Spectrophotometric assay, solubilization and purification of brain 2':3'-cyclic nucleotide 3'-phosphodiesterase

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1. A spectrophotometric assay of 2':3'-cyclic nucleotide 3'-phosphodiesterase (EC 3.1.4.37) based on the use of an acid–base indicator and a buffer having identical pK_a values is described. The assay is simple and rapid; it was particularly convenient for monitoring the enzyme activity at various stages of purification. 2. Several proteinases were examined for their ability to solubilize 2':3'-cyclic nucleotide 3'-phosphodiesterase from delipidated brain white matter. Trypsin (EC 3.4.21.4) and elastase (EC 3.4.21.11) appeared to be more effective than the other proteinases examined. Trypsin, however, caused inactivation; elastase was therefore chosen to solubilize 2':3'-cyclic nucleotide 3'-phosphodiesterase. When a partially purified preparation of 2':3'-cyclic nucleotide 3'-phosphodiesterase was treated with elastase, 2':3'-cyclic nucleotide 3'-phosphodiesterase was solubilized nearly quantitatively. Elastatin, a specific inhibitor of elastase, specifically inhibited the solubilization with elastase. 3. 2':3'-Cyclic nucleotide 3'-phosphodiesterase was purified from bovine brain white matter by: (i) delipidation; (ii) solubilization with hexadecyltrimethylammonium bromide; (iii) gel chromatography on Sepharose; (iv) ethanol precipitation and resolubilization by digestion with elastase; (v) chromatography on DEAE-Sephadex; (vi) affinity chromatography on 8-(6-aminohexyl)amino-2'-AMP–Sepharose. 4. The purified enzyme migrated as a single protein band on polyacrylamide-gel electrophoresis at pH 4.3 and on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis; the estimated mol.wt. in the latter electrophoresis was 27000–31000. Gel filtration of the purified enzyme through Sephadex G-150 indicated a mol.wt. of 31000. Therefore the purified enzyme is a monomer protein with a mol.wt. of approx. 30000.

2':3'-Cyclic nucleotide 3'-phosphodiesterase (EC 3.1.4.37) has become to be noticed since its relation to myelin was first suggested about 10 years ago (Kurihara & Tsukada, 1967, 1968; Kurihara et al., 1969, 1970, 1971). Although the enzyme has been used as a marker of myelination in central nervous tissues, its physiological function remains unknown. The enzyme is firmly bound to the membrane structures of brain white matter and has hitherto resisted purification; little is therefore known on the chemical properties of the enzyme. We found that the enzyme was solubilized effectively with hexadecyltrimethylammonium bromide and was partially purified in the presence of the detergent. Although the enzyme was precipitated as an insoluble aggregate on removal of the detergent, digestion with elastase (EC 3.4.21.11) resolubilized this aggregate completely. Subsequent purification was greatly facilitated by the solubilization with elastase, and 2':3'-cyclic nucleotide 3'-phosphodiesterase was isolated as a homogeneous protein after further two-step purification. The present paper describes the solubilization and purification of 2':3'-cyclic nucleotide 3'-phosphodiesterase from bovine brain white matter and the molecular properties of the purified enzyme. A spectrophotometric assay of 2':3'-cyclic nucleotide 3'-phosphodiesterase that has been routinely used for the solubilization and purification of the enzyme is also described.

Materials and methods

Materials
2'(3')-AMP (mixed isomers; free acid), 2'-AMP

Abbreviation used: SDS, sodium dodecyl sulphate.

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(free acid), serum albumin (bovine; fraction V), serum albumin (bovine; crystallized and freeze-dried), egg albumin (chicken; crystallized and freeze-dried), cytochrome c (horse heart; type VI), elastase (pig pancreas; twice-crystallized), papain (EC 3.4.22.2; twice-crystallized) and elastin–Congo Red were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Trypsin (EC 3.4.21.4; bovine pancreas; twice-crystallized, dialysed and freeze-dried) and α-chymotrypsin (EC 3.4.21.1; bovine pancreas; three-times-crystallized, dialysed and freeze-dried) were purchased from Worthington Biochemical Corp., Freehold NJ, U.S.A. γ-Globulin (human; fraction II) was a product from Nutritional Biochemicals Corp., Cleveland, OH, U.S.A. Hexacycltrimethylammonium bromide and Bromothymol Blue were obtained from Wako Pure Chemicals Co., Osaka, Japan, and imidazole was from Daiichi Pure Chemicals Co., Tokyo, Japan. Dicyclohexylcarbodi-imide, elastinal and leupeptin were purchased from Protein Research Foundation, Minoh, Osaka, Japan. Sepharose CL-4B, DEAE-Sephadex A-50, Sephadex G-25 and Sephadex G-150 were obtained from Pharmacia Japan, Tokyo, Japan.

2′:3′-Cyclic AMP. The spectrophotometric assay of 2′:3′-cyclic nucleotide 3′-phosphodiesterase required a large quantity of 2′:3′-cyclic AMP as the substrate. A standard procedure now available for the preparation of 2′:3′-cyclic AMP (Smith et al., 1958) involves a precipitation step with BaI₂; when the scale was increased, the product sometimes contained iodide ion, which interfered with the assay. The procedure described below involves no precipitation step and was convenient for the large-scale preparation of halide-ion-free 2′:3′-cyclic AMP. Acid form of 2′(3′)-AMP (20 mmol; 7 g) was dissolved in water (40 ml) containing triethylamine (3 ml), and methanol (300 ml) was added. The mixture was filtered and refluxed with dicyclohexylcarbodi-imide (21 g) for 2 h. The solvent was removed by evaporation at 45°C under reduced pressure and the residue was dissolved in water (200 ml). The solution was then extracted three times with diethyl ether (200 ml); the insoluble dicyclohexylurea was removed each time by filtration. The aqueous phase was concentrated by evaporation under reduced pressure; after the diethyl ether dissolved in the aqueous phase had been removed, the temperature was raised to 55°C. The concentrated solution was then passed through a column (4 cm × 45 cm) of Dowex 50W X8 (50–100 mesh; K⁺ form), and the effluent was used as potassium 2′:3′-cyclic AMP. The purity of the product was checked by paper chromatography in saturated (NH₄)₂SO₄/0.5 M-sodium acetate/propan-2-ol (40:9:1, by vol.) and in propan-2-ol/aq. NH₃ (sp.gr. 0.880)/water (7:1:2, by vol.). The concentration of 2′:3′-cyclic AMP was determined as described previously (Kurihara & Takahashi, 1973); the yield was about 17 mmol.

