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STIMULATION OF MUSCLE GLYCOGEN SYNTHASE PHOSPHATASE BY POLYAMINES

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Summary

The naturally-occurring polyamines were found to stimulate glycogen synthase phosphatase from rat skeletal muscle. The sequence of effectiveness in the stimulation was spermine > spermidine > putrescine. It was shown that the spermine-sensitive phosphatase was present primarily in the soluble fraction of the muscle extract. In the presence of spermine, the phosphatase activity can be further stimulated by Mn^{2+}; however, a lower Mn^{2+} concentration is required for the activation of the enzyme in comparison with that in the absence of spermine. Kinetic studies indicated that activation of glycogen synthase phosphatase by spermine was achieved by an increase in its V without significant alteration in the K_m, suggesting that spermine directly stimulated the catalytic efficiency of the phosphatase enzyme.

Introduction

The naturally-occurring aliphatic polyamines, spermine, spermidine, and putrescine have been implicated to have regulatory effects on protein synthesis and cell proliferation [1]. Furthermore, some of the diverse biological effects of insulin can be mimicked by polyamines. For instance, stimulation of the intracellular utilization of glucose in adipocytes appears to be a metabolic effect shared by insulin and polyamine [2,3].

In liver and skeletal muscle, insulin exerts a glycogenic effect [4,5]. This is caused likely by a conversion of glycogen synthase D to its I form, via dephos-

Abbreviation: Mes, 2-[*N*-morpholino]ethanesulfonic acid.
The D and I forms of glycogen synthase are referred to in IUB nomenclature as *a* and *b*, respectively.

phorylation by glycogen synthase phosphatase (phosphoprotein phosphatase) [6]. Since polyamines mimic several aspects of insulin action, it is of interest to examine whether the enzymatic activity of phosphoprotein phosphatase, catalyzing the conversion of synthase D to I, can be affected by polyamines. In this communication, we report that polyamines stimulate the activity of glycogen synthase phosphatase (phosphoprotein phosphatase). The polyamine order of effectiveness is: spermine > spermidine > putrescine.

Materials and Methods

Preparation of glycogen synthase D form

Glycogen synthase D was purified from rabbit skeletal muscle according to the method of Smith et al. [7].

Preparation of crude phosphatase from rat skeletal muscle

Wistar strain male rats (140—160 g) were used. The rats were first anesthetized with pentobarbital and then the hind leg muscle was removed. The muscle was quickly frozen in liquid nitrogen and powdered. 1 g frozen powder of rat skeletal muscle was homogenized with 3 ml 50 mM 2-[*N*-morpholino]ethanesulfonic acid (Mes)/1 mM EDTA, pH 7.0, in a Teflon glass homogenizer.

The homogenate was centrifuged at $700 \times g$ for 15 min. The supernatant fluid was then passed through a layer of glass wool and used as phosphatase source. In the case of column-treated enzyme, 2 ml crude phosphatase was passed through a Sephadex G-50 column (1.5 × 20 cm) equilibrated with 50 mM Mes, 1 mM EDTA, pH 7.0. The main protein peak was collected and used as enzyme source.

Assay of phosphatase activity

Method I. Phosphatase activity was measured by the rate of conversion of glycogen synthase D to I form. Glycogen synthase activity was determined by the amount of glucose transferred from UDP[^{14}C]glucose to glycogen by the method of Thomas et al. [8]. 1 unit of glycogen synthase was the amount of enzyme catalyzing the incorporation of 1 μmol glucose into glycogen. The phosphatase assay mixture composed of 100 μl phosphatase preparation, 20 μl 0.5 M Mes (pH 7.0), 10 mM EDTA, 30 μl glycogen synthase D (0.17 unit), and polyamines, in a final volume of 330 μl. In a routine assay, polyamines and Mes buffer were first mixed in a test tube, phosphatase was added immediately before the assay. The reaction was initiated by the addition of synthase D. 150 μl of the assay mixture were withdrawn at zero time and kept in ice, while the remaining mixture was incubated at 30°C for 2 min and then kept in ice. Glycogen synthase I and total activity in the zero time and 2 min samples were measured. Phosphatase activity is represented by increase in synthase I activity expressed as nmol glycose incorporated into glycogen.

