Biochemical Pharmacology, Vol. 35, No. 18, pp. 3103–3107, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

EVIDENCE FOR DIRECT EFFECT OF TOLBUTAMIDE ON HEPATIC GLYCOGENOLYSIS INDUCED BY Ca^{2+}-DEPENDENT HORMONES

TETSUYA MINE,*† SATOSHI KIMURA,‡ HITOSHI OHSAWA* and ETSURO OGATA*

*Fourth Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo; and ‡Endocrinology Division, National Cancer Center Research Institute, Tokyo, Japan

(*Received* 7 *October* 1985; *accepted* 5 *February* 1986)

Abstract—The effects of tolbutamide and glibenclamide on hepatic glycogenolysis in perfused rat liver were investigated. Tolbutamide *per se* did not influence glucose output from the liver, but at therapeutic concentrations (about 350 μM) it significantly inhibited the glycogenolysis induced by phenylephrine, vasopressin and angiotensin II, while glibenclamide did not. Neither tolbutamide nor glibenclamide inhibited the glycogenolysis induced by glucagon. Tolbutamide potentiated the inhibitory effect of submaximal concentrations of insulin on glycogenolysis induced by phenylephrine. This effect of tolbutamide was elicitable even in the absence of calcium in the perfusate, and was additive to that of trifluoperazine. However, tolbutamide did not potentiate the inhibitory effect of insulin on glucagon-induced glycogenolysis. Tolbutamide inhibited the glycogenolysis induced by A23187, a calcium ionophore. These results indicate that, in addition to its known effect on insulin secretion, tolbutamide has a direct effect on the liver to inhibit glycogenolysis induced by Ca^{2+}-dependent hormones (catecholamines, vasopressin and angiotensin II) and A23187. Thus, it is likely that tolbutamide inhibits the effect of Ca^{2+} mobilized by Ca^{2+}-dependent hormones to stimulate glycogenolysis.

Sulfonylurea agents have been in use as hypoglycemics for many years; the mechanisms of their extrapancreatic actions remain to be established. Numerous studies, both *in vitro* and *in vivo*, have demonstrated that the main effect of short-term sulfonylurea treatment is a prompt increase in insulin secretion [1–3]. On the other hand, Altzuler *et al.* [4] reported that tolbutamide decreases hepatic glucose output even in the absence of an increase in the serum insulin level. Moreover, acute administration of the drug *in vitro* [5] and *in vivo* [6] does not potentiate the anti-glycogenolytic, glucose-utilization stimulating and anti-lipolytic actions of insulin in the peripheral tissues including the liver. Therefore, sulfonylurea may act directly to inhibit glucose production by the liver.

The present study was performed to investigate whether or not the sulfonylureas, tolbutamide and glibenclamide, have direct effects on hepatic glycogenolysis.

MATERIALS AND METHODS

Animals. Fed, male Wistar rats (200 g) were used. Experiments were performed between 8:00 and 11:00 a.m. to ensure sufficient reserve of liver glycogen.

Liver perfusion. The animals were anesthetized with pentobarbital (50 mg/kg, i.p.). The liver was perfused at 37° in a flow-through system as described previously [7–9]. After oxygenation with 100% O_2, a Krebs–Ringer–Tris (KRT) buffer was infused continuously via the portal vein into the liver at a rate of 25 ml/min. The composition of the KRT buffer was the same as that of Krebs–Ringer–bicarbonate buffer except that the concentration of $CaCl_2$ was 1 mM because the concentration of Ca^{2+} in the extracellular fluid under physiological condition is close to 1 mM. Bicarbonate was replaced by 20 mM Tris–HCl (pH 7.40) to obtain a stable pH during perfusion [7–9]. Twenty minutes after the start of perfusion, sulfonylurea, insulin or control vehicle for either of these agents was added to the perfusate; this was followed 10 min later by the addition of glucagon or other agents. The effluent from the hepatic vein was collected at designated intervals. Under these conditions, the amounts of glucose released into the effluent reflect mainly the activity of glycogenolysis when the liver is perfused with either phenylephrine [7] or glucagon [9].

Determination of glucose. Glucose concentration in the effluent was measured by the glucose oxidase-peroxidase method (Boehringer-Mannheim Corp., Mannheim, West Germany).

Chemicals. Phenylephrine, vasopressin, dibutyryl cyclic AMP (DBCA) and isoproterenol were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Glucagon and insulin (Actrapid MC) were obtained from Novo Industri (Copenhagen, Denmark). A23187 was obtained from Calbiochem-Behring (Frankfurt, Germany). Tolbutamide, glibenclamide and trifluoperazine were donated by the Hoechst Japan Co., Ltd. (Tokyo, Japan), the Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan), and the Yoshitomi Seiyaku Co. (Osaka,

† Address reprint requests to: Dr. Tetsuya Mine, Fourth Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 3-28-6 Mejirodai, Bunkyo-ku, Tokyo 112, Japan.

Table 1. Effects of sulfonylurea on glucagon-induced glucose output and on glucagon–insulin interaction

	Basal glucose output rate (μmoles·g^{-1}·min^{-1})	Net increase in glucose output* (μmoles·g^{-1}·(11 min)$^{-1}$)
Glucagon only	1.14 ± 0.14†	12.0 ± 1.0
+ tolbutamide	1.20 ± 0.11	12.2 ± 0.3
+ glibenclamide	1.11 ± 0.06	11.2 ± 0.5
Glucagon plus insulin	1.10 ± 0.07	6.2 ± 0.5‡
+ tolbutamimde	1.06 ± 0.23	7.1 ± 0.7
+ glibenclamide	1.04 ± 0.04	6.8 ± 0.8

Experiments were carried out as described in Materials and Methods. Glucagon (5.7×10^{-11} M) was added 30 min after the beginning of the perfusion. Tolbutamide (7×10^{-4} M), glibenclamide (2×10^{-5} M) or insulin (0.1 mU/ml) was added 10 min before glucagon infusion when indicated.

* Net increase in glucose output above the basal output rate was cumulated for 11 min after the addition of glucagon.

† N = 4–6.

‡ $P < 0.01$ vs glucagon only.

Japan) respectively. All other chemicals were of reagent grade.

Statistics. Data are presented as mean ± S.E.M., and the Student's *t*-test was used for statistical analyses.

RESULTS

Effects of tolbutamide and glibenclamide on basal glucose production. The effect of sulfonylurea on the basal glucose output was determined with a wide range of concentrations. Tolbutamide did not affect the basal glucose output even at the highest concentration tested (2×10^{-3} M); glibenclamide slightly enhanced glucose output at concentrations above 4×10^{-5} M. The net amount of glucose released by 4×10^{-5} M glibenclamide was 1.50 ± 0.10 μmoles·(g liver)$^{-1}$·(11 min)$^{-1}$ (N = 3).

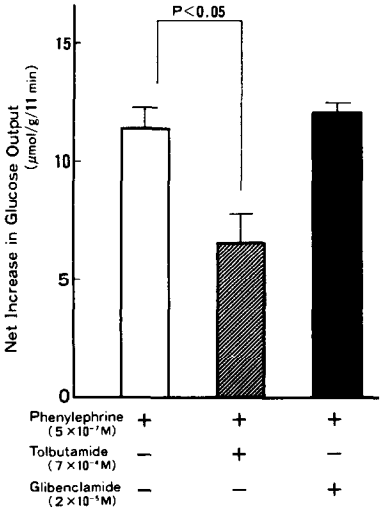

Fig. 1. Effects of tolbutamide and glibenclamide on net glucose output during 11 min of phenylephrine infusion. Livers from fed rats were perfused with KRT buffer. Twenty minutes after the start of perfusion, tolbutamide or glibenclamide was added to the perfusate, followed 10 min later by the addition of phenylephrine. The net increase in glucose output values, above basal, brought about by an 11-min period of phenylephrine infusion, is shown. Each bar and vertical line indicate mean and S.E.M. from five independent experiments for all conditions.

Effects of tolbutamide and glibenclamide on glucagon-induced glycogenolysis and glucagon–insulin interaction. The effect of sulfonylurea on the glucose output induced by half-maximal concentration of glucagon (5.7×10^{-11} M) [7, 9] was examined. As shown in Table 1, neither tolbutamide (7×10^{-4} M) nor glibenclamide (2×10^{-5} M) affected the basal or glucagon-induced glucose output. The half-maximal dose of insulin (0.1 mU/ml) [10, 11] significantly inhibited glycogenolysis induced by glucagon. The inhibitory effect of insulin on glucagon-induced glycogenolysis was not potentiated by the addition of tolbutamide or glibenclamide (Table 1).

Effects of tolbutamide and glibenclamide on phenylephrine-induced glycogenolysis and phenylephrine–insulin interaction. As shown in Fig. 1, tolbutamide (7×10^{-4} M) significantly inhibited glycogenolysis induced by a half-maximal concentration of phenylephrine (5×10^{-7} M) [7]. Even in the Ca^{2+}-free perfusate, tolbutamide inhibited the glucose output induced by phenylephrine (5×10^{-7} M) (5.43 ± 0.50 vs 2.36 ± 0.39 μmoles·(g liver)$^{-1}$·(11 min)$^{-1}$, N = 3, $P < 0.01$). Glibenclamide (2×10^{-5} M) did not inhibit the glycogenolysis induced by phenylephrine (Fig. 1).

The inhibitory effect of tolbutamide was signficant at concentrations above 3.5×10^{-4} M, although the effect was apparent at a concentration as low as 7×10^{-5} M (Fig. 2). Concentrations higher than 2×10^{-3} M could not be tested because of the insolubility of the agent. When insulin (0.1 mU/ml) was added after the infusion of submaximal concentration of tolbutamide (7×10^{-4} M), the glycogenolysis induced by phenylephrine was inhibited further (Fig. 3). However, the effect of a maximal dose of insulin (2 mU/ml) was not potentiated even by a maximum concentration of tolbutamide (2×10^{-3} M) (data not shown). Glibenclamide (2×10^{-5} M) showed no effect on the inhibitory

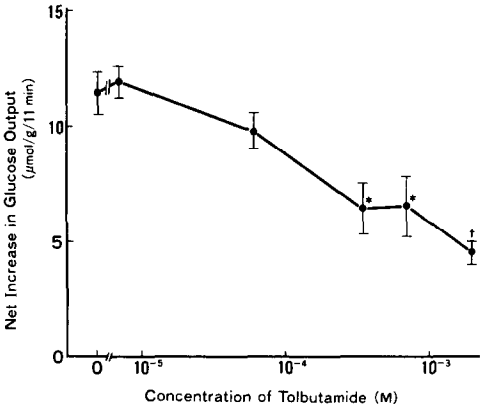

Fig. 2. Dose dependency of the inhibitory effect of tolbutamide on phenylephrine-induced glycogenolysis. The net increase in the glucose output during 11 min of phenylephrine (5×10^{-7} M) perfusion with various concentrations of tolbutamide is plotted. Each point and vertical line indicate mean and S.E.M. from three or five independent experiments. Key: (*) $P < 0.05$, and (†) $P < 0.01$ vs phenylephrine only.

effect of insulin glycogenolysis induced by phenylephrine.

Effects of tolbutamide on the dose–response curve of phenylephrine-induced glycogenolysis. Tolbutamide (7×10^{-4} M) inhibited glycogenolysis induced by high doses of phenylephrine as well as lower doses of the agonist (Fig. 4) and, as shown in the insert, the double-reciprocal plots indicate that tolbutamide inhibited the maximum response to phenylephrine without changing the half-maximal concentration.

