Inhibition of glycolysis by 5-amino-4-imidazolecarboxamide riboside in isolated rat hepatocytes

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5-Amino-4-imidazolecarboxamide riboside (AICArriboside; Z-riboside), the nucleotide corresponding to AICArribotide (AICAR or ZMP), an intermediate of the 'de novo' pathway of purine nucleotide biosynthesis, has been shown to inhibit gluconeogenesis in isolated rat hepatocytes [Vincent, Marangos, Gruber & Van den Berghe (1991) *Diabetes* 40, 1259–1266]. We now report that glycolysis is also inhibited and even more sensitive to AICArriboside in these cells. In hepatocyte suspensions from fasted rats, production of lactate from 15 mM-glucose was half-maximally inhibited by 25–50 μ M-AICArriboside. AICArriboside influenced two regulatory steps of glycolysis: (1) it decreased the release of $^3\text{H}_2\text{O}$ from [2- ^3H]glucose and the concentrations of both glucose 6-phosphate and fructose 6-phosphate, indicating that it diminished the phosphorylation of glucose by glucokinase; (2) it decreased the concentration of fructose 2,6-bisphosphate (Fru-2,6- P_2), the main physiological stimulator of liver 6-phosphofructo-1-kinase. Further studies showed that AICArriboside induced an inactivation of 6-phosphofructo-2-kinase, the enzyme that produces Fru-2,6- P_2 , without affecting the concentration of cyclic AMP. Similarly to the inhibition of gluconeogenesis by AICArriboside, the inhibition of glycolysis became apparent after an approx. 10 min latency and persisted when the cells were washed after addition of AICArriboside, strongly suggesting that the effects were also exerted by the Z-nucleotides, which accumulate after addition of AICArriboside to hepatocytes. An increased uptake of lactate was evident when 50–200 μ M-AICArriboside was added 15 min after addition of glucose. This can be explained by the higher sensitivity of glycolysis, as compared with gluconeogenesis, to inhibition by AICArriboside, and reveals the simultaneous operation of both processes.

INTRODUCTION

AICArriboside (5-amino-4-imidazolecarboxamide riboside, Z-riboside) is the nucleoside corresponding to AICArribotide (AICAR or ZMP), an intermediate of the 'de novo' pathway of purine nucleotide biosynthesis. We have previously reported [1] that addition of AICArriboside to isolated rat hepatocytes results in a potent dose-dependent inhibition of gluconeogenesis from various substrates. Production of glucose from lactate/pyruvate mixtures was half-maximally inhibited by approx. 100 μ M-, and completely suppressed by 500 μ M-AICArriboside. Incubation of isolated rat hepatocytes with AICArriboside resulted in the intracellular accumulation, most likely by way of adenosine kinase, of up to millimolar concentrations of ZMP. The latter were found to potentially inhibit fructose-1,6-bisphosphatase, a key enzyme of the gluconeogenic pathway. The inhibition might be explained by the structural similarity of ZMP to AMP [2], one of the physiological inhibitors of fructose-1,6-bisphosphatase.

We now report that glycolysis in isolated rat hepatocytes is even more sensitive than gluconeogenesis to inhibition by AICArriboside. The effect is exerted at the level of two of the regulatory enzymes of the glycolytic pathway, glucokinase and 6-phosphofructo-1-kinase (PFK 1). A preliminary report of this work has been presented at a symposium [3].

MATERIALS AND METHODS

Materials

AICArriboside, AICA base. 3-O-methyl-D-glucose and BSA (Fraction V, essentially fatty-acid-free) were purchased from

Sigma. BSA was dialysed overnight against 25 vol. of Krebs–Ringer bicarbonate buffer with two buffer changes. [2- ^3H]Glucose (17.5 Ci/mmol), 3-O-methyl-D-[U- ^{14}C]glucose (154 mCi/mmol) and [^3H]inulin (1.78 Ci/mmol) were from Amersham International, Amersham, Bucks., U.K. [2- ^3H]Glucose was repurified by paper chromatography before use, to free it from a metabolizable contaminant [4]. N-Acetyl-D-glucosamine was from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Purified glucokinase from rat liver was a gift from Dr. E. Van Schaftingen. Auxiliary enzymes used in the spectrophotometric assays were from Boehringer, Mannheim, Germany. Sephadex G-25 (fine grade) was from Pharmacia, Uppsala, Sweden. Other compounds were the highest grade commercially available.

