Inhibition of hepatic gluconeogenesis by the R_p -diastereomer of adenosine cyclic 3',5'-phosphorothioate

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The specific intracellular cyclic AMP-dependent protein kinase antagonist, the R_p -diastereomer of adenosine cyclic 3',5'-phosphorothioate (R_p -cAMPS), inhibited both basal and cyclic AMP-agonist-induced rates of gluconeogenesis in hepatocytes isolated from fasted rats. Incubation of the cells in the presence of pyruvate and lactate and either the S_p -diastereomer of adenosine cyclic 3',5'-phosphorothioate (S_p -cAMPS) or glucagon produced a concentration-dependent increase in the rate of gluconeogenic glucose production which was shifted to higher concentrations of S_p -cAMPS or glucagon in the presence of R_p -cAMPS. Incubation of the cells with R_p -cAMPS in the absence of agonist produced no increase in the rate of glucose production and, in most cases, 100 μ M- R_p -cAMPS resulted in a 14–20% decrease in the substrate-stimulated rate of glucose production. S_p -cAMPS-induced gluconeogenesis was inhibited half-maximally at 1 μ M- R_p -cAMPS and glucagon-induced gluconeogenesis was inhibited half-maximally at 12 μ M- R_p -cAMPS. Approx. 10–15% of the inhibition of gluconeogenesis observed in the presence of R_p -cAMPS was due to conversion of glucose 6-phosphate to liver glycogen, consistent with R_p -cAMPS-induced reactivation of glycogen synthase. The remaining 85–90% inhibition of gluconeogenic glucose production resulted from the action of R_p -cAMPS on the cyclic AMP-sensitive enzymes controlling the rate of gluconeogenesis.

INTRODUCTION

The diastereomers of adenosine cyclic 3',5' phosphorothioate, S_p -cAMPS and R_p -cAMPS, have been shown to have differential effects on the activity of cyclic AMP-dependent protein kinase (deWit *et al.*, 1982; O'Brien *et al.*, 1982; Rothermel *et al.*, 1983), phosphorylase and glycogen synthase (Rothermel *et al.*, 1985), and pyruvate kinase (Meserve *et al.*, 1985). S_p -cAMPS is a cyclic AMP-dependent protein kinase agonist which binds to, and activates, both Type I and Type II holoenzymes. R_p -cAMPS is a cyclic AMP-dependent protein kinase antagonist which is unique in being the first reported cyclic AMP analogue which competes with cyclic AMP for the binding sites on the regulatory subunit without causing dissociation of the holoenzyme. Since this analogue also crosses the hepatocyte membrane rapidly, R_p -cAMPS is the first available inhibitor of cyclic AMP-dependent protein kinase (Rothermel *et al.*, 1985) which can be added to cell preparations to inhibit specifically intracellular cyclic AMP-dependent biochemical events. In hepatocytes isolated from fed rats, R_p -cAMPS inhibits the S_p -cAMPS-induced (Rothermel *et al.*, 1983) and glucagon-induced activation of cyclic AMP-dependent protein kinase and the consequent effects of phosphorylation of the enzymes controlling both the glycogenolytic cascade (Rothermel *et al.*, 1984, 1985) and glycolysis (Meserve *et al.*, 1985). R_p -cAMPS alone does not affect the basal activities of cyclic AMP-dependent protein kinase, phosphorylase, glycogen synthase or pyruvate kinase in cells isolated from fed rats (Meserve *et al.*, 1985; Rothermel *et al.*, 1983, 1984, 1985).

The present paper presents the effects of R_p -cAMPS on gluconeogenesis in hepatocytes isolated from fasted rats.

It is argued that the antagonistic effects of R_p -cAMPS on basal and agonist-induced rates of gluconeogenesis are due to the inhibition of cyclic AMP-dependent protein kinase and the subsequent effects of this inhibition on pyruvate kinase activity, glycogen synthase activity and glycogenesis.

