Glucose Production by Isolated Rat Liver Cells

AN AMYLASE-OLIGOGLUCOSIDASE PATHWAY FOR GLYCOGEN BREAKDOWN*

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The ability of the liver to release glucose into the extracellular medium is well known. This phenomenon has been demonstrated in the intact animal (1-3), in the isolated perfused liver (4, 5), and in liver slices (6, 7).

The classic studies on phosphorylase (8-10), phosphoglucomutase (10-12), the specific glucose 6-phosphatase (13, 14), and the discovery of uridine diphosphate-glucose-glycogen transglucosylase (15) provide an enzymatic basis for the formation by liver tissue of glucose from intracellular glycogen. The demonstration of this phenomenon at the enzymatic level. In addition to the phosphorylase system, the liver cells contain an active α-amylase together with relatively low levels of oligoglucosidase (maltase). Under the conditions of these experiments, the liver cells produce predominantly oligosaccharides rather than glucose. The dialyzed serum contains an active oligosaccharidase (maltase) which facilitates the conversion of the oligosaccharides to glucose.

The subsequent papers (20, 21) are concerned with the description of the properties of the liver amylase, and with the evaluation of the role of the amylase pathway in glucose production by liver tissue.

EXPERIMENTAL PROCEDURE

Materials—Maltotriose, maltotetraose, and amylase were generously donated by Dr. R. L. Whistler. Glucose oxidase (crude) and horseradish peroxidase were procured from the Sigma Chemical Company. Crystalline glucagon and glucagon-free zinc insulin were kindly provided by Dr. O. K. Behrens of Eli Lilly and Company. Hydrocortisone was obtained through the courtesy of Dr. Karl Folkers of Merck and Company, Inc. Aldosterone was generously donated by Dr. A. Wettstein of Ciba Pharmaceutical Products, Inc. Bovine growth hormone (Somar, Armour) was a gift of the Endocrinology Study Section of the National Institutes of Health, United States Public Health Service.

Dialyzed horse serum was prepared by dialysis against 5 volumes of 0.9% NaCl for 3 to 4 hours, then against 5 volumes of phosphate-buffered NaCl (22) for 5 hours, and finally against 10 volumes of the latter solution for 10 hours.

Medium 2 is the balanced salts medium of Krebs (23), except that glucose is omitted. Medium 2A is Medium 2 with the glucose replaced by ethylenediaminetetraacetate.

Chemical Determinations—Glucose was determined by the glucose oxidase reaction coupled to the dye o-dianisidine with peroxidase (24). The reagent mixture contained 62.5 mg of crude glucose oxidase preparation, 5.0 mg of peroxidase, 1 ml of o-dianisidine in methanol (1 g per 100 ml), and 99 ml of 0.1 M acetate buffer, pH 4.3. The reagent solution 2.5 ml was pipetted into 2.5 ml of solution containing 0.1 to 1.0 μmole of glucose, and after 10 minutes at room temperature, 1 drop of 4 N HCl was added to stop the reaction. The optical density was measured at 401 mμ, and the level of glucose determined by comparison with appropriate standards. The crude glucose oxidase preparation exhibited significant maltase activity; in optimal concentrations maltase was about 40% as active as glucose in this test. The response to maltose could be virtually eliminated by substituting 1 mg of purified glucose oxidase for the crude glucose oxidase and increasing the incubation period to 40 minutes. Measurements of glucose in the presence of maltose were performed by this modification.

Glycogen was determined by a modification of the method of Kemp and Kits van Heijningen (25). After an initial extraction of low molecular weight carbohydrates with methanol at room temperature, the glycogen was extracted with hot trichloroacetic acid and measured with the anthrone reagent (26). Total carbohydrate amount of cells was determined in the same manner as glycogen, except that the cold methanol extraction was eliminated. Lac-tate was measured by the modified method of Barker and Summerson (27), and protein by the biuret method (28).

