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1 E. E. SNELL, *Vitamins Hormones*, 16 (1958) 77.
2 A. E. BRAUNSTEIN, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 2, Academic Press, New York, 1960, p. 113.
3 A. M. PERAULT, B. PULLMAN AND L. VALDEMORO, *Biochim. Biophys. Acta*, 46 (1961) 555.
4 W. T. JENKINS, D. A. YPHANTIS AND I. W. SIZER, *J. Biol. Chem.*, 234 (1959) 51.
5 H. WADA AND E. E. SNELL, *J. Biol. Chem.*, 237 (1962) 127.
6 A. S. KONIKOVA, N. N. DOBBERT AND A. E. BRAUNSTEIN, *Nature*, 159 (1947) 67.

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Acid and alkaline ribonucleases of plasma

A study of the properties of plasma ribonuclease was undertaken to differentiate the activity present in lysates of erythroid cells from that of the plasma ribonuclease[1]. Two earlier studies on the ribonuclease activity of mammalian plasma have shown that plasma has an ribonuclease with an optimum pH of 7.4 and is devoid of "acid" ribonuclease[2,3]. Both studies suggested that the "alkaline" ribonuclease is due to circulating pancreatic ribonuclease. Our findings indicate that most of the pH 7.4 ribonuclease of mouse plasma has an apparent molecular weight in excess of 200 000 and cannot, therefore, be pancreatic ribonuclease. We also report that there is a potent "acid" ribonuclease in mouse plasma with a pH optimum of 4.0. The pH 4.0 enzyme is activated by certain divalent cations especially Ni^{2+}.

Blood was obtained by cardiac puncture of mice of the C_3H strain into heparinized syringes. After removal of the cells by centrifugation ribonuclease activity was determined at pH 7.4 in a 0.04 M Tris buffer, 0.15 M NaCl, and 0.64 mg/ml tRNA. The solution was brought to 37° and mouse plasma was added to a concentration of 1–2 mg (plasma protein) per ml. Aliquots of 0.50 ml were removed at zero time and at intervals thereafter and transferred to tubes containing 0.70 ml of 1.0 M $HClO_4$ at 0°. The tubes were kept at 0° for 10 min. They were then centrifuged at $2000 \times g$, for each tube the supernatant was decanted, and its absorbance at 260 mμ was determined with a Beckman DU spectrophotometer. The zero-time value was subtracted. A control without enzyme was run simultaneously. The conditions for assaying plasma ribonuclease at pH 4.0 were identical to those described above except that 0.04 M acetate buffer was used. One unit of enzyme activity is defined as that amount of enzyme

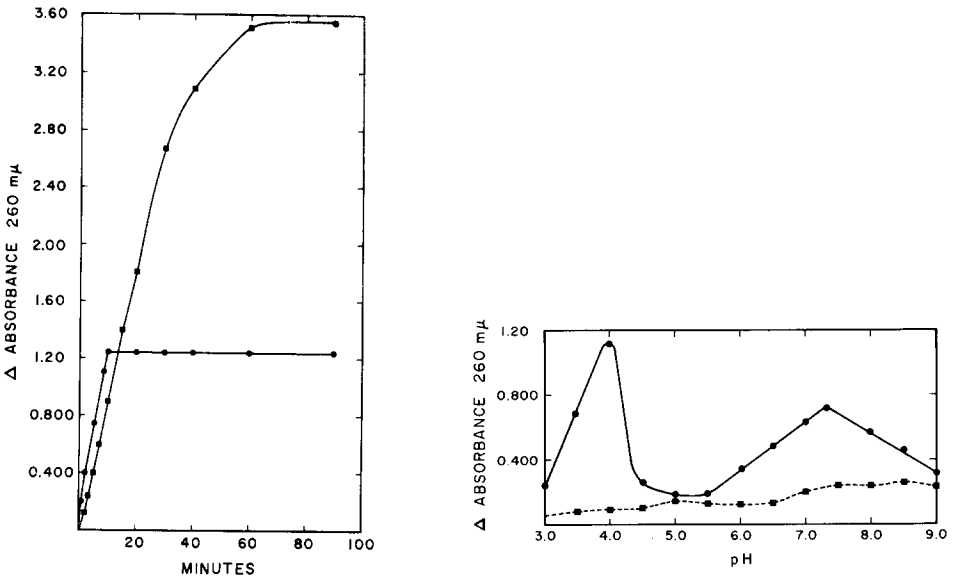

Fig. 1. Time course of RNA depolymerization by plasma ribonucleases. Samples were incubated as in the standard assay described in the text. ●, samples assayed at pH 4.0; ■, at pH 7.4.

