Inhibition by 2,5-anhydromannitol of glycolysis in isolated rat hepatocytes and in Ehrlich ascites cells

PHOSPHOFRUCTOKINASE-1/fructose 2,6-bisphosphate/2,5-anhydromannitol monophosphate/2,5-anhydromannitol bisphosphate

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ABSTRACT 2,5-Anhydromannitol decreases lactate formation and \(^{3}H_2O\) formation from \([5-^{3}H]\)glucose in isolated rat hepatocytes metabolizing high concentrations of glucose. The inhibition of glycolysis is accompanied by a slight decrease in the cellular content of fructose-6-P and a more substantial decrease in the cellular content of fructose-1,6-P\(_2\), with no change in the content of glucose-6-P. The \(^{3}H_2O\) release data and changes in hexosephosphate distribution indicate possible inhibitions at phosphofructokinase-1 and phosphoglucose isomerase. 2,5-Anhydromannitol also inhibits glycolysis in Ehrlich ascites cells, but the tumor cells, unlike hepatocytes, must be treated with 2,5-anhydromannitol prior to exposure to glucose to obtain the inhibition. The decrease in \(^{3}H_2O\) formation from \([5-^{3}H]\)glucose and the metabolite pattern from that specific due to an increase of low concentrations (\(\leq 0.25\) mM) of 2,5-anhydromannitol indicate an inhibition at phosphofructokinase-1 that cannot be attributed to a decrease in the cellular content of fructose-2,6-P\(_2\). Higher concentrations (\(\geq 0.5\) mM) of 2,5-anhydromannitol cause a substantial decrease in the cellular content of ATP that is accompanied by decreases in the content of glucose-6-P and fructose-6-P and transient increases in fructose-1,6-P\(_2\). In Ehrlich ascites cells, 2,5-anhydromannitol is metabolized to 2,5-anhydromannitol mono- and bisphosphate. The inhibition of glycolysis caused by 2,5-anhydromannitol decreases with time, because the phosphorylated metabolites formed during the preliminary incubation in the absence of glucose are rapidly dephosphorylated during the incubation in the presence of glucose.

2,5-Anhydro-D-mannitol (2,5-AM-ol) is an analog of the \(\beta\)-furanose form of \(\beta\)-fructose that lacks the C-2 hydroxyl and is thus locked in the furan ring structure (for structure, see ref. 1). Incubation of isolated rat hepatocytes with 2,5-AM-ol results in a rapid increase in 2,5-AM-ol-1-P and a slower accumulation of 2,5-AM-ol-1,6-P\(_2\). Under these conditions, the intracellular concentration of fructose-2,6-P\(_2\) (Fru-2,6-P\(_2\)), a potent activator of phosphofructokinase-1 (PFK-1), is decreased (1), probably because of the accumulation of 2,5-AM-ol-1-P. 2,5-AM-ol-1,6-P\(_2\) is a poor substrate for Fru-2,6-P\(_2\) as an activator of PFK-1, and at high concentrations it can act as a product inhibitor (2). Since the formation of either of the phosphorylated metabolites of 2,5-AM-ol could cause an inhibition of flux through PFK-1, the effects of 2,5-AM-ol on glycolysis in isolated rat hepatocytes and in Ehrlich ascites tumor cells were examined. A portion of this work has been presented (3).

MATERIALS AND METHODS

Synthesis of 2,5-AM-ol. Unlabeled 2,5-AM-ol was synthesized and purified as described (1) and crystallized as described in ref. 4. Seeding crystals of 2,5-AM-ol were a generous gift from Alan Rendina. 2,5-[1-\(^{14}C\)]AM-ol was synthesized from [1-\(^{14}C\)]glucosamine (2).

Isolation and Incubation of Hepatocytes. Cells were isolated from livers of normal rats fasted 24 h and were incubated as described in ref. 5 in the presence of 1.3 mM Ca\(^{2+}\).

