Polyhydroxybenzoates Inhibit Ascorbic Acid Activation of Mitochondrial Glycerol-3-phosphate Dehydrogenase

IMPLICATIONS FOR GLUCOSE METABOLISM AND INSULIN SECRETION*

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Glycerol-3-phosphate dehydrogenase from pig brain mitochondria was stimulated 2.2-fold by the addition of 50 μM L-ascorbic acid. Enzyme activity, dependent upon the presence of L-ascorbic acid, was inhibited by lauryl gallate, propyl gallate, protocatechuic acid ethyl ester, and salicylhydroxamic acid. Homogeneous pig brain mitochondrial glycerol-3-phosphate dehydrogenase was activated by either 150 μM L-ascorbic acid (30% or 300 μM iron (FeO or Fe2+) (62%)) and 2.6-fold by the addition of both L-ascorbic acid and iron. The addition of L-ascorbic acid and iron resulted in a significant increase of kcat from 21.1 to 64.1 s⁻¹ without significantly increasing the Km of L-glycerol-3-phosphate (10.0–14.5 mM). The activation of pure glycerol-3-phosphate dehydrogenase by either L-ascorbic acid or iron or its combination could be totally inhibited by 200 μM propyl gallate. The metabolism of [5-3H]glucose and the glucose-stimulated insulin secretion from rat insulinoma cells, INS-1, were effectively inhibited by 500 μM or 1 mM propyl gallate and to a lesser extent by 5 mM aminooxycacetate, a potent malate-aspartate shuttle inhibitor. The combined data support the conclusion that L-ascorbic acid is a physiological activator of mitochondrial glycerol-3-phosphate dehydrogenase, that the enzyme is potently inhibited by agents that specifically inhibit certain classes of di-iron metalloenzymes, and that the enzyme is chiefly responsible for the proximal signal events in INS-1 cell glucose-stimulated insulin release.

Mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH)¹ (EC 1.1.99.5) plays a critical role in the shuttle of glycolytically generated reducing equivalents into mitochondrial electron transport and oxidative phosphorylation in numerous tissues (1–4). In pancreatic islet β cells, many studies support the significant participation of mGPDH and the 1-O-glycerol-3-phosphate shuttle in the proximal events that signal the release of insulin in response to increased glucose (5–12). Recently, particular importance has been attributed to the role of NADH in the glucose-induced activation of mitochondrial metabolism and insulin secretion (13, 14). These studies emphasized the essential roles played by both the glycerol-3-phosphate and the malate-aspartate shuttles in modulating the cytosolic NADH pool.

In confirmation of the original observations of Sigal and King that scorbutic guinea pigs demonstrated abnormal glucose tolerance (15), L-ascorbic acid was shown to be essential for the release of insulin from scorbutic guinea pig pancreatic islets (16, 17). Further studies have demonstrated that L-ascorbic acid is an essential cofactor for the activation of mGPDH (oxidase) in mitochondria from guinea pig tissues and rat liver (18). In purified preparations of mGPDH from a variety of sources, both iron and acid extractable sulfur have been reported (2, 19–21), suggesting that an iron/sulfur center is involved in the catalytic mechanism of this mitochondrial inner membrane bound enzyme. No further evidence, however, was obtained to support this suggestion, and the iron center of mGPDH has remained uncharacterized.

In addition to the need to clarify the iron/L-ascorbic acid relationship of mGPDH in intact mitochondria, it was essential to examine the effect of L-ascorbic acid, iron and specific di-iron metalloenzyme inhibitors on homogeneous mGPDH. In the present study, the effects of propyl gallate and other related polyhydroxybenzoate inhibitors on pig brain mGPDH in intact mitochondria and on preparations of pure mGPDH were examined. Because of the potential role of mGPDH for shuttling reducing equivalents into the mitochondria during glucose-induced insulin release from pancreatic β cells, we also investigated the effects of a di-iron metalloenzyme inhibitor, propyl gallate, on glucose usage and glucose-induced insulin release from the rat insulinoma cell line, INS-1.

EXPERIMENTAL PROCEDURES

Materials

¹dl-α-Glycerol phosphate, mannitol, MOPS, NaN₃, Triton X-100, SHAM, PCAEE, DEAE-Sepharose (fast flow), and Sephacryl S-300 (fast flow) were purchased from Sigma. Sucrose, HEPES, Tris-base, glutathione, and NADH were from Roche Molecular Biochemicals. L-Ascorbic acid and glycerol were from J. T. Baker, Inc. l-ascorbic acid-2-phosphate (Ms⁻²) was purchased from Wako Pure Chemical Industries, Ltd. Menadione was a product of Nutritional Biochemicals Corp. Lauryl gallate, propyl gallate, and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride; GSH, glutathione; LDS-PAGE, lithium dodecyl sulfate-polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiobitol.

