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Reaction of Diethyl Pyrocarbonate with Nucleophiles

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In order to clarify the kinetic background of the biochemical and biological action pattern of diethyl pyrocarbonate (diethyl oxydiformate) the rates of reaction of this compound with amines, thiols, and compounds with nucleophilic oxygen were studied at 25°. The nucleophilic strength within each series of compounds is correlated with the basicity. The primary amines are approximately five times as reactive as the thiols and thousand times as reactive as the oxygen anions, when compared at equal pK_a values.

Diethyl pyrocarbonate has been widely used as a food preservative because of its bactericidal action.[1] More recently it has been applied as a nuclease inhibitor in the preparation of undegraded high molecular weight RNA.[2-4]

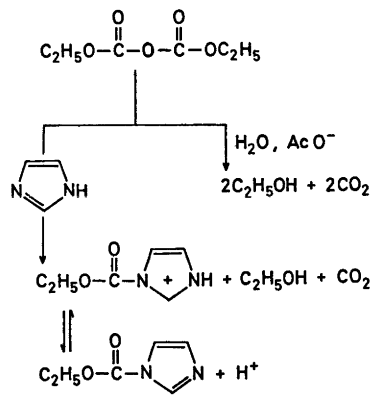
Diethyl pyrocarbonate can be regarded as the anhydride of ethoxyformic acid, and as such, although stabilized by the ethoxy group, it is very susceptible towards nucleophilic attack on a carbonyl carbon. There has been an extensive literature on the carbethoxylation by diethyl pyrocarbonate of amines, [5,6] thiols,[7] phenols,[8] amino acids,[5,9,10] and proteins.[11-13] Data on reaction rates, however, are at present very scanty.[12,14] Such data are needed for the understanding of the biological effects of diethyl pyrocarbonate,[15,16] for the optimization of the nucleic acid extraction method based on the use of diethyl pyrocarbonate, as well as for finding proper conditions for the use of diethyl pyrocarbonate in studies of the secondary structure of nucleic acids.[17]

MATERIALS AND METHODS

Diethyl pyrocarbonate was obtained from Schuchardt, München. Tris(hydroxymethyl)-aminomethane (Tris), glycylglycine, 2-mercaptoethanol, and 3-mercaptopropionic acid were purchased from Sigma, St. Louis, Mo. *p*-Nitrophenyl mercaptan was synthesized according to Augustinsson *et al.*[18] All other chemicals were obtained from Merck, Darmstadt, and were of reagent grade.

The reactions of diethyl pyrocarbonate were with a few exceptions followed in the presence of a large excess of the respective nucleophile to give pseudo first order kinetics. The temperature was maintained at $25 \pm 0.1°$ in a Hetotherm ultrathermostat. The ionic strength was kept constant ($I = 0.1$) by addition of NaCl, except in the study of buffer compounds.

Reaction with imidazole. Diethyl pyrocarbonate reacts rapidly with imidazole to form *N*-carbethoxyimidazole,[12] according to eqn. (1).



(1)

The rate of carbethoxylation was followed spectrophotometrically at 231 nm (λ_{max} for *N*-carbethoxyimidazole; $\varepsilon = 3 \times 10^3$ l mol^{-1} cm^{-1}). To a 10 – 20 mM solution of imidazole in acetate buffer (pH 4.4 – 4.9) diethyl pyrocarbonate dissolved in acetonitrile was added to give an initial concentration of 0.2 mM. The reaction was followed for ten half-lives to give the end-point A_∞. The half-time was determined from a plot of log $(A_\infty - A_t)$ against t, where A_t

is the absorbance at the time t. The observed first order rate constant was calculated from

$$k_{obs} = \ln 2/t_{1/2} \quad (2)$$

The pseudo first order rate constant for the reactions of diethyl pyrocarbonate with imidazole (IM) was obtained by subtracting the constants for hydrolysis and reaction with acetate (AcO^-), determined in separate experiments, from the observed rate constant; see eqn. (3). The second order rate constant was obtained by dividing the pseudo first order constant by the concentration of free base according to eqn. (4).

$$k_{obs} = k'_{H_2O} + k'_{AcO^-} + k'_{IM} \quad (3)$$

$$k_2 = k'_{IM}/[IM] \quad (4)$$

The fraction of base was calculated from

$$pH = pK'_a + \log \frac{[IM]}{[IMH^+]} \quad (5)$$

Reactions with water, CH_3COO^-, HPO_4^{2-}, Tris, and CO_3^{2-}. The rapid reaction with imidazole was used for the quantitative determination of diethyl pyrocarbonate. 0.1 ml samples were taken at appropriate intervals from a 2 – 3 mM solution of diethyl pyrocarbonate in the respective buffer and added to 5.0 ml of 5 mM imidazole in 0.035 M phosphate buffer (pH 8.0). The samples were carefully shaken and the absorption at 231 nm was measured after a few minutes. The errors due to parallel reaction of diethyl pyrocarbonate with water and buffers, change in background absorption of imidazole and hydrolysis of *N*-carbethoxyimidazole [12] were negligible under these conditions.