Fig. 2. Effect of pH on the rate of RNA depolymerization. ■, experiments performed in the absence of NaCl; ●, experiments performed in the presence of 0.15 M NaCl. The stock buffer was prepared by adjusting the pH of a mixed buffer of acetic acid, imidazole and Tris to the designated pH values with either HCl or NaOH. The buffer concentration in the incubation mixture was 0.04 M.

which causes an increase of 1.00 in absorption at 260 mμ in 7 min in this assay. The reaction rate was directly proportional to the amount of plasma added, both at pH 7.4 and at pH 4.0. The rate was linear with time for at least 10 min (Fig. 1). At pH 7.4 60% of the RNA was degraded to fragments soluble in $HClO_4$, but, at pH 4.0 the degradation of RNA stopped abruptly at 10 min, and 84% of the RNA remained insoluble in $HClO_4$. In another experiment with the pH 4.0 enzyme, additional RNA was added after 15 min. This RNA was partially degraded in a similar manner indicating that the cessation of RNA degradation was not due to inactivation of enzyme or accumulation of an inhibitory product. This observation indicates that many of the polynucleotides present are refractory to digestion by the pH 4.0 enzyme.

The effect of pH on the depolymerization of RNA was studied to determine optimal conditions for examining plasma ribonuclease and to determine the number of enzymes in mouse plasma that catalyze depolymerization of RNA. Fig. 2 shows that there are two peaks; one at pH 4.0 and one at pH 7.4. Fig. 2 also shows that changing the ionic strength has a marked effect on the activity of both enzymes. Pancreactic ribonuclease has a pH optimum of 7.0 when determined in this system.

The effect of the plasma ribonucleases on the rate of degradation of tRNA and different synthetic polyribonucleotides is shown in Table I. The effect of secondary structure on the rate of degradation by the two enzymes was studied by observing the rate of degradation of poly A · poly U and poly C · poly G hybrids. At pH 7.4, poly A inhibited the degradation of poly U by 75% and poly G inhibited poly C degradation

TABLE I

SUSCEPTIBILITY OF VARIOUS POLYNUCLEOTIDES TO THE PLASMA RIBONUCLEASES

Substrate	pH 7.4		pH 4.0	
	ΔA $HClO_4$ (7 min)	ΔA Ethanol* (7 min)	ΔA $HClO_4$ (7 min)	ΔA Ethanol* (7 min)
tRNA	0.602	0.063	0.995	0
Poly (A)	0	0	0	0
Poly (U)	—	0.016	—	0
Poly (G)	0.003	—	0	—
Poly (C)	0.074	0.002	0.041	0
DNA	0	—	—	—
Poly (A) · Poly (U)	—	0.004	—	0
Poly (G) · Poly (C)	0.043	—	0.008	—
Poly (AU) (5:1)	2.12	—	0.117	—

* Precipitation was carried out by addition of two volumes of ethanol.

by 45%. At pH 4.0 poly G inhibited poly C degradation by 81%. Neither enzyme hydrolyzed the 2',3'-cyclic phosphates of cytidine, guanosine or uridine.

Both ribonuclease activities were stimulated by NaCl, optimum activity was observed at 0.15 M. Neither enzyme was affected by 5.0 mM EDTA, 0.04 M iodoacetate or iodoacetamide. In the absence of NaCl the pH 4.0 enzyme was stimulated by some divalent cations especially Ni^{2+} which activated by 365% at 6.0 mM. Mg^{2+} and Ca^{2+} stimulated by 170% and 94%, respectively, at 6.0 mM.

