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Studies on the mechanism of toxicity of metrizamide—competitive inhibition of yeast hexokinase

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Metrizamide, 2-[3-acetamido-2,4,6-triiodo-5-(*N*-methylacetamido)benzamido]-2-deoxy-D-glucopyranose (Fig. 1), derived from glucosamine and metrizoic acid, is useful as a density gradient medium [1–3] and as a radiologic contrast agent [4, 5]. These applications of metrizamide take advantage of its relatively low viscosity and high molecular weight (789.1), nearly half of which is due to the iodine in the metrizoic acid portion. Although metrizamide is considered an inert substance [1–3] and is relatively safe in comparison with previous contrast media [6–9], evidence of encephalopathy is found in many patients after its intrathecal use [10]. Intrathecally administered metrizamide is known to penetrate into the cerebral cortex [11], where it could affect metabolism within the cell. Although many of the toxic effects of radiological contrast agents have been attributed [12, 13] to the iodine containing moiety, we consider here the hypothesis that the glucose moiety of metrizamide may inhibit glycolysis. Accordingly, we have compared the effects of metrizamide on hexokinase (ATP: D-hexose-6-phosphotransferase; EC 2.7.1.1) to those of glucosamine, 2-deoxyglucose, and metrizoic acid. A preliminary report of this work has been presented [14].

Materials and Methods

Metrizamide, metrizoic acid, glucosamine, 2-deoxyglucose (grade III), glucose-6-phosphate and 2-deoxyglucose-6-phosphate were obtained from the Sigma Chemical Co. (St. Louis, MO), dextrose and urea from Matheson, Coleman & Bell (Norwood, OH), mannitol from Cutter Laboratories (Berkeley, CA), and glucose single vial reagent from CalBiochem-Behring (La Jolla, CA).

Reactions were carried out at 30° in semimicrocuvettes in a temperature regulated chamber of a Gilford model 250 UV-VIS spectrophotometer connected to an Esterline Angus model 575 X-Y plotter. When reconstituted in distilled water, the single vial reagent contained 50 mM Tris buffer (pH 7.6), 0.55 mM ATP, 7.4 mM $MgCl_2$, 0.59 mM NADP, 0.33 I.U. yeast hexokinase (EC 2.7.7.1), and 0.16 I.U. yeast glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate phosphohydrolase; EC 3.1.3.9). The reactions were begun by the addition of 10 μl of 1, 2, 3, 4, 6 or 8 mM glucose in 0, 20, 50 or 100 mM metrizamide (or 2-deoxyglucose or glucosamine) to 500 μl of single vial reagent. After the cuvettes were inverted quickly twice,

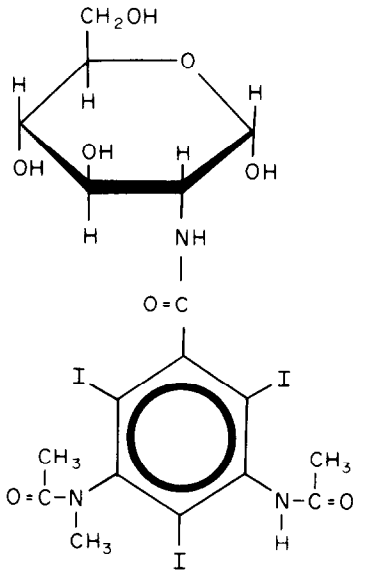

Fig. 1. Structure of metrizamide, 2-[3-acetamido-2,4,6-triiodo-5-(*N*-methyl-acetamido) benzamido]-2-deoxy-D-glucopyranose.

initial rates of NADPH formation and final NADPH concentrations were measured spectrophotometrically at 340 nm. In similar experiments, the kinetics of glucose-6-phosphate dehydrogenase were studied by adding glucose-6-phosphate instead of glucose and monitoring NADPH formation as above. In some of these studies, 10 μl of 50 mM metrizamide was added to the single vial reagent 10 min prior to addition of 10 μl of 1, 2, 3, 4, 6 or 8 mM glucose-6-phosphate. The volume of water used to prepare the single vial reagent was then adjusted so that during preincubation the reagents were all at the concentrations given above.

Results

Control results. In the absence of inhibitors, the maximum velocity (V_{max}) of NADPH formation using the standard assay conditions with glucose as added substrate was 0.20 + 0.023 (S.D.) mM/min, as calculated with a Lineweaver–Burk plot (Fig. 2). The calculated V_{max}, when hexokinase was bypassed by the use of glucose-6-phosphate as the added substrate, was 0.31 mM/min. This established that hexokinase was the rate-limiting step in NADPH formation in this system. It follows that the overall rate of NADPH formation was an excellent approximation of hexokinase activity. The K_m for the glucose–hexokinase reaction derived from Fig. 2. was 0.13 + 0.017 mM (mean + S.D. of seven separate experiments).

