Biochimica et Biophysica Acta, 341 (1974) 402–411

BBA 67183

CYCLIC 3′,5′-NUCLEOTIDE PHOSPHODIESTERASE. DISTRIBUTION AND DEVELOPMENTAL CHANGES OF THE ENZYME AND ITS PROTEIN ACTIVATOR IN MAMMALIAN TISSUES AND CELLS

JAMES ALVIN SMOAKE*, SEUNG-YIL SONG** and WAI YIU CHEUNG***

Laboratory of Biochemistry, St. Jude Children's Research Hospital, 332 North Lauderdale, P.O. Box 318, and Department of Biochemistry, University of Tennessee Medical Units, Memphis, Tenn. 38101 (U.S.A.)

(Received November 12th, 1973)

SUMMARY

The distribution of the activities of a cyclic 3′,5′-nucleotide phosphodiesterase and its protein activator was determined in mammalian tissues and cells. Changes in these activities during development were also studied. The ratio of the activity of the activator to that of the phosphodiesterase varied greatly from tissue to tissue as well as during development, suggesting that the two proteins are under separate genetic control. In contrast, the distributions of the activities of phosphodiesterase and its activator were parallel in subcellular fractions indicating possible physiological regulation of the enzyme by its activator.

INTRODUCTION

A specific protein activator of cyclic 3′,5′-nucleotide phosphodiesterase, originally identified in bovine brain [1, 2], has now been described in other mammalian tissues in several different laboratories [3–5]. In crude tissue homogenates the activator appears to be in excess of phosphodiesterase. During enzyme purification it dissociates from phosphodiesterase, and the purified enzyme requires exogenous activator for maximum activity [2]. Kinetic studies with purified phosphodiesterase have shown that the activator decreases the K_m for cyclic AMP and increases the maximum velocity (V) of the enzyme [2, 3, 5].

The presence of an activator specific for phosphodiesterase raises the possibility of physiological regulation of the enzyme by the activator, but such modulation has not been demonstrated. One way to approach this question is to determine the distribution and developmental changes of the enzyme and its activator in mammalian tissues and cells. In this communication we describe the distribution of the activities of phosphodiesterase and its activator in rat and human tissues, their localization in

* Present address: Biology Department, New Mexico Tech, Socorro, N.M. 87801.
** Present address: Jimmy Fund Building, Harvard Medical School, Boston, Mass. 02115.
*** To whom correspondence should be addressed.

subcellular fractions and their changes during development. A preliminary account of this work has appeared [6].

MATERIALS AND METHODS

Chemicals and reagents

Cyclic AMP and cyclic [^{3}H]AMP were obtained from Schwarz BioResearch, Orangeburg, N.Y. The labeled nucleotide was purified by thin-layer chromatography on cellulose sheets without fluorescent indicator (Eastman Kodak, Rochester, N.Y.). Lyophilized snake venom (*Crotalus atrox*), collagenase, hyaluronidase, and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, Mo.

Preparation of phosphodiesterase from rat tissues

Tissues were obtained from adult male Sprague–Dawley rats weighing 360–400 g. Each animal was killed by cervical dislocation and the whole body was perfused with cold physiological saline via the dorsal aorta. Two tissues were excised from each animal, placed on ice, blotted dry and weighed. The tissues were homogenized in 5 volumes of cold 20 mM Tris–Cl, pH 7.5, in a Potter–Elvehjem homogenizer with a teflon pestle turning at a speed of 1000 rev./min. Homogenates were dialyzed against the Tris–Cl buffer overnight at 4 °C before analysis of phosphodiesterase and its activator.

