Biochemical Pharmacology, Vol. 22, pp. 29–35. Pergamon Press, 1973. Printed in Great Britain.

# THE PANCREATIC β-CELL RECOGNITION OF INSULIN SECRETAGOGUES—III

## EFFECTS OF SUBSTITUTING SULPHUR FOR OXYGEN IN THE D-GLUCOSE MOLECULE

BO HELLMAN, ÅKE LERNMARK, JANOVE SEHLIN, INGE-BERT TÄLJEDAL and ROY L. WHISTLER

Department of Histology, University of Umeå, S-901 87 Umeå 6, Sweden and Department of Biochemistry, Purdue University, Lafayette, Ind. 47907, U.S.A.

(*Received* 3 *April* 1972; *accepted* 10 *August* 1972)

**Abstract**—Sulphur-containing analogues of D-glucose were tested for effects on insulin release, D-glucose transport and D-glucose oxidation in microdissected pancreatic islets of obese-hyperglycemic mice. Substituting sulphur for oxygen in the ring structure of D-glucose (5-thio-D-glucose) resulted in a total loss of insulin-releasing ability. 5-Thio-D-glucose inhibited D-glucose-stimulated insulin release, D-glucose oxidation, and to a lesser extent D-glucose transport. Another D-glucose analogue, containing sulphur bound to carbon 1 (1-thio-β-D-glucose) did not influence D-glucose transport or oxidation. Whether 1-thio-β-D-glucose affected insulin release could not be decided because this compound appeared to destroy the insulin molecule. The results are compatible with the hypothesis that D-glucose metabolism plays a role in the recognition of this sugar as an insulin secretagogue.

D-GLUCOSE is the major physiological stimulus of insulin release. It is an unresolved matter how the pancreatic β-cells are able to sense this stimulus and to respond with an adequate rate of hormone output. The current debate has focused attention both on the likelihood that D-glucose recognition involves metabolism of the sugar,[1–6] and on the possible existence of a more direct D-glucose-receptor.[2,7–11] A possible strategy for choosing between these two alternatives would be to search for D-glucose analogues which have effects on insulin release that are different from those on D-glucose metabolism. The demonstration of a few such analogues, with either inhibitory or stimulatory properties, would support the direct-receptor hypothesis. On the other hand, repeated failures do dissociate between D-glucose metabolism and insulin release by means of D-glucose analogues can be said to strengthen the idea of D-glucose metabolism as a prerequisite for D-glucose recognition. The accumulation of both types of results may necessitate the more complex hypothesis[2] that the full recognition system involves a combination of a direct receptor and glucose metabolism. It has already been reported that D-glucose analogues such as D-galactose,[5,9] and D-glucosamine[1,5,9] are able to stimulate insulin release. It is not known, however, to what extent these compounds are metabolized in the β-cells.

The present contribution to a structure–activity programme for elucidating how D-glucose stimulates insulin release was initiated by the observation that 5-thio-D-glucose is diabetogenic when administered to rats.[12] It will now be shown that this

compound has a strong inhibitory action on both insulin release and D-glucose oxidation in pancreatic islets rich in $\beta$-cells and microdissected from obese-hyperglycemic mice.

## MATERIALS AND METHODS

*Chemicals.* 5-Thio-D-glucose was prepared by the method of Nayak and Whistler:[13] m.p. 135–136°; $(\alpha)_D^{20}$ + 188° (c 1·56, water). 1-Thio-$\beta$-D-glucose (m.p. 168–169°; $(\alpha)_D^{25}$ + 17° (c 1·5, water) was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Sigma Chemical Co. also supplied theophylline. D-(U-$^{14}$C)-glucose, D-(6-$^{3}$H)-glucose, L-(1-$^{14}$C)glucose and insulin antibodies were from the Radiochemical Centre, Amersham, England.[$^{125}$I] Insulin was obtained from Farbwerke Hoechst A.G., Frankfurt/M., Germany, and human serum albumin from AB Kabi, Stockholm, Sweden.

*Animals and handling of the islets.* Adult obese-hyperglycemic mice (gene symbol: *ob/ob*) of both sexes were taken from a local colony.[14] Unless otherwise stated, the animals were starved overnight before being killed. Fresh pancreatic islets were isolated by free-hand microdissection[15] in Krebs–Ringer bicarbonate medium supplemented with human serum albumin (0·3–0·5%, w/v) and equilibrated with $O_2$ + $CO_2$ (95:5). This kind of medium was also used as basal medium during subsequent incubations, all of which were carried out at 37°. D-Glucose uptake and oxidation experiments were performed with shaking (140 strokes/min, 3·5 cm amplitude). All experiments were started by preincubating the islets for 20–40 min in D-glucose-free, basal medium.

