Inhibition of Glucose Oxidation by 6-Deoxy-D-Glucose.*† (26636)

JAMES L. BROOKS, ELEANOR CHRISTENSEN, CHARLES J. STEWART, AND ARNE N. WICK
Chemistry Department, San Diego State College, San Diego, Calif.

Considerable evidence supports the concept for some kind of membrane transport system for intracellular transfer of sugars. However, there has not been any fundamental advance in understanding this transport mechanism. It is not known whether all sugars enter cells by one process or whether there are several processes, each of which affects only one sugar or a group of structurally related sugars. To gain further information on the cell permeation mechanism of sugars, we have carried out a series of experiments to determine if the sugar analog 6-deoxy-D-glucose (6-DOG) could act as an effective inhibitor of glucose metabolism in mammalian tissue. 6-Position substituted sugars, which cannot be phosphorylated by hexokinase, are considered unlikely to affect glucose metabolism in mammalian cells beyond the hexokinase step. Thus, the inhibition, if any, produced by 6-position modified glucose analogs should be less complex than that produced by analogs modified in, for example, the 2-position.

Methods. The effect of 6-DOG on glucose metabolism was measured by means of tracer technics in rat kidney cortex slices, rat diaphragm, and mouse adipose (epididymal) tissue. Glucose metabolism was followed by collection of the $C^{14}O_2$ produced by the tissues during incubation with glucose-U-C^{14} plus varying amounts of the test substance 6-DOG. With diaphragm, the tissue incorporation of C^{14} was also determined. In one experiment adipose tissue was incubated with 6-deoxy-D-glucose-U-C^{14} to determine if this substance was oxidized to $C^{14}O_2$.

Biological systems. Standardized procedures were used throughout. The incubation medium was Krebs-Ringer-phosphate buffer (calcium free). All incubations were at 37°C for 2 hours. Kidney and diaphragm tissues were from Sprague-Dawley rats. Kidney cortex was sliced with a Stadie-Riggs microtome; 150 mg of wet tissue was used per reaction flask. Paired rat hemi-diaphragms were incubated separately, with one serving as control for the other. Adipose tissue was from White Swiss mice. Of each pair of fat pads, of approximately 150 mg wet weight, one served as a control.

Preparation of 6-deoxy-D-glucose. Beta-1,2,3,4 - tetraacetyl - 6 - deoxy - D - glucose was prepared by a modification of the method of Hardegger and Montavon(1). This compound yielded 6-DOG on acid hydrolysis. The method was modified in 2 ways. In preparation of beta-1,2,3,4-tetraacetyl-6-iodo-D-glucose from beta-1,2,3,4-tetraacetyl-6-tosyl-D-glucose, the acetic anhydride solution of the product was flash evaporated, rather than being poured into water, and the solid resulting from flash evaporation was washed with water and recrystallized from 95% ethanol. The second modification was that beta-1,2,3,4-tetraacetyl-6-deoxy-D-glucose was prepared directly from the thiouronium iodide using methanol as the solvent rather than butanol mentioned in the original procedure. To do this, 30 g of the thiouronium iodide were dissolved in 550 ml of absolute methanol, 66 g of Raney nickel added, and the mixture stirred for 3 hours. The methanol was filtered and flash evaporated, and the residue crystallized from 1-butanol.

To prepare 6-DOG, 11 g of Amberlite 120 resin, type 3 (Rohm and Haas product), were washed 5 times with 5% sulphuric acid, then with distilled water until the rinse water was neutral. The resin was placed in 100 ml of water and 10 g of beta-1,2,3,4-tetraacetyl-6-deoxy-D-glucose were added. This was refluxed for 4.5 hours with stirring. The solution was filtered and lyophilized to dryness. The 6-DOG was crystallized from absolute ethanol. The M.P. was 146 to 147°C. One

* Supported by grants from U. S. Public Health Service to Arne N. Wick.

† Taken in part from M. S. Thesis of James L. Brooks, San Diego State College, 1961.

