THE REACTION BETWEEN DIETHYLPYROCARBONATE AND SULFHYDRYL GROUPS IN CARBOXYLATE BUFFERS

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SUMMARY

Diethylpyrocarbonate reacts with sulfhydryl groups in the presence of carboxylate buffers to form a product which absorbs at 242 nm. The product is believed to be a thiol ester formed from the sulfhydryl compound and the buffer anion. This reaction interferes with the use of diethylpyrocarbonate to determine protein histidine residues when the reaction is performed in carboxylate buffers.

INTRODUCTION

Diethylpyrocarbonate (DEP, ethoxyformic anhydride) is a reagent that is commonly used in active site modification studies. Although it reacts with a number of nucleophiles at pH values of 7 and above (1-6) it is fairly specific for the imidazole ring of histidine at pH 6.0 and is commonly used as a histidine modifying reagent. The product of the reaction, N-carboethoxyhistidine, absorbs in the 230-245 nm region and the increase in the A_{242} value is used as a measure of the number of histidine residues modified (5). The carboethoxy group is easily removed with NH_2OH to regenerate the free histidine residue (7). In our studies of the reaction of this reagent with formyltetrahydrofolate synthetase in succinate buffer we discovered an anomalous reaction which led to our investigation of the reactions of the reagent with N-acetylcysteine in carboxylate buffers. In these buffers (succinate, acetate) at pH 6.0 the reaction between DEP and N-acetylcysteine produces a product which absorbs at 242 nm. Addition of NH_2OH causes the absorbance to disappear. Because this reaction mimics the reaction with histidine, a similar reaction with protein cysteine residues could cause misinterpretation of data by investigators. Since a number

of workers have used acetate buffer in such studies, it is worthwhile to seek an explanation of this apparent anomalous effect.

MATERIALS AND METHODS

N-Acetyl-L-cysteine and diethylpyrocarbonate were purchased from Sigma Chemical Company. Reactions were carried out at 22° and the absorbance was measured with a Zeiss PMQII spectrophotometer. The concentration of N-acetylcysteine used was roughly equivalent to the concentration of sulfhydryl groups in a 1 x 10^{-5} M solution of formyltetrahydrofolate synthetase, the concentration we normally use in modification studies.

RESULTS AND DISCUSSION

When N-acetylcysteine was reacted with DEP in succinate buffer the absorbance at 242 nm increased and then decreased (Fig. 1A). In a comparable experiment the concentration of sulfhydryl groups was shown to decrease rapidly and then elevate (Fig. 1A). A similar but slower reaction also occurred in acetate buffer, how-

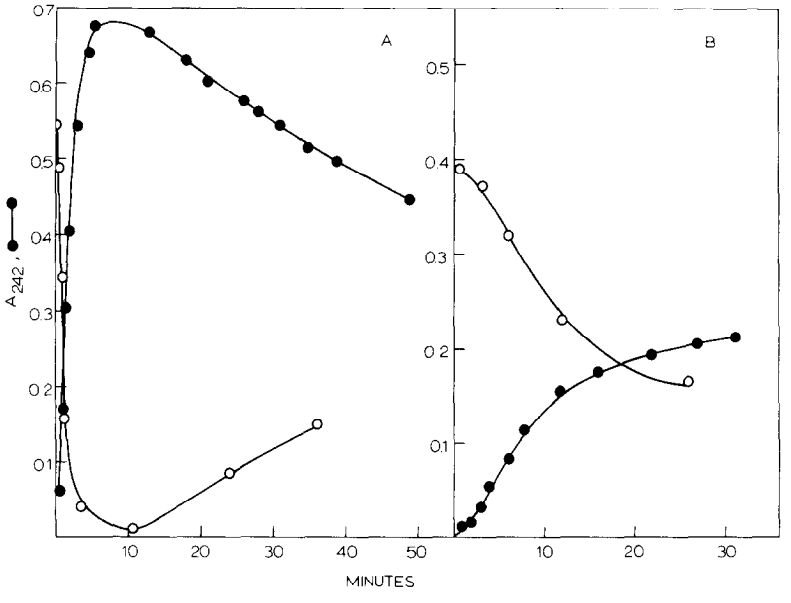

Fig. 1. Reaction of DEP with N-acetylcysteine in succinate and acetate buffers. A. DEP, 5 x 10^{-3} M and N-acetylcysteine, 2.5 x 10^{-4} M, were incubated at 22° in 0.1 M potassium succinate, pH 6.0. In one experiment the A_{242} values were measured. In another, 0.5 ml of the reaction mixture were removed at various times and added to 2.5 ml of 0.1 M imidazole, pH 8.0 containing 4.2 x 10^{-4} M DTNB. The A_{412} value was obtained immediately. B. The experiment was as described for A except that 0.2 M sodium acetate was used as the buffer.