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other chemicals used were of A.C.S. reagent grade. Pig brains were kindly provided by Thomas Fortan from Michigan State University Meats laboratory. Cerebella were removed shortly after slaughter, chilled on ice, washed in 0.25 M sucrose to remove excess blood, and frozen (~70 °C) for storage.

Methods

Isolation of Mitochondria—Frozen pig cerebellum was homogenized in an all glass Dounce homogenizer with a 10-volume homogenization fluid described by Greenawalt (22) consisting of 200 mM mannitol, 80 mM sucrose, and 10 mM potassium HEPES, pH 7.4. The mitochondrial fraction was isolated by differential centrifugation following the sedimentation protocol reported by Lai and Clark (23). Briefly, homogenization was performed on ice with eight thrusts of the Dounce plunger. All subsequent steps were conducted on ice or in a refrigerated centrifuge (Sorval-RC2-B) at 4 °C. The homogenate was first centrifuged at 1,000 × g for 10 min, and the supernatant was removed. The supernatant fraction was then centrifuged at 15,000 × g for 10 min. The upper layer was decanted off, and the pellet was gently rinsed with small amounts of homogenizing fluid to remove broken mitochondrial remnants. The pellet was gently resuspended in 5 volumes of homogenizing fluid using a Dounce homogenizer and centrifuged at 12,000 × g for 10 min. The supernatant was discarded, and the pellet was gently washed with homogenizing fluid to remove intact, but flufly mitochondrial fragments. The pellet was resuspended in 2.5 volumes of deionized water. The suspension was centrifuged at 12,000 × g for 10 min. The supernatant was discarded, and the pellet was resuspended in ~1 ml of 250 mM sucrose, 10 mM Tris-HCl, pH 7.5, per mg original tissue weight.

Electron Transport Particles—Mitochondria were isolated as described above and placed in a freezer at ~20 °C until needed. ETPs were prepared according the method of Green and Ziegler (24). The thawed mitochondrial preparations were centrifuged at 12,000 × g for 10 min, and the pellet was resuspended to a concentration of 5–6 mg protein/ml in 250 mM sucrose, Tris-HCl, pH 7.5, 1 mM GSH, 0.26 mM NADH, 0.1–0.12 mg ETP protein, with or without various concentrations of inhibitors or 50 μM t-ascorbic acid. When inhibitors or t-ascorbic acid were included, ETPs were preincubated for 3 min. Ethanol, up to 30 μl/3.0 ml reaction volume, did not affect the succinoxidase activity.

Mitochondrial Enzyme Assays—mGPDH activity in intact mitochondria was measured by oxygen uptake using a Clark oxygen electrode. mGPDH from freshly prepared mitochondrial suspensions of 5–7 g protein/ml in 250 mM sucrose, Tris-HCl, pH 7.5, from frozen pig brain was assayed as described previously (18). The Clark oxygen electrode chamber of 3.0 ml (model 53 oxygen monitor, Yellow Springs Instrument Co. Yellow Springs, Ohio) contained 250 mM sucrose, 10 mM Tris-HCl, pH 7.5, 1 mM GSH, 0.5–0.8 mg of mitochondrial protein, with or without 50 μM t-ascorbic acid. Typically, oxygen uptake was measured for 3–6 min followed by the addition of 50 mM DL-glycerol-3-phosphate, pH 7.5, at 37 °C. The rate of oxygen uptake was measured with a Linseis recorder, and the rate, in nmol oxygen uptake/min, was corrected for the basal oxygen uptake rate before addition of the substrate. Values were expressed as nmol oxygen uptake/min/mg protein. Stock solutions of the inhibitor compounds were made up in absolute ethanol. When added to the assay mixture, ethanol aliquots of 30 μl or less were preincubated with the mitochondria for 3 min followed by the sequential addition of 50 mM t-ascorbic acid (5 min of incubation) and 50 mM DL-glycerol-3-phosphate. Controls of up to 30 μl of ethanol in the 3.0-ml reaction volume had no effect on the oxygen uptake rates (data not shown).

