Presence in Beef Tissues. Tissues and fat were obtained from seven different 16–18-month-old beef heifers from three farms in two provinces. These animals were in a feedlot environment for finishing and were fed MGA-100 (Tuco) (ca. 0.4 mg/animal per day) for at least 2 months. Feed was withdrawn 48 hr before slaughter and tissue (muscle, liver, kidney, subcutaneous fat, and perirenal fat) was obtained and stored at −10°F until analyzed (up to 2 months as MGA is stable in storage). The MGA contents of these tissues using the described method are shown in Table II. The largest amount of MGA is in the fat (5–10 ppb) and this agrees with earlier work of Krzeminski et al. (1968) who found this tissue to contain most of this lipid-soluble steroid. Lesser amounts are found in the liver (1–5 ppb) and muscle with only traces in the kidney. At present, a zero tolerance has been established for MGA residues in foods, a tolerance which depends on the method sensitivity which, in turn, invariably increases with the advance of techniques. The residue values of MGA reported here in beef tissue combined with toxicological data should form a basis for a tolerance in food and a withdrawal period for this drug in animal practice.

ACKNOWLEDGMENTS

The authors are grateful to Burt Gorenstein, Agriculture Canada, Ottawa, for arrangement and collection of tissues from MGA-fed heifers, and to Walter Miles, Health Protection Branch, for the determination of mass spectra. Part of this work was presented at the Trace Analysis in Biological Materials Symposium, Halifax, Canada, Aug 21–23, 1974.

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Received for review February 14, 1975. Accepted June 16, 1975.

An Immobilized Glucose Isomerase for the Continuous Conversion of Glucose to Fructose

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The conversion of glucose to fructose in column reactors, by glucose isomerase adsorbed on the internal surface of controlled-pore alumina, has been investigated. All column reactors were evaluated with substrate solutions containing either 36 or 50 g of glucose per 100 ml of solution at 60°. Magnesium and cobalt ions were included in the feed. Sodium sulfite was employed to regulate the substrate pH. Initial column studies were performed with adsorbed enzyme derivatives having activities in the range of 200–400 glucose isomerase units (GIU) per gram of material, as determined by a static assay procedure. Column half-lives in excess of 40 days were observed for reactors operating at 80 to 85% theoretical conversion of glucose to fructose. Subsequent column reactors containing derivative with activities between 550 and 650 GIU/g of material, as measured by actual column operating parameters, also showed half-lives greater than 40 days.

The enzymatic conversion of glucose to fructose, via an isomerase extracted from *Pseudomonas hydrophila* cells grown on xylose, was reported by Marshall and Kooi (1957). Initial studies on glucose isomerase obtained from *Streptomyces* sp. were reported by Tsumura and Sato (1965). These authors demonstrated a dependency of the *Streptomyces* isomerase upon cobalt and magnesium ions for activation, and showed that cobalt ion protects the enzyme from high temperature denaturation. Takasaki (1966) and Takasaki et al. (1969) further detailed the effect of metal ions, temperature, and pH on the *Streptomyces* isomerase performance.

The glucose isomerase obtained from the *Streptomyces* organism is an endocellular enzyme, and is therefore costly to obtain because of the processing required to liberate it from cellular material. Since the sugar industry may be considered producers of commodity products, high cost enzymes must be employed in the most economical manner. Soluble enzymes are used in but one batch reaction; however, if the enzyme is immobilized, it not only can be used many times, but can also be employed in continuous reactors.

Sugar, sucrose, is at the present time in short supply. The price to the consumer has been increased from 200 to 300% during the past year. An alternative supply of a natural sweetener would relieve, to some degree, the demand for sugar and thus reduce the price to consumer. *Streptomyces* glucose isomerase can convert 50% of the glucose present in solution to fructose. This 50% glucose–50% fructose solution may be used as a substitute for invert sugar in products marketed by the soft-drink beverage and confectionary industry. Therefore, glucose isomerase immobilized by an economical procedure and judiciously employed in the conversion process is of considerable interest for such applications. This report describes the performance advantages and limitations of columns containing *Streptomyces* isomerase, adsorbed on the internal surface of controlled-pore alumina.