The reaction rates of the buffer compounds were studied at selected concentrations and pH in the following ranges: 0.05 – 0.2 M acetate buffer pH 4.5 – 5.0, 0.05 – 0.2 M phosphate buffer pH 6.5 – 7.5, 0.02 – 0.05 M Tris buffer pH 7.5 – 8.1, 0.05 – 0.1 M carbonate buffer pH 10.0. The rate constants were calculated from the stoichiometric composition of the buffer.

Reactions with glycylglycine, ammonia, ethanolamine, glycine, and β-alanine. The rate constants were determined by measuring the yield of *N*-carbethoxyimidazole in a solution of imidazole in the presence and absence of the amine under study.

The reactions were carried out in 0.02 M Tris or phosphate buffer at pH 8.0. Diethyl pyrocarbonate was dissolved in acetonitrile and added to the reaction mixture to give an initial concentration of 0.08 mM. The concentration of imidazole was 2 mM (4 mM in the reference experiment) and the amines were studied in the following concentration ranges: glycylglycine 1 – 2 mM, NH_4Cl 60 – 80 mM, ethanolamine 6 – 12 mM, glycine 5 – 20 mM, and β-alanine 15 – 50 mM. The absorption at 231 nm was measured after 5 min. The experiments were repeated for two of the compounds (glycylglycine and glycine) at pH 7.0 without buffer and with various concentrations of the amines (5 – 10 times higher than used at pH 8.0). The rate constants so determined were the same as those obtained at pH 8.0 within the limits of experimental error. The possibility of a general basic catalysis of the buffers or imidazole on the carbethoxylation of the investigated amines in the concentration ranges used could be disregarded. The stability of the *N*-carbethoxyimidazole in the presence of the amines was checked.

The concentration of the amines was large in comparison with the concentration of diethyl pyrocarbonate. Thus, the ratio of the pseudo first order rate constants could be determined directly from the ratio of the concentrations of the final products (*cf.*, *e.g.*, Ref. 19).

$$\frac{k'_{IM}}{k'_{RNH_2}} = \frac{k_{IM}[IM]}{k_{RNH_2}[RNH_2]} = \frac{[N\text{-carbethoxyimidazole}]}{[\text{urethan}]} \quad (6)$$

The second order rate constants refer to the concentrations of the free bases. The pK'_a values at $I = 0.1$ were taken from the literature (see Table 1).

Reaction with p-bromoaniline. The reaction was followed in an equimolar solution (3 – 4 mM) of diethyl pyrocarbonate and *p*-bromoaniline in 0.02 M phosphate buffer (pH 6.6). The amount of unreacted *p*-bromoaniline was determined by diazotation and coupling with histamine.[7] 0.1 ml samples were withdrawn every 10 sec and dispersed in 3.0 ml ice-cold $NaNO_2$-HCl (0.15 % $NaNO_2$, 0.1 M HCl, 1:3). After 15 min in ice-bath 1.0 ml 0.5 M borate buffer (pH 9.5) and 1.0 ml 0.01 M histamine dihydrochloride were added. The samples were carefully shaken after each addition. A yellow colour developed in 30 min at room temperature. Spectrophotometric readings were taken at 436 nm.

The second order rate constant k_2 was calculated from

$$1/c = k_2 t + C \quad (7)$$

where c is the concentration of *p*-bromoaniline (or diethyl pyrocarbonate) at time t.

Reactions with p-nitrophenyl mercaptan, 2-mercaptoethanol, and 3-mercaptopropionic acid. The reactions of diethyl pyrocarbonate with 2-mercaptoethanol and 3-mercaptopropionic acid were studied by three independent methods:

(a) By measuring the decrease of the concentration of diethyl pyrocarbonate. The reaction mixture was 1.5 mM with respect to diethyl pyrocarbonate and 15 mM with

respect to the SH compound in 0.05 M phosphate buffer (pH 7.0). 0.1 ml samples were taken at appropriate intervals and added to 3.0 ml 5 mM imidazole in phosphate buffer (pH 8). The *N*-carbethoxyimidazole was determined spectrophotometrically.

