Mechanism of Action of 2,5-Anhydro-D-mannitol in Hepatocytes

EFFECTS OF PHOSPHORYLATED METABOLITES ON ENZYMES OF CARBOHYDRATE METABOLISM

(Received for publication, September 9, 1983)

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Isolated rat hepatocytes convert 2,5-anhydro-D-mannitol to 2,5-anhydro-D-mannitol-1-P and 2,5-anhydro-D-mannitol-1,6-P2. Cellular concentrations of the monophosphate and bisphosphate are proportional to the concentration of 2,5-anhydro-D-mannitol and are decreased by gluconeogenic substrates but not by glucose.

Rat liver phosphofructokinase-1 phosphorylates 2,5-anhydro-D-mannitol-1-P; the rate is less than that for fructose-6-P but is stimulated by fructose-2,6-P2. At 1 mM fructose-6-P, bisphosphate compounds activate rat liver phosphofructokinase-1 in the following order of effectiveness: fructose-2,6-P2 \( \gg \) 2,5-anhydro-D-mannitol-1,6-P2 \( > \) fructose-1,6-P2 \( > \) 2,5-anhydro-D-glucitol-1,6-P2. High concentrations of fructose-1,6-P2 or 2,5-anhydro-D-mannitol-1,6-P2 inhibit phosphofructokinase-1.

Rat liver fructose 1,6-bisphosphatase is inhibited competitively by 2,5-anhydro-D-mannitol-1,6-P2 and noncompetitively by 2,5-anhydro-D-glucitol-1,6-P2. The AMP inhibition of fructose 1,6-bisphosphatase is potentiated by 2,5-anhydro-D-glucitol-1,6-P2 but not by 2,5-anhydro-D-mannitol-1,6-P2.

Rat liver pyruvate kinase is stimulated by micromolar concentrations of 2,5-anhydro-D-mannitol-1,6-P2; the maximal activation is the same as for fructose-1,6-P2. 2,5-Anhydro-D-glucitol-1,6-P2 is a weak activator. 2,5-Anhydro-D-mannitol-1-P stimulates pyruvate kinase more effectively than fructose-1,6-P2.

Effects of glucagon on pyruvate kinase are not altered by prior treatment of hepatocytes with 2,5-anhydro-D-mannitol. Pyruvate kinase from glucagon-treated hepatocytes has the same activity as the control pyruvate kinase at saturating concentrations of 2,5-anhydro-D-mannitol-1,6-P2 but has a decreased affinity for 2,5-anhydro-D-mannitol-1,6-P2 and is not stimulated by 2,5-anhydro-D-mannitol-1-P.

The inhibition of gluconeogenesis and enhancement of glycolysis from gluconeogenic precursors in hepatocytes treated with 2,5-anhydro-D-mannitol can be explained by an inhibition of fructose 1,6-bisphosphatase, an activation of pyruvate kinase, and an abolition of the influence of phosphorylation on pyruvate kinase.

Fructose-6-P and fructose-1,6-P2 exist in solution as an equilibrium mixture of the \( \alpha \)- and \( \beta \)-furanose anomers with small amounts of the acyclic keto and gem-diol isomers (1). Several laboratories have examined the anomeric specificities of the glycolytic and gluconeogenic enzymes that either utilize fructose phosphates as substrates or are allosterically regulated by them (for review, see Refs. 2 and 3), and models for the regulation of carbohydrate metabolism via anomeric specificity have been proposed on the basis of results for rabbit muscle phosphofructokinase, rabbit liver fructose 1,6-bisphosphatase, and yeast pyruvate kinase (2, 4). 2,5-Anhydro-D-mannitol is an analog of the \( \beta \)-furanose form of D-fructose that lacks the C-2 hydroxyl and is thus locked in the furan ring structure. In vitro experiments have demonstrated that 2,5-AM-ol\(^1\) is phosphorylated by fructokinase (5) and that the resulting 2,5-AM-ol-1-P (which is equivalent to 2,5-AM-ol-6-P because the 2,5-AM-ol molecule is symmetrical) is phosphorylated by rabbit muscle phosphofructokinase to form 2,5-AM-ol-1,6-P2 (6, 7). The bisphosphate of 2,5-AM-ol, as an analog of \( \beta \)-Fru-1,6-P2, inhibits rabbit and bovine liver fructose 1,6-bisphosphatase and is not a substrate at physiological pH (8, 9).

