Inhibition of Cellular Transport Processes by 5-Thio-D-glucopyranose

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5-Thio-D-glucopyranose, the nearest analogue of normal D-glucose, which is proving a useful tool in examinations of D-glucose biochemistry, affects active and facilitated-diffusion transport processes. 5-Thio-D-glucose is readily transported in rabbit kidney-cortex slices and reaches a tissue/medium ratio of 6.5 within 40 min. The sulphur analogue shows typical saturation kinetics with a K_m value of 2.4 mM and V_{max} value of 70 μ mol/h per g of cell water. Uptake of 5-thio-D-glucose is phlorrhizin-sensitive, Na⁺-dependent and energy-dependent. D-Galactose and methyl α -D-glucopyranoside transport is competitively inhibited by 5-thio-D-glucose with K_i values of 4.8 and 9.7 mM respectively. 5-Thio-D-glucose thus shows all of the characteristics of active transport in kidney cortex. Transport of neutral amino acids in rat kidney cortex is inhibited by 5-thio-D-glucose. Thus 5.6 mM 5-thio-D-glucose causes a 25–30% inhibition of the transport of glycine and the non-metabolized amino acids cycloleucine and α -aminoisobutyric acid. 5-Thio-D-glucose is freely taken up by the facilitated-diffusion transport system in rat diaphragm muscle. The sulphur analogue inhibits the transport of D-xylose in this tissue but has no effect on the uptake of D-arabinose. It is concluded that the ring heteroatom is not an effector of binding in the transport processes examined and causes no important alteration in the conformation of the sugar. The diabetogenic action produced by 5-thio-D-glucose is due, in part, to the ability of the analogue to interfere with cellular transport processes that use D-glucose.

5-Thio-D-glucopyranose, an analogue of natural D-glucose, was first prepared by Feather & Whistler (1962). 5-Thio-D-glucose differs from D-glucose by having a sulphur atom in place of an oxygen atom in the pyranose ring. This subtle change, and the physical and chemical properties of the resulting sugar, make 5-thio-D-glucose the nearest analogue of normal D-glucose available to date. Initial biological studies have shown that 5-thio-D-glucose is non-toxic with an LD₅₀ of 14 g/kg. Most interestingly, however, it has an unusual diabetogenic action when administered orally or intraperitoneally to rats. Rapid development of glucosuria and hyperglycaemia occurs. With a dose of 50 mg/kg, the blood D-glucose concentration increases from a basal value of 80 mg/100 ml to 300 mg/100 ml within 2.5 h and can be maintained with booster doses of 5-thio-D-glucose. Administration of insulin nullifies the diabetogenic action. Most of the 5-thio-D-glucose is excreted in the urine, indicating a low rate of metabolism (Hoffman & Whistler, 1968). Further, results *in vitro* show a marked decrease in the uptake of D-glucose in liver, kidney and diaphragm with low concentrations of 5-thio-D-glucose. 5-Thio-D-glucose does not inhibit the metabolism of D-glucose in kidney homogenates (Hoffman & Whistler, 1968). It was suggested that, because of the structural similarities of the two sugars, 5-thio-D-glucose may interfere with cellular

transport systems utilizing D-glucose. The objective of the present work was to obtain further information on the diabetogenic action of 5-thio-D-glucose. This was done through examination of the biological action of 5-thio-D-glucose on the active cellular transport of sugars and amino acids in kidney-cortex slices, and on the facilitated-diffusion transport of sugars in the intact diaphragm muscle.

Experimental

Materials

D-[U-¹⁴C]Xylose (96 mCi/mmol), D-glucose, (4.6 mCi/mmol), D-[1-¹⁴C]arabinose (4.0 mCi/mmol) and [*carboxy*-¹⁴C]inulin (2.5 μ Ci/mg) were purchased from International Chemical and Nuclear Corp., Irvine, Calif., U.S.A. α -D-[1-¹⁴C]Galactose (20.7 mCi/mmol) was obtained from Schwarz/Mann, New York, N.Y., U.S.A. Methyl α -D-[¹⁴C]glucoside (3.8 mCi/mmol), D-[U-¹⁴C]sorbitol (7.0 mCi/mmol) and [³⁵S]thiourea (31.6 mCi/mmol) were obtained from Amersham/Searle Corp., Chicago, Ill., U.S.A. [1-¹⁴C]Glycine (8.61 mCi/mmol), [*carboxy*-¹⁴C]cycloleucine (6.68 mCi/mmol) and α -amino[1-¹⁴C]isobutyric acid (10.4 mCi/mmol) were products of New England Nuclear Corp., Boston, Mass., U.S.A. Insulin (25.9 U.S. Pharmacopeia units/mg) was