(b) By measuring the decrease of the concentration of the SH compound. The reaction between diethyl pyrocarbonate and the mercaptan in equal concentrations (2 – 4 mM) in 0.035 M phosphate buffer (pH 8.0) was followed by taking 0.1 ml samples every 7 sec and adding them to 5.0 ml of Ellman's reagent, 10^{-4} M 5,5′-dithiobis(2-nitrobenzoic acid) in 0.035 M phosphate buffer (pH 8).[20] The absorption was determined at 412 nm ($\varepsilon = 13\,600$ l mol^{-1} cm^{-1} for the liberated 4-nitro-3-carboxythiophenolate ion). A disturbing fading of the colour, possibly due to a reaction of the coloured anion with the remaining diethyl pyrocarbonate made it necessary to measure every sample several times and extrapolate the absorption to the time when the sample was taken. The rate constant was calculated from a plot of $1/c$ against time [eqn. (7)].

(c) By measuring the appearance of a peak in UV at 213 nm corresponding to the formation of an ethoxyformic acid thioester.[7] The reactions were carried out at pH 8.0 in 0.035 M phosphate buffer. The initial concentrations of diethyl pyrocarbonate and mercaptan were 0.1 mM and 1.1 mM, respectively. The increase in absorbance was measured directly at 213 nm. The reactions were followed for at least ten half-lives to get an accurate end-point determination. Log $(A_\infty - A_t)$ was plotted against time t to give a straight line from which the half-life was determined.

The reaction between diethyl pyrocarbonate and *p*-nitrophenyl mercaptan was followed by measuring the decrease of the absorption at 412 nm ($\varepsilon = 9000$ l mol^{-1} cm^{-1}; Ref. 18). The reaction conditions were the same as described under (b). The samples were diluted 50 times before reading.

The purity of the SH compounds was determined with the aid of Ellman's reagent.[20]

RESULTS AND DISCUSSION

The rate constants determined for the reaction of diethyl pyrocarbonate with the different nucleophilic reagents at 25° are summarized in Table 1. Good agreement was obtained with previous studies of the reaction rates with three of the nucleophiles, *viz.* the rate of the reaction with water [k (first order) = 0.0246 min^{-1}], determined by adding samples to diethylamine solutions and titrating the diethylamine in excess;[14] the rate of the reaction with imidazole ($k = 3240$ l mol^{-1} min^{-1}), determined spectrophotometrically at 231 nm, λ_{max} for *N*-carbethoxyimidazole;[12] and the rate of the reaction with ammonia ($k = 1600$ l mol^{-1} min^{-1}) determined by quantitative gas chromatographic analysis of the urethan formed (G. Löfroth, *personal communication*).

Table 1. Rate constants for reaction of diethyl pyrocarbonate with different nucleophiles at 25°. The given constants are mean values of 3 – 10 determinations; the error (95 % confidence interval) of the constants is mostly less than ± 10 %.

Nucleophile	pK_a	k l mol^{-1} min^{-1}
Water	−1.74[a]	4.3×10^{-4}
CH_3COO^-	4.8[a]	0.35
HPO_4^{2-}	6.9[a]	0.9
CO_3^{2-}	10.4[a]	0.6×10^2
Tris	8.1[a]	24
p-Bromoaniline	3.9[b]	2.2×10^2
Imidazole	7.0[c]	3.2×10^3
Ammonia	9.29[d]	1.7×10^3
Glycylglycine	8.09[e]	1.0×10^4
Ethanolamine	9.5[f]	2.7×10^4
Glycine	9.60[g]	4.0×10^4
β-Alanine	10.3[h]	5.4×10^4
p-Nitrophenyl mercaptan	5.1[i]	1×10^{2}[k]
2-Mercaptoethanol	9.5[a]	1.0×10^4
3-Mercaptopropionic acid	10.2[j]	1.2×10^4

[a] Ref. 21 and literature quoted; [b] Ref. 30; [c] Ref. 31; [d] Ref. 32; [e] Ref. 33; [f] Ref. 34; [g] Ref. 35; [h] Ref. 36; [i] Ref. 37; [j] Ref. 38; [k] One determination only.

