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ADENOSINE 3′,5′-MONOPHOSPHATE PHOSPHODIESTERASE ASSAY IN TISSUE HOMOGENATES

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SUMMARY

1. A fast, reliable and sensitive method for the assay of 3′,5′-AMP phosphodiesterase (adenosine 3′,5′-monophosphate phosphohydrolase, EC 3.1.4.c) is described, which is also applicable to homogenates and crude enzyme preparations containing enzymes catalyzing the breakdown of the reaction product 5′-AMP. The method has been worked out for rat pancreatic homogenate.

2. The method is based on existing methods in which 3′,5′-[^{3}H]AMP is used as substrate and in which the resulting 5′-AMP is dephosphorylated in a second step by means of added 5′-nucleotidase.

3. It is shown that already during the first incubation step a mixture of radioactive products is formed: 5′-AMP, 5′-IMP, 3′,5′-IMP, adenosine, inosine, hypoxanthine and adenine. During the second incubation step 5′-AMP and 5′-IMP are converted into the corresponding nucleosides.

4. The reaction mixture after the second incubation step is applied to a column of anionic exchange resin. All products of the phosphodiesterase reaction can be eluted with 0.1 M $NaHCO_3$, while the unchanged 3′,5′-AMP and the product of the side reaction 3′,5′-IMP, are retained. After liquid scintillation counting of the eluate, the 3′,5′-AMP phosphodiesterase activity can be calculated.

INTRODUCTION

For the determination of 3′,5′-AMP phosphodiesterase activity several approaches can be used. First, the amount of 3′,5′-AMP, which remains unchanged during incubation, can be measured[1]. Secondly, the amount of 5′-AMP, the product of the enzymatic reaction, can be determined by different methods[2,3]. In the third approach this product is further hydrolysed with exogenous 5′-nucleotidase to adenosine and P_i, the latter product being determined[4,5]. Alternatively 3′,5′-[^{3}H]-AMP is used as substrate and [^{3}H]adenosine is determined. This can be done in two ways: either the reaction mixture is added to a suspension of anionic exchange resin, whereupon counting of the radioactivity of the whole suspension gives the amount

of [^{3}H]adenosine, the radioactivity from unchanged 3′,5′-[^{3}H]AMP being quenched by the resin[6], or the reaction mixture is applied to a column of the same resin and [^{3}H]adenosine is eluted with water[7].

All these methods have their disadvantages. The first approach is liable to yield relatively large errors at low conversion rates of the substrate, since it requires measuring small differences. In the third approach the measurement of P_i after hydrolysis of the resulting 5′-AMP is not sensitive enough for very low substrate concentrations, such as are necessary to determine the 3′,5′-AMP phosphodiesterase activity with very low K_m[6–11]. The measurement of [^{3}H]adenosine production is very sensitive, but cannot be applied to homogenates or other unpurified enzyme preparations. These preparations usually contain enzymes converting 5′-AMP to products other than adenosine[12,13], and these products stay on the resin, thus leading to erroneous values for 3′,5′-AMP hydrolysis. The second approach, in which the production of 5′-AMP is measured, is subject to an even greater degree to the problem caused by enzymatic conversion of 5′-AMP.

In this paper a modification of the method of Loten and Sneyd[7] is described, in which not only adenosine, but also inosine, adenine and hypoxanthine are eluted from a column of anionic exchange resin, since these compounds can also be reaction products through the reactions presented in Fig. 1. The sum of all radioactive breakdown products, which is equal to the amount of 3′,5′-AMP converted by phosphodiesterase, is then measured.

