Effect of the Exogenous Amino Acid Concentration on the Rate of Gluconeogenesis in Liver Slices[1] (35857)

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(Introduced by J. H. Annegers)
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Glucogenic amino acids are the primary substrate of gluconeogenesis, providing 57% of the carbon used for hepatic *de novo* glucose production (1). Alteration of the availability of this substrate may be an important contributor to the regulation of the rate of gluconeogenesis. Growth hormone (2), insulin (3), and glucocorticoids (4), through their influences on protein synthesis and catabolism, are capable of altering amino acid availability and thereby indirectly regulating gluconeogenesis. These, and other hormones of course, exert certain direct influences on hepatic gluconeogenesis as well. Increases in plasma amino acids of up to twice fasting levels have been reported following ingestion of a high protein meal (5), further emphasizing the potential role of this source of regulation.

With the identification of these indirect and direct contributors to gluconeogenic regulation it is desirable now to determine which of these factors are of greatest importance under what conditions, and which are of only experimental interest. Moreover, to establish a clear understanding of the relationships between the gluconeogenic process and the other processes concerned with regulation of nutrient supply and utilization, a quantitative knowledge of the gluconeogenic process must be sought. This knowledge must define the cause–effect relationships involved, or, in this case, the relationship between the rate of gluconeogenesis and the presence of the assorted gluconeogenic regulators.

Such quantitative information, obtained under steady state conditions within the *in vivo* range of the variables involved, is not available in the literature. Therefore, the purpose of this investigation was to begin by defining the steady state relationship between extracellular alanine concentration and the rate of gluconeogenesis in rat liver slices. The nature of this relationship is to be expressed by an algebraic formula. Furthermore, since a sizable fraction of the alanine taken up by the liver is oxidized, the influence of extracellular amino acid concentration on its rate of oxidation is also sought.

Methods. Male white rats (Holtzman; 75–150 g), fed *ad libitum*, were sacrificed by decapitation, and the livers were removed as quickly as possible for slicing. Slices of uniform thickness (0.3 mm) were obtained with a tissue slicer consisting of a holder in which double edged razor blades were separated by plastic shims. Slices were removed from the slicer immediately and placed in incubation flasks containing 10 ml of incubation medium previously equilibrated to 37°. The flasks were oxygenated for 5 min, the system was closed, and the 1 hr incubation period was started.

The incubation medium was Krebs–Ringer phosphate buffer containing 100 mg/100 ml of glucose, alanine (varied from 1–9 mg/100 ml), and tracer U-^{14}C-alanine (24 μCi/liter). The flasks, shaken continually in a Dubnoff shaker, were sealed with rubber stoppers from which a disposable plastic centerwell containing 2% KOH was suspended for CO_2 collection.

Following incubation, the medium was analyzed for both total glucose (Technicon, Auto Analyzer ferricyanide method) (6) and ^{14}C-labeled glucose. The quantity of glucose

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produced from alanine via gluconeogenesis was calculated from the sp act of the alanine before incubation and the radioactivity of the glucose in the incubation medium following incubation. The latter was separated from labeled alanine by thin-layer chromatography on Eastman silica gel chromatogram sheets. The sheets were developed in an Eastman chromatogram developing apparatus with a *n*-propanol, methyl ethyl ketone, and water solvent (7:1:2). Following a 3-hr migration period, the glucose and alanine were visualized with aniline–phthalate and ninhydrin sprays, respectively. Uniform portions of the chromatogram containing the glucose were cut out and counted in a liquid scintillation counter. In order to determine if any alanine contamination of the glucose existed after migration, several solutions containing the various combinations of labeled or cold glucose and alanine were chromatographed. No activity was found in the glucose spot when a solution of ^{14}C-alanine and cold glucose was tested, and no activity was found in the alanine spot when ^{14}C-glucose and cold alanine were chromatographed indicating no contamination of glucose by alanine and *vice versa*. Strip counting following migration of solutions containing either ^{14}C-alanine or ^{14}C-glucose indicated that all the activity from both substances were present in the discrete spot indicated by the visualization sprays.