Proteinases. Trypsin and α-chymotrypsin were dissolved in 1 M-HCl containing 10 mM-CaCl₂ at 1 mg/ml; the solutions were stored at 4°C and diluted with the above medium before use. Elastase was dissolved in water and passed through a DEAE-Sephadex A-50 column, as described by Sampath Narayanan & Anwar (1969). Fraction A was concentrated by freeze-drying, desalted on a Sephadex G-25 column and concentrated again by freeze-drying to approx. 2 mg of protein/ml. The preparation contained 18 units of elastase/mg of protein; 1 unit of elastase is defined as the amount that solubilizes 1 mg of elastin–Congo Red in 20 min at 37°C (Naughton & Sanger, 1961). The preparation was stored at −80°C; its ability to solubilize 2′:3′-cyclic nucleotide 3′-phosphodiesterase remained unchanged during the storage at least for several months. Papain was dissolved in 10 mM-cysteine before use.

8-Bromo-2′-AMP. 8-Bromo-2′-AMP was prepared on a large scale by a modified procedure of Ikehara & Uesugi (1969). 2′(3′)-AMP (10 g) was dissolved in 2 M-NaOH (30 ml), and acetic acid (29 ml) was added; pH was adjusted to 4.0 with 2 M-NaOH. The mixture was filtered and water was added to make the total volume 100 ml. Br₂ (2 ml) was then added, and the mixture was shaken again vigorously in a stoppered flask until most of the Br₂ was dissolved. The mixture was kept overnight at room temperature. The precipitate formed was collected by centrifugation, washed four times with acetic acid/acetone (1:1, v/v) and three times with acetone, and dried in a desiccator over silica gel. The product (3.4 g) was pure 8-bromo-2′-AMP as judged by its mobility in paper chromatography (Ikehara & Uesugi, 1969); saturated (NH₄)₂SO₄/0.5 M-sodium acetate/propan-2-ol (40:9:1, by vol.) was also used as solvent in an ascending system to distinguish 8-bromo-2′-AMP from 8-bromo-3′-AMP.

8-(6-Aminohexyl)amino-2′-AMP and its immobilization on Sepharose CL-4B. 8-Bromo-2′-AMP (3.4 g) was refluxed in 96% (v/v) ethanol (700 ml) with 1.6-diaminohexane (18 g) as described by Lee et al. (1974). After the ethanol had been removed by evaporation, the residue was dissolved in water (200 ml), and pH of the solution was adjusted to 11.5 with 5 M-NaOH. The solution was then applied to a column (4 cm × 60 cm) of Dowex 1 X8 (100–200 mesh; formate form). The column was washed with water, and 8-(6-aminohexyl)amino-2′-AMP was eluted with 0.01 M-formic acid, pH 4.0 (pH adjusted with aq. NH₃). The major fraction showing an absorbance maximum at 280 nm at pH 7 and 12 was concentrated by evaporation and freeze-dried. The product (3.0 g) gave a single u.v.-absorbing spot by paper chromatography (ascending) in butan-1-ol/
acetic acid/water (5:2:3, by vol.) and in saturated (NH₄)₂SO₄/0.5 M-sodium acetate/propan-2-ol (40:9:1, by vol.). The presence of free amino groups was detected on the spot by the procedure of Heilmann et al. (1957). 8-(6-Aminoheptyl)amino-2'-AMP was immobilized on Sepharose CL-4B by the method of Axén et al. (1967). The amount of 8-(6-aminoheptyl)amino-2'-AMP immobilized, as determined by the phosphate procedure of Mosbach (1974), was 65–78 μmol/g of dry Sepharose CL-4B.

**Spectrophotometric assay**

The following reagents were prepared for the assay: reagent A, 4 mM-Bromothymol Blue; 100 mg of Bromothymol Blue was dissolved in 40 ml of 10 mM-NaOH: reagent B, 1% (w/v) hexadecyltrimethylammonium bromide; reagent C, 0.2 M-imidazole/HCl buffer pH 6.5 (pH determined at 10 mM and at 30°C); reagent D, 50 mM-2-mercaptoethanol; reagent E, 30 mM-potassium 2':3'-cyclic AMP. The reaction mixture finally adopted for the 420 nm procedure contained 0.12 mM-Bromothymol Blue, 0.2% hexadecyltrimethylammonium bromide, 10 mM-imidazole/HCl buffer (pH 6.5 at 30°C), 0.5 mM-2-mercaptoethanol and 7.5 mM-potassium 2':3'-cyclic AMP. The final pH of the mixture was 6.6 at 30°C. This mixture was prepared by mixing 3 ml of reagent A, 20 ml of reagent B, 5 ml of reagent C, 1 ml of reagent D and 25 ml of reagent E and by making the total volume 100 ml with water. The reaction mixture finally adopted for the 630 nm procedure contained 0.08 mM-Bromothymol Blue and 15 mM-imidazole/HCl buffer (pH 6.5 at 30°C); the other constituents were the same as those for the 420 nm procedure. This mixture was prepared by mixing 2 ml of reagent A, 20 ml of reagent B, 7.5 ml of reagent C, 1 ml of reagent D and 25 ml of reagent E and by making the total volume 100 ml with water.