Isolation and Incubation of Ehrlich Ascites Cells. Ehrlich ascites tumor cells, originally obtained from Efraim Racker, were maintained in Swiss albino mice by intraperitoneal injection at 10-day intervals of 0.5 ml of ascites fluid. The cells were harvested 7–10 days after inoculation, diluted immediately with 30 ml of cold incubation buffer containing 120 mM NaCl/5 mM KCl/1 mM MgCl\(_2\)/5 mM Tris HCl, pH 7.4/5 mM K\(_2\)HPO\(_4\)/3 mM KH\(_2\)PO\(_4\), pH 7.4, washed 3 times by centrifugation at 120 × g for 2 min each, and resuspended in the buffer so the concentration of cells was equivalent to \(\approx 10\) mg of cell protein per ml. Unless otherwise indicated, the cells were incubated aerobically at 37°C in the absence or presence of 2,5-AM-ol for 15 min prior to exposure to glucose; 0.5 ml aliquots of the cell suspensions were transferred to 20-ml glass scintillation vials and the cells were incubated aerobically at 37°C with 2 mM glucose in the absence or presence of 2,5-AM-ol in a total vol of 1.5 ml for the times indicated. The reactions were stopped and samples were processed as described (5).

Measurement of Metabolites. Glucose and lactate were determined enzymatically (5). Hexose phosphates in hepatocytes (\(\approx 0.6\) g wet weight of cells incubated with 20 mM glucose in the absence or presence of 2,5-AM-ol in a total vol of 12 ml for 20 min as described in ref. 1) were determined spectrophotometrically in neutralized acid extracts of concentrated cells as described (1, 6). Glycolytic intermediates in Ehrlich ascites cells (\(\approx 5\) mg of cell protein incubated in a total vol of 1.5 ml) were assayed fluorometrically (7) in aliquots of the neutralized acid extracts. Fructose-1,6-P\(_2\) (Fru-1,6-P\(_2\)) and triose phosphates were assayed by coupling aldolase and triose phosphate isomerase to glyceraldehyde-3-phosphate dehydrogenase (7). ATP was assayed by using the luciferase method (8). Firefly luciferase was purified by extraction (9) and chromatography (8).

Fru-2,6-P\(_2\) in neutralized extracts was assayed by the activation of potato pyrophosphatase: fructose-6-phosphate phosphotransferase (1, 10), except that fructose-6-P (Fru-6-P) was treated with acid to decrease the values of the blanks (11).

The concentrations of the phosphorylated metabolites of 2,5-[1-\(^{14}C\)]AM-ol (0.1 \(\mu\)Ci/\(\mu\)mol; 1 Ci = 37 GBq) in Ehrlich ascites cells were determined as described (2).

The flux through PFK-1 was estimated by measuring the release of \(^{3}H_2O\) from \([5-^{3}H]\)glucose (12).

Materials. Sources of materials, in addition to those in ref. 2, were described firefly tails (Sigma), [1-\(^{14}C\)]glucosamine and \([5-^{3}H]\)glucose (New England Nuclear).

Abbreviations: 2,5-AM-ol, 2,5-anhydro-D-mannitol; Fru-2,6-P\(_2\), d-fructose 2,6-bisphosphate; Fru-1,6-P\(_2\), d-fructose 1,6-bisphosphate; Fru-6-P, d-fructose 6-phosphate; Glc-6-P, d-glucose 6-phosphate; PFK, phosphofructokinase.
RESULTS

Effects of 2,5-AM-ol on Glucose Metabolism in Isolated Rat Hepatocytes. In hepatocytes isolated from fasted rats, increasing concentrations of 2,5-AM-ol produce a dramatic decrease in the rate of lactate formation from high concentrations (15 or 20 mM) of glucose (Fig. 1). The inhibitory effects of 2,5-AM-ol are less apparent in the presence of 10 mM glucose when the rate of glycolysis is lower. The rate of lactate formation from 5 mM glucose is even slightly increased by 2,5-AM-ol (Fig. 1) as are the rates from gluconeogenic substrates that enter the Embden–Meyerhof pathway as triose phosphate (1, 13, 14). In all cases, the rates of lactate formation in the absence of glucose (Fig. 1, Inset) have been subtracted from those in the presence of glucose. 2,5-AM-ol also inhibits the metabolic flux through PFK-1, as indicated by its ability to decrease the production of \( \text{\textsuperscript{3}}\text{H}_2\text{O} \) from \([\text{\textsuperscript{5}}\text{H}]\)glucose (Fig. 1). Again, the inhibitory effects of 2,5-AM-ol are more apparent in the presence of higher concentrations of glucose; it has no effect on \( \text{\textsuperscript{2}}\text{H}_2\text{O} \) formation from 5 mM glucose. The decrease in the rate of lactate formation or of \( \text{\textsuperscript{2}}\text{H}_2\text{O} \) formation from \([\text{\textsuperscript{5}}\text{H}]\)glucose is almost maximal in the presence of 0.25 mM 2,5-AM-ol, a concentration that does not change the concentration of intracellular ATP (results not shown).

