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Note

Preparative purification and group separation of mono- and dinucleotides by combining charge-transfer and affinity chromatography

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The study of the binding sub-sites of the well known enzyme bovine pancreatic ribonuclease (RNAase, E.C. 3.1.27.5) has been attempted in several ways (X-ray diffraction, NMR spectroscopy, affinity labelling)[1–8]. All of these techniques require non-substrate ligands and, although most studies have been carried out with mononucleotides, it is obvious that a dinucleotide can provide a better picture of the interaction between the enzyme sub-sites and a high-molecular-weight substrate. It is for this reason that the chromatographic separation on a preparative scale of 2′-O-methylated dinucleotides which are alkali-stable was undertaken. The interaction of these compounds with the enzyme should give a closer idea of the enzyme–substrate complex than that obtained with either the phosphonate analogues of the dinucleoside monophosphates in which the critical oxygen of the 5′-phosphoester bond is replaced by a CH_2 group[1] or a 2′,5′-dinucleotide[2] with distorted geometry in the phosphodiester bond.

On the other hand, alkali-stable dinucleotides are found in a low proportion (about 3%) in RNA. The separation of the sixteen different dinucleotides has only been accomplished on a small scale by means of very complicated and tedious procedures[9,10]. In this work partial purification on the milligram scale was achieved by means of a combination of charge-transfer chromatography on acriflavin-Sephadex G-25 and affinity chromatography on Sepharose 4B-RNAase.

The existence and characteristics of charge-transfer complexes between biomolecules has been known for over 10 years[11,12]. The pioneering work of Porath and his collaborators laid the theoretical basis[13,14] and showed the applicability of this type of interaction to the purification of many substances[15,16]. The best characterized gel of the electron-acceptor type is acriflavin-Sephadex G-25. Its behaviour and response to a set of different conditions (temperature, ionic strengh, pH, buffer composition and presence of different ions) has been thoroughly studied[16,17]. These are some of the reasons that justified the choice of this gel for the partial resolution of complex dinucleotide mixtures. The charge-transfer chromatography on acriflavin-Sephadex G-25 was also used for the final purification of some brominated nucleotides from the unreacted starting materials. Halogenated nucleotides are interesting compounds for the affinity labelling of RNAase[7] and other enzymes[18].

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EXPERIMENTAL

Acriflavin-Sephadex G-25 was synthesized as described by Egly and Porath[16]. Commercial bovine pancreatic ribonuclease (crystallized twice) was obtained from Cambrian Chemicals (Croydon, Great Britain) and was immobilized on Sepharose-4B according to the method of Axén *et al.*[19]. 8-bromoadenosine 5′-monophosphate (5′-Br^{8}AMP) and 8-bromoadenosine 2′,3′ cyclic monophosphate (2′,3′-Br^{8}AMP) were synthesized from 5′-AMP (Merck, Darmstadt, G.F.R.) and 2′,3′-AMP (Cambrian Chemicals) respectively, by direct bromination according to the method of Ikehara *et al.*[20]. After the reaction, the unreacted bromine was extracted with carbon tetrachloride and the nucleotides were freeze-dried. They were stored in a desiccator at −20°C.

The fraction corresponding to the sixteen 2′-O-methylated alkali-stable dinucleotides was obtained by the method of Trim and Parker[10] from wheat germ ribosomal RNA prepared as described by Glitz and Decker[21]. The dinucleotide fraction was stored as a freeze-dried powder at −20°C.

Some of the dinucleotide peaks were identified by means of thin-layer chromatography (TLC) and UV absorbance ratios[10].

RESULTS AND DISCUSSION

Separation of halogenated from non-halogenated mononucleotides

A 3-ml volume of an equimolar mixture of 5′-AMP and 5′-Br^{8}AMP (3 mg/ml each) in 0.2 *M* ammonium acetate–acetic acid buffer (pH 6.0) were loaded on to a column (46 × 2.6 cm I.D.) of acriflavin-Sephadex G-25 at a flow-rate of 40 ml/h. Elution was carried out at 4°C with the same buffer and the corresponding V_e/V_t values are given in Table I. The temperature (4°C) and the pH (6.0) were chosen so as to obtain maximal retardation and resolution among the different nucleotides as reported by Egly and Porath[16]. In the preparative separation of the brominated nucleotides from the unreacted starting materials (5′-AMP and 2′,3′-AMP) the same chromatographic conditions were used except that the sample volume was 1.5 ml at a concentration of 150 mg/ml. The V_e/V_t values are also listed in Table I.