A Brønsted plot of the data (Fig. 1) indicates that primary amines follow the relationship[19]

$$\log k = \beta \, pK_a + C \qquad (8)$$

where $\beta = 0.4$ gives the dependence of reaction rate on the basicity of the amino group. The rate constant for the tertiary amine, imidazole, falls on the same curve; a more detailed study on strictly defined structurally related imidazoles and primary amines is required to show whether imidazole truly belongs to the group of amines in this respect or the position of k_{IM} is the fortuitous resultant of mutually counteracting factors. The strong solvation of the ammonium ion which causes an abnormally high pK_a, and the low polarizability of NH_3 nitrogen, probably account for the relatively low reactivity of ammonia.[19,21] The slow reaction of tris(hydroxymethyl)aminomethane (Tris) as compared to other primary amines of

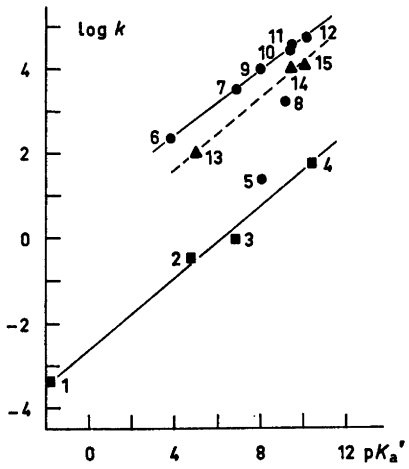
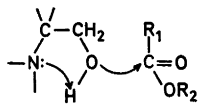


Fig. 1. Log k (k in 1 mol^{-1} min^{-1}), as a function of pK'_a of reactions of diethyl pyrocarbonate with nucleophiles at 25°. 1, water; 2, CH_3COO^-; 3, HPO_4^{2-}; 4, CO_3^{2-}; 5, Tris; 6, *p*-bromoaniline; 7, imidazole; 8, ammonia; 9, glycylglycine; 10, ethanolamine; 11, glycine; 12, β-alanine; 13, *p*-nitrophenyl mercaptan; 14, 2-mercaptoethanol; 15, 3-mercaptopropionic acid.

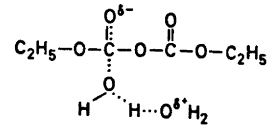
corresponding pK'_a may reflect steric hindrance at the nitrogen. It is probable, however, that this steric hindrance is comparatively large and that the reaction measured proceeds *via* an alternative mechanism, *viz.*, the intramolecular base-catalyzed attack by an oxygen according to formula (9), indicated to operate in the reactions of Tris and hydroxylamine with *p*-nitrophenyl acetate,[21] and suggested to explain the activation of the serine hydroxyl group at the active site of chymotrypsin.[22]



(9)

The three sulfhydryl compounds studied react more slowly with diethyl pyrocarbonate than primary amines with comparable dissociation constants. In this respect diethyl pyrocarbonate differs from the carefully investigated acylating agent, *p*-nitrophenyl acetate which is attacked somewhat more rapidly by thiol anions than by primary amines.[21] The difference in nucleophilic reactivity towards diethyl pyrocarbonate and *p*-nitrophenyl acetate seems to be the high reactivity of the primary amines towards diethyl pyrocarbonate, rather than the low reactivity of the thiols.

As expected, compounds with nucleophilic oxygen react slowly with diethyl pyrocarbonate. The rate constants for reaction of diethyl pyrocarbonate with water, acetate, hydrogen phosphate, and carbonate exhibit a dependence on pK_a which is approximately equal to that of the amines and the thiols. Kivinen [14] concluded that the neutral hydrolysis of diethyl pyrocarbonate in water takes place by a general base-catalyzed mechanism with water functioning as a base, and proposed the structure (10) for the transition state.



(10)

The acetate, hydrogen phosphate, and carbonate may serve either as general base catalysts or as real nucleophilic catalysts.

Preliminary studies of the carbethoxylation of RNA in phosphate buffer indicates that diethyl pyrocarbonate acts *via* a carbethoxy phosphate intermediate (which is certainly very unstable [23]), and accordingly the reaction mechanism would involve a nucleophilic attack of hydrogen phosphate on the carbonyl carbon (data to be published).

In biochemical studies, *e.g.* of enzyme inactivation with diethyl pyrocarbonate, its concentration, [DEP], decreases with time, t, according to

$$[\mathrm{DEP}] = [\mathrm{DEP}]_0 \, e^{-k't} \qquad (11)$$

where $[\mathrm{DEP}]_0$ is the initial concentration of diethyl pyrocarbonate and

$$k' = k_{H_2O}[H_2O] + \sum_i k_i c_i \qquad (12)$$

k_i and c_i being the rate constants and concentrations, respectively, of nucleophiles in the system. The rate constants of buffer components permit the calculation of k' in the reaction media and hence the dose, *i.e.* the time integral of the concentration of diethyl pyrocarbonate.