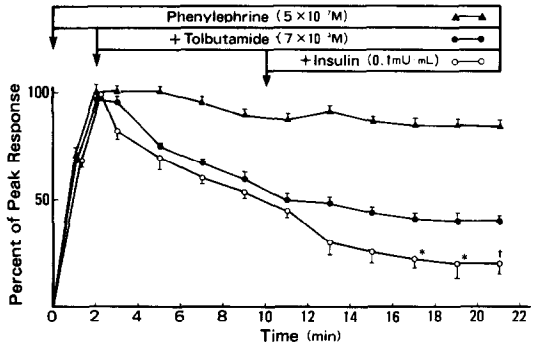

Fig. 3. Additivity of inhibitory effects of submaximal concentrations of insulin and tolbutamide. Livers from fed rats were perfused with KRT buffer. Twenty minutes after the start of perfusion, phenylephrine was added to the perfusate. When indicated, tolbutamide and insulin, respectively, were added 2 and 10 min after the phenylephrine. Results are expressed as percent of maximum reponse to phenylephrine in each experiment and N = 5 per group. Absolute values of maximum response were as follows: phenylephrine only 1.35 μmoles·(g liver)$^{-1}$·min^{-1}; phenylephrine + tolbutamide, 1.27 μmoles·(g liver)$^{-1}$·min^{-1}; and phenylephrine + tolbutamide + insulin, 1.30 μmoles·(g liver)$^{-1}$·min^{-1}. Key: (*) $P < 0.05$, and (†) $P < 0.02$ vs phenylephrine + tolbutamide at the same time point.

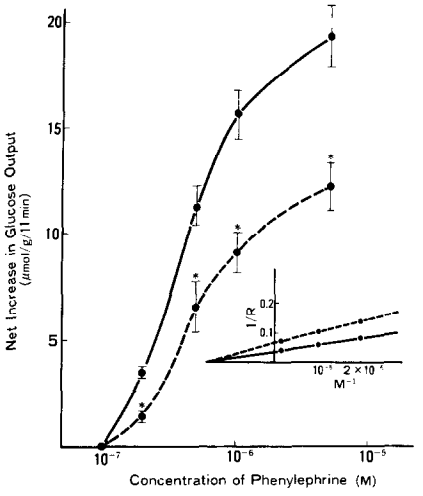

Fig. 4. Effects of tolbutamide (7×10^{-4} M) on the dose–response curve of phenylephrine. Experimental conditions were the same as those for Fig. 1 except that the concentrations of phenylephrine varied from 10^{-7} M to 5×10^{-6} M. In the insert, the double-reciprocal plots by using three of the points are shown. Each point and vertical line indicate mean and S.E.M. from three to five independent experiments. Key: (●—●) phenylephrine only; (●---●) phenylephrine + tolbutamide, and (*) $P < 0.05$.

Effects of tolbutamide on vasopressin- and angiotensin II-induced glycogenolysis. Tolbutamide (7×10^{-4} M) inhibited the glycogenolysis induced by half-maximal doses of vasopressin (0.2 mU/ml) and angiotensin II (10^{-9} M) (Table 2). The effects of vasopressin at concentrations between 0.2 and 10 mU/ml were inhibited by 7×10^{-4} M tolbutamide in such a manner as to inhibit the maximum effect of vasopressin. The effects of angiotensin II at concentrations between 10^{-7} M and 10^{-4} M were inhibited in a manner which shifted the dose-response curve of angiotensin II to the right (data not shown). Glibenclamide showed no effect on the glycogenolysis induced by either of these hormones.

Effect of tolbutamide on A23187-induced glycogenolysis. Tolbutamide inhibited glycogenolysis induced by 10^{-7} M A23187 (Table 2). Glibenclamide showed no effect on A23187-induced glycogenolysis.

Effects of tolbutamide on DBCA- and isoproterenol-induced glycogenolysis. DBCA (10^{-6} M) and isoproterenol (10^{-5} M) stimulated hepatic glycogenolysis to an extent comparable to that induced by half-maximal concentrations of glucagon and phenylephrine. Tolbutamide (7×10^{-4} M) did not inhibit the glycogenolysis induced by 10^{-6} M DBCA [DBCA only: 11.11 ± 1.36 vs DBCA + tolbutamide: 10.37 ± 0.78 μmoles·(g liver)$^{-1}$·(11 min)$^{-1}$, mean ± S.E.M., N = 4] or by 10^{-5} M isoproterenol [isoproterenol only: 10.50 ± 1.15 vs isoproterenol + tolbutamide: 9.30 ± 1.46 μmoles·(g liver)$^{-1}$·(11 min)$^{-1}$, mean ± S.E.M., N = 4].