Chromatography—Samples for chromatography were concentrated to dryness with the aid of a rotary evaporator and extracted with dry pyridine according to Malpress and Morrison (29). Separation of carbohydrates was achieved by descending chromatography at room temperature with Whatman No. 1 filter paper. Three solvent systems were used: (a) α-propanol-acetic acid-water, 3:1:1, 30 hours; (b) n-butyl alcohol- pyridine- H2O, 6:4:3, 20 hours (30); and (c) ethyl acetate-pyridine-H2O, 2:1:2 (top layer), 19 hours (30).
reagent of Lemieux and Bauer (31) was utilized for detection of glucose. The incubation mixture contained 0.8 ml of rat liver cell suspension in nutrient medium (~15 mg of protein per ml), horse serum (0, 0.004, 0.01, 0.03, 0.06, 0.1, and 0.2 ml of dialyzed serum, respectively), and nutrient medium to a total volume of 1.0 ml. Incubation was carried out in a Dubnoff metabolic incubator for 20 minutes at 37°. Aliquots were taken at zero time and at 20 minutes for analysis. The cells were removed by centrifugation and glucose was determined in an aliquot of the supernatant fluid. Glucose production was expressed as the difference between zero time and incubated samples at each level of serum.

Enzyme Assay—Amylase activity was measured in homogenates after 1 minute of sonication (20) in a 10-kc Raytheon oscillator, by following the decrease in iodine color given by the starch substrate, according to a modification of the procedure used by Schwimmer (32). To 2.0 ml of solution containing 40 mg of soluble starch and 0.3 mg of NaCl in 0.05 M histidine-chloride buffer, pH 6.5, was added an appropriate aliquot of enzyme diluted to 1.0 ml. After 0 and 20 minutes of incubation at 37°, 1.0-ml samples were removed and deproteinized with 1.0 ml of trichloroacetic acid (10 g per 100 ml). Aliquots (0.1 ml) of deproteinized samples were added to 5.0 ml of 0.25 M KI + 0.0035 M I2, and after dilution to 15 ml with water, the optical density at 550 mμ was measured. A standard curve using known starch levels was made, and the amount of starch hydrolyzed was expressed in terms of milligram equivalents of starch.

In some experiments, the amylase activity was measured by the increase of reducing power during hydrolysis (33). The enzyme activity was expressed as μmoles or reducing power (calibrated with glucose as standard) released per minute. Under the conditions used in these experiments, the activity of liver amylase, expressed in milligram equivalents of starch, obtained with the iodine color assay may be converted to μmoles of reducing power by multiplying the former by 0.8.

Phosphorylase activity was assayed by the method of Cori and Cori, adapted by Sutherland (34). Maltase (α1,4 oligo-glucosidase) activity was measured by the rate of release of glucose with maltose as substrate. The final incubation contained 0.05 M histidine, pH 6.5, 0.001 M maltose, and an appropriate quantity of enzyme. Glucose was determined at zero time and after incubation for 30 minutes at 37°.

Liver Cells—The suspensions of liver cells used in these studies were prepared by a method developed in this laboratory.2 Rats weighing 80 to 100 g were killed by decapitation and the livers immediately perfused with 30 to 50 ml of either ice-cold 0.24 M sucrose + 0.0135 M citrate (pH 5.0) or ice-cold nutrient medium (35) devoid of Ca++ and glucose, pH 5.0 with 1 g of bovine serum albumin added per 100 ml of medium. The livers were immediately suspended in cold balanced salts medium (22), pH 7.2; all further operations were performed near 0°. After removal of connective tissue, the liver was chopped with scissors and then with a battery of 10 razor blades held in a suitably designed holder. The resulting suspension was passed with the gentle action of a silicone stopper through four successive stainless steel screens of 25, 50, 80, and 100 mesh, respectively. Special care was taken not to squash the cells by shearing action of the stopper, but to allow the cells to flow freely through the screens. The cells were then centrifuged at approximately 100 X g for 5 minutes. They were resuspended and centrifuged several times (40 X g) with Medium 2, until contaminating red cells and debris were virtually eliminated. The cells were finally suspended in Krebs Medium 2 or 2A (to a concentration of about 10 to 15 mg of protein per ml). Generally there was little clumping or debris in the final suspension. The cells were relatively large polygonal, parenchymal-type cells. The yield of cells was 10 to 30% of the total liver.

