*Biochimica et Biophysica Acta*, 315 (1973) 384–393


BBA 66957

# ADENOSINE 3′,5′-MONOPHOSPHATE PHOSPHODIESTERASE IN RAT PANCREAS

W. J. RUTTEN, B. M. SCHOOT, J. J. H. H. M. DE PONT AND S. L. BONTING

*Department of Biochemistry, University of Nijmegen, Geert Grooteplein Noord 21, Nijmegen (The Netherlands)*

(Received January 25th, 1973)

## SUMMARY

1. The 3′,5′-AMP phosphodiesterase of rat pancreas behaves kinetically as if two separate activities exist, one with a high and the other with a low affinity for the substrate. In whole homogenates the $K_m$ values are 4.9 and 267 $\mu$M, respectively, and the maximal rates are 0.8 and 4.2 nmoles 3′,5′-AMP hydrolysed per min per mg protein, respectively.

2. Properties of the activity with low $K_m$ were determined at a 3′,5′-AMP concentration of 4 $\mu$M and of the activity with high $K_m$ at a concentration of 2 mM. In these experiments the contribution of each activity to the total substrate conversion rate was calculated.

3. At both substrate concentrations an optimal activity at pH 8.1–8.2 is found. $Mg^{2+}$ is required for enzyme activity. The affinity at low substrate concentration ($K_a$ = 48 $\mu$M), is somewhat higher than at high substrate concentration ($K_a$ = 110 $\mu$M). $Ca^{2+}$ in concentrations of 0.1–10 mM inhibits both activities.

4. Both enzyme activities are inhibited to the same degree by the methylxanthines, caffeine and theophylline, and more potently by papaverine. In the case of the low $K_m$ activity the inhibition by theophylline and papaverine is of the competitive type with $K_i$ values of 630 and 3 $\mu$M, respectively.

## INTRODUCTION

Mammalian pancreas consists for the greatest part of exocrine tissue. In this tissue two functionally and morphologically different types of cells occur, the acinoductular and the ductular cells which secrete fluid and electrolytes and the acinar cells which secrete enzymes. The intermediate function of 3′,5′-AMP in fluid and electrolyte secretion has been well established[1–3]. A similar role of this compound in the process of enzyme secretion is still debated[1–7]. The function of 3′,5′-AMP as a hormonal messenger requires the presence of adenylate cyclase, which synthesizes the compound, and also of 3′,5′-AMP phosphodiesterase, which removes it again.

In a previous paper[3] we have reported the presence and properties of adenylate cyclase in rat pancreas. In the present paper we report the presence and properties of 3′,5′-AMP phosphodiesterase in this tissue.

Since in other tissues phosphodiesterase activities with very low $K_m$ and with high $K_m$ for 3′,5′-AMP have been described[8–14], we have made a detailed study of the kinetic parameters. We found that the phosphodiesterase behaves kinetically as if consisting of two distinct activities, one with high and one with low substrate affinity. Various properties of these two activities including the effects of some compounds affecting exocrine pancreatic secretion, are described.

## METHODS

2–3-month-old Wistar rats, which had free access to food and water, are sacrificed and the pancreas is removed. After removal of fat and connective tissue, the tissue is minced and homogenized in 4 vol. of 0.9% NaCl in a Potter–Elvehjem homogenizer. The homogenate is then filtered through four layers of medical gauze and eventually stored at −20 °C for up to a week.

3′,5′-AMP phosphodiesterase activity is measured by the method of Loten and Sneyd[9], as modified by Rutten *et al.*[15].

Protein is measured by the method of Lowry *et al.*[16], using bovine serum albumin as a standard.