The quantity of carbon dioxide produced by oxidation of exogenous alanine was calculated from the radioactivity of the CO_2 trapped in the KOH from the center well and the sp act of the buffer before incubation. The KOH was counted by placing the entire center well in a counting vial with liquid scintillation fluid and Cab-O-Sil.

Since a time lag existed between the release of CO_2 by the tissue and its eventual appearance in KOH, it was necessary to either mathematically correct for the lag or eliminate the effect of the lag by acidifying the medium at the conclusion of incubation. Acidification sufficient to immediately release all carbonates as gaseous CO_2 interfered with the chromatographic separation of glucose and alanine after neutralization. Therefore, accessory experiments were conducted to determine the proper mathematical correction. The time course of labeled CO_2 trapping was studied in flasks following an injection of $Na_2{}^{14}CO_3$ into the incubation medium. The trapping process was found to be exponential with a time constant of 79 min. As shown in the Appendix, only 30% of the CO_2 actually produced during a 60-min incubation period could be trapped in the centerwell. The quantities of $^{14}CO_2$ measured were therefore multiplied by 3.33 to obtain quantities produced.

Results. Preliminary experiments to determine the duration of constancy of tissue viability and nutrient concentrations, mandatory conditions for steady state experimentation, were conducted as described before with the exception that the flasks were affixed to a Warburg respiration apparatus; oxygen consumption rate, an index of tissue viability, remained constant for at least 4.5 hr. The production rate of labeled CO_2, measured in flasks incubated for various times, was constant during at least 3 hr. Labeled glucose production, however, seemed to falter somewhat after 60 min. Therefore, all further incubations were conducted for a 1-hr period. Although relatively large volumes of incubation medium (10 ml) were employed to minimize changes in glucose or alanine concentrations due to production or utilization, the glucose concentration of the medium increased from 100 $\pm$ 1 mg/100 ml to from 100 to 132 mg/100 ml after 1 hr. The increase was proportional to the wet weight of tissue and was likely due to glycogenolysis. Alanine concentration remained within 2.2% of its initial level after 1 hr as determined from alanine-^{14}C activity.

To study the rate of glucose production at various alanine concentrations, slices from each rat used were incubated in media containing 1, 2, 3, 4, 5, 6, 7, 8, or 9 mg of alanine/100 ml. Following a 1-hr incubation period, the glucose in the medium and the CO_2 in the center well were analyzed for radioactivity. Linear covariance analysis of the data relating alanine concentration and rate of labeled glucose production revealed no significant difference between the slopes ($p>.25$), but there was a significant difference

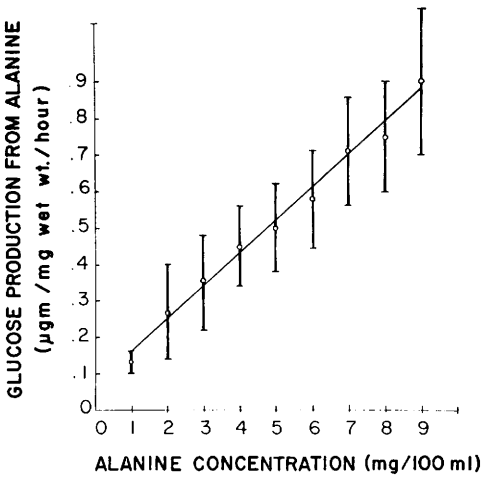

FIG. 1. The rate of gluconeogenesis as a function of the extracellular alanine concentration: Rate of gluconeogenesis = 0.071 + 0.090 [alanine]; (s_b = 0.0084, $p<.001$); (O) the mean; and (brackets) standard error.

between the adjusted mean glucose production for each animal. Tests for curvature were negative, therefore the difference between adjusted means probably is indicative of differences in basal values between rats.

The data from the 9 rats were pooled and a single regression equation was determined (Fig. 1). Over the range of alanine concentrations employed, the rate of gluconeogenesis (μg/mg wet wt/hr) was linearly related to the alanine concentration, and rose significantly with increases in the alanine concentration: Rate of gluconeogenesis = 0.071 + 0.090 [alanine]. Tests for significance found the slope significantly different from zero ($p<.001$) and the intercept not significantly different from zero (t = 1.58, df = 73).