Both sample and reference cells in a Hitachi-323 recording spectrophotometer contained 2 ml of either of the above mixtures. The mixture had been equilibrated at 30°C and was maintained at 30°C in the spectrophotometer by a thermostatically controlled cell-holder. The wavelength was set at 420 nm and the reaction was started by adding an enzyme solution to the mixture in the sample cell. Alternatively, the wavelength was set at 630 nm and the reaction was started by adding an enzyme solution to the mixture in the reference cell. A plastic rod with a flat end was used for immediate mixing. A_{420} or A_{630} was then automatically recorded with appropriate scale expansion: a scale of 0–0.2 A or 0–0.4 A was used. Recording for 1–2 min after the start of the reaction was usually sufficient. The calibrations are given in the Results and discussion section. The 420 nm procedure was routinely used: an A_{420} change of 1.00 was assumed to correspond to 11.1 μmol. One unit of 2':3'-cyclic nucleotide 3'-phosphodiesterase is defined as the amount hydrolysing 1 μmol of 2':3'-cyclic AMP/min.

**Solubilization by proteolytic digestion**

Trypsin, chymotrypsin, elastase and papain were examined for their ability to solubilize 2':3'-cyclic nucleotide 3'-phosphodiesterase as follows. The delipidated brain white matter (60 mg; Kurihara et al., 1977) was homogenized at 0°C in water (10 ml) with a Potter–Elvehjem-type Teflon homogenizer. To 200 μl of the homogenate, 100 μl of 0.2 M-Tris/HCl buffer, pH 7.5 (pH 8.8 for elastase), and 5–100 μl of a proteinase solution were added. The total volume was adjusted to 400 μl with water. The mixture was incubated at 23°C with occasional stirring for 2.5–21 h. 2':3'-Cyclic nucleotide 3'-phosphodiesterase activity in the mixture and in the supernatant fluid was then assayed. The supernatant fluid was obtained by centrifugation of the mixture at 75 000 g for 40 min.

Incubation conditions for the elastase digestion were further studied by changing the concentration of Tris/HCl buffer and that of the delipidated white matter. The delipidated white matter (150 mg) was homogenized at 0°C in 10 ml of 15 mM-Tris/HCl buffer, pH 8.8 at 23°C, with a Potter–Elvehjem-type Teflon homogenizer. To 200 μl of the homogenate 5–100 μl of an elastase solution was added, and the total volume was adjusted to 300 μl with water. The mixture was incubated for 2–5 h. The supernatant fluid after centrifugation at 100 000 g for 30 min at 4°C was used.

**Purification**

Corpus callosum and centrum semiovale were dissected from bovine brain, and attached grey matter was removed completely. Pure white matter was then delipidated as described previously (Kurihara et al., 1977). Chloroform, methanol and diethyl ether, all of analytical grade, were used without prior redistillation. The procedure was modified to meet the requirements of large-scale preparation, and after the delipidation an insoluble fraction was used. Freeze-dried pure white matter (50 g in 5 g portions) was homogenized at 0°C with 3.5 litres (0.35-litre portions) of chloroform/methanol (2:1, v/v) in a Waring Blendor. The blender was rotated for a total
of 3 min with two intervals of 1 min. The residue was collected at 0°C by centrifugation at 7500 g for 20 min and then washed with 2.1 litres (0.35-litre portions) of diethyl ether by homogenization and centrifugation as above. The residue was suspended at 0°C in 1 litre of 20 mm-imidazole/HCl buffer, pH 6.7, with a Potter–Elvehjem-type Teflon homogenizer and the suspension was centrifuged at 7500 g for 20 min. The precipitate was suspended in 180 ml of water and freeze-dried. The yield was approx. 10 g.

2' :3'-Cyclic nucleotide 3'-phosphodiesterase was purified from the insoluble fraction of the delipidated residue as described below. The enzyme volume indicated in each step is that of a typical run presented in Table 1. The buffer pH indicated is the pH at 23°C; where necessary, the pH at the temperature used is also indicated.

The insoluble fraction of the delipidated residue (9 g) was homogenized at 0°C with 120 ml of 20 mm-imidazole/HCl buffer (pH 6.7) in a Potter–Elvehjem-type Teflon homogenizer. The homogenate was warmed to 30°C, and 30 ml of 10% (w/v) hexadecyltrimethylammonium bromide was added; the mixture was poured into centrifuge tubes with an additional 15 ml of 20 mm-imidazole/HCl buffer, pH 6.7. The mixture was incubated at 30°C for 20 min with vigorous shaking and centrifuged at 75 000 g for 90 min at 20°C. The supernatant fluid (150 ml) was chromatographed successively on two Sepharose CL-4B columns at 23°C. The first column was a large Sepharose CL-4B column (8 cm × 60 cm) equilibrated with 20 mm-imidazole/HCl buffer (pH 6.7) containing 0.5% hexadecyltrimethylammonium bromide. After application of the enzyme solution (150 ml) the column was developed at 80–100 ml/h with the above buffer/detergent solution, and the major enzyme peak (440 ml) near the front was collected. The second column was a Sepharose CL-4B column (4 cm × 90 cm) to which a 0–0.2% linear gradient of hexadecyltrimethylammonium bromide in 20 mm-imidazole/HCl buffer, pH 6.7 (400 ml), had been applied. The enzyme solution (440 ml) was applied, and the column was developed at 20 ml/h with 0.2% hexadecyltrimethylammonium bromide in 20 mm-imidazole/HCl buffer, pH 6.7. The enzyme peak (88 ml) eluted with the gradient of the detergent was collected; the preparation contained 0.3–0.4% hexadecyltrimethylammonium bromide. In the presence of hexadecyltrimethylammonium bromide, the enzyme was inactivated when stored by freezing. The enzyme, however, can be stored at 4°C; the enzyme solution was warmed to 30°C before use, if precipitates appeared or the solution became gel.