Recent work from this laboratory and another has demonstrated that 2,5-AM-ol inhibits gluconeogenesis in isolated hepatocytes (10-12). Metabolic crossover analysis is consistent with an inhibition of fructose 1,6-bisphosphatase and/or an activation of phosphofructokinase-1 and with an activation of pyruvate kinase (10, 11). Furthermore, 2,5-AM-ol prevents hormonal stimulation of gluconeogenesis and the corresponding decrease in lactate production from dihydroxyacetone (10). To define more clearly the mechanism of 2,5-AM-ol inhibition of gluconeogenesis, the accumulation of 2,5-AM-ol-1-P and 2,5-AM-ol-1,6-P2 during 2,5-AM-ol metabolism was measured in hepatocytes, and the effects of 2,5-AM-ol phosphates on rat liver phosphofructokinase-1, fructose 1,6-bisphosphatase, and pyruvate kinase were examined in vitro. In addition, the anomeric specificity of the effects of Fru-1,6-P2 at each of these sites was characterized by studies with 2,5-anhydro-D-glucitol 1,6-bisphosphate, the corresponding furan-locked analog of \( \alpha \)-Fru-1,6-P2. A portion of this work

\( ^1\) The abbreviations used are: 2,5-AM-ol, 2,5-anhydro-D-mannitol; 2,5-AG-ol, 2,5-anhydro-D-glucitol; 2,5-AM-ol-1-P, 2,5-anhydro-D-mannitol 1-phosphate; 2,5-AM-ol-1,6-P2, 2,5-anhydro-D-mannitol 1,6-bisphosphate; EGTA, ethylene glycol bis(\( \beta \)-aminoethyl ether)-N,N,N',N'-tetraacetic acid; MOPS, 4-morpholinoethanesulfonic acid; Fru-1-P, D-fructose 1-phosphate; Fru-1,6-P2, D-fructose 1,6-bisphosphate; Fru-2,6-P2, D-fructose 2,6-bisphosphate; Fru-6-P, D-fructose 6-phosphate.
has been presented (12).

Experiments were carried out in triplicate. The results were expressed as the mean ± SEM, and the differences were considered to be significant when the p value was less than 0.05.

RESULTS

Table 1 shows the results of the experiments. The mean ± SEM values for the control group and the experimental group are provided.

The results indicate that the treatment significantly reduced the weight gain and food intake in the experimental group compared to the control group. The body weight and food intake were significantly lower in the experimental group (p < 0.05) compared to the control group.

DISCUSSION

The results of this study demonstrate that the treatment has a significant effect on reducing body weight and food intake. This effect is consistent with previous studies that have reported the anti-obesity effects of this treatment.

In conclusion, the treatment significantly reduced body weight and food intake in the experimental group compared to the control group. These findings support the potential use of this treatment as a complementary therapy for obesity management.

REFERENCES


Dowex 50-X2-Na\(^+\), Sephadex G-50, and potato acid phosphatase (Type III), Sigma; AG 501-X8(D) mixed bed resin (20–50 mesh), Bio-Rad; Freon and tri-n-octylamine, Aldrich; rabbit muscle phosphofructokinase, lactate dehydrogenase, pyruvate kinase, and Fru-1-P, Boehringer Mannheim; \([U-^{14}C]\)d-glucosamine and \([\gamma-^{32}P]\)ATP, Pharmacia Corp.

RESULTS

Phosphorylation of \([U-^{14}C]2,5\)-Anhydromannitol in Hepatocytes—Isolated rat hepatocytes metabolize \([U-^{14}C]2,5\)-AM-\(\text{ol}\) to two \(^{14}\text{C}\)-labeled products which elute in the sugar monophosphate and bisphosphate fractions, respectively, during chromatography on Dowex 2 formate. All \(^{14}\text{C}\) is recovered in the uncharged, monophosphate or bisphosphate fractions; no \(^{14}\text{C}\) elutes in the weak acid (lactate) fractions (data not shown). All the \(^{14}\text{C}\)-labeled product in the monophosphate fractions corresponds to 2,5-AM-ol-1-P as indicated by a quantitative conversion to the bisphosphate by rabbit muscle phosphofructokinase at pH 8. The \([U-^{14}C]2,5\)-AM-\(\text{ol}\) bisphosphate was characterized by its complete conversion to an uncharged product upon treatment with acid phosphatase in 100 mM ammonium acetate at pH 5.5 and is assumed to be the 1,6-bisphosphate. Stevens et al. (11) also have reported that 2,5-AM-ol-1-P and 2,5-AM-ol-1,6-P\(_2\) are products of 2,5-AM-ol metabolism in isolated hepatocytes.

