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### Research Article

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# Glucagon-Stimulating Activity of 20 Amino Acids in Dogs

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**ABSTRACT** The effect of 20 L-amino acids upon pancreatic glucagon secretion has been studied in conscious dogs. Each amino acid was administered intravenously over a 15 min period in a dose of 1 mmole/kg of body weight to a group of four or five dogs. Pancreatic glucagon and insulin were measured by radioimmunoassay. 17 of the 20 amino acids caused a substantial increase in plasma glucagon. Asparagine had the most glucagon-stimulating activity (GSA), followed by glycine, phenylalanine, serine, aspartate, cysteine, tryptophan, alanine, glutamate, threonine, glutamine, arginine, ornithine, proline, methionine, lysine, and histidine. Only valine, leucine, and isoleucine failed to stimulate glucagon secretion, and isoleucine may have reduced it. No relationship between glucagon-stimulating activity and insulin-stimulating activity was observed. The amino acids which enter the gluconeogenic pathway as pyruvate and, which are believed to provide most of the amino acid-derived glucose, had a significantly greater GSA than the amino acids which enter as succinyl CoA or as  $\alpha$ -ketoglutarate. However, pyruvate itself did not stimulate glucagon secretion. The R-chain structure of the amino acid did not appear to be related to its GSA, except that the aliphatic branched chain amino acids, valine, leucine, and isoleucine, were devoid of GSA.

## INTRODUCTION

The ingestion of a protein meal is accompanied in man by a prompt and substantial increase in the secretion of pancreatic glucagon (1, 2). Earlier studies in dogs had shown a similar glucagon response to the i.v. administration of a 10 amino acid mixture, thus establishing hyper-

aminoacidemia as an important stimulus of the pancreatic alpha cell (3). However, the effects of most of the individual amino acids upon pancreatic glucagon secretion have not been studied; arginine has been reported to stimulate glucagon secretion in humans (4-6) and in dogs (7), and alanine (8) and histidine (7) in dogs. Alanine, arginine, valine, leucine, tryptophan, tyrosine, glycine, and glutamate have been studied in rats by Assan and Hanoune<sup>1</sup> and all were reported to stimulate glucagon in the large doses employed.

The present studies were designed to determine the relative glucagon-stimulating activity of each of 20 amino acids and their effect upon insulin secretion, and to search for relationships between the hormone-stimulating activities of the amino acids, their metabolic fates, and their chemical structures.

## METHODS

Each amino acid was administered at pH 7.4 to a group of four or five conscious dogs after an 18 hr fast in a dose of 1 mmole/kg of body weight in 80 ml of water<sup>2</sup> for 15 min through indwelling catheters previously implanted in a crural vein. One-quarter of the dose was given as a rapid priming injection, and the remaining three-quarters of the dose administered at a constant rate thereafter. For many of the amino acids, the dose employed probably exceeded the maximal amount of an individual amino acid ingested during a normal protein meal, and the concentrations produced are probably unphysiologically high. In the case of aspartate, tryptophan, glutamate, and cysteine, the dose employed induced vomiting in all dogs, possible evidence that toxic concentrations were attained.

Each dog was used for two amino acid experiments with an interval of at least 2 days between experiments.

The amino acids were purchased from Sigma Chemical

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<sup>1</sup> Assan, R., and J. Hanoune. 1972. Effects on rat plasma glucagon and glucagon-like immunoreactivity of eight amino acids (intra-gastric and intra-peritoneal loads); comparison with intra-gastric loads of six sugars. Submitted for publication.

<sup>2</sup> The amino acids tryptophan, aspartate, methionine, cysteine, phenylalanine, and leucine were relatively insoluble and were, therefore, administered in a volume of 160 ml.

Co., St. Louis, Mo.; ICN Nutritional Biochemicals Div., Cleveland, Ohio; Mann Research Labs, Inc., New York; Pfanstiehl Chemical Corp., Waukegan, Ill.; Fisher Scientific Company, Pittsburgh, Pa.; and pyruvate from Matheson Coleman & Bell, Norwood, Ohio.

