Biochimica et Biophysica Acta, 384 (1975) 159—167

BBA 67438

HYDROLYSIS OF GUANOSINE AND ADENOSINE 3',5'-MONOPHOSPHATES BY RAT BLOOD

W.D. PATTERSON, J.G. HARDMAN and E.W. SUTHERLAND*

Department of Physiology, Vanderbilt University, School of Medicine, Nashville, Tenn. 37232 (U.S.A.)

(Received September 30th, 1974)

Summary

Cyclic nucleotide phosphodiesterase activity was measured in whole blood, plasma, and suspensions of platelets and erythrocytes from rats. In fresh whole blood, apparent phosphodiesterase activity was low, but it rose strikingly during the hour after blood withdrawal. The apparent phosphodiesterase activity in platelet-free plasma showed no such increase, but that in platelet-enriched plasma increased in parallel with that in whole blood. The apparent phosphodiesterase activity of blood or of platelet-enriched plasma also was increased markedly by sonication. The increase in rat blood phosphodiesterase activity with aging thus appeared to be due to damage of platelets.

Most of the phosphodiesterase activity in rat erythrocytes and platelets was located in the soluble fraction of sonicated preparations, but the total enzyme activities from the two sources exhibited marked differences in substrate specificity. With erythrocyte preparations, the rate of hydrolysis of μM concentrations of cyclic AMP was approx. 50 times that of cyclic GMP, while with platelet preparations, cyclic GMP was hydrolyzed about 20 times faster than cyclic AMP at μM levels. The activity of phosphodiesterase in platelets was much greater than that in erythrocytes at all concentrations of both substrates.

Introduction

Along with the growth of interest in measuring cyclic nucleotides in extracellular fluids has grown the need to recognize possible sources of error in making these measurements. One such pitfall could be the failure to recognize or to correct for degradation of the nucleotides during handling or storage of

* Career Investigator, American Heart Association; Department of Biochemistry, University of Miami, Florida, deceased.

the extracellular fluid. Studies with injected tritiated cyclic nucleotides in humans and rats have suggested that the clearance of cyclic AMP or cyclic GMP from the plasma in vivo cannot be accounted for by metabolism or uptake by blood cells [1,2]. However, as Broadus and co-workers [1,3] have shown, cyclic nucleotide phosphodiesterase activity can be detected in vitro in whole blood from several species, and the activity in rat blood appears to be much greater than that in human and dog blood. The presence of this degradative activity in blood may present serious problems in the determination of levels of cyclic nucleotides in plasma or whole blood unless steps are taken to minimize and to correct for its effects.

We have conducted studies to identify the cellular sources and to estimate the magnitude of the phosphodiesterase activity that is detectable in whole and damaged blood from rats. These studies show that enzyme activity progressively increased following withdrawal of the blood from the animal. This apparent increase in phosphodiesterase activity with aging is paralleled by a similar increase in enzyme activity in platelet-enriched plasma, but not in platelet-free plasma, implicating platelets as the major source of the degradative activity seen in whole blood. However, rat erythrocytes also possess a relatively large amount of phosphodiesterase activity, and there are pronounced differences between enzyme activities from this cell type and from platelets.

Materials and Methods

Measurement of cyclic nucleotide phosphodiesterase activity

Phosphodiesterase activity in whole and fractionated blood was measured in vitro at 37°C by two methods. In one of these procedures, the disappearance of cyclic [^{3}H]nucleotides with time of incubation was determined. Assay incubations were initiated by addition of 5 ml of undiluted whole blood, plasma, or buffered cell suspensions to vials containing 5 nmoles of cyclic [^{3}H]AMP or cyclic [^{3}H]GMP (0.02—0.05 μCi) and were terminated at various times by addition of concentrated $HClO_4$ to give a final concentration of 0.3 M. The amounts of the cyclic[^{3}H]nucleotides remaining in the acid extracts were determined by isolating cyclic AMP and cyclic GMP by Dowex-50 column chromatography [4]. In selected experiments, total amounts of both cyclic nucleotides contained in the column fractions were assayed by enzymatic cycling techniques [4,5]. When this method was employed, the exact Mg^{2+} concentrations of undiluted whole blood or plasma were not determined. The levels of cyclic AMP and cyclic GMP in rat plasma and whole blood were found to be in the range of 10^{-8} M [6]. Thus the amounts of these nucleotides introduced into the incubation medium with plasma and whole blood were negligible compared to the amount of cyclic AMP and cyclic GMP added as substrates.