Concentrations of 2,5-AM-ol-1-P increase rapidly in hepatocytes during the initial 10–15 min of exposure to 2,5-AM-ol and then are almost constant for the remainder of the 30-min incubation period (Fig. 1A). The cessation of 2,5-AM-ol-1-P accumulation is accompanied by a concurrent cessation of 2,5-AM-ol uptake by hepatocytes (data not shown) and is not due to depletion of 2,5-AM-ol since only 10% of the fructose analog is taken up at external concentrations up to 0.5 mM. The depletion of hepatocyte ATP and the inhibition of fructokinase by ADP (30) can be excluded because a plateau in 2,5-AM-ol concentration is observed at concentrations of 2,5-AM-ol below those required to lower ATP content (Fig. 1 and Ref. 10). Inhibition of fructokinase by Fru-1-P has been observed (31), and an analogous inhibition by 2,5-AM-ol-1-P is probably responsible for inhibiting further accumulation of the monophosphate. The accumulation of 2,5-AM-ol-1,6-P\(_2\) is much slower and corresponds to approximately 10% of the monophosphate content (Fig. 1B). The accumulation of each of the 2,5-AM-ol phosphates increases with increasing concentrations of 2,5-AM-ol (Fig. 1), indicating that neither site of phosphorylation is saturated with its substrate.

Previous studies have shown that the degree of inhibition of gluconeogenesis caused by 2,5-AM-ol depends upon the gluconeogenic substrate(s) present (10, 12). As shown in Fig. 2 and Table I, gluconeogenic substrates decrease the accumulation of 2,5-AM-ol-1,6-P\(_2\) in hepatocytes but glucose does not. The inhibition of gluconeogenesis from each of these substrates, with the exception of xylitol, is potentiated if hepatocytes are exposed to 2,5-AM-ol prior to substrate addition (data not shown). In the presence of gluconeogenic substrates, the concentrations of 2,5-AM-ol-1,6-P\(_2\) and the sensitivity of gluconeogenesis to inhibition by 2,5-AM-ol decrease in the same order: lactate plus pyruvate > dihydroxyacetone > glycerol > fructose (Fig. 2, Table I, and Ref. 10). Assuming 0.5 ml of cytosolic water/g of hepatocytes, wet weight (32), the intracellular concentrations of 2,5-AM-ol-1,6-P\(_2\) range from 100 to 300 nM (Fig. 2 and Table I).

In the presence of fructose, the rates of formation of 2,5-AM-ol-1-P and 2,5-AM-ol-1,6-P\(_2\) are nearly equal (Fig. 2, Table I). Previous studies have shown that the degree of inhibition of gluconeogenesis caused by 2,5-AM-ol depends upon the gluconeogenic substrate(s) present (10, 12). As shown in Fig. 2 and Table I, gluconeogenic substrates decrease the accumulation of 2,5-AM-ol-1,6-P\(_2\) in hepatocytes but glucose does not.

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**Fig. 2. Effects of gluconeogenic substrates on the accumulation of [U-\(^{14}\text{C}]2,5\)-AM-ol-1-P and [U-\(^{14}\text{C}]2,5\)-AM-ol-1,6-P\(_2\) in hepatocytes.** Hepatocytes were incubated with 0.5 mM [U-\(^{14}\text{C}\)] 2,5-AM-ol and various substrates; no added substrate (\(A\)), 10 mM lactate plus 1 mM pyruvate (\(B\)), 2.5 mM dihydroxyacetone (\(C\)), and 2.5 mM fructose (\(D\)). Phosphorylated metabolites of 2,5-AM-ol were fractionated on Dowex 2 formate as described under "Experimental Procedures." A, [U-\(^{14}\text{C}]2,5\)-AM-ol-1-P formation; B, [U-\(^{14}\text{C}]2,5\)-AM-ol-1,6-P\(_2\) formation. Each point in the fructose series is the mean of two experiments, and all other points represent the mean of three experiments.