## MATERIALS

The following materials and reagents have been used with the source indicated between parentheses. Adenosine 3′,5′-monophosphoric acid (3′,5′-AMP) and guanosine 3′,5′-monophosphoric acid (3′,5′-GMP) (Boehringer, Mannheim, Germany). [8-$^3$H]Adenosine 3′,5′-cyclic phosphate, ammonium salt, 6.5 Ci/mmole (The Radiochemical Centre, Amersham, England). 5′-Nucleotidase from *Crotalus adamanteus* venom, Grade II (Sigma Chemical Co., St. Louis, Mo., U.S.A.). Dowex AG 1-X2, 200–400 mesh, $Cl^-$ form (Bio-Rad Laboratories, Richmond, Calif., U.S.A.). Bovine serum albumin, dried purified (Behringwerke A.G., Marburg/Lahn, Germany). Clinical preparations of secretion are from Boots Pure Drug Co. Ltd, Nottingham, England. The synthetic C-terminal octapeptide of pancreozymin (activity 16 000 Ivy Dog units/mg) is a gift from Dr M. Ondetti, The Squibb Institute for Medical Research, New Brunswick, N.Y., U.S.A.

All other reagents are commercial preparations of the highest obtainable purity.

## RESULTS

### *Linearity of 3′,5′-AMP phosphodiesterase assay*

Incubation conditions as time and enzyme concentration must be carefully chosen to ensure linearity of 3′,5′-AMP hydrolysis with respect to these parameters. At a substrate concentration of 2 mM, linearity exists up to at least 45 min and 4 mg protein per ml of incubation medium. Up to 22% substrate hydrolysis the reaction rate remained constant. At this substrate concentration incubations were therefore

routinely carried out for 15 or 30 min at enzyme concentrations of 2–3 mg protein per ml of incubation medium.

At a substrate concentration of 4 $\mu$M we did not exceed incubation times of 10 min and enzyme concentrations of 0.35 mg protein per ml of incubation medium. Up to 40% of the substrate can be hydrolysed under these conditions without significant decline in reaction rate. At this substrate concentration, enzyme concentrations of 0.1 mg protein per ml of incubation mixture and an incubation time of 10 min were maintained routinely; 3′,5′-AMP hydrolysis under these conditions does not exceed 10%.

*Effect of substrate concentration*

A number of recent publications[8–14] concerning phosphodiesterases in various tissues attributes the observed relationship between activity and substrate concentration to the occurrence of two enzymes with different affinity for 3′,5′-AMP. Therefore, we made a detailed analysis of this relationship for the activity present in rat pancreas. Fig. 1 shows a Lineweaver–Burk plot for the relation between enzyme activity and substrate concentration. At low substrate concentrations a straight line is obtained, which at extrapolation yields a $K_m$ for 3′,5′-AMP of 5.8 $\mu$M and a $V$ of 1.09 nmoles 3′,5′-AMP hydrolysed per min per mg protein. At substrate concentrations above 10 $\mu$M the curve deviates from this linear relationship, suggesting the additional presence of an activity with high $K_m$ value. The points at high substrate concentrations can be corrected for the contribution of the low $K_m$ activity to substrate hydrolysis (see also the Appendix). Plotting the corrected values in the substrate concentration range 0.1–2 mM in a Lineweaver–Burk plot yields a straight line from which a $K_m$ value for 3′,5′-AMP of 282 $\mu$M and a $V$ of 3.91 nmoles 3′,5′-AMP hydrolysed per min per mg protein can be calculated.

Knowing the $V$ and $K_m$ values of the two enzyme activities, we can also correct the activities at low substrate concentration for the contribution of the high-$K_m$ enzyme. This cross-correction can be repeated in order to obtain more accurate

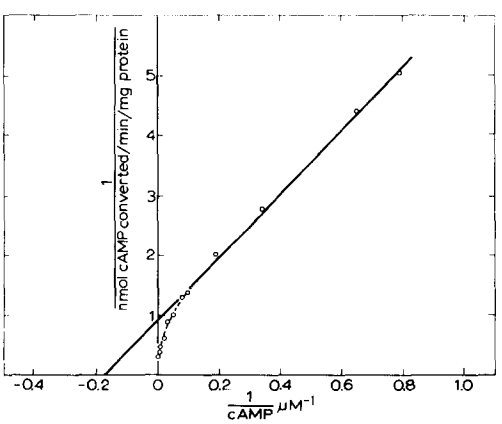



Fig. 1 Lineweaver–Burk plot for the relation of pancreatic phosphodiesterase activity and 3′,5′-AMP concentration. All incubations were carried out for 10 min. The homogenate contained 3.24 mg protein per ml at 3′,5′-AMP concentrations from 2.3–2085 $\mu$M and 1.65 mg protein per ml at 3′,5′-AMP concentrations of 1.27–257 $\mu$M.