Hexadecyltrimethylammonium bromide was removed from the above preparation by ethanol precipitation as follows. The preparation (88 ml) was placed in ice, and an equal volume of ethanol chilled to −20°C was added slowly with stirring over 10 min. The mixture was stirred for a further 10 min and centrifuged at 13000 g for 10 min at 0°C. The precipitate was suspended in 90 ml of chilled ethanol with a Potter–Elvehjem-type Teflon homogenizer and centrifuged as above. The precipitate was washed again with 45 ml of chilled ethanol. The precipitate was finally suspended at 0°C in 10 mm-Tris/HCl buffer, pH 8.8 at 23°C, with a Potter–Elvehjem-type Teflon homogenizer. The precipitate was an insoluble aggregate; it was stored at −80°C. At this stage the enzyme can be stored by freezing. After thawing, the suspension (7.5 ml) was re-homogenized at 0°C with 22.5 ml of 10 mm-Tris/HCl buffer, pH 8.8, in a Potter–Elvehjem-type Teflon homogenizer and then solubilized by digestion with elastase as described above under ‘Solubilization by proteolytic digestion’.

After the solubilization with elastase all the purification was performed at 4°C. The elastase-solubilized preparation (30 ml) was applied to a DEAE-Sephadex A-50 column (3 cm × 21 cm) equilibrated with 20 mm-Tris/HCl buffer, pH 8.8, and the column was washed with the same buffer. The fraction not retained on the column (40 ml) was adjusted to pH 6.3 with 0.5 M-HCl and applied to a column (1.4 cm × 8.5 cm) of 8-(6-aminohexyl)amino-2'-AMP–Sepharose CL-4B equilibrated with 10 mm-imidazole/HCl buffer, pH 5.7 (pH 6.3 at 4°C) (Fig. 4). The column was washed with the same buffer and then with 10 mm-imidazole/HCl buffer, pH 5.7, containing 50 mm-NaCl. After the NaCl concentration in the buffer had been lowered to 40 mm, the enzyme was eluted with 10 mm-imidazole/HCl buffer, pH 5.7, containing both 40 mm-NaCl and 1 mm-sodium 2'-AMP. The fraction of a small shoulder above 200 ml of elution volume (Fig. 4) was not homogeneous on SDS/polyacrylamide-gel electrophoresis and hence was discarded. The purified enzyme was stored at 4°C. If necessary, the purified enzyme was passed through a Sephadex G-25 column in 10 mm-imidazole/HCl buffer, pH 5.7, to remove NaCl and 2'-AMP.

**Other analytical methods**

Potentiometric and colorimetric assays of 2':3'-cyclic nucleotide 3'-phosphodiesterase. These were done at pH 6.6 and at 30°C in the presence of 0.2% hexadecyltrimethylammonium bromide, as described previously (Kurihara & Takahashi, 1973).

**Determination of protein.** The method of Lowry et al. (1951) was used; bovine serum albumin (fraction V) was used as a standard. For samples containing hexadecyltrimethylammonium bromide, excess SDS was added. To reconcile the effect of imidazole, a corresponding concentration of imidazole/HCl buffer was added to the standard.

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Determination of hexadecyltrimethylammonium bromide. The erythrocyan method (Aoki & Iwayama, 1959) was simplified. The sample mixtures contained 2–10 µ.p.m. of hexadecyltrimethylammonium bromide and 0.02% erythrocyan in 40 mM-sodium citrate buffer, pH 5.0; the reference mixture contained 0.02% erythrocyan in 40 mM-sodium citrate buffer, pH 5.0. An A₄₅₀ of the sample mixtures was read against the reference mixture in a Hitachi-323 spectrophotometer.

Electrophoresis. Polyacrylamide-gel electrophoresis was performed at pH 4.3 and at pH 9.5 as described by Reisfeld et al. (1962) and by Davis (1964) respectively. SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli (1970) with 4.5% polyacrylamide as the stacking gel and 10% polyacrylamide as the separation gel. SDS/polyacrylamide-gel electrophoresis was also performed as described by Weber & Osborn (1969); before electrophoresis the samples were heated for 2 min at 100°C with 1% SDS in the presence of 1% 2-mercaptoethanol (Weber & Osborn, 1975). The gels were stained with Comassie Brilliant Blue (Weber & Osborn, 1969). For determination of molecular weight, γ-globulin [mol.wt. 23500 (L-chain) and 50000 (H-chain)] and serum albumin (crystallized) (mol.wt. 68000) were used as marker proteins.

Gel filtration. Gel filtration for determination of molecular weight was performed at 4°C with a Sephadex G-150 column (2.2 cm x 55 cm) in 20 mM-imidazole/HCl buffer, pH 6.7 at 23°C, containing 0.1 M NaCl. The other conditions were as described by Andrews (1964, 1965). Cytochrome c [mol.wts. 12400 (monomer) and 24800 (dimer)], egg albumin (mol.wt. 45000) and serum albumin (crystallized) (mol.wt. 68000) were used as marker proteins.