Preparation and incubation of isolated hepatocytes

Adult male Wistar rats were fasted overnight unless stated otherwise. Isolated hepatocytes were prepared as described previously [5]. Cells (50–70 mg/ml) were incubated at 37 °C in Krebs–Ringer bicarbonate buffer containing 1% BSA. Unless stated otherwise, the cells were preincubated for 15 min with 15 mM-glucose before addition of AICArriboside. For measurement of the release, in the form of $^3\text{H}_2\text{O}$, of ^3H from [2- ^3H]glucose, 0.5 μ Ci of labelled glucose/ml of cell suspension was added simultaneously with AICArriboside. For the assessment of sugar uptake, 3-O-methyl-D-[U- ^{14}C]glucose (0.125 μ Ci/ml) was added 15 min after AICArriboside. [^3H]inulin (0.4 μ Ci/ml) was added simultaneously with the nucleoside for calculation of contamination of the cellular fraction by extracellular medium. Lactate, nucleotides, nucleosides including adenosine, allantoin and $^3\text{H}_2\text{O}$ were measured in HClO_4 extracts prepared from the

Abbreviations used: AICA, 5-amino-4-imidazolecarboxamide (Z-riboside); ZMP, AICArribotide; Fru-2,6- P_2 , fructose 2,6-bisphosphate; cAMP, cyclic AMP; PFK 1, 6-phosphofructo-1-kinase; PFK 2, 6-phosphofructo-2-kinase; PEP, phosphoenolpyruvate.

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whole cell suspension as described previously [5]. Intracellular 3-*O*-methyl-D-[U- 14 C]glucose and glycolytic intermediates were determined in extracts obtained by centrifuging 0.5 ml of the hepatocyte suspension through a 0.5 ml layer of silicone (DC 550; Serva, Heidelberg, Germany) into 0.5 ml of ice-cold 10% HClO_4 [6]. For measurements of fructose 2,6-bisphosphate (Fru-2,6- P_2), cyclic AMP (cAMP) and enzyme activities, samples of the cell suspension were withdrawn at the times indicated, immediately frozen in tubes cooled with a mixture of solid CO_2 and acetone, and stored at -80°C until further processed as given below.

Analytical methods

Lactate, hexose 6-phosphates, phosphoenolpyruvate (PEP), glycerol 3-phosphate, nucleotides, nucleosides and allantoin were measured as in [1]. $^3\text{H}_2\text{O}$ was separated from [2- ^3H]glucose on columns of Dowex AG-1 (borate form) as described in [4]. For the determination of Fru-2,6- P_2 , the frozen cell suspensions were thawed in alkali and the fructose ester was measured by its stimulatory effect on potato pyrophosphate:D-fructose-6-phosphate 1-phosphotransferase as described by Van Schaftingen [7]. Trichloroacetic acid extracts were prepared from the frozen cell suspensions for the determination of cAMP with a [8- ^3H]cAMP-binding assay (Amersham International).

For the measurement of the active form of 6-phosphofructo-2-kinase (PFK 2), hepatocyte extracts, prepared by thawing the frozen cell suspension, and assays were made as described by Bartrons *et al.* [8]. Similar extracts were prepared for the assay of pyruvate kinase, which was measured as described by Feliu *et al.* [9], except that the extraction buffer was supplemented with 5 mM- P_i . The proportion of the enzyme in the active dephosphorylated form was assessed by measuring the ratio of the activity at 0.2 mM-PEP to that at 5 mM. In some experiments, as mentioned in the Results section, the homogenates were gel-filtered by centrifugation through a 0.4 cm $\times$ 6 cm column of Sephadex G-25 (fine grade), before the enzyme assays.

Glucokinase was measured as described by Bontemps *et al.* [4]. The activity of PFK 2 in Sephadex filtrates of rat liver was measured as described by Bartrons *et al.* [8], except for modification of the pH to 7.4 and addition of 1 mM-dithiothreitol.