A solution of each amino acid in a concentration of 0.1 M was tested to determine if it affected directly any of the measurements. Cysteine gave a reading of >300 mg/100 ml for glucose and 4  $\mu$ U/ml for insulin. Tryptophan gave a reading of 217 mg/100 ml for glucose. Otherwise, none influenced either the insulin or glucagon assays.

Blood specimens were obtained at frequent intervals before, during, and after the amino acid infusion from a previously implanted indwelling catheter reposing in the inferior vena cava. Pancreatic glucagon was measured by radioimmunoassay (9). The immunoassay system for glucagon employed in these studies was as follows: 15  $\mu$ g glucagon-<sup>125</sup>I,<sup>3</sup> 1000 U Trasylol<sup>4</sup> 0.2 ml of plasma sample or standard, and antiserum 30K, which is highly specific for pancreatic glucagon, at a final dilution of 1:60,000, in a total volume of 1.2 ml. The tubes were incubated for 4 days at 4°C. Free and bound glucagon-<sup>125</sup>I were separated with dextran-coated charcoal (10). Insulin was measured by the radioimmunoassay of Yalow and Berson (11), as modified by Herbert, Lau, Gottlieb, and Bleicher (10). Glucose was determined by the Hoffman ferricyanide method (12), using the Technicon Autoanalyzer.<sup>5</sup>

All dogs appeared to be well at the time of their experiment two days or more after the surgical placement of the indwelling catheters. Dogs with a temperature above normal (103°), leucocyte count above 30,000 per mm<sup>3</sup>, or a hematocrit below 35%, were not employed in these studies.

## RESULTS

**Glucagon-stimulating activity.** The mean levels of glucagon, insulin, and glucose before, during, and after the infusion of each of the 20 amino acids appear in Table I. Except for valine, leucine, and isoleucine, all of the amino acids tested appeared to stimulate a prompt rise in plasma glucagon. The significance of these changes is indicated for each amino acid in Table I.

In order to obtain a single value which would reflect the increase in glucagon secretion attributable to an amino acid infusion, the area above the baseline for 30 min after the start of the infusion was calculated in each experiment; the mean of the "areas" of each group of experiments was divided by the 30 min, to give a mean increment in plasma glucagon. This value is henceforth referred to as the "glucagon-stimulating activity" or GSA<sup>6</sup> of the amino acid.

The results are shown in Fig. 1, grouped in decreasing order of GSA. Alanine, regarded as a powerful glucagon-stimulating amino acid, had a GSA of 58.5 pg/ml (SEM  $\pm$  6.3) and ranked eighth in this scheme. Asparagine ap-

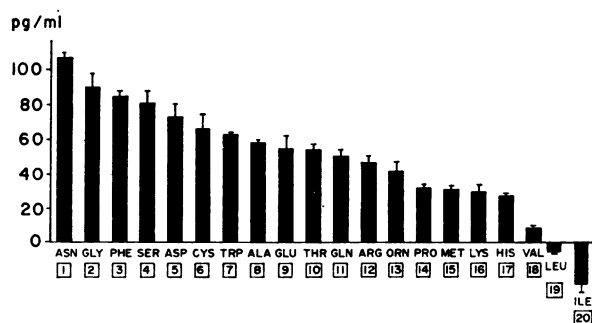


FIGURE 1 Glucagon-stimulating activity (GSA) of 1 mmole/kg of 20 L-amino acids in the dog in decreasing order of activity. Numbers refer to rank of GSA.

peared to be the most potent glucagon stimulator and histidine the weakest, while valine, leucine, and isoleucine were devoid of GSA. It is clear then, that in the doses employed 17 of the 20 amino acids examined had GSA ranging from 27 pg/ml (SEM  $\pm$  5) to 117 pg/ml (SEM  $\pm$  17).

**Insulin-stimulating activity (ISA).** In order to determine if a relationship between the glucagon-stimulating activity and the insulin-stimulating activity of these amino acids could be demonstrated, a calculation similar to that used for obtaining GSA was made for insulin-stimulating activity (ISA) of the 20 amino acids (Fig. 2). Only five amino acids, tryptophan, leucine, aspartate, isoleucine, and glutamate had ISA of 5  $\mu$ U/ml or greater. From the rank of each amino acid in terms of ISA and GSA (Fig. 2) it is apparent that no relationship between them exists, an impression which is born out by correlation analysis ( $r = -0.358$ ). Leucine and isoleucine, which rank very high as insulin stimulators, are devoid of GSA, while asparagine, serine, and alanine, which rank very high in GSA, are very weak insulin stimulators.

