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Diazoacetyl Subtilisin

(labeled enzyme/photolyses)

YURI STEFANOVSKY* AND F. H. WESTHEIMER†

James Bryant Conant Laboratories of the Chemistry Department, Harvard University, Cambridge, Massachusetts 02138

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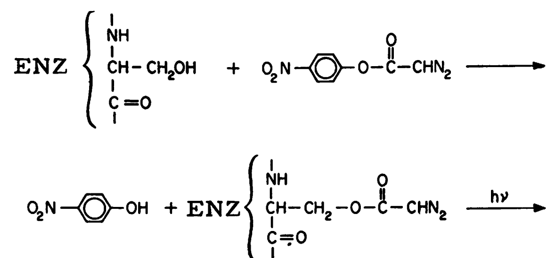
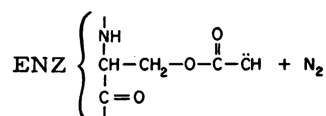
ABSTRACT **Subtilisin reacts at pH 6.8–7.8 with *p*-nitrophenyl diazoacetate to release *p*-nitrophenol and form diazoacetyl subtilisin. Although at pH 7.8 this derivative rapidly undergoes hydrolytic cleavage to regenerate active enzyme, the derivative can be trapped by rapidly lowering the pH to 5. Similarly, with ^{14}C-labeled *p*-nitrophenyl diazoacetate, the corresponding radiochemically labeled diazoacetyl enzyme can be prepared. Photolysis of this radioactive derivative incorporates radioactivity into the protein, and subsequent hydrolysis gives rise to several radioactive components, which, however, have not yet been identified.**

A chemical method of mapping the active sites of enzymes is at least a desirable, and perhaps an essential, adjunct to x-ray crystallography. One of the methods for such mapping involves the preparation of a diazoacyl derivative at the active site of an enzyme, followed by photolysis of the derivatized protein. Photolysis of the diazo group yields nitrogen plus a highly reactive carbene (Scheme 1), which then inserts into the physically adjacent residues. In this way, it "scans" the region in the neighborhood of the active site. Diazoacetyl and diazomalonyl chymotrypsin and trypsin have been prepared and photolyzed; the products so far identified have included nearby tyrosine, cystine, alanine, and histidine residues, in addition to the active-site serine (1–5). Similar "photoaffinity labeling" (6) with and without formation of a covalent bond between reagent and protein, has been applied to cyclic AMP (7), to a DPN analog (8), and to antibodies (9); furthermore, azides (6, 10) (which photolyze to nitrenes) have been used in place of diazo compounds. Subtilisin, like chymotrypsin and trypsin, is a serine esterase, and therefore presents an opportunity to attach a carbene reagent at the enzymically active site. The present paper reports the preparation of diazoacetyl subtilisin, and its photolysis, in accordance with Scheme 1.





In an ideal case the reaction between *p*-nitrophenyl diazoacetate and an enzyme would be rapid and quantitative, and the subsequent hydrolysis of the acylated enzyme would be slow. *p*-Nitrophenyl diazoacetate reacts rapidly and stoichiometrically with chymotrypsin, and the isolated derivative is stable (in the absence of hydroxylamine) even at pH 7. However, enzyme derivatives can be isolated even under less-ideal conditions. The "burst" of *p*-nitrophenol produced from *p*-nitrophenyl acetate with chymotrypsin (11) implies that acetylchymotrypsin has been formed in solution, even though this derivative is unstable at neutral pH. Acetylchymotrypsin was first isolated (12) by allowing the enzyme and *p*-nitrophenyl acetate to react at pH 5.0 (where no real "burst" of *p*-nitrophenol was obtained), and then stabilizing the product to self-hydrolysis by lowering the pH to 3.8. A similar scheme has proved effective for the preparation of diazoacetyl subtilisin.

p-Nitrophenyl diazoacetate is hydrolyzed by subtilisin at neutral or alkaline pH. At pH 7.8, the reaction is biphasic, but the "burst" is not clearly demarcated, and its magnitude (determined by extrapolation to zero time) falls short of quantitative formation of the derivative (Fig. 1). Nevertheless, diazoacetyl subtilisin can be prepared by allowing the reaction between the enzyme and *p*-nitrophenyl acetate to attain a steady-state at pH 7, and then lowering the pH to 5.0, where the diazoacetyl derivative is relatively stable. The derivative can then be subjected to photolysis.