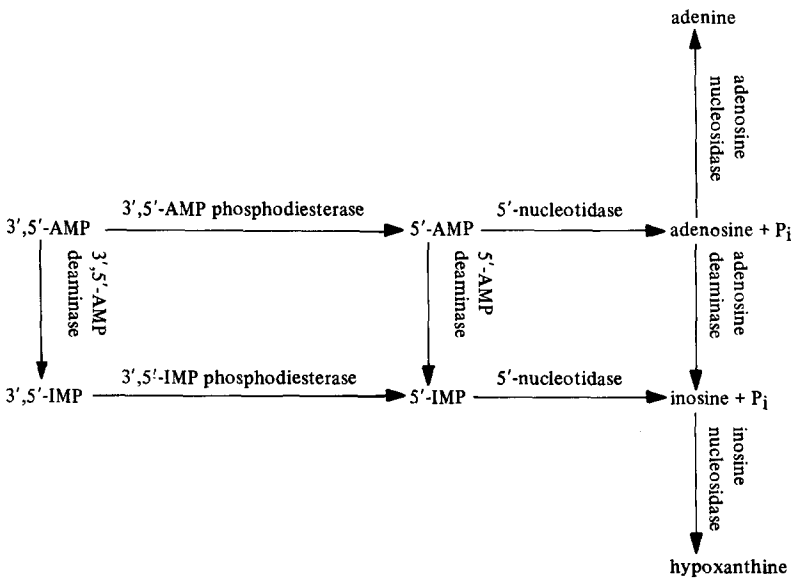

Fig. 1. Pathways of 3′,5′-AMP conversion upon incubation with tissue homogenates.

METHODS, RESULTS AND DISCUSSION

Homogenate of rat pancreas in 0.9% NaCl has been used as the enzyme preparation. Unless otherwise specified, the incubation medium contains 66 mM Tris–HCl

(pH 8.5), 1 mM $MgCl_2$, either 2 mM or 4 μM 3′,5′-[^{3}H]AMP (about 0.2 μCi; The Radiochemical Centre, Amersham, England), and 25 μl homogenate in a total volume of 125 μl. After 15 min incubation at 37 °C the reaction is stopped by placing the tubes in boiling water for 2 min. After addition of 25 μl of a solution of 100 μg 5′-nucleotidase (Sigma Chemical Co., St. Louis, Mo., U.S.A.) in 1 ml of 50 mM $MgCl_2$, the mixture is incubated for 30 min at 37 °C, whereupon the mixture is centrifuged and the supernatant is used for determination of the radioactive reaction products. It can also be stored overnight at −20 °C before centrifugation and further treatment.

Estimation of phosphodiesterase activity by measuring [^{3}H]adenosine production according to Thompson and Appleman[6] or Loten and Sneyd[7] yields very low results. Hence, the identity of the labeled products, present before and after the second incubation step, has been determined by thin-layer chromatography[14] of the supernatant on Chromar-Sheet 500 (Malinckrodt Chemical Works, St. Louis, Mo., U.S.A.). After developing the thin layers with a mixture of 2-propanol, ethyl acetate and 13 M ammonia (59:29:16, by vol.), the spots are visualized under 254-nm ultraviolet light. The spots are then cut out and transferred to 5 ml 0.5 M NH_4OH in a scintillation vial. After 1 h the mixture is neutralized with 3 M HCl and 10 ml of Insta-Gel scintillation fluid (Packard Instruments) is added. Radioactivity is counted in a liquid scintillation spectrometer (Packard TriCarb, model 3380).

The results of such experiments, both at low and high substrate concentrations are shown in Fig. 2. In both cases even before addition of 5′-nucleotidase the major part of the resulting 5′-AMP has already been converted, which is probably due to the presence of a high 5′-nucleotidase activity in rat pancreas[15]. The second incubation step leads to nearly complete conversion of 5′-AMP to adenosine. Of the breakdown products inosine is the most important, followed by adenosine, when the high substrate concentration (2 mM) is used, as has also been found in rat heart supernatant[12]. At low substrate concentration (4 μM) this situation is reversed. This may be explained by a relatively high K_m value for the enzyme adenosine deaminase, as was found in calf intestinal mucosa (K_m = 35 μM)[16]. Hypoxanthine is another break-

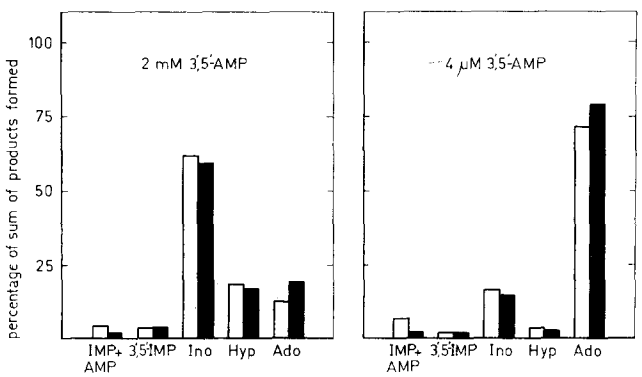

Fig. 2. Relative amounts of radioactive products formed from 3′,5′-[^{3}H]AMP before (open bars) and after (closed bars) incubation with 5′-nucleotidase. Separation of the products was performed by thin-layer chromatography as described in the text. Results are expressed as percentages of the total radioactivity recovered from the thin layers. Recovery of radioactivity, applied to the thin layers, was in all cases better than 95%. IMP = 5′-IMP, AMP = 5′-AMP, Ino = inosine, Hyp = hypoxanthine, Ado = adenosine.