Interaction between the effects of tolbutamide and trifluoperazine on the glycogenolysis induced by phenylephrine. The interaction between high concentrations of tolbutamide (2×10^{-3} M) and a maxi-

Table 2. Effects of tolbutamide on vasopressin-, angiotensin II- and A23187-induced glucose output

	Basal glucose output rate (μmoles·g·min^{-1})	Net increase in glucose output* (μmoles·g^{-1}·(11 min)$^{-1}$)
Vasopressin only	1.07 ± 0.07†	7.41 ± 0.74
+ tolbutamide	1.00 ± 0.01	5.00 ± 0.19‡
Angiotensin II only	1.05 ± 0.15	7.69 ± 0.46
+ tolbutamide	0.96 ± 0.03	3.33 ± 0.19§
A23187 only	1.02 ± 0.10	9.60 ± 0.90
+ tolbutamide	1.02 ± 0.10	3.10 ± 1.00‖

Experiments were carried out as described in Materials and Methods. Vasopressin (0.2 mU/ml), angiotensin II (1×10^{-9} M) or A23187 (1×10^{-7} M) was added 30 min after the beginning of the perfusion. Tolbutamide (7×10^{-4} M) was added 10 min before vasopressin, angiotensin II or A23187 infusion when indicated.

* Net increase in glucose output above the basal output rate was cumulated for 11 min after the addition of vasopressin, angiotensin II or A23187.

† N = 4.

‡ $P < 0.05$, §$P < 0.02$, and ‖$P < 0.01$ vs vasopressin, angiotensin II, and A23187 only, respectively.

mal concentration of trifluoperazine (10^{-4} M) [12] on glycogenolysis induced by phenylephrine was investigated to determine whether or not the mechanisms of action of tolbutamide and trifluoperazine are the same. Figure 5 shows that tolbutamide and trifluoperazine potentiated the inhibitory effects of each other on glycogenolysis induced by phenylephrine.

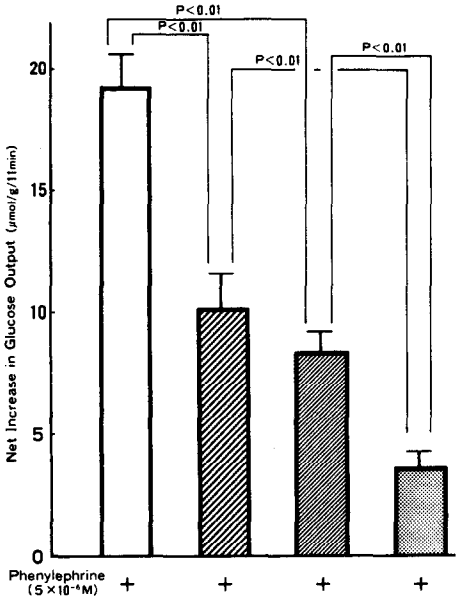

Fig. 5. Interaction between the effects of tolbutamide and trifluoperazine on the glycogenolysis induced by phenylephrine. Livers from fed rats were perfused with KRT buffer. Twenty minutes after the start of perfusion, maximum doses of tolbutamide and/or trifluoperazine were added to the perfusate, followed 10 min later by the addition of phenylephrine. The net increase in glucose output induced by an 11-min period of phenylephrine infusion is shown. Each bar and vertical line indicate mean and S.E.M. from three or four independent experiments for all conditions.

