrations of copper (0.002 μM/mg dry tissue equivalent) there was minimal splitting of the diglycine residue. Manganese ions produced a similar effect in all concentrations tested. The effect of Co^{++} was most impressive in that they produced a splitting of both peptide bonds leading to almost complete hydrolysis of triglycine to 3 moles of glycine. This was confirmed by paper chromatography of the end-products of enzymatic hydrolysis of triglycine in the presence of cobalt ions. The hydrolysis of diglycine alone, which is minimal in the absence of added activator, was shown to proceed to 90% completion on addition of extremely small amounts of cobalt (Fig. 3).

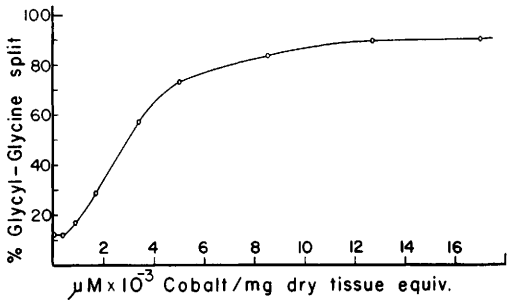

FIG. 3. Activation of cerebral diglycinase by cobalt ions.

In view of the fact that diglycine represents the first in the series of N-glycyl dipeptides with no aromatic or aliphatic non-polar side chain adjacent to the free carboxyl group, and because this group of peptides had been shown not to be split by cerebral peptidases (2), the effect of cobalt ions on the hydrolysis of other members of this group was tested. When glycyl-γ-aminobutyric acid, glycyl-L-aspartic acid, and glycyl-L-glutamic acid were incubated with enzyme, with and without addition of different concentrations of Co ions, no increase of hydrolysis could be demonstrated by titration or chromatography. It therefore appears that the effect of cobalt is

specific for hydrolysis of the dipeptide glycyl-glycine.

Discussion. Any attempt at quantitating the inhibition of a metal ion on an enzymatic system in which the bulk of the protein is probably not the enzyme, as in our case, has to make allowance for the fact that a certain proportion of the metal added will be non-specifically bound to inert protein, and only a portion will be free to react with the enzyme or the substrate. This non-specific binding leads to greatest errors in low metal concentration and is of less importance at higher concentration. The curves depicting the inhibitory effect of metals on enzymatic hydrolysis of glycyl-L-leucine and triglycine (Figs. 1 and 2) are therefore open to question as to whether inhibition actually does occur with much lower concentrations of each of the metal ions studied. This cannot be answered until each of the cerebral peptidases can be tested in pure form. The extremely low concentrations of cobalt ions needed to activate diglycinase activity would tend to argue against any significant portion of this metal being non-specifically bound by inert proteins. The data do show that there is a relative difference in the inhibitory effects of the different metals, some like Hg^{++} and Cd^{++} being more potent inhibitors by several orders of magnitude. The effect of cobalt ions on the hydrolysis of the diglycine residue of triglycine after the N-terminal glycine is removed by a separate enzymatic system strongly suggests that the cerebral enzyme responsible for diglycinase action is very similar to that described by Smith(4) in other tissues, and as such represents a distinct and separate enzyme in the mixture of enzymes operating in our cerebral peptidase system. The very low but measurable diglycinase activity without addition of cobalt observed here (Fig. 3) and in earlier studies (2) probably reflects the activity resulting from the pre-existing tissue cobalt. It is anticipated that extension of these studies to other peptidase systems may provide clues to the nature of the "toxic" effects of some of the metal ions studied here in disase processes like Wilson's disease, lead and mrcury poisoning(5,6).

Summary. The effect of Ca^{++}, Mg^{++}, Cu^{++}, Zn^{++}, Cd^{++}, Co^{++}, Mn^{++}, $MoO_4^{=}$, Ni^{++}, Fe^{+++} and Al^{+++} was studied on the enzymatic hydrolysis, by cerebral peptidases, of glycyl-L-leucine and glycyl-diglycine. The inhibition was shown to be of different orders of magnitude for different metals. Only cobalt ions were shown to enhance the hydrolysis of glycyl-diglycine by promoting the hydrolysis of the diglycine residue in the tripeptide which normally resists hydrolysis. This effect was specific for diglycine and not applicable to the other N-glycyl dipeptides which are normally not hydrolyzed by brain.

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Lack of Relationship Between Sialic Acid Content, Toxicity, and Lethality of *Escherichia coli.* (26837)

M. FORBES AND N. A. KUCK (Introduced by E. H. Dearborn)
Experimental Therapeutics Research, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

Some strains of *E. coli* are more lethal than others for mice. The reason for this difference is not known. The possibility that sialic acid which occurs in cells of some strains may be involved in their lethality was suggested by the following considerations: