Lack of Inhibition by L-Tryptophan or Quinolinate of Gluconeogenesis in Diabetic Rats*

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FREDERICK L. ALVARES AND PAUL D. RAY†

From the Guy and Bertha Ireland Research Laboratory, Department of Biochemistry, University of North Dakota School of Medicine, Grand Forks, North Dakota 58201

SUMMARY

Administration of L-tryptophan to normal fed rats inhibits hepatic gluconeogenesis at the level of P-enolpyruvate formation but nonetheless causes a paradoxical increase in the assayable specific activity of P-enolpyruvate carboxykinase. Similar results obtain if livers isolated from normal rats are perfused with tryptophan, 3-hydroxyanthranilate or quinolinate.

However, we find that no such inhibition of gluconeogenesis occurs in intact, fed, alloxan- or streptozotocin-diabetic rats following treatment with tryptophan or 3-hydroxyanthranilic acid, nor is there any increase in the already elevated assayable specific activity of P-enolpyruvate carboxykinase. Furthermore, perfusion of livers isolated from alloxan- or streptozotocin-diabetic rats with tryptophan, 3-hydroxyanthranilate or quinolinate does not produce a metabolite crossover between P-enolpyruvate and its precursors nor is there any significant enhancement of assayable specific activity of P-enolpyruvate carboxykinase. Additionally, perfusion of diabetic livers with tryptophan or quinolinate does not inhibit the conversion of radioactive lactate to glucose although such inhibition does occur in livers from normal rats.

The results suggest that tryptophan and certain of its metabolites, including quinolinate, are unable to inhibit gluconeogenesis in diabetic rats under conditions known to cause such inhibition in normal rats.

A convincing body of evidence demonstrates that L-tryptophan is capable of inhibiting hepatic gluconeogenesis in normal rats (2-6), but it has been found to enhance the assayable specific activity of a key gluconeogenic enzyme, P-enolpyruvate carboxy-

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† To whom reprint requests should be addressed.
3-hydroxyanthranilic acid (38 mg per ml) were suspended in 0.9% NaCl and administered intraperitoneally at a dose of 3.7 mmole per kg body weight. Controls received an appropriate volume of vehicle. Three or 4 hours after injection, all rats were killed and portions of their livers removed and quick frozen in a tissue press precooled in liquid N2. Perchloric acid extracts of frozen liver samples were prepared and stored as previously described (6). Levels of hepatic metabolites (except citrate) were assayed by enzymatic methods which employ either the oxidation of NADH or the reduction of NAD (22). Citrate was assayed according to Moeljering and Gruber (23). Liver samples for assay of enzyme activity were removed concomitantly with samples taken for metabolite determinations. Supernatant fractions were prepared and assayed for P-enolpyruvate carboxykinase according to Nordlie and Lardy (24) as modified by Foster et al. (7, 25). Protein concentrations in the supernatant fractions were determined by the Biuret method (26).

Technique of Liver Perfusion—Livers were isolated from 12-hour fasted rats (under light ether anesthesia) and the blood was allowed to flow for 5 min. The liver was removed, weighed, and immersed in 37°C Krebs-Ringer bicarbonate buffer, pH 7.45, consisting of rat erythrocytes suspended in Krebs-Ringer bicarbonate buffer (30) containing 3% bovine serum albumin (Cohn Fraction V, Sigma Chemical Company) and oxygenated with 95% O2 and 5% CO2.

Protocol for Liver Perfusion Experiments—Livers were "preperfused" for 30 min to permit their equilibration. All lobes flushed out rapidly and resumption of bile flow was almost immediate. Perfusate flow rates were maintained at 3.5 ml per min per g of liver.

Following the "preperfusion" period, an aliquot of perfusate (usually 0.2 ml) was removed for determination of glucose. Perfusion was subsequently sampled at 10-min intervals. At 30 min after start of the experiment, a second sample of tissue were taken from the left lobe. One sample was rapidly homogenized (within 10 s) in 5% perchloric acid, processed, and assayed for metabolites as described above. The other sample was homogenized in ice-cold 0.25 M sucrose and centrifuged at 105,000 g for 1 hr, and the supernatant fraction was assayed for P-enolpyruvate carboxykinase as described above.