NADH dehydrogenase (oxidase) from pig brain ETPs was assayed spectrophotometrically by a modification of the method of Singer (25). The reaction mixture contained 100 mM potassium phosphate, pH 7.4, 1 mM GSH, 0.26 mM NADH, 0.1–0.12 mg ETP protein/ml, with or without 3 min of preincubation with various levels of inhibitors or 50 μM t-ascorbic acid at 30 °C in a total of 500 μl. The reaction was initiated by the addition of NADH, and the decrease in absorbance at 340 nm was recorded in a Gilford Response II recording spectrophotometer. NADH dehydrogenase activity (nmol/min/mg protein) were calculated from the extinction coefficient of NADH of 6.22 × 10³ M⁻¹ cm⁻¹. Because 5 μl of ethanol were without effect on enzyme activity, all additions of inhibitors were made with less than 5 μl of ethanol.

Succinocinase activity in ETP preparations was assayed by a modification of the method of Green and Ziegler (24). The rate of succinate oxidation at 37 °C was measured by oxygen uptake analysis using a Clark oxygen electrode. The reaction mixture contained 100 mM potassium phosphate, pH 7.4, 1 mM GSH, 0.3 mg of ETP protein, 50 mM potassium succinate, and with or without various concentrations of inhibitors or 50 μM t-ascorbic acid. When inhibitors or t-ascorbic acid were included, ETPs were preincubated for 3 min. Ethanol, up to 30 μl/3.0 ml reaction volume, did not affect the succinocinase activity.

Mitochondrial enzyme activities were determined by the bicinechonic acid protocol described above. The purification of mGPDH was a modification of the method of Garrib and McMurray (27). In a typical preparation, 65–100 g of frozen pig brain were thawed and homogenized at 4 °C in 650–1000 ml of homogenizing buffer consisting of 80 mM mannose, 0.2 mM t-ascorbic acid, 1 mM DTT, and 10 mM HEPES, pH 7.4, in a Waring blender for 1 min at low speed. The suspension was centrifuged at 1000 × g for 10 min in a Sorval RC2-B refrigerated centrifuge at 4 °C. The supernatant was stored on ice or kept at 4 °C for all subsequent steps, and the pellet was resuspended in 300 ml of homogenizing buffer and homogenized with an all glass Dounce homogenizer using five strokes of the pestle. The homogenate was diluted with 300 ml of homogenizing buffer, stirred for 5 min, and then centrifuged at 1000 × g for 10 min. The supernatant fractions of the previous steps were combined and centrifuged at 15,000 × g for 10 min. The supernatant was discarded, and the pellets were resuspended in 300 ml of 0.15 M KCl containing 1 mM DTT and centrifuged at 15,000 × g for 10 min. The pellets were resuspended in 40 ml of 10 mM Tris-HCl, pH 7.5, containing 250 mM sucrose followed by dilution 10-fold with 1 mM DTT. The slurry was stirred for 60 min on ice, and the diluted suspension was centrifuged at 15,000 × g for 15 min. The pellet was washed with 300 ml of 50 mM potassium phosphate, pH 7.5, 0.1 mM DTT, and 0.02% NaN₃ followed by centrifugation at 15,000 × g for 15 min. The pellets were resuspended in 50 mM potassium phosphate, 0.1 mM DTT, and 0.02% NaN₃, and the protein content was determined by the BCA method described previously. The suspension was stored at ~70 °C until required.

DEAE-Sepharose Chromatography—Washed and stored mitochondrial fragments were thawed at 37 °C, chilled, and kept at 4 °C throughout each subsequent purification step. The mGPDH was solubilized by addition of appropriate amounts of 10% Triton X-100 (Triton X-100/protein = ~0.5) for ~60 min. Ethanol up to 30 μl/3.0 ml reaction volume, did not affect the succinoxidase activity. The concentrated enzyme fraction was applied to a Sephacryl S-300 (fast flow) column (2.5 × 12 cm) equilibrated with 10 mM potassium phosphate, pH 7.5, 0.1% Triton X-100, 0.02% NaN₃, 0.1 mM DTT, and 10% glycerol. After loading the sample, the column was washed with buffer A, and the enzyme was eluted with a linear NaCl gradient consisting of 150 ml of buffer A and 150 ml of buffer A containing 0.6 M NaCl at a flow rate of 1 ml/min. The fractions with enzyme activity were pooled and diluted with triple distilled water to 10 mM potassium phosphate, pH 7.5, 0.1 mM DTT, 0.1% Triton X-100, 2% glycerol, and 0.02% NaN₃.