In the other procedure, phosphodiesterase activity was measured by determining both the amount of ^{3}H-labelled product formed and ^{3}H-labelled substrate remaining using anion-exchange chromatography as described by Beavo et al. [7]. Enzyme preparations were incubated for 30 min with various concentrations of cyclic AMP or cyclic GMP (in the presence of 0.1—0.2 μCi of the appropriate ^{3}H-labelled nucleotide) in a medium containing 10 μmol of Tris · HCl buffer (pH 7.5) and 0.5 μmol of $MgCl_2$ in a final volume of 0.25 ml. A

dilution of the enzyme preparation was chosen to produce 10—30% conversion of substrate to product.

Isolation of platelets and erythrocytes from rat blood

Rat blood was collected in centrifuge tubes containing EDTA (final concn 6 mM) at room temperature as described previously [6]. When phosphodiesterase activity in whole blood or platelet-enriched plasma was to be determined, blood was collected with 10 units/ml of heparin instead of the EDTA. Platelet-enriched plasma was separated from erythrocytes and white cells by centrifugation of the blood at 150 × *g* for 20 min. The centrifugation was repeated until contamination of platelets with other cell types constituted less than five cells in ten random high power microscope fields. Platelets were separated from plasma by centrifugation at 2500 × *g* for 10 min and resuspended to their plasma density in a medium containing in mmoles per liter: 145 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 10 mM glucose, 20 mM glycylglycine buffer (pH 7.5), and 1 mg/ml crystallized bovine serum albumin. All of the above steps were performed at 22 °C since platelet survival in vitro is reported to be enhanced at this temperature [8]. Platelets were disrupted by ultrasonic vibration for 30 s at 4 °C using a Branson sonifier. These sonicated preparations could be stored for over a year at —70 °C without loss of phosphodiesterase activity.

Preparations of essentially uncontaminated erythrocytes were obtained by two procedures. After removal of the platelet-enriched plasma as described above, red cells were isolated by three successive washings at 4 °C in 0.9% NaCl solution with aspiration of the upper 5% of the packed cells after each centrifugation. Finally the erythrocytes were washed twice in the buffered solution described above.

In an alternate procedure, red cells were obtained by gradient centrifugation of diluted whole blood [9]. Four volumes of blood that had been diluted 4-fold in 0.9% NaCl solution were layered in a centrifuge bottle over 1 vol. of a solution prepared by mixing 10 parts of 34% (w/v) sodium diatrizoate with 24 parts of 9% Ficoll. After centrifugation for 20 min at 400 × *g* applied to the interface, red cells were collected free of other cell types at the bottom of the bottle and washed several times at 4 °C in the buffer described above. The washed erythrocytes then were suspended in this buffer to a final hematocrit of 40% and sonicated for 1 min at 4 °C. Phosphodiesterase activity was virtually the same in red cell homogenates prepared by either procedure and was not diminished during storage for 1 year at —70 °C. The protein concentration of platelet and erythrocyte preparations corrected for added bovine serum albumin was determined by a modification of the method of Lowry et al. [10] using bovine serum albumin as a standard.

Materials

Cyclic[^{3}H]GMP (4.47 and 24.6 Ci/mmol) was purchased from New England Nuclear Corp. and from ICN and cyclic[^{3}H]AMP (16.5 and 24.1 Ci/mmol) from Schwarz BioResearch and from New England Nuclear. The cyclic[^{3}H]nucleotides were purified on columns of Dowex-50 and stored as neutral aqueous solutions at —70 °C. Cyclic AMP as the free acid was purchased from Schwarz BioResearch, and cyclic GMP as the Na^+ salt was from Boehrin-

ger Mannheim and Nutritional Biochemicals. Neutral aqueous solutions were prepared without further purification and stored at −20 °C.