When l-tryptophan, 3-hydroxyanthranilic acid, or quinolinic acid was added to the perfusate, such addition (unless otherwise stated) was made immediately after removal of tissue samples (that is, at 30 min). Tryptophan was dissolved in 0.9% NaCl; 3-hydroxyanthranilic acid or quinolinic acid were added as their sodium salts at neutral pH; all were added to the perfusate to a final concentration of 2.4 μM. Unless indicated otherwise, sodium L-lactate was added 90 min after the start of the experiment to a perfusate concentration of 20 mM. In experiments involving radioactive lactate, a tracer dose of sodium [3-3H]lactate (50 μCi total radioactivity with a specific radioactivity of 20,000 ± 29 mCi per mmole) was mixed with the unlabeled sodium L-lactate.

All additions were made in 5 ml or smaller volumes. Two samples of tissue were removed from the median lobe at 120 min of perfusion (that is, 60 min after addition of lactate and 90 min after addition of tryptophan or a metabolite thereof). Soon after, fructose was added to a perfusate concentration of 10 mM, and its conversion to glucose was used as an additional criterion of viability of the liver, particularly in cases where glucose production from lactate had been blocked. Subsequently, the experiment was terminated, and the liver was weighed.

Four criteria of viability of perfused livers are, in order of importance: (a) glucose production from lactate and fructose, or both; (b) constant perfusate flow rate; (c) general macroscopic appearance of the liver (compared to its appearance in the living animal); and (d) production of bile. There was no evidence of localized or generalized anoxia or visible edema in livers perfused for 4 hours. Terminal pH measurements on the perfusate ranged from 7.3 to 7.6. Perfusion flow rate was constant; although a slight fall in flow rate was observed after removal of tissue samples, the original rate was rapidly regained. The hematoctit did not decrease by more than 1%. Bile was produced at a rate similar to that reported by others (61); more importantly, perhaps, the rate of bile production by any given liver remained constant.

Analysis of Perfusate Glucose—Glucose was determined by the glucose oxidase method (Glucostat, Worthington Biochemical Corp.). Radioactive glucose in the perfusate was separated from its labeled precursor (lactate) and other charged products by chromatography on AG-1-X8, chloride form, 200 to 400 mesh (Bio-Rad Laboratories, Richmond, Calif.). This procedure was found to be reliable in accurate determination of radioactive glucose in the sample. Aliquots of the eluate were counted in a liquid scintillation spectrometer. Quenching, determined with the internal standard [7-14C]benzoic acid, was less than 5%. Values for radioactivity appearing in perfusate glucose are adjusted to take into account the wet weight of liver at the time of perfusate sampling; the results are thus expressed as radioactivity in glucose produced per g of perfused liver.

Materials—Bovine serum albumin (Cohn Fraction V) powder, heparin (sodium salt, Grade I, 165 USP units per mg), 3-hydroxyanthranilic acid, and l(+)-lactic acid (30% solution Grade L-III) were obtained from Sigma Chemical Company. St. Louis, Mo. Quinolinic acid (99 to 100% pure obtained from Sigma) was reconstituted once from 1 M acetic acid. l-Tryptophan was from Mann Research Laboratories (Division of Becton, Dickinson and Company) New York, N. Y., Purified enzymes were from Bio-Ringer Mannheim Corporation, New York, N. Y., and nucleotides were from P-L Biochemicals, Inc., Milwaukee, Wisc. 3-[3H]-Lactic acid (sodium salt) was purchased from Amersham-Searle, Arlington Heights, Ill. Streptozotocin (Lot 9548-1392-21, U-2898) was a generous gift from Dr. W. A. Dulin, The Upjohn Company, Kalamazoo, Mich.

All chemicals were of analytical reagent grade and solutions were prepared in deionized, glass-distilled water.

Expression of Results—Values are expressed as means ± 1 S.D. The significance of differences between means was established by the Student's t test. Values for p > 0.05 are not considered significant.

