Glutamate formation via the leucine-to-glutamate pathway of rat pancreas

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Submitted 19 November 2013; accepted in final form 31 March 2014

Schachter D, Buteau J. Glutamate formation via the leucine-to-glutamate pathway of rat pancreas. Am J Physiol Gastrointest Liver Physiol 306: G938–G946, 2014. First published April 3, 2014; doi:10.1152/ajpgi.00394.2013.—The leucine-to-glutamate (Leu→Glu) pathway, which metabolizes the carbon atoms of l-leucine to form l-glutamate, was studied by incubation of rat tissue segments with 1-[U-14C]leucine and estimation of the [14C]glutamate formed. Metabolism of the leucine carbon chain occurs in most rat tissues, but maximal activity of the Leu→Glu pathway for glutamate formation is limited to the thoracic aorta and pancreas. In rat aorta, the Leu→Glu pathway functions to relax the underlying smooth muscle; its functions in the pancreas are unknown. This report characterizes the Leu→Glu pathway of rat pancreas and develops methods to examine its functions. Pancreatic segments effect net formation of glutamate on incubation with l-leucine, l-glutamine, or a mix of 18 other plasma amino acids at their concentrations in normal rat plasma. Glutamate formed from leucine remains mainly in the tissue, whereas that from glutamine enters the medium. The pancreatic Leu→Glu pathway uses the leucine carbons for net glutamate formation; the α-amino group is not used; the stoichiometry is as follows: 1 mol of leucine yields 2 mol of glutamate (2 leucine carbons per glutamate) plus 2 mol of CO2. Comparison of the Leu→Glu pathway in preparations of whole pancreatic segments, isolated acini, and islets of Langerhans localizes it in the acini; relatively high activity is found in cultures of the AR42J cell line and very little in the INS-1 832/13 cell line. Pancreatic tissue glutamate concentration is homeostatically regulated in the range of ~1–3 μmol/g wet wt. l-Valine and leucine ethyl, benzyl, and tert-butyl esters inhibit the Leu→Glu pathway without decreasing tissue total glutamate.

Our prior reports (33–36) identify and characterize the leucine-to-glutamate (Leu→Glu) pathway in the endothelium of thoracic aorta. Studied by incubation of tissue segments in vitro with 1-[U-14C]leucine and estimation of the net [14C]glutamate formed, the peak aortic activity is limited sharply to the “windkessel” region, just distal to the aortic arch. Tissue differentiation for the pathway was further observed in 14 rat tissues (34, 35). The highest specific activities were limited to the endothelium of the proximal thoracic aorta and to the pancreas; much lower activity levels were found in the lung and testis, and little or no activity was observed in the liver, kidney, skeletal muscle, brain, or remaining tissues. Systematic characterization of the aortic Leu→Glu pathway defined experimental conditions for its inhibition, which made possible the subsequent demonstration of its function in regulation of aortic smooth muscle contractility (33, 34). The comparable role(s) of the Leu→Glu pathway in pancreatic physiology is unknown, but it is likely to be significant in view of its high specific activity. Accordingly, this report describes studies characterizing the pancreatic Leu→Glu pathway and defining experimental conditions for its inhibition.

In rat aorta, the Leu→Glu pathway functions as one of at least four endothelial pathways regulating the contractility of the underlying vascular smooth muscle. Glutamate formed via the Leu→Glu pathway enters the underlying smooth muscle layer (35) and increases cGMP to cause relaxation. Additionally, nitric oxide formed in the endothelium from l-arginine (1) and increased by l-glutamine (33) also increases the smooth muscle cGMP and relaxation. By contrast, prostaglandin H2 originating in the endothelium decreases the smooth muscle cGMP and elicits contraction (19, 36). We proposed (35) that these pathways function in concert to regulate dynamically the contractility and capacitance of the aortic wall, thereby modulating hemodynamic parameters such as the arterial systolic and pulse pressure levels and the work of the left ventricle.

Studies described in a number of comprehensive reviews (4, 7, 9, 15, 32) support the generalization that the metabolic pathways for leucine, glutamate, and glutamine in mammalian organisms are differentiated in specific tissues for specialized functions. The rat aortic Leu→Glu pathway is an example of such a specialized pathway within one tissue. Net glutamate synthesis via the Leu→Glu pathway occurs by 1) metabolism of the branched-chain carbon skeleton of leucine to yield acetyl coenzyme A (CoA), a well-studied and described reaction sequence found broadly distributed in many rat tissues (18, 24, 25, 38), followed in the limited, specific tissues listed above by 2) net glutamate synthesis via mitochondrial utilization of acetyl CoA to form α-ketoglutarate, which can be converted to glutamate by transamination or by the glutamate dehydrogenase reaction (18, 39). The Leu→Glu sequence begins with leucine transfer across the plasma membrane into the cell cytosol followed by its transamination to α-ketoisocaprate (α-KIC) mediated by the mitochondrial branched-chain aminotransferase (BCAT) (14, 16). α-KIC is a substrate for oxidative decarboxylation by the branched-chain α-ketoadid dehydrogenase complex of the mitochondrial membrane, and the product α-ketoisovaleryl CoA is further processed by mitochondrial reactions to yield acetyl CoA and acetoacetate (18, 25). Acetyl CoA so formed can enter the tricarboxylic acid cycle via the condensation reaction with oxaloacetate and, thereby, increase the synthesis of α-ketoglutarate.

Leucine, an essential dietary amino acid, is recognized as an important nutritional precursor for tissue glutamate synthesis (15). Leucine can supply its carbon atoms for the Leu→Glu pathway, as noted above, as well as its α-amino group for transamination of α-ketoglutarate to glutamate mediated by a BCAT. In the central nervous system, the latter synthesis using the leucine α-amino group predominates (8, 20, 27), whereas...
the Leu→Glu pathway, using the leucine carbons, predominates in the aortic endothelium. The studies described below test the hypothesis that pancreatic segments in vitro, when tested with leucine at its concentration in normal rat plasma, ~0.2 mM, utilize predominantly the carbons of leucine for net glutamate formation. In addition, the studies characterize basic features of the pancreatic Leu→Glu pathway to establish conditions for exploring its functions in pancreatic physiology.

