strate complex, $6{\cdot}30 \pm 0{\cdot}09$ and $8{\cdot}10 \pm 0{\cdot}09$. The groups involved are probably histidine residues.

3. Inhibition constants for cytidine 2′-phosphate and cytidine 3′-phosphate are reported. The descending order of affinity of cytidine nucleotides for the enzyme is: 2′-phosphate; 3′-phosphate; 2′,3′-phosphate.

We are indebted to the Central Research Fund, University of London, for a grant for the purchase of automatic titration equipment and to the Wellcome Trust for a grant for the purchase of a Cary recording spectrophotometer. We also acknowledge a personal grant to D. G. H. from the Department of Scientific and Industrial Research.

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The Active Site and Mechanism of Action of Bovine Pancreatic Ribonuclease

4. THE ACTIVITY IN INERT ORGANIC SOLVENTS AND ALCOHOLS

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Bovine pancreatic ribonuclease is active in high concentrations of organic solvents (Findlay, Mathias & Rabin, 1960). In the presence of methanol, both cytidine 3′-phosphate methyl ester and cytidine 3′-phosphate are formed from cytidine 2′,3′-phosphate (Heppel & Whitfeld, 1955). We have investigated the alcoholysis reaction and compared the reactivity of various alcohols. The effects of inert solvents on these reactions and on the hydrolysis of cytidine 2′,3′-phosphate methyl ester have also been investigated.

EXPERIMENTAL

Chromatography. The ascending technique was employed with propan-2-ol–water–aq. ammonia (sp.gr. 0·88) (14:5:1, by vol.) as solvent.

Paper electrophoresis. This was carried out at 320 v for 4 hr. on acid-washed Whatman no. 4 paper saturated with 0·02M-sodium phosphate buffer, pH 7·4.

Ribonuclease and cytidine 2′-3′-phosphate. These were as described by Herries, Mathias & Rabin (1962).

Cytidine 3′-phosphate methyl ester. This was prepared enzymically by incubating cytidine 2′,3′-phosphate (350 mg.), ethylenediamine buffer, pH 7·0 (0·1M with respect to ethylenediamine; 2 ml.), methanol (16 ml.) and ribonuclease (0·35 mg./ml. of water; 2 ml.) at 25° for 4 hr. The incubation mixture was streaked on acid-washed Whatman no. 3 MM paper (0·035 ml./cm.), which was developed by ascending chromatography in propan-2-ol–water–aq. ammonia for 18 hr. After removal of the solvent three major components were visible under ultraviolet light; in order of decreasing R_F values, they were: cytidine 3′-phosphate methyl ester, cytidine 2′,3′-phosphate and cytidine 3′-phosphate. The ester band was cut into small pieces and triturated with ion-free water (400 ml.). After filtration, the trituration was repeated (200 ml. of water) and the combined filtrates were freeze-dried. The residue was dissolved in anhydrous methanol, filtered and the ester precipitated by the addition of 3 vol. of anhydrous diethyl ether. The product was collected by centrifuging, washed with anhydrous ether and dried *in vacuo* over P_2O_5. The yield was 180 mg., which was presumed to be of the ammonium salt. Chromatography showed the presence of less than 2% of ultraviolet-absorbing contaminants. The absorption spectrum was almost identical with that of cytidine 3′-phosphate under the conditions of Crook, Mathias & Rabin (1960). Cytidine 2′,3′-phosphate, cytidine 3′-phosphate and cytidine 3′-phosphate methyl ester have an isosbestic point at 265 mμ.

Solvents. Formamide was purified by the method of Verhoek (1936). *p*-Dioxan was AnalaR and dried over sodium before use. Ethylenediamine was dried over NaOH pellets and redistilled as described by Weissberger, Proskauer, Riddick & Toops (1955, p. 444). Methanol and ethanol were purified by the method of Lund and Bjerrum (Weissberger *et al.* 1955, p. 334). Propan-1-ol was dried over anhydrous Na_2SO_4 and twice redistilled. Other solvents were dried over anhydrous Na_2SO_4 and redistilled under reduced pressure.

Kinetic runs. These were performed at 25°. Except for the inhibition studies, the assay procedure was similar to that of Heppel & Whitfeld (1955), which depends on the chromatographic separation of substrates and products.

