Biochemical Pharmacology, Vol. 31, No. 7, pp. 1227–1231, 1982.
Printed in Great Britain.

0006-2952/82/071227-05 $03.00/0

EFFECTS OF TOLBUTAMIDE ON INSULIN BINDING TO ISOLATED FAT CELLS OF THE RAT

HANS-GEORG JOOST*, WOLFGANG AREND and SUSANNE A. HOLZE
Institute of Pharmacology and Toxicology, University of Göttingen, D-3400 Göttingen, West Germany

(*Received* 22 *July* 1981; *accepted* 16 *October* 1981)

Abstract—In isolated fat cells of the rat the *in vitro* and *in vivo* effects of tolbutamide on insulin binding and insulin response were studied. 450 mg tolbutamide/kg/day given for 7 days significantly increased the binding of insulin to isolated adipocytes. The binding curves reflected an increase in the number of receptor sites rather than in the affinity. The effect was associated with an enhanced response to insulin of the adipose tissue, since the fat cells obtained from animals treated with tolbutamide converted significantly more glucose to lipids in the presence of insulin than those obtained from the control group. However, the augmentation of insulin binding sites was observed only at a large tolbutamide dosage, which reduced the pancreatic insulin content, the secretory response of the isolated pancreas, and the serum insulin levels. Smaller doses, sufficient to produce metabolic effects via a stimulation of insulin secretion, did not provide additional insulin binding sites. When added *in vitro* to the binding assay or to adipose tissue incubated for 16 h, tolbutamide failed to increase insulin binding of the fat cells. It is suggested, therefore, that the effects produced by tolbutamide after *in vivo* treatment reflect an indirect rather than a direct action of the sulphonylurea.

The hypoglycemic effect produced by an acute application of sulphonylurea derivatives may sufficiently be explained by their stimulatory action on the endogenous insulin secretion. Additional extrapancreatic actions, however, have been supposed to contribute to the metabolic effect of these agents, since clinical investigations revealed a dissociation of the metabolic effects and the stimulation of insulin secretion. The improved carbohydrate tolerance persisted, although the effect on serum insulin levels faded after a chronic therapy with chlorpropamide [1, 2], tolbutamide [3], or glibenclamide [4].

The prolonged application of sulphonylurea might therefore rather enhance the sensitivity of peripheral tissues to insulin than augment the amounts of insulin available. Accordingly, an increase of the insulin receptors was observed in monocytes from patients treated with chlorpropamide [5] or glibenclamide [6].

In animal experiments extrapancreatic actions of sulphonylureas could be established *in vivo* as well as *in vitro* [7–9]. Recently several groups have reported that sulphonylurea treatment increased the number of insulin receptors in liver membranes of rats [10], normal [11], and diabetic mice [12]. Moreover, a direct *in vitro* effect of a sulphonylurea derivative on insulin receptors in cultured human fibroblasts was observed [13]. The previous investigations [10–13] contained no data on the response of tissues to insulin. Thus it is still an open question, whether, and if so, to what extent, the augmentation of insulin receptor sites may enhance the metabolic effects of insulin.

In the present study, the effects of a sulphonylurea derivative on insulin receptors and insulin effects in one of the main target tissues, the adipose tissue, were investigated. Tolbutamide was applied to the animals during an *in vivo* treatment period, and was added to the medium of incubated adipose tissue in order to elucidate whether the sulphonylurea might directly modify insulin receptors of the fat cell. Some of the results have been previously published in abstract form [14, 15]. The results suggest that large doses of tolbutamide applied *in vivo* may give rise to an increase of insulin binding and response to insulin in fat cells, probably by indirect mechanisms.

MATERIALS AND METHODS

Materials. ^{125}I-labeled insulin was purchased from Behringwerke AG (Frankfurt, West Germany). Cristalline pork insulin and tolbutamide were gifts of the Farbwerke Hoechst AG (Frankfurt, West Germany). Bovine serum albumin (Cohns' fraction V), Eagle's minimum essential medium, and fetal calf serum were obtained from Serva (Heidelberg, West Germany). [U-^{14}C]Glucose was purchased from Amersham-Buchler (Braunschweig, West Germany), crude collagenase from Worthington (NJ). All other reagents (analytical grade) were from Merck AG (Darmstadt, West Germany).

Animals. Male albino Wistar rats (200–300 g) bred in our institute were used throughout. The animals had been fed a standard pellet diet (Altromin, Lage/Lippe), and had free access to food and water. During the treatment period the same diet was given in powder form mixed with tolbutamide to yield doses as indicated in the figures and tables.