Method II. Phosphatase activity was also assayed by the release of ^{32}P from ^{32}P-labeled glycogen synthase D. ^{32}P-labeled synthase D was prepared according to the method of Binstock and Li [9] using purified synthase I, [γ-^{32}P]ATP, and the catalytic subunit of rabbit skeletal muscle cyclic AMP-dependent protein kinase. The phosphatase activity of rat skeletal muscle extract was mea-

sured by incubating ^{32}P-labeled-synthase D (0.06 mg containing 20 000 cpm), 50 mM Mes, 1 mM EDTA (pH 7.0), and phosphatase preparations in a total volume of 100 μl at 30°C for 10 min. The reaction was terminated by the addition of 1 ml 10% trichloroacetic acid, 0.25% sodium tungstate, 0.06 N H_2SO_4 as described by Kuo and Greengard [10]. Then 1 mg bovine serum albumin was added to the mixture and the protein precipitate was separated by centrifugation. The clear supernatant was transferred to a scintillation counting vial and counted in a Beckman 7000 scintilation counter. The increase in radioactivity in the supernatant over reagent blank represents the phosphatase activity.

Separation of soluble fraction from mitochondrial and microsomal fractions

1 g of frozen rat muscle powder was homogenized with 9 ml 0.25 M sucrose in 10 mM Mes, pH 7.0 [11]. The homogenate was then centrifuged at 700 × *g* for 15 min to remove cell debris. The supernatant was then centrifuged at 57 000 × *g* for 1 h. After centrifugation, the supernatant was used as soluble fraction while the precipitate was resuspended in the original volume of 0.25 M sucrose, 10 mM Mes (pH 7.0) as particulate fraction.

Incubation of rat diaphragm with polyamines and insulin

An intact diaphragm of a Wistar rat (weighing 140—160 g), fasted for 24 h was first incubated in 20 ml Gey and Gey buffer [12] containing no glucose for 30 min at 37°C as described by Shen et al. [13]. The diaphragm was then transferred to 20 ml of the same buffer containing 2 mM polyamines or 0.1 unit/ml amorphous insulin. After incubation, the diaphragm was rapidly frozen in liquid nitrogen. A duplicate experiment was performed under identical conditions. The frozen muscle was removed from the rib cage and powdered. The powdered muscle was homogenized with 10 vols of 10 mM EDTA/50 mM KF (pH 7.8), as described by Shen et al. [13]. Glycogen synthase activity of the homogenate was assayed according to the method of Thomas et al. [8].

Other methods

Protein concentration was determined according to the method of Lowry et al. [14] using bovine serum albumin as standard.

Chemicals

Polyamines were purchased from Sigma Chemical Company. Mes was obtained from Calbiochem. UDP[^{14}C]glucose was prepared from [^{14}C]glucose according to the method of Wright and Robbins [15] as modified by DePaoli-Roach (personal communication). Insulin was purchased from Novo Laboratories, Copenhagen, Denmark. Other chemicals were of reagent grade.

Results

Effect of polyamines on phosphatase activity

The crude phosphatase preparation isolated from rat skeletal muscle was used to examine the effect of various polyamines on its activity. In these experiments, purified glycogen synthase D was the substrate of phosphoprotein phosphatase in the crude enzyme preparation, and the activity of phosphatase

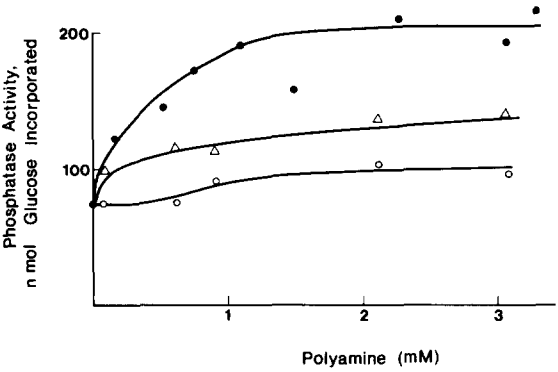

Fig. 1. Effect of polyamines on phosphatase activity. Phosphatase activity was assayed by Method I (see Materials and Methods). Phosphatase activity is presented as the increase in glycogen synthase I activity which is expressed as nmol glucose incorporated into glycogen. The polyamines were added at the indicated concentrations. ●———●, spermine was added; △———△, spermidine was added; ○———○, putrescine was added.

was expressed, via the coupled glycogen synthase according to Method I, in terms of incorporation of [^{14}C]glucose into glycogen from UDP[^{14}C]glucose.

Results show that the amount of [^{14}C]glucose incorporation assayed in the absence of phosphoprotein phosphatase is not affected by polyamines, indicating that the activity of purified glycogen synthase D remains constant upon addition of polyamines. However, in the presence of phosphoprotein phosphatase, the glucose incorporation was found to be enhanced by polyamines (Fig. 1). This enhanced incorporation can, therefore, be reasonably assumed to be mediated via the stimulation of phosphatase activity by polyamines. Fig. 1 shows that the enhanced glucose incorporation which corresponds to the phosphatase activity can be stimulated significantly by spermine with a half-saturation concentration of about 0.4 mM. Stimulation by spermidine was also apparent. Putrescine, however, was the least effective polyamine in activating the phosphatase.

The activation of phosphatase, as measured from the enhancement in the glucose incorporation by a fixed concentration (2 mM) of spermine, was found to be linearly dependent on the concentration of the crude enzyme used, ranging from 0.4 to 1.5 mg of the total protein (data not shown). Furthermore, the total glycogen synthase assayed in the presence of glucose 6-phosphate was unchanged during the course of phosphatase reaction in the presence and absence of spermine.

Glycogen synthase phosphatase activity was also assayed directly by the release of ^{32}P from ^{32}P-labeled synthase according to Method II. In the absence of spermine, the amount of ^{32}P released was 71 pmol/mg protein; in the presence of 2 mM spermine, however, the amount of ^{32}P released was increased to 135 pmol/mg protein. These data support our results obtained by Method I, indicating that glycosen synthase phosphatase was activated by polyamines.

Localization of the spermine-sensitive phosphatase activity

It is known that two classes of phosphoprotein phosphatase exist: the soluble fraction and the microsome-bound or particulate glycogen-bound frac-

tions [16]. The phosphoprotein phosphatase activity in the soluble and particulate fractions of muscle homogenate, separated according to the method of Schneider and Hogeboon [11], was measured. Also, the endogenous glycogen synthase activity was determined. Results (data not shown) indicate that 97% of the total phosphoprotein phosphatase activity was present in the soluble fraction, whereas glycogen synthase was present mostly in the particulate fraction. Moreover, it was the soluble form of phosphoprotein phosphatase that can be stimulated 2.5-fold by spermine. These results rule out the possibility that the spermine may activate the phosphoprotein phosphatase in the crude preparation by converting the latent particulate form of phosphatase into the active soluble form of phosphatase.

Effects of spermine on the activation of phosphatase by Mn^{2+}

It is known that divalent cations are required for glycogen synthase phosphatase to show optimal activity [17]. We have, therefore, designed experiments to examine whether spermine can substitute Mn^{2+} for glycogen synthase phosphatase. In order to remove endogenous kinase activity, the crude phosphoprotein phosphatase was subjected to gel filtration on a Sephadex G-50 column which can separate the endogenous ATP, a substrate of protein kinase, from the protein. With this ATP-free phosphatase, it was observed (Fig. 2) that the phosphatase activity was stimulated by Mn^{2+} within a very narrow range of concentration (3—4 mM). In the presence of 2 mM spermine, the phosphatase activity assayed in the absence of Mn^{2+} showed an activity higher than the maximal activity stimulated by Mn^{2+} (Fig. 2). Addition of small amounts of Mn^{2+} further stimulated the spermine-activated enzyme and maximum was reached at about 1—2 mM Mn^{2+}. It should be mentioned that the relative increase in phosphatase activity by Mn^{2+} in the absence and presence of spermine was different (Fig. 2).

Noncompetitive activation of phosphatase by spermine

The phosphatase activities assay in the presence and absence of spermine showed a typical Michaelis-Menten type saturation curve with respect to the

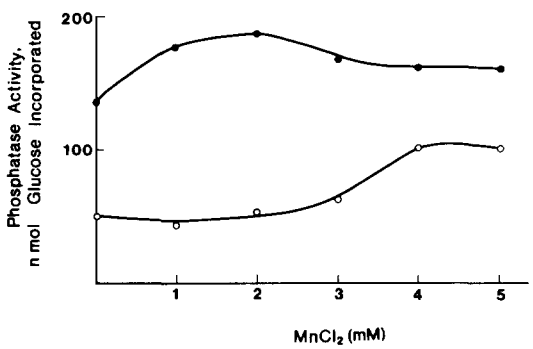

Fig. 2. Effect of Mn^{2+} on phosphatase activity in the presence and absence of spermine. The phosphatase activity was assayed by Method I (see Materials and Methods). The phosphatase was the column-treated extract. $MnCl_2$ was added as indicated. o———o, no spermine was added; •———•, 2 mM spermine was added.

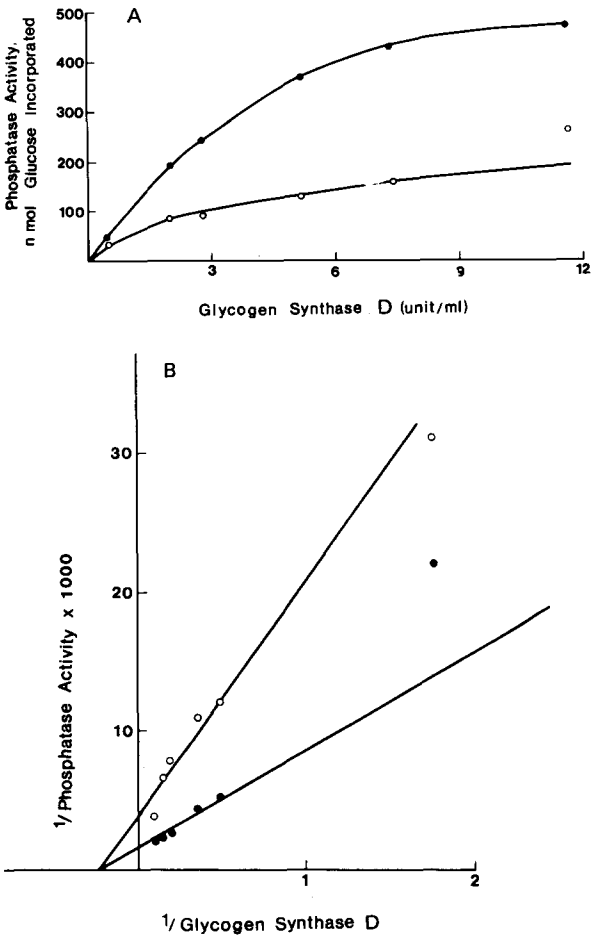

Fig. 3. (A) Effect of glycogen synthase D concentration on phosphatase activity in the presence and absence of spermine. Phosphatase activity was measured by Method I (see Materials and Methods) except synthase D was varied at the concentration indicated. ○———○, enzyme alone, no spermine added. ●———●, 2 mM spermine was added. (B) Lineweaver-Burk plot of the data presented in (A). Symbols are the same as in (A).

substrate concentration (Fig. 3A). The Lineweaver-Burk plot indicated that spermine behaved as simple noncompetitive activator (Fig. 3B). The K_m value estimated from the horizontal intercept was virtually identical for the two lines corresponding to 4.2 unit/ml, while the V is increased from 316 nmol/mg obtained in the absence of spermidine to 835 nmol/mg obtained in the presence of 2 mM spermine.

Effect of polyamines and insulin on glycogen synthase in the intact diaphragm

Since polyamines have been shown to stimulate the conversion of glycogen synthase D to I in vitro, experiments were designed to test if the stimulatory effect can also occur in the intact muscle. Intact rat diaphragms were incubated with polyamines in the absence of glucose and their glycogen synthase activities were determined. Table I shows that when the diaphragms were incubated with

TABLE I

EFFECT OF POLYAMINES AND INSULIN ON THE GLYCOGEN SYNTHASE ACTIVITIES IN THE INTACT DIAPHRAGMS

Rat diaphragms were incubated with 2 mM polyamines or 0.1 unit/ml insulin. Two diaphragms were used for each experimental condition and their muscle was pooled and powdered. The synthase I and total activities in each muscle extract were determined and expressed as synthase I percent. Means of three experiments ± S.D. are presented.

Incubation conditions	Glycogen synthase I percent
Control	26.7 ± 3.9
Spermine	28.3 ± 3.2
Spermidine	41.6 ± 6.9 *
Putrescine	54.9 ± 4.6 **
Insulin	51.2 ± 9.1 **

* Comparison of the difference of the means by Student's paired *t*-test shows different from control at the $0.1 > P > 0.05$ level.
** $P < 0.01$.

spermidine or putrescine, their synthase I percentage was significantly increased. It also demonstrated that putrescine elevated the synthase I percentage to the same extent as that of insulin. It was noted that spermine failed to exert any effect on synthase I percentage in the intact diaphragm. It is possible that spermine cannot enter the muscle cell membrane as readily as putrescine, due to its size, charge, or both. Clearly, more work is needed to clarify this point. However, these experiments do demonstrate that some polyamines can stimulate glycogen synthase in intact muscle. Furthermore, this stimulation is similar to the action of insulin.

Discussion

Although insulin interacts directly with its receptor at cell surface, it is well known that insulin exerts diverse biological effects on cell function. At the level of cell membrane, insulin is known to promote glucose transport. Within the cell, the glycogenic effect of insulin is well documented. Polyamines have been demonstrated to mimic several metabolic effects of insulin, but not at the transport level [2,3,18]. Polyamines have also been reported to enhance the affinity of phosphorylase *b* toward AMP, in vitro [19]; the physiological significance of this observation, however, has not been demonstrated in intact cells. In this study we have shown using rat skeletal muscle extract and purified rabbit glycogen synthase D that in vitro synthesis of glycogen as measured by the [^{14}C]glycose incorporation in stimulated by polyamines. Furthermore, glycogen synthase activity in the intact muscle can also be shown to be stimulated by polyamines. This enhanced glycogen synthesis can be attributed primarily to the increase in phosphoprotein phosphatase activity by polyamines based on the following reasons. (1) The glycogen synthase D activity is not affected by the addition of polyamines. (2) The enhanced glycogen synthesis persists under the condition that ATP has been removed. If the effect of polyamines is to inhibit the endogenous protein kinase which, in turn, promotes the glycogen synthesis, then the polyamines effect would be dependent on the presence of

ATP, a substrate of protein kinase. The fact that polyamine together with the ATP-free glycogen synthase D can stimulate the glycogen synthesis can be taken as evidence to argue against the involvement of protein kinase in the observed enhancement of glucose incorporation. (3) Phosphoprotein phosphatase activity is known to be stimulated by Mn^{2+}. The enhanced glycogen synthesis is also stimulated by Mn^{2+}, although the relative stimulation is lowered somewhat in the presence of polyamines (Fig. 2). (4) The release of ^{32}P from labeled glycogen synthase in the presence of rat skeletal muscle extract is stimulated by spermine, suggesting strongly that the glycogen synthase phosphatase in the muscle is activated by spermine. We have further shown that the presumptive increase activity cannot be attributed to the shift in equilibrium concentration of phosphoprotein phosphatase by polyamine between the soluble and the particulate forms.