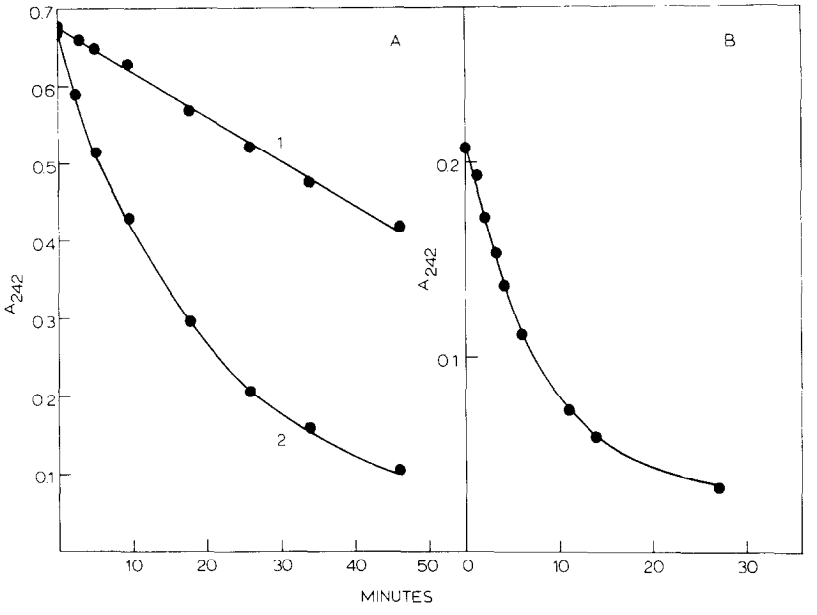

Fig. 2. Effect of NH_2OH on the A_{242} value. A. The reaction of DEP with N-acetylcysteine was carried out in succinate buffer as described in Fig. 1A. for 13 min. in two separate cuvettes. At this time one sample was made 0.1 M in NH_2OH, pH 6.0. 1, without NH_2OH addition. 2, with NH_2OH addition. B. The reaction was carried out in acetate buffer as described in Fig. 1B. After 70 min. NH_2OH, pH 6.0, (final concentration, 0.1M) was added. There was no change in A_{242} in the absence of NH_2OH. In A and B zero time refers to when NH_2OH was added.

ever, after increasing, the A_{242} value remained constant. Likewise the concentration of sulfhydryl groups did not increase after the initial decrease (Fig. 1B). In experiments not shown the final A_{242} value produced in acetate was stable for at least 2 hours. The addition of NH_2OH caused the disappearance of the absorbance at 242 nm in the case of the acetate reaction and accelerated the rate of decrease of the absorbance in the succinate reaction (Fig. 2A&B). Moreover, the addition of NH_2OH increased the rate of return of sulfhydryl groups in succinate buffer.

The results cited above were observed only in the presence of the buffer, DEP and N-acetylcysteine. Both the rate and extent of reaction were elevated by increasing the concentration of thiol. No change in A_{242} was observed in the absence of the amino acid derivative. Nor did an observable increase occur

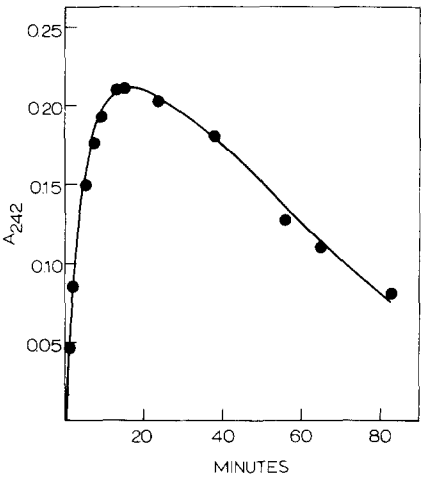

Fig. 3. Reaction of succinic anhydride with N-acetylcysteine. Succinic anhydride (1 x 10^{-2} M) and 2.5 x 10^{-4} M N-acetylcysteine were incubated in 0.1 M potassium phosphate pH 6.0 at 22°.

when DEP and N-acetylcysteine were incubated in 0.1 M [2-(N-morpholino)ethane sulfonic acid] buffer, pH 6.4, or in 0.1 M phosphate buffer, pH 6.0 and 7.0, in spite of the fact that in the latter case there was a rapid decrease in sulfhydryl concentration ($t_{1/2} \sim 3.5$ min). At pH 6.0 in phosphate buffer the disappearance of thiol groups was quite slow ($t_{1/2} \sim 35$ min). This latter value compares with the very rapid disappearnace of thiol groups in succinate buffer (Fig. 1A).

It occurred to us that the reaction of DEP with N-acetylcysteine (a similar reaction was seen with 2-mercaptoethanol) in the presence of carboxylate buffers was leading to the formation of a thiol ester. Thiol esters absorb in the 230-245 nm region and indeed the product of the above reaction in acetate buffer displayed an absorption maximum at 232 mm, the same as that reported for thiol esters (8). Our conclusion is supported by the fact that we could duplicate the results obtained in succinate buffer by combining succinic anhydride and N-acetylcysteine in phosphate buffer without DEP (Fig. 3). Succinyl CoA dissolved in 0.1 M phosphate buffer, pH 6.0, also showed a continuous decrease in the absorbance at 242 nm.

The results in this report are best explained by assuming a reaction between the carboxylate buffers and DEP to form a mixed anhydride. A subsequent reaction with a sulfhydryl compound would produce a thiol ester (succinyl CoA or acetyl CoA). Thiol esters have a characteristic absorption band in the 230-245 region (8), thus the increase in A_{242}. Moreover, such a reaction would account for the disappearance of sulfhydryl groups. The later decrease in A_{242} and return of sulfhydryl groups which occurs in succinate buffer is explained by a further intramolecular reaction of the succinyl thiol ester leading to succinic anhydride and N-acetylcysteine. The fact that the increase and subsequent decrease could be duplicated with succinic anhydride and N-acetylchysteine in phosphate buffer supports this view. Splitting of the thiol ester by NH_2OH also leads to a disappearance of the 242 nm absorbance.

It is clear from these studies that DEP could easily modify carboxylate groups on proteins at pH 6.0. More important, however, the use of carboxylate buffers such as acetate should be avoided. It is possible that loss of enzymic activity and increases in A_{242} observed by some investigators upon the reaction of proteins with DEP in this buffer could have been due to the conversion of protein thiol groups to thiol esters. Restoration of enzymic activity by adding NH_2OH would also be explained by this possibility.

ACKNOWLEDGEMENT

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