values of the kinetic parameters. Three consecutive cross-corrections proved to be sufficient. This results in $K_m$ values of 4.9 and 267 $\mu$M, respectively, and $V$ values of 0.83 and 4.16 nmoles 3′,5′-AMP hydrolysed per min per mg protein, respectively. Figs 2a and 2b show Lineweaver–Burk plots, in which the corrected activities have been used. Subsequent experiments have all been carried out at substrate concentrations of 4 $\mu$M and 2 mM, respectively. At 4 $\mu$M the activity with high substrate affinity is mainly measured; calculation by means of the above kinetic parameters shows that at this substrate concentration the low affinity activity contributes approximately 14% to the observed activity. Conversely, the contribution of the low $K_m$ activity at the high substrate concentration of 2 mM is 16%.

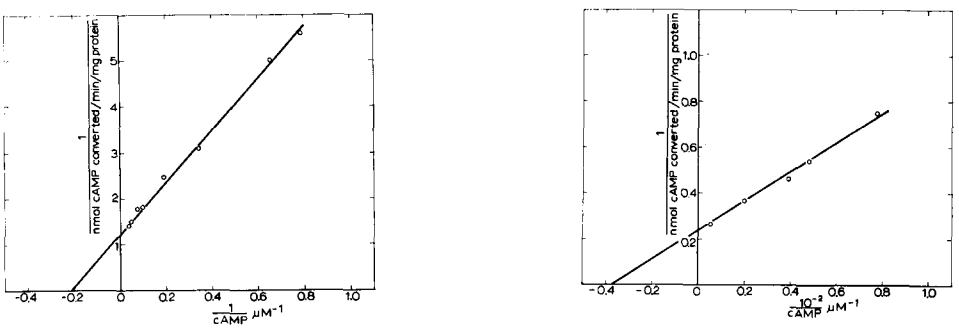



Fig. 2. Corrected Lineweaver–Burk plots at low (a) and high (b) substrate concentration, obtained from the data in Fig. 1 after triple cross-correction as described in the text for the contributions of the high and low $K_m$ activities.

Although the kinetic data do not definitely prove that the two activities are two different enzymes, they at least suggest that the two activities are distinct and behave independently of each other.

*Influence of $Mg^{2+}$ and $Ca^{2+}$*

The presence of $Mg^{2+}$ is an absolute requirement for most, if not all 3′,5′-AMP phosphodiesterases investigated so far, although $Mn^{2+}$ can replace $Mg^{2+}$ in many cases[10,17,18]. In rat pancreas both activities increase sharply with rising $Mg^{2+}$ concentration; maximal activities are reached at approximately 0.5 mM at low substrate concentration and at approximately 0.75 mM at the high substrate concentration. If the reciprocal values of substrate hydrolysis velocity at low substrate concentrations are plotted against the reciprocal $Mg^{2+}$ concentration, a straight line is obtained (Fig. 3a). The intercept with the axis yields the negative reciprocal value of the $K_a$ for $Mg^{2+}$: this value is 48 $\mu$M. When the results at high substrate concentration are plotted in the same way, no straight line is obtained. However, after correction of the observed values for the contribution of the low $K_m$ enzyme a straight line is obtained (Fig. 3b), from which the $K_a$ for $Mg^{2+}$ for the high $K_m$ enzyme is calculated to be 110 $\mu$M.