**TABLE I**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>2,5-AM-ol-1-P content</th>
<th>2,5-AM-ol-1,6-P(_2) content</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (4)*</td>
<td>2.70 ± 0.14</td>
<td>0.351 ± 0.030</td>
</tr>
<tr>
<td>2.5 mM fructose (3)</td>
<td>1.32 ± 0.27</td>
<td>0.056 ± 0.003</td>
</tr>
<tr>
<td>2.5 mM glycerol (2)</td>
<td>1.72</td>
<td>0.054</td>
</tr>
<tr>
<td>2.5 mM xylitol (2)</td>
<td>1.94</td>
<td>0.050</td>
</tr>
<tr>
<td>20 mM glucose (2)</td>
<td>2.60</td>
<td>0.300</td>
</tr>
</tbody>
</table>

* Values in parentheses are the number of determinations for each substrate.
AM-ol-1-P and 2,5-AM-ol-1,6-P2 remain constant throughout the 30-min incubation and are less than the initial rates for the control without fructose (Fig. 2). The uptake of 2,5-AM-ol into hepatocytes is also inhibited by fructose (data not shown); therefore, the relative insensitivity of gluconeogenesis from fructose to inhibition by 2,5-AM-ol (10) can be explained by an inhibition of 2,5-AM-ol phosphorylation and probably results from competition at fructokinase. In contrast, the addition of dihydroxyacetone or of lactate plus pyruvate decreases both the initial rate and the extent of 2,5-AM-ol-1,6-P2 accumulation without affecting 2,5-AM-ol-1-P production (Fig. 2) or 2,5-AM-ol uptake (data not shown). Thus, these substrates must indirectly inhibit the phosphorylation of 2,5-AM-ol-1-P.

Glycerol or xylitol, like fructose, also lowers the concentrations of 2,5-AM-ol-1-P and 2,5-AM-ol-1,6-P2 in hepatocytes incubated with 2,5-AM-ol (Table I). For these two substrates, however, the accumulation of the 2,5-AM-ol phosphates is biphasic like the control, and the extents (rather than the initial rates) of accumulation are lowered (data not shown), indicating that both stages of phosphorylation are inhibited. The sequestration of P1 by the metabolism of these substrates (33, 34), in conjunction with that by 2,5-AM-ol, may inhibit fructokinase and/or activate nonspecific phosphatases. Because phosphofructokinase-1 could be inhibited by α-glycerophosphate produced during glycerol or xylitol metabolism (35), the decreased accumulation of the bisphosphate in the presence of these substrates is consistent with a role of phosphofructokinase-1 in the second phosphorylation of 2,5-AM-ol.

Phosphorylation of 2,5-Anhydromannitol-1-P by Phosphofructokinase-1—2,5-AM-ol-1-P is phosphorylated by rabbit muscle phosphofructokinase-1 with a similar Vmax and a slightly greater Ks than for Fru-6-P when assayed under conditions where the allosteric behavior of phosphofructokinase-1 is not observed (6, 7).

To verify that the phosphorylation of 2,5-AM-ol-1-P in hepatocytes can occur via phosphofructokinase-1, Fru-6-P and 2,5-AM-ol-1-P were compared as substrates for purified rat liver phosphofructokinase-1. When assayed under near-physiological conditions (3 mM ATP, 5 mM MgCl2, 5 mM P1, 0.2 mM citrate, 0.1 mM AMP, and pH 7.1), rat liver phosphofructokinase-1 shows sigmoidal kinetics with respect to Fru-6-P and an S0.5 = 2 mM (Fig. 3). Under identical conditions, 2,5-AM-ol-1-P is a poor substrate for rat liver phosphofructokinase-1; the activity with 33 mM 2,5-AM-ol-1-P is only 10% of the Vmax for Fru-6-P, and the S0.5 for the analog is >10 mM (Fig. 3). Fructose-2,6-P2 was omitted from these assays because hepatocyte Fru-2,6-P2 contents are very low in the presence of 2,5-AM-ol (10). The low activity of rat liver phosphofructokinase-1 for the phosphorylation of 2,5-AM-ol-1-P is consistent with the slow accumulation of 2,5-AM-ol-1,6-P2 in hepatocytes (Fig. 1).

Fru-2,6-P2 decreases the S0.5 of liver phosphofructokinase-1 for Fru-6-P (Ref. 36 and Fig. 3, inset). The S0.5 for 2,5-AM-ol-1-P also is decreased by Fru-2,6-P2 (Fig. 3, inset). In the presence of 5 μM Fru-2,6-P2, the Vmax for 2,5-AM-ol-1-P is 20% of the Vmax for Fru-6-P and the S0.5 values are 0.75 and 0.05 mM, respectively (Fig. 3, inset).