**MATERIALS AND METHODS**

**Animals.** Sprague-Dawley male rats (~250 g body wt) were obtained from Charles River Laboratories (Wilmington, MA) and maintained on a nutritionally complete pellet diet (Purina rat chow 50001) and water ad libitum. Rats weighed 250–450 g when used in the experiments. When indicated, some rats were deprived of food for 15 h prior to euthanasia.

**Preparation and incubation of pancreatic segments.** Rats were euthanized by inhalation of CO₂ and rapidly exsanguinated; the pancreas was excised, immediately immersed in cold 145 mM NaCl-5 mM KCl, and maintained at 2°C until the onset of incubation ~10–20 min later. Approximately 1.5 cm of pancreas originating at the splenic end was cut off, adherent membranes were removed, and the tissue was sliced into ~2–3-mm² segments. Aliquots of three to five segments, combined wet weight ~25–60 mg, were placed in each incubation flask containing 2.0 ml of chilled incubation medium of the following general composition: 118 mM NaCl, 4.7 mM KCl, 2.0 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 10 mM D-glucose, and 0.1 mg/ml soybean trypsin inhibitor (Sigma). In some experiments, the medium also contained the plasma amino acid mix (AAmix) described previously (33), a mixture of 18 amino acids, ketogenic and glucogenic, at their concentrations in normal rat plasma, to approximate in vivo conditions more closely. The AAmix was prepared free of L-leucine, L-glutamine, and L-glutamate. For study of [¹⁴C]glutamate formation, the medium also contained unlabelled L-leucine, usually 0.20 mM [its concentration in normal rat plasma (29)] plus 0.05–0.10 μCi/ml L-[U-¹⁴C]leucine (specific radioactivity 306 mCi/mmol; Amersham). Tissue suspensions were shaken at 37°C in an atmosphere of 5% CO₂–95% O₂. Thereafter, tissues were removed, blotted briefly to remove adherent medium, and placed in tared vials for rapid weighing, and the vials were frozen in dry ice and maintained at −15°C until the samples were assayed.

**Estimation of L-glutamate and [¹⁴C]glutamate.** Free L-glutamate in tissues and media was estimated using the microbiplate-fluorometric method described previously (33). To estimate the [¹⁴C]glutamate content of aortic tissues (33), extracts are resolved on a Dowex 1X8 anion-exchange column, and a pure [¹⁴C]glutamate fraction was eluted and counted. The same procedure applied to pancreatic extracts yields an eluate containing [¹⁴C]glutamate plus other radioactive components. Accordingly, the following method was developed to treat, in parallel, two aliquots, A and B, of each sample extract. *Aliquot B* was treated with a bacterial L-glutamate oxidase to remove glutamate specifically and efficiently. *Aliquot A* was a control not exposed to the enzyme. After resolution of *aliquots A* and *B* separately on the anion-exchange columns, the ¹⁴C content of the A eluate (designated *a*) minus that of the B eluate (designated *b*) yielded an uncorrected [¹⁴C]glutamate content for each sample, Vₐ, where Vₐ = a − b. The detailed procedure is as follows. Pancreatic segments frozen as described above were suspended in 0.5 ml of H₂O₂ heated at 100°C for 7 min, cooled on ice, homogenized, and centrifuged for 15 min at 11,500 g. An aliquot of 120 μl of each supernatant was placed in each of two wells (A and B) of a 96-well microtiter plate. To the well B, 25 μl of 0.1 M Tris-HCl buffer (pH 7.5) containing 25 μM of *Streptomyces* L-glutamate oxidase (US Biologicals) were added; the same buffer alone was added to well A. After the microplates were incubated for 4 h at 37°C, 100 μl of each well were placed on a separate column (2-ml bed volume) of Dowex 1X8–200 anion-exchange resin (Sigma), as previously described (33). Each loaded column was washed with 10 ml of 20 mM L-leucine; then the anionic fraction containing glutamate was eluted with 3 ml of 0.5 M acetic acid, and its radioactive content was estimated by liquid scintillation spectrometry.

To estimate and correct for the recovery of glutamate and for the efficiency of the enzymatic elimination of glutamate in the procedure, a standard solution of 3.5 μCi/ml authentic L-[¹⁴C]glutamate (specific activity 218 mCi/mmol; Moravek) was prepared in the same vehicle as the unknown samples. Aliquots of 120 μl of this standard solution were included on each microtiter plate of unknowns and carried through the entire procedure described above. For the tissue samples, the number of ¹⁴C counts in the anionic fractions eluted from the standard solution control (A) and enzyme-treated (B) columns are designated *a’* and *b’*, respectively, and the [¹⁴C]glutamate value (Vₐ), is given by Vₐ = a’ − b’. If the number of ¹⁴C counts loaded on the control (A) column is designated c, the fractional recovery of the standard solution glutamate is a’/c. The fraction of glutamate escaping enzymatic elimination is b’/a’, and the efficiency of the enzyme treatment is 1 − b’/a’. The corrected value (Vₐ) of [¹⁴C]glutamate for each unknown sample was then calculated as follows: Vₐ = Vₐ/d(a’/c) = 1 − b’/a’. The mean ± SE values of 27 fractional recoveries (a’/c) and enzyme efficiencies (1 − b’/a’) obtained in one year of work were 0.833 ± 0.018 and 0.834 ± 0.007, respectively. The [¹⁴C]glutamate content was calculated as Vₐ-specific radioactivity of one acetyl group of the precursor L-[¹⁴C]leucine (see stoichiometry studies below) and expressed as nmol/g or μmol/g wet wt of tissue. Samples of media were also assayed for [¹⁴C]glutamate by the foregoing procedure, but they required an initial fivefold concentration by centrifugation under vacuum (Speed Vac Concentrator, Savant) owing to much lower concentrations.