obtained from Calbiochem, Chicago, Ill., U.S.A. 5-[^{35}S]Thio-D-glucose (0.13 mCi/mmol) and unlabelled 5-thio-D-glucose were prepared by the method of Nayak & Whistler (1969). All other reagents were commercial preparations of A. R. grade.

Methods

Active sugar transport in kidney-cortex slices. Adult New Zealand white rabbits weighing 2–4 kg were used in all experiments. The animals were fed on a stock laboratory diet and water *ad libitum* until they were killed by stunning and decapitation. The kidneys were quickly removed and placed in an ice-cooled dish. Each kidney was bisected transversely and the capsule stripped off. Slices were prepared by hand. The incubation procedure was essentially that described by Kleinzeller *et al.* (1967b).

Six tissue slices (approx. 40 mg each) were placed in a 50 ml Erlenmeyer flask and subjected to an aerobic (O_2) preincubation at 25°C for 30 min in 8 ml of sugar-free Krebs–Ringer-type phosphate buffer, pH 7.4 (Kleinzeller, 1970). This preincubation was necessary to minimize the possible interference of D-glucose formed from endogenous substrate. The slices were then transferred to a flask containing 8 ml of the same buffer with the sugar to be investigated. The total radioactivity added to each flask was 0.1 $\mu\text{Ci/ml}$ irrespective of the quantity of sugar. The flasks were incubated aerobically (O_2) on a Dubnoff metabolic shaker for 60 min at 25°C. This time was sufficient to obtain a steady-state distribution of the sugar. After the incubation, the slices were removed, rinsed twice with water and blotted with filter paper. Each slice was then weighed and the tissue was homogenized and deproteinized (Somogyi, 1945). The deproteinized extract was then used for sugar determinations. A sample of the incubation medium was also deproteinized and used for the determination of the final sugar concentration ($[\text{S}]_0$) in the incubation medium.

In experiments where the rate of sugar transport, v , was measured, the rate of change in $[\text{S}]_i$ (intracellular sugar concentration) was assumed to be equal to v if the transport was linear over the time-course of the experiment. The tissue was usually incubated for 20 min in the presence of the test sugar.

Where Na^+ -free media were required, the NaCl was replaced with an equivalent amount of tris–HCl buffer, pH 7.4 (tris $^+$ –saline). Tris–HCl buffer does not harm the sugar transport system (Kleinzeller *et al.*, 1967b). To ensure the removal of endogenous Na^+ from the tissue, and a steady-state distribution of tissue water and electrolytes, the tissue slices were placed into tris $^+$ –saline and maintained at 0°C for 2.5 h with repeated washings. The usual

incubation procedure as indicated above was then followed.

Radioactivity was measured by pipetting 2 ml of the deproteinized tissue extract or incubation medium into counting vials followed by 10 ml of a solution composed of 0.5% 2,5-diphenyloxazole and 10% naphthalene in dioxan. The radioactivities in the vials were counted in a Beckman CPM 100 liquid-scintillation spectrophotometer.

Total tissue water was determined by the difference between the tissue weight after blotting and the weight after drying at 100°C for 24 h. From these results the intracellular sugar concentration $[\text{S}]_i$ was calculated (Kleinzeller *et al.*, 1967a). The $[\text{S}]_i/[\text{S}]_0$ ratio expresses the degree of intracellular accumulation; values above 1.0 indicate transport against the concentration gradient.

Amino acid transport in kidney-cortex slices. Wistar rats from our colonies, weighing 100–150 g, were fed on a stock laboratory diet and water *ad libitum* until they were killed by stunning and decapitation. Slices of renal cortex were cut by hand. Groups of three kidney slices were then incubated for 90 min in the presence of various amounts of 5-thio-D-glucose and radioactive amino acids (Genel *et al.*, 1971). The tissue amino acid pool was equilibrated with 2 ml of water by boiling for 15 min and radioactivity was then measured as described above.