The fact that the cyclic nucleotides are more retarded than the non-cyclic nucleotides is in agreement with published results on the analytical separation of 5′-AMP and 3′,5′-AMP[17]. Nevertheless, the high retardation of the brominated nucleotides with respect to the non-brominated ones would, in principle, be in disagreement

TABLE I

REDUCED ELUTION VOLUMES OF SOME MONONUCLEOTIDES ON ACRIFLAVIN-SEPHADEXG-25

Nucleotide	V_e/V_t*
5′-AMP	3.0
5′-Br^{8}AMP	7.5
2′,3′-AMP	5.4
2′,3′-Br^{8}AMP	12.6

* V_e is the elution volume, V_t the bed volume and V_e/V_t the reduced elution volume. The values were obtained in 0.2 *M* ammonium acetate buffer (pH 6.0).

with the behaviour of acriflavin-Sephadex G-25 as an acceptor gel[16,17]. The bromine, an electron-withdrawing substituent, should diminish the interaction of the halogenated nucleotide with the gel compared with the non-halogenated nucleotide. The results are, however, in agreement with the strong retardation of iodinated compared with non-iodinated tyrosines in dinitrophenyl-Sephadex G-25 (another π-electron acceptor gel)[13]. This effect was explained by the participation of the halogen itself in an electron-transfer interaction with the gel[13]. This could also be the case in the present experiments. An alternative explanation for the unexpected result could be the fact that acriflavin is situated in an intermediate position in the scale of donor and acceptor molecules[14]. Acriflavin could then act as an electron acceptor with 5'-AMP and as an electron donor with 5'-Br^{8}AMP, although other explanations are also possible.

If this behaviour of the halogenated nucleotides in acriflavin-Sephadex G-25 columns were general it would provide a powerful tool for separating halogenated from non-halogenated nucleotides in complex mixtures. This procedure has the advantage of being quick and suitable for scaling-up.

Fractionation of complex alkali-stable dinucleotide mixtures

The chromatographic fractionation of the 2'-O-methylated alkali-stable dinucleotides was attempted by combining charge-transfer chromatography on acriflavin-Sephadex G-25 and affinity chromatography on Sepharose 4B-RNAase. A mixture of the sixteen dinucleotides (3 ml at a total concentration of 7 mg/ml) in 0.2 *M* am-

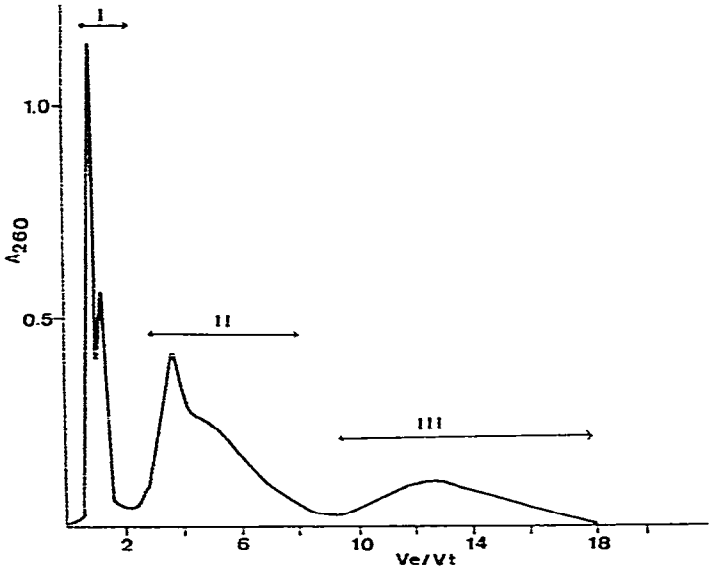

Fig. 1. Chromatography of a mixture of alkali-stable dinucleotides on acriflavin-Sephadex G-25. A 3-ml volume of 2'-O-methylated alkali-stable dinucleotides (7 mg/ml) was loaded on an acriflavin-Sephadex G-25 column (46 × 2.6 cm I.D.) in 0.2 *M* ammonium acetate buffer (pH 6.0). Elution was carried out with the same buffer at a flow-rate of 40 ml/h. V_e is the elution volume, V_t is the total volume and V_e/V_t is the reduced elution volume. Peak I represents dinucleotides of the PyrpPyrp type, peak II contains PyrpPurp and PurpPyrp dinucleotides and peak III dinucleotides of the PurpPurp type.

