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THE EFFECT OF pH, UREA AND GUANIDINE ON ADENOSINE DEAMINASE ISOLATED FROM BOVINE MUCOSA

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SUMMARY

1. Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) from bovine duodenal mucosa is stable between pH 5 and 9 when incubated for 24 h at 37°.

2. Urea and guanidine markedly reduce the pH stability of the enzyme. Both substances also exhibit a minimum in denaturation rate in the region of 20°.

3. Concentrations of urea greater than 6 M and of guanidine above 0.5 M produce an irreversible denaturation resulting in a protein which is eluted earlier from a Sephadex column than the active enzyme.

4. The viscosity of urea- and guanidine-denatured enzyme increases slowly with time.

5. Low concentrations of urea and guanidine are competitive inhibitors of adenosine deaminase.

INTRODUCTION

In the present paper a study is made of the stability of adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4), isolated from bovine duodenal mucosa, over the pH range 3.5–11 and the effect upon it of urea and guanidine under varying conditions of pH, concentration and temperature. A short account of a portion of this work has already appeared[1].

MATERIALS AND METHODS

Enzyme

Purified preparations of adenosine deaminase[2] with specific activities between 380 and 430 were used in all experiments.

Urea

A commercial sample of urea (Analar), at a strength of 8 M caused an instantaneous inactivation of about 10% of the added enzyme due probably to cyanate in

equilibrium with the urea[3]. Two-fold recrystallisation from 30% ethanol and use of freshly prepared solutions almost completely eliminated this inhibition.

Guanidine

An instantaneous inactivation of 25% of the added enzyme was encountered in 1 M guanidine·HCl whether it was prepared from guanidine carbonate or from commercially available pure salt (Dr. T. Schuchardt, München). Neither 6-fold recrystallisation from 30% aqueous ethanol nor purification by the procedure of SPACKMANN *et al.*[4] were successful in removing this inhibition completely, but they did reduce it to about 5%.

Enzyme assays

The activity was measured either by NH_3 determination or, by spectrophotometry at 265 mμ, as previously described[2].

Substrate

Chromatographically pure anhydrous adenosine obtained from Schwarz Bio Research Inc., U.S.A., was used in all experiments.

RESULTS

Effect of pH upon the activity of adenosine deaminase

Solutions of the enzyme containing 2 mg of enzyme per ml were made up in 0.1 M citrate or 0.1 M phosphate buffers at pH intervals of approx. 0.5 from 3.5 to 11.0, and incubated at 37° ± 0.1° for 24 h. Activity was determined after the solutions had been diluted and adjusted to pH 7.0. The results after 24 h incubation are shown in Fig. 1, from which it can be seen that the enzyme is stable between pH 5 and 9,

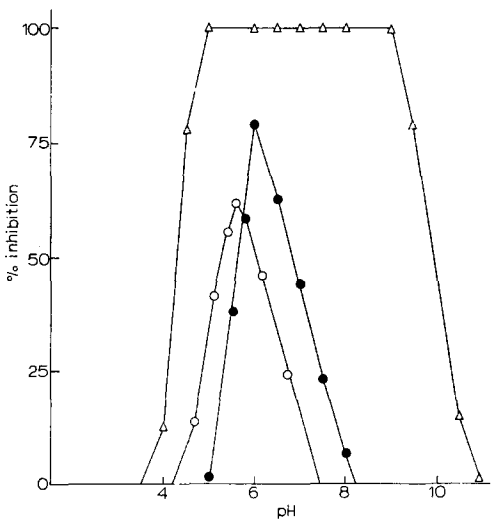

Fig. 1. Effect of pH on stability of adenosine deaminase: △—△, in buffer; ●—●, in 8 M urea; ○—○, in 2 M guanidine. 0.1 M citrate buffer used from pH 3 to pH 5; 0.1 M phosphate from pH 6 to pH 10. For assay conditions see text.

but decreases rapidly in activity above or below these values and is completely inactivated at approx. 1 pH unit on either side. The inactivation curves are symmetrical, the pH values at which 50% inactivation occurs being 4.25 and 9.75, from which it appears that the suggestion originally proposed by LEWIS[5] for oxyhaemoglobin that H^+ and OH^- have equal effects upon denaturation holds good for this enzyme.