Preparation of rat liver cells

Liver cells were obtained by a slight modification of the method of Berry and Friend [7]. Briefly, animals were anesthetized by intraperitoneal injection of Nembutal (5 mg/100 g of body weight). The livers were perfused with Hank's Ca^{2+}-free buffer to remove blood and, after excision, were perfused for 30 min with this buffer containing 0.05% collagenase and 0.1% hyaluronidase. Liver cells were obtained by shaking. filtering and repeated washings. Subcellular fractions were prepared from isolated parenchymal cells by differential centrifugation [8]. Since phosphodiesterase and the activator lost activity upon dialysis, the subcellular fractions were assayed for these two proteins without dialysis.

Isolation of human blood elements

Erythrocytes, leukocytes, platelets and plasma were isolated at 4 °C from freshly drawn human blood as previously described [9]. Contamination of the platelet preparation by leukocytes and erythrocytes was minimized by repeating the centrifugation at $120 \times g$ for 10 min. The morphology of the platelets and leukocytes appeared intact under a phase-contrast microscope. Cells were counted manually under a phase-contrast microscope. Isolated elements were suspended in 0.25 M sucrose containing 1 mM EDTA. The platelets were adjusted to 1×10^{10} cells/ml and the leukocytes to 5×10^8 per ml. The cells were disrupted by sonication in an Ultrasonifier Model W185 for 10 s at a setting of 4. The process was repeated twice with 1-min intervals for cooling. The time lapse between the collection of blood and assay of the enzyme and activator was about 4 h.

Preparation of organelles from platelets

Organelles from platelets were isolated by a slight modification of the method of Holmsen et al. [10]. Platelets isolated from 2 units of blood were adjusted to 1×10^{10} cells/ml in 0.44 M sucrose containing 0.001 M EDTA and homogenized in a Kontes homogenizer fitted with a teflon pestle with "no clearance". The homogenizer and pestle were washed twice with 1 ml of the medium, and the washings were combined with the homogenates. The combined sample was centrifuged at $2250 \times g$ for 15 min to obtain a sediment composed mainly of intact or partially disrupted platelets. The sediment was homogenized and centrifuged again, and the supernatants were combined. This step was repeated if the quantity of sediment was significant. One ml of the supernatant was applied to a continuous sucrose gradient ranging from 0.89–1.97 M.

The gradients were centrifuged at $130\,000 \times g$ for 2 h and the sucrose density was routinely monitored with a refractometer. Five fractions were collected and each was examined by electron microscopy. The fractions contained soluble proteins (A), membranes (B), membranes, alpha granules and mitochondria (C), mitochondria and alpha granules (D), and alpha granules with bull's eye and very dense bodies (E). Fraction E contained intact or partially ruptured platelets which were removed by centrifugation at $2250 \times g$ for 15 min. The densities of the borders were 1.100 between A and B, 1.127 between B and C, 1.150 between C and D and 1.190 between D and E.

The fractions were diluted with 20 mM Tris–Cl, pH 7.5, containing 0.001 M EDTA to a final sucrose concentration of 0.25 M. All fractions were then centrifuged at $166\,700 \times g$ for 45 min and the sediments were suspended in the Tris–EDTA buffer. Fraction A was diluted with this buffer to a sucrose concentration of 0.1 M and then was centrifuged at $30\,000 \times g$ for 45 min. The precipitate contained membranes and was added to Fraction B. All fractions were rapidly frozen, thawed twice, and assayed immediately for phosphodiesterase and its activator.

Assay of phosphodiesterase

Three methods, each a two-stage assay, were used to determine phosphodiesterase activity. Method I was a modified procedure [2] of Butcher and Sutherland [11] in which inorganic phosphate was determined after its release from 5′-AMP via the action of snake venom 5′-nucleotidase. Method II, an isotopic assay, was essentially that of Thompson and Appleman [12]. Method III, also an isotopic assay, was described in an earlier report [8]. The reaction mixture in each assay method contained 40 mM Tris–Cl, pH 8, 2 mM cyclic AMP and an optimal concentration of Mn^{2+}. With 2 mM of cyclic AMP, the total activities of phosphodiesterase (low and high K_m values) were determined. The Mn^{2+} concentration required for maximum phosphodiesterase activity was determined for each tissue examined. The following concentrations (mM) of Mn^{2+} were found to give maximum activity and were therefore used: brain, 0.1; adrenal gland, 0.2; liver, 2.0; testis, 2.0; bone marrow, 2.0; blood, 2.0; kidney, 4.0; epididymal fat pad, 4.0; thymus, 4.0. Specific activity was expressed as nmoles of cyclic AMP hydrolyzed per mg protein per min.