RESULTS

Differential Responses of Intact Normal versus Diabetic Rats to l-Tryptophan or 3-Hydroxyanthranilic Acid

Effects on Hepatic Gluconeogenic Metabolites—Accumulations of intermediary metabolites in fed normal rats treated with tryptophan were initially compared to those found in fed alloxan-diabetic rats similarly treated. Fig. 1 depicts the results obtained with normal fed rats. Accumulations of citrate, aspartate, and malate to levels of 6-, 5-, and 9-fold, respectively, are concomitant with subnormal levels of P-enolpyruvate and 3-phosphoglycerate. These data indicate a crossover-type phenomenon similar to that previously reported by Ray et al. (5). In contrast, no accumulation of hepatic metabolites or a metabolite crossover is seen following administration of either tryptophan or 3-hydroxyanthranilic acid to fed alloxan diabetic rats (Fig. 2). Although data are not presented, there is a similar absence of hepatic metabolite accumulation and of a metabolite crossover in fed streptozotocin-diabetic rats treated with either tryptophan or 3-hydroxyanthranilic acid.

These data indicate that in the fed, intact normal rat, trypto-
Effects on Assayable Specific Activity of Hepatic P-enolpyruvate Carboxykinase—It has previously been shown that the assayable specific activity of this enzyme from livers of alloxan-diabetic rats is approximately 4-fold higher than that of normal rat livers (2, 25). Additionally, the paradoxical increase in the specific activity of P-enolpyruvate carboxykinase which occurs following tryptophan or 3-hydroxyanthranilate treatment of normal, fasted rats (2, 3) does not occur upon administration of tryptophan to alloxan-diabetic rats (2).

In view of the failure of either tryptophan or 3-hydroxyanthranilic acid to elicit the phenomena of hepatic metabolite accumulation and crossover in either alloxan- or streptozotocin-diabetic rats, we investigated further the effects of these two compounds on the assayable specific activity of hepatic P-enolpyruvate carboxykinase of normal and diabetic rats. Data in Table I show that administration of either tryptophan or 3-hydroxyanthranilic acid causes a 2.5- to 3-fold elevation in the specific activity of this enzyme in normal rats but neither compound elicits a significant increase in carboxykinase activity from alloxan- or streptozotocin-diabetic rats. Thus our data confirm and extend those previously reported (2).

The abbreviations used are: L-Trp, L-tryptophan; 3-HAA, 3-hydroxyanthranilic acid.

### Table I

<table>
<thead>
<tr>
<th>Condition</th>
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<th>L-Trp</th>
<th>3-HAA</th>
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<tr>
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<td>56.5</td>
<td>164</td>
</tr>
<tr>
<td>Normal</td>
<td>3</td>
<td>63</td>
<td>170</td>
</tr>
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<td>243 ± 25</td>
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<tr>
<td>Streptozotocin-diabetic</td>
<td>4</td>
<td>243 ± 19</td>
<td>250 ± 61</td>
</tr>
</tbody>
</table>

* The abbreviations used are: L-Trp, l-tryptophan; 3-HAA, 3-hydroxyanthranilic acid; PEP, P-enolpyruvate; SPG, 3-phosphoglycerate.

- Four hours prior to killing, 3.7 mmole of inhibitor per 200 g body wt were given intraperitoneally.
- Data taken from Ref. 2.
- Data taken from Ref. 3.
- Significant difference from control.

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**Effect of Tryptophan and Its Metabolites on Gluconeogenesis by Perfused Livers Isolated from Normal or Diabetic Rats**

### General Characteristics of Perfused Livers

In the absence of exogenous substrate, glucose production averaged 11 pmoles per hour per g of liver wet weight. Following 60 min of perfusion without substrate, the addition of 20 mM L-lactate caused an increase in the mean rate of glucose formation to 32 pmoles per hour per g of liver. Addition of 10 mM fructose affected an even further increase in the rate of gluconeogenesis to an average of 75 pmoles per hour per g of liver. The rates of gluconeogenesis from 20 mM lactate in livers from diabetic rats are from 1.5 to 2 times higher than those obtained with livers from normal rats.