It should be pointed out that in contrast to our data, Killilea et al. [20] reported that phosphorylase phosphatase with M_r 35 000 from rabbit liver and beef heart can be inhibited in vitro by polyamines. The inhibitory effect of polyamines was due to their binding to the substrate rather than binding to the M_r 35 000 enzyme itself [20]. Furthermore, these workers showed that the dephosphorylation of ^{32}P-labeled lysine-rich histone by bovine heart phosphorylase phosphatase was not affected by polyamines. As reported here, we observed that the ^{32}P release from labeled glycogen synthase was markedly increased by polyamines and, more importantly, the stimulatory effect of polyamines on glycogen synthase phosphatase has been demonstrated not only in vitro, but also in intact cells. One possible explanation of the discrepancy is that phosphorylase phosphatase may exist as a complex multi-subunit form and the purified M_r 35 000 species of Killilea et al. may be a catalytic subunit of the holoenzyme [21]. Polyamines may activate the holoenzyme by interacting with the binding subunit followed by the release of catalytic subunit (M_r 35 000); however, in the absence of the binding subunit of the holoenzyme, polyamines interact preferentially with the substrate or phosphorylase. This polyamine-substrate complex renders the M_r 35 000 phosphatase a poor enzyme as suggested by Killilea et al. [20]. In fact, polyamines have been reported to stimulate the activity of a histone phosphatase with a molecular weight of 250000. In constrast, the activity of a smaller histone phosphatase (M_r 30 000) was inhibited [22]. These data keep in line with our hypothesis that the M_r 35 000 subunit interacts differently with polyamines from its holoenzyme. An alternative explanation is that phosphorylase phosphatase of M_r 35 000 is an enzyme distinctly different from glycogen synthase phosphatase of M_r 49 000 [9]. Thus, a different interaction with polyamines is expected. Clearly, the explanations given above are merely speculative and the true explanation must await further experimentation.

The stimulation of glycogen synthesis by insulin has been explained, at least in part, as a result of the activation of glycogen synthase [23,24]. There are at least two ways by which the activation can be brought about: one is the inhibition of a protein kinase which phosphorylates glycogen synthase [25], while the other is the activation of a phosphoprotein phosphatase which dephosphorylates glycogen synthase. There is evidence to suggest that phosphoprotein phosphatase can be activated by insulin [6,25]. Here, we present evidence to

show that the glycogen synthesis in vitro can be stimulated by polyamines, and the stimulation is best explained by the activation of a soluble form of phosphoprotein phosphatase which, in turn, dephosphorylates its substrate, glycogen synthase D, resulting in a more active form of glycogen synthase.

The fact that polyamines can mimic several aspects of insulin action in the regulation of carbohydrate metabolism may be, in part, due to their common stimulating effect on the activity of phosphoprotein phosphatase. Since insulin may exert its effects without actually entering cells, it is quite possible that insulin may, through an unknown mechanism, cause an increase in intracellular level of polyamines which by stimulating phosphoprotein phosphatase, favors glycogenesis as well as lipogenesis. In fact, it has been recently postulated by Younkin [27] that insulin binds to liver membranes in liver cells and eventually causes an increase in the activity of intracellular ornithine decarboxylase. Since ornithine decarboxylase is the first, and probably rate-limiting step in the biosynthesis of polyamines from ornithine [28,29], it is thus quite probable that the biosynthesis of polyamines may be stimulated by insulin. Alternatively, insulin may, via an unknown mechanism, shift the intracellular concentrations of polyamines from bound to free form which, in turn, activate phosphoprotein phosphatase. Hence, the glycogenesis and lipogenesis promoted by insulin may be mediated by polyamines through their stimulatory effects on phosphoprotein phosphatase. It should be emphasized, however, that the insulin mechanism proposed by Younkin [27] was based on studies using liver cells and soluble enzymes in liver cells. Here, our studies were carried out with muscle phosphatase. More studies are clearly needed to establish the connection between insulin action and the polyamines function.

