

## The Pepsin-catalysed Hydrolysis of Sulphite Esters. Oxygen-18 Studies providing Direct Evidence for Involvement of a Carboxyl Group in Sulphite Esterase Activity

By T. PETER STEIN and DAVID FAHRNEY\*

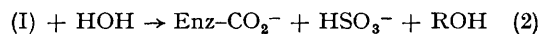
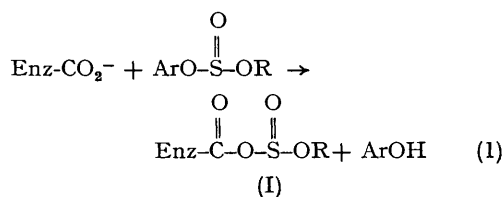
(Department of Chemistry and Molecular Biology Institute, University of California, Los Angeles, California 90024)

CERTAIN organic sulphites are excellent substrates for pepsin and kinetic evidence indicates that peptidase and sulphite-esterase activities probably involve the same catalytic site.<sup>1</sup> A carboxyl group probably acts as a catalytic group in peptidase<sup>2-13</sup> and sulphite esterase<sup>1</sup> reactions. We now present results of a direct <sup>18</sup>O-tracer experimental test for participation of a carboxyl group in the bond-breaking process.

In recent years enzyme chemists have devised sophisticated approaches to the problem of identifying catalytic functional groups in the active site of enzymes, such as the use of active-site-directed irreversible inhibitors to attach a covalent marker at or near the active site.<sup>14</sup> Several groups<sup>7-13</sup> have found such stoichiometric irreversible inhibitors for pepsin, using the affinity of the enzyme for substrate-like molecules as the "driving force" for the selective labelling of a group at the active site. However, there is always the possibility that inactivation is due to overlap with the active site (preventing access of substrate), or to changes in the conformation of the protein

which preclude proper functioning of the active site.

A plausible pathway for the pepsin-catalysed hydrolysis of sulphite esters would involve formation of a mixed anhydride intermediate:



An analogous anhydride intermediate has been suggested for peptidase reactions. The absence of a deuterium solvent isotope effect on the catalytic constant  $k_{\text{cat}}$  for both peptidase<sup>5</sup> and sulphite esterase<sup>1</sup> reactions is consistent with formation of an anhydride intermediate as the rate-limiting

step. The two electrophilic centres in a sulphite-enzyme anhydride (I) are dissimilar enough so that water might preferentially attack at the enzyme acyl carbon atom.  $^{18}\text{O}$ -Tracer studies afford a direct test of this hypothesis, and thus direct evidence that the carboxyl group is an essential catalytic group.

Pepsin (1 g.,  $3 \times 10^{-5}$  mol.) was allowed to react at pH 3.5 (25°, 30 min.) with a 15-fold excess of *p*-bromophenyl methyl sulphite in 50 ml. of  $^{18}\text{O}$ -water (1.5 atom %). The steady-state kinetic parameters evaluated from initial velocity studies are  $K_m$  (app) = 3 mM and  $k_{\text{cat}} = 7.5 \text{ min.}^{-1}$ ; thus the enzyme recycles until  $^{18}\text{O}$  exchange into the active-site carboxyl group is complete. Then the reaction mixture was freeze-dried to remove  $\text{H}^{18}\text{OH}$ . The protein was purified further by twice freeze-drying from unenriched water. Finally, the enzyme was dissolved in 50 ml. of glycine buffer (0.1 M, pH 3.7) and dialysed against 3 l. of the same buffer at 4° for 3 hr., to remove inorganic sulphite.

The solution was adjusted to pH 4.8, and the labelled pepsin was allowed to interact once with *p*-bromophenyl methyl sulphite (12.5 mg.,  $5 \times 10^{-5}$  mol.). After 1 min. the enzymic reaction was quenched and the excess of substrate hydrolysed by adding 50% NaOH (carbonate-free) to about pH 12. Pepsin denatures almost instantaneously at alkaline pH, but does not precipitate. The solution was adjusted to pH 9.5 with 1M-perchloric acid, and 5 ml. of saturated barium chloride solution was added; barium sulphite was collected by (centrifuge) and washed once with 0.1 mM-NaOH and once with water. The barium sulphite was dried at 110° for 3 hr. and analysed for  $^{18}\text{O}$  by pyrolysis with guanidinium chloride.<sup>15</sup>

Barium sulphite isolated from five experiments with  $^{18}\text{O}$ -pepsin contained  $0.256 \pm 0.015$  atom %  $^{18}\text{O}$ ; the theoretical value is 0.46. Two types of control experiments were performed; one was the

entire experiment without enriched water, and the other was also identical except that no sulphite ester was added to the pepsin- $\text{H}^{18}\text{OH}$  solution. Barium sulphite from control experiments had  $0.205 \pm 0.004$  atom %.

These results provide evidence that an oxygen atom from the enzyme is incorporated into the bisulphite ion formed during a single turnover of the  $^{18}\text{O}$ -labelled enzyme. Loss of  $^{18}\text{O}$  from labelled bisulphite ion *via* an exchange reaction with water is probably the main reason for the 20% yield. We have found that the exchange reaction has a half-life of about 30 sec. at pH 4.4, 25° (*cf.* ref. 16.)

Recently Bayliss and Knowles<sup>7</sup> have isolated a heptapeptide containing a single aspartic acid residue which had been esterified at the  $\beta$ -carboxyl group by reaction of pepsin with *N*-diazoacetyl-L-phenylalanine methyl ester. Experiments to determine whether the same residue is labelled during sulphite ester hydrolysis are in progress. This is a likely possibility because the similar diazo-reagent of Rajagopalan, Stein, and Moore<sup>8</sup> completely abolishes both peptidase and sulphite-esterase activity.<sup>1</sup>

In hydrolysis of *N*-benzyloxycarbonyl-L-phenylalanyl-L-tyrosine by pepsin in  $\text{H}^{18}\text{OH}$ , the initial *N*-benzyloxycarbonyl-L-phenylalanine released in amounts stoichiometric with the enzyme contains  $^{18}\text{O}$  as expected for direct incorporation of one water oxygen atom into the phenylalanine carboxyl group.<sup>17</sup> Thus this approach has not revealed an anhydride intermediate and suggests that, if such an intermediate forms in peptide-bond cleavage, water attacks at the substrate acyl carbon atom.

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