Results and discussion

Spectrophotometric assay

The present method is based on the use of an acid–base indicator and a buffer having identical pKₐ values. The amount of acid produced in the reaction is equivalent to the amount of basic buffer neutralized, which in turn is proportional to the amount of basic indicator neutralized. By reading at a wavelength at which only the basic form of the indicator absorbs light, the decrease in absorbance becomes a quantitative measure of the acid produced (Darrow & Colowick, 1962). Since imidazole/HCl buffer (pKₐ 6.9 at 30°C) has been used as an assay medium for 2':3'-cyclic nucleotide 3'-phosphodiesterase (Kurihara & Takahashi, 1973), a combination of Bromothymol Blue (pKₐ 7.0) and imidazole/HCl buffer was introduced. An enzyme solution was added to a mixture of Bromothymol Blue, hexadecyltrimethylammonium bromide, imidazole/HCl buffer, 2-mercaptoethanol and 2':3'-cyclic AMP; the rate of decrease in absorbance at 630 nm, or more conveniently the rate of increase in absorbance at 420 nm, was then measured. Hexadecyltrimethylammonium bromide was included in the mixture as an activator of 2':3'-cyclic nucleotide 3'-phosphodiesterase and also as a reagent to prevent precipitate formation between Bromothymol Blue and imidazole. The assay was performed at pH 6.6, where the amount of proton released was maximum (Kurihara & Takahashi, 1973).

The absorption spectrum of Bromothymol Blue was recorded at pH 6.2, 6.6 and 7.0 in the presence of hexadecyltrimethylammonium bromide, imidazole/HCl buffer and 2':3'-cyclic AMP; the absorption of the acidic form was maximum at 400–420 nm and that of the basic form was at 630 nm. The wavelength of 420 nm or 630 nm was therefore used for the assay at the following Bromothymol Blue concentrations: 0.12 mM (A₄₂₀ 1.35) for 420 nm and 0.08 mM (A₆₃₀ 1.61) for 630 nm. A white-matter homogenate was dispersed with hexadecyltrimethylammonium bromide (see the legend to Fig. 1a) and the resulting mixture was used as an enzyme solution. The enzyme solution was added to the reaction mixture containing various concentrations of imidazole/HCl buffer, and the absorbance change at 420 nm or 630 nm was recorded. For each concentration of the buffer, linearity between the rate of absorbance change and the enzyme concentration was checked. The following buffer concentrations were required for the assay: 10 mM for 420 nm and 15 mM for 630 nm.

Under the standard assay conditions, A₄₂₀ increased and A₆₃₀ decreased linearly with time until an absorbance change reached 0.25. Fig. 1(a) shows the relationship between the rate of absorbance change and the enzyme concentration; the linear relationship was observed when the rate of absorbance change was below 0.17/min. An increase in volume of the reaction mixture due to the enzyme solution added did not affect the activity value if it was no more than 100 µl. Fig. 1(b) shows the absorbance change of the reaction mixture when 2':3'-cyclic AMP was partially replaced by 2'(3')-AMP and equivalent amounts of HCl were added. An absorbance change of 1.00 corresponded to the hydrolysis of 10.3 µmol for 420 nm and of 7.1 µmol for 630 nm. From the parallel assay of the same enzyme solution by the colorimetric method (Kurihara & Takahashi, 1973), an absorbance change of 1.00 corresponded to the hydrolysis of 11.1 µmol for 420 nm and of 7.5 µmol for 630 nm. It should be noted that a smaller but definite absorbance change was found when 2':3'-cyclic AMP was partially replaced by 2'(3')-AMP without addition of HCl (Fig. 1c). Furthermore, the absorbance change
varied with the concentration of 2':3'-cyclic AMP (Fig. 1d); therefore, if the substrate concentration is varied, the absorbance change must be corrected. The Lineweaver–Burk plot after this correction showed a $K_m$ of 0.25 mM for the enzyme solution used. The potentiometric method (Kurihara & Takahashi, 1973) gave an identical $K_m$ value for the same enzyme solution.

The present method required a constant temperature during the assay, since a small rise in temperature could simulate acid formation (see Darrow & Colowick, 1962). The reaction mixture and the cell were equilibrated to 30°C before use, and the reaction was performed in a temperature-controlled cell-holder. The pH of imidazole/HCl buffer varied considerably with temperature and must be determined at 30°C. If care was taken on these points, the assay was easy and reproducible. The present method was particularly convenient for monitoring 2':3'-cyclic nucleotide 3'-phosphodiesterase activity.

\[ \text{Rate of absorbance change} = \frac{dA}{dt} \]

**Fig. 1. Spectrophotometric assay of 2':3'-cyclic nucleotide 3'-phosphodiesterase**

(a) Effect of enzyme concentration. The rate of absorbance change at 420 nm (O) and at 630 nm (●) was measured under the standard assay conditions. Bovine brain white matter was homogenized at 0°C with 9 vol. of 30 mM-imidazole/HCl buffer, pH 6.7, in a Potter–Elvehjem-type Teflon homogenizer. To 0.5 ml of the homogenate 4.5 ml of 1% (w/v) hexadecyltrimethylammonium bromide was added at 25°C. The resulting mixture was used as the enzyme solution. (b) and (c) Calibration by addition of HCl. 2':3'-Cyclic AMP in the reaction mixture was partially replaced by 2'(3')-AMP with (b) or without (c) addition of equivalent amounts of HCl and the absorbance change was determined at 30°C. 2'(3')-AMP (free acid) was neutralized with NaOH to pH 6.6 at 30°C. The dissociation of the secondary phosphate group of 2'-AMP was assumed to be 80% at pH 6.6 (Kurihara & Takahashi, 1973). O, 420 nm; ●, 630 nm. (d) Effect of substrate concentration on the calibration. At various concentrations of 2':3'-cyclic AMP, 0.5 μmol of 2';3'-cyclic AMP in the reaction mixture for the assay at 420 nm was replaced by 0.5 μmol of 2'(3')-AMP and equivalent amount of HCl (0.4 μmol) was added. The absorbance change at 420 nm was determined at 30°C. For full details see the text.
at various stages of its purification. Dilution of samples to an appropriate enzyme concentration was unnecessary. The reaction mixture can be stored in a stoppered flask at room temperature, and the enzyme activity for several samples was determined immediately. When the enzyme activity in tissue homogenates was determined, the homogenates were dispersed with hexadecltrimethylammonium bromide before assay for complete activation. Other enzymes and other proteins in central nervous tissues seemed not to affect the assay. Since only a small volume of enzyme solution was used for the assay, buffers or salts that enzyme samples contained usually did not affect the assay. For example, 10–20 μl of enzyme solutions containing 10–50 mm-Tris/HCl buffer, pH 8.8, was added to the reaction mixture when the enzyme was solubilized from delipidated brain white matter by digestion with elastase. A slight shift of absorbance at the start of the reaction did not interfere with the assay. Even if enzyme samples contained 2'-AMP, as in the affinity chromatography described below, the enzyme activity could be determined with appropriate correction of activity values.