MATERIALS AND METHODS

Materials. Subtilisin (Protease Type VII), purchased from the Sigma Chemical Co., corresponds to subtilisin DPN or Novo (13). The crude enzyme was partially purified by passage over Sephadex. Typically, 15 mg of subtilisin in 5 ml of 0.05 M (pH 5.0) acetate buffer was introduced onto a 1.5 × 30 cm column containing 15 g of Sephadex G-25, saturated with the same buffer. The column was eluted with this pH 5.0 buffer; 3.8-ml fractions were collected and monitored for protein at 278 nm. Fractions 4, 5, and 6 (11.4 ml) contained the enzyme. In a similar experiment with 50 mg of enzyme the solution was assayed spectrophotometrically; the extinction coefficient for the pure enzyme was taken as 1.17 ml/mg·cm (13). On this basis, the recovery of protein was 31.2

* Present address: Institute of Organic Chemistry, Bulgarian Academy of Sciences, Sofia 13, Bulgaria.
† To whom reprint requests are to be sent.

mg, or 62%; since 98% of the total activity was recovered, the chromatographed enzyme was about 75–80% pure.

p-Nitrophenyl chloroformate (14) and *p*-nitrophenyldiazoacetate (2) were prepared by published procedures, except that the latter was crystallized from hexane instead of from chloroform–hexane.

p-Nitrophenyldiazo-[^{14}C]acetate was prepared by the same general procedure as that used for the nonradioactive compound. [^{14}C]Phosgene (1 mCi; 19.8 mg) in 10 ml of benzene was purchased from New England Nuclear Corp. Sodium *p*-nitrophenolate (35 mg) was added and the mixture was stirred at 55° for 1 hr. After isolation (2), the crude chloroester was diluted with 300 mg of unlabeled *p*-nitrophenyl chloroformate and crystallized from hot benzene to yield 225 mg of pure product, with 0.25 mCi of ^{14}C per mmol. An ether solution of 100 mg of the radioactive *p*-nitrophenyl chloroformate was added slowly with stirring to 20 ml of a cold ether solution of 0.08 mol of diazomethane, and the resulting mixture was allowed to stand overnight at 4°. Excess diazomethane was removed in a stream of nitrogen and the ether was evaporated under reduced pressure. The yellow oil that remained was dissolved in 4 ml of benzene and chromatographed on a column (1 × 10 cm) containing 10 g of Woelm Grade IV neutral alumina. The column was eluted with benzene, and the eluate was evaportated to yield 100 mg of crude *p*-nitrophenyl diazoacetate. After recrystallization from 5 ml of hexane, the product (70 mg) melted at 91–93° and assayed for 0.246 mCi of ^{14}C per mmol.

Methods. Samples dissolved or suspended in 15 ml of polyether scintillation fluid (2) were counted with a Nuclear-Chicago liquid scintillation system (720 series). Amino-acid analyses were done with a Beckman model 120 B amino-acid analyzer, equipped with 6.6-mm cuvettes and a 0–5 mv slidewire. Photolyses were conducted in a Rayonet photochemical reactor model RPR 100 with RPR 3500-Å lamps as the light source. These lamps have maximum emission at 3500 Å, with essentially none below 3100 Å.

Subtilisin was assayed (15) with *p*-nitrophenylacetate and its purity was also determined with cinnamoyl imidazole (16). Specifically, for assay, 50–100 μl of 10 mM *p*-nitrophenylacetate in acetonitrile was introduced into 3.00 ml of 1 μM enzyme in pH 7.82 phosphate buffer at 25°. The increase in absorbancy at 400 nm was recorded with a Gilford model 240 spectrophotometer. The extinction coefficient of the equilibrium mixture of *p*-nitrophenol and *p*-nitrophenolate ion at pH 7.82 was 16,500 cm^{-1} M^{-1}. The purity of the enzyme as supplied by Sigma was 46% by the *p*-nitrophenyl acetate assay, and 49% by the cinnamoylation procedure.