Assay of activator

The activator was assayed for its ability to stimulate the activity of phosphodiesterase partially purified from bovine brain [2]. The partially purified phospho-

diesterase in our experiments was relatively inactive due to loss of the activator during enzyme purification, but was activated 5- to 10-fold by exogenous activator.

Activator activity was expressed either as stimulated activity or as specific activity. Stimulated activity was determined as follows: a tube with 0.1 mM Mn^{2+}, 40 mM Tris–Cl, pH 8, and a sample containing the activator was heated in a boiling bath for 3 min. Boiling inactivates phosphodiesterase but not the activator [2]. Twenty-five micrograms of a purified phosphodiesterase was then added to the tube and the reaction was initiated by the addition of cyclic AMP. Subsequent steps were identical to those for the phosphodiesterase assay. The basal activity of the purified phosphodiesterase was subtracted from the total activity and the corrected value was defined as stimulated activity.

Specific activity of the activator was determined by titrating partially purified phosphodiesterase with the boiled homogenate of a tissue or a subcellular fraction. The reaction mixture contained 40 mM Tris–Cl, pH 8.0, 0.1 mM Mn^{2+} and partially purified phosphodiesterase (50 μg for assay of activator in a tissue homogenate and 5 μg for assay of activator in a subcellular fraction) in a final volume of 0.5 ml. The reaction was initiated by adding 2 mM cyclic AMP. The rest of the procedure was identical to that described for the assay of phosphodiesterase. The amount of protein (μg) required to produce a 50% activation of partially purified phosphodiesterase was defined as 1 unit of activator. Specific activity was expressed as units per mg protein.

Protein determination

Proteins were determined either by use of the biuret reagent containing sodium desoxycholate or by the method of Lowry et al. [13]. Bovine serum albumin was used as a standard.

All assays were duplicated.

RESULTS

Tissue distribution of phosphodiesterase and its activator

The distribution of the phosphodiesterase and its activator was determined in 8 different tissues from adult male rats. As shown by the results in Table I, the activities of the two proteins were not parallel. In the brain, for example, phosphodiesterase and activators activities were both high, whereas testicular enzyme activity was low but activator activity high. 6 of the tissues could be paired on the basis of comparable phosphodiesterase activities: (a) adrenal gland and kidney, (b) epididymal fat pad and bone marrow, and (c) liver and testis. However, activator activities in these groups differed by factors of 2, 3, and 9, respectively. The variability of the ratios of the two activities in different tissues may be a result of cellular heterogeneity.

Developmental aspects of phosphodiesterase and its activator

Changes in the activities of phosphodiesterase and its activator during development were examined in rat brain, testis, thymus and liver. Fig. 1a shows the changes in these activities in brain from 8 days before birth through adulthood. During this period the activity of the enzyme increased 25-fold while that of its activator doubled. Testicular phosphodiesterase (Fig. 1b) displayed a different pattern: enzyme activity decreased from birth to adulthood while activator activity increased 4-fold. The

TABLE I

DISTRIBUTION OF PHOSPHODIESTERASE AND ITS ACTIVATOR IN ADULT RAT TISSUES

Phosphodiesterase activity was determined by Method I as described in the text. About 0.2–1 g of each tissue was used in each experiment. Nine adrenal glands were pooled for one assay. The bone marrow was obtained from the femur.