**Solubilization by proteolytic digestion**

Membrane-bound enzymes are often solubilized by proteolytic digestion (Phillips & Langdon, 1962; Dahlqvist, 1963; Auricchio et al., 1963; Takesue & Omura, 1970; Danner & Morrison, 1971; Maroux et al., 1973; Curthoys & Kuhlenschmidt, 1975). Once a membrane-bound enzyme is solubilized by proteolytic digestion, the enzyme can be purified much more easily than if a detergent, in place of a proteinase, is used for solubilization. According to an early study on 2':3'-cyclic nucleotide 3'-phosphodiesterase in pancreas (Davis & Allen, 1956), the enzyme extracted appears soluble. This can be interpreted as that the enzyme has been solubilized by proteinases in pancreas during the long-term extraction. The interpretation prompted us to use proteinases for the solubilization of 2':3'-cyclic nucleotide 3'-phosphodiesterase in brain white matter, the richest source of the enzyme.

Three major proteinases of pancreas (trypsin, chymotrypsin and elastase) and papain were examined for their ability to solubilize 2':3'-cyclic nucleotide 3'-phosphodiesterase from delipidated brain white matter. These proteinases were found to solubilize, and also inactivate, the enzyme to various extents depending on their concentrations; trypsin and elastase appeared to be more effective than the others. Trypsin solubilized 45% of the enzyme activity at 0.7 μg/mg of the delipidated white matter but inactivated the enzyme markedly at higher concentrations. Elastase solubilized 40–45% of the enzyme activity at 1–6 μg/mg and caused little inactivation. These findings led us to use elastase for the solubilization of 2':3'-cyclic nucleotide 3'-phosphodiesterase, and incubation conditions for the elastase digestion were further studied. If the buffer concentration was decreased, the recovery of the solubilized enzyme increased: approx. 75% of the enzyme activity was solubilized at 0.5–3 μg/mg of the delipidated white matter by incubation for 21 h (Fig. 2). Inactivation did not occur at 0.5–10 μg/mg; however, less activity was solubilized at 10 μg/mg than at 0.5–3 μg/mg by incubation for 6–21 h. This was contradictory but reproducible.

When a partially purified preparation of 2':3'-cyclic nucleotide 3'-phosphodiesterase, in place of the delipidated white matter, was treated with elastase, 2':3'-cyclic nucleotide 3'-phosphodiesterase was solubilized nearly quantitatively (Table 1). On addition of elastase the turbid suspension gradually became transparent; the solubilization was completed within several hours as judged by transparency of the solution. 2':3'-Cyclic nucleotide 3'-phosphodiesterase activity was recovered in the supernatant fluid in a 98–100% yield. For the solubilization with elastase, pH of the incubation mixture was critical; little solubilization occurred below pH 8.0.

Because elastase preparations usually contain other proteinase activities (Sampath Narayanan & Anwar, 1969), the possibility remained that 2':3'-cyclic nucleotide 3'-phosphodiesterase was solubilized by other proteinases in the elastase. To exclude this possibility, effects of proteinase inhibi-

![Graph](https://via.placeholder.com/150)

**Fig. 2. Solubilization of 2':3'-cyclic nucleotide 3'-phosphodiesterase by digestion with elastase**

The homogenate of the delipidated white matter was incubated at 23°C with elastase in 10 mm-Tris/HCl buffer, pH 8.8. The final concentration of the delipidated white matter was 10 mg/ml. After incubation for 2.5 h (● and ○), 6 h (△ and △) or 21 h (■ and □), 2':3'-cyclic nucleotide 3'-phosphodiesterase activity in the mixture (●, △ and ■) and in the supernatant fluid of the mixture (○, △ and □) was assayed. For full details see the text.
tors were studied. Elastatinal, which is a specific inhibitor of elastase (Umezawa et al., 1973), specifically inhibited the solubilization (Fig. 3). In contrast, leupeptin, which inhibits trypsin, plasmin and cathepsin B (Aoyagi et al., 1969; Ikezawa et al., 1971), had no effect on the solubilization (Fig. 3). These results indicate that the solubilization is caused by the proteolytic action of elastase and not by the proteolytic action of other proteinases that might have been contained in the elastase.

Among proteinases trypsin and papain have been frequently used for the solubilization of membrane-bound enzymes. To our knowledge elastase has rarely been used for this purpose, though Maestracci (1976) has observed that elastase releases enzymes from brush-border membranes. Elastate hydrolyses elastin, a highly cross-linked insoluble protein; it also hydrolyses insulin and other soluble proteins. Elastase seems to split peptide bonds between neutral amino acids (Sampath Narayanan & Anwar, 1969), and the best synthetic substrate is N-acetyl-L-Ala-L-Ala-L-Ala methyl ester (Gertler & Hofmann, 1970). Therefore it is possible that elastase attacks preferentially a hydrophobic region of 2':3'-cyclic nucleotide 3'-phosphodiesterase, leaving the catalytic site of the enzyme intact.