The total volume of the reaction was usually 0·1 ml. and was contained in a small glass vessel fitted with a ground-glass cap. The joint was lubricated with silicone grease to prevent the loss of volatile solvents. The reaction was started by the addition of 0·01 ml. of ribonuclease solution to a solution of the substrate and buffer in water or in water plus organic solvent (0·09 ml.). Samples of 0·005 ml. were withdrawn immediately after mixing, and subsequently at 10 min. intervals, and applied to Whatman no. 4 paper and the spots rapidly dried in a current of warm air. After chromatography, the spots were detected under ultraviolet light, cut out and triturated with ethylenediamine buffer, pH 7·0 (0·01M; 4 ml.). The paper was removed by centrifuging and the extinction at 265 mμ of the supernatant determined by using a blank consisting of the eluate from a piece of paper of the same size and R_F. For alcoholysis experiments with polyhydric alcohols the products and reactants were separated by paper electrophoresis in place of chromatography.

Identification of ester products. For the methyl and ethyl esters of cytidine 3′-phosphate this was based on the greater stability towards acid hydrolysis of these esters compared with cytidine 2′,3′-phosphate. They were unaffected by treatment with 0·1N-HCl for 3 hr. at 25° and incubation of the esters with ribonuclease completely converted them into cytidine 3′-phosphate via cytidine 2′,3′-phosphate. The glycerol ester of cytidine 3′-phosphate gave a positive reaction with periodate–Schiff reagent (Baddiley, Buchanan, Handschumacher & Prescott, 1956). Incubation of both the glycerol ester and the ethylene glycol ester with ribonuclease completely converted them into cytidine 3′-phosphate. The liberated polyhydric alcohols, after electrophoresis, were detected by the periodate–Schiff reagent.

Inhibition studies. The titrimetric procedure described by Herries *et al.* (1962) was used.

RESULTS AND DISCUSSION

Alcohols. Ribonuclease is active in the presence of very high concentrations of alcohols and other solvents. In aqueous alcohols the products formed from cytidine 2′,3′-phosphate are cytidine 3′-phosphate and the ester of cytidine 3′-phosphate. The rates in aqueous methanol and glycerol as functions of the mole fraction of the alcohol are shown in Figs. 1 and 2. The rates shown are relative values, setting the rate of hydrolysis in the absence of alcohol as unity. The linear relationships shown have been found with all the alcohols tested and are consistent with competition between the alcohol and water. The slopes vary with the nature of the alcohol and are shown in Table 1.

Attempts to obtain alcoholysis of cytidine 2′,3′-phosphate in the complete absence of water (mixtures of formamide and alcohols or alcohols alone) were unsuccessful. This was not due to inactivation in the solvent mixtures since full activity could be recovered on dilution with water. A minimal quantity of water may be necessary for retention of the active conformation.

The inhibition of the hydrolysis of cytidine 2′,3′-phosphate by glycerol or ethylene glycol is non-competitive, as shown in Figs. 3 and 4, with K_i equal to $K_{i'}$ in the following expression:

$$v = \frac{V[\mathrm{S}]}{K_m\left(1+\frac{[\mathrm{I}]}{K_i}\right)+[\mathrm{S}]\left(1+\frac{[\mathrm{I}]}{K_{i'}}\right)}$$

where v is the initial reaction velocity for concentrations of substrate and inhibitor [S] and [I] respectively. The values of K_i and $K_{i'}$ determined by least-squares procedures are shown in Table 2. The form of the inhibition is further evidence for competition between water and alcohol for a site on the enzyme, and according to the general theorem

of Morales (1955), the Michaelis constant is the true dissociation constant of the enzyme–substrate complex. The lack of variation of the Michaelis constant for the hydrolysis as a function of the alcohol concentration is in sharp contrast with the findings of Bender & Glasson (1960) for chymotrypsin.