Tissue*	Specific activity (mean ± S.E.)	
	Phosphodiesterase (nmoles/mg protein per min)	Activator (units/mg protein)
Brain (4)	24.5 ± 2.9	22.9 ± 2.8
Adrenal gland (2)	3.9	7.4
Kidney (4)	3.6 ± 0.4	4.4 ± 0.3
Epididymal fat pad (4)	2.0 ± 0.3	8.9 ± 0.6
Bone marrow (3)	1.8 ± 0.06	3.0 ± 0.4
Liver (4)	1.2 ± 0.1	4.7 ± 0.3
Testis (4)	1.1 ± 0.2	43.5 ± 2.9
Thymus (4)	0.6 ± 0.02	9.9 ± 1.3

* Number of experiments given in parentheses.

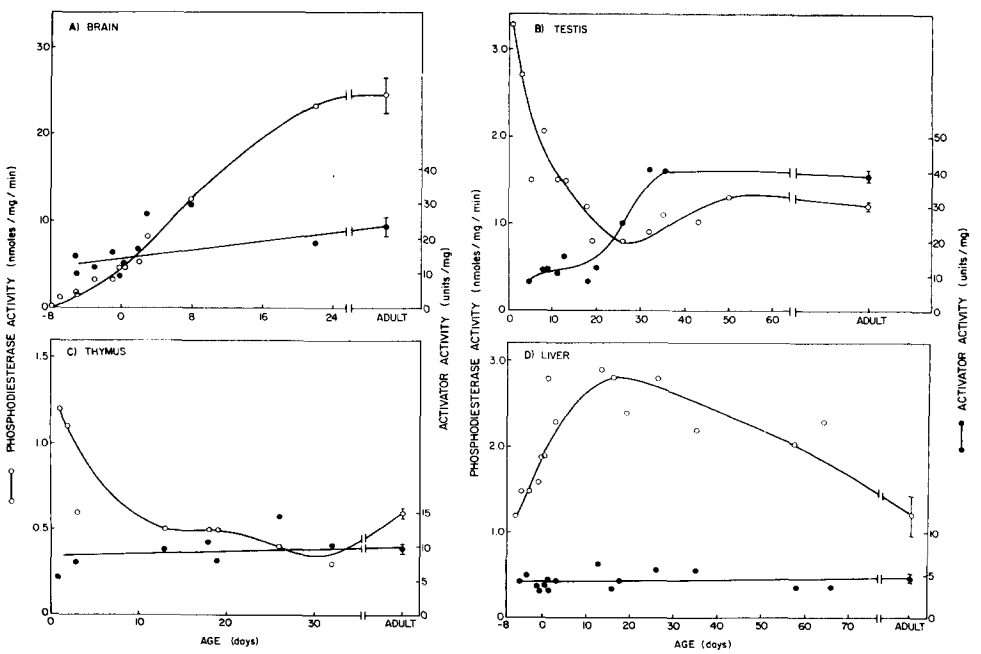

Fig. 1. Developmental changes of phosphodiesterase and its activator in rat tissues. Phosphodiesterase activity (○—○) was determined by Method I as described in the text. Activator activity (●—●) is expressed as specific activity. Each point on this curve represents all the tissues pooled from 1 litter of 10 to 12 fetuses or animals in the earlier stage of development. Part or all of one tissue was used in subsequent stages.

developmental pattern of phosphodiesterase activity in the thymus (Fig. 1c) appeared biphasic in an upward concave direction; the activator showed virtually no change from birth to adulthood. The liver (Fig. 1d) exhibited still a different pattern: the changes in phosphodiesterase were biphasic in a downward concave direction, whereas the activator remained constant throughout this period.

This apparent lack of correlation between the activities of phosphodiesterase and its activator in a given tissue during development may indicate separate genetic regulation of the two proteins. Alternately, it may be a result of cellular heterogeneity.

Phosphodiesterase and its activator in human blood

Blood is a tissue composed of several types of cells suspended in plasma. Because its elements can be separated into relatively homogeneous fractions by differential centrifugation, blood offers a good system with which to examine the distribution of the activities of phosphodiesterase and its activator in each cell population.