**Purification and molecular properties of the purified enzyme**

The experimental details of the purification procedure are given in the Materials and methods section. Table 1 summarizes the results of a typical run. From 9 g of the insoluble fraction of the delipidated residue, 7.4 mg of a homogeneous 2':3'-cyclic nucleotide 3'-phosphodiesterase protein was obtained in a 9% yield; specific activity increased 110-fold.

2':3'-Cyclic nucleotide 3'-phosphodiesterase was solubilized from the insoluble fraction of the delipidated residue with hexadecyltrimethylammonium bromide: 75–90% of the original activity was solubilized and specific activity increased 2-fold. The enzyme was partially purified by gel chromatography on successive two columns of Sepharose CL-4B. From the second Sepharose column, a concentrated enzyme solution was eluted with the gradient of hexadecyltrimethylammonium bromide, presumably at a minimum detergent/protein ratio.

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**Table 1. Purification of 2':3'-cyclic nucleotide 3'-phosphodiesterase**

For full details see the text. Abbreviation used: HTAB, hexadecyltrimethylammonium bromide.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg of protein)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insoluble fraction of the delipidated residue</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HTAB-soluble fraction</td>
<td>150</td>
<td>3090</td>
<td>162000</td>
<td>52.4</td>
<td>75</td>
</tr>
<tr>
<td>Sepharose CL-4B (0.5% HTAB)</td>
<td>440</td>
<td>924</td>
<td>98900</td>
<td>107</td>
<td>46</td>
</tr>
<tr>
<td>Sepharose CL-4B (HTAB gradient)</td>
<td>88</td>
<td>274</td>
<td>72800</td>
<td>266</td>
<td>34</td>
</tr>
<tr>
<td>Ethanol precipitation</td>
<td>7.5</td>
<td>232</td>
<td>47400</td>
<td>204</td>
<td>22</td>
</tr>
<tr>
<td>Supernatant after elastase digestion</td>
<td>30</td>
<td>230</td>
<td>46700</td>
<td>203</td>
<td>22</td>
</tr>
<tr>
<td>DEAE-Sephadex A-50</td>
<td>40</td>
<td>45</td>
<td>21300</td>
<td>473</td>
<td>10</td>
</tr>
<tr>
<td>8-(6-Aminohexyl)amino-2'-AMP-Sepharose CL-4B</td>
<td>60</td>
<td>7.4</td>
<td>19800</td>
<td>2680</td>
<td>9</td>
</tr>
</tbody>
</table>
The eluent contained 30–40% of the original activity; an overall increase in specific activity was 10–15-fold. 2':3'-Cyclic nucleotide 3'-phosphodiesterase solubilized with hexadecyltrimethylammonium bromide behaved as micelles of high molecular weight. Even after gel chromatography on the second Sepharose column, the enzyme was precipitated as an insoluble aggregate on removal of the detergent. However, this aggregate was resolubilized by digestion with elastase (see above under 'Solubilization by proteolytic digestion')

The elastase-resolubilized preparation was passed through a DEAE-Sephadex A-50 column, and the enzyme was finally purified to homogeneity by affinity chromatography on 8-(6-aminohexyl)-amino-2'-AMP-Sepharose CL-4B (Fig. 4). During passage through the DEAE-Sephadex column, about half of the enzyme activity was retained on the column and lost; however, if this step was omitted, subsequent purification was unsuccessful. The enzyme activity retained on the column was eluted with a gradient of NaCl; it is possible that the enzyme fractions retained on the column are different in molecular species from the enzyme fraction not retained on the column. The affinity chromatography gave a homogeneous enzyme protein if appropriate elution conditions were used. The enzyme was eluted with 1 mM-2'-AMP almost quantitatively, and specific activity increased 5–7-fold at this step.

The purified enzyme was stable at 4°C. When the eluate from the affinity column was stored at 4°C for 6 months, more than 90% of the enzyme activity was preserved. If 2'-AMP and NaCl were removed, the enzyme was less stable during the storage at 4°C. The purified enzyme was seriously inactivated when exposed to freezing-and-thawing or dilution. If 0.1% serum albumin (fraction V) was present in the medium, no such inactivation occurred; glycerol was less effective. Therefore the purified enzyme can be stored by freezing in the presence of 0.1% serum albumin and can be diluted with 0.1% serum albumin without loss of enzyme activity. The purified enzyme was also seriously inactivated by shearing with a glass pipette. For the purified enzyme, therefore, plastic pipettes were used and agitation of the enzyme solution was avoided.

The purified enzyme migrated rapidly towards the cathode as a single protein band on polyacrylamide-gel electrophoresis at pH 4.3 by the pro-

![Fig. 4. Affinity chromatography of 2':3'-cyclic nucleotide 3'-phosphodiesterase](attachment)

The arrows indicate eluent changes: A, 50 mM-NaCl; B, 40 mM-NaCl; C, 1 mM-2'-AMP in 40 mM-NaCl. All the eluents contained 10 mM-imidazole/HCl buffer, pH 5.7. O, Enzyme activity; ●, protein. For full details see the text.