down product present in appreciable amounts, although its relative amount is higher at high substrate concentration. This is probably due to its being formed from inosine by the action of inosine nucleosidase. Adenine, which would be formed by adenosine nucleosidase from adenosine, is only found in trace amounts (± 0.5% of the 3',5'-AMP converted), when high substrate concentrations are used.

A hitherto-unreported observation is the formation of a small amount of 3',5'-IMP. This may be due to the action of a specific 3',5'-AMP deaminase or possibly to the less specific action of 5'-AMP deaminase. With the aid of another developing system (2-propanol–ethyl acetate–8.5 M ammonia; 30:44.5:25.5, by vol.) which is able to separate 5'-AMP and 5'-IMP, it could be shown that the amount of 5'-IMP is not more than about 10–20% of the sum of both compounds at either substrate concentration.

The pathways by which 3',5'-AMP can be broken down are shown in Fig. 1. The question now arises whether 3',5'-IMP is an important intermediate in the breakdown of 3',5'-AMP. This could only be the case if 3',5'-IMP is rapidly converted *via* 5'-IMP into inosine. This is improbable for the following reasons. First, at low substrate concentrations the main breakdown product is adenosine, which cannot be formed *via* 3',5'-IMP. Secondly, upon shorter incubation times at high substrate concentration the larger amount of adenosine formed and a smaller amount of inosine suggest that the hydrolytic pathway of 3',5'-AMP *via* 5'-AMP and adenosine is the dominant one. Finally, upon inhibition of 3',5'-AMP phosphodiesterase by addition of 10 mM theophylline or 0.1 mM papaverine no increase in 3',5'-IMP is found.

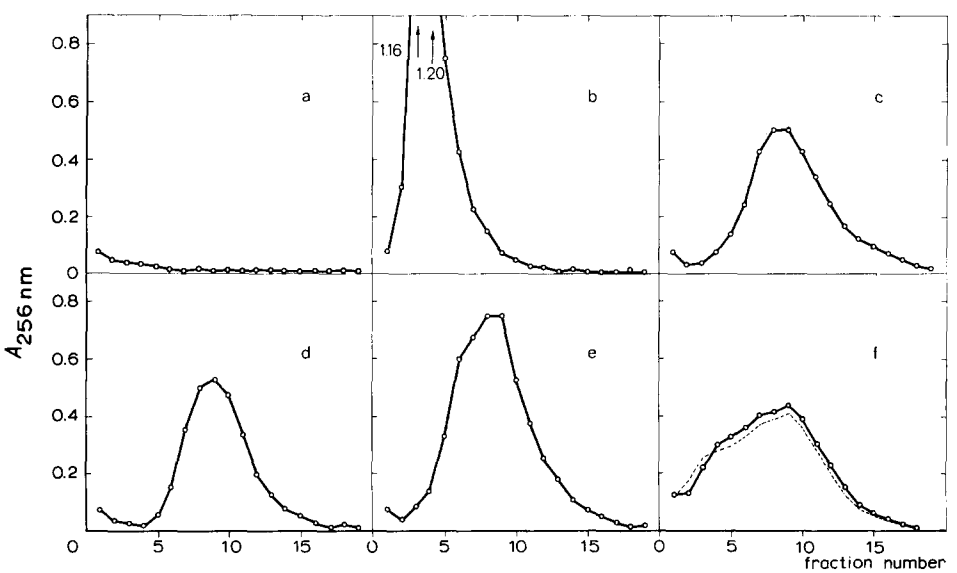

Fig. 3. Elution patterns of 3',5'-AMP (10.1 mM; a) adenosine (0.37 mM; b) inosine (0.37 mM; c) hypoxanthine (0.73 mM; d) adenine (0.74 mM; e) and a mixture of equal volumes of these five solutions (f) from Bio-Rad AG 1-X2 200–400 mesh, Cl^- form. Length of the columns in Pasteur pipettes (diameter, 7 mm), is 15 mm; a 0.5-ml aliquot of solutions of the compounds in 66 mM Tris–HCl (pH 8.5) is placed on the top of the column, which is then eluted with 18 × 0.5 ml 0.1 M $NaHCO_3$. The absorbance of the eluted fractions is measured at 256 nm. Absorbance pattern of the Tris buffer virtually coincides with Pattern a. The calculated absorbance pattern for the mixture is indicated by the dotted line in f.