EXPERIMENTAL

Materials

Crystalline glucagon, UDP-glucose and glycogen were obtained from Sigma. Collagenase (EC 3.4.24.3; clostridiopeptidase A) was purchased from Worthington (CLS II, 133 units/mg; 1 enzyme unit equals 1 μ mol of L-leucine equivalents from collagen in 5 h at 37 °C and pH 7.5). The phosphorothioates R_p -cAMPS and S_p -cAMPS were prepared as published previously by Stec and co-workers (Baraniak *et al.*, 1979), and in order to reach diastereomeric purity and to eliminate traces of cyclic AMP, were repurified by h.p.l.c. on a reverse phase C-18 column using separation conditions optimized according to Van Haastert (1981). UDP-[U-¹⁴C]glucose and Aquasol were obtained from New England Nuclear. All other chemicals were reagent grade.

Methods

Liver parenchymal cells were isolated from 18–20 h fasted male Sprague–Dawley rats that were maintained on a 12 h light/12 h dark cycle in an environmentally controlled room. Cells were prepared according to a modification of a procedure of Berry & Friend (1969). The stock solution used for cell isolation was a Ca²⁺-free Hepes (20 mM, pH 7.4) buffer containing 136 mM-NaCl.

Abbreviation used: cAMPS, adenosine cyclic 3',5'-phosphorothioate.

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4.7 mM-KCl, 1.2 mM-KH₂PO₄, 0.7 mM-MgSO₄, 24 mM-NaHCO₃ and 20 mM-glucose. This solution was gassed with O₂/CO₂ (19:1) for 1 h at 35 °C immediately prior to use. The liver was perfused via the hepatic portal vein first with the stock Hepes buffer containing 0.5 mM-EGTA for 10 min and then with the stock buffer containing collagenase (26.6 units/ml) and 5 mM-CaCl₂ for 20 min. The tissue was then removed and rapidly minced in the stock Hepes buffer and filtered through nylon mesh. The hepatocyte suspension was centrifuged at slow speed and the resulting soft pellet was washed three times in an incubation buffer comprised of 20 mM-Hepes (pH 7.4), 123 mM-NaCl, 5 mM-KCl, 2 mM-MgSO₄, 2.5 mM-K₂HPO₄, 0.8 mM-CaCl₂, 24 mM-NaHCO₃, 60 µM-penicillin, 2% (w/v) bovine serum albumin and 3.25% (w/v) dextran. Only preparations demonstrating greater than 90% cell viability as determined by Trypan Blue exclusion and microscopic observation of the cell membranes were used in the experiments described in this paper.

Each 20 ml vial contained hepatocytes at a concentration of 1×10^7 cells/ml of incubation buffer and all experiments were performed at 30 °C in a Dubnoff metabolic shaker after a 30 min equilibration period under O₂/CO₂ (19:1). All additions to the cell suspensions were prepared in the incubation buffer. R_p-cAMPS was added to the cell suspensions at -20 min when glucagon was used as the cyclic AMP-dependent protein kinase agonist, and at either -20 min or 0 min when S_p-cAMPS was used as the agonist. At -5 min, a mixture of the gluconeogenic substrates, pyruvate and lactate, were added so that their incubation concentrations were 8 mM and 2 mM, respectively. Glucagon or S_p-cAMPS was added at 0 min. Cells were incubated for 1 h, during which time aliquots of cell suspension were removed for determinations of glycogen synthase, cyclic AMP-dependent protein kinase, glucose, lactate and glycogen.

For the determination of glycogen synthase activity, 70 µl aliquots of cell suspension were transferred to an equal volume of ice-cold buffer containing 10 mM-Mops (pH 7.0), 400 mM-sucrose, 5 mM-dithiothreitol, 0.03% (v/v) Triton X-100, 20 mM-EDTA and 150 mM-NaF at a final concentration of 5.0×10^3 cells/µl and were immediately frozen in an acetone/CO₂ bath. Glycogen synthase activity was assayed according to Thomas *et al.* (1968) with some modifications as follows: 50 µl of thawed, suspended cell mixture (equivalent to 0.165 mg dry weight) were incubated in an equal volume of buffer containing 20 mM-Mops (pH 7.0), 80 mM-sucrose, 1 mM-dithiothreitol, 4 mM-EDTA, 30 mM-NaF, 1% (w/v) glycogen, 6.7 mM-UDP-glucose with UDP-[¹⁴C]glucose (0.04 µCi/µmol). Reactions were terminated as described by Gilboe *et al.* (1972) and counted in Aquasol.

For the determination of cyclic AMP-dependent protein kinase activity, 300 µl aliquots of cell suspension were treated as described previously (Rothermel *et al.*, 1984).

For the determination of glucose or lactate and glycogen, 400 µl aliquots of cell suspension were removed to ice-cold Microfuge tubes and centrifuged at 8000 *g* for 5 s. The supernatant was immediately removed and assayed for glucose and lactate concentrations with a Technicon AutoAnalyzer II. The pellet was immediately frozen in an acetone/CO₂ bath for glycogen determination according to Huijing (1970).

The mixture of gluconeogenic substrates chosen maintained a cytosolic NADH:NAD⁺ ratio sufficient for linear rates of gluconeogenesis over a 60 min time course (Williamson *et al.*, 1969). It has been shown that rates of gluconeogenesis and the magnitude of hormonal stimulation remain constant in spite of changes in the ratio of lactate to pyruvate over a wide range of concentration, but the use of either agent as the sole substrate leads to abnormal effects. For example, the lag period observed in the rate of glucose production with 15–20 mM-lactate can be overcome by adding as little as 2 mM-pyruvate, and the initial inhibition of 20 mM-pyruvate-induced gluconeogenesis obtained in the presence of cyclic AMP or glucagon can be overcome by the addition of as little as 3 mM-lactate (Clark & Janett, 1978; Garrison & Haynes, 1973; Rognstad, 1975; Zahlten *et al.*, 1973). Preliminary experiments established that, of several concentration combinations tried, 8 mM-pyruvate and 2 mM-lactate gave the highest rate of linear glucose production over 60 min in the presence of 0.3 µM-S_p-cAMPS or 0.3 nM-glucagon.

Fig. 1. R_p-cAMPS inhibition of S_p-cAMPS-induced activation of gluconeogenic glucose production in hepatocytes isolated from fasted rats

Cells prepared from fasted rats were preincubated for 5 min in the presence of pyruvate and lactate, and then incubated with S_p-cAMPS at concentrations ranging from 30 nM to 100 µM in the absence (●) or presence of 10 µM (Δ) or 100 µM (○) R_p-cAMPS. Samples were removed at 0, 20, 40 and 60 min and glucose production (nmol/10⁶ cells) was measured. Data shown are from the 20 min time point for two separate experiments and are representative of the results obtained from five separate experiments run in cell preparations obtained from different rats. Each point in the Figure represents the average of the values obtained from four vials of cells. The deviation of the individual values from their average was less than 2% if error bars are not present.

Fig. 2. R_p -cAMPS inhibition of glucagon-induced activation of gluconeogenic glucose production in hepatocytes isolated from fasted rats

Cells prepared from fasted rats were preincubated for 20 min in the presence of buffer (●), 10 μ M- R_p -cAMPS (△) or 100 μ M- R_p -cAMPS (○). At -5 min, pyruvate and lactate were added to give final concentrations of 8 mM and 2 mM, respectively, followed by glucagon (final concentrations 0.03–30 nM) at zero time. Samples were removed at 0, 20, 40 and 60 min and glucose production (nmol/10⁶ cells) was measured. Data shown are from the 60 min time point, and are representative of two separate experiments from two different rats. Each point in the Figure represents the average of the values obtained from two separate vials of cells. The deviation of the individual values from their average was less than 1% if error bars are not present.

RESULTS

Cells prepared from fasted rats were incubated in the presence of a mixture of pyruvate and lactate at varying concentrations of S_p -cAMPS in the absence or presence of two different concentrations of R_p -cAMPS. Samples were removed periodically for analysis of glucose (nmol/10⁶ cells). The data in Fig. 1 represent results from three separate experiments at the 20 min point. Maximal glucose production was obtained at 3 μ M- S_p -cAMPS and half-maximal glucose production was obtained at 0.3 μ M- S_p -cAMPS in the absence of R_p -cAMPS. This value was shifted to 3-fold higher concentrations of S_p -cAMPS in the presence of 10 μ M- R_p -cAMPS and to 30-fold higher concentrations of S_p -cAMPS in the presence of 100 μ M- R_p -cAMPS. At concentrations of 10 μ M- and 100 μ M- R_p -cAMPS, substrate-induced gluconeogenesis in the absence of S_p -cAMPS was inhibited 5% and 16 $\pm$ 5%, respectively. The decrease in the control values for the samples containing R_p -cAMPS alone was

not due to a loss of cell viability, since the cells treated with either 10 μ M- or 100 μ M- R_p -cAMPS reached the same maximal rate of S_p -cAMPS-induced glucose production, but at higher concentrations of S_p -cAMPS. Glucose production was measured at 20 min, since S_p -cAMPS-induced gluconeogenesis was not linear at later time points in several treatment groups. As can be seen from Fig. 1, glucose production rate decreased at concentrations of S_p -cAMPS greater than 3 μ M in the absence of R_p -cAMPS and at concentrations of S_p -cAMPS greater than 30 μ M in the presence of 10 μ M- R_p -cAMPS, but was linear up to an S_p -cAMPS concentration of 100 μ M in the presence of 100 μ M- R_p -cAMPS.

Glucagon was used as the agonist in a similar series of experiments. Maximal glucose production obtained in the presence of 1 nM-glucagon was 47–144% higher than control levels measured over a 60 min time course in eight separate experiments. Half-maximal activation of glucose production was obtained at 0.07 nM-glucagon and this value was shifted to 7-fold higher concentrations of glucagon in the presence of 10 μ M- R_p -cAMPS and to 31-fold higher concentrations of glucagon in the presence of 100 μ M- R_p -cAMPS. In the experiment shown in Fig. 2, a concentration of 100 μ M- R_p -cAMPS inhibited the basal rate of substrate-induced gluconeogenesis by 20% in the absence of glucagon. The glucose production data shown in Fig. 2 are from the 60 min point.

The relative importance of inhibition of cyclic AMP-dependent protein kinase versus activation of glycogen synthase and, hence, the extent of gluconeogenic glucose production versus storage of glucose as glycogen, was determined with pyruvate and lactate plus glucagon, S_p -cAMPS or buffer in the absence or presence of R_p -cAMPS. Samples were removed for the determination of cyclic AMP-dependent protein kinase activity, glycogen synthase activity, glucose production and glycogen production. The data appear in Table 1. The basal (control) activity ratio of cyclic AMP-dependent protein kinase was 0.33 in the hepatocytes isolated from fasted rats, about double that of hepatocytes isolated from fed rats (Rothermel *et al.*, 1983). Addition of the cyclic AMP agonists S_p -cAMPS or glucagon increased the activity ratios to 0.43 and 0.44, respectively, which resulted in a corresponding increase in glucose production. R_p -cAMPS not only inhibited the agonist-induced activities by 100%, but reduced them below basal levels. The basal glycogen synthase activity ratio of 0.43 was depressed to a level of 0.22 in the presence of S_p -cAMPS and to a level of 0.28 in the presence of glucagon. The addition of R_p -cAMPS did not significantly affect the basal level of glycogen synthase, but the agonist-depressed levels were both returned to near basal. Table 1 also shows that R_p -cAMPS inhibited gluconeogenic glucose production in the absence or presence of the two agonists to approximately the same level. R_p -cAMPS did not increase the glycogen content of the hepatocytes in the absence of agonist, but did increase the glycogen content in the presence of S_p -cAMPS. Analysis of the amount of glucose measured as free glucose in the supernatant versus the amount of glucose stored as glycogen showed that 85–90% of the R_p -cAMPS inhibition of gluconeogenic glucose production was due to inhibition of the gluconeogenic pathway to glucose 6-phosphate and that the other 10–15% was due to an activation of the pathway from glucose 6-phosphate to glycogen.

Table 1. Effects of R_p -cAMPS on S_p -cAMPS-induced and glucagon-induced changes in cyclic AMP-dependent protein kinase activity, glycogen synthase activity, gluconeogenic glucose production and glycogen production

Hepatocytes isolated from fasted rats were preincubated for 5 min with 8 mM-pyruvate and 2 mM-lactate and then incubated in the presence of buffer (control), 0.3 μ M- S_p -cAMPS or 0.3 nM-glucagon in the absence or presence of 100 μ M- R_p -cAMPS (with a 20 min preincubation in the case of glucagon). Column 1 shows the cyclic AMP-dependent protein kinase activity ratios measured at 2 min, column 2 shows the glycogen synthase activity ratios measured at 15 min, column 3 shows the cell supernatant glucose (nmol/ 10^6 cells) measured at 30 min, and column 4 shows the pellet glycogen (μ g/ 10^6 cells) measured at 30 min. The S.E.M. for the activity ratios is ± 0.01 and for the glucose and glycogen values ± 0.07 . Each value represents the average of data obtained from five vials of cells per treatment. The experiment was repeated four times with separate liver cell preparations.

Treatment	Cyclic AMP-dependent protein kinase activity ratio	Glycogen synthase activity ratio	Glucose (nmol/ 10^6 cells)	Glycogen (μ g/ 10^6 cells)
Control	0.33	0.43	163	3.84
Control + R_p -cAMPS	0.17	0.42	137	3.64
S_p -cAMPS	0.43	0.22	182	1.91
S_p -cAMPS + R_p -cAMPS	0.15	0.38	134	2.81
Glucagon	0.44	0.28	172	3.01
Glucagon + R_p -cAMPS	0.28	0.44	133	3.17

Fig. 3. R_p -cAMPS concentration-dependent inhibition of S_p -cAMPS-induced or glucagon-induced gluconeogenesis in hepatocytes isolated from fasted rats

Cells were preincubated for 20 min with 100 nM–300 μ M- R_p -cAMPS; 8 mM-pyruvate and 2 mM-lactate were added at –5 min and agonist was added at zero min. Free glucose (nmol/ 10^6 cells) was measured in the cell supernatant 30 min after addition of 1 μ M- S_p -cAMPS (a) or 0.3 nM-glucagon (b). Results are expressed as percentage inhibition of S_p -cAMPS- or glucagon-stimulated glucose production. These data were combined from four experiments. Error bars are shown where the deviation of the individual values from their average was more than 5%.

R_p -cAMPS inhibited the S_p -cAMPS and glucagon-induced activation of glucose production with the concentration dependence shown in Fig. 3. Cells from fasted rats were preincubated for 20 min with R_p -cAMPS and for 5 min with the pyruvate and lactate mixture.

S_p -cAMPS or glucagon was added at zero min and samples were removed for glucose analysis at 30 min. S_p -cAMPS stimulated a 79% increase and glucagon a 56% increase in substrate-induced gluconeogenesis. R_p -cAMPS inhibited S_p -cAMPS-induced glucose pro-

duction by 50% at $1\ \mu\text{M}$ and by 125% (maximal inhibition) at $100\ \mu\text{M}$ (Fig. 3a). R_p -cAMPS inhibited glucagon-induced glucose production by 50% at $12\ \mu\text{M}$ and by 95% (maximal inhibition) at $300\ \mu\text{M}$ (Fig. 3b). The concentrations of S_p -cAMPS and glucagon chosen for this experiment were those observed to produce 80–100% of maximal glucose production.

DISCUSSION

The cyclic AMP antagonist R_p -cAMPS inhibited agonist-stimulated rates of gluconeogenesis and depressed the basal rate of gluconeogenesis in hepatocytes isolated from fasted rats. The inhibition of glucagon- and S_p -cAMPS-stimulated gluconeogenesis in the presence of added pyruvate and lactate is consistent with inhibition of cyclic AMP-induced effects on the rate-limiting enzymes which control gluconeogenic glucose production. The correspondence between the increased cyclic AMP-dependent protein kinase activity following glucagon challenge and the increased rates of gluconeogenesis has been thoroughly examined (for a comprehensive review see Hers & Hue, 1983) and all phosphoprotein substrates of cyclic AMP-dependent protein kinase have been considered as candidates for the rate-controlling step in the gluconeogenic pathway. Five such substrates are rate-limiting enzymes controlling two important substrate cycles in the gluconeogenic pathway, namely, the fructose 1,6-bisphosphate/fructose 6-phosphate cycle and the pyruvate/phosphoenolpyruvate cycle.