DISCUSSION

Sulfonylurea agents stimulate insulin release from pancreatic islets [1–3] and are widely used for the treatment of non-insulin-dependent diabetes mellitus. However, Altzuler *et al.* [4] found that tolbutamide decreases the hepatic glucose output even if no increase in the serum level of insulin is observed. Schambaye and Tarding [13, 14] reported that infusion of tolbutamide into the portal vein decreases glucose output in the dog. In support of this concept, it is clearly shown, in the present study, that tolbutamide at therapeutic concentrations inhibited the glucose output induced by the α-adrenergic agonist, vasopressin, angiotensin II and A23187, while it does not inhibit the glucose output induced by glucagon. As previously described [7, 9], when glucagon or phenylephrine is perfused, the decease in hepatic glycogen is nearly equivalent to the amount of glucose released into the effluent. Therefore, the increase in glucose output induced by either glucagon or phenylephrine is generated mainly through glycogenolysis. It can be considered that in this system tolbutamide inhibits glycogenolysis induced by phenylephrine, vasopressin and angiotensin II. The effective concentrations of tolbutamide in this study were well within the range of clinical significance (the peripheral concentration about 100 μg/ml = 3.5×10^{-4} M) described previously [6, 15, 16], while the concentration of this drug in the portal vein seems to be slightly higher than that in the peripheral vein. Although at high concentrations, glibenclamide slightly increased glucose output, the concentrations were far above the range clinically attained in the peripheral blood [17].

Miller *et al.* [16] showed that tolbutamide does not inhibit glucagon-induced glycogenolysis, while Blumenthal [18] reported that chlorpropramide potentiates the inhibitory effects of insulin on glu-

cagon-stimulated cyclic AMP production and glucose production in perfused rat liver. In our system, it was shown that tolbutamide and glibenclamide did not inhibit glucagon-induced glycogenolysis and that they did not potentiate the inhibitory action of insulin on this process.

The glycogenolytic effect of glucagon is believed to be mediated by cyclic AMP-dependent mechanisms [19, 20] and that of α-adrenergic stimulation, vasopressin and angiotensin II by Ca^{2+}-dependent mechanisms [21, 22]. Because tolbutamide inhibited only the glycogenolysis induced by these Ca^{2+}-dependent hormones and A23187 but not that induced by glucagon, tolbutamide seems to interfere with the Ca^{2+}-mediated mechanism. This concept is further supported by the data that tolbutamide inhibited neither DBCA-induced nor isoproterenol-induced glycogenolysis. The steps mediating the action of a cyclic AMP-dependent hormone (glucagon) and Ca^{2+}-dependent hormones may be confluent after the step of the activation of phosphorylase kinase. Therefore, the step(s) which tolbutamide affects may be in the process before or at the activation of phosphorylase kinase.

There seem to be several possibilities. First, tolbutamide may inhibit the binding of the Ca^{2+}-mediating hormones to their receptors. This possibility is unlikely because tolbutamide inhibited the glycogenolytic effect of the calcium ionophore A23187 and because it is not conceivable that tolbutamide would inhibit the binding of these three quite different hormones (α-adrenergic stimulation, vasopressin and angiotensin II) to their distinct receptors.

It is reported that insulin inhibits the phenylephrine-induced mobilization of calcium from the intracellular pool and inhibits glycogenolysis induced by phenylephrine [10, 23]. In the present study, it is shown that the effect of tolbutamide on phenylephrine action was additive to that of insulin when the concentration of insulin was half-maximal; it was no longer additive when the concentration of insulin was super-maximal. Accordingly, the mechanism of action of tolbutamide on phenylephrine may be closely related to that of insulin. Therefore, the second possibility is that tolbutamide inhibits Ca^{2+} mobilization induced by Ca^{2+}-dependent hormones. However, this is unlikely because tolbutamide inhibited A23187-induced glycogenolysis.

The third possibility is that tolbutamide interferes with the function of calmodulin. Trifluoperazine is reported to inhibit the expression of the biological activity of calmodulin, though its specificity is now questioned [24]. In the perfused rat liver system, trifluoperazine inhibits glycogenolysis induced by phenylephrine [12, 25], vasopressin and angiotensin II but not that induced by glucagon [12]. These effects of trifluoperazine are very similar to those obtained with tolbutamide in the present study. However, the modes by which tolbutamide inhibits the glycogenolysis induced by vasopressin and angiotensin II are different from those of trifluoperazine; trifluoperazine inhibits the glycogenolytic effect of vasopressin by shifting the dose-response curve to the right and that of angiotensin II by lowering the maximal response [12]. In contrast, tolbutamide inhibited the glycogenolytic effect of vasopressin by lowering the maximal response and that of angiotensin II by shifting the dose–response curve to the right. Therefore, the mechanism of action of tolbutamide may be different from that of trifluoperazine. This concept is supported by the finding in this study that both maximal doses of tolbutamide and trifluoperazine were additive in their inhibitory effects on phenylephrine-induced glycogenolysis (Fig. 5). Thus, although the detailed mechanism of action of tolbutamide is not clarified in the present study, it is most likely that tolbutamide inhibits the function of mobilized calcium in a calmodulin-independent manner.