Extracellular-space measurements were made by using inulin. Determination of total tissue water then allowed calculation of the distribution ratio $[\text{S}]_i/[\text{S}]_0$, i.e. the ratio of the sugar concentration in the intracellular space to the sugar concentration in the incubation medium.

Facilitated diffusion of sugars in diaphragm muscle. Diaphragm muscle was obtained from male or female Wistar rats of 100–150 g. The animals were fed on a stock laboratory diet and food was withheld for 18–20 h before each experiment. The procedure described by Randle & Smith (1958) was used to remove intact diaphragms. Each diaphragm was incubated for various time-periods in 10 ml of Krebs–Ringer bicarbonate buffer (Umbreit *et al.*, 1945) containing 0.1 unit of insulin/ml, 5-thio-D-glucose, and the sugar to be tested. Total radioactivity added to each flask was 0.1 $\mu\text{Ci/ml}$ irrespective of the quantity of sugar. After incubation the diaphragm muscle was excised away from the ribs, weighed, and frozen with solid CO_2 . Radioactive sugars were extracted from the tissue by boiling the tissue in 2 ml of Krebs–Ringer bicarbonate buffer for 15 min. Tubes containing the tissue and buffer were capped with marbles during boiling to decrease loss of water. The extracts were deproteinized (Somogyi, 1945) and the radioactivity was assayed as described above. Because of the limited quantities of 5-[^{35}S]thio-D-glucose available, the transport of this sugar was assayed by using the phenol–sulphuric acid

method described by Dubois *et al.* (1956). After incubation the tissue was homogenized in 4ml of buffer and the homogenate was deproteinized. A sample (1 ml) was then used for the sugar assay. After the addition of the phenol-sulphuric acid solution, samples were left for 60min before measuring the E_{491} with a Beckman DB spectrophotometer. Samples were corrected for endogenous sugar by subtracting the value of a tissue blank. Extracellular sugar space was measured with sorbitol. Results were expressed as the sugar space (Randle & Smith, 1958) where:

$$\text{Sugar space (ml/100 g)} = \frac{\text{muscle content (c.p.m./100 g wet wt. of muscle)}}{\text{medium concentration (c.p.m./ml)}}$$

Intracellular sugar space = sugar space less extracellular sorbitol space.

Results

Active sugar transport in kidney-cortex slices

5-Thio-D-glucose was examined as a possible inhibitor of D-galactose and methyl α -D-glucopyranoside transport in kidney-cortex slices. 5-Thio-D-glucose was also tested as a substrate for this transport system, and for other criteria of the active-transport process, including Na^+ -dependence, energy-dependence and phlorrhizin-sensitivity. Transport of D-galactose (Fig. 1) and methyl α -D-glucopyranoside (Fig. 2) were competitively inhibited by 5-thio-D-glucose. Values for K_i were 4.85mM and 9.7mM respectively. When cortex slices were incubated in the presence of 1mM-5-thio-D-glucose for various time-intervals, the sulphur sugar was rapidly transported against the concentration gradient and reached a tissue/medium ratio of approx. 6.5 within 40min (Fig. 3). A Lineweaver-Burk plot of the accumulation of 5-thio-D-glucose (Fig. 4) yielded a K_m of 2.4mM and $V_{\max}$ of 70 $\mu\text{mol/h}$ per g of cell water. Replacement of Na^+ by tris^+ or the addition of 0.4mM-phlorrhizin, a specific inhibitor of hexose transport, decreased the tissue/medium ratio to a value of less than 1, indicating an inhibition of 5-thio-D-glucose transport. In the presence of 1.0mM-2,4-dinitrophenol the active accumulation of 5-thio-D-glucose was inhibited by 70% (Table 1).

Amino acid transport in kidney-cortex slices

Interaction between 5-thio-D-glucose and the transport system for neutral amino acids was investigated by using rat kidney-cortex slices. Addition of 5-thio-D-glucose to a concentration of 5.6mM caused a 25–30% inhibition in the uptake of glycine and the non-metabolized amino acids cycloleucine and α -aminoisobutyrate (Table 2). At 11.2mM and 16.8mM,

5-thio-D-glucose caused no increased inhibition over that observed with 5.6mM-5-thio-D-glucose.