Dowex resins (100—200 mesh) were purchased from Bio-Rad Laboratories as AG-50 X8 (H^+ form) and AG-2 X8 (Cl^- form) and prepared for use as described by Hardman et al. [4]. Crystalline bovine serum albumin and Tris were obtained from Sigma. Lyophilized *Crotalus atrox* venom was from Ross Allen's Reptile Institute. Sodium diatrizoate (Hypaque, Lot. No. N238FF) was purchased from Winthrop Laboratories and Ficoll from Pharmacia. All other chemicals were of reagent grade from commercial sources. Glass-redistilled water was used for all procedures.

Results

Cyclic nucleotide phosphodiesterase activity in whole blood from rats

The detectable cyclic nucleotide phosphodiesterase activity in fresh whole blood from rats was comparatively low. However, during the first hour after blood withdrawal there was a striking increase in this activity. Fig. 1 depicts the disappearance of both cyclic[^{3}H]nucleotides at 37 °C after their addition in 1 μM concentration to blood that had been kept at 4 °C for varying times after collection. The rate of hydrolysis of 1 μM cyclic GMP was consistently some 2—3-fold greater than that of 1 μM cyclic AMP. As the blood aged in vitro at 4 °C before being incubated at 37 °C cyclic nucleotide phosphodiesterase activity increased progressively for up to 60 min. Little or no further increase in enzyme activity was noted with additional time of preincubation. Phosphodiesterase activity in fresh whole blood was completely inhibited by 6 mM EDTA.

When snake venom nucleotidase was added to the incubation mixtures, the disappearance of ^{3}H-labelled substrate was accompanied by the appearance of stoichiometric amounts of ^{3}H-labelled nucleoside as determined following Dowex-2 chromatography [7]. The specific activity of the cyclic nucleotides measured at each time point remained constant; thus the disappearance of cyclic [^{3}H]nucleotide reflected net loss of total cyclic nucleotide.

Cyclic nucleotide phosphodiesterase activity of platelet-enriched plasma

An apparent explanation for the increase in whole blood phosphodiester-

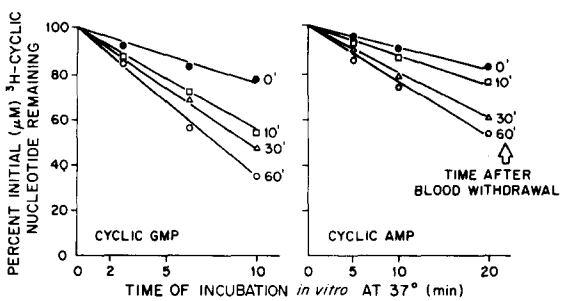

Fig. 1. Degradation of cyclic nucleotides by rat blood in vitro. Rat blood was collected in a chilled vessel and incubated at 4 °C. Aliquots of blood then were taken immediately (0 min) and at 10, 30, or 60 min after withdrawal from the animal and assayed for cyclic nucleotide phosphodiesterase activity at 37 °C by the first procedure as described in Materials and Methods. Note the difference in scales on the abscissas.