Recent experiments using a rat liver cell perfusion technique (Groen *et al.*, 1983) showed that glucagon stimulation of gluconeogenesis in the presence of subsaturating concentrations of gluconeogenic substrates can be accounted for almost completely by inhibition of pyruvate kinase, with no measurable effect on the kinetic parameters of the enzymes operating in the gluconeogenic pathway between phosphoenolpyruvate and glucose. In the absence of glucagon, the rate of glucose production was maintained by both pyruvate kinase and pyruvate carboxylase. No rate-limiting control was observed at the mitochondrial pyruvate translocator step. The major control point has been postulated by these authors to be inhibition of pyruvate kinase.

In the present study, with hepatocytes isolated from 24 h fasted rats, glucagon and S_p -cAMPS increased the rates of gluconeogenic glucose production and the cyclic AMP antagonist R_p -cAMPS inhibited these effects. R_p -cAMPS has previously been shown to inhibit glucagon-induced and S_p -cAMPS-induced kinetic changes in pyruvate kinase activity (Meserve *et al.*, 1985) and in glycogen synthase and phosphorylase activities through inhibition of cyclic AMP-dependent protein kinase (Rothermel *et al.*, 1983, 1984, 1985). Thus, in cells from fasted animals where gluconeogenesis dominates and the intensity of cycling at both the fructose 6-phosphate to fructose 1,6-bisphosphate step and the phosphoenolpyruvate to pyruvate step is controlled by cyclic AMP-dependent mechanisms (Hers & Hue, 1983), the inhibition of cyclic AMP-dependent protein kinase by R_p -cAMPS would be expected, in turn, to inhibit gluconeogenic glucose production. Analysis of the inhibitory effects of R_p -cAMPS shows that 85–90% of the R_p -cAMPS inhibition of S_p -cAMPS- and glucagon-induced gluconeogenic glucose production could be accounted for through inhibition of the pyruvate to

glucose 6-phosphate pathway and an additional 10–15% through activation of glycogen synthesis (Table 1). Thus, R_p -cAMPS inhibition of the flux through the gluconeogenic pathway to glucose 6-phosphate probably occurs through activation of pyruvate kinase and/or inhibition of fructose 1,6-bisphosphatase. Additionally, R_p -cAMPS diverted glucose 6-phosphate to glycogen, consistent with the notion that a three-carbon intermediate entering hepatic metabolism at pyruvate or lactate can be a proximate precursor of liver glycogen (Katz *et al.*, 1983; Newgard *et al.*, 1984; Radziuk & Inculet, 1983). That R_p -cAMPS had a greater effect on the amount of glucose stored as glycogen in the S_p -cAMPS-treated cells versus the glucagon-treated cells seems to be a result of the greater inhibitory effect on the cyclic AMP-dependent protein kinase activity ratio in the S_p -cAMPS samples, despite the fact that the effect on glycogen synthase was not as great.

The R_p -cAMPS-induced effect on gluconeogenic glucose production from a mixture of pyruvate and lactate was not limited to inhibition of only the agonist-induced rates, but resulted in an additional 14–20% decrease in the unstimulated rate of glucose production. This additional inhibitory effect on the basal rates of glucose production resulted from R_p -cAMPS inhibition of the higher endogenous cyclic AMP-dependent protein kinase activity (Table 1) resulting from the increased cyclic AMP levels in hepatocytes isolated from fasted rats (Goldstein & Curnow, 1978).