Statistics

Unless noted otherwise, experiments shown are representative of at least three studies that gave similar results. Results of averaged experiments are given as means $\pm$ S.E.M. Significance was tested for by Student's *t* test.

RESULTS

Influence of AICArriboside on the production of lactate from glucose

Isolated hepatocytes, prepared from fasted rats and incubated in the presence of 15 mM-glucose, produced lactate at the rate of 0.2 $\mu\text{mol}/\text{min}$ per g wet wt. When 10–100 μM -AICArriboside was added simultaneously with glucose, the production of lactate was inhibited in a dose-dependent manner (Fig. 1, upper curve). Half-maximal inhibition was reached at approx. 50 μM , and complete suppression at 100 μM of the nucleoside. Surprisingly, at higher concentration (200 μM) of AICArriboside, production of lactate resumed, and reached about half the control rate at 500 μM of the nucleoside. No production of lactate was recorded with cells incubated in the absence of glucose (Fig. 1, $\circ$ symbols). Also, if AICArriboside was increased to 200–500 μM , production of lactate became apparent, which was the same as that measured

when 15 mM-glucose had been added simultaneously with 200–500 μM -AICArriboside.

When the cells were preincubated with glucose for 15 min before the addition of AICArriboside, and had already accumulated lactate in the incubation medium, 10–25 μM -AICArriboside also inhibited the production of lactate (Fig. 1, lower curve). At higher concentrations of AICArriboside utilization of lactate became apparent, which reached a maximum of approx. 0.3 $\mu\text{mol}/\text{min}$ per g of cells at 100 μM of the nucleoside. At still higher concentrations of AICArriboside, this utilization was progressively suppressed, and at 500 μM -AICArriboside production of lactate again became apparent. The time course of the accumulation of lactate, as a function of the concentration of AICArriboside, in a cell suspension which had been preincubated for 15 min in the presence of 15 mM-glucose before addition of the nucleoside, is shown in Fig. 2. Characteristically, both inhibition of the production of lactate and enhancement of its utilization were preceded by a latency of approx. 10 min.

The inhibitory effect of AICArriboside on the production of lactate was also observed with hepatocytes prepared from fed

Fig. 1. Influence of AICArriboside on the production of lactate by isolated rat hepatocytes

Cells were incubated in the absence of glucose ($\circ$), or in the presence of 15 mM-glucose, which had been added either 15 min before zero time ($\blacktriangle$), or at 0 min ($\bullet$) simultaneously with various concentrations of AICArriboside. Production of lactate was calculated over the 10–30 min time interval following addition of AICArriboside.

Fig. 2. Time course of the effect of AICArriboside on the production of lactate

Cells were incubated for 15 min in the presence of 15 mM-glucose before the addition, at zero time, of various concentrations of AICArriboside.

Fig. 3. Effect of AICArriboside on the release of ^3H from $[2\text{-}^3\text{H}]\text{glucose}$

Cells were preincubated for 15 min in the presence of 15 mM-glucose, before the addition of $[2\text{-}^3\text{H}]\text{glucose}$ ($0.5\text{ }\mu\text{Ci/ml}$) and various concentrations of AICArriboside at zero time.

Fig. 4. Influence of AICArriboside on the concentration of Fru-2,6- P_2

Cells were preincubated for 15 min in the presence of 15 mM-glucose, before the addition of various concentrations of AICArriboside at zero time.

animals: the accumulation of lactate measured in the absence of added glucose as a result of the degradation of glycogen (approx. $0.2\text{ }\mu\text{mol/min per g}$ of cells), and that recorded in the presence of 15 mM-glucose (about $0.8\text{ }\mu\text{mol/min per g}$), were both completely inhibited by $100\text{ }\mu\text{M}$ -AICArriboside (results not shown).

Since concentrations of intermediates of the glycolytic pathway and of Fru-2,6- P_2 , its principal stimulator in liver (reviewed in [10,11]), are very low in hepatocytes before incubation with glucose, all further experiments were performed with cells which had been preincubated for 15 min in the presence of 15 mM-glucose before addition of AICArriboside.