EXPERIMENTAL SECTION

Materials. The glucose isomerase utilized for these studies was derived from a *Streptomyces* sp. The activity of the various native enzyme preparations ranged from 270 to 450 glucose isomerase units (GIU)/g.

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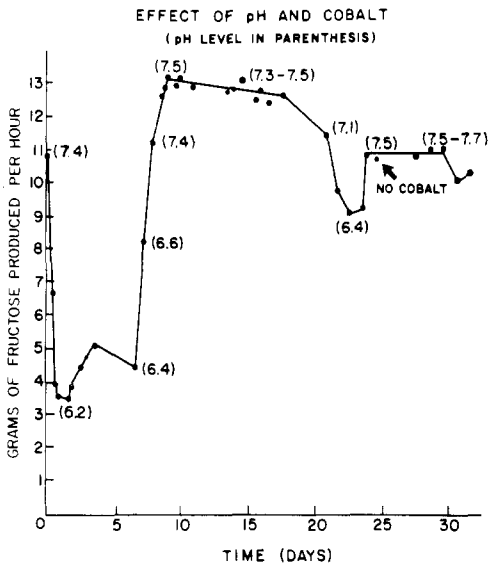

Figure 1. Effect of pH on isomerization reaction.

All immobilized enzyme preparations were made with controlled-pore alumina as a carrier. This carrier was selected from a spectrum of controlled-pore ceramics recently developed in our laboratories. Its physical characteristics were: average pore diameter, 175 Å (range, 140–220 Å); pore volume, 0.6 cm^3/g, surface area, 100 m^2/g; theoretical density, 3.7 g/cm^3; particle size, 25–65 mesh.

Standard laboratory chemicals used in these investigations (e.g., cobalt chloride, magnesium sulfate) were of reagent grade.

Preparation of Immobilized Glucose Isomerase. Prior to undertaking the actual enzyme coupling of glucose isomerase to the porous alumina, the carrier was pretreated with a solution of 0.05 *M* magnesium acetate and 0.01 *M* cobalt acetate at pH 7.7. The support (Mg^{2+}–Co^{2+} solution) mixture was agitated at 60° for 15 min. Following decantation of the Mg^{2+}–Co^{2+} solution, the pretreated carrier was held at room temperature until ready for enzyme coupling.

For all of the enzyme coupling reactions, glucose isomerase derived from *Streptomyces* sp. was employed. Enzyme solution (568 GIU/ml) at a 1000 GIU/g ratio of enzyme to carrier was employed in the preliminary studies; however, 1000 GIU/ml of enzyme solution at a 2000 GIU/g ratio of enzyme activity to solid carrier was found to be more effective and these conditions were utilized to prepare derivatives for column performance studies. The pH of the enzyme solution was adjusted to 7.5. Pretreated porous alumina was combined with the enzyme solution and the mixture was placed in a water bath at 60° and then agitated periodically at temperature for 2 hr. The alumina–enzyme mixture was then removed from the 60° bath, allowed to cool to room temperature, and held for 16 hr.

Following the elapsed 16-hr time period, the enzyme solution was decanted from the newly formed enzyme derivative, and the immobilized glucose isomerase product was washed with water in a fluidized bed to remove fines that had been generated during the coupling procedure. The derivative was then washed once with 0.5 *M* NaCl solution and twice with 0.05 *M* cobalt–magnesium solution.

Static Assay Technique. Batches of immobilized enzyme, 0.3–0.4 g in weight, were assayed with 36% glucose solution at 60°. Maleate at a concentration of 0.1 *M* was used as a buffer at pH 6.9; 0.001 *M* cobalt chloride and 0.005 *M* magnesium sulfate salts were employed as activating and stabilizing electrolytes. The activities of the immobilized enzyme derivatives were expressed in glucose isomerase units (GIU), where the GIU is the quantity of enzyme activity required to convert 1 μmol of glucose to fructose per minute at 60° and at pH 6.85.