Over the range of alanine concentrations employed the rate of alanine oxidation was also linearly related to the alanine concentration in the medium. Figure 2 shows the rate of production of CO_2 from alanine by liver slices from 7 rats. Although covariance analysis revealed slight differences between rats in the relationship of labeled CO_2 production to alanine concentration, the data of all rats were pooled to obtain an average estimate of the relationship:

Rate of labeled CO_2 production (μl/mg/hr) = 0.0096 + 0.014 [alanine].

There is evidence in the literature for a direct inhibition of gluconeogenesis by high plasma glucose concentraton (7, 8). Since the final glucose concentration of the medium did vary slightly, a multiple linear regression analysis was run on both the gluconeogenesis data and the alanine oxidation data to assess the effect of variation in the glucose concentration. No significant effect of glucose concentration was detected, perhaps due to the limited range of glucose concentrations involved.

Discussion. The steady state rate of gluconeogenesis from exogenous alanine by the rat liver slice is linearly related to the exogenous alanine concentration over a range of alanine concentrations from 1 to 9 mg/100 ml, with no evidence of saturation. In addition to the amino acid converted to glucose, a constant fraction is oxidized to CO_2, accounting for 5.98% of the total oxygen consumption at an alanine concentration of 9 mg/100 ml.

Information on the effect of changes in the amino acid concentration on the rate of gluconeogenesis in perfused rat livers has been reported by two investigators. Herrera *et al.* (7) reported a greater rate of gluconeogenesis with an initial perfusate alanine concentration of 89 mg/100 ml than with 44.5 mg/100 ml alanine in a recirculating system

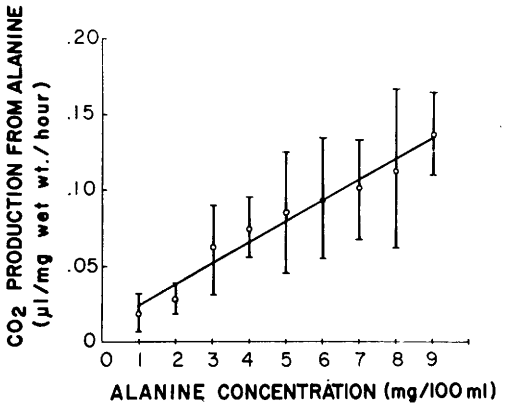

FIG. 2. The rate of carbon dioxide production from exogenous alanine as a function of the alanine concentration: CO_2 production = 0.0096 + 0.014 [alanine]; (s_b = 0.0019, $p<.001$); (O) the mean; and (brackets) standard error.

which did not permit steady state operation. Both production rates were much below those predicted by extrapolation of the above equation. Mallette *et al.* (10) reported parabolically increasing rates of gluconeogenesis as perfusate alanine concentration was increased from 4 to 80 mg/100 ml in a nonrecirculating system. Still higher alanine concentrations, up to 140 mg/100 ml, did not produce further increases in the rate of gluconeogenesis. Those experiments covered a much greater range of alanine concentration than the present slice experiments, which were carried out within the alanine concentration range encountered *in vivo*. The only gluconeogenic rate data obtained at a comparable alanine concentration, 4 mg/100 ml, were only 10% of those found in the slices. The reason for this discrepancy is not apparent.

Since the rate of oxidation of alanine to CO_2 was proportional to the alanine concentration, the ratio of alanine oxidized to CO_2 to alanine converted to glucose (0.13:1.0) was constant. Therefore, over the range of alanine concentrations used in these experiments, the increased rate of gluconeogenesis seen with increased alanine concentration was not a result of decreasing the fraction of available alanine which was directly metabolized to CO_2 by the tissue.

The equation obtained from this investigation indicates that the threefold increase in plasma alanine concentration, which has been shown to follow a 5-g high protein meal in rats (11), could result in a 172% increase in the rate of glucose production. Thus, the ability of other factors to indirectly influence the rate of gluconeogenesis by altering availability of amino acids is potentially an important part of gluconeogenic regulation.