The bacterial glutamate oxidase deaminates [¹⁴C]glutamate to [¹⁴C]α-ketoglutarate. If the latter eluted from the Dowex column in the same fraction as glutamate, the values obtained for glutamate would be falsely low. The reasonably high values of the fractional efficiencies above indicated that this was not a significant error, and additional studies confirmed this conclusion. Samples of L-[¹⁴C]glutamate were incubated with the *Streptomyces* glutamate oxidase, the products were resolved on the Dowex columns as described above, and the anionic fraction containing glutamate was eluted. Examination of this fraction by thin-layer chromatography and autoradiography detected no α-ketoglutarate. [Chromatography was carried out on Whatman flexible silica gel plates (250 μm) developed with 2 solvent systems: 70:30 (vol/vol) ethanol-ammonia water (28%) and 80:20:20 (vol/vol/vol) 1-butanol-glacial acetic acid-water, as previously described (35). RF values for glutamate and α-ketoglutarate were 0.49 and 0.59, respectively, in the former system and 0.57 and 0.81, respectively, in the latter.]

**Quantification of ¹⁴CO₂ evolved.** To estimate the amount of ¹⁴CO₂ formed via the metabolism of L-[¹⁴C]leucine or L-[¹⁴C]leucine, ~35 mg wet weight of pancreatic segments were incubated in 2.0 ml of the following medium: 142 mM NaCl, 4.7 mM KCl, 1.0 mM CaCl₂, 1.2 mM MgSO₄, 4.0 mM sodium phosphate (pH 7.4). 10 mM D-glucose, 0.2 mM L-leucine, tracer quantities of one of the labeled leucine precursors, and 0.1 mg/ml soybean trypsin inhibitor. Tissues were incubated with shaking for various times up to 60 min in closed vessels containing a KOH trap, under an atmosphere of 100% O₂, at 37°C. Thereafter, tissues were removed, weighed, and frozen as described above, and the media were acidified by addition of 0.2 ml of 2.0 N HCl and shaken in the closed vessels for an additional 30 min. The ¹⁴CO₂ evolved and trapped was estimated as the carbonate by liquid scintillation counting in ScintiSafe Plus 50% counting solution (Fisher). For stoichiometry and carbon balance studies, the tissues were also assayed for [¹⁴C]glutamate and total glutamate, as described above. The acidified media were neutralized to pH 7 by
Pancreatic preparations and cell lines. The following preparations and cultured cell lines were assayed for formation of [14C]glutamate from 0.2 mM L-[U-14C]leucine and for total glutamate content, as described above for whole pancreatic segments. The results are expressed per milligram of tissue protein as determined by the method of Lowry et al. (22). Suspensions of pancreatic acini were prepared as described by Williams et al. (40) and Louie et al. (21). Islets of Langerhans were isolated via the method of pancreatic ductal injection of collagenase described by Gotoh et al. (11). Rats were anesthetized for this procedure by intraperitoneal injection of ketamine (70–80 mg/kg) + xylazine (5 mg/kg) according to protocol ACAAAD 1453 approved by our Institutional Animal Care Utilization Committee.

The AR42J pancreatic acinar cell line (6), a rat acinar cell line derived from azaserine-induced tumors, was cultured in flasks under DMEM containing 10% fetal bovine serum. The INS-1 832/13 pancreatic beta-cell line (12), which secretes insulin in response to physiological glucose concentrations, was cultured in RPMI 1640 medium supplemented with 10 mM HEPES, 10% fetal bovine serum, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol. For the assays, the culture medium was replaced with the medium containing 0.2 mM L-[U-14C]leucine described above for incubating pancreatic whole segments. Flasks were incubated at 37°C in a tissue culture incubator gassed with 5% CO2-95% O2, and cells and media were harvested after 1 h.

Materials. L-[U-14C]leucine (306 mCi/mmol) was purchased from Amersham; L-[1-14C]leucine (55 mCi/mmol) and L-[U-14C]glutamate (218 mCi/mmol) were obtained from Moravek. L-Leucine ethyl ester hydrochloride; L-leucine tert-butyl ester hydrochloride; L-leucine benzyl ester hydrochloride; trypsin inhibitor type II-S: soybean; collagenase from Clostridium histolyticum, type V; α-ketoisovalerate (3-methyl-2-oxobutanoic acid, Na+ salt); L-leucine; L-valine; L-glutamine; and the α-amino acids of the plasma AAmix were purchased from Sigma-Aldrich (33).

Statistical significance. Statistical significance (P < 0.05) was evaluated by the t-test of unpaired or paired comparisons or by ANOVA and Tukey’s least significant difference posttest. Unless otherwise indicated, values are means ± SE, and tissue concentrations are listed per gram of tissue wet weight or per milligram of tissue protein.

RESULTS

Time course of glutamate formation from L-leucine and of [14C]glutamate formation from L-[U-14C]leucine in vitro. Pancreatic segments were incubated for up to 60 min in Krebs-Ringer bicarbonate (KRB) without and with 0.2 mM L-[U-14C]leucine, and the tissue and medium total glutamate and [14C]glutamate were quantified. The values of total (tissue + medium) glutamate monitor the net of glutamate formation vs. metabolic alteration or utilization and are plotted in Fig. 1.1

The area between curve B (tissues in KRB alone) and curve A (tissues in KRB + leucine) over the 0- to 60-min interval is equivalent to a leucine-dependent glutamate increment of 0.80 μmol/g wet wt (2-way ANOVA, F = 24.7, P < 0.0001).