Table I. Glucose Isomerase Sodium Sulfite Electrolyte System (50% Glucose; 0.005 *M* $MgSO_4$; 0.001 *M* $CoCl_2$; 0.004 *M* Na_2SO_3; pH 7.8; 60°, 10.07 g)

Time, days	a.m. flow, ml/hr	a.m. % conv.	p.m. flow, ml/hr	p.m. % conv.
1	122	86.5	129	82.5
2			122	82.2
3	122	84.5		
4	122	82.8	123	82.0
5			114	84.8
6	112	82.8	108	85.5
7	108	85.5	108	87.0
8	104	82.0		
9	108	83.2		
10	104	88.0		
11			108	82.0
12	105	82.6	98	83.5
13	99	83.5	99	82.5
14	94	85.0	96	91.0
15	86	92.8	92	90.5
16	99	89.0		
17	96	88.8		
18	99	87.0	96	87.0
19	102	86.0	92	86.8
20	108	82.5	100	83.2
21	102	84.0	106	81.8
22	105	79.0		
23	100	82.5		
25	96	87.6		
26	98	82.8	98	81
27	84	84.0		
28	80	87.0		
29	83	87.4		
30				
31				
32				
33	78	82.0		
34	62	87.4		
35	72	82.8		

Dynamic Assay Technique. Column activities can be calculated through use of the following formula:

$$E = 27.9[(F/W) \ln (1 - X)]$$

where E = column activity in glucose isomerase units/gram, F = flow rate of substrate, W = weight of immobilized enzyme used, and X = percent conversion G → F. This equation was evolved from experimental comparisons of batches run under assay and column conditions (Havewala and Pitcher, 1974). Product samples were collected periodically from the column, and a Bendix recording automatic polarimeter was used to determine the enzymatic conversion (G → F) which occurred.

Column Evaluations of Immobilized Glucose Isomerase. To evaluate the performance of glucose isomerase adsorbed upon the internal surface of controlled-pore alumina, column studies were initiated. The water-jacketed columns employed for this work were 1.5 cm in diameter (i.d.) and 40 cm in length. At least 10 g of enzyme derivative was used for each column evaluation. All columns were run with feed that contained either 36 or 50 g of glucose per 100 ml of solution. Magnesium ion was used as an activator and cobalt ion as a temperature stabilizer. Sulfite and bicarbonate were evaluated as buffers.

Table II. Column 071119702 Glucose Isomerase Ammonium Bicarbonate Electrolyte System (50% Glucose; 0.005 *M* $MgCl_2$; 0.001 *M* $CoCl_2$; 0.004 *M* $NaHCO_3$; pH 7.8; 60°)

Time, days	a.m. flow, ml/hr	a.m. % conv.	p.m. flow, ml/hr	p.m. % conv.
1	107	86.5	112	83.0
2	114	85.0	112	83.5
3	111	84.0	114	81.2
4	88	84.4		
5	113	79.8		
6	111	77.0	103	78.5
7	112	76.2	112	75.0
8	96	79.2	109	74.0
9	108	73.0	91	80.0
10	110	70.0		
11	107	68.0		
12	108	67.2		
13	90	73.6	108	67.8
14	108	68.0	109	68.6
15	110	66.2	105	66.6
16			92	78.0
17	88	78.0	92	75.5
18	104	70.0		
19	79	77.8		
20	111	64.4	111	63.5
21	109	63.2	112	62.2
22	107	63.8		

Data gathered from the columns included the flow rate to obtain the required conversion of 42% of the glucose to fructose, immobilized enzyme activities as measured by column parameters, and the pH variations of the feed as it flowed through the column.