Urea denaturation of adenosine deaminase

Effects of concentration on denaturation: Enzyme solutions of final concentration 1 mg/ml made up in 2, 4, 6 and 8 M urea and adjusted with 0.2 M citrate buffer to pH 5.5, were compared with enzyme controls buffered at the same pH, after incubation at 20° for 24 h. pH 5.5 was chosen, because preliminary experiments had shown that at this pH 8 M urea gave considerable, but not complete, inactivation after 24 h. In the present experiment 8 M urea produced a 60.6% inactivation while the lower concentrations of urea had no effect upon enzyme activity.

Effect of pH on denaturation in 8 M urea: It has been pointed out by a number of investigators[6-8] that the exact significance of pH measurements in concentrated urea solutions is open to question; and considerable alterations in the pH of buffers when dissolved in 8 M urea have been noted. The values recorded in these experiments were made on the final mixtures with a Radiometer pH meter.

It can be seen from Fig. 1 that although denaturation occurs at all values, it is minimal at pH 6.0 where 78.5% of the original activity is retained after 24 h at 37°. Almost complete inactivation occurs below pH 5 and above pH 8, clearly demonstrating that the pH stability of the enzyme is much reduced in concentrated urea solution.

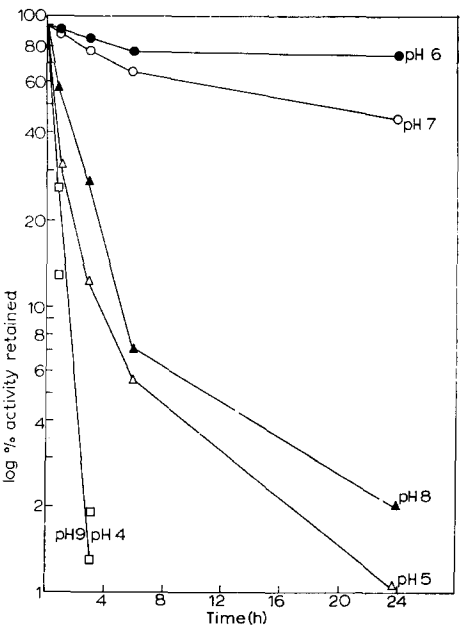

Fig. 2. Time-dependant urea denaturation of adenosine deaminase at 37° at different pH values.

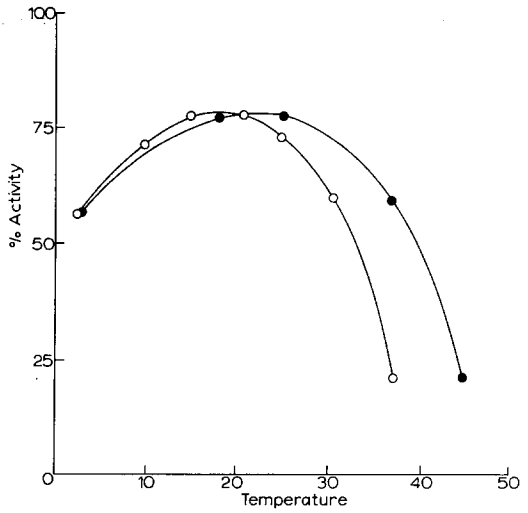

Fig. 3. Effect of temperature on rate of denaturation of adenosine deaminase in 8 M urea (○—○) and in 2 M guanidine (●—●).

Order of reaction of denaturation: It has already been shown (Fig. 1) that the degree of inactivation of adenosine deaminase by urea varies with pH. In Fig. 2 the course of inactivation plotted on a logarithmic scale against time on a linear scale at a number of pH values is shown. If the inactivation followed first-order kinetics, then straight lines would be obtained, but this is not so, except at the extremes of pH, 4 and 9, where inactivation is very rapid. At intermediate pH values the rate of inactivation decreases as the reaction proceeds. WRIGHT AND SCHOMAKER[9] found that diphtheria antitoxin behaved in the same way. However, pepsin[10] and tobacco mosaic virus protein showed first-order reaction rates at all pH values tested.