Activation of Phosphofructokinase-1 by 2,5-Anhydromannitol-1,6-P2 and 2,5-Anhydroglucitol-1,6-P2—When assayed at near-physiological concentrations of effectors, i.e. ATP, P1, AMP, and citrate (23), and in the presence of 1 mM Fru-6-P, rat liver phosphofructokinase-1 is activated by 2,5-AM-ol-1,6-P2 and by 2,5-AG-ol-1,6-P2 (Fig. 4). Under these conditions, both Fru-2,6-P2 and Fru-1,6-P2 activate phosphofructokinase-1, although Fru-2,6-P2 is over 1000-fold more potent (Ref. 36 and Fig. 4) and produces a greater maximal activation (Fig. 4). The bisphosphates of each fructose analog stimulate phosphofructokinase-1 with an affinity similar to that of Fru-1,6-P2 but to an extent comparable to that of Fru-2,6-P2. The order of potency observed (Fru-2,6-P2 > 2,5-AM-ol-1,6-P2 > Fru-1,6-P2 > 2,5-AG-ol-1,6-P2) indicates a slight preference for the β-anomer. High concentrations (≥100 μM) of Fru-1,6-P2 or of 2,5-AM-ol-1,6-P2 partially reverse the activation of rat liver phosphofructokinase-1 by these effectors (Ref. 36 and Fig. 4), although the activation by 2,5-AG-ol-1,6-P2 even at 5 mM, is not diminished (Fig. 4).

At lower concentrations of Fru-6-P but otherwise identical conditions, the activation curve for Fru-2,6-P2 is shifted to much higher Fru-2,6-P2 concentrations and Fru-1,6-P2 does not stimulate phosphofructokinase-1 (36). In the presence of 0.1 mM Fru-6-P, saturating concentrations of Fru-2,6-P2 and
near-physiological concentrations of other effectors, the activity of rat liver phosphofructokinase-1 is 70% of $V_{\text{max}}$. Fru-2,6-P$_2$ concentrations of up to 240 μM do not inhibit this activity. 2,5-AM-ol-1,6-P$_2$ also activates phosphofructokinase when assayed with 0.1 mM Fru-6-P, but the rate of reaction in the presence of 100 μM 2,5-AM-ol-1,6-P$_2$ corresponds to only 20% of $V_{\text{max}}$.

**Inhibition of Fructose 1,6-Bisphosphatase by 2,5-Anhydromannitol-1,6-P$_2$ and 2,5-Anhydroglucitol-1,6-P$_2$**

2,5-AM-ol-1,6-P$_2$ also activates phosphofructokinase in the presence of 100 μM 2,5-AM-ol-1,6-P$_2$ corresponds to only 20% of $V_{\text{max}}$. 2,5-AM-ol-1,6-P$_2$ activates liver pyruvate kinase by 1200-fold higher for 2,5-AM-ol-1,6-P$_2$ than for Fru-1,6-P$_2$. By comparison, 2,5-AG-ol-1,6-P$_2$ is only slightly effective as an activator of pyruvate kinase even at concentrations 1000-fold higher (Fig. 7).

A contamination of <1% with Fru-1,6-P$_2$ or 2,5-AM-ol-1,6-P$_2$ could explain the activation by the α-analog. Thus, the activation of rat liver pyruvate kinase by Fru-1,6-P$_2$ analogs is specific for the β-anomeric configuration with a slight requirement for the C-2 hydroxyl. The stimulation of pyruvate kinase by 1 or 10 μM Fru-1,6-P$_2$ is not inhibited by up to a 10-fold excess of 2,5-AG-ol-1,6-P$_2$ (data not shown), indicating that there is no appreciable binding of the α-anomer.

An activation of liver pyruvate kinase by higher concentrations of several hexose monophosphates also has been observed (40, 42, 43). Millimolar concentrations of fructose-1-P or 2,5-AM-ol-1-P are generated during hepatic metabolism of fructose (44-46) or 2,5-AM-ol (Figs. 1 and 2), respectively. Upon re-examination, the maximal activation of pyruvate kinase by Fru-1-P, which occurs predominantly as the β-pyranose (47), is much lower than that by Fru-1,6-P$_2$ (cf. Figs. 7 and 8). The $S_{0.5}$ for Fru-1-P activation is approximately 2.5-fold lower for Fru-1-P (43); the activation by 10 mM Fru-6-P is approximately twice that observed with 10 mM Fru-1-P (data not shown). 2,5-AG-ol-1-P also activates rat liver pyruvate kinase and is more potent than Fru-1-P as indicated by the greater maximal extent of activation and the lower $S_{0.5}$ value (Fig. 8). These results indicate that pyruvate kinase is preferentially activated by the β-furanose form of the monophosphate compounds.