The cooperation of these various direct and indirect regulators is indicated in Fig. 3, which shows, in diagrammatic form, the functional relationships involved. The availability of amino acid, expressed as a homogeneous extracellular concentration, is determined by the relative rates at which these amino acids are added to and removed from the free amino acid pool of the body, and the volume of this extracellular pool. The pool is in reality

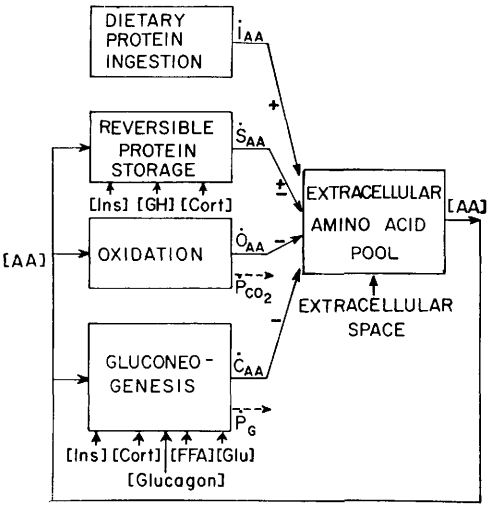

FIG. 3. Functional diagram indicating direct and indirect gluconeogenic regulation: [AA], extracellular amino acid concentration; [Ins], insulin concentration; [GH], growth hormone concentration; [Cort], corticosteroid concentration; [FFA], free fatty acid concentration; [Glu], glucose concentration; $\dot{I}_{AA}$, rate of amino acid ingestion; $\dot{S}_{AA}$, rate of amino acid storage; $\dot{O}_{AA}$, rate of amino acid oxidation; $\dot{C}_{AA}$, rate of amino acid conversion; $\dot{P}_G$, rate of glucose production; $\dot{P}_{CO_2}$, rate of CO_2 production.

an integration process. Ingestion of protein adds to the pool, gluconeogenesis and amino acid oxidation remove from the pool, and protein storage may either add to or take from the pool. The rates at which some of these four processes contribute or remove amino acids are in turn partly dependent upon the concentration of amino acids, as well as the presence or absence of certain hormones. Growth hormone enhances the storage (2), while insulin inhibits (3) and glucocorticoids facilitate (4) the depletion of protein stores. Glucagon (12, 13) and glucocorticoids (14) act directly on liver to stimulate gluconeogenesis, while insulin directly inhibits the regenesis of glucose from lactic acid; its direct action on gluconeogenesis from amino acid is uncertain. Increases in plasma free fatty acids (7) and glucose (8) appear to stimulate and inhibit gluconeogenesis respectively, probably acting directly on the liver.

Thus, substrate supply may be considered a primary gluconeogenic regulator, which can

focus the influence of several indirect factors on the gluconeogenic mechanism. The quantitation of this primary gluconeogenic regulator now makes quantitative exploration of the other mechanisms of regulation possible.

Summary. The steady state rate of gluconeogenesis in rat liver slices incubated in U-^{14}C-alanine was linearly related to the extracellular alanine concentration over a range of 1–9 mg of alanine/100 ml. The rate of oxidation of alanine to CO_2 was also linearly related to the extracellular alanine concentration and the ratio of alanine oxidized to CO_2 to the alanine converted to glucose was constant with changes in the alanine concentration.

Appendix: CO_2 trapping efficiency correction. Since the quantity of labeled carbon dioxide found in the centerwell of closed incubation flasks following a test injection (step forcing) of $Na_2{}^{14}CO_3$ increased exponentially with time, the trapping mechanism was a first order process (15). During incubation the liver slice produced carbon dioxide continuously, therefore, under experimental conditions the first order carbon dioxide trapping process was subjected to a ramp, rather than a step forcing. The equation, predicting the fraction of released $^{14}CO_2$ that would be trapped in the centerwell, is that of a ramp response of a first order process:

F = fraction of $^{14}CO_2$ recovered = $1/t[t\text{-}\tau(1\text{-}e^{-t/\tau})]$, where: τ is the time constant of 79 min determined from the test experiment; t is the incubation period of 60 min; e is the base of natural logarithms. The equation predicts that at the end of a 60-min incubation period only 30% of the total carbon dioxide released during that time must have been trapped in the centerwell:

$$F = 1/60\ [60 - 79\ (1\text{-}e^{-60/79})] = 0.30.$$

Therefore, the $^{14}CO_2$ data obtained must be corrected by multiplying by 3.33.

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