Table II shows the cellular distribution of phosphodiesterase and activator activities in human blood. The erythrocytes contained 29% of the phosphodiesterase and 87% of the activator, while the platelets contained 66% of the phosphodiesterase and 10% of the activator. The leukocytes had trace levels of enzyme activity but no activator activity. The plasma had neither.

TABLE II

CELLULAR DISTRIBUTION OF PHOSPHODIESTERASE AND ITS ACTIVATOR IN HUMAN BLOOD

Phosphodiesterase activity was determined by Method III as described in the text. The cells in each fraction were counted manually under a phase microscope. The isolated platelets were free of erythrocytes and leukocytes. However, erythrocyte and leukocyte fractions were contaminated with formed elements. Therefore, the data on phosphodiesterase and activator activity in these two fractions were corrected for these contaminants on the basis of cell counts as described previously [9].

Fraction	Phosphodiesterase		Activator	
	Total activity (pmoles/ml of blood per min)	Distribution (%)	Stimulated activity (nmoles/ml of blood per min)	Distribution (%)
Whole blood	8700	100	306	100
Erythrocytes	2500	29	270	88
Leukocytes	246	3	0	0
Platelets	5700	66	30	10
Plasma	0	0	0	0

Table III shows the specific activities of the two proteins in the blood elements. The ratio of activator activity to enzyme activity varied greatly in the different cell populations.

Most tissues consist of several cell types and their ratios may vary from tissue to tissue. Each cell type may have its own ratio of the enzyme to its activator. This may account for the apparent lack of parallel distribution of the activities of the two proteins in different tissues (Table I) and during development of the same tissue (Fig. 1).

TABLE III

PHOSPHODIESTERASE AND ITS ACTIVATOR IN HUMAN BLOOD ELEMENTS

Phosphodiesterase activity was determined by Method II as described in the text. Isolated platelets were free of erythrocytes and leukocytes. Activity in erythrocytes and leukocytes has been corrected for contamination as described in the legend to Table II.

Cell type	Specific activity	
	Phosphodiesterase (nmoles/mg protein per min)	Activator (units/mg protein)
Erythrocytes	0.01	6.4
Leukocytes	0.74	0
Platelets	3.60	74

Subcellular distribution of phosphodiesterase and its activator

Since platelets contain both phosphodiesterase and activator and can be obtained in relatively large quantities free of contamination of other blood elements, they were examined for the subcellular distribution of these proteins. Table IV shows that about 70% of the activities of phosphodiesterase and its activator were present in the soluble fraction. Trace activities of phosphodiesterase were distributed in other fractions. In contrast to their tissue distribution, the activities of phosphodiesterase

TABLE IV

SUBCELLULAR DISTRIBUTION OF PHOSPHODIESTERASE AND ITS ACTIVATOR IN HUMAN BLOOD PLATELETS

The 5 fractions, A to E, were prepared from 2 units of blood as described under "Materials and Methods" and were defined therein. Phosphodiesterase activity was determined by Method III as described in the text.

Fractions	Phosphodiesterase		Activator	
	Total activity (pmoles/fraction per min)	Distribution (%)	Stimulated activity (pmoles/fraction per min)	Distribution (%)
Homogenate	1230	100	4700	100
A	900	73	3300	70
B	33	2.8	197	4.2
C	20	1.7	128	2.7
D	22	1.8	220	4.6
E	28	2.3	130	2.7

and its activator in these subcellular fractions appeared parallel to each other. Because blood platelets are not "true cells," these results may not be typical of most cell types.