Hydroxyapatite Chromatography—A column of hydroxyapatite (2.5 × 8 or 12.0 cm) was equilibrated with buffer B consisting of 110 mM potassium phosphate, pH 7.5, 0.1 mM DTT, 0.1% Triton X-100, 100 mM NaCl, 5% glycerol, and 0.02% NaN₃. The diluted enzyme sample from the DEAE column was applied to the hydroxyapatite and washed with buffer B. The enzyme was eluted by a linear sodium phosphate gradient consisting of 50 ml of buffer B and 50 ml of buffer B with 150 mM sodium phosphate, pH 7.5. The enzyme containing fractions were subjected to LDS-PAGE to monitor purity. The fractions with highest activity and purity were pooled and concentrated to 1.5 ml by an Amicon Centiprep YM-10 apparatus.

Sephacryl S-300-HR Chromatography—The concentrated enzyme fraction was applied to a Sephacryl S-300 (fast flow) column (2.5 × 92 cm) equilibrated with buffer C (50 mM potassium phosphate, pH 7.5, 0.05% Triton X-100, 0.02% NaN₃, 0.1 mM DTT, 20% glycerol, and 0.2 mM ethanol) without effect on enzyme activity, all additions of inhibitors were made with less than 5 μl of ethanol.

Succinocinase activity in ETP preparations was assayed by a modification of the method of Green and Ziegler (24). The rate of succinate oxidation at 37 °C was measured by oxygen uptake analysis using a Clark oxygen electrode. The reaction mixture contained 100 mM potassium phosphate, pH 7.4, 1 mM GSH, 0.3 mg of ETP protein, 50 mM potassium succinate, and with or without various concentrations of inhibitors or 50 μM t-ascorbic acid. When inhibitors or t-ascorbic acid were included, ETPs were preincubated for 3 min. Ethanol, up to 30 μl/3.0 ml reaction volume, did not affect the succinocinase activity.
NaCl at a flow rate of 0.25 ml/min. The fractions containing mGPDH activity were pooled, concentrated as described above, and subjected to LDS-PAGE analysis to assess purity.

LDS-PAGE—LDS-PAGE was performed on samples of purified mGPDH by a modification (28) of the basic method of Laemmli (29). The stacking and separating gels (0.75 mm) had polyacrylamide concentrations of 5 and 8%, respectively. The gels were run at a constant 100V in a Bio-Rad minigel apparatus, and the gel was stained with Coomassie Brilliant Blue R-250.

Homogeneous mGPDH Assay—For measurement of the mGPDH specific activity and kinetic properties, preparations of the pure enzyme were assayed by oxygen uptake using the Clark electrode following a modification of the assay described by Beleznai et al. (30). Homogeneous mGPDH (2–5 μg), stabilized in a solution of 50 mM HEPES, pH 7.5, 0.1 mM DTT, 0.05% Triton X-100, 0.02% NaN₃, and 20% glycerol, was placed in a 3.0-ml chamber at 37 °C containing 100 mM potassium phosphate, pH 7.6, 0.6 mM GSH, 0.05% Triton X-100, and 0.15 mM FeCl₃. Reaction rates after substrate addition were corrected by dividing V_{max(app)} by the molar concentration of pig brain mGPDH with the molecular weight taken as 75,000 (21).

INS-1 Cell Culture—INS-1 cells (kindly provided by Dr. C. B. Wohlen (31)) were routinely cultured in CO₂/air (1:19) at 37 °C in RPMI 1640 medium containing 11.1 mM glucose and supplemented with 10% fetal bovine serum, 1 μM pyruvate, 10 mM HEPES, pH 7.4, 50 μM 2-mercaptoethanol, 100 units penicillin/ml, and 100 μg streptomycin/ml. Cells were passed weekly by trypsin-EDTA detachment. All experiments were performed on INS-1 cells between passages 70–85.

Insulin Secretion Studies—For static secretion studies, INS-1 cells were plated onto 12-well plates at a density of 1.5 × 10⁵ cells/well in RPMI 1640 medium plus supplements and grown for 24 h. The growth medium was then changed to RPMI 1640 containing 4 mM glucose plus the supplements described above, and cells were cultured for an additional 30 h. Cells were then incubated for 60 min at 37 °C in Krebs Ringer bicarbonate buffer (KRB buffer) (118.5 mM NaCl, 2.54 mM CaCl₂, 1.19 mM KH₂PO₄, 4.74 mM KCl, 25 mM NaHCO₃, 1.19 mM MgCl₂, 10 mM HEPES, pH 7.4, 0.1% bovine serum albumin) containing 4.0 mM glucose. Cells were then incubated for 20 min at 37 °C in KRB buffer containing 100 μM 3-isobutyl-1-methylxanthine and either 4 or 16.7 mM glucose in the absence or presence of metabolic inhibitors as indicated in the figure legends. Propyl gallate was dissolved in absolute ethanol, and therefore all controls contained equal amounts of ethanol. Concentrations of insulin released into the medium were determined by insulin enzyme-linked immunosorbent assay using a modification of the procedure described by Kekow et al. (32). Insulin released into the medium was normalized to cellular protein concentrations determined according to Lowery et al. (33).