Since some authors report that 3′,5′-AMP phosphodiesterase is at least in part dependent on low concentrations of $Ca^{2+}$ (refs 19–21), we studied the effects of $Ca^{2+}$ and of EGTA, which should eliminate $Ca^{2+}$ present in the homogenate. $Ca^{2+}$ in low concentrations (< 0.1 mM) has no effect on the enzyme activity, but in concen-

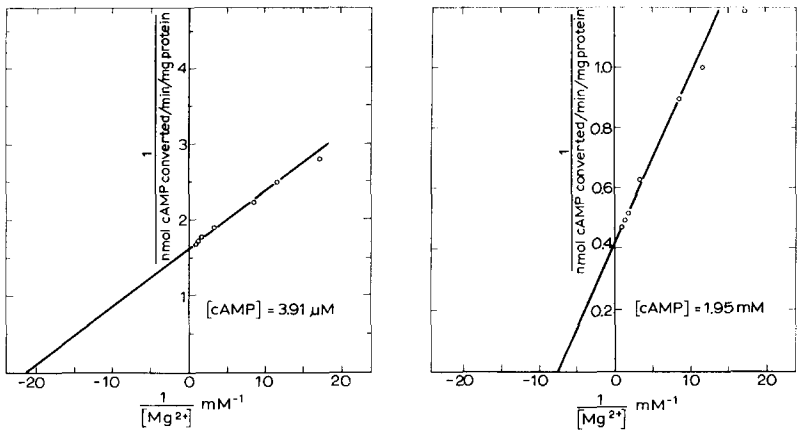



Fig. 3. Influence of $Mg^{2+}$ on 3′,5′-AMP phosphodiesterase activity at high (Fig. 3a) and low (Fig. 3b) substrate concentration, plotted in a double reciprocal way. Pancreatic homogenate was prepared in a solution of 0.2 mM EDTA in 0.9% NaCl and then dialysed against 0.9% NaCl for 4 h at 0 °C. The diluted homogenate contained 9.6 mg protein per ml (Fig. 3a) or 0.46 mg protein per ml (Fig. 3b). Incubation times were 30 and 10 min, respectively.

trations of 0.1–10 mM $Ca^{2+}$ increasingly inhibits enzyme activity, both at low and high substrate concentration (Figs 4a and 4b). Addition of EGTA has no effect, except that at high concentrations (10 mM) some inhibition is seen, which may, however, be due to complexation of $Mg^{2+}$.

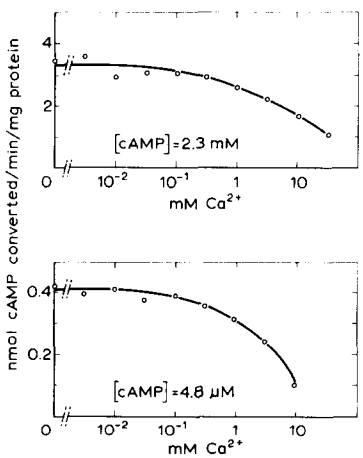
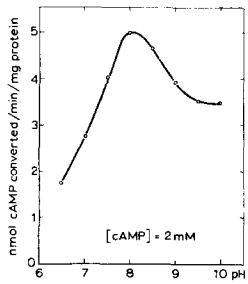
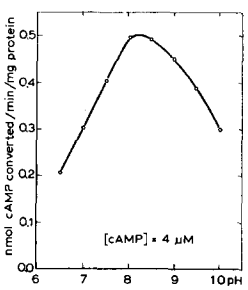



Fig. 4. Inhibition of 3′,5′-AMP phosphodiesterase in rat pancreas by $Ca^{2+}$. Freshly prepared pancreatic homogenate was dialysed during 3 periods of 1 h against 0.9% NaCl at 0 °C and diluted to a concentration of 15.8 mg protein per ml for 30-min incubations at high substrate concentration (Fig. 4a) or 0.74 mg protein per ml for 10-min incubations at low substrate concentration (Fig. 4b).