In reactions with proteins no absolute specificity, *e. g.* towards histidine, occurs. Reaction is expected to occur in a random fashion with various groups (*cf.* Ref. 24), if sterically accessible, and proportionally to the respective rate constants and the degrees of dissociation of these groups at the pH of the treatment. Fig. 2 illustrates the selectivity of the reaction with imidazole groups. It gives the expected total rates of carbethoxylation at pH 7 of amino ($\equiv N + \equiv NH^+$) and thiol ($-S^- + -SH$)

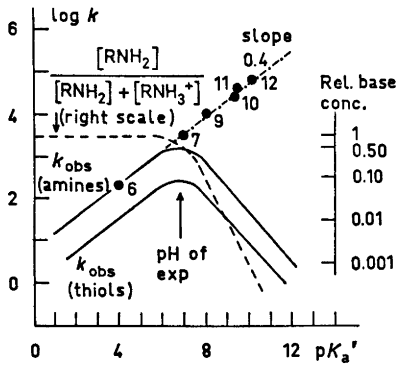


Fig. 2. The total observed reactivities at pH 7 (log k, scale to the left) of diethyl pyrocarbonate towards amines and thiols [solid lines marked k_{obs} (amines) and k_{obs} (thiols), respectively] as a function of pK'_a of the nucleophiles. For nucleophiles with $pK'_a <$ pH of the experiment (in the figure pH 7) the concentration of the reactive species, *i.e.* free base, approaches the total concentration of the nucleophile, whereas the relative concentration of the free base of nucleophiles with higher pK'_a approaches a line with slope -1 (shown for amines in curve – – – marked $[RNH_2]/([RNH_2]+[RNH_3^+])$; right scale). k_{obs} of weak bases ($pK'_a <$ pH) approach the respective Brønsted curve with slope 0.4 (–·–· curve for amines from Fig. 1). k_{obs} of strong bases approach the resultants of the Brønsted slope and the slope -1. *Cf.* Ref. 19 p. 84.

groups as a function of their dissociation constants. The rate of carbethoxylation of (total) imidazole exhibits a maximum aroung pK_a. For similar reasons, enzyme histidines will be carbethoxylated with a certain selectivity at or below pK_a (around 6.5).[12] The rate of inactivation of ribonuclease [13,15] by diethyl pyrocarbonate in the pH range 4 – 7 agrees well with the expected rate of reaction with histidine. The rate constants for inactivation of ATP-creatine phosphotransferase and ATP-L-arginine phosphotransferase [25] in 0.05 M phosphate buffer (pH 6.1) correspond to $k_{his} = 1000$ l mol^{-1} min^{-1} in agreement with expectation.

Lysine ε-amino groups (pK_a about 10) will react about 50 times slower than histidines in neutral solution but their reaction rates will increase rapidly with pH.

The second order rate constant for the reaction of diethyl pyrocarbonate with RNA was found to be about 0.003 l (mol nucleotide)$^{-1}$ min^{-1} in 0.05 M phosphate buffer (pH 6.5 – 7.5) and 25°. Since the reaction rate was found to depend on the concentration of phosphate, indicating a role of transcarbethoxylation *via* carbethoxy phosphate, the value of the constant is preliminary. It agrees acceptably with earlier data for the inactivation of virus RNA [26-28] and transfer RNA[29] by diethyl pyrocarbonate.

An RNA molecule containing 1000 nucleotides (in single-stranded regions [26-29]) will, under the conditions given, be inactivated at a rate of about 3 l (mol RNA)$^{-1}$ min^{-1}, *i.e.* two orders of magnitude more slowly than enzymes inactivated by carbethoxylation of histidine.

Changes in the ultraviolet spectra of diethyl pyrocarbonate-treated nucleic acids indicate [17,39] that diethyl pyrocarbonate reacts with single-stranded regions of RNA and DNA. Isotope studies using ^{3}H-labelled diethyl pyrocarbonate and homopolymers have revealed [40] that in the polynucleotide chain it is adenosine whose reaction rate with diethyl pyrocarbonate is the highest. If we now assume that in *E. coli* rRNA it is also adenosine which reacts fastest and that about 40 %[41] of its adenosine residues are in single-stranded regions, the rate constant at 25° for the reaction with RNA-adenosine will be about 0.03 l mol^{-1} min^{-1} in 0.05 M phosphate buffer and some three times higher in 0.2 M buffer. This rate is at least one order of magnitude lower than the value of 1.1 ($\pm$0.2) l mol^{-1} min^{-1} determined from ultraviolet spectral studies (our unpublished data *) for the reaction of

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diethyl pyrocarbonate with adenosine in phosphate buffers, independently of buffer concentration. Adenosine residues in RNA are therefore less reactive towards diethyl pyrocarbonate than the free nucleoside and it remains to be shown whether the two reactions proceed by the same mechanism.

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