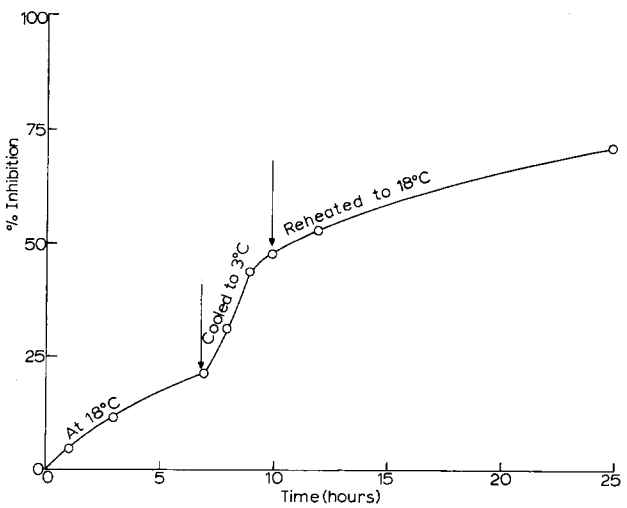

Fig. 4. Rate of denaturation of adenosine deaminase in 8 M urea at 18° and at 3° in 0.1 M phosphate buffer.

Effect of temperature on urea denaturation: It can be seen from Fig. 3 that when the enzyme is incubated in 8 M urea at pH 7.5 for 24 h, at temperatures between 3° and 43°, the degree of denaturation follows a curve with a minimum in the region of 18°. This is similar to the findings of LAUFFER[11] with tobacco mosaic virus protein and of SIMPSON AND KAUZMANN[12] with ovalbumin, but CLARK[13] measuring denaturation by another method recorded a positive temperature coefficient for ovalbumin between 4° and 40°. Other proteins such as pepsin[10] and diphtheria antitoxin[9] showed progressive inactivation with increase in temperature when measured by enzyme and biological assay methods, respectively.

The negative temperature coefficient of this enzyme was studied further by incubating a solution containing 1 mg protein per ml in 8 M urea at pH 7.5 initially at 18°, then after rapid cooling at 3° for 3 h, followed by reheating to 18°. The results are shown graphically in Fig. 4 from which it can be seen that denaturation was accelerated by cooling. Although re-heating caused a return to the slower rate there was no reversal of the acceleration produced by cooling as was found by JACOBSON AND CHRISTENSEN[14] with β-lactoglobulin.

Irreversibility of urea denaturation: The previous experiment (Fig. 4) indicated that urea denaturation was probably irreversible. This was confirmed by the failure to achieve reactivation by reducing the urea concentration through dilution or by dialysis.

Guanidine denaturation of adenosine deaminase

Guanidine, as was expected, caused denaturation at lower concentrations than urea. Another difference, shown by the results in Table I was the effect which changes in both ionic strength and constitution of buffer have upon the extent of denaturation with guanidine.

Effect of pH on guanidine denaturation: It can be seen from Fig. 1 that guanidine denaturation is also pH dependant. The pH of maximum stability at 5.6 is slightly

TABLE I

EFFECT OF GUANIDINE CONCENTRATION AND BUFFER CONSTITUTION ON DENATURATION OF BOVINE ADENOSINE DEAMINASE

The enzyme at a concentration of 2 mg protein per ml was incubated at 37° for 24 h. Prior to assay the solutions were adjusted to pH 7.0.

Guanidine final concn. (M)	*% inhibition*			
	Citrate buffer (pH 5.2)		*Phosphate buffer (pH 5.2)*	
	0.2 M	*0.5 M*	*0.05 M*	*0.1 M*
0.5	0	—	—	—
1.0	8	—	52	62
1.5	17	—	—	—
2.0	36	—	—	—
3.0	100	—	—	—

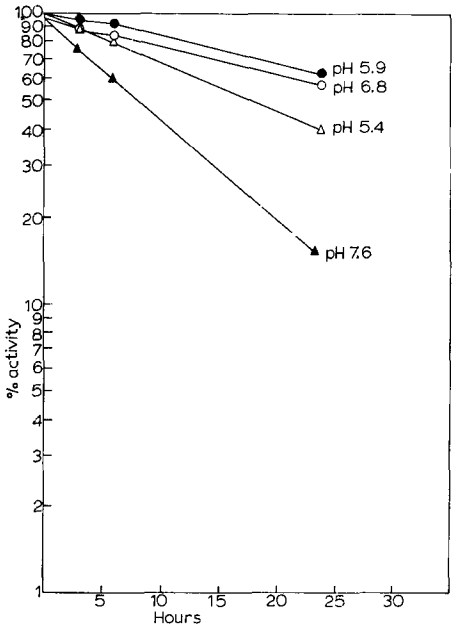

Fig. 5. Time-dependant guanidine denaturation of adenosine deaminase at 37° at different pH values.

more acid than with urea. In these experiments 1 mg protein per ml in 2 M guanidine in 0.1 M citrate buffers was used.