Glucose Utilization Studies—Glucose usage was measured using a modification of the method of Zawalich and Matschinsky (34, 35). INS-1 cells were plated onto 12-well plates at a density of 1.5 × 10⁵ cells/well and grown for 24 h. The growth medium was then changed to RPMI 1640 containing 4 mM glucose and supplements described above and incubated for an additional 30 h. Cells were then incubated for 60 min at 37 °C in KRB buffer containing 4.0 mM glucose. Glucose utilization was then measured by incubating cells for 30 min at 37 °C in 1 ml of KRB buffer containing 100 μM 3-isobutyl-1-methylxanthine and either 4 or 16.7 mM glucose and 5-[⁴H]glucose at a final specific activity of 2.2 dpm/pmol. Vehicle controls or metabolic inhibitors were added as indicated in the figure legends. Background controls were determined by incubating the medium in the absence of cells. After incubation, duplicate 50-μl samples of the incubation medium were added to Eppendorf tubes containing 5 μl of 1 N HCl. The Eppendorf tubes were then placed in scintillation vials containing 0.5 ml of H₂O,

**FIG. 1.** Inhibition of the L-ascorbic acid-dependent mGPDH activity of pig brain mitochondria by lauryl gallate (A), propyl gallate (B), SHAM (C), and PCAEE (D). The rates are expressed as nmol oxygen consumed/min/mg protein in the presence of 50 μM L-ascorbic acid as described under “Methods.” Each value is the mean ± S.E. error for 4–11 separate experiments.
and the scintillation vials were sealed tightly and incubated at 50 °C for 18 h. After cooling, the Eppendorf tubes were removed, 10 ml of Safety-Solve scintillation mixture were added to the vials, and the samples were counted in a Beckman scintillation counter. Glucose utilization was then determined from the following formula and expressed as picomoles of glucose metabolized per min per mg protein. The equilibration coefficient (EQC) was determined with $^3$H$_2$O following the procedure outlined above. Glucose usage = (dpm - blank)/specific activity × EQC × min.

**RESULTS**

**Pig Brain mGPDH**—Pig brain mGPDH activity in isolated intact mitochondria was 28.6 ± 6.6 nmol/min/mg protein ($n = 11$). Addition of 50 μM L-ascorbic acid increased mGPDH activity to 62.9 ± 10.7 nmol/min/mg protein ($n = 11$). These activities were completely inhibited by 10 mM KCN (data not shown), indicating that a functional cytochrome $c$ oxidase was required to complete the reaction with oxygen whether L-ascorbic acid was present or not. SHAM, PCAEE, propyl gallate, and lauryl gallate were potent inhibitors of pig brain mGPDH activity in intact mitochondria stimulated by L-ascorbic acid (Fig. 1). In contrast, these agents were without effect on the basal activity, i.e. activity in the absence of L-ascorbic acid (data not shown). The concentration of each compound calculated to cause 50% inhibition of the L-ascorbic acid-stimulated activities were: SHAM, 27.7 ± 6.9 μM; PCAEE, 585 ± 203 nM; propyl gallate, 305 ± 113 nM; and lauryl gallate, 111 ± 42 nM.

NADH dehydrogenase and succinoidase activities from pig brain ETPs were compared in the presence and absence of the four hydroxybenzoic acid derivatives. Minimal concentrations of each agent, previously found to completely inhibit the L-ascorbic acid-stimulated mGPDH activity, had no effect on these two well-established iron/sulfur enzymes (data not shown). In addition, L-ascorbic acid had no stimulatory effect on the activity of either NADH dehydrogenase or succinoidase (data not shown).

**Purification of mGPDH**—The purification of mGPDH from pig brain was accomplished by a series of steps including Triton X-100 extraction of washed mitochondria, DEAE-Sepharose chromatography, Bio-Gel HTP hydroxylapatite chromatography, and Sephacyr S-300 gel chromatography (Table I). mGPDH was judged to be homogeneous based on LDS polyacrylamide gel electrophoresis (Fig. 2) with a molecular weight of 75,000. This value is in good agreement with that reported by Cottingham and Ragan (21).