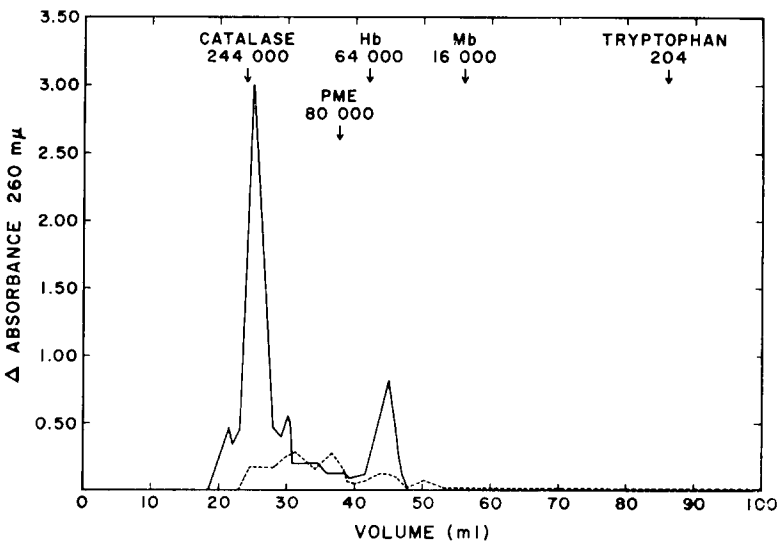

Fig. 3. Chromatography of plasma ribonucleases on Bio-Gel P-200. Mouse plasma (1.0 ml) in 3% sucrose was layered onto a 1.0 cm × 55 cm column of Bio-Gel P-200 equilibrated with 1 mM Tris–HCl buffer (pH 7.4) containing 0.13 M NaCl. The column was developed with this buffer; 0.8 ml fractions were collected. The column was calibrated with tryptophan, myoglobin, hemoglobin, *Escherichia coli* phosphomonoesterase (PME) and catalase. ———, activity at pH 7.4; — — —, at pH 4.0. The volume at which various substances used to calibrate the column were eluted is also indicated.

Mouse plasma could be heated up to 62° without any loss in activity of the pH 7.4 enzyme. At 65° the pH 7.4 enzyme was completely inactivated. The pH 4.0 enzyme retained 45% of its activity when the plasma was maintained at 95° for 7 min.

Mouse plasma was chromatographed on Bio-Gel P-200 (Fig. 3) in the presence of 0.13 M NaCl. The material in the major peak at pH 7.4 (containing 54% of the activity) has an apparent molecular weight of 230 000. There is a small peak which elutes before the major peak (7% of the total activity). There is another slight peak (9% of the total activity) with a molecular weight corresponding to 190 000 and a fourth peak (14% of the total activity) with an apparent molecular weight of 52 000. The remaining 16% of the pH 7.4 activity was eluted between the third and fourth peaks. The 1.0 ml of plasma that was layered onto the column contained 91 units at pH 7.4. However, a quantity of activity amounting to 350 units was recovered from the column indicating that there was almost 4 times as much activity recovered as was applied to the column. It is possible that the high molecular weight of the ribonuclease may be due to interaction with other plasma proteins.

When the column fractions were assayed at pH 4.0 no well defined peak was observed. The activity was distributed among fractions eluting between 24 and 51 ml. It is unlikely that this was due to an inactivation of the enzyme since 74% of the activity layered onto the column was recovered in these fractions.

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REFERENCES

1 W. R. FARKAS AND P. A. MARKS, *J. Biol. Chem.*, 243 (1968) 6464.
2 M. RABINOVITCH AND S. R. DOHI, *Arch. Biochem. Biophys.*, 70 (1957) 239.
3 G. R. BARKER AND J. G. PAVLIK, *Nature*, 215 (1967) 280.

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Purification of rabbit muscle pyruvate kinase by CM-Sephadex and evidence for an endogenous inhibitor

Pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) from rabbit muscle is most often prepared by the method of TIETZ AND OCHOA[1] or by slight modifications of this procedure. REYNARD *et al.*[2] reported that this preparation has traces of adenylate kinase as well as ATPase activities. Further attempts at purification by these authors were unsuccessful. Recently COTTAM *et al.*[3] stated that their sample