# RELATIONSHIP OF ADENOSINE-3', 5'-MONOPHOSPHATE TO OTHER ADENINE NUCLEOTIDES IN HUMAN PLATELETS

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SUMMARY

Labeled cyclic AMP and other adenine nucleotides in human platelets were distinctly separated by means of thin-layer chromatography. In lysates of human platelets, ATP was decomposed following the major route: ATP→ADP→AMP→IMP→inosine→hypoxanthine. In contrast, cyclic AMP synthesis occurred rapidly following the breakdown of ATP and leveled off after 30-60 min of incubation. Cyclic AMP synthesis in platelet lysates was 1.02 ± 0.39 nanomoles/hr/mg protein. The level of cyclic AMP formed was related to the 5'-AMP level, although the former did not exceed 5 % of the latter.

## Introduction

It has been suggested that intracellular adenosine-3', 5'-monophosphate (cyclic AMP) in human platelets is closely related to their susceptibility to aggregation. The inhibitory effect of prostaglandin $E_1$ ($PGE_1$) on platelet aggregation was presumably mediated by an increased level of cyclic AMP due to the activation of adenyl cyclase (1). This interpretation was further supported by the finding that the inhibition of platelet aggregation with $PGE_1$ was potentiated

by phosphodiesterase inhibitors such as caffeine or theophylline (2, 3). Epinephrine-induced platelet aggregation, accompanied by a decrease of the cyclic AMP level in platelets (4, 5, 6). Phentolamine, an α-adrenergic blocking agent, prevented the cyclic AMP-lowering effect of epinephrine (6, 7, 8), and inhibited the epinephrine-induced platelet aggregation (9). Based on these observations, the hypothesis was proposed that agents which produce or augument platelet aggregation reduce the level of cyclic AMP, and that agents with the opposite effect on platelet aggregation elevate platelet cyclic AMP (8). On the other hand, human platelets can be aggregated by a variety of agents as well as ADP. However, ADP released from platelets has been strongly suggested to be responsible for platelet aggregation with aggregating agents other than ADP (10). From the point of view, the relationship of cyclic AMP to adenine nucleotide metabolism in human platelets would be of importance to establish the role of cyclic AMP on platelet aggregation and its inhibition.

Recently, we described the thin-layer chromatographic separation of labeled adenine nucleotides in human platelets (11). The following method was employed in an attempt to explore the relationship of platelet cyclic AMP to other adenine nucleotides under more sensitive conditions.

## Materials and Methods

Platelet pellets were obtained by centrifugation of 50 ml of citrated platelet-rich plasma at room temperature for 40 min at 2000 g. Platelet pellets were washed once with 5 ml of washing solution (consisting of 0.139 M NaCl, 12.3 mM tris-HCl and 1.54 mM EDTA, pH 7.4). The washed pellets were

resuspended in 2 ml of buffer containing 50 mM tris-HCl, 5 mM $MgSO_4$, 5 mM EDTA, pH 7.3. The platelet suspensions contained approximately 125 x $10^8$ platelet/ml buffer. The platelet suspensions were disrupted completely by rapid freezing in a bath of dry-ice-acetone and thawing in a water bath at 37°C, twice in succession. Protein was determined by the method of Lowry (12).

Platelet lysates were preincubated for 30 min at 37°C, to complete the decomposition of endogenous ATP in lysates. The incubation mixture was composed of 0.2 ml of preincubated platelet lysate, 0.05 ml of saline containing 10 µCi/ml of $^{14}$C-ATP (50 mCi/mMole, Radiochemical Centre, Amersham, England), and 0.15 ml of tris-HCl buffer containing 0.1 mg of phosphoenolpyruvate and 2 µl of pyruvate kinase. The mixture were incubated at 37°C and the reactions were stopped by placing the tubes in boiling water for 3 min, cooled, and centrifuged at approximately 1500 g for 10 min. The supernatant (50 µl) was then spotted on cellulose layers, followed by a mixture of 40 mµmoles of each carrier ATP, ADP, AMP, cyclic AMP, IMP, inosine and hypoxanthine. After two-dimensional thin-layer chromatography and radioautography were performed as described previously (11), using the following solvents: Solvent 1, tert-amyl alcohol/formic acid/water (3:2:1, by volume) (13); Solvent 2, n-butanol/acetone/acetic acid/5 % ammonium hydroxide/water (4.5:1.5:1:1:2, by volume) (14). The areas marked under U.V. light were removed and the radioactivity of the eluates from them were counted in a Beckman LS-200B liquid scintillation spectrometor. To identify cyclic AMP spots, 0.5 ml of the eluates were precipitated with 0.05 ml each of $ZnSO_4$ (0.25 M) and Ba $(OH)_2$ (0.25 M)

