THE IRREVERSIBLE CLEAVAGE OF HISTIDINE RESIDUES BY DIETHYLPYROCARBONATE (ETHOXYFORMIC ANHYDRIDE)

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1 . Introduction

In aqueous solution at neutral or slightly acidic pH values diethyl pyrocarbonate (ethoxyformic anhydride, DEP) has been shown to modify histidine residues in proteins with considerable specificity [1–3]. For this reason DEP has been widely used to test for the presence of functional histidine residues at the active sites of enzymes; for example, phosphofructokinase [4], thermolysin [5,6], lactate dehydrogenase [7], pyruvate kinase [8], and liver alcohol dehydrogenase [9]. The reagent is convenient since it reacts with histidine to give an ultraviolet spectral change centered at 240 nm [2,3] from the intensity of which the number of modified histidine residues can be estimated and the modification reaction can be reversed by cleavage of the product, *N*-carbethoxyhistidine, with suitable nucleophiles such as hydroxylamine.

Diethyl pyrocarbonate has also been shown to modify nucleic acid components. Leonard et al. [10–12] however, have shown that in this case not only are ring nitrogen atoms and substituent amino groups carbethoxylated but also the imidazole ring of purine bases can be cleaved in a Bamberger [13] reaction. Oligonucleotides and nucleic acids are also subject to this reaction [14].

Despite this warning, many users of DEP as a protein modifying reagent appear unaware of the possibility of interference by the Bamberger reaction. In this work we demonstrate that under commonly used mild conditions for protein modification by DEP, Bamberger cleavage of imidazole rings in amino acids, peptides and proteins can and does occur.

Abbreviation: DEP, diethylpyrocarbonate

2. Materials and methods

DEP was obtained from Aldrich Chemical Co. and used without further purification. The modification reactions in 0.1 M phosphate buffer at pH 6.0 and 30°C of 0.2 mM solutions of imidazole, histidine, *N*-acetylhistidine and glycylhistidylglycine (all from Sigma Chemical Co.) on addition of DEP to a final concentration of 1–5 mM (added as 5–20 μl of an acetonitrile or ethanol solution to 3 ml of imidazole solution) were followed spectrally with a Cary 14 spectrophotometer. The products of DEP-modified imidazole were isolated by chloroform extraction of a 12 h room-temperature reaction mixture obtained by stirring in 20 ml of an acetonitrile solution of DEP to 1.0 litre of an aqueous solution of 2 mM imidazole in 0.1 M phosphate buffer, pH 6.0 (final DEP concentration 20 mM).

For the protein modification reactions 1–2 mg samples of horse-liver alcohol dehydrogenase and bovine pancreatic ribonuclease A (both Sigma Chemical Co.) were dissolved in 1.0 ml of 0.1 M phosphate buffer at pH 6.0 and treated at room temperature with 1.5 mM DEP for 12 h. The samples were then evaporated to dryness and acid hydrolyzed for amino acid analysis.

3. Results and discussion

Addition of small amounts (final concentrations, 10^{-5}–10^{-4} M) of DEP to relatively concentrated (0.01–0.1 M) imidazole solutions at neutral pH gives rise to *N*-carbethoxyimidazole in essentially stoichiometric quantities. This procedure is used for assaying

DEP [1–3]. The product is characterized by absorbance (ϵ = 3200 [2]) at 230 nm [2,3]. The converse experiment, however (i.e. addition of excess DEP to small amounts of imidazole) gives different results. Most protein modifications are, of course, done under the latter conditions. On addition of DEP (final concentration 1.5 mM) to 0.2 mM imidazole in 0.1 M phosphate buffer at pH 6.0 and at 30°C an increase in absorbance in the range 210–240 nm is observed but the spectrum differs from that of *N*-carbethoxyimidazole in having a higher extinction coefficient at all wavelengths and a maximum absorbance initially around 215 nm but increasing slowly with time.