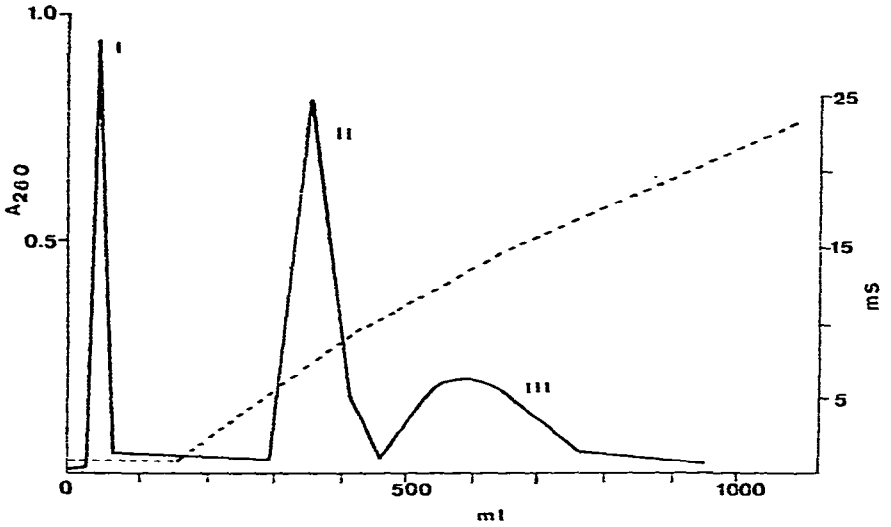

Fig. 2. Chromatography of a mixture of alkali-stable dinucleotides on Sepharose 4B-RNAase. A 1-ml volume of 2'-O-methylated alkali-stable dinucleotides (10 mg/ml) in 10 m*M* ammonium acetate buffer (pH 7.0) was loaded on a Sepharose 4B-RNAase column (35 × 1.2 cm I.D.). The flow-rate was 30 ml/h and the temperature was 4°C. Elution was carried out with a linear ionic strength gradient from 10 m*M* to 0.3 *M* (400 ml in each vessel) of ammonium acetate buffer (pH 7.0). Peak I represents PurpPurp dinucleotides, peak II a mixture of PyrpPyrp and PurpPyrp dinucleotides and peak III dinucleotides of the PyrpPurp type.

monium acetate buffer (pH 6.0) was loaded on to a column (46 × 2.6 cm I.D.) of acriflavin-Sephadex G-25. The flow-rate was 40 ml/h and the elution was carried out with the same buffer at 4°C. Three well separated fractions were obtained and identified as shown in Fig. 1. The order of elution was in agreement with the published data for some natural dinucleoside monophosphates[16].

The elution profile of a chromatogram of a similar sample of total dinucleotides applied to a Sepharose 4B-RNAase column is shown in Fig. 2. Fraction I, not retained in the conditions of equilibration of the column, was shown to be constituted by dinucleotides of the Pur–Pur type whereas the other two, eluted by means of an ionic strength gradient, followed the order expected from the known affinities for the binding sub-sites of the enzyme[1]. It should be noted that affinity chromatography has been widely used to purify proteins by immobilizing a suitable low-molecular-weight ligand[22]. In the case of nucleic acid fragments or analogues, purification has been mainly attempted by immobilizing not proteins but complementary strands of nucleic acids[22]. In this work a different approach was used, namely immobilization of the enzyme for the fractionation of a complex mixture of low-molecular-weight ligands based on the different affinity of the individual ligands towards the enzyme.

In order to fractionate further peak II of the Sepharose 4B-RNAase column it was chromatographed, after freeze-drying, through a long acriflavin-Sephadex G-25 column (110 × 1.2 cm I.D.). The experimental conditions and results are shown in Fig. 3. Experiments are in progress to fractionate further the unresolved dinucleotides by means of other chromatographic procedures.