Liver Slices—Slices from perfused livers of 150-g rats were prepared by the method of Renold et al. (7) and suspended in the high K+ salts medium (minus glucose) described in the same report.

**RESULTS**

Glucose Production by Liver Cells—Freshly prepared rat liver cells produce glucose on incubation in a balanced salts solution. The addition of a variety of substrates (fructose, lactate, malate, glutamate, acetate, aspartate, palmitate, oleate, linoleate), vitamins and cofactors did not markedly affect the rate of glucose accumulation. Moreover, in this system the following hormones, at the concentrations noted, were without appreciable effect: glucagon (0.1 to 10 μg per ml), epinephrine (10-4 to 10-7 M), insulin (0.2 μg per ml), hydrocortisone (10-5 to 10-8 M), aldosterone (10-4 to 10-6 M), growth hormone (0.1 to 10 μg per ml). The addition of dialyzed horse serum to the incubation medium, however, augmented glucose production by the liver cells markedly. As shown in Fig. 1, in the absence of serum, glucose accumulated at a rate of ~20 μmoles per g of protein per hour. There was a graded increase as a function of added serum until a maximal rate of about 140 μmoles per g of protein per hour was obtained. This rate compares with about 200 μmoles of glucose per g of protein per hour reported with a liver slice system (36). In other liver cell preparations, the endogenous rate varied from 15 to 50 μmoles per g of protein per hour, and the stimulation varied from 3- to 10-fold.

It is also evident from Fig. 1 that a linear response was approached at low levels of dialyzed horse serum. Even though the absolute values of glucose production varied somewhat in different liver cell preparations, the increment in the rate was

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1 W. J. Rutter and M. Eib, unpublished results.
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2A. Incubation temperature, 37°.

rabbit and pig liver, total volume adjusted to 1.0 ml with Medium 2A. Incubation temperature, 37°. adjusted to 1.0 ml with Medium 2A (when added), 0.1 ml of 1% glycogen added to sonic extracts from rabbit and pig liver, total volume adjusted to 1.0 ml with Medium 2A. In incubation mixture: 0.8 ml of liver cell extract in Medium 2A (when added), 0.1 ml of dialyzed horse serum (when added), 0.1 ml of 1% glycogen added to sonic extracts from rabbit and pig liver, total volume adjusted to 1.0 ml with Medium 2A. In the latter instance, the liver was not perfused and serum concentration was present.

Experimental conditions I Glucose production by sonically treated liver cell preparations

<table>
<thead>
<tr>
<th>Source of liver cells</th>
<th>Horse serum</th>
<th>Glucose production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min</td>
<td>60 min</td>
</tr>
<tr>
<td></td>
<td>μmoles/g protein</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>105</td>
</tr>
<tr>
<td>Rabbit</td>
<td>232</td>
<td>458</td>
</tr>
<tr>
<td>Pig</td>
<td>17</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>51</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>61</td>
<td>130</td>
</tr>
</tbody>
</table>

There was a substantial decrease in the glucogen level; the somewhat exaggerated glyco gen depletion in isolated rat liver cells. In the experiments with no preincubation (broken lines), the incubation mixture contained 0.9 ml of liver cell suspension in nutrient medium and 0.1 ml of either phosphate-buffered NaCl or 50 mg per ml bovine serum fraction V (Cohn) in phosphate-buffered NaCl. In the experiment with preincubation (solid line), 0.9 ml of liver cell preparation was incubated for 30 minutes, then 0.1 ml of 50 mg per ml fraction V in phosphate-buffered NaCl was added and the zero time sample taken immediately. Aliquots for glucose and glucogen determination were taken at the periods indicated. Incubation temperature, 37°.