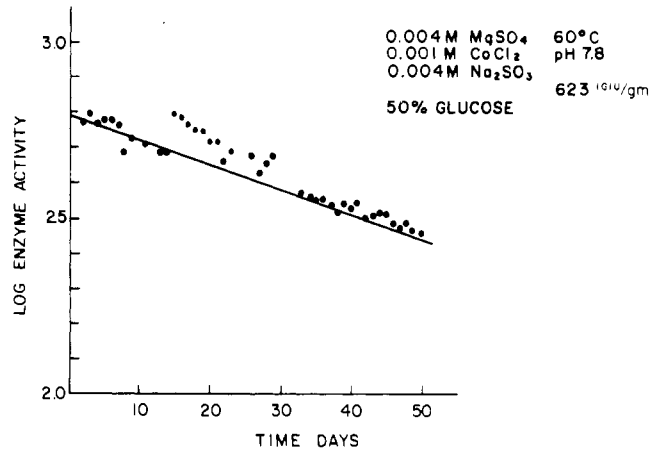

Figure 2. Half-life plot, immobilized glucose isomerase, sulfite buffer.

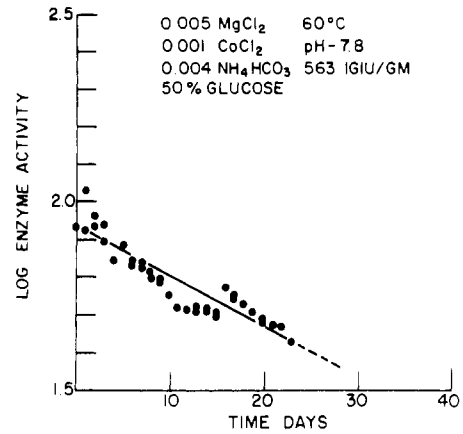

Figure 3. Half-life plot, immobilized glucose isomerase, bicarbonate buffer.

RESULTS AND DISCUSSION

Effect of pH and Co^{2+} Ion Presence upon Reactor Performance. The assay of one of the immobilized glucose isomerase preparations indicated a loading of 206 GIU/g. This derivative was placed in a column and fed a solution containing 36% glucose, 0.004 *M* sodium sulfite, 0.005 *M* magnesium sulfate, and 0.001 *M* cobalt chloride, at a pH of 7.4 and a flow rate of 100 ml/hr.

Data collected on this column are plotted in Figure 1 in terms of fructose production vs. time. Although the substrate was continually fed at a pH of 7.4 to the column, the pH of the product had dropped to 6.4 after the first day of operation and to 6.2 after 3 days of continuous operation. Column performance deteriorated with the decrease in pH of the product. When no significant rise in column pH was noted after 7 days of operation, the sulfite concentration in the feed was increased to 0.007 *M*. This caused an increase in the pH of the feed to 7.9. After 48 hr exposure of the column to feed with a higher sulfite concentration, the product pH rose to 7.45, with a concurrent 3.7-fold increase in the conversion rate of glucose to fructose.

Between the 17th and 21st day of operation, the pH of the product eluent dropped again, and reached 6.9 on the 23rd day. It was determined that the feed, after standing at room temperature for several days, had shown a significant decrease in pH, and this pH decrease had resulted in a lowering of the column conversion rate. By readjusting the pH of the feed upward again, the conversion rate, glucose to fructose, again improved.

On the 25th day of operation cobalt ion was withdrawn from the feed; magnesium ion was continued as an additive. During the next 5 days of operation, no loss in conversion resulted from the omission of cobalt from the feed.

The effect of pH on enzyme activity is dramatic, particularly in light of the pH optimum studies reported by Takasaki et al. (1969) for the native enzyme. At a pH of 6.4 (see Figure 1) the activity of the enzyme is reduced to approximately 25% of that at the optimum (7.5). Takasaki's results indicate that under the same conditions approximately 50% of the enzyme activity is retained.

When cobalt was eliminated from the substrate, no enzyme activity loss was detected. Therefore, it is possible that after an initial equilibration of the immobilized enzyme derivative with cobalt ions, addition of cobalt to the operating feed might be unnecessary.