These examples show the great potential of the combination of charge-transfer

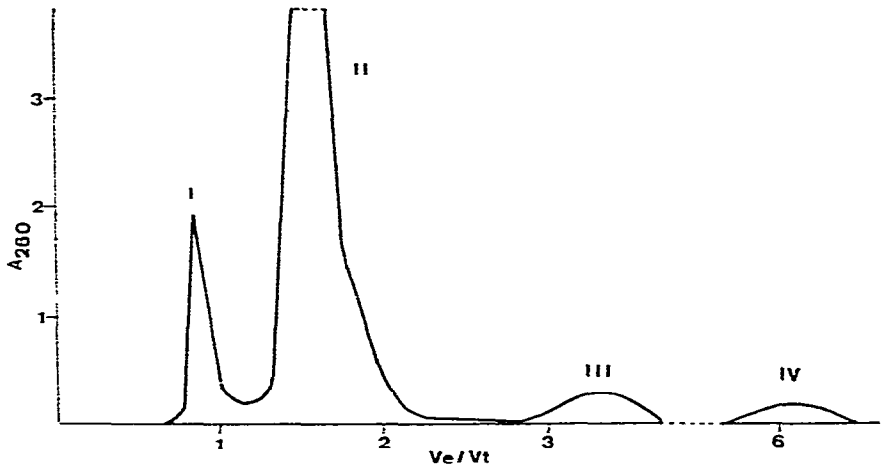

Fig. 3. Chromatography of a partially purified mixture of alkali-stable dinucleotides on acriflavin-Sephadex G-25. A 0.3-ml volume of peak II from the chromatogram in Fig. 2 at a concentration of 11 mg/ml in 0.2 *M* ammonium acetate buffer (pH 6.0) was chromatographed on an acriflavin-Sephadex G-25 column (110 × 1.2 cm I.D.) at a flow-rate of 15 ml/h at 4°C. The assignment of the peaks was as follows: I, CmpCp (V_e/V_t = 1); II, CmpUp, UmpCp, UmpUp (V_e/V_t = 1.5); III, AmpCp, GmpCp (V_e/V_t = 3.1); IV, AmpUp, GmpUp (V_e/V_t = 6). m in the above abbreviations represents a methyl group in the 2′-position of the ribose ring of the corresponding nucleoside.

and affinity chromatography for the preparative purification of complex mixtures of mono- and dinucleotides. The method is rapid and simple and avoids the tedious procedures that use ion-exchange chromatography with[23] or without[9,10] urea. Obviously, the step that uses the immobilized enzyme can be used only when the nucleotides are not substrates for the enzyme such as, in the case of RNAase, the 2′-O-methylated di- and oligonucleotides, 2′,5′-oligonucleotides or 2′-deoxyribonucleotides (single-stranded DNA).

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REFERENCES

1 F. M. Richards and H. W. Wyckoff, in P. D. Boyer (Editor), *The Enzymes*, Vol. IV, Academic Press, New York, 1971, p. 647.
2 S. Y. Wodak, M. Y. Liu and H. W. Wyckoff, *J. Mol. Biol.*, 116 (1977) 855.
3 Y. Mitsui, Y. Urata, K. Torii and M. Irie, *Biochim. Biophys. Acta*, 535 (1978) 299.
4 A. G. Pavlovsky, S. N. Borisova, V. V. Borisov, I. V. Antonov and M. Ya. Karpeisky, *FEBS Lett.*, 92 (1978) 258.
5 J. H. Griffin, A. N. Schechter and J. S. Cohen, *Ann. N.Y. Acad. Sci.*, 222 (1973) 693.
6 I. V. Antonov, A. Z. Gurevich, S. M. Dudkin, M. Ya. Karpeisky, V. G. Sakharovsky and G. I. Yakolev, *Eur. J. Biochem.*, 87 (1978) 45.
7 X. Parés, R. Llorens, C. Arús and C. M. Cuchillo, *Eur. J. Biochem.*, 105 (1980) 571.

8 C. Arús, L. Paolillo, R. Llorens, R. Napolitano, X. Parés and C. M. Cuchillo, *Biochim. Biophys. Acta*, 660 (1981) 117.
9 H. Singh and B. G. Lane, *Can. J. Biochem.*, 42 (1964) 1011.
10 A. R. Trim and J. E. Parker, *Biochem. J.*, 116 (1970) 589.
11 M. A. Slifkin, *Charge Transfer Interactions of Biomolecules*, Academic Press, London, 1971.
12 R. S. Mulliken and W. B. Person, *Molecular Complexes —A Lecture and Reprint Volume*, Wiley-Interscience, New York, 1969.
13 J. Porath and K. D. Caldwell, *J. Chromatogr.*, 133 (1977) 180.
14 J. Porath, *J. Chromatogr.*, 159 (1978) 13.
15 J. Porath and B. Larsson, *J. Chromatogr.*, 155 (1978) 47.
16 J.-M. Egly and J. Porath, *J. Chromatogr.*, 168 (1979) 35.
17 C. Rochette-Egly and J.-M. Egly, *J. Cyclic Nucleotide Res.*, 6 (1980) 335.
18 L. W. Brox and A. Hampton, *Biochemistry*, 7 (1968) 398.
19 R. Axén, J. Porath and S. Ernback, *Nature (London)*, 214 1967) 1302.
20 M. Ikehara, T. Tazawa and T. Fukui, *Chem. Pharm. Bull.*, 17 (1969) 1019.
21 D. C. Glitz and C. A. Decker, *Biochemistry*, 2 (1963) 1185.
22 J. Turková, *Affinity Chromatography* (*Journal of Chromatography Library*, Vol. 12), Elsevier, Amsterdam, 1978, p. 301.
23 R. V. Tomlison and G. M. Tener, *Biochemistry*, 2 (1963) 697.