Order of reaction: The plot of inactivation with time shown in Fig. 5 gives a straight-line relationship for all pH values from which it may be concluded that guanidine denaturation follows first-order reaction kinetics.

Temperature effect: It is evident from Fig. 3 that the degree of denaturation produced by guanidine passes through a temperature minimum, slightly higher than with urea.

Irreversibility of guanidine denaturation: Removal of guanidine by dialysis or reduction in its concentration by dilution failed to restore enzyme activity, and an experiment, identical with the one shown in Fig. 4 for urea, gave similar results. Thus denaturation of this enzyme with both urea and guanidine is irreversible.

Viscosity of solutions of adenosine deaminase treated with urea and guanidine

The viscosity of native and urea-treated adenosine deaminase was studied at pH 7.2 in 0.1 M Tris–HCl buffer at 37°, and at pH 5.0 in 0.1 M citrate buffer at 30°, at a final protein concn. of 10 mg/ml in each case. A lower concn. of 3 mg/ml at pH 7.2 and 37° was also included to determine the effect of protein concn. on viscosity. An Ostwald viscometer of 2 ml capacity with a flow rate of 53.6 sec for water at 37° was used.

For the urea studies, the enzyme solution and a 12 M urea solution, both made up in buffer, and adjusted to pH 7.2 (5.0), were heated separately in a water bath to 37° (30°) $\pm$ 0.1°. One ml of enzyme was then rapidly added to 2 ml 12 M urea giving a final concn. of 8 M urea. Viscosity determinations were begun about 10 min after mixing.

The reduced viscosity of native adenosine deaminase was found to be 0.026 at pH 7.2 and 37° and 0.030 at pH 5.0 and 30°. At 37° urea-treated adenosine deaminase gave a steady but gradual increase in viscosity reaching a reduced viscosity of 0.200 in about 30 h after which no further increase occurred. Its behaviour at 30° and pH 5.0 was similar but it took longer to reach its maximum value.

The viscosity of adenosine deaminase in 2 M guanidine at a final concn. of 10 mg protein/ml in 0.1 M citrate buffer at pH 5.0 and 30° was also measured at various time intervals and showed the same slow increase with time as was found with urea. However, a lower maximum value 0.160 for the reduced viscosity was obtained.

It was decided to compare the behaviour of adenosine deaminase with the following commercially obtained crystalline proteins: ovalbumin and chymotrypsinogen (Worthington Biochemical Corporation, N.J.); bovine serum albumin (Sigma Chemical Co., St. Louis) and ribonuclease (Mann Research Lab. Inc. N.Y.) in 8 M urea at pH 7.2 and 37°.

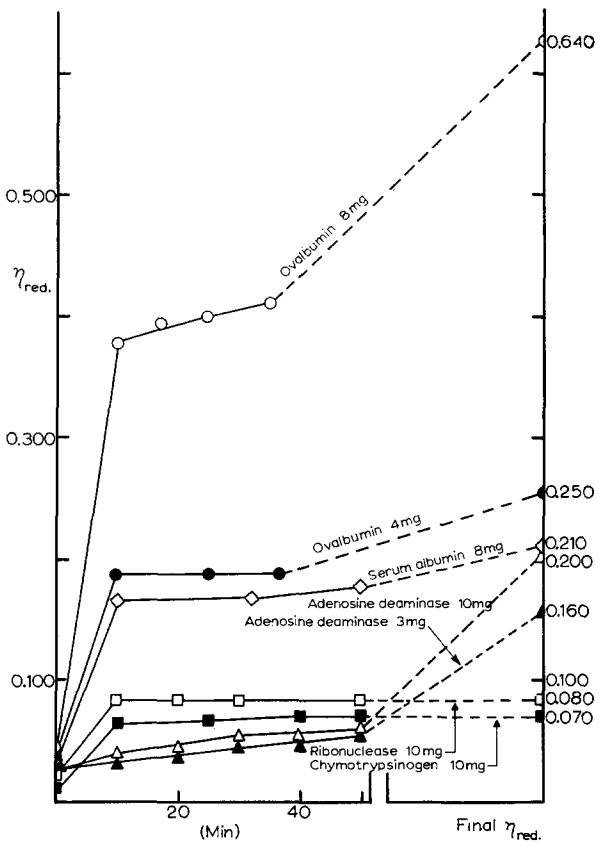

Fig. 6. Effect of time on reduced viscosity of ovalbumin (8 mg/ml) ○—○, (4 mg/ml) ●—●; bovine serum albumin (8 mg/ml) ◇—◇; ribonuclease (10 mg/ml) □—□; chymotrypsinogen (10 mg/ml) ■—■; adenosine deaminase (10 mg/ml) △—△, (3 mg/ml) ▲—▲ in 8 M urea at pH 7.2 in 0.1 M Tris –HCL buffer at 37°.