TABLE 1. *Recovery of enzymic activity during hydrolysis of diazoacetyl subtilisin*

Min	Slope (units*/ml)	Total units (in 11.2 ml)	% Activity†
10	0.228	25.3	39
60	0.438	49.1	75.6
180	0.597	66.0	91.0

* Arbitrary units, corresponding to an increase of 0.08 *A* units/min.

† 10 ml of initial solution contains 65 units.

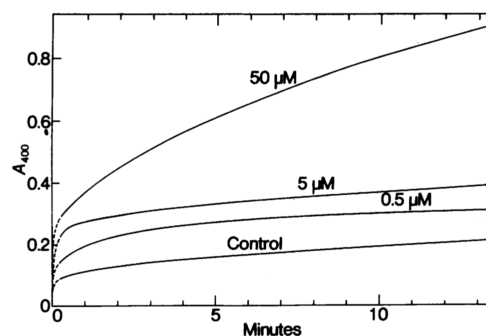


FIG. 1. Rate of the reaction of *p*-nitrophenyl acetate with subtilisin, showing the small "burst" of *p*-nitrophenol at the start of the reaction, and the subsequent enzymic hydrolysis of the substrate. The concentration of the enzyme added is given for each line.

RESULTS

Diazoacetyl Subtilisin. 25 mg of enzyme was purified by chromatography, and half of it was used for the preparation of diazoacetyl subtilisin. The pH of the solution was adjusted to 7.0 with 0.1 N NaOH solution, and 0.3 ml of a 3 mM *p*-nitrophenyl acetate in acetone was added. After 15 min the pH was lowered to 5.0 with 10% aqueous acetic acid, and the solution was extracted twice with methylene chloride. The resulting solution was passed over Sephadex G-25 in acetate buffer (pH 5.0). The diazoacetyl subtilisin was collected in 11.2 ml. 1 ml of this fraction was diluted to 10 ml with pH 7.0 buffer, and 3-ml samples were assayed as a function of time; 50 μl of 2 mM *p*-nitrophenyl acetate was used for each assay. The results are shown in Table 1 and Fig. 2.

Diazo-[^{14}C]acetyl Subtilisin. The pH of a solution of 11.2 mg of chromatographed enzyme in 7 ml of pH 5 buffer was adjusted to 7.8 by the addition of 0.1 N NaOH, and 4.16 mg of *p*-nitrophenyl diazo-[^{14}C]acetate in 0.4 ml of acetone was added. The reaction mixture was treated exactly as for the preparation of the nonradioactive derivative. After the pH had been lowered to 5.0 with 10% acetic acid, the resulting solution was extracted twice with 15 ml each of methylene chloride and chromatographed on Sephadex G-25; 4.0-ml fractions were collected and monitored for protein at 278 nm.

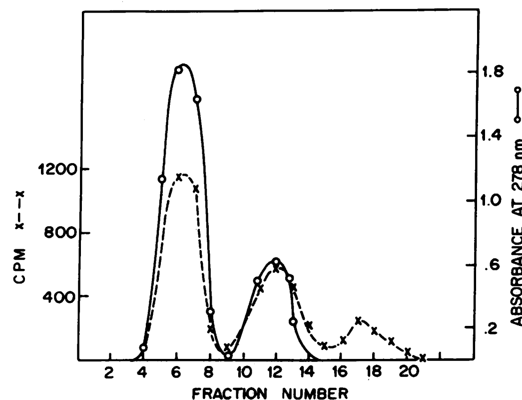


FIG. 2. Chromatography of diazo-[^{14}C]acetyl subtilisin. *Left-hand scale*, cpm above background for 0.1-ml samples of fractions. *Right-hand scale*, absorbance of these fractions. Chromatography was on Sephadex G-25 at pH 5. (*a*) The right-hand peak was measured at 268 nm, where the absorbance of these fractions was a maximum.