Further information on the interaction of ribonuclease with the attacking reagent (water or alcohol) has been obtained by studying the effects of inert organic solvents on the ratio of the initial rates of methanolysis and hydrolysis under conditions where the molar ratio of methanol to water was held constant. The addition of solvent will alter the pH of the solution, and as a preliminary the variation of the ratio of the initial rates of methanolysis to hydrolysis as a function of pH was measured. This ratio was constant in citrate buffer over the pH range 5·6–7·1. The ratio was also constant, but different, in the presence of 50% (v/v) dioxan. The substrate concentration in these experiments, 0·05 M, exceeds the Michaelis constant by at least a factor of 5, which ensures that the formation of the enzyme–substrate complex is not rate-limiting. Even if the combination of

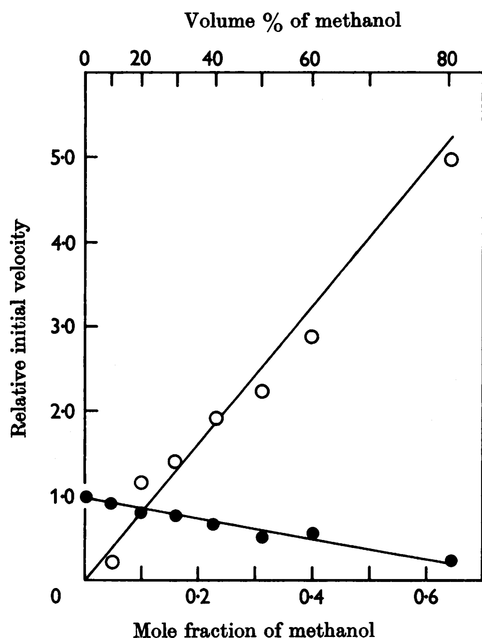

Fig. 1. Variation of rates of methanolysis and hydrolysis with methanol concentration. ○, Initial rate of formation of cytidine 3′-phosphate methyl ester; ●, initial rate of formation of cytidine 3′-phosphate. The initial concentration of cytidine 2′,3′-phosphate was 0·05 M in citrate buffer, pH 6·9. The rates are expressed relative to the initial rate of hydrolysis in the absence of methanol, which is taken as unity.

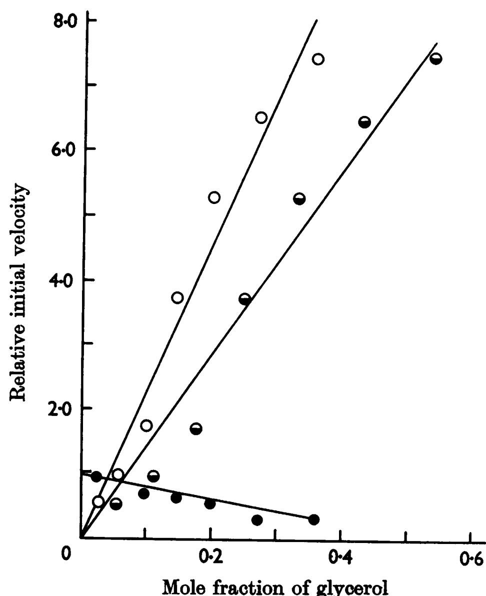

Fig. 2. Variation of rates of glycerolysis and hydrolysis with glycerol concentration. ○, Initial rate of formation of cytidine 3′-phosphate glycerol ester; ◒, initial rate of formation of cytidine 3′-phosphate glycerol ester with abscissa equal to the mole fraction of primary hydroxyl; ●, initial rate of formation of cytidine 3′-phosphate. The initial concentration of cytidine 2′,3′-phosphate was 0·05 M in ethylenediamine buffer, pH 7·0. The rates are expressed relative to the initial rate of hydrolysis in the absence of glycerol, which is taken as unity.

Table 1. *Initial rate of alcoholysis of cytidine 2′,3′-phosphate*

The rates, expressed per unit mole fraction of alcohol, are relative to the initial rate of hydrolysis in the absence of alcohol, which is taken as unity. The reaction mixture consisted of cytidine 2′,3′-phosphate (0·04 M) and ribonuclease (0·0125 mg./ml.) in ethylenediamine buffer, pH 7·0 (0·2 M).

Alcohol	Relative initial rate
Methanol*	8·0
Ethanol*	2·6
Propan-1-ol†	0·61
Propan-2-ol†	0·00
Benzyl alcohol*	0·00
Ethylene glycol†	10·0
Ethylene glycol monoethyl ether†	1·1
Glycerol†	22·5
Propane-1,3-diol†	3·5
Propane-1,2-diol†	2·8
Butane-1,4-diol†	3·1
Butane-1,3-diol†	1·9
Butane-2,3-diol†	0·0
(Hydroxylamine)*	(0·0)

* Reaction products separated by paper chromatography.