![Fig. 2. LDS-PAGE analysis of pig brain mitochondrial glycerol-3-phosphate dehydrogenase.](image)

**TABLE I**

| Purification step                  | Protein (mg) | Total mGPDH activity (nmol/min) | Specific activity (nmol/min/mg) | Yield (%) | Purification fold |
|-----------------------------------|--------------|--------------------------------|--------------------------------|-----------|-------------------|
| Solubilized inner membrane        | 89.7         | 37,819                         | 421.6                          | 100       | 1.0               |
| membrane fraction                 |              |                                |                                |           |                   |
| DEAE-Sepharose                    | 24.1         | 23,098                         | 957.6                          | 61        | 2.3               |
| Bio-Gel HTP                       | 1.69         | 20,551                         | 12,139                         | 54        | 28.8              |
| hydroxylapatite                   |              |                                |                                |           |                   |
| Sephacyr S-300 HR                 | 0.23         | 3,578                          | 15,715                         | 9.5       | 37.0              |

**TABLE II**

| Assay condition                  | Specific activity (μmol/min/mg protein) |
|----------------------------------|----------------------------------------|
| Control                          | 14.0 ± 2.2                             |
| 200 μM propyl gallate            | 9.1 ± 1.6                              |
| 150 μM L-ascorbic acid           | 21.0 ± 3.3                             |
| 150 μM L-ascorbic acid + 200 μM propyl gallate | 11.4 ± 1.6 |
| 300 μM FeSO₄                     | 22.7 ± 4.2                             |
| 150 μM L-ascorbic acid + 300 μM FeSO₄ | 36.7 ± 3.0                            |

The values are the means ± S.D. of six separate experiments as described under "Experimental Procedures."
The Fe$^{2+}$ and Fe$^{3+}$ plus L-ascorbic acid activity stoichiometrically (data not shown). When Fe$^{3+}$ was provided as 300 μM FeCl$_3$ instead of 300 μM Fe$^{2+}$, comparable results were obtained (data not shown).

mGPDH Kinetics—The kinetic constants for purified mGPDH are reported in Table III. In these studies, the values for basal control are compared with those obtained for enzyme supplemented with 150 μM L-ascorbic acid and 150 μM Fe$^{3+}$. The $K_{m(app)}$ for basal enzyme was 10.0 ± 1.2 mM L-glycerol-3-phosphate (in good agreement with reference number 3), whereas that for the stimulated enzyme was 14.5 ± 4.9 mM (not statistically different). L-Ascorbic acid and Fe$^{3+}$ supplementation increased the $V_{max(app)}$ from 56.9 ± 13.2 nmol/min to 161.9 ± 24.7 nmol/min ($p = 0.0001$). Expressed as $k_{cat}$ values, the controls were 21.1 ± 9.2 compared with 64.1 ± 25.3 s$^{-1}$. Taken together, $k_{cat}/K_m$ values were 2.1 × 10$^5$ and 4.4 × 10$^3$ M$^{-1}$ s$^{-1}$, for control and supplemented samples, respectively.

Effect of Propyl Gallate on Glucose Utilization and Insulin Secretion from INS-1 Cells—In pancreatic β cells, it is well established that there is a relationship between glucose metabolism through mGPDH and insulin release (5–9, 11, 12, 36). Therefore, agents that effectively inhibit L-ascorbic acid-induced mGPDH activity may have profound effects on glucose utilization and insulin release from pancreatic β cells. To examine this possibility, the effects of propyl gallate on glucose utilization and insulin secretion from INS-1 cells were determined.

Incubation of INS-1 cells in 16.7 mM glucose led to a 4.83 ± 0.11-fold ($n = 4$) increase in the rate of conversion of 5-[^3H]glucose to[^3H]H$_2$O compared with cells incubated in 4.0 mM glucose (Fig. 3). The addition of 500 μM propyl gallate led to a 23.64 ± 0.57% ($n = 4, p < 0.006$) and 13.61 ± 3.42% ($n = 4, p < 0.008$) reduction in glucose usage in cells incubated in 4 or 16.7 mM glucose, respectively. The addition of 1 mM propyl gallate led to a 75.20 ± 2.32% ($n = 4, p < 0.0001$) and 80.75 ± 1.23% ($n = 4, p < 0.0001$) reduction in cells incubated in 4 or 16.7 mM glucose, respectively. Treatment of cells with propyl gallate concentrations lower than 250 μM had no significant effects on glucose usage in cells incubated in 4.0 or 16.7 mM glucose. Next the ability of aminooxidase (AOA), an inhibitor of aspartateaminotransferases in the malate-aspartate shuttle (37), to potentiate the propyl gallate-mediated inhibition of glucose usage was determined. Incubation of cells in 5 mM AOA led to a 27.08 ± 1.47% ($n = 4, p < 0.004$) and 23.77 ± 3.10% ($n = 4, p < 0.005$) reduction in glucose utilization in cells cultured in 4.0 mM or 16.7 mM glucose, respectively. Combined treatment of cells with 5 mM AOA and 500 μM propyl gallate led to a further reduction in glucose usage in cells incubated in 16.7 mM glucose. Nevertheless, combined treatment of 5 mM AOA and 1 mM propyl gallate were not able to reduce glucose usage below that observed with 1 mM propyl gallate alone.