### Changes in Concentration of Metabolites in Perfused Normal or Diabetic Livers

Hepatic metabolite levels in normal rat liver perfused with lactate in the presence of previously added tryptophan or quinolinate are shown in Fig. 3. Compared to control values (obtained from livers perfused with lactate only), concentrations of citrate, aspartate, and especially malate are elevated concomitant with decreases in the levels of P-enolpyruvate and 2- and 3-phosphoglycerate. Although not shown, a similar pattern of metabolite accumulation and crossover is observed following perfusion with 3-hydroxyanthranilic acid.

**Effect on Specific Activity of P-enolpyruvate Carboxykinase**—Although perfusion of livers from normal rats for 60 min with either tryptophan, 3-hydroxyanthranilate, or quinolinate as opposed to vehicle causes statistically significant increases in the assayable specific activity of P-enolpyruvate carboxykinase, no such increase is seen following perfusion of livers from alloxan-diabetic rats with the same compounds. Similarly, no significant change is observed in carboxykinase activity following per-
metabolite, 2PG, 2-phosphoglycerate. See legend to Fig. 1 for either 2.4 mM L-tryptophan or quinolinate and for 60 min with 20 mM n-lactate. Control values for each metabolite obtained from livers perfused without inhibitor are recorded at the bottom of the figure with the standard deviations of the means. Experimental data are expressed as multiples of these control values. The number of perfusions contributing to each experimental point is shown in parentheses above the vertical line which indicates one standard deviation. The number of perfusions contributing to each control point is indicated in parentheses below the value for the particular metabolite. 2PG, 2-phosphoglycerate. See legend to Fig. 1 for definition of the other abbreviations.

Fig. 4 (right). Concentrations of glucogenic metabolites in livers isolated from alloxan-diabetic rats following perfusion for 90 min with either 2.4 mM L-tryptophan or quinolinate and for 60 min with 20 mM n-lactate. Control values for each metabolite obtained from livers perfused without inhibitor are recorded at the bottom of the figure with the standard deviations of the means. Experimental data are expressed as multiples of these control values. The number of perfusions contributing to each experimental point is shown in parentheses above the vertical line which indicates one standard deviation. The number of perfusions contributing to each control point is indicated in parentheses below the value for the particular metabolite. 2PG, 2-phosphoglycerate. See legend to Fig. 1 for definition of the other abbreviations.

Glucose Production from Isotopically Labeled Lactate by Normal or Diabetic Livers Perfused with Either Tryptophan or Quinolinate—Addition of tryptophan or quinolinate to the perfusate 30 min before the introduction of radioactive lactate greatly decreases the amount of label incorporated into glucose by perfused normal rat livers (Fig. 5). On the other hand, addition of either compound to the perfusate prior to the introduction of labeled lactate brings about a much less appreciable reduction in glucose synthesis by perfused livers from alloxan-diabetic rats (Fig. 6) or streptozotocin-diabetic rats (not shown).

When tryptophan or quinolinate is added to the perfusate 40 min after the introduction of radioactive lactate, the rate of isotope incorporation into glucose by perfused normal livers decreases and reaches a plateau (Fig. 7). In contrast, no such decrease in rate of label incorporation into glucose by alloxan-diabetic livers is seen when either tryptophan or quinolinate is added to the perfusate 40 min after the addition of labeled lactate (Fig. 8).

**DISCUSSION**

Tryptophan inhibits gluconeogenesis in intact normal rats and in their isolated livers by inhibiting the P-enolpyruvate carboxykinase-mediated conversion of oxalacetate to P-enolpyruvate (4, 5). Vennesale et al. (4) have concluded that quinolinic acid is actually responsible for the inhibition. Even though quinolinate inhibits the in vivo conversion of oxalacetate to P-enolpyruvate, it also paradoxically enhances the assayable specific activity of P-enolpyruvate carboxykinase well above its normal level (2, 4). Nevertheless, the observation by Foster et al. (2) that tryptophan does not alter the assayable specific activity of carboxykinase from alloxan-diabetic rats suggested that tryptophan might also be unable to inhibit gluconeogenesis in diabetic rats and prompted the experiments reported here.