† Reaction products separated by paper electrophoresis.

enzyme and substrate becomes rate-limiting in the presence of the solvent, the experiments are still valid because this factor would be common to both reactions. Fig. 5 shows the effect of formamide and dioxan on the ratio of the initial velocities of methanolysis and hydrolysis at pH 6·9 (pH measured in water) when the ratio of water to methanol was 3:1 (v/v). Dioxan favours hydrolysis as compared with methanolysis; formamide tends to favour methanolysis rather than hydrolysis.

We have also studied the effect of solvents on the reverse of the methanolysis reaction, i.e. the cyclization of cytidine 3′-phosphate methyl ester. This reaction, which does not involve water, has been compared with the hydrolysis of cytidine 2′,3′-phosphate (Fig. 6). As would have been predicted from the previous experiments, dioxan favours cyclization of the esters as compared with hydrolysis of the cyclic phosphate. However, slight reservations have to be made about this experiment owing to the variation in the concentration of water. To overcome this objection we have measured this ratio, under conditions where the water concentration was held constant, in various proportions of formamide and dioxan. As shown in Fig. 7, dioxan favours the cyclization reaction compared with hydrolysis of the cyclic phosphate. We conclude from these experiments that there is a Van der Waals' interaction between methanol and the protein which is interfered with by dioxan. This interaction tends to position the methanolic oxygen atom near the phosphorus atom and thus favours methanolysis of the cyclic phosphate; it would also tend to impede cyclization of the methyl ester by preventing the departure of methoxide group. A markedly hydrophilic solvent like formamide

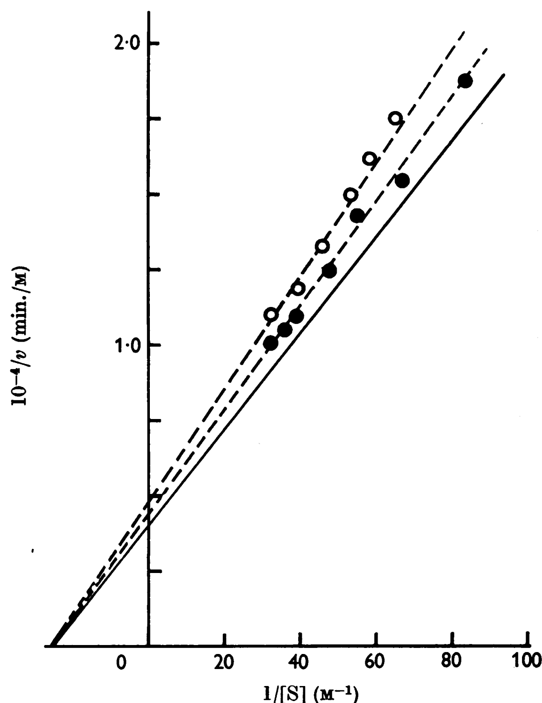

Fig. 3. Non-competitive inhibition by glycerol of the hydrolysis of cytidine 2′,3′-phosphate. Full line, no glycerol; broken lines: ●, 1·4M-glycerol; ○, 2·7M-glycerol. The reactions were carried out at I 0·2 and pH 8·0; the concentration of ribonuclease was 0·365 μM. The results were obtained by titrimetric assay.

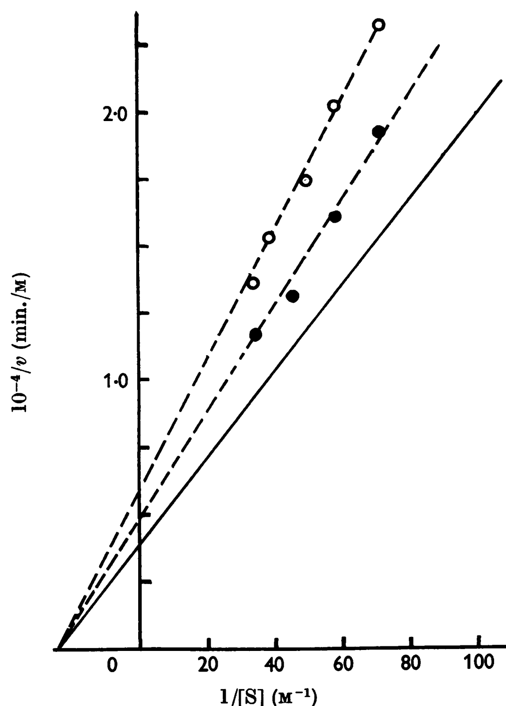

Fig. 4. Non-competitive inhibition by ethylene glycol of the hydrolysis of cytidine 2′,3′-phosphate. Full line, no ethylene glycol; broken lines: ●, 1·8M-ethylene glycol; ○, 3·6M-ethylene glycol. The reactions were carried out at I 0·2 and pH 8·0; the concentration of ribonuclease was 0·365 μM. The results were obtained by titrimetric assay.