The ability of propyl gallate to inhibit both purified mGPDH activity and glucose usage in INS-1 cells suggests that propyl gallate may also inhibit glucose-induced-insulin release. Incubation of INS-1 cells in 16.7 mM glucose led to a 3.15 ± 0.34-fold ($n = 4, p < 0.0006$) increase in insulin release compared with cells incubated in 4.0 mM glucose (Fig. 4). The addition of 250 μM, 500 μM and 1 mM propyl gallate led to a 65.93 ± 2.59% ($n = 4, p < 0.003$), 66.16 ± 7.19% ($n = 4, p < 0.004$), and 74.11 ± 4.06% ($n = 4, p < 0.002$) reduction in insulin secretion, respectively, from cells incubated in 4 mM. Importantly, the addition of 500 μM and 1 mM propyl gallate led to a 89.22 ± 1.67% ($n = 4, p < 0.0001$) and 91.18 ± 3.66% ($n = 4, p < 0.0001$) reduction in insulin release, respectively, from cells incubated in 16.7 mM glucose.

Treatment of cells with 250 μM propyl gallate had no significant effects on the ability of 16.7 mM to induce insulin release. Next the ability of AOA to potentiate the propyl gallate-mediated inhibition of insulin release was determined. Incubation of cells in 5 mM AOA led to a 36.62 ± 3.35% ($n = 4, p < 0.03$) and 58.75 ± 3.78% ($n = 4, p < 0.001$) reduction in insulin release from cells cultured in 4.0 or 16.7 mM glucose, respectively. Combined treatment of cells with 5 mM AOA and 500 μM or 1 mM propyl gallate were not able to further reduce insulin release from cells incubated in either 4.0 or 16.7 mM glucose when compared with cells incubated with 500 μM or 1 mM propyl gallate alone.

**DISCUSSION**

mGPDH of mammalian origin has been purified to homogeneity by other laboratories with results, suggesting that the inner mitochondrial membrane bound enzyme contains iron and acid releasable sulfur (2, 19–21). However, the amount of iron found after purification ranged between 1 mol of iron/100,000–350,000 g of enzyme protein. Because pig brain mGPDH is a monomer of 75 kDa, these iron level estimations suggested that significant loss of iron occurred during the pu-

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**TABLE III**

*Homogeneous mitochondrial glycerol-3-phosphate dehydrogenase kinetic parameters*

| Parameter                  | Control | Ascorbic acid/iron activation |
|----------------------------|---------|------------------------------|
| $K_{m(app)}$ (mM)          | 10.0 ± 1.2 | 14.5 ± 4.9                  |
| $V_{max(app)}$ (nmol/min)  | 56.9 ± 13.2 | 161.9 ± 24.7               |
| $k_{cat}$ (s$^{-1}$)       | 21.1 ± 9.2  | 64.1 ± 25.3                |
| $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$) | 2.1 × 10$^5$ | 4.4 × 10$^3$               |

$aK_{m(app)}$ and $V_{max(app)}$ values were calculated by initial velocity versus substrate concentration values using the Response II advanced kinetics program. Differences in $K_m$ were not statistically different at $p = 0.005$, whereas $V_{max}$ values were extremely different at $p = 0.0005$.

$bK_{cat}$ values were calculated by dividing $V_{max(app)}$ by the molar concentration of the enzyme.

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![Fig. 3](http://www.jbc.org/) Effect of propyl gallate and/or AOA on glucose utilization in INS-1 cells. Glucose utilization was measured by incubating cells for 30 min at 37°C in KRB buffer containing either 4 or 16.7 mM glucose and [5-[^3H]glucose (final specific activity, 2.2 dpm/pmol) in the presence or absence of metabolic inhibitors. Conversion of [5-[^3H]glucose to[^3H]H$_2$O was determined as described under "Experimental Procedures." Data represent the means ± S.E. of four independent experiments.
Polyhydroxybenzoate Inhibition of mGPDH

without producing severe toxic side effects in the host (46–49).