*Effect of pH*

The relation between activity and pH was determined at both low and high substrate concentrations. The pH optima for both cases differed little from each



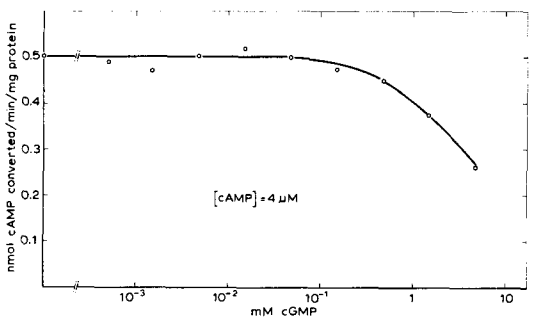




Fig. 5. Effect of pH on 3',5'-AMP phosphodiesterase activity at high (a) and low (b) substrate concentration. The pancreatic homogenate contained 17.0 mg protein per ml at high and 0.31 mg protein per ml at low substrate concentration. Incubations were carried out in media, prepared with 30 mM Tris–30 mM histidine buffers. In these experiments the pH was brought to pH 8.5 before 5'-nucleotidase treatment. Incubation times were 30 min (a) or 10 min (b).

Fig. 6. Inhibition of 3',5'-AMP phosphodiesterase in rat pancreas by 3',5'-GMP. Diluted homogenate, containing 0.32 mg protein per ml was incubated for 10 min in media containing 4 $\mu$M 3',5'-AMP and varying concentrations of 3',5'-GMP.

other. In the experiments shown in Fig. 5 the optima were 8.1 and 8.2 for the high $K_m$ and the low $K_m$ activity, respectively.

*Effect of 3',5'-GMP*

Addition to the incubation medium of 3',5'-GMP in concentrations up to 100 $\mu$M did not influence the hydrolysis velocity of low concentrations (1.3–2.0 $\mu$M) of 3',5'-AMP. Only at higher concentrations of 3',5'-GMP (1 mM) did significant inhibition of 3',5'-AMP hydrolysis occur (Fig. 6).

*Effects of methylxanthines and papaverine*

The methylxanthines, theophylline and caffeine, are known inhibitors of 3',5'-AMP phosphodiesterase activity. More recently, papaverine was reported to be a more potent inhibitor than the methylxanthines[22–24]. The two methylxanthines and papaverine were therefore tested for possible inhibition of 3',5'-AMP hydrolysis by rat pancreas homogenates. Both methylxanthines cause virtually equal inhibition of the 3',5'-AMP phosphodiesterase activity at both substrate concentrations (Fig. 7). The low $K_m$ activity (half inhibition at 1 mM) is much more sensitive than the high $K_m$ activity (half inhibition at $>$ 10 mM). Papaverine is an even stronger inhibitor: half-maximal inhibition occurs at 3 $\mu$M for the low $K_m$ activity. It also inhibits the low $K_m$ activity at much higher concentrations (half inhibition at 2 mM). A Dixon

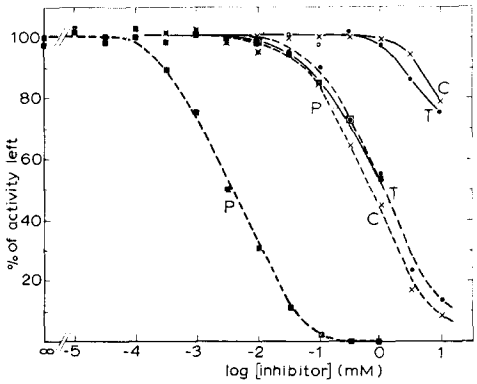



Fig. 7. Inhibition of 3′,5′-AMP phosphodiesterase in rat pancreas by caffeine, theophylline and papaverine. Homogenate dilutions contained 25 mg protein per ml for 30-min incubation at 2 mM 3′,5′-AMP concentration (solid lines) in the presence of varying amounts of caffeine (C), theophylline (T) or papaverine (P). At 4 $\mu$M 3′,5′-AMP concentration (broken lines) the homogenate dilution contained 0.8 mg protein per ml for 10-min incubations.