Influence of AICArriboside on the formation of $^3\text{H}_2\text{O}$ from $[2\text{-}^3\text{H}]\text{glucose}$

Glycolysis from glucose is regulated at three levels: (1) phosphorylation of glucose, catalysed mainly by glucokinase in the liver; (2) phosphorylation of fructose 6-phosphate to Fru-1,6- P_2 , catalysed by PFK 1; (3) dephosphorylation of PEP to

pyruvate, catalysed by pyruvate kinase. In order to evaluate the effect of AICArriboside on the first regulatory step, the release of $^3\text{H}_2\text{O}$ from $[2\text{-}^3\text{H}]\text{glucose}$ was measured. This release, which occurs at the level of phosphohexose isomerase, assesses the rate of the initial metabolism of glucose, involving successively its transport, phosphorylation by glucokinase, and isomerization by phosphohexose isomerase [12]. As shown in Fig. 3, AICArriboside decreased the rate of release of ^3H from $[2\text{-}^3\text{H}]\text{glucose}$ in a dose-dependent manner after a latency of approx. 10 min. At $500\text{ }\mu\text{M}$ -AICArriboside, inhibition was approx. 80%. To assess specifically the effect of AICArriboside on the transport of glucose, we measured the entry into the hepatocytes of its non-metabolizable analogue, 3-O-methyl-D-glucose, which is known to be transported by the same carrier as glucose. The amount of 3-O-methyl-D-[U- $^{14}\text{C}]\text{glucose}$ recovered inside the hepatocytes within 4 min after addition was not influenced by preincubation of the cells for 15 min with $500\text{ }\mu\text{M}$ -AICArriboside (results not shown). Taken together, these data suggested that AICArriboside decreases the initial metabolism of glucose by inhibiting glucokinase and/or phosphohexose isomerase.

Influence of AICArriboside on the concentration of hexose phosphates

In order to locate more precisely the site of inhibition of the initial metabolism of glucose, the concentrations of glucose 6-phosphate and fructose 6-phosphate were measured inside the cells. Upon addition of $100\text{ }\mu\text{M}$ -AICArriboside, glucose 6-phosphate decreased within 10 min from a control value of 69 ± 13 to $33 \pm 9\text{ nmol/g}$ of cells (means $\pm$ S.E.M., $n = 4$; $0.01 > P > 0.001$). Fructose 6-phosphate decreased nearly in parallel, from a control value of 35 ± 9 to $13 \pm 3\text{ nmol/g}$ of cells ($0.05 > P > 0.02$). These decreases indicated that AICArriboside induced an inhibition of glucokinase. The fact that glucose 6-phosphate and fructose 6-phosphate remained close to equilibrium rendered an inhibition of phosphohexose isomerase unlikely.

Influence of AICArriboside on the concentration of Fru-2,6- P_2

Fru-2,6- P_2 is the recently discovered principal physiological stimulator of liver PFK 1, and therefore of hepatic glycolysis (reviewed in [10,11]). As shown in Fig. 4, addition of AICArriboside to hepatocytes in which the concentration of Fru-2,6- P_2 has been elevated by preincubation for 15 min in the presence of 15 mM-glucose decreased the concentration of Fru-2,6- P_2 in a dose-dependent manner. At $500\text{ }\mu\text{M}$, AICArriboside provoked the complete disappearance of Fru-2,6- P_2 within 10 min. When $100\text{ }\mu\text{M}$ -AICArriboside was added, together with 10 mM-glucose, to cells which had been preincubated in the absence of glucose, the glucose-induced accumulation of Fru-2,6- P_2 (from 0.2 to 14 nmol/g of cells) was suppressed. AICArriboside also decreased Fru-2,6- P_2 in hepatocytes prepared from fed rats (results not shown). These data indicated that AICArriboside also exerts an inhibitory effect at the level of PFK 1. They prompted further studies to try to explain the effect of AICArriboside on the concentration of Fru-2,6- P_2 .