Facilitated diffusion of sugars in rat diaphragm muscle

By using intact rat diaphragm muscle, 5-thio-D-glucose was investigated as an inhibitor of the uptake of D-xylose and D-arabinose. Table 3 shows that 5-thio-D-glucose at a concentration of 26mM decreased the uptake of D-xylose but had no effect upon the transport of D-arabinose. These results show

clearly that the rate of uptake of D-xylose was affected during the first 10min of the experiment, but that during the remaining time-periods the rate of uptake was essentially the same, in the presence or the absence of 5-thio-D-glucose. 5-Thio-D-glucose was

Fig. 1. Lineweaver-Burk plot of the effect of 5-thio-D-glucose on the rate of D-galactose transport into kidney-cortex cells

Slices were incubated aerobically at 25°C for 20min. For further details see the text. Transport parameters were: K_m 1.61mM; $V_{\max}$ 16 $\mu\text{mol/h}$ per g of cell water; K_i 4.85mM. ○, 2mM-5-Thio-D-glucose; △, 0.5mM-5-thio-D-glucose; ●, control. v is in $\mu\text{mol/20min}$ per g of cell water.

accumulated quite readily in the intact rat diaphragm muscle (Fig. 5).

Discussion

With the initial biological testing of 5-thio-D-glucose, it quickly became obvious that the sulphur analogue was capable of acting as an antagonist of the ubiquitous metabolite D-glucose. Shankland *et al.* (1968) found that 5-thio-D-glucose in very low concentrations effectively interfered with the utilization of D-glucose for development of *Drosophila melanogaster*. 5-Thio-D-glucose/glucose molar ratios of 0.31 and above prevented development from the larval to the pupal stage. This inhibition was attributed to interference of enzyme(s) and/or transport of D-glucose across cell membranes, because of the structural similarity of the two sugars. The observation (Hoffman & Whistler, 1968) that the intraperitoneal injection of 5-thio-D-glucose into rats caused glucosuria and hyperglycaemia, which was overcome by the administration of insulin, gave even stronger support for the theory that 5-thio-D-glucose

Fig. 2. Lineweaver-Burk plot of the effect of 5-thio-D-glucose on the rate of methyl α -D-glucoside transport into kidney-cortex cells

Slices were incubated aerobically at 25°C for 20 min. For further details see the text. Transport parameters were: K_m 3.7 mM; V_{max} 33.3 $\mu\text{mol/h}$ per g of cell water; K_i 9.7 mM. $\blacktriangle$, 2 mM-5-Thio-D-glucose; $\circ$, control. v is in $\mu\text{mol/20 min}$ per g of cell water.

Fig. 3. Uptake of 5-thio-D-glucose by kidney-cortex slices

The initial concentration of 5-thio-D-glucose was 1 mM. For further details see the text.

Fig. 4. Lineweaver-Burk plot of 5-thio-D-glucose transport in kidney-cortex slices

Slices were incubated aerobically at 25°C for 20 min. For further details see the text. Transport parameters were: K_m 2.4 mM; V_{max} 70 $\mu\text{mol/h}$ per g of cell water. v is in $\mu\text{mol/20 min}$ per g of cell water.

Table 1. *Effect of phlorrhizin, Na⁺, and 2,4-dinitrophenol on the accumulation of 5-thio-D-glucose in kidney-cortex slices*

Slices were incubated aerobically for 60min at 25°C in Na⁺- or tris⁺-saline containing 1 mM-5-thio-D-glucose. Each value is the mean $\pm$ S.E.M. of 12 analyses. For further details see the text.

Conditions	[S] _i /[S] _o
5-Thio-D-glucose, 135 mM-Na ⁺	6.52 $\pm$ 0.32
5-Thio-D-glucose, tris ⁺ medium, [Na ⁺] _o = 0	0.78 $\pm$ 0.4
5-Thio-D-glucose, phlorrhizin (0.4 mM)	0.96 $\pm$ 0.21
5-Thio-D-glucose, 2,4-dinitrophenol (0.1 mM)	2.31 $\pm$ 0.62

Table 2. *Effect of 5-thio-D-glucose on the accumulation of amino acids by rat kidney-cortex slices*

Slices were incubated aerobically for 90min at 37°C. The 5-thio-D-glucose concentration was 5.6 mM. Each value is the mean $\pm$ S.E.M. For further details see the text.