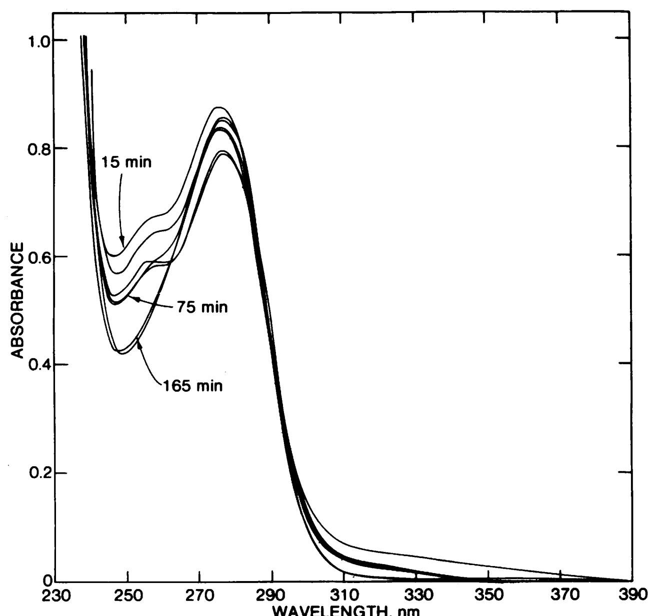


FIG. 3. Optical absorption of diazoacetyl subtilisin, at various times during its photolysis with light of about 350 nm. The final spectrum (after 165 min) corresponds well to that of subtilisin or any other pure protein.

Fractions 5, 6, and 7 (12.0 ml) contained the radioactive protein. The radiochemical data for 0.1-ml samples are shown in Fig. 2.

Based on the assumption that the extinction coefficient of diazoacetyl subtilisin at 278 nm is the same as that of subtilisin itself, the radioactive fractions contained 15.4 mg of protein; fractions 5–7 were combined and their radioactivity was measured at 1045 cpm above background, or about 1400 dpm. This corresponds to 0.307 μmol of ^{14}C in 12 ml; on the basis of a molecular weight of 27,600 for subtilisin (13), the solution contains 8.5 mg of singly acylated enzyme. The cinnamoyl imidazole assay showed that only about 0.6 mg of active enzyme was still present. These results, in combination, show that the trapped enzyme is almost completely acylated, although considerable additional protein is present but enzymically inactive. When the diazo-[^{14}C]acetyl subtilisin was allowed to hydrolyze at pH 7.0 for 3 hr under the same conditions as for the nonradioactive derivatized enzyme, almost all the activity that could arise from 7.5 mg of acylated enzyme was regenerated.

Spectra and Photolysis of Diazoacetyl Subtilisn. The spectrum of diazoacetyl subtilisin shows, in addition to the protein absorption at 278 nm, strong absorption in the region between 240 and 260 nm, an absorption that appears as a shoulder on the 278-nm protein band (Fig. 3). This band has its maximum near 250 nm, and corresponds to the known strong short-wavelength absorption of the diazo esters. In particular, the extinction coefficient for the absorption of ethyl diazoacetate (17) in ethanol at 247 nm is 13,500 cm^{-1} M^{-1}. The presence of this same band in the derivatized enzyme shows that the diazo group is present, and demonstrates that the reaction of *p*-nitrophenyl diazo acetate with subtilisin proceeds, as it does with chymotrypsin (1, 2), without loss of the diazo function; furthermore, treatment of the diazoacetyl enzyme with 10% acetic acid, to pH 5.0, has not destroyed the diazo function.

A solution of diazo[^{14}C]acetyl subtilisin containing 4.06 mg of the diazoacetyl enzyme in 9 ml of acetate buffer (pH 5), was photolyzed at 5° for 3.5 hr in a water-jacketed Pyrex tube. Little short-wavelength radiation can penetrate the Pyrex of the photolysis vessel. In general, diazo compounds, such as ethyl diazoacetate, have a weak absorption band around 370 nm, and diazoacyl enzymes (1–5) undergo photolysis with light in the near ultraviolet that must be absorbed by this weak band. Such light cannot, of course, be absorbed either by the strong diazo absorption band at 250 nm or by the normal protein chromophores, so that photolyses at long wavelength, although they obviate the complications that might be introduced by absorption of light into the aromatic residues of the protein, are necessarily slow. Photolysis leads to the destruction of the diazo band; after about 3 hr the absorption in the 250-nm region was eliminated, and the ultraviolet spectrum again resembled that of ordinary proteins.