Glucose production by sonic extracts of liver cells from fasted rats

Liver cells were prepared from rats fasted for 18 hours. Incubation mixture: 0.8 ml of liver cell extract in Medium 2A (when added), 0.1 ml of dialyzed horse serum (when added), 0.1 ml of glycogen, 10 mg per ml, in Medium 2A (when added), total volume adjusted to 1.0 ml with Medium 2A. In incubation mixture: 0.8 ml of sonicated liver cell preparation (~50% increase) were obtained with pig liver cell preparations.

Table II

Glucose production by sonic extracts of liver cells from fasted rats

<table>
<thead>
<tr>
<th>Sonic extract</th>
<th>Horse serum</th>
<th>Glycogen</th>
<th>Glucose production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min</td>
<td>60 min</td>
<td>μmoles/g protein</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>21</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>5</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>317</td>
<td>712</td>
<td></td>
</tr>
</tbody>
</table>

Since the total carbohydrate as measured by the anthrone reaction remained essentially constant during the incubation, it was likely that the glucose precursor was carbohydrate. The appropriate analysis indicated the presence of a considerable quantity of cold methanol-soluble (nonglucose) carbohydrate which was apparently a prominent precursor of glucose in this system. The possibility that maltose or similar glucosides were present in the latter fraction was strengthened by the observations presented in Table III that added maltose caused the glucose production by sonicated rat liver cell preparations, especially in the presence of horse serum.

Identification of Serum Factor as Maltase—The serum factor was shown to be nondialyzable and heat-labile (100% inactivation by 5-minute treatment at 100°). Moreover, the system exhibited kinetics similar to that expected for an enzyme, viz. at low concentrations of serum the increase in glucose production was directly proportional to serum concentration (see Fig. 1). These facts, together with the observed effect of maltose in this system, suggested that the serum factor was maltase. A comparison of the maltase level and glucogenic factor activity was therefore made in various sera, in an ammonium sulfate fraction of rat serum, and in the highly purified horse serum maltase preparation of Lieberman and Eto (37). The horse serum mal-
10 pmoles of maltose were added to the tubes indicated. Under "Experimental procedure." Maltase activity was estimated as described in the present experiments, glycogen could not be the sole substrate for added α-1,4-oligoglucosidase, since the absolute increase in glucose production by liver cells observed in the presence of a limiting amount of purified oligoglucosidase was at least an order of magnitude greater than its ability to hydrolyze glycogen. It is concluded from these data that oligoglucosidases are produced by the liver cell preparations, and that added oligoglucosidase facilitates the conversion of these substances to glucose.

Oligoglucoside Production by Liver Slices—The demonstration of the accumulation of oligoglucosides during incubation of isolated liver cells made it desirable to determine whether this phenomenon occurred in other liver preparations. Accordingly, slices from carefully perfused livers were incubated for 60 minutes at 37° in the high potassium medium of Renold et al. (7). The incubation medium was then analyzed for glucose and oligoglucosides with the glucose oxidase test before and after incubation with purified oligoglucosidase. During this period 155 pmoles of glucose and 325 pmoles of oligoglucosides were produced per g of protein during this period. Approximately two-thirds of the carbohydrate accumulating in the medium was oligoglucosides. Paper chromatography of the medium in three solvent systems confirmed the above analysis, and indicated the presence of a limiting amount of purified oligoglucosidase.
presence of substantial quantities of maltose, maltotriose, and maltotetraose in the medium.

