at the C(2)-O(3) bond of the metallocycle to yield carbonyl and carbonate containing fragments prior to product formation. Likewise, the report by Aresta et al.,[2] that Ni(PCy$_3$)$_2$(CO$_2$) upon decomposition affords carbonyl and carbonate species, may reflect a mechanism like that of Scheme I.

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Detection of Covalent Intermediates by Nucleophile Trapping in the Hydrolysis of Phenyl Tetrahydrofurfuryl Sulfite Catalyzed by Pepsin

Sir:

From a consideration of both kinetic studies and chemical modification data, the mechanism illustrated in Scheme I involving the formation of an anhydride intermediate, V, has been proposed for the pepsin-catalyzed hydrolysis of sulfite esters.[1,2] Recently, it has been demonstrated that in the hydrolysis of sulfite esters mediated by model carboxylate catalysts mixed anhydride intermediates can be detected by the use of nucleophile trapping agents.[3] This finding encouraged us to test the application of this approach to the elucidation of the mechanism of the pepsin-catalyzed hydrolysis of sulfite esters. We now wish to report that with the use of hydroxylamine as a trapping agent we have been able to obtain the first direct evidence for the intermediacy of mixed anhydrides in the pepsin-catalyzed hydrolysis of sulfite ester and to identify the active site carboxyl groups involved in the formation of the anhydrides.

Scheme I. Proposed Mechanism for the Pepsin-catalyzed Hydrolysis of Sulfite Esters

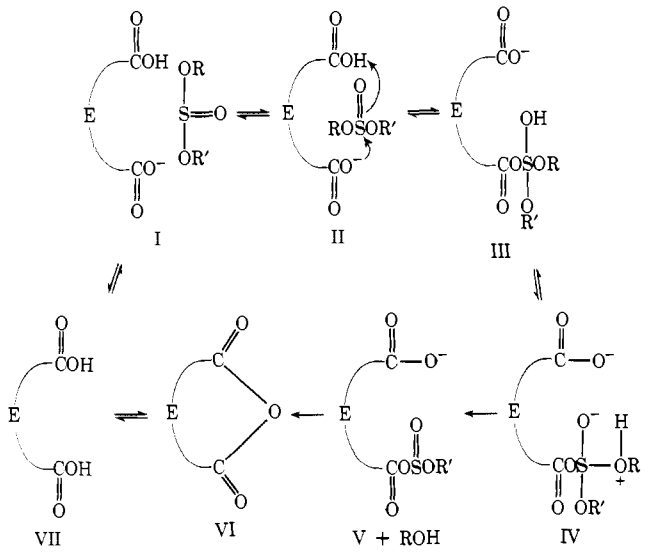
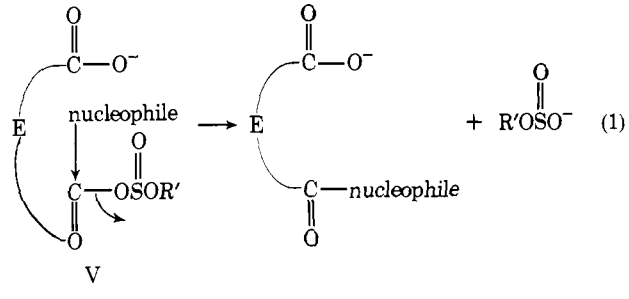


Table I. Hydroxamate Incorporation and Remaining Catalytic Activity in the Pepsin-Catalyzed Hydrolysis of Phenyl Tetrahydrofurfuryl Sulfite in the Presence of Hydroxylamine[a]

Mole ratio of PTFS to pepsin	No. of incubations[b]	Remaining pepsin activity %	Mol of hydroxamate/ mol of pepsin
2.5	1	81.9	1.2
2.2	2	61.2	3.3
5.0	1	59.5	1.4
5.0	2	52.0	3.8
9.2	1	57.2	1.4
11.7	9	53.3	3.2

[a] Solutions containing porcine pepsin (Worthington PM 8HA and PM 2LB) were purified by passing them through a column of Sephadex G-25 which had been equilibrated with 0.01 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 5.3. Phenyl tetrahydrofurfuryl sulfite (PTFS) in CH_3CN was added to the pepsin solution containing 0.01 M hydroxylamine (pH 5.3), and the resultant solution was equilibrated for 30 min at room temperature. Control experiments for the measurement of pepsin activity were carried out in parallel in the presence of CH_3CN only. Experiments on the incubation of hydroxylamine with pepsin in the absence of the sulfite ester revealed no significant incorporation of hydroxylamine during time periods comparable to those employed for the experiments summarized in this table. [b] Additional substrate was added at 30-min intervals for multiple incubation experiments. At the end of the experiments the enzyme solution was dialyzed against 3 l. of 0.01 M sodium acetate buffer, pH 4.0, for 72 h with five changes. Hydroxamic acid was quantitatively analyzed by the method of F. Bergmann and R. Segal, *Biochem. J.*, **62**, 542 (1956). Pepsin activity was determined by the hemoglobin assay method described by R. B. Chow and B. Kassell, *J. Biol. Chem.*, **243**, 1718 (1968).