Effects of metrizamide. It was initially determined that metrizamide in the absence of glucose did not lead to product formation under these conditions. The effect of metrizamide on hexokinase is depicted in the Lineweaver–Burk plots in Fig. 2. The intersection near the abscissa of the lines of data points derived from the various metrizamide concentrations is consistent with competitive inhibition by metrizamide. The reciprocal of the *y* intercept designates the V_{max} values as 0.20, 0.28, 0.26 and 0.43 mM for 0, 0.4, 1.0 and 2.0 mM metrizamide respectively. Thus, there was no reduction of V_{max} with metrizamide, but there was an apparent increase at higher metrizamide concentrations that is of unknown significance. The negative

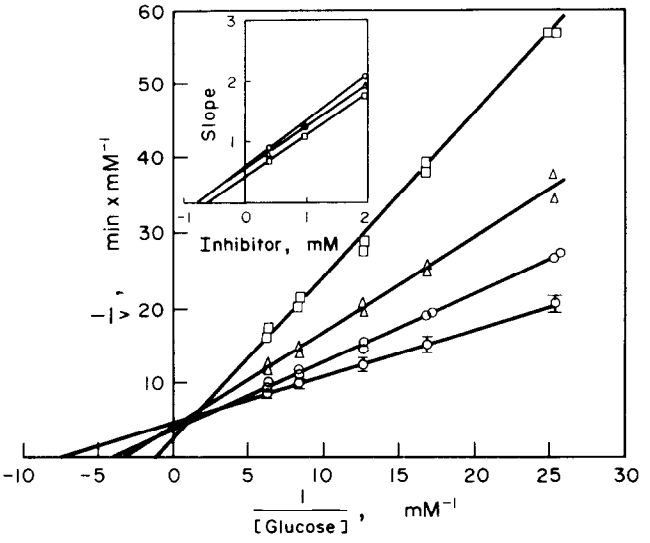

Fig. 2. Effect of metrizamide on hexokinase activity. Lineweaver–Burk plots of reciprocal initial rates of NADPH formation ($min \cdot mM^{-1}$) are depicted as a function of reciprocal initial glucose concentrations (mM^{-1}) in the presence of 0 (♀), 0.4 (○), 1.0 (△), and 2.0 (□) mM metrizamide. Linear regression was used to determine V_{max} and K_m values. Mean values and S.D. are shown for seven separate control experiments. The V_{max} was 0.20, 0.28, 0.26 and 0.43 $mM \cdot min^{-1}$ and the K_m for glucose was 0.13, 0.25, 0.33 and 0.91 mM for 0, 0.4, 1.0 and 2.0 mM metrizamide respectively. Reactions were monitored at 340 nm at 30° after addition of 10 μl of 1, 2, 3, 4, 6 or 8 mM glucose in 0, 20, 50 and 100 mM metrizamide to 500 μl of single vial reagent which contained 50 mM Tris buffer (pH 7.6), 0.55 mM ATP, 7.4 mM $MgCl_2$, 0.59 mM NADP, 0.33 I.U. yeast hexokinase, and 0.16 I.U. yeast glucose-6-phosphate dehydrogenase. Inset: The slopes of Lineweaver–Burk plots of yeast hexokinase activity in the presence of metrizamide (○), 2-deoxyglucose (△) and glucosamine (□) are plotted as a function of final inhibitor concentration (mM). The X intercept represents $-K_i$. The K_i falls in the range of 0.6 to 0.8 mM.

reciprocal of the x intercept, representing the K_m for the glucose–hexokinase reaction, was 0.13, 0.25, 0.33 and 0.91 mM with 0, 0.4, 1.0 and 2.0 mM metrizamide respectively. There was no effect of 2 mM metrizamide on NADPH production with glucose-6-phosphate as the variable substrate: the K_m was 0.082 mM and the V_{max} was 0.32 mM/min versus control values of 0.076 mM and 0.31 mM/min.