Binding studies. Fat cells were prepared by the method of Rodbell [16]. Epididymal adipose tissue (approximately 1 g) obtained from anaesthetized animals was incubated in Krebs–bicarbonate buffer

* Author to whom correspondence should be addressed.

containing 1 mg/ml collagenase and 10 mg/ml albumin for 60 min at 37° in a metabolic shaker. The isolated cells were separated by flotation, washed five times with fresh buffer, and a diluted sample was counted in a Fuchs–Rosenthal counting chamber. The yield was approximately 5×10^6 cells per g adipose tissue. Samples containing $2–5 \times 10^5$ cells, 1.7×10^{-10} M [^{125}I]insulin, and increasing concentrations of unlabeled insulin ($10^{-9}–10^{-5}$ M) were prepared in a final volume of 300 μl and incubated for 40 min at room temperature (20°) in a shaking water bath. The equilibration period was stopped by rapid centrifugation of the cells through a layer of dinonylphthalate [17]. The cells were separated, and the radioactivity was determined (Autogamma 5100, Packard instruments).

Lipogenesis from glucose. The conversion of glucose to lipids was determined as described by Moody *et al.* [18]. A suspension of approximately 60,000 fat cells was incubated in 1 ml Krebs–bicarbonate buffer containing 1 mM glucose, 10 mg/ml albumin, 0.25 μCi [3-^{3}H]glucose, and 0.1–2 ng/ml insulin. The incubation was stopped by addition of a liquid scintillation cocktail (5 ml, Lipoluma, a toluene-based scintillator supplied by Baker Groß-Gerau). The vials (polypropylene scintillation vials) were shaken for 2 hr (60 strokes per min) to extract the lipids into the toluene layer, allowed to equilibrate for 4 hr, and the radioactivity was determined in a liquid scintillation counter. Blanks (a cell suspension immediately extracted with scintillator) were 150–200 cpm, and incubated cells usually yielded 500–3000 cpm per vial, depending on the concentration of insulin.

Insulin determinations. Insulin was measured by radioimmunoassay using an antibody obtained from guinea pigs. The antibody was diluted (1:750.000) to bind 50% of total insulin. The bound fraction was precipitated by ammonium sulfate in a final concentration of 21% (w/v). Unspecific binding was about 15% of the total radioactivity. Intraassay variation was less than 5%, and 0.1 ng/ml was the smallest concentration that could be detected. To measure the insulin content of the pancreas, animals were anaesthetized and desanguinized. The pancreas was removed and quickly homogenized in ice-cold acid–alcohol (75% ethanol, 0.4% hydrochloric acid). The homogenate was centrifuged, the supernatant was neutralized with 10% ammonium hydroxide and was again centrifuged. The final supernatant was immediately diluted (1:5,000–1:20,000) in Tris buffer (pH 7) containing 1% albumin. Insulin in the diluted samples was stable, and the samples were stored at −20° until assayed. In control experiments it was assured that the extracts contained no substances that interfered with the assay. Insulin added to the extracts was recovered correctly.

Perfusion of pancreas. The animals were anaesthetized by an intraperitoneal injection of pentobarbital (45 mg/kg body weight). Pancreas, spleen, stomach, and the proximal part of the duodenum were removed according to the method of Grodsky *et al.* [19], and perfused through the cannulated abdominal aorta. The perfusion media consisted of Krebs–bicarbonate buffer containing albumin (0.4%) and 5 or 20 mM glucose, and were gassed with oxygen/carbon dioxide 95:5. The flow rate was 4 ml/min and provided a constant perfusion pressure of 80–100 mm Hg. Total dead time for medium change was 20 sec. The perfusate was collected in 1 or 5 min intervals, and samples were diluted immediately in Tris buffer (pH 7.4) containing 0.1% albumin.

Blood glucose was assayed using the glucose oxidase reaction (Test kit by Boehringer, Mannheim, West Germany).

Non-esterified fatty acids were determined by the method of Duncombe [20].

Statistical analysis. The results were calculated as means ± S.E.M. and tested for statistical significance by means of the *U*-test of Wilcoxon, Mann and Whitney, or by the D_i-test of Wilcoxon and Wilcox.

