THE INACTIVATION OF PEPSIN BY AN EQUIMOLAR AMOUNT OF 1-DIAZO-4-PHENYLBUTANONE-2

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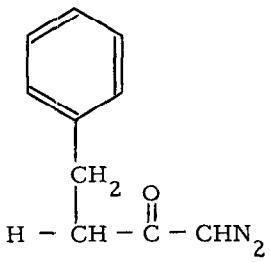
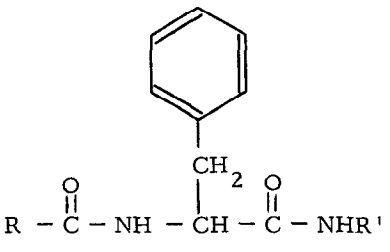
The recent report by Rajagopalan, Stein, and Moore (1966) on the Cu(II) catalyzed inactivation of pepsin by diazoacetylnorleucine methyl ester prompts us to communicate our results on the inhibition of pepsin catalyzed reactions by another compound, 1-diazo-4-phenylbutanone-2 (DPB). We were lead to investigate the possible inhibitory properties of this compound because pepsin



DPB



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catalyzes transpeptidation reactions in which the amino portion of the cleaved peptide bond is transferred to another amino acid residue (Bovey and Yanari, 1960; Neumann, Levin, Berger, and Katchalski, 1959; Fruton, Fujii, and Knappenberger, 1961). In this respect it is similar to glutamine amidotransferases, and these are known to be inhibited by specific diazoketones or esters in which the diazomethyl group replaces the amide amino group (French, Dawid, and Buchanan, 1963). DPB was prepared as a similar diazomethyl

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analog of phenylalanine amides (I) which are specific substrates for pepsin. The results summarized in this communication indicate that DPB is a potent inactivator of pepsin (Crowell, 1966) and it reacts in a one to one molar stoichiometry with the enzyme.

Material and Methods

DPB and DPB-2-C^{14} were synthesized from 3-phenylpropionic acid and 3-phenylpropionic-1-C^{14} acid (New England Nuclear Corp.) by the procedure described by Birkofer (1947). They were purified by distillation twice at 0.1 mm (95-100°C). The DPB-2-C^{14} had a specific activity of 3.2×10^{7} dpm/mmole. 2-diazocyclohexanone was synthesized by the procedure of Stetter and Kiehs (1965). Swine pepsin (lot numbers J2211, P2589, and 3466, 2 or 3 times crystallized from dilute alcohol) and swine pepsinogen (lot number K2298, crystalline, lyophillized) were obtained from Mann Research Laboratories.

The pepsin content of solutions was estimated from the absorbance at 278 mμ assuming a molar extinction coefficient of 50,900 (Perlmann, 1966). Enzyme activity was determined at 38° C and pH 2 by the ninhydrin assay (Moore and Stein, 1948) using N-acetyl-L-phenylalanyl-L-tyrosine (Cyclo Chemical Corp.) as substrate. For these assays the enzyme or inhibited enzyme concentration was ca. 3×10^{-6} M and the initial substrate concentration was 3×10^{-3} M. The percent inhibition was determined after 1.5 to 4 hours hydrolysis and in each case 0% inhibition refers to the enzyme which was treated in the same way as the inhibited enzyme except that no inhibitor was added. However, extended dialysis at 5° C or incubation at 38° C and pH 5.5 for a few hours had no appreciable effect on the enzyme activity.

For the reaction of pepsin with DPB and DPB-2-C^{14} the enzyme was dissolved in 0.04 M acetate buffer, pH 5.5 (containing $CuSO_4$ for the reactions

catalyzed by Cu(II)). A small aliquot (ca. 10 μl) of an ether solution of DPB or DPB-2-C^{14} was added to 10 ml of the pepsin solution and the solution was incubated at 38°C for various lengths of time. Following incubation the solutions which initially contained DPB-2-C^{14} were dialyzed at 5°C; usually 2 to 5 ml were dialyzed versus 4 liters of 0.04 M acetate buffer (pH 5.5) for at least 20 hours with at least 2 changes of buffer. Following dialysis the extinction coefficient of the inhibited pepsin at 278 mμ is essentially the same as that of pepsin. For the radioactivity measurements, a 0.1 or 0.2 ml aliquot of the dialyzed solution was added to 15 ml of Bray's solution (Bray, 1960) and the radioactivity determined using a Parkard Tri-Carb Liquid Scintillation Counter.