On photolysis, the absorbance at 250 nm, as shown in Fig. 3, decreased by about 0.02 *A* units; this absorbance must then correspond to the short-wavelength absorption of the diazo group. If one assumes that the extinction coefficient at 250 nm of the diazo group in diazoacetyl subtilisin is roughly the same as that in ethyl diazoacetate (13,600 M^{-1} cm^{-1}), one calculates that the concentration of diazo groups in the solution shown in Fig. 3 is 15 μM. The total concentration of protein can be calculated as 27 μM from the optical absorption at 278 nm, using the extinction coefficient for subtilisin (13) of 1.17 ml/mg·cm and a molecular weight of 27,600.

It had been shown by radiochemical analysis that this same solution contained 8.5 mg of singly acylated protein, of a total of 15.4 mg determined spectrophotometrically. The percentage of total protein present as the diazoacetyl derivative is then 55% as determined radiochemically and 56% as determined by the ultraviolet absorption of the diazo group. These numbers are fortuitously close, since the absorption of the diazo group at 250 nm cannot be so precisely estimated, but the results nevertheless strongly support the claim for the preparation of diazoacetyl subtilisin.

Products from the Photolysis of Diazo-[^{14}C]acetyl Subtilisin. In an experiment similar to that described above, a solution of diazo-[^{14}C]acetyl subtilisin in 22.5 ml of pH 5.0 acetate buffer was prepared; its radioactivity corresponded to the presence of

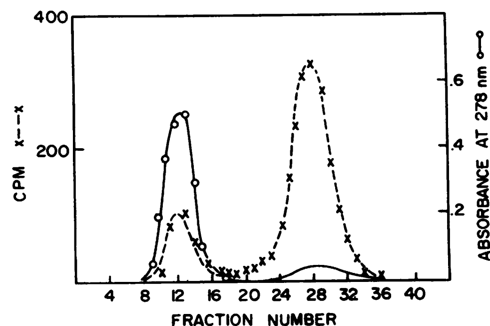


FIG. 4. Chromatography of photolyzed diazo-[^{14}C]acetyl subtilisin. *Left-hand scale,* cpm above background for 0.1-ml samples of fractions. *Right-hand scale,* absorbance of these fractions. Chromatography was on Sephadex G-25 at pH 5.