Table 2. *Inhibition constants for polyhydric alcohols for the hydrolysis of cytidine 2′,3′-phosphate*

The inhibition constants were obtained from Figs. 3 and 4.

	K_i (M)	$K_{i'}$ (M)
Glycerol (1·4M)	0·06	0·04
Glycerol (2·7M)	0·06	0·08
Ethylene glycol (1·8M)	0·13	0·12
Ethylene glycol (3·6M)	0·14	0·13

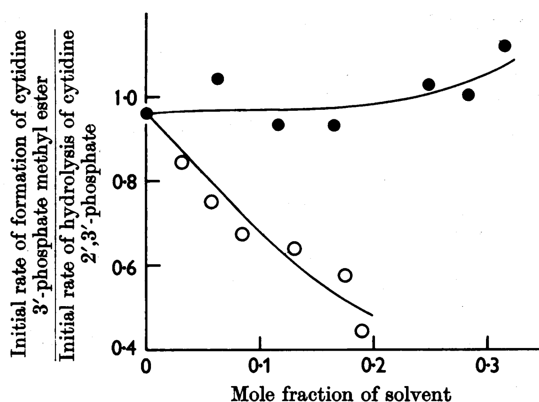

Fig. 5. Effects of organic solvents on the ratio of the initial velocities of methanolysis and hydrolysis. ●, Formamide; ○, dioxan. The substrate was cytidine 2′,3′-phosphate (0·05M) in citrate buffer, pH 6·9. The ratio of water to methanol was 3:1 (v/v) throughout.

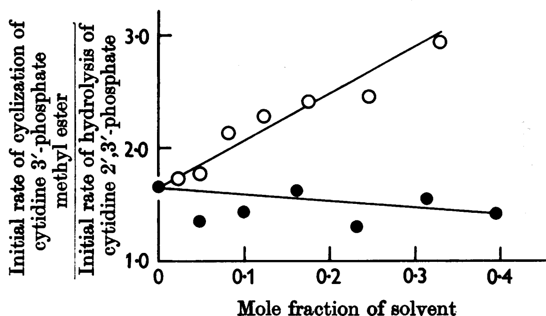

Fig. 6. Effects of organic solvents on the ratio of the initial velocity of cyclization of cytidine 3′-phosphate methyl ester to the initial rate of hydrolysis of cytidine 2′,3′-phosphate. ●, Formamide; ○, dioxan. The substrate concentration was 0·05M in citrate buffer, pH 6·9.

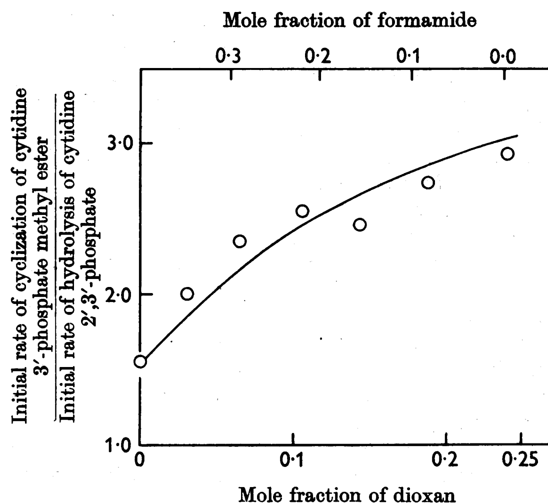

Fig. 7. Effect of replacing formamide by dioxan at constant water concentration on the ratio of initial velocities of cyclization of cytidine 3′-phosphate methyl ester to the hydrolysis of cytidine 2′,3′-phosphate. The substrate concentration was 0·05M in citrate buffer, pH 6·9. The water concentration was 40 % (v/v).

would not be expected to interfere with Van der Waals' interactions of this type. The effect of dioxan may be pictured as being due to the solvation of both methanol and one of the sites on the protein with which it interacts. These experiments provide further evidence for the existence of a site binding alcohols or water. Moreover, the existence of such a site would seem to be the only reasonable way of explaining the variation of rate with the structure of the alcohol (Table 1).