In summary, the intracellular inhibitor of cyclic AMP-dependent protein kinase, R_p -cAMPS, inhibited substrate-induced gluconeogenesis in hepatocytes isolated from fasted rats in both the absence of cyclic AMP-stimulatory agents and in the presence of glucagon and the cyclic AMP agonist, S_p -cAMPS. R_p -cAMPS inhibited the production of glucose from added pyruvate and lactate both through inhibition of the synthesis of glucose 6-phosphate and through activation of the glycogen synthesis pathway. All of the effects on gluconeogenic glucose production may be attributed to inhibition of cyclic AMP-dependent protein kinase.

We thank: Stephen Tillery and Mark Quagliotti for performing the glucose and lactate analyses; Dr. Robert V. Coombs and James Fraser for synthesizing R_p and S_p -cAMPS; Dr. Donald K. Webster for assisting in the preparation of the manuscript; Professor Bernd Jastorff for many helpful and insightful discussions.

REFERENCES

- Baraniak, J., Kinas, R. W., Lesiak, K. & Stec, W. J. (1979) *J. Chem. Soc. Chem. Commun.* 940–941
- Berry, M. N. & Friend, D. S. (1969) *J. Cell. Biol.* **43**, 506–520
- Clark, M. G. & Janett, I. G. (1978) *Biochem. J.* **176**, 805–816
- de Wit, R. J., Hoppe, J., Stec, W. J., Baraniak, J. & Jastorff, B. (1982) *Eur. J. Biochem.* **122**, 95–99
- Garrison, J. C. & Haynes, R. C. Jr., (1973) *J. Biol. Chem.* **248**, 5333–5343
- Gilboe, D. P., Larson, K. L. & Nuttall, F. Q. (1972) *Anal. Biochem.* **47**, 20–27
- Goldstein, D. E. & Curnow, R. T. (1978) *Metabolism* **27**, 315–323
- Groen, A. K., Vervoorn, R. C., VanderMeer, R. & Tager, J. (1983) *J. Biol. Chem.* **258**, 14346–14353
- Hers, H. G. & Hue, L. (1983) *Annu. Rev. Biochem.* **52**, 617–653

- Huijing, F. (1970) *Clin. Chim. Acta* **30**, 567–572
- Katz, L. D., Glickman, M. G., Rapoport, S., Ferrannini, E. & Defronzo, R. A. (1983) *Diabetes* **32**, 675–679
- Meserve, C. D., Rothermel, J. D., Houlihan, M. & Botelho, L. H. P. (1985) *J. Cyclic Nucleotide Protein Phosphorylation Res.* **10**, 371–382
- Newgard, C. B., Moore, S. V., Foster, D. W. & McGarry, J. D. (1984) *J. Biol. Chem.* **259**, 6958–6963
- O'Brien, C. A., Rocznik, S. O., Bramson, N., Baraniak, J., Stec, W. J. & Kaiser, E. T. (1982) *Biochemistry* **21**, 4371–4376
- Radziuk, J. & Inculet, R. (1983) *Diabetes* **32**, 977–981
- Rognstad, R. (1975) *Biochem. Biophys. Res. Commun.* **63**, 900–905
- Rothermel, J. D., Stec, W. J., Baraniak, J., Jastorff, B. & Botelho, L. H. P. (1983) *J. Biol. Chem.* **258**, 12125–12128
- Rothermel, J. D., Jastorff, B. & Botelho, L. H. P. (1984) *J. Biol. Chem.* **259**, 8151–8155
- Rothermel, J. D., Perillo, N. L., Marks, J. S. & Botelho, L. H. P. (1985) *J. Biol. Chem.* **259**, 15294–15300
- Thomas, J. A., Schlender, K. K. & Larner, J. (1968) *Anal. Biochem.* **25**, 486–499
- Van Haastert, P. J. M. (1981) *J. Chromatogr.* **210**, 229–254
- Williamson, J. R., Browning, E. T. & Scholtz, R. (1969) *J. Biol. Chem.* **244**, 4607–4616
- Zahlten, R. N., Stratman, F. W. & Lardy, H. A. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3213–3218

Received 15 October 1985/6 February 1986; accepted 19 March 1986