D-*Glucose transport.* After preincubation, batches of 3 islets were incubated for 45 sec in 200 $\mu$l medium supplemented with 2·0 mM D-(6-$^{3}$H)glucose (1·6 mCi/m-mole) and 2·5 mM L-(1-$^{14}$C) glucose (3·0 mCi/m-mole). This double label procedure permitted correction for label in the extracellular (L-glucose) space using each batch of islets as its own control.[16] After completed incubation the islets were placed on pieces of aluminium foil and were immediately freed of contaminating fluid with the aid of a micropipette. After freeze-drying (−40°, 0·001 mm Hg) overnight, the islets were weighed on a quartz fibre balance[17] and were dissolved by incubation for 45 min in 100 $\mu$l hyamine at room temperature. Ten ml of scintillation fluid (5 g of 2,5-diphenyloxazole and 50 mg of 1,4-*bis*-2-[5-phenyloxazolyl]benzene in 1·1 of toluene) was added and counting was carried out in a liquid scintillation spectrometer (Packard, Model 3375). The setting of the discriminators was such that less than 0·5 per cent of the $^{3}$H-counts were also counted in the $^{14}$C-channel. Spillover to the $^{3}$H-channel was 25 per cent of the counts in the $^{14}$C-channel. In all experiments the observed counts/min (corrected for background) were translated into m-mole of glucose taken up by comparison with external standards counted in parallel with the islets. These standards consisted of 5 $\mu$l incubation medium dissolved in 100 $\mu$l hyamine.

D-*Glucose oxidation.* After preincubation, islets were individually incubated for 60 min in 100 $\mu$l medium using liquid scintillation vials equipped with a small glass centre well.[18] The medium contained 10·0 mM D-[U-$^{14}$C]glucose (1·7 mCi/m-mole). Blank values were obtained by incubating medium without tissue. Metabolism was stopped and $^{14}CO_2$ was determined as previously.[19] The observed counts/min values were translated into m-mole of D-glucose equivalents completely oxidized per $\mu$g dry islet after freeze-drying and weighing the islets as described above.

*Insulin release.* The techniques for measuring insulin release from microdissected mouse islets have recently been dealt with in detail.[20] After preincubation, the islets were individually exposed to test substances by incubation in 115 μl of medium for 5 min (early phase) followed by incubation in 315 μl of medium for 60 min (late phase). The amounts of insulin released were measured in samples of medium taken from the preincubation as well as from the following incubation periods. Correction was made for insulin present in a minimum volume left from the preceding incubation periods. Insulin was radioimmunologically determined, using ethanol to separate free and antibody-bound insulin.[21] Crystalline mouse insulin was used as reference. The procedure of freeze-drying and weighing the islets as described above made it possible to express the amounts of insulin released per unit dry wt of islet tissue.

*Evaluation of data.* Parallel control incubations were routinely performed with islets isolated from the same animals as used for test incubations. This design made it possible to use each pancreas as its own control and to estimate statistical significances from the mean differences between paired test and control incubations over a series of repeated experiments. In this paper the expression "experiment" refers to a set of incubations with islets from a single animal. Within each experiment all factors were tested in multiple incubations, the mean result of which was entered as one observation in the statistical treatment.

## RESULTS

D-*Glucose transport and oxidation.* Table 1 shows the effects of sulphur-containing D-glucose analogues on D-glucose transport. At a D-glucose concentration of 2 mM, 10 mM 5-thio-D-glucose inhibited the 45-sec uptake of D-glucose by 30 per cent ($P < 0.05$). 5-Thio-D-glucose had an even stronger effect on D-glucose oxidation (Table 2). Islets incubated with 10 mM D-glucose and 10 mM 5-thio-D-glucose produced only 20 per cent as much $^{14}CO_2$ as islets incubated with 10 mM D-glucose alone ($P < 0.005$). 1-Thio-β-D-glucose had no significant effects on D-glucose transport (Table 1) or oxidation (Table 2).