Effects of 2,5-AM-ol on Hexose Phosphate Content in Rat Hepatocytes. At 0.5 or 2 mM, 2,5-AM-ol does not decrease the glucose 6-P (Glc-6-P) content in hepatocytes incubated with 20 mM glucose (Table 1); thus, phosphorylation of glucose mediated by glucokinase is not impaired in spite of the fact that 0.5 or 2 mM 2,5-AM-ol produces a 20% or 50% decrease, respectively, in ATP content (results not shown). There is a slight decrease in the content of Fru-6-P that could result from an inhibition of phosphoglucone isomerase or from the decrease in the content of Fru-6-P that, after acid extraction of the cells, contributes to the Fru-6-P pool. There is a greater decrease in the content of Fru-1,6-P\(_2\) that is consistent with the decrease in the flux through PFK-1.

Effects of 2,5-AM-ol on Glucose Utilization, Lactate Formation, and \( \text{\textsuperscript{3}}\text{H}_2\text{O} \) Formation from \([\text{\textsuperscript{5}}\text{H}]\)Glucose in Ascites Cells. To observe the inhibitory effects of 2,5-AM-ol on glycolysis in Ehrlich ascites cells, the tumor cells, unlike isolated rat hepatocytes, require preliminary treatment with 2,5-AM-ol prior to exposure to glucose (Fig. 2). This is consistent with a competition of 2,5-AM-ol with glucose for hexokinase and indicates that 2,5-AM-ol is a poor substrate for Ehrlich ascites hexokinase. Thus, in all of the experiments that follow, ascites cells routinely were treated with 2,5-AM-ol for 15 min before incubation with glucose. Under these conditions, 2,5-AM-ol decreases the rates of glucose utilization, lactate formation, and flux through PFK-1, as estimated by \( \text{\textsuperscript{3}}\text{H}_2\text{O} \) formation from \([\text{\textsuperscript{5}}\text{H}]\)glucose (Fig. 3). The inhibitory effect of 2,5-AM-ol on the above parameters decreases with time throughout the incubation period with glucose and is more apparent at concentrations of 2,5-AM-ol >0.25 mM. At concentrations up to 0.25 mM, 2,5-AM-ol does not decrease the intracellular ATP concentration significantly, but at higher concentrations, it can decrease the ATP content to 30% that of the control (Fig. 4).

Effects of 2,5-AM-ol on Glycolytic Intermediates. The metabolite pattern in Ehrlich ascites cells incubated with 2 mM glucose in the presence of increasing concentrations of 2,5-AM-ol was determined by neutralized extracts of concentrated cells. Each value is mean ± SEM from a minimum of four experiments.

<table>
<thead>
<tr>
<th>Glucose control, ( \mu\text{mol/g} )</th>
<th>Glucose with 0.5 mM 2,5-AM-ol, % control</th>
<th>Glucose with 2 mM 2,5-AM-ol, % control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glc-6-P</td>
<td>0.049 ± 0.004</td>
<td>104 ± 3</td>
</tr>
<tr>
<td>Fru-6-P</td>
<td>0.031 ± 0.002</td>
<td>73 ± 1</td>
</tr>
<tr>
<td>Fru-1,6-P(_2)</td>
<td>0.025 ± 0.005</td>
<td>40 ± 5</td>
</tr>
</tbody>
</table>

Hepatocytes were incubated for 20 min with 20 mM glucose in the absence or presence of 2.5-AM-ol and then concentrated. Hexose phosphates were determined in neutralized extracts of concentrated cells. Each value is mean ± SEM from a minimum of four experiments.