The rat liver is made up predominantly of parenchymal cells. A homogeneous population of these hepatocytes can be isolated with retention of morphological and physiological attributes. Subcellular fractions were therefore prepared from isolated parenchymal cells and the distribution of the activities of phosphodiesterase and its activator were examined in each fraction. Table V shows that the activities of the two proteins were localized in the supernatant, nuclear and microsomal fractions. The

TABLE V

SUBCELLULAR DISTRIBUTION OF PHOSPHODIESTERASE AND ITS ACTIVATOR IN LIVER PARENCHYMAL CELLS

The liver of an anesthetized rat was excised and perfused with a Hank's Ca^{2+} free buffer containing collagenase and hyaluronidase. Subcellular fractions were prepared from the isolated parenchymal cells as described in the text. Phosphodiesterase activity was determined by Method II. The data represent the average of two separate experiments.

Fractions	Phosphodiesterase		Activator	
	Total activity (nmoles/fraction per min)	Distribution (%)	Total activity (units/fraction)	Distribution (%)
Homogenate	149	100	2139	100
Supernatant	52	35	950	44
Nuclear	39.5	27	342	16
Mitochondrial	4.7	3	0	0
Microsomal-membrane	17.1	11.5	246	12

mitochondrial fraction had traces of phosphodiesterase activity and little or no activator activity, reminiscent of the result obtained with human leukocytes (Table II). Table V also shows that the distribution of the activities of the two enzymes was parallel, in agreement with the results obtained from human blood platelets.

DISCUSSION

The present study has shown that the ratios of the activities of phosphodiesterase and its activator vary from tissue to tissue as well as during development of a given tissue. We have considered that cellular heterogeneity may cause variable ratios of these activities.

The presence of multiple forms of phosphodiesterase in different tissues may be another factor. Several studies have demonstrated more than one form of phosphodiesterase, on the basis of either different Michaelis constants [12, 14], gel filtration [15] or electrophoretic mobility [16, 17]. Weiss and Strada [18] found that the ratio of the high to low K_m phosphodiesterase was markedly different in various areas of rat brain. Uzunov and Weiss [17] reported six distinct fractions of phosphodiesterase activity in preparative polyacrylamide gel electrophoresis of rat cerebellar homogenates, but only two of these fractions were responsive to the activator, suggesting the existence of activator-dependent and activator-independent forms of the enzyme. The existence of two forms of the enzyme has been postulated previously [2, 19]. The distribution of the activities of phosphodiesterase and its activator in the blood appears consistent with this notion. We show in Tables II and III that leukocytes contain phosphodiesterase but no activator activity, indicating a form of enzyme independent of the activator.

The specific activity of phosphodiesterase was very low in erythrocytes. The trace of activity may be due to contamination by platelets, leukocytes or reticulocytes. However, the specific activity of the erythrocyte activator was more than can be accounted for by such contaminants. The apparent marked variance of these two

activities in the erythrocytes may suggest differential los of activities of the two proteins.

Multiple forms of phosphodiesterase have been reported in the platelets [20]. The preponderance of the activity of the enzyme over that of the activator in the platelets may mean either an inherently relative excess of the activator-independent form, or a differential loss of activator activity. Although the half lives of the enzyme and its activator have not been determined, a faster loss of activator activity in the platelets appears unlikely. First, the life span of human blood platelets in circulation is about 10 days [21], and is much shorter than that of the erythrocytes (120 days). Nevertheless, the erythrocytes account for the majority of the activator in the blood. Second, while phosphodiesterase activity in the several tissues studied changes during development, the activator remains relatively constant.

Several studies on the developmental changes of phosphodiesterase activity in brain [22] and testis [23] have been reported, but work on activator activity during development has not. Our data on phosphodiesterase from brain and testis indicate patterns of change similar to those reported earlier [22, 23]. Although the activator activity in brain and testis changed during development, the changes were not striking compared to those of phosphodiesterase. The variability of the ratios of the two activities may indicate independent genetic regulation of the two proteins.