The absence of a metabolite crossover in data from alloxan-diabetic rats presented in Fig. 2 indicates that neither tryptophan nor 3-hydroxyanthranilic acid can inhibit gluconeogenesis in intact alloxan-diabetic rats. The fact that similar data are obtained with both alloxan- and streptozotocin-diabetic rats suggests that the inability of tryptophan or 3-hydroxyanthranilic acid to inhibit gluconeogenesis in these animals relates to the diabetic condition rather than to the nature of the diabeticogenic agent used. Data in Table I indicate that neither tryptophan
nor 3-hydroxyanthranilate can significantly alter the assayable activity of hepatic P-enolpyruvate carboxykinase from specific activity of the enhanced glucose formation seen in diabetes mellitus and suggest that the two paradoxical effects seen after tryptophan treatment of normal rats may be directly related and thus inseparable. Such a suggestion is consistent with the explanation of these paradoxical effects by Snoke et al. (8).

Data presented in Figs. 4, 6, and 8, as well as additional data not presented, all indicate that neither tryptophan nor 3-hydroxyanthranilate can inhibit gluconeogenesis nor can they significantly alter the in vitro assayable activity of P-enolpyruvate carboxykinase in livers isolated from either alloxan- or streptozotocin-diabetic rats. Data obtained with intact diabetic rats and their isolated livers are thus in good agreement.

Many of our results would appear to be explainable on the basis of reports that tryptophan metabolism is aberrant in both alloxan-diabetic (18-21) and depancreatized rats (19, 32-34) and, interestingly, also in alloxan-diabetic rabbits (35) and in humans with diabetes mellitus (36). For example, urinary excretion of N'-methylbiotinomide is diminished in alloxan-diabetic (18) and depancreatized rats (32) and there is a decreased production of 3-hydroxyanthranilate and quinolinate by liver slices from depancreatized rats (34). Furthermore, the activity of picolinic carboxylase which shunts tryptophan metabolism toward glutarate and away from quinolinate is substantially elevated in alloxan-diabetic (19-21) and depancreatized rats (19, 33). Thus, in intact diabetic rats, tryptophan metabolism could be sufficiently aberrant to diminish the amount of quinolinate produced from tryptophan to levels below inhibitory concentrations. If so, presentation of quinolinate to isolated diabetic livers should result in inhibition of gluconeogenesis and enhancement of the in vitro specific activity of carboxykinase.

However, as is apparent from a comparison of metabolite data presented in Fig. 4 versus those in Fig. 3, quinolinate also does not inhibit gluconeogenesis (based on the absence of a metabolite crossover) in alloxan-diabetic rats (or for that matter in streptozotocin-diabetic rats). A similar conclusion is arrived at on the bases of data presented in Figs. 6 and 8, which indicate that addition of quinolinate, either prior to or subsequent to addition of labeled lactate, exerts little if any effect on isotope incorporation into perfusate glucose by diabetic livers as opposed to quinolinate's inhibition of labeled lactate's conversion to glucose in normal livers (Figs. 5 and 7).

These latter data preclude the explanation that the inability of tryptophan to inhibit gluconeogenesis in diabetic rats is simply due to its aberrant metabolism. Nonetheless, the reasons for the inability of quinolinate to inhibit glucose formation in isolated perfused livers are not readily apparent at this time.

Our observations suggest the intriguing possibility that if tryptophan does play a role in the regulation of gluconeogenesis in the normal rat (as has been suggested (5)), then alteration or loss of its role in the diabetic rat could be at least partially responsible for the enhanced rate of glucose formation seen in diabetes mellitus. After examining the effects of quinolinate in isolated perfused rat, guinea pig, and pigeon livers, Soling et al. (37) have concluded that it inhibits only cytosolic P-enolpyruvate carboxykinase activity. Thus, if tryptophan normally participates in regulation of glucose synthesis in other species (such as the human) which possess cytosolic carboxykinase, then loss or modification of its regulatory role could be at least partially responsible for the enhanced glucose formation seen in diabetes mellitus.
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