![Fig. 1. Effects of 2,5-AM-ol on lactate formation and \( \text{\textsuperscript{3}}\text{H}_2\text{O} \) formation from \([\text{\textsuperscript{5}}\text{H}]\)glucose in hepatocytes.](image1.png)
AM-ol varies with the concentration of 2,5-AM-ol and with the incubation time (Fig. 5). After a 10- or 30-min period of incubation with glucose, low concentrations of 2,5-AM-ol induce a decrease in Fru-1,6-P_2 consistent with an inhibition of PFK-1, but higher concentrations of 2,5-AM-ol (>0.25 mM) cause a decrease in Glc-6-P that would be consistent with an inhibition of hexokinase. In addition, after 10 min of incubation with glucose, 2 mM 2,5-AM-ol causes an increase in the content of Fru-1,6-P_2 that indicates an inhibition of aldolase. This effect is not apparent at 30 min, however.

**Effects of 2,5-AM-ol on Fru-2,6-P_2 Content in Ehrlich Ascites Cells.** Fru-2,6-P_2 is a potent physiological activator of PFK-1 (15), and, thus, an effect of 2,5-AM-ol to decrease the cellular concentration of this regulator could cause a decrease in the flux through PFK-1. Whereas both low and high concentrations of 2,5-AM-ol induce a dramatic decrease of Fru-2,6-P_2 in isolated rat hepatocytes (1), low concentrations of 2,5-AM-ol induce an increase of Fru-2,6-P_2 in Ehrlich ascites cells (Fig. 6) and higher concentrations produce a decrease. The decrease probably occurs via an indirect mechanism that results from the decrease in the ATP content (Fig. 4), which in turn results in a decrease in the phosphorylation of glucose and a subsequent decrease in the formation of Fru-6-P (Fig. 5), the direct precursor of Fru-2,6-P_2 (15). The differential effects of increasing concentrations of 2,5-AM-ol on Fru-2,6-P_2 content are observed after either a 10- or 30-min period of incubation with glucose, but as the period of incubation increases, there also is an increase in the formation of Fru-2,6-P_2.

**Intracellular Content of 2,5-AM-ol Mono- and Bisphos-**
cites cells. Incubation conditions were as described in Fig. 4. Values are means ± SEM from three experiments. * P < 0.05 in comparison with no added 2,5-AM-ol.

Phosphates in Ehrlich Ascites Cells. When ascites cells that have been metabolizing 2,5[14C]AM-ol for 15 min are exposed to 2 mM glucose and increasing concentrations of 2,5[14C]AM-ol, the concentrations of the 14C-labeled mono- and bisphosphorylated products are altered as shown in Fig. 7. In contrast to the results obtained in isolated rat hepatocytes incubated with glucose and 2,5-AM-ol (2), there is a continual decrease in the content of both phosphorylated metabolites during the 30-min incubation period, except for the increase in 2,5-AM-ol bisphosphate observed at the highest concentration of 2,5-AM-ol tested. The slight transient increase in the content of 2,5-AM-ol monophosphate observed in the presence of 0.5 mM 2,5-AM-ol can be accounted for by the degradation of 2,5-AM-ol bisphosphate. The increase in 2,5-AM-ol bisphosphate observed in the presence of 2 mM 2,5-AM-ol indicates that, although the major metabolic fate of 2,5-AM-ol monophosphate is dephosphorylation, some of it is being further phosphorylated.

Control experiments in which ascites cells were incubated with 0.5 mM 2,5-AM-ol in the absence of glucose for 15 min indicate that there is a continual increase in the intracellular content of 2,5-AM-ol mono- and bisphosphates, although the rate of accumulation of the latter is approximately one-half of that of the monophosphate (results not shown). When the cells are incubated for an additional 30 min in the absence of glucose, the content of 2,5-AM-ol monophosphate decreases 20%, but the content of 2,5-AM-ol bisphosphate increases 4- to 7-fold; under the same incubation conditions but in the presence of 2 mM glucose, both the mono- and bisphosphates decrease ≈40%.