The metal chelation properties of these drugs have been implicated in their inhibitory action (46). Alternatively, the effect of hydroxamic acids on the glycerol phosphate oxidase system of trypanosomes was proposed to be due to their ability to competitively displace ubiquinol, the putative electron carrier, from the dehydrogenase to the terminal oxidase of the glycerol phosphate oxidase complex (50). Whether either of these two unique systems, plant alternative oxidase and trypanosome terminal oxidase, are activated by l-ascorbic acid remains to be explored.

If mGPDH is indeed an essential enzyme in the proximal events linked to glucose-stimulated glucose metabolism and insulin release as previously reported (5, 8, 11, 51), specific binuclear iron inhibitors such as propyl gallate should significantly inhibit the process. This hypothesis was tested in the INS-1 cell line because they have been previously reported to have high levels of mGPDH activity similar to that observed in rat pancreatic β cells (12, 53). Incubation of INS-1 cells with propyl gallate at concentrations of 500 μM and 1 mM effectively reduced [5-3H]β-glucose metabolism and glucose-induced insulin release. The ability of propyl gallate to reduce glucose utilization is consistent with the hypothesis that mGPDH inhibition would reduce the reoxidation of cytosolic NADH and thereby inhibit glycolysis at the level of triose phosphates. Blockage of mGPDH would also cause a reduction in shuttling of cytosolic NADH generated from glycolysis and pyruvate into the mitochondria, thus leading to an overall reduction in ATP generation and thereby markedly reducing insulin release. Under similar culture conditions, 5 mM AOA was only partially effective in inhibiting glucose utilization and led to a 50% reduction in glucose-induced insulin release. The ability of AOA to inhibit glucose-induced insulin release in INS-1 cells is consistent with previous reports showing that millimolar concentrations of AOA inhibit insulin secretion from rat islets by 50% (37, 54). Our results suggest that in INS-1 cells the glycerol phosphate shuttle is more active than the malate-aspartate shuttle in the regeneration of NAD" consumed during glycolysis because propyl gallate is more effective than AOA at suppressing both glucose utilization and glucose-induced insulin release. This conclusion, however, directly contradicts Ishihara et al. (52) results that suggest that the malate-aspartate shuttle is more active than the glycerol phosphate shuttle in INS-1 cells. Our observation also contradicts results from Eto et al. (14) demonstrating that in mouse islets glucose-induced insulin release is only markedly suppressed when activities of both the glycerol phosphate and malate/aspartate shuttles are impaired. Nevertheless, our results are in general agreement with those of Sekine et al. (12), Dukes et al. (13), and Eto et al. (14) regarding the importance of cytosolic NADH and its subsequent oxidation through the glycerol-3-phosphate and malate-aspartate shuttles.

Our previous studies have shown l-ascorbic acid is essential for insulin release from scrobicutean guinea pig islets (16, 17) and that l-ascorbic acid serves as an essential cofactor for mGPDH (oxidase) activation in mitochondria isolated from guinea pig tissues and rat liver (18). However, the requirement for ascorbic acid in glucose-induced insulin release from INS-1 cells has been difficult to directly access. In unpublished studies, we have established that INS-1 cells are capable of dephosphorylating exogenously added ascorbic acid 2-phosphate, thus releasing ample amounts of vitamin C. Addition of 1 mM ascorbic acid 2-phosphate, however, did not enhance glucose utilization or glucose-induced insulin release from INS-1 cells.²

² W. W. Wells, H. K. Cirrito, and L. K. Olson, unpublished data.
ability of exogenously added ascorbic acid 2-phosphate to stimulate both glucose utilization and insulin release is most likely due to the difficulty of establishing scorbutic INS-1 cells because these cells are capable of scavenging trace amounts of ascorbic acid present in our commercial source of fetal bovine serum. Nevertheless, the ability of propyl gallate to block ascorbic acid-activated mGPDH activity in vitro and to reduce both glucose utilization and insulin release from INS-1 cells suggests that ascorbic acid plays an essential role in glucose-induced insulin release. Overall we conclude that mGPDH is activated by 1-ascorbic acid via a potential di-iron reactive center and is effectively inhibited by propyl gallate and other polyhydroxybenzoic acid derivatives. Furthermore, the glycerol phosphate shuttle, in comparison with the malate-aspartate shuttle, is crucial to the release of insulin from INS-1 cells in response to elevated glucose levels. Use of propyl gallate and other related polyhydroxybenzoate inhibitors may serve as effective tools for studying the role of mGPDH in glucose-induced insulin secretion.

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Polyhydroxybenzoates Inhibit Ascorbic Acid Activation of Mitochondrial Glycerol-3-phosphate Dehydrogenase: IMPLICATIONS FOR GLUCOSE METABOLISM AND INSULIN SECRETION

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