RESULTS

In rats treated with a daily dose of 450 mg/kg tolbutamide, insulin binding to isolated fat cells was significantly increased (Table 1). Serum insulin levels were significantly lowered, whereas the blood glucose levels were unaffected. A moderate decrease of the serum non-esterified fatty acids was observed (11%), and insulin content of the pancreas was reduced by 17%. The size of the fat cells, food intake, and weight gain during the treatment period were not altered.

More detailed information on the effect of tolbutamide on insulin binding to adipocytes was obtained from the binding curves (Fig. 1). As is well recognized for the insulin receptor, the Scatchard plots of the data showed curvilinearity [21]. Tolbutamide shifted the Scatchard curve without altering its slope, suggesting that the number of receptors rather than their affinity was increased.

The response of adipocytes to insulin was largely enhanced by the tolbutamide treatment (Fig. 2). The maximal effect produced by insulin was approximately doubled, whereas insulin concentrations giving rise to half maximal stimulation of lipogenesis were not affected.

The effects of tolbutamide on insulin binding to fat cells was restricted to large sulphonylurea doses, as is shown in Fig. 3. In a smaller dosage sufficient to lower the blood sugar (50 mg/kg), tolbutamide failed to augment insulin receptors of adipocytes. The doses of tolbutamide that did increase insulin binding (400 and 600 mg/kg) simultaneously reduced the insulin content of the endocrine pancreas.

In order to test whether the depletion of the endocrine pancreas is paralleled by an impaired secretory function that might lower serum insulin levels (Table 1), we studied the response of the pancreas gland to a glucose stimulus (Fig. 4). The secretory response of the perfused rat pancreas to the glucose stimulus was significantly delayed and reduced, when the donor rats had been treated with a large tolbutamide dose (450 mg/kg for 7 days).

Tolbutamide failed to increase the specific binding of insulin in the *in vitro* system. After addition of the sulfonylurea in concentrations attainable *in vivo*, the specific as well as the unspecific binding were not altered at 37° and at 20° (Fig. 5). An incubation of the fat pads for 16 hr prior to the preparation of

Table 1. Effects of tolbutamide treatment (450 mg/kg/day) for seven days*

	Control	Tolbutamide	
Insulin binding (fmoles/10^6 cells)	5.4 ± 0.7	8.6 ± 0.4	$P < 0.001$
Cell volume ($l \times 10^{-12}$)	66.4 ± 4	65.3 ± 5	n.s.
Blood glucose (mM)	7.2 ± 0.2	7.0 ± 0.2	n.s.
Serum insulin (ng/ml)	1.27 ± 0.1	0.98 ± 0.1	$P < 0.025$
Pancreatic insulin content (μg)	113 ± 7	94 ± 4	$P < 0.025$
Non-esterified fatty acids (μM)	281 ± 14	249 ± 12	$P < 0.025$
Daily food intake (g)	19.7 ± 0.4	20.4 ± 0.5	n.s.
Initial body weight (g)	239 ± 2	238 ± 3	n.s.
Final body weight (g)	268 ± 4	262 ± 3	n.s.

* The results represent means ± S.E.M. of 12 experiments. Insulin binding represents the amount of tracer insulin (1.7×10^{-10} M) bound minus the tracer bound in the presence of excess (10^{-5} M) unlabeled insulin (non-specific binding).

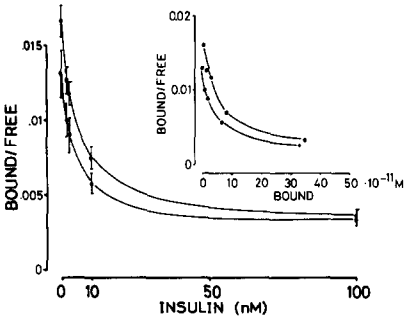

Fig. 1. Binding of insulin of adipocytes from rats treated with tolbutamide (300 mg/kg daily for 9 days). The bound/free ratio of tracer insulin is plotted against the concentrations of unlabeled insulin added. Data from the control experiments are represented by open circles, the smaller graph is the Scatchard-transformation of the results. (n=6).

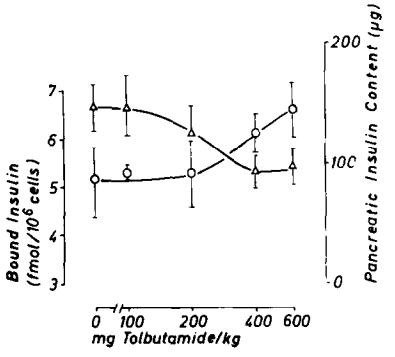

Fig. 3. Dose–response curves for the effects of tolbutamide on the binding of insulin (1.7×10^{-10} M, tracer insulin only) to adipocytes (circles) and pancreatic insulin content (triangles). The rats had received the dosage of tolbutamide indicated for seven days (n = 6). The effect on insulin binding was significant ($P < 0.05$) at 600 mg/kg, the effect on pancreatic insulin content at 400 and 600 mg/kg.