Influence of inhibition of glucokinase on the concentration of Fru-2,6- P_2

Fru-2,6- P_2 is formed from fructose 6-phosphate and ATP by PFK 2, and degraded to fructose 6-phosphate and P_i by fructose-2,6-bisphosphatase. Glucose elevates the concentration of Fru-2,6- P_2 by increasing the concentration of fructose 6-phosphate, the substrate of PFK 2 [10,11]. To evaluate if the AICArriboside-induced diminution of the phosphorylation of glucose, resulting in a decrease in fructose 6-phosphate, could by itself be responsible for the decrease in the concentration of Fru-2,6- P_2 ,

Fig. 5. Comparison of the effects of *N*-acetylglucosamine and AICArriboside on the release of ^3H from $[2\text{-}^3\text{H}]\text{glucose}$ (a) and on the concentration of Fru-2,6- P_2 (b)

Cells were preincubated for 15 min in the presence of 15 mM-glucose before the addition, at zero time, of $[2\text{-}^3\text{H}]\text{glucose}$ ($0.5 \mu\text{Ci/ml}$) alone, together with 50 mM-*N*-acetylglucosamine (GlcNAc), or together with $100 \mu\text{M}$ -AICArriboside.

Fig. 6. Influence of AICArriboside on the activity of PFK 2

Cells were preincubated for 15 min in the presence of 15 mM-glucose, before the addition of various concentrations of AICArriboside at zero time. The active form of PFK 2 was measured at the times indicated.

experiments were conducted with *N*-acetylglucosamine, a known inhibitor of glucokinase [13]. As shown in Fig. 5(a), 50 mM-*N*-acetylglucosamine had a more pronounced inhibitory effect than $100 \mu\text{M}$ -AICArriboside on the release of ^3H from $[2\text{-}^3\text{H}]\text{glucose}$. However, whereas $100 \mu\text{M}$ -AICArriboside induced the disappearance of 80% of Fru-2,6- P_2 , the glucokinase inhibitor *N*-acetylglucosamine decreased Fru-2,6- P_2 by no more than 50% (Fig. 5b). This result indicates that the decrease in the phos-

phorylation of glucose induced by AICArriboside is not a sufficient explanation for its lowering effect on Fru-2,6- P_2 .

Influence of AICArriboside on the concentrations of adenosine and cAMP

The hepatic concentration of Fru-2,6- P_2 is decreased by compounds that increase the concentration of cAMP, such as glucagon [10] and adenosine [14]. cAMP acts by inactivating PFK 2 and activating fructose-2,6-bisphosphatase. Both processes occur by phosphorylation, catalysed by cAMP-dependent protein kinase [10,11]. Since AICArriboside treatment of dogs had been shown to result in an increase in the release of adenosine in the coronary veins during ischaemia [15,16], we also measured adenosine in hepatocyte suspensions after the addition of AICArriboside. However, no modifications of the concentration of adenosine (0.2 nmol/ml of cell suspension) were recorded. The concentration of cAMP (approx. 1 nmol/g of cells) was also not modified by addition of up to $500 \mu\text{M}$ -AICArriboside to the hepatocyte suspensions. These results indicate that AICArriboside does not influence glycolysis in hepatocytes via adenosine- or cAMP-dependent mechanisms.

Influence of incubation of hepatocytes with AICArriboside on the activities of PFK 2 and pyruvate kinase

To explore further the mechanism(s) whereby AICArriboside decreases the concentration of Fru-2,6- P_2 in hepatocytes, we studied the influence of the nucleoside on the activation state of PFK 2. The latter was measured in extracts prepared from hepatocytes which had been incubated for various times in the presence of AICArriboside. As depicted in Fig. 6, AICArriboside induced a dose-dependent decrease in the activity of PFK 2. That this decrease was recorded in 50-fold-diluted extracts, and persisted after their filtration through Sephadex G-25 (result not shown), indicates that it was not due to the accumulation of a small-molecular-mass ligand or regulator, but rather to a conversion of PFK 2 from its active into its inactive form.

The influence of AICArriboside on the last regulatory step of glycolysis was assessed by measuring the activation state of pyruvate kinase (v at 0.15 mM-PEP/V at 5 mM-PEP) also in hepatocyte extracts prepared at various time intervals. Incubation of the cells in the presence of up to $500 \mu\text{M}$ -AICArriboside did not modify this ratio (about 0.5 in control conditions), indicating that AICArriboside did not affect the interconversion of pyruvate kinase.