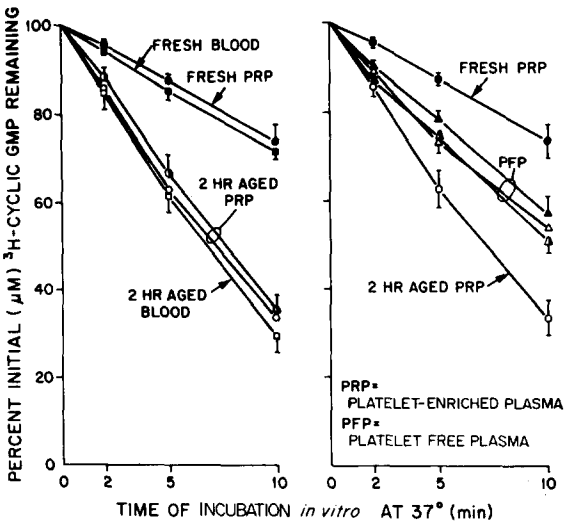

Fig. 2. Degradation of cyclic GMP in whole blood and in platelet enriched plasma. Rat blood was collected and an aliquot was immediately centrifuged at 1400 × *g* for 3 min. The resulting platelet-enriched plasma was separated from the packed cells and diluted in buffered salt solution to bring the platelet density to that in the plasma of the original blood sample. Relatively cell-free plasma was obtained by centrifuging aliquots of the blood sample at 4000 × *g* for 5 min. Cyclic GMP phosphodiesterase activity was determined on aliquots of whole blood, platelet-enriched plasma, or cell-free plasma that were freshly obtained or that were incubated at 4°C for 2 h. Vertical bars through each time point represent standard errors of the mean for four determinations. ■, fresh whole blood; □, aged whole blood; ●, fresh platelet-enriched plasma; ○, platelet-enriched plasma from aged blood; ◑, fresh platelet-enriched plasma aged for 2 h at 4°C; ▲, fresh cell-free plasma; △, cell-free plasma from aged blood; ◭, fresh cell-free plasma aged for 2 h at 4°C.

ase activity was derived from studies with platelet-enriched plasma. As depicted in the left hand panel of Fig. 2, the increase in whole blood phosphodiesterase activity against cyclic GMP was paralleled by a similar increase in platelet-enriched plasma phosphodiesterase activity. This increased activity with aging was observed when the platelet-enriched plasma was obtained from blood which had been preincubated at 4°C for 2 h as well as when fresh platelet-enriched plasma itself was preincubated for this length of time. A variable amount of balanced salt solution containing 2 mM of $MgCl_2$ was added to platelet-enriched plasma to bring the cell density to that of the original whole blood sample. Thus exact comparisons of the phosphodiesterase activities in the two preparations may not be possible.

As seen in the right hand panel of Fig. 2, the apparent phosphodiesterase activity of platelet-free plasma from fresh blood was greater than that in platelet-enriched plasma from fresh blood. Presumably the higher centrifugal forces employed to obtain platelet-free plasma caused platelet damage which resulted in the release of some intracellular phosphodiesterase activity. In contrast to platelet-enriched plasma, there was no further increase in phosphodiesterase activity in platelet-free plasma regardless of the age of the blood from which it was obtained. Although a contribution by other cellular elements is possible, it appears that the increase in cyclic nucleotide phosphodiesterase activity that occurs in whole blood while standing in vitro at 4°C is mainly the result of structural alterations or damage to platelets.

The apparent phosphodiesterase activity in aged platelet-enriched plasma could be increased an additional 10—20-fold by sonication, indicating that only a small fraction of the total enzyme activity was exposed by aging. Hydrolysis of cyclic GMP by sonicated preparations was some 10—20 times faster than that of cyclic AMP in the 1—10-μM range. Thus extensive platelet damage could lead to a disproportionate loss of plasma cyclic GMP compared to cyclic AMP.

Cyclic nucleotide phosphodiesterase activity of isolated platelets

Approx. 90% of the detectable cyclic nucleotide phosphodiesterase activity in sonicated preparations of isolated platelets was in the 30 000 × *g* supernatant fractions. The hydrolysis of cyclic AMP and cyclic GMP by these supernatant fractions was studied over a wide range of substrate concentrations (Fig. 3). At μM order substrate concentrations, cyclic GMP was hydrolyzed up to 15—20 times faster than cyclic AMP, while at 0.2 mM concentrations, cyclic AMP was hydrolyzed faster than cyclic GMP (44 vs 36 nmol/min per mg protein, respectively).

Cyclic nucleotide phosphodiesterase activity of isolated rat erythrocytes

Sheppard and Brughardt [11] first reported the existence of a predominantly soluble cyclic AMP phosphodiesterase activity in the erythrocytes of rats and some other mammalian species. Conceivably then, hemolysis is another form of cell damage that could lead to an increase in apparent phosphodiesterase activity of whole blood. In order to assess the possible effect of the presence of this phosphodiesterase activity on cyclic nucleotide measurements in damaged blood, studies were undertaken to determine the substrate specificity of the enzyme activity in isolated rat erythrocytes.