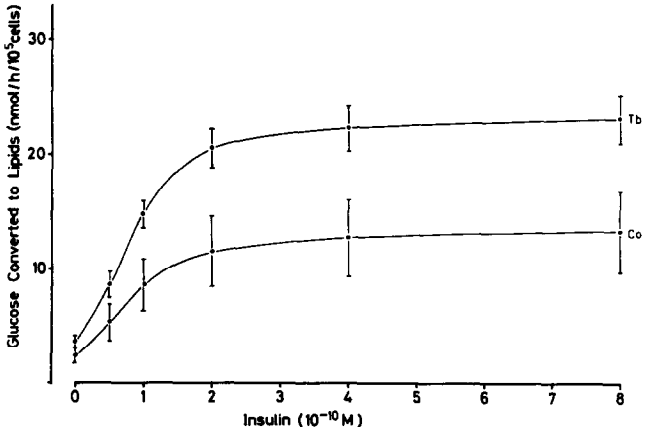

Fig. 2. Effects of insulin on the conversion of glucose to lipids in fat cells from rats treated with tolbutamide (450 mg/kg/day for 7 days, filled circles). Means ± S.E.M. of 8 experiments. The differences to controls were significant ($P < 0.025$) at 2, 4 and 8×10^{-10} M insulin.

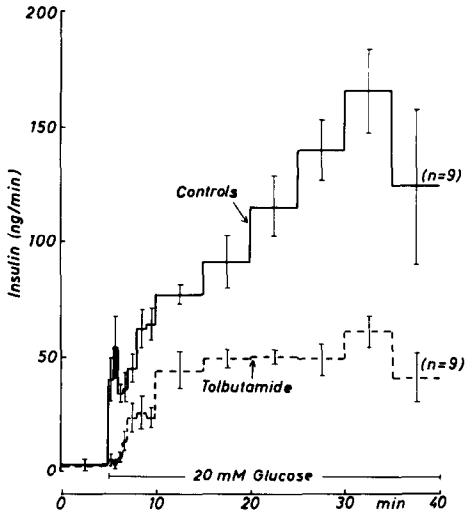

Fig. 4. Insulin release from the perfused rat pancreas of tolbutamide treated rats (450 mg/kg for seven days). The results represent means ± S.E.M. of 9 experiments. The glucose concentration infused was 5 mM from min 0 to 5, and 20 mM from min 5 to 40.

isolated cells failed to increase insulin binding, and also failed to antagonize the receptor loss [22] induced by added unlabeled insulin (Table 2).

DISCUSSION

In the present study tolbutamide treatment *in vivo* induced an increase of the insulin receptors in rat fat cells. Accordingly, the effect of insulin on lipogenesis of adipocytes from the treated animals was enhanced. In spite of reduced serum insulin levels, the blood glucose was normal, and the serum fatty acids were decreased. These effects suggest an enhanced responsiveness of the tissues to the endogenous insulin, paralleled by an increase in the number of the insulin receptors.

It is thus tempting to conclude that the augmentation of insulin receptors had produced an increased response of the fat cell to insulin. In view of the current hypotheses on the relationship of insulin

Table 2. Binding of insulin to fat cells from adipose tissue which had been previously kept in culture in the presence of tolbutamide and insulin*

	Control	Tolbutamide (0.2 mg/ml)
Without insulin during the culture period ($n = 8$)	9.68 ± 1.2	9.28 ± 0.5
2×10^{-8} M insulin ($n = 12$)	2.57 ± 0.4	2.68 ± 1.2

* The fat pads had been incubated for 16 hr in Eagle's minimum essential medium which contained fetal calf serum, streptomycin (0.1 mg/ml), tolbutamide, and insulin as indicated. The incubation flasks were continously gassed with oxygen/carbon dioxide 95:5. Fat cells were prepared thereafter in the absence of tolbutamide and insulin. The results represent means ± S.E.M. and are calculated as percent of the bound insulin per 10^6 fat cells (bound tracer at 1.7×10^{-10} M corrected for non-specific binding).