In Table I the results obtained by incubating various amounts of phenyl tetrahydrofurfuryl sulfite (PTFS) with pepsin in the presence of 0.01 M hydroxylamine at pH 5.3 are given. At a molar ratio of PTFS/pepsin = 2.5 in a single incubation experiment about 20% of the peptic activity was lost and concomitantly 1 mol of hydroxamate per mole of pepsin was found. This observation is consistent with the scheme of eq 1 in which the potent nucleophile hydroxylamine is postulated to attack the mixed anhydride intermediate V. With increases in the number of incubations performed with PTFS or increases in the concentration of PTFS, the peptic activity was seen to decrease and there was an increase in the number of enzyme-bound hydroxamate groups produced, up to a maximum value of 3–4 mol per mole of pepsin.



Previously, many investigators have reported evidence from chemical modification studies with various esterifying agents that there are at least two catalytically essential aspartate β-carboxyl groups in the active site of pepsin.[4-8] Additionally, Erlanger et al.[9] and Gross and Morell[10] have demonstrated that an aspartate β-carboxyl group which is not crucial for catalysis is esterified by *p*-bromophenacyl bromide. It was, of course, of interest to determine the relationship of the carboxyl groups involved in hydroxamate formation to those identified as important residues in the esterification experiments. Therefore, following the procedure of Gross and Morell,[10] the hydroxamate-containing pepsin species obtained in the hydroxylamine-trapping experiments illustrated in Table I were subjected to Lossen rearrangement, followed by acid hydrolysis. With all samples studied, the amino acid analyses performed on the hydrolyzates revealed that only 2,3-diaminopropionic acid was formed. No 2,4-diaminobutyric acid was detected. Thus, the PTFS–pepsin intermediates trapped with hydroxylamine must have been formed at the β-carboxyl groups of aspartate residues.

Having demonstrated the nature of the groups involved in anhydride formation in the pepsin-catalyzed hydrolysis of PTFS, we wished to identify the particular residues taking part in catalysis. In a typical experiment a solution of the hydroxamate-containing pepsin (3.3 mol of hydroxamate per mole of pepsin) obtained from two incubations of PTFS with pepsin (mole ratios PTFS to pepsin in each incubation = 2.2, see Table I) was digested by native pepsin at pH 3.5, subjected to gel filtration through Sephadex G-25, high voltage electrophoresis (3000 V at pH values of 3.5 and 6.5), and descending paper chromatography (*tert*-butyl alcohol:methyl ethyl ketone:water, 2:2:1 by volume). Three hydroxamate-containing peptides and their amino acid compositions were determined. One peptide contained Asp_1, Thr_1, Ser_1, Gly_1, Val_1, Ile_1, Phe_1. This amino acid analysis is consistent with the composition of a fragment of pepsin (Val-29 → Ser-35) containing the active site residue Asp-32, the group modified by 1,2-epoxy-3-(*p*-nitrophenoxy)propane.[7,8,11] The second peptide had the composition Asp_1, Thr_1, Ser_1, Gly_3, Ala_1, Glu_1, Val_1, Ile_1, Leu_1 which seems to correspond to a fragment of pepsin (Gly-208 → Leu-220)[12] containing the active site residue Asp-215, the group modified by diazocarbonyl reagents.[4-6,11] The third peptide was a large one, and we still do not know its location in the amino acid sequence of pepsin.

Three principal conclusions can be drawn from our experiments. (1) The detection of hydroxamate formation in the pepsin-catalyzed hydrolysis of PTFS in the presence of hydroxylamine provides the first direct demonstration that anhydrides are intermediates in the hydrolysis reactions of sulfite esters. This finding is in good agreement with circumstantial evidence that acyl enzymes (anhydrides) may be intermediates in at least some pepsin-catalyzed transpeptidation reactions.[13,14] (2) The correspondence of two of the aspartate residues involved in hydroxamate formation to those (Asp-32 and Asp-215), the esterification of which is known to inactivate pepsin as a peptidase, not only shows that these residues are indeed direct participants in the hydrolysis of sulfite esters but also gives strong support to the hypothesis that the active site of pepsin as a sulfite esterase overlaps with that for its action as peptidase. (3) Finally, the observation that as many as 3–4 mol of hydroxamate can be incorporated per mole of pepsin shows that anhydride formation can occur at several carboxyl groups in pepsin-catalyzed sulfite ester hydrolysis. This is consistent with the earlier discovery that pepsin esterified either at Asp-32 or at Asp-215 can function as a catalytically active species toward sulfite esters.[2]

A major question which remains is to determine why pepsin in which hydroxamate groups have been introduced at the active site carboxyl groups can still act quite effectively as a catalyst for the hydrolysis of hemoglobin (see Table I). Two alternative explanations are either that a carboxyl group other than the β-carboxyls of Asp-32 or Asp-215 can participate in the peptidase action of pepsin or that the hydroxamate groups themselves are catalytically active. Experiments are now in progress in our laboratory to resolve this problem.

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Isotope Effects in Hydrophobic Binding Measured by High-Pressure Liquid Chromatography[1]

Sir:

We wish to report the first isotopic separations by hydrophobic high-pressure liquid chromatography. Several deuterated substances are completely separated from the corresponding protiated substances by this method. These results are of interest in four ways: (1) The possibility of quantitative analysis of isotopic content is demonstrated. (2) Extension to preparative-scale separation of isotopic species seems feasible. (3) Isotope effects on binding can be measured accurately. (4) By study of isotope effects under different conditions, the nature of the hydrophobic effect may be probed.

The ability to make such separations on a column only 30 cm long is related to the fact that the hydrophobic effect directly involves the CH(CD) bonds. Our results (Table I) show isotope effects on binding which are greater than unity, so that H is favored in the stationary phase relative to