Acknowledgements—This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education. We are grateful to Mrs. Tatsuko Kuroda and Mrs. Kunimi Yonezawa for their excellent assistance.

REFERENCES

1. S. E. Fineberg and S. H. Schneider, *Diabetologia* **18**, 49 (1980).
2. R. S. Yalow, H. Black, M. Villazon and S. A. Berson, *Diabetes* **9**, 36 (1960).
3. C. F. Gotfredsen, *Diabetologia* **12**, 339 (1976).
4. N. Altzuler, E. Morau and J. Hampshire, *Diabetes* **26** (Suppl.), 386 (1977).
5. G. F. Cahill, Jr., A. B. Hastings and J. Ashmore, *Diabetes* **6**, 26 (1957).
6. B. Zinman and R. I. Ogilvie, *J. clin. Endocr. Metab.* **35**, 299 (1972).
7. S. Kimura, Y. Koide, R. Tada, K. Abe and E. Ogata, *Endocr. Japan* **28**, 69 (1981).
8. S. Kimura, N. Kugai, R. Tada, I. Kojima, K. Abe and E. Ogata, *Hormone Metab. Res.* **14**, 133 (1982).
9. S. Kimura, T. Matsumoto, R. Tada, E. Ogata and K. Abe, *Acta endocr., Copenh.* **99**, 559 (1982).
10. P. F. Blackmore, F. Assimacopoulos-Jeannet, T. M. Chan and J. H. Exton, *J. biol. Chem.* **254**, 2828 (1979).
11. S. Kimura, R. Tada, T. Mine, E. Ogata and K. Abe, *Clin. Endocr.* **9**, 1083 (1981) (in Japanese).
12. Y. Koide, S. Kimura, R. Tada, N. Kugai and K. Yamashita, *Endocr. Japan* **29**, 369 (1982).
13. P. Schambye and F. Tarding, *Ann. N.Y. Acad. Sci.* **72**, 557 (1958).
14. F. Tarding and P. Schambaye, *Endokrinologie* **36**, 223 (1958).
15. W. Creutzfeldt, F. Andrew-Kern and R. Discher, *Ann. N.Y. Acad. Sci.* **82**, 537 (1959).
16. L. L. Miller, J. E. Sokal and E. J. Sarcione, *Am. J. Physiol.* **197**, 286 (1959).
17. H. A. E. Schmidt and P. Petrides, *Arzneimittel-Forsch.* **19**, 1422 (1969).
18. S. A. Blumenthal, *Diabetes* **26**, 485 (1977).
19. E. W. Sutherland and T. W. Rall, *J. biol. Chem.* **232**, 1077 (1958).
20. J. H. Exton, G. A. Robinson, E. W. Sutherland and C. R. Park, *J. biol. Chem.* **246**, 6166 (1971).
21. P. F. Blackmore, F. T. Brumley, J. L. Marks and J. H. Exton, *J. biol. Chem.* **253**, 4851 (1978).
22. J-L. J. Chen, D. F. Babcock and H. A. Lardy, *Proc. natn. Acad. Sci. U.S.A.* **75**, 2234 (1978).
23. J-P. Dehaye, B. P. Hughes, P. E. Blackmore and J. H. Exton, *Biochem. J.* **194**, 949 (1981).
24. P. F. Blackmore, M. F. El-Refai, J. P. Dehaye, W. G. Strickland, B. P. Hughes and J. H. Exton, *Fedn Eur. Biochem. Soc. Lett.* **123**, 245 (1981).
25. P. H. Reinhart, M. W. Taylor and F. L. Bygrave, *Fedn Eur. Biochem. Soc. Lett.* **120**, 71 (1980).