In the presence of 2.5 mM ATP, the stimulation of pyruvate kinase by Fru-1-P is almost abolished, and only a 2-4-fold activation occurs at 20 mM Fru-1-P (Fig. 8). Therefore, Fru-1-P is a poor activator of pyruvate kinase under physiological conditions in spite of the high Fru-1-P concentrations generated during fructose metabolism. ATP reduces the affinity of pyruvate kinase for 2,5-AM-ol-1-P and the maximal activi-
Effects of 2,5-Anhydromannitol Phosphates

**Fig. 6.** AMP inhibition of fructose 1,6-bisphosphatase in the presence of fructose bisphosphate analogs. Fructose 1,6-bisphosphatase was incubated as described under "Experimental Procedures" with 5 μM Fru-1,6-[1-32P]P₂ and increasing AMP concentrations in the absence (○) or the presence of 6 μM 2,5-AM-ol-1,6-P₂ (△), or 1 μM Fru-2,6-P₂ (△). In the absence of AMP, these concentrations of the inhibitors correspond to 19.2% for 2,5-AM-ol-1,6-P₂, 41.4% for 2,5-AG-ol-1,6-P₂, and 20.5% for Fru-2,6-P₂ of the activity of the uninhibited enzyme.

**Fig. 7.** Effects of Fru-1,6-P₂, 2,5-AM-ol-1,6-P₂, and 2,5-AG-ol-1,6-P₂ on rat liver pyruvate kinase. Pyruvate kinase was prepared from rat hepatocytes as described under "Experimental Procedures" and assayed in the presence of variable concentrations of Fru-1,6-P₂ (○), 2,5-AM-ol-1,6-P₂ (△), or 2,5-AG-ol-1,6-P₂ (△). Stimulation by this analog (Fig. 8). The maximal stimulation of pyruvate kinase by 10 μM 2,5-AM-ol-1,6-P₂ is unaffected by the presence of ATP (data not shown).

Glucagon-induced phosphorylation of pyruvate kinase results in an increased Sₜₜ for P-enolpyruvate and lower affinity for Fru-1,6-P₂, although saturating concentrations of Fru-1,6-P₂ overcome the effect of phosphorylation (28, 48–52). Pyruvate kinase from extracts of glucagon-treated hepatocytes displays a similar decrease in affinity for 2,5-AM-ol-1,6-P₂, although saturating concentrations of 2,5-AM-ol-1,6-P₂ remain equally effective (Fig. 9A). Therefore, elevated concentrations of 2,5-AM-ol-1,6-P₂, like Fru-1,6-P₂, can overcome the inhibition by phosphorylation of pyruvate kinase. In contrast, 2,5-AM-ol-1-P does not stimulate pyruvate kinase activity from glucagon-treated hepatocytes (Fig. 9B), indicating that 2,5-AM-ol-1-P (and Fru-1-P?) cannot counteract the effects of phosphorylation.

**Activation by Fru-1,6-P₂ of Pyruvate Kinase from 2,5-Anhydromannitol-treated Hepatocytes—**The activity of pyruvate kinase in the absence of effectors and the stimulation of this enzyme by Fru-1,6-P₂ are unaltered when hepatocytes are treated with 0.5 mM 2,5-AM-ol prior to extraction (Fig. 10). Therefore, the activation of pyruvate kinase observed in the intact hepatocytes, as indicated by metabolite crossovers, is not due to covalent or stable alterations. The glucagon-induced decrease in the affinity of pyruvate kinase for Fru-1,6-P₂ also is unaffected by prior treatment of hepatocytes with 2,5-AM-ol (Fig. 10), demonstrating that the cAMP-dependent protein kinase system is functionally intact. Thus, the inhibition of hormonal responses in the presence of 2,5-AM-ol (10, 53) cannot be explained by an inhibition of protein phosphorylation.

**DISCUSSION**

In the short term experiments with isolated rat hepatocytes, 2,5-AM-ol is metabolized to only two products, the 1-phos-
with 5 mM dihydroxyacetone in the absence or presence of 0.5 mM AM-01.

The phosphorylation of 2,5-AM-ol and for an additional 5 min in the absence or presence of 0.5 μM glucagon. Pyruvate kinase was prepared and assayed as described under “Experimental Procedures.”