solutions as indicated by Krishna (15). More than 80 % radioactivity was detected in the supernatant fraction. In addition, more than 30 % of radioactivity of purified $^{3}$H-adenosine-3', 5'-monophosphate (Radiochemical Centre, Amersham, England) was recovered in a spot of cyclic AMP on chromatogram.

## Results and Discussion

A typical example of the autoradiographic results is shown in Fig. 1, in which the chromatography was carried out

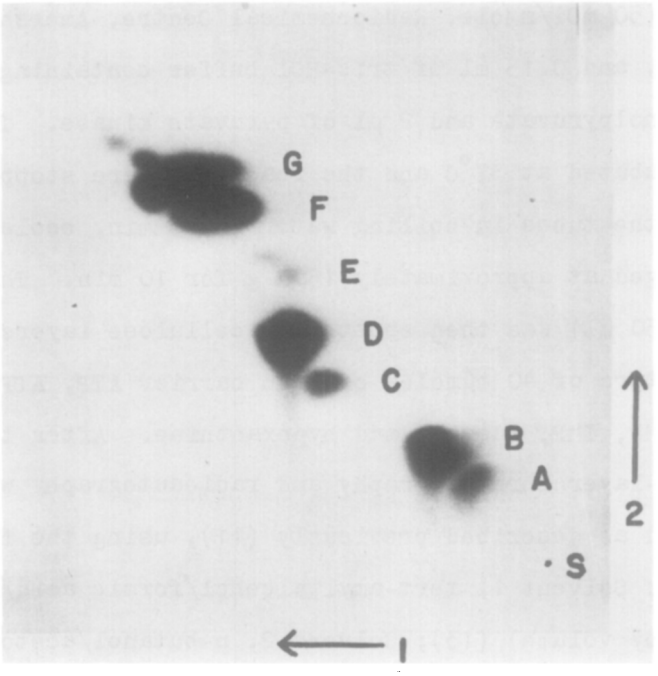


Figure 1. Radioautogram of a two-dimensional chromatogram. 1-, first dimension; 2-, second dimension; S, starting point; A, ATP; B, ADP; C, IMP; D, 5'-AMP; E, cyclic AMP; F, inosine; G, hypoxanthine.

after 60 min of incubation. It would be more satisfactory separation of cyclic AMP compared with the column chrom-

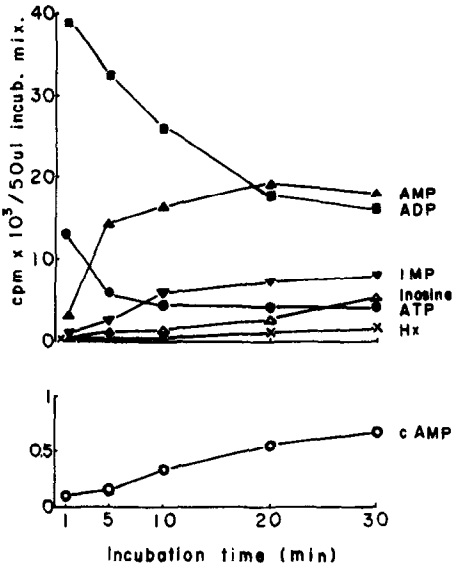


Figure 2. Cyclic AMP synthesis during incubation.