Additions	α -Aminoisobutyrate (0.065 mM)	Cycloleucine (0.08 mM)	Glycine (0.03 mM)
None	5.90 $\pm$ 0.20	4.89 $\pm$ 0.19	8.29 $\pm$ 0.29
5-Thio-D-glucose	4.48 $\pm$ 0.17	3.60 $\pm$ 0.17	5.88 $\pm$ 0.16

Table 3. *Effect of 5-thio-D-glucose on the accumulation of D-xylose and D-arabinose in intact rat diaphragm muscle*

Slices were incubated aerobically for the time-periods shown in the presence and the absence of 5-thio-D-glucose. Each flask contained insulin at a concentration of 0.1 unit/ml. Extracellular sugar space was measured, with D-[¹⁴C]sorbitol, as 24 ml/100g.

Total sugar space (ml/100g of muscle)

Time (min)	D-Xylose	D-Xylose + 5-thio- D-glucose (26 mM)	D-Arabinose	D-Arabinose + 5-thio- D-glucose (26 mM)
10	28.3	24.3	24.7	24.0
20	40.5	35.8	28.3	28.1
30	47.8	41.9	32.7	32.7
40	51.3	45.8	32.6	32.5

Intracellular sugar space (ml/100g of muscle)

Time (min)	D-Xylose	D-Xylose + 5-thio- D-glucose (26 mM)	D-Arabinose	D-Arabinose + 5-thio- D-glucose (26 mM)
10	4.3	0.3	0.7	0.0
20	16.5	11.8	4.3	4.1
30	23.8	17.9	8.7	8.7
40	27.3	21.8	8.6	8.5

inhibited the transport of D-glucose across cell membranes. Critchley *et al.* (1970) and Barnett *et al.* (1970) used 5-thio-D-glucose to examine the transport of the sulphur sugar in hamster small intestine. 5-Thio-D-glucose exhibited all of the criteria necessary for it to be accepted as a substrate for the D-glucose active-transport system. Critchley *et al.* (1970) also found that the specific binding of

D-[³H]glucose to intestinal brush border was inhibited by 5-thio-D-glucose.

In rabbit kidney-cortex slices, which accumulate sugar by an active-transport process, 5-thio-D-glucose is a competitive inhibitor of D-galactose and methyl α -D-glucopyranoside transport. Competitive inhibition reported here and in the intestinal transport work done by Critchley *et al.* (1970) indicates that the

Fig. 5. Transport of 5-thio-D-glucose by intact rat diaphragm

Flasks contained 8.3 mM-5-thio-D-glucose and 0.1 U.S. Pharmacopeia unit of insulin/ml. For further details see the text.

5-thio-D-glucose is bound to the D-glucose transport carrier in these tissues. Values for K_m , V_{max} , and K_i for 5-thio-D-glucose are of the same order as those found for D-glucose and other sugars (Kleinzeller *et al.*, 1967a; Kleinzeller, 1970). The competitive nature of the inhibition leads to the question of whether 5-thio-D-glucose is exerting its effect simply through reversibly binding to the transport carrier or is itself being transported. The observation that 5-thio-D-glucose is rapidly accumulated against the concentration gradient (Fig. 3) indicates that the sulphur sugar is acting as a true substrate for the transport carrier. In fact, 5-thio-D-glucose exhibits all of the criteria required for it to be accepted as a substrate for the active-transport system. These include accumulation against the concentration gradient, energy-dependence, Na^+ -dependence, substrate inhibition and phlorrhizin-sensitivity.

A relationship between the renal transport of hexoses and of amino acids is suggested by the occurrence of aminoaciduria with galactosaemia, fructose intolerance, Fanconi syndrome, and glucosuria in diabetes mellitus (Thier *et al.*, 1964). Infusion of sugar into humans and feeding of large amounts of D-galactose to rats also results in aminoaciduria (Thier *et al.*, 1964). There is a mutual inhibition of transport between amino acids and hexoses that use the D-glucose-D-galactose transport systems in kidney cortex (Genel *et al.*, 1971; Thier *et al.*, 1964; Segal *et al.*, 1962). Inhibition is non-