Curve C plots the values of the [14C]glutamate synthesized over the same time interval, and the area subtended is equivalent to 0.77 μmol/g wet wt. Thus, under these experimental conditions, glutamate synthesis using the carbon atoms of leucine via the Leu→Glu pathway accounted for 96% of the leucine-dependent synthesis. This observation, similar to that previously reported for rat aortic endothelium (33, 34), is in accord with the stoichiometry and carbon balance studies described below. As noted above, it differs from leucine-dependent glutamate synthesis in the central nervous system, where mainly the leucine α-amino group is used for glutamate formation (8, 20, 27).

In the foregoing experiments for Fig. 1, a relatively narrow range of tissue glutamate concentrations was observed following various incubation times, suggesting the effects of regulatory mechanisms. From 10 to 60 min of incubation, the tissue content of [14C]glutamate increased from 0.23 ± 0.04 to 0.80 ± 0.05 μmol/g (P < 0.001); at 60 min, [14C]glutamate comprised 43% of the tissue total (14C-labeled + nonradioactive) glutamate. Yet the tissue total glutamate concentration at 60 min, 1.84 ± 0.17 μmol/g, was not significantly greater than that at 10 min, 1.64 ± 0.07 (P = 0.28), owing to a decrease in nonradioactive glutamate. In additional experiments, segments from each pancreas were incubated as described above in medium containing 0.2 or 7.0 mM L-[U-14C]leucine. After 30 min of incubation, the tissue contents of [14C]glutamate formed from 0.2 and 7.0 mM leucine were 0.65 ± 0.05 and 2.07 ± 0.15 μmol/g (5 rats, P < 0.001), respectively, whereas the corresponding total glutamate contents of the tissues, 2.55 ± 0.20 and 2.97 ± 0.09 μmol/g, respectively, were not significantly different (P = 0.1). Further evidence for the regulation of pancreatic tissue glutamate concentration is summarized and discussed below.

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1 Glutamate content of the incubated tissues alone, i.e., not including the content in the ambient medium, results not only from formation vs. metabolic alteration, but also from glutamate transfer to the ambient medium. Just prior to incubation in the experiments described above, tissue glutamate content was 4.70 ± 0.20 μmol/g wet wt (11 rats). In the initial 5 min of incubation, this value decreased by ~53% in the incubations without or with L-leucine, owing to transfer of accessible glutamate pools from tissue to medium. These pools likely include fluids of the duct system, other extracellular fluids, and leakage from cut surfaces.

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Fig. 1. Time course of total glutamate content of pancreatic segments plus media incubated without or with L-leucine and content of [14C]glutamate formed from L-[U-14C]leucine. Pancreatic segments were prepared and incubated in Krebs-Ringer bicarbonate (KRB) without (curve B) and with (curve A) 0.2 mM L-leucine. Values are means ± SE for 11 rats at 0 min; 6, 5, 11, and 5 rats at 5, 10, 20, and 60 min, respectively, in curve B; and 4, 3, 8, and 4 rats at 5, 10, 20, and 60 min, respectively, in curve A. Formation of [14C]glutamate from 0.2 mM L-[U-14C]leucine is shown in curve C. Values are means ± SE for 3, 4, 2, and 3 rats at incubation times of 5, 10, 20, and 60 min, respectively. A net change in total glutamate content of tissue + ambient medium, as plotted above, monitors formation vs. metabolic alteration or disposal of glutamate.

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AJP-Gastrointest Liver Physiol • doi:10.1152/ajpgi.00394.2013 • www.ajpgi.org
Table 1. Effects of L-leucine, L-glutamine, and AAmix on net glutamate formation and tissue glutamate content of pancreatic segments incubated in vitro

<table>
<thead>
<tr>
<th>Expt No.</th>
<th>Medium</th>
<th>n</th>
<th>Tissue Glutamate Content, μmol/g</th>
<th>Medium Glutamate Content, μmol/g</th>
<th>Tissue + Medium Glutamate Content, μmol/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>KRB</td>
<td>5</td>
<td>1.83 ± 0.21</td>
<td>1.41 ± 0.13</td>
<td>3.25 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>KRB + AAmix</td>
<td></td>
<td>2.01 ± 0.22</td>
<td>1.77 ± 0.09</td>
<td>3.78 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>(P = NS)</td>
<td></td>
<td>(P &lt; 0.05)*</td>
<td>(P &lt; 0.01)*</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>KRB</td>
<td>7</td>
<td>1.46 ± 0.13</td>
<td>1.74 ± 0.20</td>
<td>3.34 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>KRB + leucine</td>
<td></td>
<td>2.17 ± 0.21</td>
<td>1.78 ± 0.17</td>
<td>3.95 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>(P &lt; 0.01)*</td>
<td></td>
<td>(P = NS)</td>
<td>(P &lt; 0.015)*</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>KRB</td>
<td>4</td>
<td>1.67 ± 0.21</td>
<td>1.32 ± 0.06</td>
<td>2.98 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>KRB + glutamine</td>
<td></td>
<td>1.64 ± 0.13</td>
<td>2.72 ± 0.26</td>
<td>4.36 ± 0.26</td>
</tr>
<tr>
<td></td>
<td>(P = NS)</td>
<td></td>
<td>(P &lt; 0.001)*</td>
<td>(P &lt; 0.01)*</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>KRB + AAmix</td>
<td>4</td>
<td>1.71 ± 0.26</td>
<td>1.72 ± 0.31</td>
<td>3.43 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>KRB + AAmix + leucine</td>
<td></td>
<td>2.11 ± 0.11</td>
<td>1.82 ± 0.37</td>
<td>3.93 ± 0.41</td>
</tr>
<tr>
<td></td>
<td>(P &lt; 0.03)*</td>
<td></td>
<td>(P = NS)</td>
<td>(P &lt; 0.033)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KRB + AAmix + glutamine</td>
<td></td>
<td>2.13 ± 0.20</td>
<td>2.70 ± 0.36</td>
<td>4.83 ± 0.53</td>
</tr>
<tr>
<td></td>
<td>(P = NS)</td>
<td></td>
<td>(P &lt; 0.045)*</td>
<td>(P &lt; 0.025)*</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. Pancreatic segments were incubated in Krebs-Ringer-bicarbonate (KRB) without or with plasma amino acid mix (AAmix) (lacking leucine and glutamine, as described in MATERIALS AND METHODS), L-leucine (0.2 mM), or L-glutamine (0.5 mM) at 37°C for 20 min under 5% CO2-95% O2. *P values determined by unpaired t-test. †P values determined by t-test of paired comparisons. NS, not significant.