**DISCUSSION**

The effects of 2,5-AM-ol in hepatocytes isolated from fasted rats are of a dual nature. In cells metabolizing gluconeogenic precursors that enter the Embden–Meyerhof pathway as triose phosphate, 2,5-AM-ol inhibits gluconeogenesis and simultaneously stimulates lactate formation via an activation of pyruvate kinase and an inhibition of fructose-1,1-bisphosphate that result from the formation and accumulation of 2,5-AM-ol-1,6-P2 (1, 2, 13, 14). In cells metabolizing high concentrations of glucose, however, 2,5-AM-ol inhibits lactate formation (Fig. 1). The inhibition of glycolysis from glucose probably occurs at or before the PFK-1 step, and under these conditions, the role of 2,5-AM-ol monophosphate may be more important than that of 2,5-AM-ol bisphosphate.

The inhibitory effects of 2,5-AM-ol are observed at two sites in the glycolytic pathway. The decreased cellular content of Fructose-6-P with no change in the content of Glc-6-P (Table 1) indicates an inhibition at phosphoglucose isomerase. While an inhibition at this site cannot be ruled out, 2,5-AM-ol does not inhibit gluconeogenesis from xylitol (1), a substrate that enters the gluconeogenic pathway primarily as Fructose-6-P, and thus, 2,5-AM-ol appears not to affect phosphoglucose isomerase under those conditions. The decreased flux through PFK-1, as estimated by measuring 1H2O release from [5-3H]glucose (Fig. 1), and the decreased cellular content of Fructose-1-6-P2 (Table 1) indicate an inhibition at PFK-1. An inhibition at this site seems likely because it could result, whether directly or indirectly, from the accumulation of the phosphorylated metabolites of 2,5-AM-ol in hepatocytes (1, 2).

Previous studies from this laboratory have indicated that 2,5-AM-ol-1-P could inhibit the phosphorylation of Fructose-6-P because it is an alternative substrate for PFK-1 (2) and that it could be responsible, via an inhibition of PFK-2, for the ability of 2,5-AM-ol to decrease cellular concentrations of Fructose-2,6-P2 (1, 2). Competition of 2,5-AM-ol monophosphate with Fructose-6-P for phosphorilation probably does not account for the 80% inhibition of lactate formation from 20 mM glucose in the presence of 0.5 mM 2,5-AM-ol, however, for the rate of lactate formation in the absence of 2,5-AM-ol is 250 nmol/min per g wet weight (Fig. 1), and the rate of phosphorylation of 2,5-AM-ol monophosphate is only 10 nmol/min per g wet weight (calculated from data in ref. 2). Thus, the most likely explanation for the inhibitory effects of 2,5-AM-ol on glycolysis from glucose in isolated rat hepatocytes is the ability of 2,5-AM-ol to decrease the cellular content of Fructose-2,6-P2 (1), a potent activator of PFK-1 (15). When hepatocytes metabolize high concentrations of glucose, the cellular concentration of Fructose-2,6-P2 is increased, rates of glycolysis are increased, and inhibitory effects of 2,5-AM-ol become apparent (Fig. 1). The cellular concentration of Fructose-2,6-P2 is very low in the presence of 5 mM glucose (16). Under these conditions, 2,5-AM-ol has no effect on the already low rate of glycolysis.

*2,5-AM-ol monophosphate, an analog of β-D-Fru-6-P that lacks the C-2 hydroxyl, cannot be phosphorylated in the 2 position but it might act as an inhibitor of PFK-2.
2,5-AM-ol has inhibitory effects on glycolysis in Ehrlich ascites tumor cells that are similar to those in hepatocytes, but there are major differences in the metabolism and mechanism of action of 2,5-AM-ol in the two cell types. The requirement of ascites cells for a preliminary treatment with 2,5-AM-ol before incubation with glucose to obtain inhibition of glycolysis (Fig. 2) indicates that hexokinase is involved in the metabolism of 2,5-AM-ol but that 2,5-AM-ol does not compete effectively with glucose for phosphorylation.