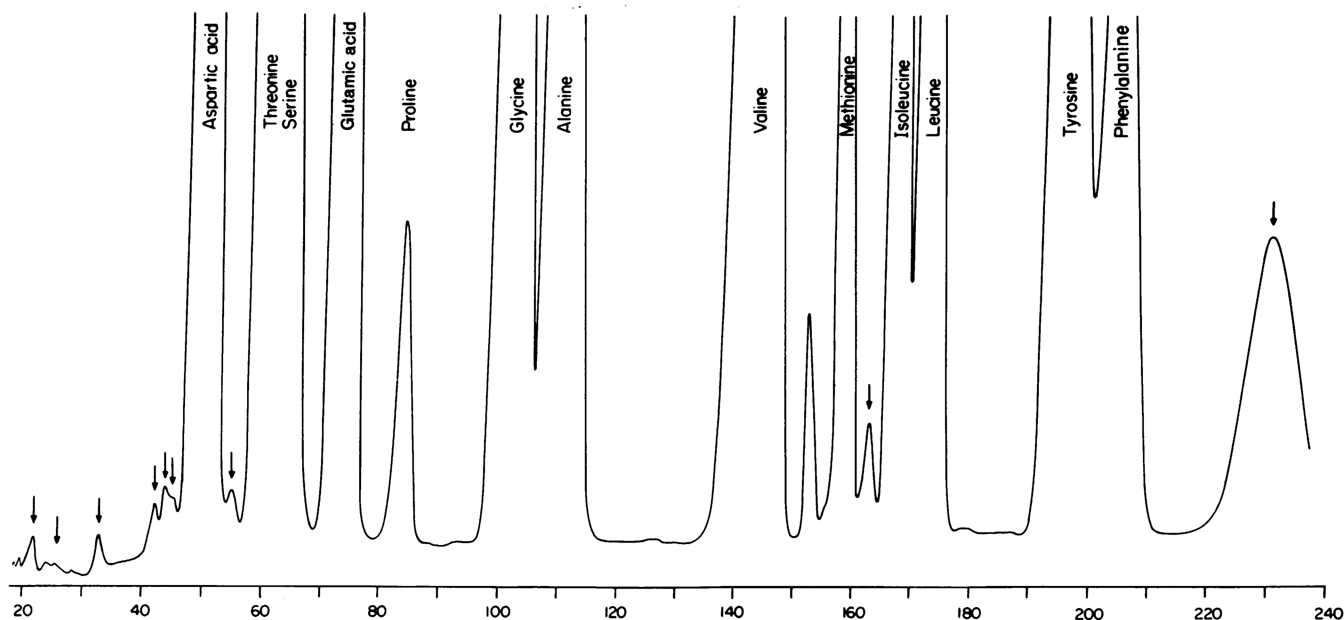


FIG. 5. Amino-acid analysis of the solution of photolyzed and hydrolyzed diazoacetyl subtilisin. The *arrows* point to radioactive ninhydrin-positive peaks other than those of the normal amino-acid complement of proteins.

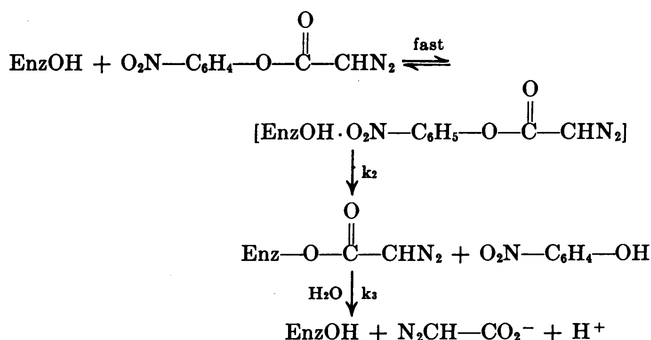
9.2 mg of the derivatized enzyme. The product, on assay with cinnamoyl imidazole, showed only about 6.5% residual active enzyme. The product was photolyzed as described above, and again assayed with cinnamoyl imidazole; the product was now 77% active. The photolysis then has presumably resulted in regeneration of 77% of the subtilisin, and has led to the insertion of the carbene into 23% of the protein in such a way as to produce inactive products (Scheme 1).

12 ml of the photolyzed solution, containing 75,200 cpm, was chromatographed at pH 5.0 on Sephadex G-25; about 45 fractions of 3.3 ml (each) were collected. Small samples of each fraction were counted; the recovery of total radioactivity was quantitative. Two radioactive regions were observed in the effluent from the chromatographic column. The first comprised fractions 9–16; it contained about 19% of the counts, and absorbed in the ultraviolet. The second peak comprised fractions 23–34; it contained 81% of the counts, but little absorbance at 278 nm (Fig. 4). The introduction of 19% of the radioactivity into the UV-absorbing fraction (the protein) corresponds reasonably well with the 23% loss in enzymic activity that results from derivatizing and photolyzing the enzyme, whereas the recovery of 81% of the radioactivity in the nonprotein fraction corresponds reasonably well with the 77% recovery of activity on photolysis. The regeneration of enzymic activity is presumably accompanied by the release of the radioactivity from the protein, (see *Discussion*), so that the amount of radioactivity released corresponds to the amount of enzymic activity recovered.

Hydrolysis of the Radioactive Protein. Fractions 9–16 (Fig. 4) were combined and lyophilized. The lyophilized product was dissolved in 6 ml of 6 N HCl containing 1 g of phenol per liter and hydrolyzed for 24 hr in a sealed tube at 110°. The contents of the tube were then evaporated to dryness at room temperature. The residue was dissolved in 1.5 ml of citrate "diluter buffer", and 0.5 ml was applied to the 50-cm column of an amino-acid analyzer; amino acids were detected with ninhydrin as usual. Another 0.5 ml was applied to the same column, arranged for the direct collection of 1-ml fractions without introduction of ninhydrin. Portions (0.2 ml) of each fraction were then added to scintillation fluid and counted. The distribution of radioactivity and of ninhydrin-positive peaks are shown in Fig. 5.