Returning to the methods of Thompson and Appleman[6] and Loten and Sneyd[7], we can show that the former method is unsuitable since the reaction products remain absorbed to the resin in different proportions. This was tested by mixing 500 μl adenosine solution in 66 mM Tris–HCl (pH 8.5) with 500 μl of a 1:4 aqueous suspension of Dowex AG 1-X2 200–400 mesh (Bio-Rad Laboratories, Richmond, U.S.A.). Measurement of the absorbance at 256 nm of the supernatant after centrifugation reveals that 30–40% of the adenosine and 80% of the inosine have been absorbed to the resin. This leads to erroneously low values for [^{3}H]adenosine, which makes this method unsuitable for phosphodiesterase assays in pancreas homogenates.

Loten and Sneyd[7] apply the reaction mixture to a column of the same resin and elute this column with water. However, we have observed that moderate volumes of water elute only adenosine, while inosine, adenine and hypoxanthine are retained on the column. Therefore, we tested solutions of various electrolytes at different ionic strengths as eluent. Best results are obtained with 10 ml 0.1 M $NaHCO_3$, as shown in Fig. 3. This figure shows the elution patterns of the tested compounds, alone and in combination, for each compound in the total eluate. It is clear that, while 3',5'-AMP is completely retained, the other substances are virtually completely eluted. Other nucleotides, 3',5'-IMP, 5'-AMP and 5'-IMP, are retained like 3',5'-AMP.

These findings led us to the following definitive procedure. A 100-μl aliquot of the supernatant obtained after 5'-nucleotidase treatment is applied to a 15 mm long column of Dowex AG 1-X2 resin in a Pasteur pipette (diameter, 7 mm). The column is eluted with 10 ml 0.1 M $NaHCO_3$. The total eluate is mixed with 10 ml Insta-Gel and the radioactivity is counted. In Fig. 4 our method with $NaHCO_3$ elution is compared with that of Loten and Sneyd[7] involving elution with water. It is obvious that measurement of adenosine production by elution of the column

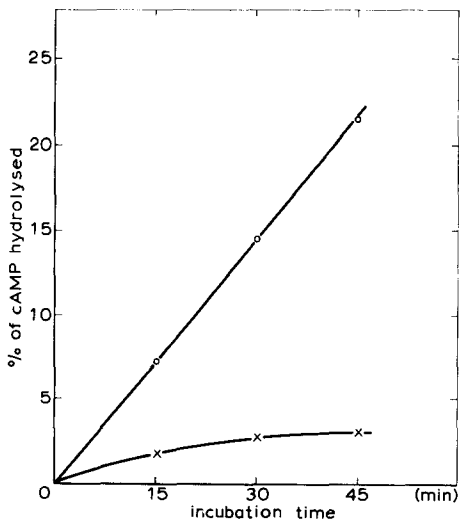

Fig. 4. Time course of 3',5'-AMP hydrolysis by rat pancreas homogenate. The upper line (○—○) was obtained with method described in the text, column elution with 10 ml 0.1 M $NaHCO_3$. The lower curve (×—×) was obtained by elution with 10 ml of water. Initial 3',5'-AMP concentration, 2 mM.

with water gives—at least for rat pancreas—erroneously low and non-linear results. Elution with 0.1 M $NaHCO_3$, on the other hand, yields a linear relationship between 3′,5′-AMP conversion and time.

The results obtained by our modified procedure are quite reproducible (duplicate agreement expressed as coefficient of variation: 3%) and they agree within the experimental error with the results obtained by thin-layer chromatography. Our method is suitable for the assay of 3′,5′-AMP phosphodiesterase in tissue homogenates and crude enzyme preparations at micromolar to millimolar substrate concentrations. Its use is recommended for tissues with an appreciable deaminase activity. Detailed results of our studies on 3′,5′-AMP phosphodiesterase in rat pancreas are published in an accompanying paper[17].

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