![Fig. 5. Polyacrylamide-gel electrophoresis of 2':3'-cyclic nucleotide 3'-phosphodiesterase](attachment)

The eluate from the affinity column was passed through Sephadex G-25 and electrophoresed in 7.5% polyacrylamide by the procedure of Reisfeld et al. (1962). A sample containing 30 μg of protein in 0.2 ml, to which sucrose had been added to increase the density, was layered over the spacer gel. Electrophoresis was performed at 4°C by applying a current of 2 mA per gel. (a) The gel was stained with Coomassie Brilliant Blue. The arrow indicates the β-alanine front. (b) The gel was cut into 0.5 cm-wide slices. Each slice was immersed in 0.2 ml of 20 mM-imidazole/HCl buffer, pH 6.7, containing 0.1% bovine serum albumin (fraction V) and kept at 4°C for 3 days with stirring at intervals. 2':3'-Cyclic nucleotide 3'-phosphodiesterase activity in the supernatant fluid was then determined. For full details see the text.
procedure of Reisfeld et al. (1962) (Fig. 5); the position where the enzyme activity was found coincided with that of the protein band. The purified enzyme did not enter the gel on polyacrylamide-gel electrophoresis by the procedure of Davis (1964). These findings indicate that the purified enzyme is a homogeneous basic protein. Homogeneity of the purified enzyme was also shown by SDS/polyacrylamide-gel electrophoresis. If the procedure of Laemmli (1970) was used, the purified enzyme migrated as a single protein band under both reducing and non-reducing conditions (Figs. 6a and 6b). The position of the protein band was almost the same irrespective of whether electrophoresis was done under reducing or non-reducing conditions. However, if the procedure of Weber & Osborn (1969) was used, the electrophoretic pattern varied depending on the conditions used. Whereas under reducing conditions only a single protein band was seen (Fig. 6c), under non-reducing conditions three additional minor bands, one at the position of a high-molecular-weight protein and two overlapping the upper and lower parts respectively of the major band, appeared (Figs. 6d and 6e). These additional bands may be artifacts produced by a combined action of SDS and phosphate buffer in the absence of 2-mercaptoethanol: they seemed to become denser during the prior heat treatment in SDS and phosphate buffer (compare Figs. 6d and 6e).

SDS/polyacrylamide-gel electrophoresis by the procedures of Laemmli (1970) and of Weber & Osborn (1969) indicated mol.wts. of 27000–30000 and of 28000–31000 respectively for the purified enzyme (Fig. 7a). Gel filtration through Sephadex G-150 showed that the purified enzyme has a mol.wt. of 31000 under non-denaturing conditions (Fig. 7b). These results indicate that the purified enzyme is a monomer protein with a mol.wt. of approx. 30000. Repeated purification gave a product with identical molecular weight, as judged by mobility in SDS/polyacrylamide-gel electrophoresis.

In the present study 2':3'-cyclic nucleotide 3'-phosphodiesterase was purified by: (1) delipidation; (2) solubilization (hexadecyltrimethylammonium bromide); (3) gel chromatography; (4) resolubilization (elastase); (5) ion-exchange chromatography; (6) affinity chromatography. Step 4 and step 6 seem to be key steps for the purification. When the treatment with elastase (step 4) has not yet been introduced, all attempts to purify the enzyme to homogeneity failed. The use of affinity chromatography in step 6 was effective: the enzyme was specifically adsorbed on and desorbed from the affinity column under appropriate conditions. Inherent in the use of a proteinase is that the purified enzyme may be a product of proteolytic degradation. For some membrane-bound enzymes evidence has been given that a hydrophobic segment is left on the membranes when a soluble enzyme protein is released by proteolytic digestion (Spatz & Strittmatter, 1971, 1973; Sigrist et al., 1975). It remains to be shown whether a similar hydrophobic segment is removed when 2':3'-cyclic nucleotide 3'-phosphodiesterase is solubilized by digestion with elastase.

While the present work was in progress, Drummond et al. (1978) reported that they have obtained a nearly homogeneous preparation of 2':3'-cyclic nucleotide 3'-phosphodiesterase from bovine brain. The major protein in their preparation was a dimer protein composed of two identical subunits, subunit mol.wt. being 50000. Our preparation from the same source was homogeneous and, in contrast with the preparation obtained by Drummond et al. (1978), consisted of a monomer protein with a mol.wt. of 30000. The difference between both preparations with respect to molecular structure, however, can be accounted for by the use of a proteinase in our procedure.

Fig. 6. SDS/polyacrylamide-gel electrophoresis of 2':3'-cyclic nucleotide 3'-phosphodiesterase
The eluate from the affinity column was electrophoresed by the procedures of Laemmli (1970) (a and b) and of Weber & Osborn (1969) (c, d and e), as described in the Materials and methods section. Approx. 40 μg of protein/gel was electrophoresed. (a) and (c) The standard procedures were used. (b) and (d) Electrophoresis and prior heating with SDS were done in the absence of 2-mercaptoethanol. (e) Electrophoresis was done in the absence of 2-mercaptoethanol, and prior heating with SDS was omitted. The arrows indicate the position of Bromophenol Blue. For full details see the text.
2',3'-Cyclic nucleotide 3'-phosphodiesterase

Fig. 7. Determination of the molecular weight of 2',3'-cyclic nucleotide 3'-phosphodiesterase by SDS/polyacrylamide-gel electrophoresis (a) and by gel filtration through Sephadex G-150 (b)

(a) The procedures of Laemmli (1970) (A) and of Weber & Osborn (1969) (B) were used, as described in the Materials and methods section. Each point represents single determination. O, 2',3'-Cyclic nucleotide 3'-phosphodiesterase; ●, marker proteins. (b) The eluate from the affinity column (approx. 300 μg of protein) and the mixed marker proteins were applied separately (O and ●) or together (∆ and △) on a Sephadex G-150 column. Each point represents single determination. O and ∆, 2',3'-Cyclic nucleotide 3'-phosphodiesterase; ● and △, marker proteins. For full details see the text.

Although the purified enzyme reported here may be a fragment of the native enzyme, its use will have several advantages. First, a homogeneous enzyme protein can be prepared easily, since the solubilization with elastase greatly facilitates subsequent purification. Secondly, an antibody against the enzyme can be raised effectively by injection of the preparation into rabbits (Y. Nishizawa & T. Kurihara, unpublished work). The antibody will be useful in studying the localization and disposition of the enzyme in cell membranes. Thirdly, this preparation may be useful for structural studies because of its low molecular weight. As far as we have examined in preliminary studies, the catalytic properties of the enzyme remain unchanged during the purification.

References


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