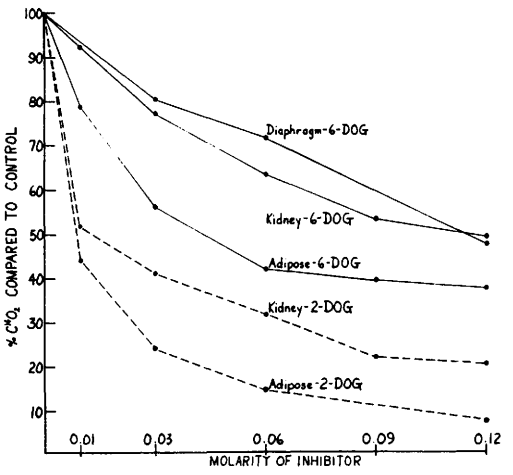

FIG. 1. Effect of 6-DOG and 2-DOG on oxidation of glucose-U-C^{14} by adipose, kidney, and diaphragm tissues.

batch was subjected to carbon and hydrogen analysis.‡ Found: 43.43% carbon and 7.53% hydrogen. Calculated for $C_6H_{12}O_5$: 43.90% carbon and 7.37% hydrogen. The osozone melted at 186°C as compared to the value 186 to 187°C given by Fischer and Zach(2). The labeled compound was prepared by starting was glucose-U-C^{14}.

Results. One experiment run with quadruplicate samples of adipose tissue (with and without insulin) and using 6-DOG-U-C^{14} as the sole substrate indicated that this compound is not oxidized to $C^{14}O_2$. No significant count above background was detected using the tagged 6-DOG at a concentration of 0.03M. A rate of 6-DOG oxidation 3% that of glucose would have given a counting rate twice normal background. The data are not included in this report.

Fig. 1§ shows the effect of 6-DOG on oxidation of glucose (0.01M). Experiments carried out simultaneously with 2-deoxy-D-glucose (2-DOG) are also shown for comparison purposes. It is apparent that 6-DOG inhibits glucose oxidation to a considerable degree, although the inhibition is not equivalent to that observed with equimolar 2-DOG. With the mammalian systems studied, 6-DOG inhibits oxidation of glucose 50 to 60% at a concentration ratio for 6-DOG to glucose of 12:1. Mouse adipose tissue is a much more sensitive tissue than etiher rat kidney slices or diaphragm tissue. With adipose tissue, a 6:1 ratio of 6-DOG to glucose inhibits glucose oxidation 55%.

To enhance the inhibiting effect of 6-DOG and to demonstrate the reversible nature of the inhibition, the effect of 0.06M 6-DOG on oxidation of glucose by mouse adipose tissue was examined at several different glucose concentrations. The data in Table I indicate that the inhibition produced by 6-DOG is reversible.

In Table II are results of 2 experiments concerning the effect of 6-DOG on glucose metabolism in rat diaphragm tissue. Besides showing that 6-DOG to glucose ratios of 6:1 and 12:1 inhibit glucose oxidation 26 and 52% respectively, the data show that the reduction in amount of C^{14} activity retained within this tissue was approximately proportional to the reduction in rate of glucose oxidation. The tissue incorporation of C^{14} was measured by technics involving wet combustion of the rinsed incubated tissue.

Discussion. 6-Deoxyglucose has been reported by Woodward *et al.*(3) to have no effect on glucose metabolism in yeast. In their studies the highest ratio of 6-DOG to glucose used was 2:1. The data presented here with mammalian tissue for higher ratios of analog to glucose show that this analog

TABLE I. Competitive Effect of 6-Deoxy-D-Glucose (0.06M) on Rate of Oxidation of Glucose-U-C^{14} to $C^{14}O_2$ by Mouse Adipose Tissue at Different Concentrations of Glucose.

Molarity of glucose	% inhibition*
.020	13
.010	43
.005	62
.0025	72

* Inhibition refers to the ratio of rate of glucose oxidation in presence of 6-DOG to rate in its absence, concentration of glucose being the same in both cases. Results are avg values of 2 experiments.

‡ Analysis performed by Elek Micro Analytical Laboratories, Los Angeles.