TABLE 1. EFFECTS OF THIOGLUCOSE COMPOUNDS ON D-GLUCOSE TRANSPORT

| | Cellular uptake of labelled D-glucose (m-mole/kg dry islet) | |
|---|---|---|
| Test substance | Test | Test minus control |
| 5-thio-D-glucose | 0·89 ± 0·09 (6) | −0·35 ± 0·13* (6) |
| 1-thio-β-D-glucose | 1·42 ± 0·09 (8) | 0·01 ± 0·11 (8) |

After 30 min of preincubation in the absence of D-glucose, the islets were incubated for 45 sec with 10 mM thioglucose, 2·0 mM D-(6-$^3$H)glucose (1·6 mCi/m-mole) and 2·5 mM L-(1-$^{14}$C)glucose (3·0 mCi/m-mole). After correction for label in the L-glucose space, uptake of D-glucose was expressed as m-mole/kg dry islet. Results are given as mean values ±S.E. for incubations with thioglucose (test) as well as for the differences between parallel test and control incubations. The numbers of experiments are given within parenthesis.

* $P < 0.05$.

TABLE 2. EFFECTS OF THIOGLUCOSE COMPOUNDS ON D-GLUCOSE OXIDATION

| Test substance | Rate of D-glucose oxidation (m-mole/kg dry islet/hr) | |
|---|---|---|
| | Test | Test minus control |
| 5-thio-D-glucose | 4·65 ± 1·69 (5) | −18·06 ± 3·09* (5) |
| 1-thio-β-D-glucose | 18·34 ± 1·81 (6) | −4·80 ± 2·53 (6) |

After 30 min of preincubation in the absence of glucose, the islets were incubated for 60 min with 10·0 mM D-(U-$^{14}$C)glucose (1·7 mCi/m-mole) and 10·0 mM thioglucose. Oxidation rates are expressed as glucose equivalents completely oxidized per kilogram dry islet and hr. Results are given as mean values ±S.E. for incubations with thioglucose (test) as well as for the differences between parallel test and control incubations. The numbers of experiments are given within parentheses.

* $P < 0.005$.

*Insulin release.* The effects of 10 mM 5-thio-D-glucose are shown in Table 3. When islets were incubated in the absence of D-glucose, 5-thio-D-glucose had no significant effect on insulin release whether or not 5 mM theophylline was present. 5-Thio-D-glucose was, however, a strong inhibitor of D-glucose-stimulated insulin release.

In contrast to 5-thio-D-glucose, 1-thio-β-D-glucose carries its sulphur in a sulphydryl group. Because of the reactivity of sulphydryl groups, it was anticapated that 1-thio-β-D-glucose might invalidate the insulin measurements, for example by splitting the

TABLE 3. EFFECT OF 5-THIO-D-GLUCOSE ON INSULIN RELEASE

| Compounds added (mM) | | | Insulin release (ng/μg dry islet) | |
|---|---|---|---|---|
| D-glucose | 5-thio-D-glucose | Theophylline | Early phase | Late phase |
| 0 | 0 | 0 | 0·16 ± 0·03 (6) | 0·48 ± 0·15 (7) |
| 0 | 10 | 0 | 0·76 ± 0·34 (7) | 0·76 ± 0·20 (7) |
| 0 | 0 | 5 | 0·36 ± 0·10 (7) | 1·43 ± 0·51 (7) |
| 0 | 10 | 5 | 0·41 ± 0·12 (7) | 1·17 ± 0·30 (7) |
| 10 | 0 | 0 | 1·03 ± 0·22 (6) | 6·31 ± 1·38 (7) |
| 10 | 10 | 0 | 0·40 ± 0·11* (7) | 0·93 ± 0·19† (7) |

After preincubation for 40 min in the absence of glucose, islets from fed mice were incubated for 65 min with 10 mM D-glucose, 10 mM 5-thio-D-glucose and/or 5 mM theophylline. Results are given as nanograms insulin released/μg dry islet during the first 5 min (early phase) and subsequent 60 min (late phase) of incubation with glucose. Mean values ±S.E. for the numbers of experiments given within parentheses. Comparison versus incubations with 10 mM D-glucose alone.

* $P < 0.05$.

† $P < 0.01$.