As shown in Fig. 4 suspensions of apparently intact red cells were capable of hydrolyzing both cyclic AMP and cyclic GMP at a slow rate. However, when the cells were disrupted by sonication, the rate of hydrolysis of cyclic AMP was

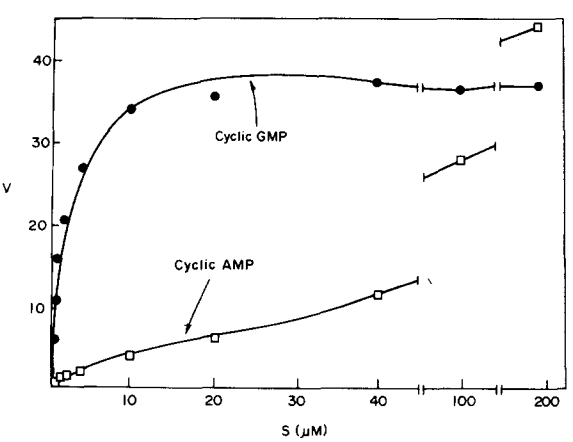

Fig. 3. Hydrolysis of cyclic AMP and cyclic GMP by a 30 000 × *g* supernatant fraction from rat platelets. Cyclic nucleotide phosphodiesterase activity was measured by the second procedure described in Materials and Methods at concentrations of cyclic AMP and cyclic GMP extending from 0.1 to 200 μM. The unit of velocity is nmol of cyclic nucleotide hydrolyzed per min per mg protein.

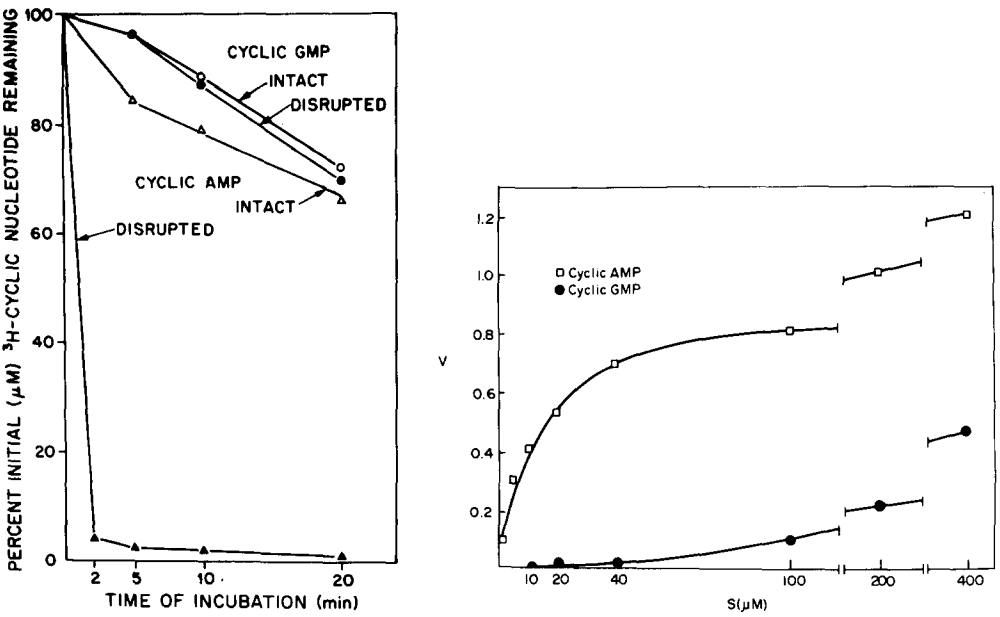

Fig. 4. Hydrolysis of cyclic nucleotides by intact and disrupted rat erythrocytes. Suspensions of intact and sonicated washed erythrocytes were assayed for cyclic nucleotide phosphodiesterase activity by the first procedure described in Materials and Methods.