Column Performance. Performance data for two columns containing glucose isomerase adsorbed onto the internal surface controlled-pore alumina are presented in Tables I and II. Figures 2 and 3 show plots of log of enzyme activity (or production) vs. time for columns containing immobilized enzyme derivatives. Column operating conditions are indicated on each figure, as are electrolyte additives and initial column activities for the immobilized enzyme as measured by column parameters. Table III is a summary of the pertinent results obtained on each column. Included are the initial column activities and column half-lives as determined by the least-squares method. In all cases there was minimal pressure drop observed when using the plug-flow reactors.

Carrier and Buffer. Previous studies (Messing, 1974) of enzymes immobilized in the pores of inorganic carriers have indicated that the major dimension of either the enzyme or the substrate, whichever is larger, is one of the

most important limiting factors relative to enzyme loading and stability. In this study, since the substrate, glucose, is dimensionally smaller than the enzyme, the size of the enzyme is the determinant in optimizing the pore morphology of the carrier. To reduce the effect of turbulence in the environment external to the carrier pore, and to maximize the utilization of internal surface for enzyme attachment, it was previously determined (Messing, 1974) that the average pore diameter of the carrier should be twice the major dimension of the enzyme unit cell.

Since the unit cell dimensions of glucose isomerase have not been reported in the literature, optimization of carrier pore diameter relative to enzyme size was determined empirically. Initial screening of carriers indicated that little or no glucose isomerase was adsorbed on the internal surface of 140-Å pore diameter alumina. Alumina with pores 175 Å in diameter readily accepted the enzyme, however. Based on these data, it is estimated that the major axis of the unit cell of glucose isomerase is between 70 and 90 Å.

The chemical composition of the carrier to be used for the enzyme immobilization effort is also important. Controlled-pore glass and controlled-pore silica yielded immobilized enzyme derivatives that had relatively poor half-lives, compared to those prepared with the controlled-pore alumina. Both the glass and the silica dissolve (Eaton, 1974) when used in the alkaline environment required for glucose isomerase activity; alumina is durable (Eaton, 1974) when employed in the basic pH range.

Relative to the contributing electrolytes employed for these reactor studies, the sulfite system is clearly superior to the bicarbonate. Two factors may contribute to the advantage in using sodium sulfite rather than ammonium bicarbonate as the contributing electrolyte. Our studies indicate that ammonium bicarbonate interferes with the adsorption process, and, therefore, it may actively participate in the desorption of the enzyme from the alumina surface. The ammonium ion thus formed then competes with the amine group of the protein as the carbonate ion competes with the isomerase's carboxyl groups for the adsorption sites on the surface of the alumina. If this salt, at the concentration utilized, successfully replaces only a few of the adsorption sites of the protein over a unit of time, this would explain the gradual removal of the enzyme from the alumina surface.

Table III. Column Results Summary

Column no.	Enzyme	Electrolyte	Additives	E_0, GIU/g	$t_{1/2}$, days
1	Glucose isomerase	0.004 *M* Na_2SO_3	0.005 *M* $MgSO_4$ 0.001 *M* $CoCl_2$	623	40
2	Glucose isomerase	0.004 *M* NH_4HCO_3	0.005 *M* $MgCl_2$ 0.001 *M* $CoCl_2$	563	23

In addition, sulfite dissociates to form SO_2, which behaves as an antimicrobial agent to reduce the effects of the microbial population which is responsible for the degradation on the immobilized enzyme derivative. To assume that the microbes themselves are directly responsible for the derivative's deterioration is unacceptable however, since no microbe could enter a pore 175 Å in diameter. It is more likely that the microorganisms elaborate extracellular proteases which then attack the immobilized glucose isomerase. One such proteolytic enzyme, alkaline *Bacillus subtilis* (major dimension 50-Å diameter), is produced by *B. subtilis*, and is capable of hydrolyzing in an alkaline pH range. The spin diameter of this protease would be expected to be 100 Å in diameter, and its entry to a 175-Å diameter pore would be unhindered.

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Received for review November 25, 1974. Accepted May 8, 1975.