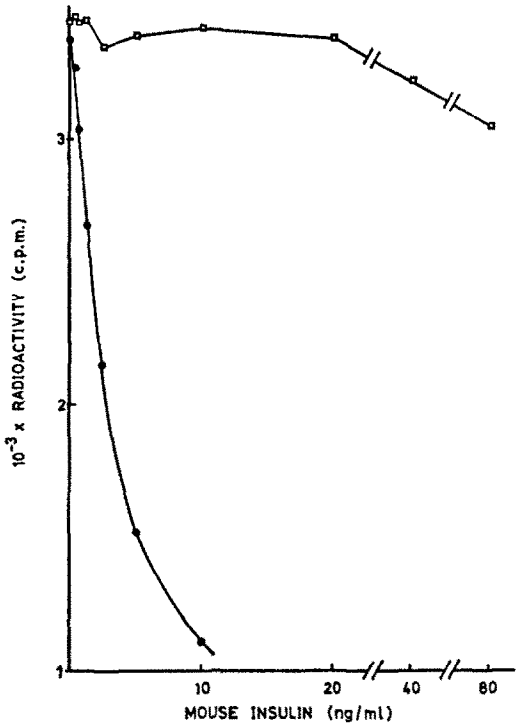


FIG. 1. Appearance of immunoassay standard curves after incubating the mouse insulin for 60 min at 37° in phosphate buffer containing (□) or lacking (●) 10 mM 1-thio-β-D-glucose.

disulphide bridges of the insulin molecule. Control experiments were therefore performed to evaluate the possibility of assaying insulin in the presence of 1-thio-β-D-glucose. When 0·6–80 ng/ml mouse insulin was incubated with 10 mM 1-thio-β-D-glucose at 37° for 60 min, almost none of the insulin was detectable by the immunoassay (Fig. 1). For this reason it was not possible to obtain reliable data on the effect of 1-thio-β-D-glucose on insulin release.

## DISCUSSION

It is clear from the present results that substituting sulphur for oxygen in the ring structure of D-glucose results in an apparently total loss of insulin-releasing ability. This might indicate that the oxygen atom in itself is essential for the stimulation of insulin release. Alternatively, distortion or puckering of the D-glucose molecule[22] may render it unfit for the stereochemical requirements of the system for recognition of D-glucose as an insulin secretagogue.

It has been shown that 5-thio-D-glucose is a substrate for the D-glucose transport system in the hamster small intestine[22,23] and inhibits D-glucose transport in rat diaphragm and kidney.[12,24] Our observation that 5-thio-D-glucose inhibited the 45-sec uptake of D-glucose suggests that 5-thio-D-glucose is a substrate for the D-glucose transport system in pancreatic β-cells as well. Studies on the effects of mannoheptulose[16,25] and phlorizin[11,16] on D-glucose transport and insulin release have led us

to conclude that the transport system in β-cells is not identical with the system for the recognition of D-glucose as an insulin secretagogue. This conclusion is supported by the present results, which indicate that substituting sulphur for oxygen in the D-glucose ring does not prevent the reaction with transport sites in the β-cells although abolishing the insulin-releasing ability.

In contrast to 5-thio-D-glucose, 1-thio-β-D-glucose had no effect on D-glucose transport or oxidation. This probably means that 1-thio-β-D-glucose is a poor substrate for the D-glucose transport system in the β-cells and may suggest that a hydroxyl group at carbon 1 is essential for transport into these cells.

The marked effects of 5-thio-D-glucose on both D-glucose oxidation and insulin release are in line with the hypothesis that metabolism plays a role in the recognition of D-glucose as an insulin secretagogue. For the sake of clarity, it should be stressed that this conclusion does not exclude the existence of a direct receptor mechanism as well. Since the D-glucose transport system in the investigated islets has a capacity that by far exceeds the rate of oxidation,[11,16] the drastic inhibition of D-glucose oxidation can hardly be explained by the rather moderate effect on D-glucose transport. The effects of 5-thio-D-glucose are qualitatively similar to those exerted by the seven-carbon sugar mannoheptulose.[1,3,25,26] The action of mannoheptulose has been thought to involve inhibition of D-glucose phosphorylation,[26,27] and it is of interest that 5-thio-D-glucose inhibits a particulate hexokinase purified from ox heart.[28] Caution is, however, warranted in extrapolating the latter finding to the β-cells, since 5-thio-D-glucose does not inhibit D-glucose phosphorylation by yeast hexokinase.[12] Whatever the molecular mechanism, the strong inhibition of insulin release exerted by 5-thio-D-glucose makes it possible to explain at least in part why this substance is diabetogenic when administered to rats.[12]

*Acknowledgements*—This work was supported by the Swedish Medical Research Council (12P-3870; 12x-562; 12x-2288) and the United States Public Health Service (AM 15641). Journal paper designation 4707 of the Purdue University Agricultural Experiment Station.