Comparison of amino acid precursors for pancreatic glutamate formation in vitro. Pancreatic segments were incubated as described above for 20 min in KRB without or with L-leucine (0.2 mM), L-glutamine (0.5 mM), or AA mix. Values of the resulting glutamate contents of tissues, media, and tissues plus media are listed in Table 1 and support the following conclusions. All three precursors, compared with their respective KRB controls, significantly increased net glutamate formation and total (tissue + medium) glutamate content. The localization of the glutamate increments, however, differed. The increment owing to the Leu -> Glu pathway was almost entirely in the tissue (P < 0.01), consistent with its mitochondrial site of origin. The increment owing to L-glutamine was in the medium (P < 0.001), with no change in tissue content; the increment dependent on AA mix was mainly (~68%) in the medium. The results highlight the differences in cellular handling of glutamate formed from leucine vs. that from glutamine or AA mix precursors, and it is reasonable to suggest that the resulting glutamate pools serve different functions. The mean tissue glutamate concentrations resulting from each of the studies listed in Table 1 ranged from ~1.5 to 2.2 μmol/g wet wt, further evidence of homeostatic regulation.

When tested in the presence of AA mix (4 rats), the mean glutamate increments owing to leucine or glutamine were not statistically significantly different from those in the absence of AA mix (7 rats). The glutamate increments owing to addition of leucine or glutamine to KRB containing AA mix (4 rats) compared with AA mix alone were significant as calculated by the t-test of paired comparisons. The glutamate increments owing to leucine were significant for tissue (P < 0.03) and tissue plus medium (P < 0.033), and those for glutamine were significant for medium (P < 0.045) and tissue plus medium (P < 0.025).

Stoichiometry and carbon balance of the pancreatic Leu -> Glu pathway. To establish the stoichiometry of the pathway, segments of each pancreas were incubated separately in Krebs-Ringer phosphate (KRP) alone, KRP containing 0.2 mM L-[1-14C]leucine, and KRP containing 0.2 mM L-[U-14C]leucine.

After incubation for 5, 10, 20, or 60 min, the tissues were removed for estimation of [14C]glutamate and total glutamate and media were acidified, and the evolved 14CO2 was collected and quantified. The biochemical reactions that metabolize the leucine carbon chain to form acetyl CoA and, subsequently, glutamate and CO2 are described above. After leucine transport across the plasma cell membrane and removal of the amino group by transamination via action of the mitochondrial BCAT (16, 38), the product, α-ketoisocaproate, is oxidatively decarboxylated by the mitochondrial branched-chain dehydrogenase complex. Thus the 14CO2 so evolved from carbon 1 of L-[1-14C]leucine quantifies the moles of leucine entering the Leu -> Glu pathway. (Leucine carbon 1 is not incorporated into the [14C]glutamate product of the pathway (34).) Further metabolism of leucine carbons 2–6 yields [14C]glutamate and 14CO2 derived from the L-[U-14C]leucine precursor.

The experimental results yield molar ratio parameters that indicate the following stoichiometry: 1 mol of [U-14C]leucine (6 carbons) entering the pancreatic Leu -> Glu pathway yields 2 mol of 14CO2 plus 2 mol of [14C]glutamate (each glutamate containing a 2-carbon acetyl group from leucine). Table 2 shows the values of three molar ratios predicted by this stoichiometry and their close correspondence to the values observed experimentally. The values for [14C]glutamate in the ratios are those for incubation periods up to 10 min to minimize decreases owing to its further metabolism with time (see below).

The foregoing experimental methods also yielded values for the balance of the number of leucine carbon atoms entering the Leu -> Glu pathway vs. the number in the total products of the pathway, and these values are listed in Table 3. At each incubation time up to 60 min, the ratio of carbons in products to carbons entering the pathway was close to 1, particularly at the shorter incubations. The value for all four of the incubation periods was 0.98 ± 0.07 (SE). The radioactive products of further metabolism of the [14C]glutamate formed initially (Table 3) increase with longer incubation times. They were quantified as the remaining total radioactivity in a column anionic
of Langerhans, respectively. Accordingly, preparations and exocrine and endocrine secretory functions of the mammalian metabolism of leucine under these experimental conditions.

In the products of the pathway, Leu-Glu pathway is substantial in the overall intracellular incubation, \( \text{Leu} \rightarrow \text{Glu} \) pathway is then entered the Leu-Glu pathway in the same fraction as glutamate. The possibility that they include remain to be identified. They are anionic and elute from the Dowex 1X8 40% at 20 and 60 min, respectively. Products formed via this metabolism compounds was found.

Thin-layer chromatography on silica gel plates, and no evidence of these metabolized by segments of rat pancreas incubated in vitro. And methods (4)

Balance of the number of carbon atoms entering the pancreatic Leu-Glu pathway was decreased by the branched-chain dehydrogenase complex to enter the Leu-Glu pathway (\( \text{Leu} \rightarrow \text{Glu} \)), nanomoles times 2 of \([14C]\text{glutamate} \) estimated as described in materials and methods.

