THE INHIBITION OF RIBONUCLEASE BY IODOACETATE.

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The inhibition of bovine pancreatic ribonuclease by halogenated acetic acids has been reported by various authors (Zittle, 1946; Barnard & Stein, 1959; Gundlach, Stein & Moore, 1959). At pH values near 6.0 the major site of alkylation is histidine residue 119, though some reaction at histidine 12 also occurs (Moore, 1961). No inhibition is observed with iodoacetamide (Gundlach, Moore & Stein, 1959) suggesting that electrostatic factors are involved. The limited data available on the pH dependence of the inactivation suggested that a histidine residue in the cationic form serves as a binding site for iodoacetate. Since we have postulated (Findlay et al. 1961) that two histidine residues form part of the catalytic site of the enzyme, a careful kinetic study of the alkylation reaction has been undertaken.

MATERIALS AND METHODS

Ribonuclease A was prepared by the method of Hirs, Moore and Stein (1953) as modified by Crestfield (1961) with the further modification that the final dialysis step was replaced by treatment with Amberlite monobed resin MB-3.

Iodoacetic acid was five times recrystallised and had no detectable iodide or iodine.

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The alkylation reaction was carried out at 40°C in a pH-stat. A jacketed cell provided with a sidearm, to contain the reference electrode, was used. The solution was stirred using a magnet; nitrogen gas, freed from CO_2 and saturated with water at 40°C was passed over the surface. The iodoacetic acid and ribonuclease were separately taken to the required pH and mixed in the pH stat cell at zero time. The total volume was 2.5 ml and the ionic strength adjusted with sodium chloride (Johnson & Matthey, "Specpure"). At suitable times 0.05 ml samples were removed from the reaction mixture and pipetted into tris buffer (3 ml, pH 7.18, I, 0.2). The activity of these diluted samples was determined at 25°C in a Cary Model 14 Spectrophotometer at 284 mμ. 2.5 ml of the diluted sample was placed in the spectrophotometer cell and the reaction started by the addition of substrate (0.5 ml, 0.6 mg cytidine 2':3'-phosphate/ml tris buffer). The initial slope of the progress curve was used as a measure of the quantity of active enzyme remaining. The logarithm of this slope was found to be a linear function of time, enabling the pseudo-first order rate constant to be determined without obtaining a zero time sample.

RESULTS

The rate of reaction of ribonuclease with iodoacetate was found to be a linear function of the concentration of each component at fixed pH and ionic strength. The pseudo-first order rate constant at I = 0.02 and iodoacetate concentration 0.02 M was determined as a function of pH. The initial ribonuclease concentration was 1 mg/ml. The results are shown in Figure 1.

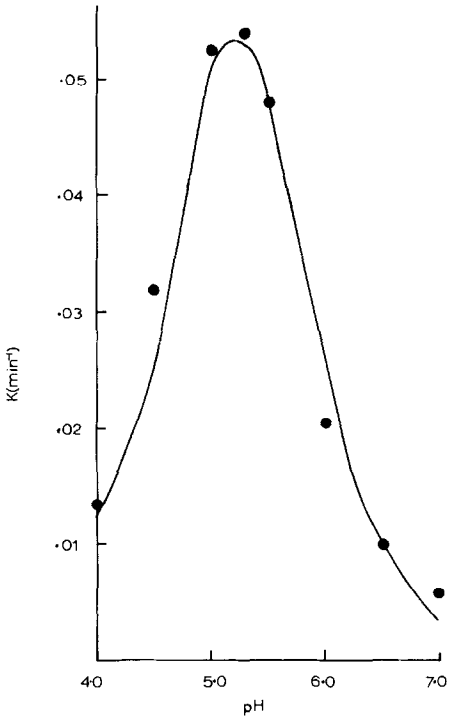

Fig. 1. Variation of rate of inactivation of ribonuclease by iodoacetate with pH. Conditions as in text. The points are experimental and the line calculated using the values in Table 1.

ANALYSIS AND INTERPRETATION.

The results can be interpreted satisfactorily in terms of the following scheme:

$$EH_2 \underset{}{\overset{K_b}{\rightleftharpoons}} EH \overset{K_a}{\rightleftharpoons} E$$

$$+$$

Iodoacetate

$$\downarrow \bar{k}$$

Inactive enzyme

K_b and K_a are dissociation constants of the groups involved in the alkylation reaction and $\bar{k}$ is the pH-independent pseudo-first order rate constant. The measured pseudo-first order rate constant at any pH, k, is given by the expression:

$$k = \bar{k} / (1 + [H] / K_b + K_a / [H]) \qquad (1)$$

It can be shown that,

$$K_a K_b = [H_{max}]^2 \qquad (2)$$

where H_{max} is the hydrogen ion concentration corresponding to the maximum rate.

Rearrangement of (1) gives:

$$\frac{1}{k} = \frac{([H] + K_b K_a / [H])}{K_b \cdot \bar{k}} + \frac{1}{\bar{k}} \qquad (3)$$

The individual parameters were determined by regression of 1/k against $([H] + K_b K_a / [H])$ and the data are shown in Figure 2.

The value of the constants obtained are given in Table 1. The curve in Figure 1 was calculated assuming these values and it is in satisfactory agreement with the experimentally determined points.

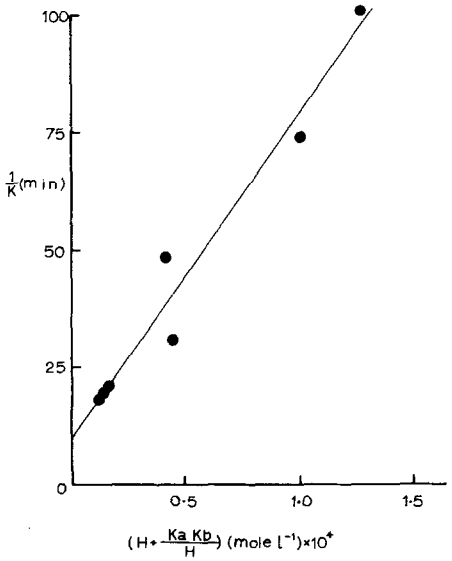

Fig. 2. Method of determining the pK values of the groups concerned in the reaction of ribonuclease with iodoacetate.

TABLE I.

Experimentally determined parameters of the alkylation reaction of 40°C, I = 0.02 and (Iodoacetate) = 0.02M.

$\bar{k} = 0.11\ min^{-1}$

$pK_b = 4.85$

$pK_a = 5.55$

DISCUSSION

The regular nature of the kinetics between pH values 4.0 and 7.0 and the excellent agreement between theory and experiment suggest that the inactivation is a single chemical process and the nature of the group alkylated does not change over this pH range. This is in agreement with the findings of Gundlach, Stein & Moore (1959). The measured pK values are at the lower end of the range expected for imidazole groups of histidine at this temperature and ionic strength. The fact that alkylation is associated with activity loss leads naturally to the supposition that reaction occurs with groups forming part of the active site. It is of interest, therefore, to compare the pK values of the active site of the free enzyme as determined by alkylation with those obtained by hydrolysis of cytidine 2':3'-phosphate. The data of Findlay et al (1961) was obtained at 25°C and I=0.2 and direct comparison of the constants is not possible. However, the measured value of $pK_a - pK_b$, which should vary little with temperature and ionic strength, was 1.56 from substrate hydrolysis and 0.70 from iodoacetate inactivation. This discrepancy would indicate the need for caution in equating the histidine residues at the active site with those involved in the reaction with iodoacetate. This reservation is reinforced by the existence of compounds which inhibit the alkylation reaction but not the hydrolysis of the substrate.

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