The variation of the product spectrum with time suggests a more than single-phase process and this is clearly seen when the reaction is followed at 212 nm as shown in fig.1. Three phases, *a,b* and *c*, are evident here requiring two intermediates building up to significant concentrations in solution.

The nature of the observed reactions can be deduced from the results of a preparative reaction. The elemental analyses, NMR, mass, infared and ultraviolet spectra of the two isolated products are in accord with their being cis-*N,N'*-dicarbethoxy-*N*-formyl-1,2-diaminoethene (I) and cis-*N.N'*-dicarbethoxy-1,2-diaminoethene (II).

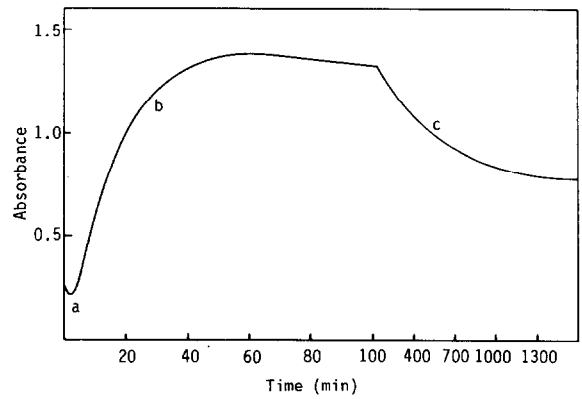
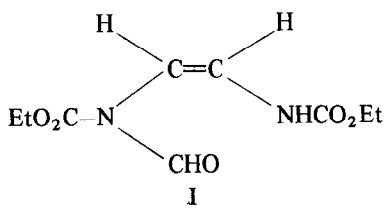
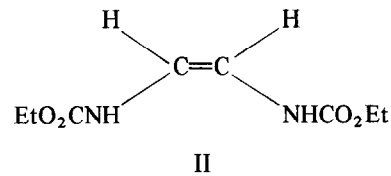


Fig.1. Absorbance changes at 212 nm as a function of time after the addition of DEP (final concentration 1.3 mM) to 0.2 mM imidazole in 0.1 M phosphate buffer, pH 6.0, 30°C, showing the three phases of reaction *a,b* and *c* (see text). Note the change in time scale along the abscissa.

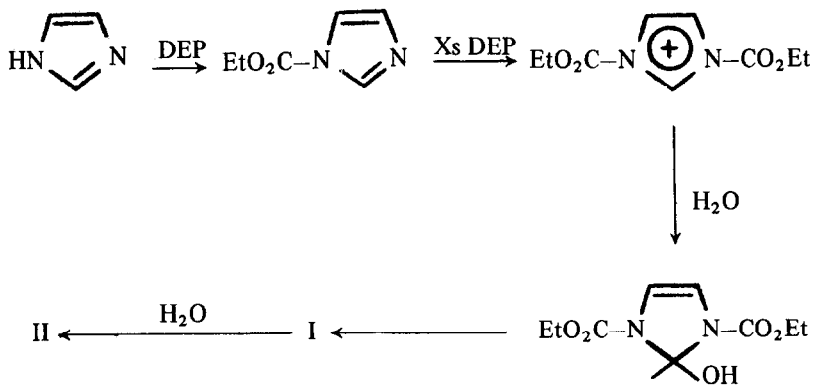
These products indicate that a Bamberger cleavage [13] of the imidazole ring has occurred, presumably by attack of water on C-2 of the imidazole ring of a *N,N'*-dicarbethoxyimidazolium ion [15] as shown below. The initial product of ring opening would be I which yields II on further hydrolysis.



I



II



In fig.1 then, phase *a* can be correlated with the first step here, the carbethoxylation of imidazole, phase *b* with the ring cleavage (i.e. the appearance of I and phase *c* with the hydrolysis of I – II). The other intermediates would be unstable and probably not observed.