Liver Amylase—The demonstration of the accumulation of oligoglucosides in the extracellular medium by liver cells and slices suggested the presence of an intracellular amylase. As recorded in Table VI, amylase activity was observed in homogenates prepared from isolated liver cells of well perfused rat liver, as well as from livers of pig, cow, and rabbit; the level was especially high in rat liver. These results are in qualitative agreement with the reported assays of several animals by McGeachin et al. (38).

Phosphorylase assays of the same preparations were performed. The assays varied considerably, but the conclusion can be drawn that in rat liver the amylase and phosphorylase activities are of the same order of magnitude, whereas in the other species tested the amylase activity is lower by at least an order of magnitude.

Oligoglucosidase (Maltase) Levels in Liver Cells—The presence of amylase in both liver and serum and of oligoglucosidase in serum prompted the assay of the latter enzyme in liver cells. A rather constant value of 1.5 μmoles per minute per g of protein was found in sonically treated fresh rat liver cell preparations, whereas in sonic extracts of perfused liver the results were somewhat more variable and averaged 2.3 μmoles per minute per g of protein. The latter preparations apparently were contaminated with traces of the serum enzyme.

Since oligoglucosidase is not detectable in rabbit serum, the level of this enzyme in fresh rabbit liver sonic extracts was also investigated. The activity was very close to that found in similar preparations from rat liver.

The observation that oligoglucosidase activity (~1.5 μmoles per minute per g of protein) present in rat liver cells is much lower than that of the liver amylase (~60 μmoles of reducing power per minute per g of protein) is consistent with the observation that added serum oligoglucosidase increases the accumulation of glucose by liver cells.

Several attempts to measure a maltose phosphorylase producing either α- or β-glucose 1-phosphate were unsuccessful, and it is concluded that significant levels of this enzyme are not present in liver parenchymal cells.

**DISCUSSION**

The data presented in this paper allow the definition at the enzymatic level of certain aspects of the production of glucose or extracellular carbohydrate by isolated rat liver cells. In addition to the “phosphorylase pathway” for glucose production (Equation 1) which has been clearly demonstrated in rat liver slices by Sutherland and Cort (36),

(a) Glycogen → P↓ → glucose 1-phosphate

(b) Glucose 1-phosphate → glucose 6-phosphate

(c) Glucose 6-phosphate → glucose + inorganic phosphate

the enzymatically catalyzed conversions described in Equation 2 are present.

(a) Glycogen → oligoglucosides

(b) Oligoglucosides → glucose

**Table VI**

AMYLASE AND PHOSPHORYLASE LEVELS IN LIVER CELL SONIC EXTRACTS

Only rat liver was well perfused before homogenization, so that amylase values for other livers are maximal. In Experiment 1, the livers were homogenized and sonicated in 0.005 M histidine-chloride, pH 6.5. In Experiment 2 livers were homogenized in 0.005 M histidine-chloride, pH 6.5, plus 0.1 M NaF. Amylase activity was measured by the iodine color assay and the values were converted to equivalents of reducing power formed as described under "Experimental Procedure."

<table>
<thead>
<tr>
<th>Liver</th>
<th>Amylase: reducing power released</th>
<th>Phosphorylase: inorganic phosphate released</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Rat</td>
<td>40</td>
<td>80</td>
</tr>
<tr>
<td>Sheep</td>
<td>0.9</td>
<td>0.22</td>
</tr>
<tr>
<td>Pig</td>
<td>2.4</td>
<td>4.0</td>
</tr>
<tr>
<td>Rabbit</td>
<td>8.0</td>
<td>80</td>
</tr>
</tbody>
</table>

Isolated rat liver cells exhibit considerable amylase activity and an order of magnitude lower than oligoglucosidase activity. Horse serum, on the other hand, exhibits little if any amylase and considerable oligoglucosidase activity. The stimulation of "glucose production" by horse serum can clearly be attributed to the addition of the latter enzyme, which hydrolyzes the excess oligoglucosides produced by the liver cell system.