Fig. 5. Hydrolysis of cyclic AMP and cyclic GMP by a 30 000 × *g* supernatant fraction from rat erythrocytes. Cyclic nucleotide phosphodiesterase activity was measured by the second procedure described in Materials and Methods at concentrations of cyclic AMP and cyclic GMP extending from 0.1 to 400 μM. The unit of velocity is nmol of cyclic nucleotide hydrolyzed per min per mg protein.

greatly increased while that of cyclic GMP was virtually unchanged with respect to intact cell suspensions. Thus erythrocyte damage alone could lead to a disproportionate loss of plasma cyclic AMP compared to cyclic GMP.

Approx. 90% of the enzyme activity against either cyclic nucleotide was located in the 30 000 × *g* supernatant fractions of sonicated erythrocyte preparations. When the hydrolysis of cyclic AMP and cyclic GMP by these preparations was studied over a wide range of substrate concentrations, the results were in striking contrast to those obtained with platelet extracts. The rate of hydrolysis of cyclic AMP was 50—70 times that of cyclic GMP at μM-order substrate levels, and velocities at 0.4 mM were approx. 1.2 nmol per min per mg protein for cyclic AMP and 0.5 for cyclic GMP (Fig. 5).

Additional experiments indicated that the slow hydrolysis of cyclic [^{3}H]nucleotides by suspensions of apparently intact erythrocytes was not the result of uptake of the intact compounds from the surrounding medium. Red cell suspensions were incubated for various lengths of time at 37 °C with 1 μM cyclic [^{3}H]AMP or cyclic [^{3}H]GMP. Aliquots of the red cell suspensions were removed from the incubation mixtures, diluted 6-fold in cold 0.9% NaCl solution, and layered over 1 ml of 10% albumin dissolved in 0.9% NaCl in a centrifuge tube. After centrifugation at 2500 × *g* for 10 min, the cell pellets were recovered and deproteinized with $HClO_4$. Cyclic [^{3}H]nucleotides were determined following Dowex-50 chromatography [4] as described previously [6].

There was no detectable entry of cyclic [^{3}H]AMP or cyclic [^{3}H]GMP from the surrounding medium into rat erythrocytes at any time during 90 min of incubation.

Discussion

As is true for other tissues, cyclic AMP and cyclic GMP degradative activity must be reckoned with in collecting and handling blood for determinations of the two nucleotides. Phosphodiesterase activity has been found in rat, dog and human whole blood [1,3], in human platelets [12—14] and white cells [15], and, as results in this and other papers show, in rat erythrocytes [11,16] and platelets. Since damage of these cells leads to the release of considerable phosphodiesterase activity and since some phosphodiesterases can retain considerable activity at low temperatures [17,18], careful storage of blood, plasma, or other material in which enzyme activity is suspected is necessary.

The cyclic nucleotide degradative activity measured in vitro in rat blood can be influenced significantly by the formed elements. Freshly obtained rat blood displays phosphodiesterase activity which increases with time following withdrawal. Platelets contribute significantly to this increase, probably as a result of structural alterations which increase the access of extracellular cyclic nucleotides to the enzyme. Electron microscopic studies have shown that platelet structural changes caused by chilling closely resemble those signalling the development of stickiness and aggregation [19]. Formation of aggregates would normally lead to the disruption of some cells, thus exposing intracellular phosphodiesterase to cyclic nucleotides in the surrounding medium. In studies with human platelets, only 70% of the cells were found to be viable after reinfusion into the donor without a storage interval [8]. Perhaps the increase in phosphodiesterase activity observed in whole rat blood or platelet-enriched plasma is related to a similar "lesion" of blood withdrawal and handling.

In human platelets, phosphodiesterase activity is intracellular and can not be demonstrated in carefully prepared platelet-enriched plasma [20]. However, centrifugation and resuspension of the cells is associated with the appearance of enzyme activity which is platelet bound, the supernatant plasma containing no phosphodiesterase [21,22]. This finding is in contrast to our results with cell-free plasma of the rat, which does contain demonstrable phosphodiesterase activity.

Hemington et al. [16] also have shown cyclic nucleotide phosphodiesterase activity in rat plasma. These workers reported that the enzyme activity was unrelated to the plasma heme concentration but they could not rule out the possibility that it was due to disruption of platelets or other formed elements in the plasma. They also showed evidence for considerable cyclic GMP phosphodiesterase activity in erythrocytes at 10^{-7} M substrate concentrations, which is in contrast to the data presented in this paper. However, it is not clear that the erythrocytes used by these workers were uncontaminated with other blood cells.