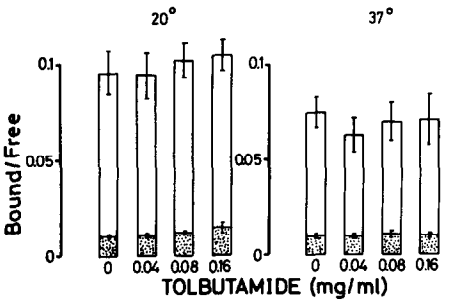

Fig. 5. Binding of insulin (1.7×10^{-10} M tracer insulin) to fat cells in the presence of tolbutamide. Means ± S.E.M. of 8 experiments. The shaded columns represent bound insulin in the presence of excess unlabeled insulin (10^{-5} M).

effects and insulin receptors, however, this conclusion may be questioned. According to the spare receptor concept [22] an augmentation of insulin binding sites is expected to shift the concentration–response-curve to lower insulin concentrations, thus lowering the insulin concentration which produces half maximal effects. An increase of the maximally obtainable response, as was seen in the present experiments, is attributed to post-receptor mechanisms rather than to an alteration of the receptor sites. Post-receptor events may therefore at least participate in the increased responsiveness of adipocytes to insulin after tolbutamide treatment.

Metabolic effects of sulphonylureas that are independent from the endogenous insulin secretion have been a matter of debate since the discovery of their hypoglycemic action. In view of animal experiments, the existence of such effects cannot be doubted. The *in vitro* lipolysis is inhibited [23], and glucose oxidation is stimulated by tolbutamide [24]. In the eviscerated rat the blood glucose lowering effect of insulin is enhanced by tolbutamide [8]. Chronic treatment of mice with tolbutamide (750 mg/kg) increased the glucose uptake into the incubated diaphragm, if both insulin and tolbutamide had been added to the incubation medium [25]. However, the concentration or dosage of tolbutamide that was necessary to produce extrapancreatic effects (0.15–0.8 mg/ml *in vitro* [23, 24], 400–800 mg/kg *in vivo*) was considerably higher than that necessary to stimulate insulin release *in vitro* (0.001–0.05 mg/ml [26]) or *in vivo* (25–200 mg/kg in the rat [27]). In the present experiments comparably large doses of tolbutamide (300–600 mg/kg) were necessary to increase the insulin binding sites of fat cells. As is shown in Fig. 3, the rise of insulin binding correlated with a depletion of the endocrine pancreas, an effect attributable to excessive dosage of sulphonylurea [28]. It is thus unlikely that, as far as the adipose tissue is concerned, the increase in insulin binding might contribute to the metabolic effects of tolbutamide when a dosage sufficient to stimulate insulin release is applied.

In cultured human fibroblasts glibenclamide increased the number of insulin receptors, and, more pronouncedly, antagonized the insulin induced receptor loss [13]. Loss of receptors is supposed to reflect an endocytotic internalization of occupied

receptors [31]. This process is facilitated by transglutaminase, an enzyme that covalently links the occupied receptors to form coated pits which are more easily internalized [32, 33]. Sulphonylureas inhibit transglutaminase [32], suggesting that the *in vitro* effect of glibenclamide at cultured fibroblasts is a consequence of inhibited receptor internalization [13]. In our experiments, however, a similar direct effect on the insulin receptor site could not be observed. Tolbutamide failed to directly modify the insulin binding and to reverse the insulin induced receptor loss when added to the binding assay or to the medium of adipose tissue incubated for 16 hours. It may be speculated, therefore, that in the fat cell transglutaminase is insensitive to sulphonylureas, or plays a minor role in regulating the receptor density of the cell surface.

The failure of tolbutamide to directly augment insulin binding sites suggests that indirect mechanisms are responsible for the effects observed after *in vivo* treatment. A possible link between the action of tolbutamide and insulin binding might be the serum insulin levels. The inverse relationship between insulin levels and insulin receptors is well established: decreased insulin levels induce a counterregulation of the receptors [34]. The dosage of tolbutamide applied in our study to increase the binding of insulin had obviously depleted the endocrine pancreas. This effect of large sulphonylurea doses has been described previously [28–30]. The depletion of the pancrease parallels a reduced response to a glucose stimulus [28–30], as was confirmed in the perfused pancreas preparation (Fig. 4), and might thus have lowered the serum insulin levels. Finally, the reduced availability of insulin might then have caused a counterregulatory effect on insulin receptors and insulin responsiveness.

Acknowledgements—The authors are indebted to Christoph Schmitz-Salue for excellent technical assistance, and to David Schluckebier for his help in the preparation of the manuscript.