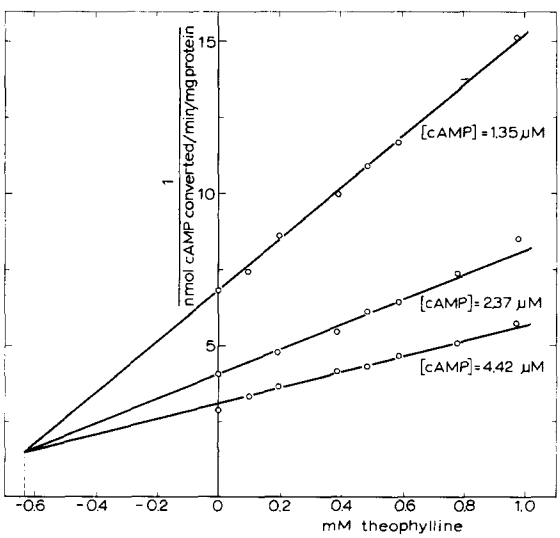



Fig. 8. Dixon plot of the inhibition of 3′,5′-AMP phosphodiesterase in rat pancreas by theophylline. The diluted homogenate contained 0.35 mg protein per ml. Incubations were carried out for 10 min.

plot of the theophylline inhibition of the low $K_m$ activity (Fig. 8) demonstrates that the inhibition is of the competitive type ($K_i$ 0.63 mM). Papaverine (not shown) also inhibits the low $K_m$ activity in a competitive way ($K_i$ 3 $\mu$M).

*Effect of secretory stimulants*

No change in either 3′,5′-AMP phosphodiesterase activity was found when carbachol (10 $\mu$M), secretin (10 units/ml) or pancreozyminoctapeptide (300 units/ml) were added to the incubation medium.

*Solubility of the 3′,5′-AMP phosphodiesterase activity*

The filtered homogenate was centrifuged 1 h at at 225 000 × $g_{max}$ at 0 °C in a Spinco Ti 50 rotor. The sediment was washed twice with 0.9% NaCl. The enzyme activity of the washed sediment was determined and compared with that of the homogenate. The 3′,5′-AMP phosphodiesterase activity of the sediment at 4 $\mu$M and 2 mM substrate concentration amounted to 21 and 16%, respectively, of the corresponding activity in the homogenate. This indicates that at least part of the enzyme activity is particulate.

## DISCUSSION

The 3′,5′-AMP phosphodiesterase activity of rat pancreas was characterized. In the present experiments total pancreas homogenate was used, notwithstanding the fact that this organ contains different cell types. Since the endocrine (islet) tissue comprises only a small percentage of the organ, the results would seem to represent primarily the enzyme occurring in the exocrine tissue. Our finding that about 20% of both activities is apparently membrane bound also needs to be taken into account in further experiments aimed at identifying the location of the enzyme activities.

Our results suggest that two phosphodiesterase activities are present in rat pancreas, one with a high and the other with a low substrate affinity. This phenomenon has already been reported in a number of other tissues in various species[8–14]. The kinetic analysis of the data in these reports is not always adequate. In several cases[10–14] the kinetic parameters $K_m$ and $V$ are simply obtained by plotting $1/v$ *vs* $1/[S]$ or $v$ *vs* $v/[S]$. Such a simplification can lead to considerable errors, as has been shown by Spears *et al.*[25] It will only give correct values when both the $K_m$ and the $V$ values differ greatly. If the $V$ values are not greatly different, reasonably accurate values can only be obtained without correction for the low $K_m$ activity (see Eqn 2 in the Appendix). Accurate values for the other activity can only be obtained by correcting the velocities at high substrate concentrations for the contribution of the low $K_m$ activity. The accuracy of the resulting values for $K_m$ and $V$ can be further improved by repeated cross-correction.

The $K_m$ values for rat pancreas (4.9 and 267 $\mu$M) are of the same order of magnitude as found in other tissues, *e.g.* rat brain[8,27], rat kidney[13,26] and rat pancreatic islets[12]. In rat adipose tissue three different phosphodiesterase fractions were separated, each of which showed different kinetic characteristics[11,28].

In rat pancreas maximal phosphodiesterase activity is reached at lower concentrations of $Mg^{2+}$ (0.5 and 0.75 mM for low $K_m$ and high $K_m$ activities, respectively) than reported in other tissues, where $Mg^{2+}$ concentrations of over 1 mM are necessary. Only Huang and Kemp[10] reported full activation at 0.1 mM $Mg^{2+}$ in rabbit skeletal muscle.

