Short Communications

A paper chromatographic assay for ribonucleases using cyclic mononucleotides as substrates

Certain ribonucleases (RNases) have been shown to have two distinct diesterase activities: one effecting the depolymerisation of ribonucleic acid (RNA) to give nucleotides which may include nucleoside 2′,3′-cyclic phosphates, the other effecting the ring opening of such cyclic mononucleotides to give specifically the 2′ or 3′ isomer. The RNase activity of tissues is usually measured by a spectrophotometric method based on the first of these two activities[1,2], which has the disadvantage that the apparent activity varies with the batch of RNA used, and that reproducibility is poor unless conditions are rigorously standardized. A spectrophotometric assay method using uridine 2′,3′-cyclic phosphate as substrate was developed by RICHARDS[3] but, in contrast with the method now described, was obviously not practicable if whole tissue preparations were used as the source of the enzyme, as the assay was performed at a wavelength of 280 mμ, at which there is interfering absorption by tissue proteins.

The present method of assay was developed in the course of work on the specificities of the two RNases known to be present in rat-liver homogenates[4,5]. As in liver only the acid RNase attacks cyclic-nucleotide substrates[6,7] the new method provides an assay for acid RNase unaffected by alkaline RNase. (The possibility that there are two "acid RNases" is supported by the finding[6] that the products are adenosine 2′-phosphate and cytidine 3′-phosphate with adenosine 2′,3′-cyclic phosphate and cytidine 2′,3′-cyclic phosphate respectively.) On the other hand, with RNA as substrate[1,2] alkaline RNase contributes significantly to the "acid RNase" activity observed at the pH optimum of the latter enzyme. The present method would presumably be applicable to any RNase which attacks cyclic mononucleotides, such as pancreatic RNase (using a cyclic pyrimidine nucleotide as substrate for the latter).

An alternative assay procedure was tried (following a suggestion by Dr. L. A. HEPPEL), based on the release of inorganic phosphate by human prostatic acid phosphatase from the open-chain nucleotide formed in the incubation. The conditions of assay were similar to the paper chromatographic method, except that excess prostatic acid phosphatase was added to the incubation mixture so that open-chain nucleotides, produced by the enzymic hydrolysis of cyclic nucleotides, would be further hydrolysed to nucleosides releasing inorganic phosphate which could be quantitatively measured. The method, although promising, was not developed, as it was found difficult to obtain a prostatic phosphatase preparation completely free of diesterase activity, and also because the method could not be used outside the pH range 4.5–6.5, due to the phosphatase activity becoming the limiting factor in the reaction.

The present experiments were carried out on mitochondrial fractions (which are rich in both RNases) isolated in 0.25 *M* sucrose by the procedure of REID[8] based on

that of SCHNEIDER[9]. The mitochondrial pellets so obtained were resuspended in 0.25 *M* sucrose so that 2 ml of the suspension corresponded to 1 g-equivalent of liver. The fraction was diluted with an equal vol. water and freeze-thawed 8 times to release the bound enzymic activity[2]. To 0.1 ml of a 0.1 *M* acetate buffer (pH 6.0) were added 0.1 ml of the diluted mitochondrial fraction, and 0.1 ml of a solution of 15 mg/ml of adenosine (or cytidine) 2',3'-cyclic phosphate (Schwarz Laboratories, Mount Vernon, N.Y.). As the hydrolysis of adenosine 2',3'-cyclic phosphate proceeds at about 5 times the rate of the hydrolysis of cytidine 2',3'-cyclic phosphate, the mixture was incubated at 37° for 45 min in the case of adenosine 2',3'-cyclic phosphate, and 120 min in the case of cytidine 2',3'-cyclic phosphate. Controls were incubated for the same period, the tissue extract being added immediately before terminating the incubation. The reaction was stopped with 0.6 ml of ethanol–ether–chloroform (2:2:1, v/v/v). (In early experiments in which 8% trichloroacetic acid (w/v) or 10% $HClO_4$ (w/v) were used as protein precipitants, much non-specific breakdown of the cyclic mononucleotide substrates occurred.)

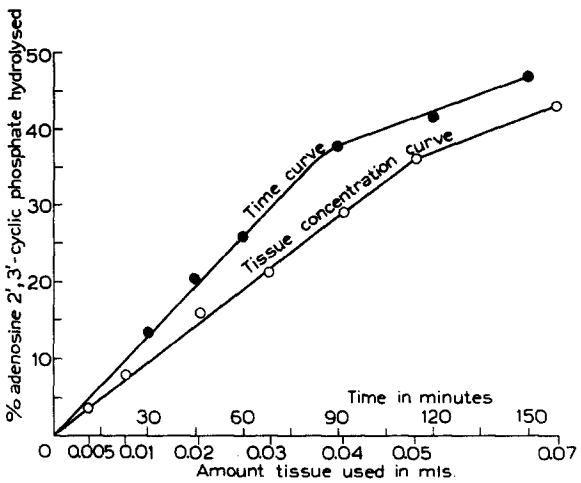

Fig. 1. Enzymic hydrolysis of adenosine 2',3'-cyclic phosphate in relation to time ●, and in relation to enzyme concentration ○. The aim was to demonstrate linearity in each case; the curves are not expected to coincide. Conditions of assay as in text.