competitive (Genel *et al.*, 1971) and there are evidently separate transport systems for sugars and amino acids in this tissue (Segal *et al.*, 1971). Because of this we examined the interaction between 5-thio-D-glucose and the transport system for neutral amino acids in rat kidney cortex. Inhibition of amino acid transport caused by 5-thio-D-glucose is non-competitive and is approximately the same as the inhibition caused by the addition of the same amount of D-glucose (Thier *et al.*, 1964). Inhibition of the transport of the non-metabolized cycloleucine and α -aminoisobutyric acid by the apparently non-metabolized 5-thio-D-glucose confirms the observation (Genel *et al.*, 1971) that the amino acid-sugar interaction does not depend upon metabolism of either substance in rat kidney cortex. Thus, 5-thio-D-glucose antagonizes active transport of amino acids and sugars in rat kidney cortex.

Uptake of 5-thio-D-glucose in rat diaphragm is nearly linear over the first 30 min and then levels off (Fig. 5). Since the intracellular volume of rat diaphragm is about 50 ml/100 g (calculated from total water less sorbitol space), it would appear that 64% of the intracellular water is available for penetration by 5-thio-D-glucose. This compares with 56% available to D-xylose and only 15% available to D-arabinose. The lack of inhibition of this low accumulation of D-arabinose by 5-thio-D-glucose suggests that D-arabinose enters the 15% intracellular space by diffusion rather than transport.

The observation that the rate of uptake of D-xylose is inhibited by 5-thio-D-glucose only during the first 10 min of the experiment indicates that the inhibition is not of a simple competitive nature. One can observe the same phenomenon in the results reported by Battaglia & Randle (1960). The significant finding here is that 5-thio-D-glucose antagonizes the membrane transport of sugars in a facilitated-diffusion transport system.

Our results provide strong evidence that the inhibition of membrane transport processes by 5-thio-D-glucose is a general phenomenon.

From the observations, especially the similar K_m and V_{max} values of D-glucose and its sulphur analogue, it appears that the heteroatom of the D-glucose ring is not an important effector of binding in the membrane transport systems examined. This is interesting since replacement of the ring oxygen atom by the much larger but much less negative sulphur atom could be expected to affect ring binding. However, the shape of the two rings are the same with both apparently existing in the C1 chair conformation, where all substituents other than hydrogen can be equatorially oriented (Suzuki & Whistler, 1972).

In explaining the diabetogenic effect of 5-thio-D-glucose, the ability of the analogue to greatly decrease the transport of sugars in all of the tissues examined

is an important factor. However, the biological activity of 5-thio-D-glucose is more complex and other factors must also be considered. Hellman *et al.* (1972) found that 5-thio-D-glucose inhibited the uptake of D-glucose in pancreatic islets by 30%, but the sulphur sugar was also a strong inhibitor of D-glucose-stimulated insulin release. Insulin is known to greatly facilitate the transport of sugars in many tissues. When islets of Langerhans are incubated in the absence of D-glucose, 5-thio-D-glucose has no effect on insulin release. It is concluded that substituting a sulphur atom for an oxygen atom in the ring structure of D-glucose results in an apparently total loss of insulin-releasing ability. In addition to the inhibition of D-glucose-stimulated insulin release, Hellman *et al.* (1972) found that pancreatic islets incubated with D-glucose and 5-thio-D-glucose produce only 20% as much $^{14}\text{CO}_2$ from D-glucose as islets incubated with D-glucose alone. This indicates that 5-thio-D-glucose also interferes with the metabolism of transported D-glucose.

Paranjpe & Jagannathan (1971) reported that 5-thio-D-glucose inhibits ox heart hexokinase. If the hexokinase in other tissues is also inhibited, the inability of the cells to phosphorylate D-glucose would result in high blood sugar concentrations. In contrast to these findings, 5-thio-D-glucose is a poor substrate for yeast hexokinase and does not inhibit the action of this enzyme (Hoffman & Whistler, 1968; Critchley *et al.*, 1970). 5-Thio-D-glucose also does not inhibit the metabolism of D-glucose in kidney homogenates (Hoffman & Whistler, 1968).

Diabetogenic action of 5-thio-D-glucose is almost certainly caused by a combination of its effect on membrane transport and insulin release, and may also involve inhibition of the cellular metabolism of D-glucose. The question of the extent to which 5-thio-D-glucose may interfere with the metabolism of D-glucose is unanswered, but this should be shown by future work.

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