Calcium, in low concentrations, stimulates phosphodiesterase activity in rat brain in the presence of $Mg^{2+}$ (refs 19–21), but such a stimulation was not found in other rat tissues[19]. We found that $Ca^{2+}$ does not stimulate the enzymes in rat pancreas, and has an inhibitory effect on the $Mg^{2+}$-stimulated activity, when added in concentrations above 0.1 mM.

The 3′,5′-AMP phosphodiesterase of many tissues is able to hydrolyse 3′,5′-GMP [8,10,11,21,29,30]. In several cases this nucleotide competitively inhibits the hydro-

lysis of 3′,5′-AMP [8,11,14,29]. In rat liver, however, 0.08–50 $\mu$M 3′,5′-GMP stimulates 3′,5′-AMP hydrolysis (initial concentration: 1 $\mu$M), while it inhibits at concentrations above 50 $\mu$M (ref. 31). In rat thymic lymphocytes the same phenomenon has been observed[14]. In rat pancreas homogenate the hydrolysis of 4 $\mu$M 3′,5′-AMP is not influenced by 3′,5′-GMP in concentrations below 100 $\mu$M, while higher concentrations become inhibitory.

The inhibition of 3′,5′-AMP phosphodiesterase by methylxanthines and by papaverine is a well-known phenomenon. The finding that the inhibition by theophylline is of the competitive type is also in agreement with earlier reports[23]. For papaverine both non-competitive and competitive inhibition have been described[23]. The relative potency of papaverine and theophylline is approximately the same as in other tissues[22–24].

In conclusion, some remarks about the physiological significance of these findings are in order. The low $K_m$ activity would seem to be more important than the high $K_m$ activity for the physiological events in the pancreas, since the 3′,5′-AMP levels in the resting and the stimulated pancreas vary between 0.3 and 10 $\mu$moles/mg (refs 1, 7). The high $K_m$ activity may function as a safeguard against incidentally and locally higher concentrations of 3′,5′-AMP inside the cell, or it may represent a phosphodiesterase specific towards other nucleotides. Another possibility would be that both activities belong to separate enzymes from different types of cells in the pancreas. The total capacity of the phosphodiesterase system is very large compared to that of the adenylate cyclase, even when the latter is stimulated maximally. From our previous data[3] it can be calculated that the rate of hydrolysis at a 3′,5′-AMP concentration of 4 $\mu$M is about 50 times that of the rate of formation. This finding may explain why the 3′,5′-AMP levels in isolated cat pancreas rise only very briefly after stimulation[1].

The relatively high potency of papaverine as an inhibitor of phosphodiesterase in comparison with theophylline agrees with our observation that in the isolated rabbit pancreas papaverine is a more potent stimulant of enzyme secretion than theophylline (unpublished).

## APPENDIX

For two separate enzyme activities I ($V_1$, $K_{m,1}$) and II ($V_2$, $K_{m,2}$), which convert the same substrate, the following relation is valid at any given substrate concentration:

$$v_{\text{total}} = v_1 + v_2 = \frac{V_1 \cdot [S]}{K_{m,1} + [S]} + \frac{V_2 \cdot [S]}{K_{m,2} + [S]} \tag{1}$$

If $K_{m,1} \ll K_{m,2}$ and $V_1 \approx V_2$, Eqn 1 can be simplified for very low $[S]$:

$$v_{\text{total}} = \frac{V_1 \cdot [S]}{K_{m,1} + [S]} = v_1 \tag{2}$$

and for high $[S]$:

$$v_{\text{total}} = V_1 + \frac{V_2 \cdot [S]}{K_{m,2} + [S]} = V_1 + v_2 \tag{3}$$

ACKNOWLEDGEMENT

The excellent technical assistance of Miss Elly Roose is gratefully acknowledged. The authors wish to thank Dr M. Ondetti for his generous gift of pancreozymin-octapeptide.