After shaking, the tubes were heated in a water bath for about 10 min at 85° whereby the volume was reduced to about 0.4 ml, and were cooled and centrifuged. A portion of each supernatant and of a solution of appropriate markers, were applied at 4-cm intervals to a sheet (30 cm long) of Whatman No. 1 paper to give spots of about 2 cm diameter. Descending chromatography was carried out for 16 h with the satd. $(NH_4)_2SO_4$–isopropanol–1 *M* sodium acetate (80:2:20, v/v/v) solvent of MARKHAM AND SMITH[10], allowing the solvent front to run off the serrated edge of the paper. The chromatograms were dried at room temperature, and spots were localised under u.v. light. This solvent system will separate adenosine 2',3'-cyclic phosphate, adenosine 3'-phosphate and adenosine 2'-phosphate in that order, and will similarly separate guanosine or cytidine nucleotides. With uridine nucleotides, however, the 2' and 3' isomers are not separable from each other.

Corresponding spots were cut from the paper in the form of squares of identical size from both experimental and control runs. Blank squares were cut at the same level as the experimental spots. All squares were placed in test tubes with 5 ml of distilled water and allowed to stand for several hours at room temperature. The extracts were read in a Unicam spectrophotometer near the λ_{max} (260 mμ for adenosine compounds, 270 mμ for cytidine compounds), against a blank of distilled water. Paper blank values were subtracted from their respective experimental spots before comparison of the optical densities. As the amount of material initially applied in each spot was not kept constant, the net readings of spots from each experimental run were summated so that the percentage of cyclic substrate hydrolysed could be calculated. For example, if an experimental run furnished adenosine 2',3'-cyclic phosphate and adenosine 2'-phosphate with net readings of 0.60 and 0.20 respectively, evidently 25 % of the cyclic substrate had been attacked. Controls usually showed little non-specific breakdown of the cyclic nucleotides, but where this did occur then the corresponding experimental readings were adjusted accordingly. The reaction is linear up to about 35 % degradation of the cyclic substrates under these assay conditions (Fig. 1), and there is good agreement between duplicates.

With some tissue preparations there occurred further degradation of the open-chain nucleotide to nucleoside, which ran very close to the 3' isomer, and which could, in routine assays, be treated as equivalent to nucleotide, as the molar absorbancy is the same for both. However, in the study of specificities[6], two-dimensional chromatography was employed using a second solvent, *n*-butanol–water (860:140, v/v[11]), to distinguish such nucleosides.

A similar assay procedure has been used in studies of crystalline pancreatic RNase[12], with electrophoresis in place of chromatography, but the procedure would probably require modification if used for crude tissue preparations.

Thanks are expressed to Dr. E. REID for help and advice during the course of these studies. The work was supported by grants to this Institute from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Institutes of Health, U.S. Public Health Service.

The Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London (Great Britain) J. T. NODES

[1] W. C. SCHNEIDER AND G. H. HOGEBOOM, *J. Biol. Chem.*, 198 (1952) 155.
[2] C. DE DUVE, B. C. PRESSMAN, R. GIANETTO, R. WATTIAUX AND F. APPELMANS, *Biochem. J.*, 60 (1955) 604.
[3] F. M. RICHARDS, *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, 29 (1955) 315.
[4] G. DE LAMIRANDE, C. ALLARD, H. C. DA COSTA AND A. CANTERO, *Science*, 119 (1954) 351.
[5] J. S. ROTH, *J. Biol. Chem.*, 208 (1954) 181.
[6] J. T. NODES AND E. REID, *Biochem. J.*, 69 (1958) 52P.
[7] J. ZYTKO, G. DE LAMIRANDE, C. ALLARD AND A. CANTERO, *Biochim. Biophys. Acta*, 27 (1958) 495.
[8] E. REID, *Nature*, 175 (1955) 461.
[9] W. C. SCHNEIDER, *J. Biol. Chem.*, 176 (1948) 259.
[10] R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 52 (1952) 558.
[11] R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 45 (1949) 294.
[12] F. F. DAVIS AND F. W. ALLEN, *J. Biol. Chem.*, 217 (1955) 13.

Received September 1st, 1958