Values are means ± SE; \( n \), number of rats. Values were estimated as follows: nanomoles times 6 of \([U-14C]\text{leucine} \) (0.2 mM) transferred from ambient medium to the Leu-Glu pathway via oxidative decarboxylation, but its subsequent metabolism does not yield acetyl CoA or glutamate. Pancreatic segments were incubated with 0.2 mM \( [\text{L-14C}]\text{leucine} \) and \( 0.5-10 \) mM \( \text{l-valine} \) or \( \alpha\)-ketoisovalerate. Formation of \([14C]\text{glutamate} \) and total \((14C\)-labeled + nonradioactive\) glutamate contents of tissues and media were estimated, and the resulting tissue values are illustrated in Fig. 2. Valine and \( \alpha\)-ketoisovalerate effectively inhibited the Leu-Glu pathway, i.e., decreased the cell lines consisting of or derived from each region were incubated in vitro with 0.2 mM \( [\text{L-14C}]\text{leucine} \), and formation of \([14C]\text{glutamate} \) was quantified. The results in Table 4 locate the pathway predominantly in the acinar portion of the pancreas. Thus the mean activity for formation of \([14C]\text{glutamate} \) by preparations of acini (21, 40) was ~3-fold that of whole pancreatic segments (\( P < 0.001 \)) and 12-fold that of the isolated islet preparations (\( P < 0.001 \)). In the acinar preparations, \([14C]\text{glutamate} \) formed via the Leu-Glu pathway comprised >90% of total tissue glutamate after 60 min of incubation. Relatively high activity was also found in the AR42J pancreatic acinar cell line. Its activity was ~10-fold greater than that of the INS-1 832/13 cell line (\( P < 0.001 \)), and it was >5-fold greater than that of the isolated islet preparations. Of further interest, although not explained by our results, is the very high total glutamate content of the AR42J cells, approximately fivefold that of the whole pancreatic segments.

Inhibitors of the Leu-Glu pathway. A number of competitive inhibitors of the Leu-Glu pathway, notably leucine esters, \( \text{l-valine} \), and its deaminated product, \( \alpha\)-ketoisovalerate, were identified in prior studies of rat aorta (33, 34). The last compound is a substrate of the mitochondrial branched-chain dehydrogenase complex and enters the Leu-Glu pathway via oxidative decarboxylation, but its subsequent metabolism does not yield acetyl CoA or glutamate. Pancreatic segments were incubated with 0.2 mM \( [\text{l-14C}]\text{leucine} \) and \( 0.5-10 \) mM \( \text{l-valine} \) or \( \alpha\)-ketoisovalerate.

Table 3. Balance of the number of carbon atoms entering the pancreatic Leu-Glu pathway vs. the number of carbon atoms in the products of the pathway

<table>
<thead>
<tr>
<th>Incubation Time, min</th>
<th>(^{14}C) Atoms, nmol/g wet wt tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2.894 306</td>
</tr>
<tr>
<td>10</td>
<td>4.698 1,072</td>
</tr>
<tr>
<td>20</td>
<td>10.938 3,696</td>
</tr>
<tr>
<td>60</td>
<td>16.290 10,194</td>
</tr>
</tbody>
</table>

Values are means; \( n \), number of rats. Values were estimated as follows: nanomoles times 6 of \([U-14C]\text{leucine} \) (0.2 mM) transferred from ambient medium to tissue (\( a \)), nanomoles times 6 of \( ^{14}\text{CO}_2 \) evolved from \([1-14C]\text{leucine} \) owing to its conversion to \([1-14C]\text{\alpha-ketoisocaproate} \), which is oxidatively decarboxylated by the branched-chain dehydrogenase complex to enter the Leu-Glu pathway (\( b \)), nanomoles times 2 of \([14C]\text{glutamate} \) estimated as described in materials and methods (\( c \)), nanomoles of \( ^{14}C \) in products of the further metabolism of \([14C]\text{glutamate} \) as described in materials and methods (\( d \)), and \( ^{14}\text{CO}_2 \) evolved from metabolism of \([U-14C]\text{leucine} \) via the Leu-Glu pathway (\( e \)). The ratio \( \beta/\beta \) of total \(^{14}C \) in the products to \(^{14}C \) entering the Leu-Glu pathway is close to 1 at early time points and 0.98 ± 0.07 (SE) for all times tested. ND, none detected.

Table 4. Synthesis in vitro of \([14C]\text{glutamate} \) from \([U-14C]\text{leucine} \) by preparations and cell lines of rat pancreas

<table>
<thead>
<tr>
<th>Preparation</th>
<th>(^{14}C) Glutamate Formed, mmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole segments</td>
<td>2.04 ± 1.2</td>
</tr>
<tr>
<td>Acini preparations</td>
<td>3.73 ± 1.2</td>
</tr>
<tr>
<td>AR42J cell line</td>
<td>10.9 ± 1.6</td>
</tr>
<tr>
<td>Islet preparations</td>
<td>13.0 ± 1.6</td>
</tr>
<tr>
<td>INS 832/13 cell line</td>
<td>13.5 ± 1.3</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n \), no. of rats, cell preparations, or cell line cultures. Preparations were assayed by incubation for 60 min with \([U-14C]\text{leucine} \).
formation and final content of tissue plus medium \( ^{14}\text{C}\)glutamate (1-way ANOVA, \( P < 0.001 \)), with 50% inhibitory concentrations of \( \sim 3 \) and \( 2 \) mM, respectively. Their effects on total \( ^{14}\text{C}\)-labeled (nonradioactive) glutamate content, however, differed. \( \alpha\)-Ketoisovalerate lowered the total glutamate values of tissues plus medium and tissues alone, in parallel with reductions in \( ^{14}\text{C}\)glutamate (Fig. 2A; 1-way ANOVA, \( P < 0.01 \)). Valine did not decrease the tissue content significantly (Fig. 2A) and modestly increased the total (tissue plus medium) value by \( \sim 19\% \) (1-way ANOVA, \( P < 0.03 \)). Thus the tissue can use the \( \alpha\)-amino group of valine to maintain the control glutamate content, e.g., by transamination of \( \alpha\)-keto-glutamate mediated by a BCAT. Lacking the \( \alpha\)-amino group, \( \alpha\)-ketoisovalerate cannot. It is noteworthy that valine and leucine can compete for transport via the \( L \)-amino acid (system L) transporter (LAT), but such competition did not influence the results in Fig. 2, A and B, inasmuch as valine and \( \alpha\)-ketoisovalerate (not described as an LAT substrate) were approximately equally effective inhibitors of the Leu–Glu pathway.