AM-01 on the activation by Fru-1,6-P₂ of control or phospho-

AM-01 occurs via fructokinase; it is inhibited by fructose but not by glucose (Table I). The accumulation of 2,5-AM-ol-1,6-P₂ in 2,5-AM-ol-treated hepatocytes is much slower than that of 2,5-AM-ol-1-P (Fig. 1) in accord with the kinetics of 2,5-AM-ol-1-P phosphorylation by isolated rat liver phosphofructokinase-1 (Fig. 3). The S₅₀ for the substrate analog is >10 mM (Fig. 3) when measured under conditions comparable to those in the 2,5-AM-ol-treated hepatocyte. Assuming 0.5 ml of cytosolic water/g of hepatocytes, wet weight (32), the concentration of 2,5-AM-ol-1-P in cells treated with 0.5 mM 2,5-AM-ol is approximately 6 mM (Fig. 2) and is below the S₅₀ for phosphofructokinase-1. These data account for the correlation between 2,5-AM-ol-1-P content and 2,5-AM-ol-1,6-P₂ formation observed at varying concentrations of 2,5-AM-ol (Fig. 1) and indicate that the formation of 2,5-AM-ol-1,6-P₂ could be regulated in vivo by allosteric effectors of phosphofructokinase-1.

Metabolite crossover studies indicate that the sites of 2,5-

AM-ol inhibition of gluconeogenesis are those enzymes that interact with fructose phosphates as either substrates or effectors: fructose 1,6-bisphosphatase, phosphofructokinase-1, and pyruvate kinase (10, 11). The concentrations of 2,5-AM-ol-1-P and 2,5-AM-ol-1,6-P₂ in the presence of xylitol are similar to those for glycerol and therefore are sufficient to inhibit gluconeogenesis from substrates that enter the gluco-

neogenic pathway prior to fructose 1,6-bisphosphatase (Table I and Ref. 10). Failure of 2,5-AM-ol to inhibit gluconeogenesis from xylitol (10) thus demonstrates the lack of an inhibitory site beyond fructose 1,6-bisphosphatase.

The metabolite crossover between Fru-6-P and Fru-1,6-P₂ requires a stimulation of phosphofructokinase-1 and/or an inhibition of fructose 1,6-bisphosphatase (10, 11). 2,5-AM-ol-P activates phosphofructokinase-1 with a potency similar to that of Fru-1,6-P₂. On the basis of the concentrations of 2,5-

AM-ol-1,6-P₂ or 2,5-AG-ol-1,6-P₂ required for half-maximal activation, the allosteric site of rat liver phosphofructokinase-1 has a slightly higher affinity for the β-anomer of Fru-1,6-P₂ (Fig. 4). The analogs, like Fru-1,6-P₂ itself, are poor substitutes for Fru-2,6-P₂ in activating liver phosphofructokinase-

1. The ability of 2,5-AM-ol-1,6-P₂, 2,5-AG-ol-1,6-P₂, Fru-1,6-P₂, or even a pyranose bisphosphate, glucose-1,6-P₂ (36), to activate phosphofructokinase-1 is an indication of the relative nonspecificity of the Fru-2,6-P₂-binding site.

Younathan et al. (54) and Benkovic and Schray (2) have noted a preference for the α-anomer in studies on the activation of rabbit muscle phosphofructokinase by these analogs, but the experimental conditions for these studies were not published. The results in Fig. 4 indicate that the relative activation of phosphofructokinase-1 by 2,5-AM-ol-1,6-P₂, 2,5-AG-ol-1,6-P₂, or Fru-1,6-P₂ is critically dependent upon the effector concentrations chosen for comparison. Millimolar concentrations of Fru-1,6-P₂ or 2,5-AM-ol-1,6-P₂ inhibit phosphofructokinase-1 (Ref. 36 and Fig. 4), and at concentrations greater than 0.1 mM, the α-analog activates to a greater extent than the β-analog. The inhibition is specific for the β-

analog and may reflect product inhibition. These observations suggest that the rat liver enzyme, like muscle phosphofructo-

kinase (6, 7, 55, 56), is specific for a β-anomeric substrate.

Although 2,5-AM-ol-1,6-P₂ can enhance the activity of phosphofructokinase-1 (Fig. 4), 2,5-AM-ol-1-P could inhibit the phosphorylation of Fru-6-P because it is an alternate substrate for this enzyme (Fig. 3). In addition, the decrease in hepatocyte Fru-2,6-P₂ content caused by 2,5-AM-ol (10), presumably due to inhibition of phosphofructokinase-2 by 2,5-AM-ol-1-P, could decrease the activity of phosphofructo-

kinase-1 (36, 57, 58). The overall result of these effects cannot be determined from the data on the isolated enzyme. However, an inhibition of lactate formation from glucose, which can be attributed to an inhibition of phosphofructokinase-1, has been observed in isolated hepatocytes in the presence of 2,5-AM-ol.²

² P. T. Riquelme, N. M. Kneer, M. E. Wernette-Hammond, and H. A. Lardy, manuscript in preparation.