The initial viscosity changes resulting from urea treatment and the final values for the reduced viscosity of the 5 proteins are shown in Fig. 6 from which it is apparent that there is a marked difference in the behaviour of adenosine deaminase in the early stages from that of the other proteins. Ribonuclease reaches its maximum reduced viscosity within 10 min. Chymotrypsinogen and bovine serum albumin are within 80% of their maxima, and ovalbumin reaches 60% at 8 mg, and 70% at 4 mg protein/ml in 10 min. In the same time interval adenosine deaminase at 10 mg and 3 mg/ml gives only 20% approx. of its final value. The subsequent increase in viscosity with time follows a similar course at the 2 concentrations but the final value of 0.160 at 3 mg/ml is 20% less that at the higher concentration.

It can be seen from Fig. 6 that ovalbumin shows a much greater concentration dependance, both in respect to initial viscosity rise and the final value attained than adenosine deaminase. McKENZIE, SMITH AND WAKE[15] have shown that the concentration dependant increase in viscosity of ovalbumin is due to aggregation of the denatured protein. The behaviour of adenosine deaminase is so different to that of ovalbumin that aggregation as a cause for the time dependant viscosity increase with this protein appears untenable. A more plausible explanation is that conc. solutions of urea and guanidine produce a very slow unfolding of the molecule. The results described in the next section lend support to this view.

Effect of urea and guanidine upon the behaviour of adenosine deaminase on a column of Sephadex

A method developed by ANDREWS[16] for the determination of the molecular weights of proteins was used. A 50 cm × 3 cm column of Sephadex G-75 (Pharmacia Uppsala, Sweden) was standardised with the following pure proteins: albumin, ovalbumin, chymotrypsin, myoglobin, ribonuclease and glucagon. The peak elution volume plotted against the logarithm of the molecular weight gave a linear relationship between ovalbumin (mol. wt. 45 000) and glucagon (mol. wt. 3485). A solution of adenosine deaminase of specific activity 380 (88.4% pure on the basis of a maxi-

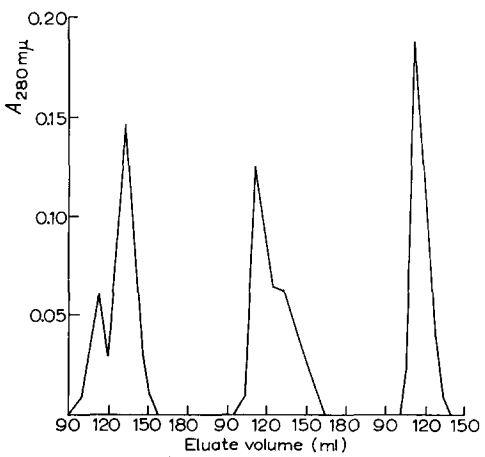

Fig. 7. Elution of adenosine deaminase from a Sephadex G-75 column (50 cm × 3 cm) in 8 M urea buffered with 0.04 M Tris–HCl plus 0.24 M KCl (pH 7.8). a, Immediately after mixing; b, after incubation for 24 h at 37°; c, in 10 M urea, after incubation for 24 h at 37°.

mum activity of 430 (see ref. 2)) in 0.04 M Tris–HCl buffer (pH 7.8) gave a small peak with low deaminase activity, corresponding to a mol. wt. of approx. 70 000 followed by a large peak with high deaminase activity of mol. wt. 35 000 ± 2000.

When this enzyme was made up in 8 M urea and passed immediately through the column the same pattern, Fig. 7a, was obtained. After standing for 24 h in urea the elution pattern altered to that shown in Fig. 7b in which the first peak was increased while the second had almost disappeared. Assay of this solution showed that it had lost 95% of its original activity. A solution of the deaminase in 10 M urea gave, after incubation for 24 h, a single protein (Fig. 7c), which eluted at the same volume as the inactive protein in Fig. 7a. This solution had lost all enzyme activity. Repetition of these experiments with a Sephadex G-75 column equilibrated with 8 M urea and elution with 0.01 M Tris–HCl buffer (pH 7.8) containing 8 M urea gave essentially the same results but the peaks emerged at a lower elution volume due probably to swelling of the granules by urea.