atographic procedure (15). The cyclic AMP synthesis from added $^{14}$C-ATP was observed in relation to other ATP metabolites during incubation (Fig. 2). Labeled ATP was breaking down continuously in platelet lysates following the major route: ATP→ADP→AMP→IMP→inosine→hypoxanthine. It was also confirmed that the breakdown process of exogenous ATP was consistent with that of endogenous ATP, which was formed from $^{14}$C-adenine or $^{14}$C-adenosine in intact platelets and then disrupted by freezing and thawing. In contrast, cyclic AMP synthesis occurred rapidly following the decomposition of $^{14}$C-ATP, as shown in the lower part of Fig. 2, and leveled off after 30-60 min of incubation. In 11 experiments, cyclic AMP synthesis in platelet lysates was estimated 1.02 ± 0.39 nanomoles/hr/mg protein. The relation of cyclic AMP synthesis to the concentration of platelet lysates in the incubation mixture is shown in Fig. 3. Mixtures were incubated at 37°C for 60 min. The breakdown of $^{14}$C-ATP was

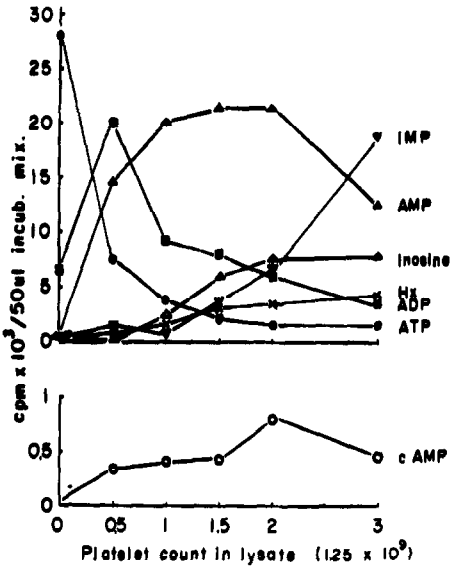


Figure 3. Cyclic AMP synthesis in the platelet lysate with various concentrations.

more rapid in the higher concentration of platelet lysates. More than half of the adenine nucleotides (ATP + ADP + AMP) were converted already to other metabolites (IMP + inosine + hypoxanthine) in the lysate containing $3.75 \times 10^9$ platelets. The synthesis of cyclic AMP was increased in the higher concentration of lysate. The level of cyclic AMP formed appeared to be related to the 5'-AMP level rather than to the ATP level, although the cyclic AMP did not exceed 5 % of the 5'-AMP level. From this observation, it can be concluded that ATP-ase was far predominant over adenyl cyclase in decomposing ATP, the substrate of both enzymes, in the lysate system. The effect of various agents on the cyclic AMP level in platelets in relation to the effect on adenine nucleotide metabolism is presently under investigation. Analysis of the kinetics of enzymes regulating intracellular cyclic AMP, adenyl cyclase and phosphodiesterase, and the effect of

various stimuli on them can be approached also in other tissues by the above thin-layer chromatographic method, in sofar as purification of the enzymes is not attained.

## REFERENCES


1. Wolfe, S.M., and Shulman, N.R., Biochem. Biophys. Res. Commun., 35, 265 (1969).
2. Marquis, N.R., Vighahl, R.L., and Tavormina, P.A., Biochem. Biophys. Res. Commun., 36, 965 (1969).
3. Cole, B., Robinson, G.A., and Hartmann, R.C., Fed. Proc. 29, 316 (1970).
4. Salzman, E.W., and Neri, L.L., Nature, 224, 609 (1969).
5. Zieve, P.D., and Greenough, W.B., Biochem. Biophys. Res. Commun., 35, 462 (1969).
6. Marquis, N.R., Becker, J.A., and Vigdahl, R.L., Biochem. Biophys. Res. Commun., 39, 783 (1970).
7. Robinson, G.A., Arnold, A., and Hartman, R.C., Pharmacol. Res. Commun., 1, 325 (1969).
8. Salzman, E.W., and Levine, L., J. Clin Invest., 50, 131 (1971).
9. O'Brien, J.R., Nature, 200, 763 (1963).
10. Haslam, R.J., Nature, 202, 765 (1964).
11. Murakami, M., and Odake, K., Thromb. Diath. Haemorrh. in press
12. Lowry, O.H., Rosebrough, N.J., Farr, A.J., and Randall, R.J., J. Biol. Chem., 193, 265 (1951).
13. Randerath, K., Biochem. Biophys. Res. Commun., 6, 452 (1961/1962).
14. Randerath, K., J. Chromat., 6, 365 (1961).
15. Krishna, G., Weiss, B., and Brodie, B.B., J. Pharm. Exp. Therap., 163, 379 (1968).