SUMMARY

1. Ribonuclease is active in high concentrations of organic solvents.

2. In aqueous alcohols the products formed from cytidine 2′,3′-phosphate are cytidine 3′-phosphate and the ester of cytidine 3′-phosphate. The relative rates of alcoholysis by a large number of alcohols are reported. Glycerol is the most effective of the alcohols studied; no ester formation occurred with propan-2-ol or benzyl alcohol.

3. Alcohols inhibit the hydrolysis of cytidine 2′,3′-phosphate in a non-competitive manner with respect to the nucleotide, but competitively with respect to water.

4. The rate of ester formation from cytidine 2′,3′-phosphate is a linear function of the molar fraction of alcohol.

5. At a fixed ratio of [alcohol] to [water], the ratio of the rates of formation of cytidine 3′-phosphate and cytidine 3′-phosphate ester from cytidine 2′,3′-phosphate is independent of pH, but is a function of the concentration of other added solvents.

6. The effect of the medium, at constant [water], on the ratio of initial velocities of cyclization of cytidine 3′-phosphate methyl ester to hydrolysis of cytidine 2′,3′-phosphate has been investigated.

7. Evidence for the existence of a water- or alcohol-binding site is presented.

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The Active Site and Mechanism of Action of Bovine Pancreatic Ribonuclease

5. THE CHARGE TYPES AT THE ACTIVE CENTRE

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An understanding of the effects of solvents on the acid dissociation constants of buffers and ionizing groups on proteins is an essential prerequisite for interpreting solvent effects on enzyme reactions. According to Mandel (1958) the shift in the pK of an acid in passing from water to another solvent depends primarily on the nature of the solvent and the ionizing group (CO_2H, OH, NH_3^+) and rather little on the exact nature of the complete molecule. This conclusion is substantiated by the work of Mandel & Decroly (1960) on carboxylic acids in formamide. Although dioxan and formamide differ sharply in their physical properties, both cause the pK of acetic acid to increase, but to different extents.

Acids may conveniently be divided into two main groups: (*a*) neutral acids, whose dissociation involves charge separation, e.g. carboxylic acids:

$$AH = A^- + H^+$$

(*b*) cationic acids, whose dissociation does not involve charge separation, e.g. amines:

$$BH^+ = B + H^+$$

The pK values of neutral acids have been measured by a number of workers (Bacarella, Grunwald, Marshall & Purlee, 1955, 1958; Bates & Schwarzenbach, 1955; Harned & Owen, 1950; Mandel & Decroly, 1960; Shedlovsky & Kay, 1956; Verhoek, 1936) in a variety of solvents and mixed solvent systems. In all instances the pK increased as water was replaced by an organic solvent. The magnitude of this increase depends on the solvent, its concentration and to a minor extent on the acid itself. The pK of acetic acid increases from 4·76 in water to 6·82 in formamide (Mandel & Decroly, 1960), 9·72 in anhydrous methanol (Bacarella *et al.* 1955) and 10·14 in aq. 82 % (w/w) dioxan (Harned & Owen, 1950, p. 581).

Cationic acids have been studied less thoroughly than carboxylic acids. The pK values of substituted anilines decrease on the addition of dioxan (Willi, 1957) or methanol (Bacarella *et al.* 1955), and the pK of triethanolamine decreases on the addition of ethanol (Bates & Schwarzenbach, 1955). Verhoek (1936) found that the pK values of neutral acids increased whereas those of cationic acids decreased on the addition of formamide. The dissociation constants of some amino acids and related compounds have been determined in water and in aq. 20 % (w/w) dioxan by Duggan & Schmidt (1942). They found that the decrease in the pK of the amino group was much less than the increase in the pK of the carboxyl group.

The existence of a pH optimum in an enzyme reaction implies a requirement for at least two ionizing groups for activity, one in the base form and the other in the acid form. Solvents will in general affect the binding of the substrate to the enzyme in a non-predictable fashion. Accordingly it is best to conduct experiments involving solvents under conditions where the substrate concentration is not rate-limiting. The effects of solvents will then be due to: (*a*) changes in the conformation of the protein; (*b*) changes in the pK values of buffers and the groups at the catalytic site; (*c*) effects on