Results and Discussion

Some of the characteristics of the inhibition of pepsin catalysis by DPB are shown by the results summarized in Table 1. Although Cu(II) alone has no effect on the enzyme catalysis, it markedly catalyzes the inactivation of the enzyme by DPB. In the absence of Cu(II) there is a slow inactivation of the enzyme by DPB, but its rate is not very reproducible, possibly because it is catalyzed by trace impurities of Cu(II) which are present in varying amounts. In the absence of Cu(II), a specific substrate can protect the enzyme from inhibition; thus, presumably DPB exerts its effect at the active site. In the presence of Cu(II), such protection is not observed; this result is expected because of the rapidity of the Cu(II) catalyzed reaction. Additional evidence that binding at a specific site on the enzyme is necessary for inactivation is the observation that 2-diazocyclohexanone, which is a diazoketone that is not an analog of phenylalanine, does not inhibit the enzyme.

Although cyclohexane extracts DPB almost quantitatively from aqueous

Table 1

The Inhibition of Pepsin Activity by DPB

Concentration ($M \times 10^4$) during Incubation of pepsin with DPB at pH 5.5, 38°C			Time of Incubation (minutes)	% Enzyme inhibited (from ninhydrin assay at pH 2, 38°C)
Pepsin	DPB	Cu(II)		
0.3	0.83	0.0	5	0
0.3	0.83	0.0	120	40[a]
0.3	0.83	0.1	5	84
0.3	0.83	0.1	120	80
0.3	0.00	1.0	120	0
0.3	0.83[b]	0.0	120	0
0.3	0.83[b]	1.0	120	76
0.3	0.00[c]	0.5	120	0

[a] This value is not too reproducible; however, it was usually between 20 and 50. [b] During incubation the solution also contained 1.5×10^{-2} M N-acetyl-L-phenylalanyl-L-tyrosine. [c] 2-diazocyclohexanone (6.6×10^{-5} M) was used instead of DPB.

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solution, cyclohexane extraction of the inhibited enzyme solution following incubation does not reverse the inhibition. In Table 2 are summarized some results using DPB-2-C^{14} which indicate that one molecule of DPB reacts with one molecule of pepsin to give a modified pepsin which is catalytically inactive. At high concentrations of inhibitor the percent inhibition appears to decrease, possibly because the inhibitor reacts with itself. However, even though the inhibitor is present in excess only one mole reacts with pepsin per mole of enzyme. Thioethanol and hydroxylamine do not remove the label from the enzyme at pH 5.5. The thioethanol result is in contrast to that observed by Erlanger, Vratsanos, Wasserman, and Cooper, (1965) for pepsin inhibited by p-bromophenacyl bromide. The observation that

Table 2

The Reaction of Pepsin with DPB-2-C^{14}

Concentrations ($M \times 10^4$) during incubation of pepsin with DPB-2-C^{14} at pH 5.5 38° C			Time of incubation (min)	Radioactivity following dialysis (dpm/mmole of pepsin $\times 10^{-7}$)	% pepsin with inhibitor attached	% pepsin inhibited (from ninhydrin assay at pH 2, 38°C)
pepsin	DPB-2-C^{14}	Cu(II)				
6.5	18	7.0	120	2.3	72	86
9.3	10	1.0	30	2.6	81	91
				2.5[a]	78[a]	-
				2.4[b]	75[b]	-
9.3	100	1.0	30	1.9	60	60
0.0	10	1.0	30	0.0[c]	-	-
4.6[d]	5	0.5	120	0.1	3	-[e]

[a] Following dialysis the inhibited protein solution was incubated with 0.1 M thioethanol at pH 5.5, 38°C for 24 hours and then dialyzed again for 40 hours versus 0.04 M acetate buffer, pH 5.5. [b] Same as footnote 'a' except hydroxylamine (0.1 M) used instead of thioethanol. [c] dpm/mmole of initial DPB-2-C^{14}; the experiment indicates that all the inhibitor which does not react with the enzyme is removed under the dialysis conditions. [d] Pepsinogen used instead of pepsin. [e] The pepsinogen showed no catalytic activity either before of after the attempted reaction with DPB-2-C^{14}.

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pepsinogen does not react with the inhibitor again indicates that the inhibitor is reacting at the active site of pepsin.

The inhibited (with DPB-2-C^{14}) enzyme can be precipitated with 5 N trichloroacetic acid (TCA) and the precipitated material washed several times with acetone and dioxane with no loss of radioactivity. However, the radioactivity is removed from the TCA precipitated material on treatment with 6 NHCl, or incubation for a few hours in tris buffer (pH 8) at 25° C (these observations were made by Dr. K. T. Fry). If the attachment of the inhibitor to the enzyme is by an ester linkage (Erlanger, Vratsanos, Wasserman, and Cooper, 1966; Gross and Morell, 1966; Rajagopalan,

Stein, and Moore, 1966; Delpierre and Fruton, 1965) then the rate of the base catalyzed ester hydrolysis must be increased dramatically by the carbonyl group. Newman and Hishida (1962) and Bender and Silver (1962) have observed some rate acceleration in related model systems. However, diazoketones are also known to react with alcohols, phenols, amines, and thiols when copper is present as a catalyst (Yates, 1952). The site and mode of attachment of the inhibitor to the enzyme are currently under further investigation.

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