## REFERENCES

1. H. G. COORE and P. J. RANDLE, *Biochem. J.* **93,** 66 (1964).
2. P. J. RANDLE, S. J. H. ASHCROFT and J. R. GILL, in *Carbohydrate Metabolism and its Disorders*, (Eds. F. DICKENS, P. J. RANDLE and W. J. WHELAN), Vol. 1, p. 427. Academic Press, New York (1968).
3. S. J. H. ASHCROFT, C. J. HEDESKOV and P. J. RANDLE, *Biochem. J.* **118,** 143 (1970).
4. W. MONTAGUE and K. W. TAYLOR, *Biochem. J.* **115,** 257 (1969).
5. A. E. LAMBERT, A. JUNOD, W. STAUFFACHER, B. JEANRENAUD and A. E. RENOLD, *Biochim. biophys. Acta* **184,** 529 (1969).
6. B. HELLMAN, *Diabetologia* **6,** 110 (1970).
7. E. K. MATTHEWS and P. M. DEAN, in *The Structure and Metabolism of the Pancreatic Islets*, (Eds. B. HELLMAN, S. FALKMER and I.-B. TÄLJEDAL), p. 305. Pergamon Press, Oxford (1970).
8. F. M. MATSCHINSKY, J. E. ELLERMAN, J. KRZANOWSKI, J. KOTLER-BRAJTBURG, R. LANDGRAF and R. FERTEL, *J. biol. Chem.* **246,** 1007 (1971).
9. R. LANDGRAF, J. KOTLER-BRAJTBURG and F. M. MATSCHINSKY, *Proc. natn. Acad. Sci.* **68,** 536 (1971).
10. Y. KANAZAWA, L. ORCI and A. E. LAMBERT, *Endocrinology* **89,** 576 (1971).
11. B. HELLMAN, Å. LERNMARK, J. SEHLIN and I.-B. TÄLJEDAL, *Metabolism* **21,** 60 (1972).
12. D. J. HOFFMAN and R. L. WHISTLER, *Biochemistry* **7,** 4479 (1968).
13. U. G. NAYAK and R. L. WHISTLER, *J. Org. Chem.* **34,** 97 (1969).
14. B. HELLMAN, *Ann. N.Y. Acad. Sci.* **131,** 541 (1965).
15. C. HELLERSTRÖM, *Acta endocr. (Kbh.)* **45,** 122 (1964).
16. B. HELLMAN, J. SEHLIN and I.-B. TÄLJEDAL, *Biochim. biophys. Acta* **241,** 147 (1971).

17. O. H. LOWRY, *J. Histochem. Cytochem.* **1,** 420 (1953).
18. H. KEEN, J. B. FIELD and I. H. PASTAN, *Metabolism* **12,** 143 (1963).
19. B. HELLMAN, J. SEHLIN and I.-B. TÄLJEDAL, *Biochem. J.* **123,** 513 (1971).
20. Å. LERNMARK, *Acta diabet. lat.* **8,** 649 (1971).
21. L. G. HEDING, *Horm. Metab. Res.* **1,** 145 (1969).
22. D. R. CRITCHLEY, A. EICHHOLTZ and R. K. CRANE, *Biochim. biophys. Acta* **211,** 244 (1970).
23. J. E. G. BARNETT, A. RALPH and K. A. MUNDAY, *Biochem. J.* **118,** 843 (1970).
24. W. C. LAKE and R. L. WHISTLER, *J. biol. Chem.*, in press.
25. B. HELLMAN, L.-Å. IDAHL, Å. LERNMARK, J. SEHLIN, E. SIMON and I.-B. TÄLJEDAL, *Molec. Pharmac.* **8,** 1 (1972).
26. W. J. MALAISSE, M. A. LEA and F. MALAISSE-LAGAE, *Metabolism* **17,** 126 (1968).
27. S. J. H. ASHCROFT and P. J. RANDLE, *Biochem. J.* **119,** 5 (1970).
28. S. V. PARANJPE and V. JAGANNATHAN, *Ind. J. Biochem.* **8,** 227 (1971).