Influence of the cell washing and of AICA

When cell suspensions were incubated for 5–10 min in the presence of $200 \mu\text{M}$ -AICArriboside, and thereafter washed free of the nucleoside, the inhibition of the production of lactate and the other effects persisted, namely the diminution of the release of ^3H from $[2\text{-}^3\text{H}]\text{glucose}$, the decrease in Fru-2,6- P_2 and the inactivation of PFK 2 (results not shown). AICA, at $100 \mu\text{M}$ concentration, did not influence the production of lactate, indicating that the effect of AICArriboside did not involve its degradation to the corresponding base (results not shown).

Formation and metabolism of Z-nucleotides

In hepatocytes incubated with 15 mM-glucose and $10\text{--}500 \mu\text{M}$ -AICArriboside, dose-dependent accumulations of ZMP (up to $3 \mu\text{mol/g}$ of cells after 20 min), of ZDP (up to $0.1 \mu\text{mol/g}$) and of ZTP (up to $0.3 \mu\text{mol/g}$) were recorded (results not shown), which were closely similar to those observed in cells incubated with lactate/pyruvate mixtures [1]. In cells incubated for 5–10 min with AICArriboside, and washed thereafter, the nucleoside was no longer detectable, but Z-nucleotides remained stable over 30 min. In hepatocytes incubated with 15 mM-glucose, as in cells

incubated with lactate/pyruvate [1], the addition of up to 500 μM -AICArriboside did not significantly modify either the concentrations of adenine and guanine nucleotides or the production of allantoin (results not shown). These data indicate that in glycolytic, as in gluconeogenic, conditions the metabolism of AICArriboside in isolated hepatocytes is mainly restricted to its conversion into Z-nucleotides, which are not further metabolized to a significant extent.

Influence of AICArriboside and Z-nucleotides on enzyme activities measured in cell-free systems

To explain further the inhibition of glycolysis by AICArriboside, its effects and those of its main metabolites were investigated directly on glucokinase, PFK 2 and pyruvate kinase. Purified glucokinase from rat liver was 20% inhibited by 2.5 mM-, and 50% inhibited by 7.5 mM-ZMP. It was not influenced by 0.5 mM-AICArriboside, 2.5 mM-ZDP or 2.5 mM-ZTP. The activity of PFK 2 measured in Sephadex G-25-filtered high-speed supernatants of rat liver homogenates was not significantly influenced by AICArriboside (0.5 mM), ZMP, ZDP or ZTP (all at 2.5 mM). The activity of pyruvate kinase, measured at 0.2 mM-PEP in hepatocyte extracts, was also not influenced by the addition of AICArriboside (0.5 mM), ZMP (2 mM), ZDP (1 mM) or ZTP (1 mM).

DISCUSSION

AICArriboside exerts a biphasic effect on the production of lactate by isolated rat hepatocytes. This can be explained by inhibition, with different sensitivities, of both glycolysis and gluconeogenesis [1]. That the effects became apparent after an approx. 10 min latency, and persisted when the cells were washed after addition of the nucleoside, strongly suggests that they are exerted by the Z-nucleotides which accumulate after addition of AICArriboside to hepatocytes [1]. The mechanisms whereby AICArriboside inhibits glycolysis are discussed below, as well as the simultaneous operation of glycolysis and gluconeogenesis, evidenced by AICArriboside.

Mechanisms of inhibition of glycolysis by AICArriboside

The inhibition of the production of lactate from glucose, provoked by the addition of 10–100 μM -AICArriboside, is effected at the level of both glucokinase and PFK 1. Inhibition of glucokinase was suggested by diminution of the loss of ^3H from $[2\text{-}^3\text{H}]\text{glucose}$, and by the decrease in glucose 6-phosphate. ZMP at 2.5 mM, a concentration reached in hepatocytes exposed to 300–500 μM -AICArriboside [1], inhibited the activity of glucokinase by 20%. This is probably not sufficient to explain the decreased phosphorylation of glucose. Interaction of ZMP with the newly discovered protein regulator of glucokinase [17] remains to be investigated.