DISCUSSION

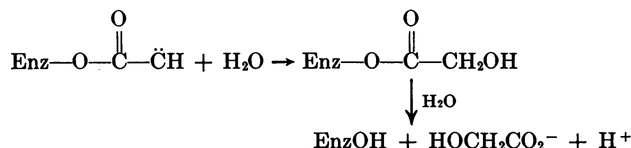
Whereas *p*-nitrophenyl diazoacetate reacts nearly irreversibly with chymotrypsin to form a diazoacetyl enzyme, and ethyl *p*-nitrophenyl diazomalonate reacts similarly with trypsin, both of these *p*-nitrophenyl diazoesters act as substrates for subtilisin. The burst of *p*-nitrophenol produced is not pronounced, and in the two-step mechanism (11) for hydrolysis, the step controlled by k_2 is not substantially faster than the step controlled by k_3.



The magnitude of the "burst" of *p*-nitrophenol, shown in Fig. 1, corresponds to the acylation of about 60% of the enzyme, based on an assay of the crude enzyme as 47% pure. (The concentrations of enzyme shown in Fig. 1 are based on assay, not weight.) From this (approximate) datum, one may estimate (18) that the ratio k_2/k_3 is about 4. Consistent with this ratio, and with the modest value for the "burst" of *p*-nitrophenol shown in Fig. 1, the enzyme must be largely—

but incompletely—converted at steady-state to its acylated derivative. Then, when the pH is abruptly lowered (here by the addition of 10% acetic acid), the rate of hydrolysis of the diazoacyl enzyme is reduced substantially; the acylated enzyme can be, and has been, purified from excess reagent (by extraction of the solution with methylene chloride and subsequent chromatography over Sephadex G-25) and obtained in solution. The assay of these solutions with cinnamoyl imidazole shows that very little active protein remains; the protein is either acylated or otherwise inactivated. Furthermore, the ultraviolet absorption spectrum of the ester shows clearly that the treatment with acetic acid, and the reduction of the pH to 5.0, do not result in the acid-catalyzed decomposition of the diazoacyl enzyme; solutions of the derivative are optically stable at and below room temperature for many hours. A further proof of the formation of the diazoacetyl enzyme is offered by changes in the UV spectrum during the photochemical decomposition, where irradiation leads to the loss of the diazo absorption. Most significantly, consistent quantitative estimates of the amounts of diazoacetyl enzyme formed can be obtained in two entirely independent ways: by measurment of radioactivity of the "Sephadexed" derivative and by measurement of the quantitative loss of UV absorption at 250 nm on photolysis of the diazoacetyl enzyme at long wavelengths.

The photolysis also leads to a considerable reactivation of the enzyme. The same phenomenon had been observed in the photolysis of diazoacetyl chymotrypsin (1, 2, 4), where 80% activity is regenerated on photolysis. In that example, the reactivation of the enzyme was accompanied by the formation of glycolic acid, and this fact strongly implies that the carbene, generated on photolysis from the diazo grouping, has largely inserted into water to produce the glycolate ester of the enzyme at the active serine residue. This ester is then self-hydrolyzed by the normal enzymic mechanism, producing glycolic acid and regenerating active enzyme. In all probability the same mechanism is operating with diazoacetyl subtilisin.



On the other hand, the radioactive, inert protein corresponds to insertion of the carbene into some other residue of the enzyme. Since the extent of the radioactivity incorporated into the protein (19%) is similar to, but less than, the percent of inactivation (23%), presumably not very much radioactivity has been incorporated into protein molecules that retain enzymic capability. The hydrolysis of the radioactive protein produced at least nine new ninhydrin-positive radioactive peaks. One of these is presumably carboxymethyl serine, produced by the photochemical Wolff rearrangement of the diazoester (2, 19–23). The other peaks must represent insertion products into the residues of subtilisin surrounding the active site.

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