§ The data presented here are average values for a large series of experiments. The data from control experiments using sucrose, sorbitol, and mannitol are omitted to conserve space. Studies were also carried out with yeast and Ehrlich ascites cells. The reader is referred to the original thesis for detailed studies.

TABLE II. Effects of 6-Deoxy-D-Glucose on Rate of Oxidation of Glucose-U-C^{14} to $C^{14}O_2$ and on Amount of Glucose Derived Carbon Accumulated in Rat Diaphragm. Results expressed as percentage of control.*

Compound	Molarity	$C^{14}O_2$	C^{14} in tissues
6-DOG	.03	81	81
"	.06	74	71
"	.12	48	62
2-DOG	.12	30	78

* % of control refers to rate at which rat diaphragm tissue oxidized 0.01M glucose-U-C^{14} to $C^{14}O_2$ and accumulated C^{14} compounds in the tissue during incubation in presence of the indicated concentration of test compounds relative to rate in absence of the test compound.

inhibits oxidation of glucose and that the inhibition is reversible. The fact that 6-DOG inhibits glucose utilization in mammalian tissue, provides a second 6-position substituted glucose analog for study of glucose metabolism, particularly those steps prior to and including the hexokinase step. This compound offers a means of comparing directly results formerly observed with 6-fluoro-D-glucose.

As far as we are aware, 6-DOG has not been used previously with mammalian tissue as in this study. However, 6-DOG has been used as a tool for studying the mechanism of intestinal absorption of sugars. Crane(4) reported that this sugar is actively absorbed from the intestinal tract, and Wilson *et al.* (5) have reported that glucose inhibits intestinal transport of 6-DOG and the inhibition is reversible.

The evidence presented here provides little information concerning the site or sites of action of 6-DOG in inhibiting glucose oxidation. The observation that 6-DOG is not oxidized to CO_2 in mouse adipose tissue indicates that the observed inhibition is not merely the effect of the tissue metabolizing 6-DOG instead of glucose.

The site (sites) at which 6-DOG blocks glucose metabolism depends largely on whether the analog is altered within the cell. Although 6-DOG cannot be phosphorylated by hexokinase, kinases which will phosphorylate sugars in positions other than the 6-position are known to occur in mammalian tissues(6,7). 2-DOG inhibits glucose metabolism at a multiplicity of sites, due to accumulation of its phosphorylated derivative in the cells. The production of oxidized derivatives of 6-DOG within the cell offers the possibility of these compounds acting as actual blocking agents. However, if 6-DOG is not acted upon by the tissues in which it inhibits glucose oxidation, the most probable points at which metabolism will be blocked are those of cell entry and/or the hexokinase step. 6-DOG has been shown to be an inhibitor of glucose phosphorylation by brain hexokinase(8). At an inhibitor to substrate molar ratio of 100:1, we have found that 6-DOG also competitively inhibits yeast hexokinase by 59%. However, at a ratio consistent with the tissue results, *i.e.*, 12:1, 6-DOG has no effect upon rate of phosphorylation of glucose by yeast hexokinase.

Summary. 6-Deoxy-D-glucose has been shown to be an inhibitor of glucose oxidation in rat kidney slices, mouse adipose tissue, and rat diaphragm. The ratio of 6-DOG to glucose which gave a 50% inhibition of glucose oxidation corresponded to 12:1 in rat kidney slices and rat diaphragm and 4:1 in mouse adipose tissue. The inhibition of glucose oxidation in mouse adipose tissue was shown to be reversible. Experiments using rat diaphragm tissue showed that 6-DOG inhibited uptake of glucose in proportion to decrease in glucose oxidation. Incubation of mouse adipose tissue with 6-DOG uniformly labeled with C^{14} did not result in formation of measurable amounts of $C^{14}O_2$. It is concluded that this compound is incapable of complete oxidation in this tissue. It is believed that the site of competitive inhibition is either at the cell entry mechanism or at the hexokinase reaction. Thus, the metabolic block produced by 6-DOG should be less complex than that produced by the 2-position modified sugars. The use of 6-DOG may be very useful for studying cell permeation mechanisms.