Incubation of the deaminase with 2 M guanidine had the same effect as urea in transforming the active enzyme into an inactive form which emerged earlier from the column. Inactivation by acid or alkali *i.e.*, by incubating the enzyme at pH 3.0 or at pH 10 gave a single peak on Sephadex emerging in the same position as the urea- or guanidine-denatured protein.

The behaviour of denatured proteins on Sephadex columns has not, as far as the authors are aware, been systematically investigated. The results obtained with denatured adenosine deaminase are however consistent with the view that 'an increased molecular asymmetry associated with an unfolding of a polypeptide chain'[17] occurs which leads to a reduced retention of the protein on the column. The sharpness of the peak given by the denatured protein indicates a uniformity of molecular size which would not be obtained if aggregation of the molecules occurred.

Inhibition of adenosine deaminase activity by dilute solutions of urea and guanidine

Urea concentrations less than 6 M and guanidine less than 0.5 M were without

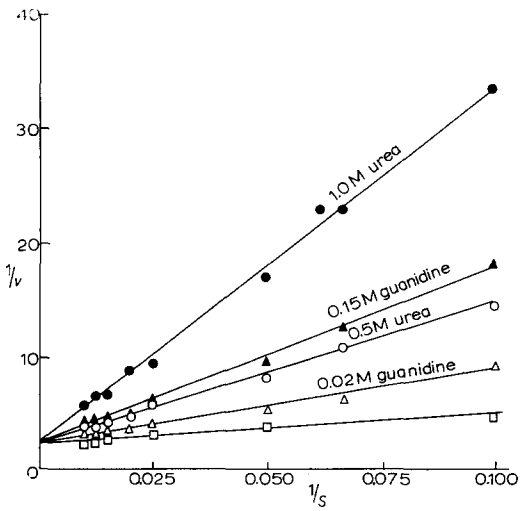

Fig. 8. Lineweaver–Burk plot of adenosine deaminase activity in dilute urea and guanidine solutions in citrate buffer (pH 7.0). Enzyme assayed by the spectrophotometric method at 265 mμ.

effect upon the activity of the enzyme when assays were performed in the presence of excess substrate. When, however, the concentration of substrate was reduced urea and guanidine both exerted a competitive inhibition of the enzyme at concentrations much below that causing denaturation. This is illustrated in Fig. 8 by the Lineweaver–Burk plots of enzyme activity against substrate concentration in the presence and absence of urea and guanidine. This competitive inhibition indicates that these substrates become attached to the active site of the enzyme and is possibly related to their effect in high concentration which results in irreversible denaturation. RAJAGOPALAN, FRIDOVICH AND HANDLER[18] found that a large number of enzymes which act on organic substrates are competively inhibited by low concentrations of urea and guanidine, while enzymes which act on inorganic substrates are non-competitively inhibited.

DISCUSSION

While bovine adenosine deaminase is optimally active at pH 7.0 it is stable at 37° for at least 24 h over the pH range 5–9. Inactivation occurs above or below these values resulting in almost complete loss of activity within a pH unit on either side.

Both urea and guanidine reduce the pH stability of the enzyme. As can be seen from Fig. 1, a peak rather than a plateau of maximum activity, occurs, at pH 6.0 with urea, and at 5.6 with guanidine.

The order of reaction for urea denaturation (Fig. 2) does not obey first-order kinetics. For a similar result with ovalbumin SIMPSON AND KAUZMANN[7] considered 2 possibilities: (a) inhomogeneity of the protein, (b) the existence of a series of stages in the denaturation of homogeneous protein. The second hypothesis appears the more likely for adenosine deaminase because guanidine denaturation should be similarly affected if protein inhomogeneity were the cause, but, as can be seen from Fig. 5, this is not so. Although a systematic investigation of the effect of ions upon denaturation has not been undertaken it was found that the extent of guanidine denaturation in citrate and phosphate buffers differed (Table I) while urea denaturation was unaffected by buffer composition.

The slow increase in viscosity of adenosine deaminase in solutions of urea and guanidine compared with the other proteins examined, together with the symmetrical peak obtained on gel filtration of the fully denatured protein, is taken as evidence that these reagents bring about a slow unfolding of the protein which does not show a tendency to aggregate.

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