The effects of leucine ethyl, benzyl, and tert-butyl esters were tested similarly in vitro. Addition of 1 or 7 mM leucine ethyl ester decreased the \( ^{14}\text{C}\)glutamate formed (2 rats, 3 samples) from the control value of \( 0.69 \pm 0.03 \) \( \mu\text{mol/g} \) to \( 0.41 \pm 0.01 \) (a decrease of \( 41\% , \quad P < 0.01 \)) and \( 0.09 \pm 0.02 \) \( \mu\text{mol/g} \) (a decrease of \( 87\% , \quad P < 0.0001 \)), respectively. Total \( ^{14}\text{C}\)-labeled (nonradioactive) glutamate levels (tissue plus medium), however, were not decreased at either concentration. The untreated, control value of \( 3.68 \pm 0.23 \) \( \mu\text{mol/g} \) was somewhat increased by 7 mM leucine ethyl ester to \( 4.35 \pm 0.36 \) (an increment of \( 18.3\% , \quad \text{paired} \, t = 7.03, \quad P < 0.01 \)). Treatment in vitro with 7 mM leucine benzyl ester or leucine tert-butyl ester (1 rat each) also decreased the \( ^{14}\text{C}\)glutamate formed by 93% and 37%, respectively, whereas total (tissue + medium) glutamate was not decreased.

**Regulation of rat pancreatic tissue glutamate concentration in vitro.** We have provided evidence that significant increases or decreases in tissue \( ^{14}\text{C}\)glutamate formed via the Leu–Glu pathway do not affect total tissue glutamate concentrations significantly. Thus the observed tissue glutamate levels were maintained within a range of \( \sim 1–3 \) \( \mu\text{mol/g} \) wet wt, owing to compensatory, regulatory tissue mechanisms. Figure 3 shows a
scatter plot of the pancreatic tissue content of total (\(^{14}\text{C}\)-labeled + nonradioactive) glutamate vs. \([^{14}\text{C}]\)glutamate in studies of 37 rats described above. The content of tissue glutamate in Fig. 3 is \(1.94 \pm 0.49\) (SD) \(\mu\text{mol/g} \text{ wet wt and does not correlate significantly with increased or decreased Leu→Glu metabolic flow. Tissue mechanisms responsible for this regulation are discussed below.}

**DISCUSSION**

The foregoing results demonstrate net formation of \(\text{L-glutamate by rat pancreatic segments in vitro from each of three amino acid sources: L-leucine via the Leu→Glu pathway, L-glutamine, and AAmix. These amino acid precursors of glutamate were effective at their basal concentrations in normal rat plasma (Table 1) and are independent of short-term fluctuations owing to feeding. There are notable differences between the Leu→Glu pathway for glutamate formation, described in this report, and glutamate formation from glutamine by action of the phosphate-dependent glutaminase isozymes (EC 3.5.1.2) described previously (7, 26). The Leu→Glu pathway is intramitochondrial and localized in the acinar portion of the pancreas (Table 4); the glutaminase isozyme activities, also intramitochondrial (7), are mainly in the islets of Langerhans (3, 28), and immunohistochemical evidence indicates that the kidney-type isozyme predominates and is highly localized in the glucagon-expressing pancreatic alpha-cells (30). The dispositions of the net glutamate moieties formed in vitro from leucine and glutamine, respectively, also differ, with the former retained in the tissue and the latter mostly transferred to the ambient medium (Table 1). Thus functions fulfilled by pancreatic glutamate formed from leucine via the Leu→Glu pathway are likely different from those fulfilled by leucine formed from glutamine.**

Comparison of the Leu→Glu pathways of rat pancreas and thoracic aorta, the two organs of highest Leu→Glu specific activity, is instructive, and the following similarities were noted. 1) Segments of aorta (33, 34) and pancreas incubated in vitro can utilize \(\text{L-leucine or L-glutamine for net glutamate formation. 2) Net glutamate so formed from leucine by aortic and pancreatic segments was \sim 67\% (33) and entirely (Table 1), respectively, retained in the tissue. 3) Net glutamate formed from glutamine by aortic and pancreatic segments was \sim 90\% (33) and entirely (Table 1), respectively, transferred to the ambient medium. 4) Net synthesis of \([^{14}\text{C}]\)glutamate from \([U-^{14}\text{C}]\)leucine via the Leu→Glu pathway accounted fully for the net synthesis of total (radioactive + nonradioactive) glutamate by aortic segments (33) and by pancreatic segments (Fig. 1); the \(\alpha\)-amino group of leucine did not contribute significantly to net glutamate formation via transamination of \(\alpha\)-ketoglutarate. 5) Despite substantial increases in the percentage of \([^{14}\text{C}]\)glutamate per total tissue glutamate owing to glutamate synthesis from leucine and despite substantial decreases in that percentage owing to several inhibitors of the Leu→Glu pathway, the pretreatment control values of tissue free glutamate in aortic and pancreatic segments were maintained in the range of \(\sim 1–3\) \(\mu\text{mol/g}, indicative of homeostatic, regulatory controls. 6) L-Valine, its deaminated product \(\alpha\)-ketoisovalerate, and a number of leucine esters inhibit the Leu→Glu pathway in both tissues. 7) L-Valine inhibition of the Leu→Glu pathway in each tissue does not decrease the tissue free glutamate level, whereas \(\alpha\)-ketoisovalerate inhibition does. 8) The specific activity of the aortic or pancreatic Leu→Glu pathway tested in the absence vs. the presence of AAmix, the latter being more representative of conditions in vivo, was not significantly different. Despite the foregoing similarities, aortic and pancreatic tissues differ in the stoichiometry of leucine carbon utilization for glutamate synthesis. Pancreatic segments yield 2 mol of glutamate plus 2 mol of \(\text{CO}_2\) per mole of leucine (Table 2), whereas aortic endothelium forms 1 mol of glutamate plus 4 mol of \(\text{CO}_2\) per mole of leucine (34). In view of the predominant similarities, the function of the Leu→Glu pathway for signaling demonstrated for the aorta (33, 34) suggests the likelihood of a signaling function in the pancreas. Functional differences between the tissues could stem from signaling in the aorta for control of vascular smooth muscle contractility vs. signaling in the pancreas for control of secretion.