Spectra of relevant intermediates and products are given in fig.2. Addition of DEP to solutions of histidine, *N*-acetylhistidine and glycyl histidylglycine under the same conditions as those above gave spectral charges very similar to those suggested by figs.1 and 2. The reaction then seems general for imidazole derivatives under these conditions, leading to essentially quantitative cleavage.

Strong nucleophiles such as hydroxylamine readily cleave *N*-carbethoxyimidazoles leading to disappearance of the 240 nm absorption band and regeneration of the imidazole. This loss of the 240 nm band in the presence of hydroxylamine has been recommended for other reasons [6] as a better indicator of the number of histidine residues of a carbethoxylated protein than is the initial spectral charge. In the light of the present work, this recommendation gains further merit: I is rapidly converted into II by hydroxylamine (5–50 mM, pH 6.0) while II appears unaffected.

Indications of problems with DEP in protein modification have been observed previously (Pratt, R. F. and Vallee, B. L., Pratt, R. F. and Loosemore, M. J. unpublished observations). One sees on occasion, spectral changes at 240 nm greater than allowed by the total histidine content of the protein, difference spectra maxima lower than the 240 nm expected [2] for *N*-carbethoxyhistidine derivatives (although this is difficult to be confident of usually because of the high background absorption below 240 nm with most proteins) and non-reversal of the DEP-induced spectral change in the presence of hydroxylamine. At least one report of aberrant behavior is present in the literature [9].

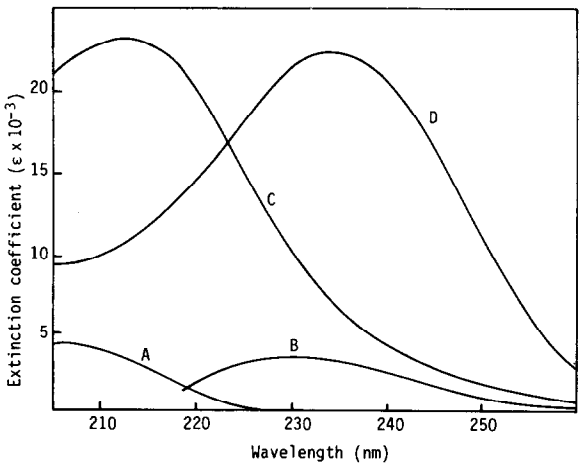


Fig.2. Ultraviolet absorption spectra (taken in 0.1 M phosphate buffer at pH 6.0, 30°C) of imidazole (A), *N*-carbethoxyimidazole (B), cis-*N,N'*-dicarbethoxy-*N*-formyl-1,2-diaminoethene (C), and cis-*N,N'*-dicarbethoxy-1,2-diaminoethene (D).

If the Bamberger reaction were responsible for these phenomena rather than say modification of amino groups [3] or protein conformational changes, its presence could be easily tested by amino acid analysis – the disappearance of histidine residues. In a preliminary experiment we treated horse-liver alcohol dehydrogenase and bovine pancreatic ribonuclease A with 1.4 mM DEP as described in the experimental section. Amino acid analysis indicated that 1.0 ± 0.3 histidine residues were lost from the dehydrogenase and 0.5 ± 0.1 residues from the ribonuclease. These losses are less than what might be predicted from the model reactions, but certainly some cleavage is indicated; the use of higher DEP concentrations (up to 10 mM is commonly used) would presumably lead to further cleavage.

It seems likely then that histidine residues in proteins can be destroyed by DEP. We do not know at present how specific this reaction might be or what factors in a protein might control specificity; these matters need attention. A variety of erroneous conclusions are obviously possible if the results of DEP protein modification are interpreted without taking the Bamberger reaction into account. We are currently investigating the conditions, specificity and mechanism of this reaction in order to delineate circumstances under which it might be either eliminated or used to advantage in protein chemistry.

After this work was completed, we noticed in an abstract [16] that similar results to these have been obtained by Russian workers; they did not appear to investigate the reaction with proteins, however.

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