The relationship of the amylase pathway (Equation 2) and the phosphorylase pathway (Equation 1) is of some importance. The sizeable stimulation by horse serum of glucose production by cells and slices attests to the prominence of oligoglucoside production in this system. It is evident, however, that the amylase pathway was emphasized in the present experiments.

1. Many of the experiments were carried out in a medium of low phosphate concentration.
2. No attempt was made to maintain "active phosphorylase" in these experiments.
3. There may have been significant concentrations of intracellular oligoglucosides which were released on incubation. This latter possibility is especially pertinent in view of the recent isolation of significant quantities of low molecular weight oligoglucosides from rat liver (39).

Although it is not possible to define the relative metabolic importance of the amylase and phosphorylase pathways simply by measurements of the maximal activity of these two enzymes under conditions in vitro, such assays do presumably place limits on the relative activities of the pathways. In the present instance, however, the measured activities of both enzymes may be particularly misleading. The phosphorylase presumably exists in active and inactive forms (17, 41), and the amylase activity in liver homogenates is partially cryptic, being activated several-fold by sonic oscillation and a variety of other treatments (20). The amylase activities measured, therefore, are probably maximal and may be several times greater than the effective intracellular activity. The data are, however, consistent with a significant role of the amylase pathway, especially in rat liver. In other species studied (rabbit, pig, sheep) the amylase activity is less by an order of magnitude, in agreement with the conclusions of Cort et al. (40).

Perhaps the most compelling argument against the ready extrapolation of the present results to the liver in vivo is the relative...
lack of sensitivity of both isolated liver cells and rat liver slices (41) to epinephrine and glucagon. Both these hormones enhance glucose production in the intact perfused liver (21, 42, 43). The rat liver cell preparations have also been shown to be abnormal in other respects. They have altered permeability properties and readily lose respiratory control (44). In a subsequent paper (21), it is shown that the isolated perfused liver does not elaborate detectable quantities of oligoglucosides into the extra cellular medium, therefore eliminating the internal amylase-external oligoglucosidase pathway as a major pathway of glucose production. The role of the amylase pathway in glucose production is probably restricted to the rate of the intracellular oligoglucosidase. A more definitive discussion of the function of liver amylase is presented in the following papers (20, 21).

The results of the experiments presented here are of relevance to studies on glucose production by isolated liver cell systems, especially slices. Hastings et al. (7, 45), for example, have reported that the total glucose output by slices cannot be accounted for by glycogen depletion and formation from lactate. They have therefore concluded that an unknown glucose precursor (compound X) is present. It is likely from the present observations that at least part of these precursors are alcohol-soluble oligoglucosides. A more detailed reinterpretation of the results of these authors is difficult, since the extracellular carbohydrate assayed by reducing power as "glucose" was undoubtedly contaminated with a significant but unknown quantity of oligoglucosides.

SUMMARY

1. Isolated liver cells elaborate glucose into the incubation medium. Although a variety of externally added substrates, cofactors, and the hormones epinephrine and glucagon are without appreciable effect on this system, added dialyzed horse serum markedly enhances the rate of glucose production.

2. Isolated liver cells contain an active amylase which produces oligoglucosides from glycogen. The activity of amylase is especially high in the rat and is considerably lower in other species tested (rabbit, pig, cow).

3. Isolated liver cells also exhibit a low order of oligoglucosidase activity. In the rat, oligoglucosidase activity is much lower than amylase activity.

4. As would be predicted from the above data, maltose, maltotriose, and maltotetraose constitute a major portion of the carbohydrate accumulating in the medium of isolated rat liver cells and slices.

5. The factor in serum responsible for enhanced glucose production is oligoglucosidase which hydrolyzes maltose and other oligoglucosides formed by the system.

6. In rat liver cell and slice systems, the amylase pathway is prominent in the production of glucose.

7. The significance of the present work relative to studies on the carbohydrate metabolism of liver cell systems is discussed.

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