Effects of glucosamine and 2-deoxyglucose. The effects of glucosamine and 2-deoxyglucose on yeast hexokinase activity were very similar to those of metrizamide. The V_{max} was relatively unaffected, being 0.20, 0.21, 0.21 and 0.27 mM/min for 0, 0.4, 1.0 and 2.0 mM glucosamine respectively; glucosamine alone did not result in any product formation under these conditions. The K_m for glucose, however, changed from 0.13 to 0.15 to 0.23 to 0.48 mM at 0, 0.4, 1.0 and 2.0 mM glucosamine respectively. Initial experiments revealed that 2-deoxyglucose alone led to NADPH formation at low rates: approximately 4.8 μM/min. It was also apparent at low glucose concentrations that an amount of NADPH was being formed that was greater than expected and greater than could be accounted for by the glucose alone. After the rates were corrected for product formation due to 0.4, 1.0 and 2.0 mM 2-deoxyglucose, linear Lineweaver–Burk plots were obtained. The V_{max} was 0.20, 0.21, 0.28 and 0.31 mM/min for 0, 0.4, 1.0 and 2.0 mM 2-deoxyglucose respectively. The K_m for glucose was 0.13, 0.17, 0.36 and 0.61 mM for 0, 0.4, 1.0 and 2.0 mM 2-deoxyglucose respectively. Again, this is most consistent with a competitive inhibition.

There was no effect of 2.0 mM glucosamine or 2-deoxyglucose on either the K_m for glucose-6-phosphate or the V_{max} of glucose-6-phosphate dehydrogenase under these conditions.

Effects of metrizoic acid and mannitol. To explore the possibilities that osmolarity alone or the metrizoic acid moiety of metrizamide had inhibitory effects upon hexokinase, these substances were also tested. There was no effect of these substances on hexokinase activity. The V_{max} of hexokinase for 2.0 mM mannitol and 2.0 mM metrizoic acid was 0.19 and 0.21 mM/min, respectively, versus a control of 0.20 mM/min. The K_m for glucose of hexokinase in the presence of 2.0 mM mannitol and metrizoic acid was 0.13 and 0.14 mM, respectively, compared to the control value of 0.13 mM.

Determination of K_i. Replots of the slopes of the Lineweaver–Burk plots for inhibition of hexokinase by metrizamide, 2-deoxyglucose, and glucosamine are shown in the inset in Fig. 2. The K_i values were approximately 0.6 to 0.8 mM for the inhibitors.

Discussion

Metrizamide is not an inert substance, contrary to previous assertions [1–3]. These results establish that it is a competitive inhibitor of hexokinase, as are glucosamine and 2-deoxyglucose which are well-known inhibitors of glucose metabolism. Harpur and Quastel [15] showed that, like glucosamine, *N*-acetyl glucosamines inhibit hexokinase *in vitro* but, unlike glucosamine, they are not themselves phosphorylated. Maley and Lardy [16] demonstrated that benzoylglucosamine compounds are powerful competitive inhibitors of hexokinase, with K_i values from 10^{-6} to 10^{-3} M although they are not phosphorylated. They showed further that hexokinase from beef brain was more susceptible to these agents than was yeast hexokinase. Since metrizamide is a substituted benzoylglucosamine, the inhibitory effects demonstrated in this report are just as expected. Further studies with brain hexokinase are required to determine the effects of metrizamide there.

Besides being a competitor with glucose for carrier mediated transport [17–19], 2-deoxyglucose is also a competitive inhibitor of hexokinase [18, 20, 21]. At a concentration of 20 mM, 2-deoxyglucose-6-phosphate causes a slight inhibition of phosphofructokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase; EC 2.7.1.11) and a marked inhibition of phosphoglucose isomerase (D-glucose-6-phosphate ketol-isomerase; EC 5.3.1.9) in cerebral homogenates [22]. It is not known which, or if, other enzymes are also inhibited by metrizamide, but since metrizamide is not phosphorylated, it probably does not affect subsequent enzymes of glycolysis as 2-deoxyglucose and glucosamine eventually do.

Since, after 6 hr of incubation under the standard conditions, the extinction change with 2 mM 2-deoxyglucose alone is equivalent to 1.7 mM glucose, contamination of 2-deoxyglucose by glucose cannot be the sole explanation of this product formation. Horton *et al.* mentioned, in passing, that yeast glucose-6-phosphate dehydrogenase preparations dehydrogenated 2-deoxyglucose-6-phosphate but that brain extracts apparently did not [22]. Further studies are required to clarify this matter.

We have concluded that metrizamide is a competitive inhibitor of yeast hexokinase *in vitro* when used in the concentrations recommended (from 0.22 to 0.45 M) for density gradient separations or in concentrations recommended (0.73 M initially, diluted to a theoretical 0.05 M) for radiological examinations. The K_i values for metrizamide, 2-deoxyglucose, and glucosamine are near 0.7 mM. Furthermore, since metrizoic acid is found not to be inhibitory to hexokinase, it is postulated that the glucosamine moiety of metrizamide is responsible for this inhibition.

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