1. Hardegger, E., Montavon, R. M., *Helv. Chim. Acta,* 1946, v29, 1199.

2. Fischer, E., Zach, K., *Chem. Ber.,* 1912, v45, 3761.

3. Woodward, G. E., Cramer, F. B., Hudson,

M. T., *J. Franklin Inst.*, 1953, v256, 577.
4. Crane, R. K., Wilson, T. H., *J. Appl. Physiol.*, 1958, v12, 145.
5. Wilson, T. H., Lin, E. C. C., Landau, B. R., Jorgensen, C. R., *Fed. Proc.*, 1960, v19, 870.
6. Staub, A., Vestling, C. S., *J. Biol. Chem.*, 1951, v191, 395.
7. Hosterlitz, H. W., *Biochem. J.*, 1937, v31, 2217.
8. Crane, R. K., Sols, A., *J. Biol. Chem.*, 1954, v210, 597.

Received April 7, 1961. P.S.E.B.M., 1961, v107.

Isolation and Identification of 17α-Hydroxyprogesterone in Adrenal Venous Plasma of Normal Dogs.* (26637)

BALWANT DAJI AGASHE,† ROBERT G. WEAVER AND KRISTEN B. EIK-NES
(Introduced by Leo T. Samuels)
Departments of Biological Chemistry and Anatomy, College of Medicine, University of Utah, Salt Lake City

Adrenal venous blood of dogs was analyzed for steroids by Farrell and Lamus(1) and 14 different fractions were isolated of which 10 appeared to be steroids. Three of these fractions were characterized as cortisol, corticosterone, and Reichstein's Substance S. Carstensen *et al.*(2) while reporting the isolation of 5-pregnene-3β.17α-diol-20-one (17α-hydroxypregnenolone) in the canine adrenal vein plasma indicated the presence of 4-pregnene-17α-ol-3,20-dione (17α-hydroxyprogesterone) in this fluid but did not characterize the material. The studies referred to were made on dogs under the 'stress' of an acute surgical operation. The present communication deals with the isolation and identification of 17α-hydroxyprogesterone from adrenal venous blood of 'unstressed' normal dogs.

Experimental. a. *Materials.* The animal preparation used for the experiment was that described by Weaver and Eik-Nes(3). An intravenous injection of 25 I.U. of adrenocorticotropin (ACTH) (Upjohn Co.) was given ½ hour before blood was collected from the adrenal pouch into a heparinized tube. No more than 70 to 80 ml of blood were withdrawn per tapping. The plasma was separated from the red cells by centrifugation for 1 hour at 1800 rpm and stored at −15°C in a deep freeze until processed.

Chemicals used were reagent grade. Solvents were purified according to standard procedures(4) and redistilled before use. All evaporations of organic solvents were done under nitrogen at 40°C.

b. *Extraction and purification of the steroids.* The plasma was extracted 4 times using an equal volume of a mixture of ethyl acetate-ethyl ether (1:1 v/v) each time. The extract was washed once with 0.1 volume of 1 N NaOH and 3 times with 0.1 volume of water, and evaporated to dryness. To the residue 0.01 μg of 17α-hydroxyprogesterone-4-C^{14},‡ equivalent to 800 cpm was added to serve as reference standard for detection on paper chromatograms and for calculation of recoveries.

The dry residue was suspended in 20 ml of 70% aqueous methanol and left overnight (15 hours) at −15°C. The suspension was then centrifuged for ½ hour at 1800 rpm in a refrigerated centrifuge and the supernatant decanted and collected(5). The residue was washed with 5 ml of ice-cold 70% methanol, and the wash after centrifugation was combined with the original supernatant. After evaporating to dryness the dilute methanolic extract, the residue was dissolved in 15 ml of n-hexane and washed thrice with equal volumes of 70% methanol (6). The hexane fraction was discarded and the methanolic solution dried down.

* This work was in part supported by Research Grants from U. S. Public Health Service, Bethesda, Md.

† Present address: Dept. of Chemistry, Osmania University, Hyderabad-7, (A.P.) India.

‡ New England Nuclear Corp., Boston, Mass.