REFERENCES

1 Case, R. M., Johnson, M., Scratcherd, T. and Sherratt, H. S. A. (1972) *J. Physiol.* London, 223, 669–684
2 Case, R. M. and Scratcherd, T. (1972) *J. Physiol.* London, 223, 649–667
3 Rutten, W. J., de Pont, J. J. H. H. M. and Bonting, S. L. (1972) *Biochim. Biophys. Acta* 274, 201–213
4 Kulka, R. G. and Sternlicht, E. (1968) *Proc. Natl. Acad. Sci. U.S.* 61, 1123–1128
5 Bauduin, H., Rochus, L., Vincent, D. and Dumont, J. E. (1971) *Biochim. Biophys. Acta* 252, 171–183
6 Ridderstap, A. S. and Bonting, S. L. (1969) *Pfluegers Arch. Ges. Physiol.* 313 ,61–70
7 Benz, L., Eckstein, B., Matthews, E. K. and Williams, J. A. (1972) *Brit. J. Pharmacol.* 46, 66–77
8 Thompson, W. J. and Appleman, M. M. (1971) *Biochemistry* 10, 311–316
9 Loten, E. G. and Sneyd, J. G. T. (1970) *Biochem. J.* 120, 187–193
10 Huang, Y. C. and Kemp, R. G. (1971) *Biochemistry* 10, 2278–2283
11 Klotz, U., Berndt, S. and Stock, K. (1972) *Life Sci.* 11 II, 7–17
12 Ashcroft, S. J. H., Randle, P. J. and Täljedal, I. B. (1972) *FEBS Lett.* 20, 263–266
13 Jard, S. and Bernard, M. (1970) *Biochem. Biophys. Res. Commun.* 41, 781–788
14 Franks, D. J. and MacManus, J. P. (1971) *Biochem. Biophys. Res. Commun.* 42, 844–849
15 Rutten, W. J., Schoot, B. M. and de Pont, J. J. H. H. M. (1973) *Biochim. Biophys. Acta* 315, 378–383
16 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
17 Cheung, W. Y. (1971) *Biochim. Biophys. Acta* 242, 395–409
18 Nair, K. G. (1966) *Biochemistry* 5, 150–157
19 Miki, N. and Yoshida, H. (1972) *Biochim. Biophys. Acta* 268, 166–174
20 Kakiuchi, S. and Yamazaki, R., (1970) *Biochem. Biophys. Res. Commun.* 41, 1104–1110
21 Kakiuchi, S., Yamazaki, R. and Teshima, Y. (1971) *Biochem. Biophys. Res. Commun.*, 42, 968–974
22 Pöch, G. (1971) *Arch. Pharmakol.* 268, 272–299
23 Schwabe, U., Berndt, E. W. and Ebert, R. (1972) *Arch. Pharmakol.* 273, 62–74
24 Triner, L., Vulliemoz, Y., Schwartz, I. and Nahas, G. G. (1970) *Biochem. Biophys. Res. Commun.* 40, 64–69
25 Spears, G., Sneyd, E. G. T. and Loten, E. G. (1971) *Biochem. J.* 125, 1149–1151
26 Dousa, T. and Rychlík, I. (1970) *Biochim. Biophys. Acta* 204, 10–17
27 Brooker, G., Thomas, L. J. and Appleman, M. M. (1968) *Biochemistry* 12, 4177–4181
28 Thompson, W. J. and Appleman, M. M. (1971) *J. Biol. Chem.* 246, 3145–3150
29 Beavo, J. A., Hardman, J. G. and Sutherland, E. W. (1970) *J. Biol. Chem.* 245, 5649–5655
30 Song, S. Y. and Cheung, W. Y. (1971) *Biochim. Biophys. Acta* 242, 593–605
31 Beavo, J. A., Hardman, J. G. and Sutherland, E. W. (1971) *J. Biol. Chem.* 246, 3841–3846
32 Hokin, L. E. (1966) *Biochim. Biophys. Acta* 115, 219–221
33 Heisler, S., Fast, D. and Tenenhouse, A. (1972) *Biochim. Biophys. Acta* 279, 561–572