The inhibition of glycolysis in ascites cells by low concentrations of 2,5-AM-ol cannot be attributed to a decrease in the cellular content of Fru-2,6-P as is the case in hepatocytes, because low concentrations of 2,5-AM-ol actually cause a slight increase in the content of Fru-2,6-P (Fig. 6). The differential effects of 2,5-AM-ol on Fru-2,6-P content in hepatocytes and ascites cells probably result from differences in the concentration of 2,5-AM-ol monophosphate relative to that of Fru-6-P. In hepatocytes metabolizing 20 mM glucose, the ratio of the concentration of 2,5-AM-ol monophosphate that accumulates in cells treated with 0.5 mM 2,5-AM-ol to the concentration of Fru-6-P that is present in control cells is 84 (5.2 mM/0.062 mM) [based on data in ref. 2 and Table 1 and assuming 0.5 ml of cytosolic water per g wet weight of cells (17)]. Thus, competition of 2,5-AM-ol monophosphate with Fru-6-P for phosphorylation by PFK-2 could inhibit the synthesis of Fru-2,6-P2. This is not the case in ascites cells, however. The ratios of 2,5-AM-ol monophosphate to Fru-6-P in cells treated with 0.1 mM 2,5-AM-ol and incubated with glucose for 10 or 30 min are 2.0 (0.055 mM/0.028 mM) or 0.1 (0.03 mM/0.028 mM), respectively; the ratios in cells treated with 0.5 mM 2,5-AM-ol are 4 (0.92 mM/0.23 mM) or 2 (0.45 mM/0.23 mM) at 10 or 30 min, respectively [based on data in Figs. 5 and 7 and assuming that the intracellular content of water is 7.6 µl per mg of cell protein (18) and that 50% of that corresponds to cytosolic water].

The inhibition of glycolysis produced by low concentrations of 2,5-AM-ol in Ehrlich ascites cells could be attributed to an inhibition of PFK-1 by 2,5-AM-ol bisphosphate. Fru-1,6-P2 and 2,5-AM-ol-1,6-P2 have dual effects on rat liver PFK-1. At low concentrations, they can act as weak allosteric activators by binding to the Fru-2,6-P2 site, but at concentrations >100 µM, they can act as inhibitors, possibly by binding to the active site of the enzyme (2, 19). Fru-2,6-P2 is a potent allosteric activator of Ehrlich ascites PFK-1, but Fru-1,6-P2 (20) and probably its β-anomer analog, 2,5-AM-ol-1,6-P2, fail to activate the enzyme. The possibility of Fru-1,6-P2 or 2,5-AM-ol-1,6-P2 binding to the active site of PFK-1 still exists, however, and at high concentrations, 2,5-AM-ol-1,6-P2 could produce product inhibition of the enzyme. While Fru-1,6-P2 is not a stimulator of yeast PFK-1 (21), Bartrons et al. (22) have demonstrated that high concentrations of Fru-1,6-P2 can counteract the activation of the enzyme induced by Fru-2,6-P2.

Reevaluation of the data in Figs. 5 and 7 indicates that the concentration of 2,5-AM-ol bisphosphate that accumulates in ascites cells is dramatically increased in comparison to the normal cellular concentration of Fru-1,6-P2. The content of Fru-1,6-P2 in cells incubated with 2 mM glucose for 10 or 30 min is 60 µM or 100 µM, respectively, and it is decreased to <50% of that of the control in cells treated with 2,5-AM-ol. On the other hand, the content of 2,5-AM-ol bisphosphate in cells treated with 0.1 mM 2,5-AM-ol and then incubated with glucose for 10 or 30 min is 410 µM or 260 µM, respectively, and it increases to 3.8 mM or 2.9 mM, respectively, in cells treated with 0.5 mM 2,5-AM-ol. Thus, the concentrations of 2,5-AM-ol bisphosphate that accumulate in the ascites cells are in the range of those that could cause product inhibition of PFK-1. Kinetic studies with purified Ehrlich ascites PFK-1 are necessary to confirm this hypothesis, however.

In summary, the inhibitory effects of 2,5-AM-ol on glycolysis in isolated rat hepatocytes appear to be due to the formation and accumulation of 2,5-AM-ol monophosphate and the ability of the monophosphorylated derivative to decrease the cellular concentration of Fru-2,6-P2 and, thus, to inhibit flux through PFK-1. The possibility of product inhibition by 2,5-AM-ol bisphosphate on the enzyme (2) cannot be completely ruled out, however. The inhibitory effects of 2,5-AM-ol on glycolysis in Ehrlich ascites cells are not related to decreases in the cellular concentration of Fru-2,6-P2, but they appear to be due to product inhibition of PFK-1 by 2,5-AM-ol bisphosphate.

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