The importance of glutamate, a metabolite at the junction of mammalian nitrogen and carbon metabolic pathways and a major signal transmitter in the central nervous system, is well documented and reviewed (4, 7, 9, 15, 31, 32). Thus the quantitative evidence for homeostatic control of pancreatic tissue glutamate concentration, described above and illustrated in Fig. 3, is not surprising. The evidence, however, poses additional relevant questions; e.g., what molecular mechanisms maintain the tissue concentrations in the range of \(\sim 1–3\) \(\mu\text{mol/g}\)? Further studies are needed to characterize fully the mechanisms of glutamate formation, utilization, metabolic degradation, and transport that are balanced to effect the regulation. However, our results indicate two such mechanisms: one mechanism to counter increased formation of glutamate and the other to counter decreased formation of glutamate. To counter an increase in tissue glutamate from treatment with \(\text{l-glutamine, the resulting glutamate is transported from the pancreatic segment to the ambient medium (Table 1). Fukushima et al. (10) described a transfer in the rat of glutamate derived from plasma glutamine to the pancreatic juice. They also identified a number of amino acid transporter classes in rat pancreas, including the excitatory amino acid transporter only in acinar cells and LAT-1 only in islets. Our results (Table 1) support their suggestion that glutamate derived from glutamine is transferred to extracellular pools such as the pancreatic juice. To counter a decrease in tissue glutamate owing to treatment with \(\text{l-valine, the amino group of valine is available to transaminase \(\alpha\)-ketoglutarate to glutamate by action of the BCAT, well studied by Sweatt et al. (38). Lacking the amino group of valine, \(\alpha\)-ketoisovalerate cannot compensate similarly. A second question is whether control of the free glutamate concentration is related to other pancreatic components and functions. One example of such influence is in the synthesis of glutathione (GSH), reported to occur in rat pancreatic acinar cells (31) as in most other cell types (23, 42). Glutathione (\(\gamma\)-glutamyl-cysteinyl-glycine) has essential protective and metabolic functions in the pancreas, including detoxification of noxious oxygen radicals (37) and, in its oxidized...
form (GSSG), as a cofactor in the organized folding of zymogen secretory proteins (17, 31). Formation of GSH in the pancreas is limited by the availability of cysteine and by the initial, rate-limiting synthesis of γ-glutamyl-cysteine mediated by the glutamate cysteine ligase (EC 6.3.2.2.). Elegant biochemical studies of this ligase show that it is a heterodimer of a heavy and a light component encoded by different genes (2, 5, 13, 23). The isolated heavy subunit has catalytic activity, but the $K_m$ for glutamate, 12.4 mM (5, 13), considerably exceeds the tissue free glutamate concentration. The light subunit is not active catalytically, but it lowers the glutamate $K_m$ of the holoenzyme to 1.4 mM, within the homeostatically regulated tissue concentration range. Regulation of the tissue free glutamate concentration to $\sim1$–3 μM/g thus maintains a lower value that is sufficient to ensure enzyme activity and a higher value that requires the evolution of a modifier subunit.

Homeostatic control of the extracellular glutamate concentration in brain is essential for its signal transmitter function and has received considerable attention. Two models for this control, the “buffer” model of Yudkoff (41) and the “shuttle” model of Hutson et al. (15), have been described. The buffer model relies on the near-equilibrium concentrations of the components of the reversible BCAT reaction in a single cellular compartment. The shuttle model takes into account the involvement of the astrocyte glial and the neuronal compartments and the glutamate-glutamine cycle of neuronal tissues. Further studies are required to determine whether these or other models best account for the regulation of tissue free glutamate concentration in rat pancreas and aorta.

In summary, this report characterizes features of the Leu$\rightarrow$Glu pathway of rat pancreas studied in vitro. The studies demonstrate that net glutamate formation from leucine at its concentration in normal rat plasma, $\sim0.2$ mM, utilizes predominantly the leucine carbons, rather than its α-amino group. Furthermore, the stoichiometry of the conversion of leucine carbons to glutamate was characterized, the localization of the Leu$\rightarrow$Glu pathway to the acinar portion of the pancreas was demonstrated, the regulation of pancreatic tissue free glutamate concentration was shown, and the ability of certain amino acids to inhibit the Leu$\rightarrow$Glu pathway was described. Because the inhibition owing to l-valine and several leucine esters can occur without decreasing the total free glutamate concentration, it is feasible to explore unique functions of the specific Leu$\rightarrow$Glu pathway in pancreatic physiology.