Numerous reports describing the properties of cyclic nucleotide phosphodiesterase activities from various sources have appeared recently (e.g. [7,23—26]). In contrast to total phosphodiesterase activity in other crude prepara-

tions, that from sonicated rat erythrocytes displays a striking preference for cyclic AMP over cyclic GMP at low concentrations. Rat platelet phosphodiesterase activity, on the other hand, displays a striking preference for low concentrations of cyclic GMP over cyclic AMP. Such distinct substrate specificities in crude extracts are rare, but similar findings with partially purified preparations from various tissues of rats or rabbits have been reported [23—26].

Acknowledgements

This work was supported by Grants GM-16811 and AM-07462 from N.I.H. W.D.P. is a recipient of a Vivian B. Allen Foundation Fellowship.

References

1 Broadus, A.E., Kaminsky, N.I., Hardman, J.G., Sutherland, E.W. and Liddle, G.W. (1970) J. Clin. Invest. 49, 2222
2 Butlen, D. and Jard, S. (1972) Pflügers Arch 331, 172
3 Broadus, A.E., Hardman, J.G., Kaminsky, N.I., Ball, J.G., Sutherland, E.W. and Liddle, G.W. (1971) Ann. N.Y. Acad. Sci. 185, 50
4 Hardman, J.G., Davis, J.W. and Sutherland, E.W. (1969) J. Biol. Chem. 244, 6354
5 Ishikawa, E., Ishikawa, S., Davis, J.W. and Sutherland, E.W. (1969) J. Biol. Chem. 244, 6371
6 Patterson, W.D., Hardman, J.G. and Sutherland, E.W. (1974) Endocrinology, 95, 325
7 Beavo, J.A., Hardman, J.G. and Sutherland, E.W. (1970) J. Biol. Chem. 245, 5649
8 Murphy, S. and Gardner, F.H. (1969) N. Engl. J. Med. 280, 1094
9 Perper, R.J., Zee, T.W. and Mickelson, M.M. (1968) J. Lab. Clin. Med. 72, 842
10 Lowry, O.H., Rosebrough, N.J. Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265
11 Sheppard, H. and Burghardt, C.R. (1970) Mol. Pharmacol. 6, 425
12 Abdullah, Y.H. (1969) J. Atheroscler. Res. 9, 171
13 Marquis, N.R., Vigdahl, R.L. and Tavormina, P.A. (1969) Biochem. Biophys. Res. Commun. 36, 965
14 Song, S. and Cheung, W.Y. (1971) Biochim. Biophys. Acta 242, 593
15 Bourne, H.R., Lehrer, R.I. Cline, M.J. and Melmon, K.L. (1971) J. Clin. Invest. 50, 920
16 Hemington, J.G., Chenoweth, M. and Dunn, A. (1973) Biochim. Biophys. Acta 304, 552
17 Davoren, P.R., and Sutherland, E.W. (1963) J. Biol. Chem. 238, 3009
18 O'Dea, R.F., Haddox, M.K. and Goldberg, N.D. (1971) J. Biol. Chem. 246, 6183
19 White, J.G. and Krivit, W. (1967) Blood 30, 625—635
20 Salzman, E.W. and Levine, L. (1971) J. Clin. Invest. 50, 131—141
21 Salzman, E.W. and Weisenberger, H. (1972) Adv. Cyclic Nucleotide Res. 1, 231—247
22 Salzman, E.W. (1972) N. Engl. J. Med. 286, 358—363
23 Thompson, W.J. and Appleman, M.M. (1971) J. Biol. Chem. 246, 3145—3150
24 Huang, Y. and Kemp, R.G. (1971) Biochemistry 10, 2278—2283
25 Russell, T.R., Terasaki, W.L. and Appleman, M.M., (1973) J. Biol. Chem. 248, 1334—1340
26 Appleman, M.M., Thompson, W.J. and Russell, T.R. (1973) Adv. Cyclic Nucleotide Res. 3, 65—98