Although distributions of the activities of the two proteins were not parallel in various tissues, or during development of several tissues examined, they were parallel in isolated rat parenchymal cells and in human blood platelets. Although parallel activities by these two proteins in subcellular fractions suggested regulation of phosphodiesterase by the activator, the mechanism of physiological modulation remains unknown. Note that parallelism does not exclude the possibility that the two proteins may exist as separate entities in the cell. Association of the enzyme and its activator may be a consequence of homogenization of the cells. The association, once formed, may not be readily reversible because of the two proteins' mutual affinity [2]. Were the activator and the enzyme present as distinct entities, it is conceivable that the activator-dependent form may be inactive in situ.

In summary, the present study reveals apparent parallel distribution of the activities of phosphodiesterase and its activity in subcellular fractions of isolated rat hepatocytes and human blood platelets, indicating possible regulation of phosphodiesterase activity by the activator. On the other hand, the ratios of the two activities are highly variable from tissue to tissue and during development. This vairability may be explicable on the basis of cellular heterogeneity or multiple forms of phosphodiesterase.

ACKNOWLEDGEMENTS

This work was supported by ALSAC and by grants NS-08059, CA-13537 and CA-08480 from the United States Public Health Service. J.A.S. is a multidisciplinary postdoctoral trainee (CA-05176) and W.Y.C. is the recipient of a United States Public Health Service Research Career Development Award (NS-42576).

REFERENCES

1 Cheung, W. Y. (1969) Biochim. Biophys. Acta 191, 303–315
2 Cheung, W. Y. (1971) J. Biol. Chem. 246, 2859–2869
3 Goren, E. N. and Rosen, O. M. (1971) Arch. Biochem. Biophys. 142, 720–723
4 Kakiuchi, S. and Yamazaki, R. (1970) Biochem. Biophys. Res. Commun. 41, 1104
5 Teo, T. S., Wang, T. H., and Wang, J. H. (1973) J. Biol. Chem. 248, 588–595
6 Lin, Y. M., Smoake, J. A., Song, S. Y. and Cheung, W. Y. (1973) Fed. Proc. 32, 609
7 Berry, M. N. and Friend, D. S. (1969) J. Cell Biol. 43, 506–520
8 Touster, O., Aronson, Jr, N. N., Dulaney, J. T. and Hendrickson, H. (1970) J. Cell Biol. 47, 604–618
9 Song, S-Y. and Cheung, W. Y. (1971) Biochim. Biophys. Acta 242, 593–605
10 Holmsen, H., Day, H. J. and Pimentel, M. A. (1969) Biochim. Biophys. Acta 186, 244–253
11 Butcher, R. W. and Sutherland, E. W. (1962) J. Biol. Chem. 237, 1244–1250
12 Thompson, W. J. and Appleman, M. M. (1971) Biochemistry 10, 311–316
13 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 264–275
14 Brooker, G., Thomas, Jr, L. J. and Appleman, M. M. (1968) Biochemistry 7, 4177–4181
15 Thompson, W. J. and Appleman, M. M. (1971) J. Biol. Chem. 246, 3145–3150
16 Monn, E. and Christiansen, R. O. (1971) Science 173, 540–542
17 Uzuno, P. and Weiss, B. (1972) Biochim. Biophys. Acta 284, 220–226
18 Weiss, B. and Strada, S. J. (1972) Advances in Cyclic Nucleotide Research I (Greengard, P. and Robison, G. A., eds), p. 357, Raven Press, New York
19 Cheung, W. Y. (1970) Advances in Biochemical Psychopharmacology 3 (Greengard, P. and Costa, A., eds), p. 51, Raven Press, New York
20 Pichard, A.-L., Hanoune, J. and Kaplan, J.-C. (1972) Biochim. Biophys. Acta 279, 217–220
21 Bloom, W. and Fawcett, D. W. (1970) A Textbook of Histology p. 128, W. B. Saunders, Philadelphia
22 Weiss, B. (1971) J. Neurochem. 18, 469–477
23 Monn, E., Desoutel, M. and Christiansen, R. O. (1972) Endocrinology 91, 716–720