The marked decrease in Fru-2,6- P_2 , the principal physiological stimulator of liver PFK 1, indicates that AICArriboside also provokes a decrease in the activity of this enzyme. Two mechanisms were shown to play a role in the decrease in Fru-2,6- P_2 : the decrease in glucose 6-phosphate and the inactivation of PFK 2. The decrease in glucose 6-phosphate, a consequence of inhibition of glucokinase, results in a decrease in fructose 6-phosphate, and plays a role in the AICArriboside-induced decrease in Fru-2,6- P_2 , since a linear correlation between the concentration of the hexose phosphates and that of Fru-2,6- P_2 has been reported [18]. However, the fact that *N*-acetylglucosamine inhibited the release of ^3H from $[2\text{-}^3\text{H}]\text{glucose}$, and thus glucokinase, more than AICArriboside did, but had a less pronounced effect on Fru-2,6- P_2 (Fig. 5), indicates that additional factors intervene.

The AICArriboside-induced inactivation of PFK 2 is caused by a stable modification of the enzyme, as indicated by its persistence upon dilution and after Sephadex G-25 filtration. It was shown to involve neither an adenosine- nor a cAMP-dependent mechanism, as also evidenced by the absence of effect on the cAMP-dependent phosphorylation state of pyruvate kinase. Whether AICArriboside induces a parallel activation of fructose-2,6-bisphosphatase, as demonstrated with other inactivators of PFK 2 [10,11], remains to be investigated.

Inhibition of glycolysis [19], associated with a decrease in Fru-2,6- P_2 [20], has also been reported upon addition of the fructose analogue 2,5-anhydro-D-mannitol to isolated hepatocytes. The decrease in Fru-2,6- P_2 is explained by inhibition of PFK 2 by 2,5-anhydro-D-mannitol 1-phosphate formed from 2,5-anhydro-D-mannitol [21]. The AICArriboside-induced inhibition of glycolysis differs in several respects from that induced by 2,5-anhydro-D-mannitol: the latter does not influence the activity of glucokinase [19], stimulates that of pyruvate kinase [22], and is less potent, since it maximally decreases the total production of lactate in the presence of 15 mM-glucose by less than 50% [19], as compared with over 90% inhibition at 100 μM -AICArriboside (Fig. 1, upper curve).

Simultaneous operation of glycolysis and gluconeogenesis evidenced by AICArriboside

Simultaneous operation of both pathways, resulting in futile recycling, has been repeatedly documented by isotopic methods (reviewed in refs. [12,23,24]). This recycling can occur at three levels: glucose/glucose 6-phosphate, fructose 6-phosphate/Fru-1,6- P_2 , and PEP/pyruvate. The data depicted in Fig. 1 allow estimation of the overall rate of recycling. In the presence of 15 mM-glucose and in the absence of AICArriboside, the rate of production of lactate was 0.2 $\mu\text{mol}/\text{min}$ per g of cells. AICArriboside (100 μM), which completely inhibited glycolysis, revealed simultaneous utilization of lactate, i.e. gluconeogenesis, at a rate of 0.3 $\mu\text{mol}/\text{min}$ per g. Glycolysis under control conditions would thus reach 0.5 $\mu\text{mol}/\text{min}$ per g, of which 0.3 $\mu\text{mol}/\text{min}$ per g is recycled. Recycling may even be higher, since 100 μM -AICArriboside already exerts an inhibitory effect on gluconeogenesis [1].

The progressive inhibition of the utilization of lactate, recorded at 200–500 μM concentrations of AICArriboside, accords with their inhibitory effect on gluconeogenesis [1]. The resumption of the accumulation of lactate at 500 μM -AICArriboside most likely results from both inhibition of gluconeogenesis from endogenous amino acids and entry of substrates into the glycolytic pathway below PFK 1. Indeed, the same production of lactate was measured when the cells were incubated in the absence of glucose. Isolated hepatocytes release amino acids (approx. 0.5 $\mu\text{mol}/\text{min}$ per g of cells), owing to their protein-catabolic state [25]. From the production of lactate recorded in the presence of 500 μM -AICArriboside, it can be concluded that of the total release of amino acids by the cells, about 0.1 $\mu\text{mol}/\text{min}$ per g is normally converted into glucose.

As discussed previously [1], accumulation of ZMP has been recorded in other cell types when deficient in hypoxanthine-guanine phosphoribosyltransferase, which causes increased synthesis of purines *de novo*, and upon inhibition of AICArriboside transformylase (EC 2.1.2.3) by methotrexate. Whether this accumulation occurs in liver and influences glycolysis therein remains to be determined.

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