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References

1 Williams-Ashman, H.G. and Canellakis, Z.N. (1979) Persp. Biol. Med. 22, 421–433
2 Czech, M.P., Lawrence, J.C., Jr. and Lynn, W.S. (1974) J. Biol. Chem. 249, 5421–5427
3 Lockwood, D.H. and East, L.E. (1974) J. Biol. Chem. 249, 7717–7722
4 Villar-Palasi, C. and Larner, J. (1970) Annu. Rev. Biochem. 39, 639–672
5 Hers, H.G. (1976) Annu. Rev. Biochem. 45, 167–189
6 Bishop, J.S. (1970) Biochim. Biophys. Acta 208, 208–218
7 Smith, C.H., Villar-Palasi, C. Brown, N.E., Schlender, K.K., Rosenkrans, A.M. and Larner, J. (1972) Methods Enzymol. 28, 520–539
8 Thomas, J.A., Schlender, K.K. and Larner, J. (1968) Anal. Biochem. 25, 486–499
9 Binstock, J.F. and Li, H.C. (1979) Biochem. Biophys. Res. Commun. 87, 1226–1234
10 Kuo, J.F. and Greengard, P. (1970) J. Biol. Chem. 245, 4067–4073
11 Schneider, W.C. and Hogeboon, G.H. (1970) J. Biol. Chem. 183, 123–128
12 Gey, G.O. and Gey, M.K. (1936) Am. J. Cancer 27, 45
13 Shen, L.C., Villar-Palasi, C. and Larner, J. (1970) Physiol. Chem. Phys. 2, 536–544
14 Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
15 Wright, A. and Robbins, P.W. (1965) Biochim. Biophys. Acta 104, 594–596

16 LaRoux, M., Stalmans, W. and Hers, H.G. (1978) Eur. J. Biochem. 92, 15—24
17 Kato, K. and Bishop, J.S. (1972) J. Biol. Chem. 247, 7420—7429
18 Livingston, J.N., Gurny, P.A. and Lockwood, D.H. (1977) J. Biol. Chem. 252, 560—562
19 Wang, J.H., Humniski, P.M. and Black, W.J. (1968) Biochemistry 7, 2037—2044
20 Killilea, S.D., Mellgren, R.L., Aylward, J.H. and Lee, E.Y.C. (1978) Biochem. Biophys. Res. Commun. 81, 1040—1046
21 Lee, E.Y.C., Mellgren, R.L., Aylward, J.H. and Killilea, S.D. (1978) Biochem. Soc. Trans. 6, 25--29
22 Usui, H., Imazu, M., Imaska, T. and Tekada, M. (1978) Biochim. Biophys. Acta 526, 163—173
23 Larner, J., Lawrence, J.C., Walkenbach, K.J., Roach, P.J. Hazen, R.J. and Huang, L.C. (1977) Adv. Cyclic Nuc. Res. 9, 425—439
24 Cohen, P. (1978) Current Top. Cell Reg. 14, 117—196
25 Larner, J., Roach, P.J., Huang, L.C., Brooker, G., Murad, F. and Hazen, R., (1979) in Hormone and Energy Metabolism (Klachko, D.M. and Anderson, R.R., eds.), pp. 103—123, Plenum Press, New York, NY
26 Nuttall, F.Q., Gannon, M.C., Corbett, V.A. and Wheeler, M.P. (1976) J. Biol. Chem. 25, 6724—6729
27 Younkin, B. (1979) XI International Congress of Biochemistry Abstracts, p. 610, 8—13 July, Toronto, Canada
28 Pegg, A.E. and Williams-Ashman, H.G. (1968) Biochem. J. 108, 533—539
29 Russell, D.H. and Snyder, S.H. (1968) Proc. Natl. Acad. Sci. U.S.A. 60, 1420—1427