REFERENCES

1. G. M. Reaven and J. Dray, *Diabetes* **16**, 487 (1967).
2. A. J. Barnes, K. J. T. Garbien, M. F. Crowley and A. Bloom, *Lancet* **ii**, 69 (1974).
3. B. R. Boshell, O. J. Fox, R. F. Roddam and P. S. Hills, in *Tolbutamide after Ten Years* (Eds. W. H. J. Butterfield, and W. von Westering), p. 286. Excerpta Medica, Amsterdam (1967).
4. J. M. Feldman and H. E. Lebovitz, *Diabetes* **20**, 745 (1971).
5. J. M. Olefsky and G. M. Reaven, *Am. J. Med.* **60**, 89 (1976).
6. H. Beck-Nielson, O. Pederson and H. O. Linskov, *Acta endocr. Copenh.* **90**, 451 (1979).
7. J. M. Feldman, and H. E. Lebovitz, *Archs intern. Med.* **123**, 314 (1969).
8. W. Creutzfeldt, U. Deuticke, and H. D. Söling, *Klin. Wschr.* **39**, 790 (1961).
9. J. Beyer, U. Cordes, H. Krall, G. Sell and K. Schöffling, *Arzneimittel Forsch.* **22**, 2167 (1972).
10. W. Bachmann, I. Böttger, M. Haslbeck and H. Mehnert, *Eur. J. clin. Invest.* **9**, 411 (1979).
11. M. N. Feinglos and H. E. Lebovitz, *Nature, Lond.* **276**, 184 (1978).
12. B. D. Greenstein, *Br. J. Pharmac.* **66**, 317 (1979).
13. M. J. Prince and J. M. Olefsky, *J. clin. Invest.* **66**, 608 (1980).
14. H. G. Joost and W. Arend, *Naunyn-Schmiedeberg's Arch. Pharmac.* **311**, R 51 (1980).
15. H. G. Joost, W. Arend and E. Heise, *Diabetologia* **19**, 286 (1980).
16. M. Rodbell, *J. biol. Chem.* **239**, 375 (1964).
17. S. Gammeltoft and J. Glieman, *Biochim. biophys. Acta* **320**, 16 (1973).
18. A. J. Moody, M. A. Stan, M. Stan and J. Gliemann, *Hormone Metab. Res.* **6**, 12 (1974).
19. G. M. Grodsky, L. L. Batts, C. Vcella, N. B. McWilliams and D. F. Smith, *Am. J. Physiol.* **205**, 638 (1963).
20. W. G. Duncombe, *Clin. Chim. Acta* **9**, 122 (1964).
21. C. R. Kahn, P. Freychet and J. Roth, *J. biol. Chem.* **249**, 2249 (1974).
22. J. M. Olefsky and O. G. Koltermann, *Am. J. Med.* **70**, 151 (1981).
23. D. B. Stone, J. D. Brown and C. P. Fox, *Am. J. Physiol.* **210**, 26 (1966).
24. A. E. Renold, G. R. Zahnd, B. Jeanrenaud and B. R. Boshell, *Ann. N.Y. Acad. Sci.* **74**, 490 (1959).
25. J. M. Feldmann and H. E. Lebovitz, *Diabetes* **18**, 84 (1969).
26. H. G. Joost and A. Hasselblatt, *Naunyn-Schmiedeberg's Arch. Pharmac.* **306**, 185 (1979).
27. A. Bänder, W. Pfaff and G. Schesmer, *Arzneimittel Forsch.* **19**, 1363 (1969).
28. J. C. Sodoyez, F. Sodoyez-Goffaux, J. C. Dunbar and P. P. Foà, *Diabetes* **19**, 603 (1970).
29. J. C. Dunbar and P. P. Foà, *Diabetologia* **10**, 27 (1974).
30. P. Schauder and H. Frerichs, *Diabetologia* **11**, 301 (1975).
31. J. Schlessinger, Y. Shechter, M. C. Willingham and I. Pastan, *Proc. natn. Acad. Sci. U.S.A.* **75**, 2659 (1978).
32. P. J. A. Davies, D. R. Davies, A. Levitzky, F. R. Maxfield, P. Milhaud, M. C. Willingham and I. H. Pastan, *Nature, Lond.* **283**, 162 (1980).
33. J. R. Gavin, J. Roth, D. M. Neville, P. De Meyts and D. N. Buell, *Proc. natn. Acad. Sci. U.S.A.* **71**, 84 (1974).