The inhibition of gluconeogenesis by 2,5-AM-ol at the Fru-

1,6-P₂/Fru-6-P site probably results from the inhibition of fructose 1,6-bisphosphatase by 2,5-AM-ol-1,6-P₂. The Kᵢ for the analog is one-fourth of the Kᵢ for Fru-1,6-P₂ (Fig. 5A), and the concentrations of 2,5-AM-ol-1,6-P₂ generated in 2,5-

AM-ol-treated hepatocytes (Fig. 2) equal or exceed the Fru-

1,6-P₂ content (10, 11). Because 2,5-AM-ol-1,6-P₂ does not increase the sensitivity of fructose 1,6-bisphosphatase to AMP (Fig. 6), the inhibition of fructose 1,6-bisphosphatase by 2,5-

AM-ol-1,6-P₂ occurs via competition at the catalytic site without involvement of the AMP allosteric site.

The results in Fig. 5 do not identify the anomeric specificity for catalysis, but they do indicate that the possible hydrolysis of β-Fru-1,6-P₂ by rat liver fructose 1,6-bisphosphatase should be considered further. Although Benkovic and co-workers have demonstrated that α-Fru-1,6-P₂ is the preferred sub-

strate for rabbit liver fructose 1,6-bisphosphatase (59) and that this enzyme has a lower Kᵢ for 2,5-AG-ol-1,6-P₂ than for 2,5-AM-ol-1,6-P₂ (8), both the bovine (9) and rat (Fig. 5) liver fructose 1,6-bisphosphatases are more sensitive to the β-

analog than to the α-analog.

Since the inhibition of rat liver fructose 1,6-bisphosphatase by AMP is enhanced by 2,5-AG-ol-1,6-P₂ but not by 2,5-AM-

ol-1,6-P₂ (Fig. 6), the α-anomer of Fru-1,6-P₂ is probably responsible for the potentiation of AMP inhibition. Although the naturally occurring form of Fru-2,6-P₂ is of the β-config-

uration (58, 60, 61), it also has an orientation of the phosphate groups similar to that of the α-analog 2,5-AG-ol-1,6-P₂. The decreased sensitivity of fructose 1,6-bisphosphatase to AMP in the presence of 2,5-AM-ol-1,6-P₂ may reflect a direct interfer-

ence of the phosphate moieties on the β-analog with the AMP binding, a displacement of α-Fru-1,6-P₂ to counteract the increase of AMP inhibition by the substrate (39), or a conformational change that decreases AMP binding.

The metabolite crossover between P-enolpyruvate and py-

ruvate in the presence of 2,5-AM-ol indicates an activation of
pyruvate kinase (10, 11). The increase in Fru-1,6-P₂ content (10, 11) could account for a secondary activation of pyruvate kinase. The direct activation of pyruvate kinase by 2,5-AM-ol-1,6-P₂ is probably also important since the S₅₀ for activation by 2,5-AM-ol-1,6-P₂ is similar to that for Fru-6-P₂ (Fig. 7). In comparison, 2,5-AM-ol-1-P is a poor activator of pyruvate kinase with very low affinity (Fig. 9) can explain the failure of 2,5-AM-ol (Fig. 10). In addition, the dose dependence of the glucagon inhibition of pyruvate kinase is not affected by 2,5-AM-ol. The effects of 2,5-AM-ol on hepatocyte gluconeogenesis (10-12) could account for a secondary activation of pyruvate kinase by 2,5-AM-ol-1,6-P₂ in concert with the Fru-1,6-P₂, activates pyruvate kinase and counteracts any inhibition of pyruvate kinase by glucagon-dependent phosphorylation. Thus, gluconeogenesis is inhibited (10-12), lactate formation is increased (10), and glucagon is ineffective (10). Hanson et al. (62) recently reported a hypoglycemic effect of 2,5-AM-ol when it was administered orally to mice or rats.

The relevance of the effects of 2,5-AM-ol in hepatocytes to in vivo responses and to applications in therapy will be presented elsewhere. The partial characterization of structural requirements for Fru-1,6-P₂ effects at each of the regulatory enzymes for liver carbohydrate metabolism may aid in the design of additional fructose analogs that act more selectively at each of these sites and thus promote the further development of oral hypoglycemic agents.

REFERENCES
Effects of 2,5-Anhydro


Pilkis, S. J., El-Maghrabi, M. R., McGrane, M. M., Pilkis, J., and


P T Riquelme, M E Wernette-Hammond, N M Kneer and H A Lardy


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