These results appear to differ in a quantitative sense from those of Floyd, Fajans, Conn, Knopf, and Rull obtained in humans (13) in terms of rank of ISA.

**Relationship between GSA and ISA of amino acids and their metabolic fates.** Because glucagon is widely regarded as a potent gluconeogenic hormone, an effort was made to determine if the GSA of the various amino acids bears a relationship to their participation in gluconeogenesis and thus supports a regulatory role for glucagon in this pathway. Fig. 3 separates the amino acids into groups according to the currently accepted point or points of entry of their carbon skeletons into metabolic pathways leading either to gluconeogenesis or to ketogenesis (14, 15). Five of the amino acids, threonine, glycine, alanine, serine, and cysteine, are metabolized directly to pyruvate, and pass via oxaloacetate to phospho-

<sup>3</sup> Glucagon was obtained from Cambridge Nuclear Corp., Cambridge, Mass.

<sup>4</sup> FBA Pharmaceuticals, New York.

<sup>5</sup> Technicon Corporation, Tarrytown, N. Y.

<sup>6</sup> Abbreviations used in this paper: GSA, glucagon-stimulating activity; ISA, insulin-stimulating activity.

TABLE I

The Effect of 20 Amino Acids and of Pyruvate (1 mmole/kg per 15 min) on Glucose, Insulin, and Glucagon

Amino Acid	Infusion (min)						Infusion (min)					
	Baseline	5	10	15	20	30	Baseline	5	10	15	20	30
<b>Alanine* (N = 4)</b>												
Glucose (mg/100 ml)	85	89†	96	97‡	95	91‡	11	21¶	25	30**	15	19‡
SEM ±	3	6	5	7	5	4	1	4	7	5	1	3
Insulin (μU/ml)	5	6	6	10	5	5	59	60	54	51	43**	60
SEM ±	1	3	2	7	2	4	8	19	17	19	17	18
Glucagon (pg/ml)	101	180‡	170‡	197¶	150‡	113						
SEM ±	17	35	32	32	33	36						
<b>Arginine (N = 4)</b>												
Glucose (mg/100 ml)	84	88	85	88	85	84						
SEM ±	1	2	2	3	2	3						
Insulin (μU/ml)	6	12†	8	7	5	5						
SEM ±	1	1	1	2	1	1						
Glucagon (pg/ml)	129	215‡	178‡	183‡	143	101						
SEM ±	13	43	34	37	24	14						
<b>Asparagine (N = 4)</b>												
Glucose (mg/100 ml)	76	79	83	81	86†	85						
SEM ±	5	6	8	7	7	7						
Insulin (μU/ml)	9	15	8	10	7	8						
SEM ±	1	3	1	1	1	1						
Glucagon (pg/ml)	197	287‡	397	377‡	338**	254						
SEM ±	12	14	80	60	30	19						
<b>Aspartate (N = 4)</b>												
Glucose (mg/100 ml)	79	83	88†	87	86	86						
SEM ±	6	10	6	9	4	8						
Insulin (μU/ml)	11	13	15	31†	22	19						
SEM ±	1	1	4	5	6	1						
Glucagon (pg/ml)	113	150	193	183	154	170						
SEM ±	16	30	43	26	33	30						
<b>Cysteine (N = 4)</b>												
Glucose (mg/100 ml)	85	82	91†	92	89	93						
SEM ±	5	10	6	9	11	5						
Insulin (μU/ml)	9	13	11‡	13	15	10						
SEM ±	1	2	1	3	5	1						
Glucagon (pg/ml)	150	203	268	251	200	200						
SEM ±	21	34	77	71	47	73						
<b>Glutamate (N = 4)</b>												
Glucose (mg/100 ml)	83	90	96†	101‡	102	100†						
SEM ±	4	8	7	7	6	3						
Insulin (μU/ml)	15	17	29	19	21	16						
SEM ±	2	5	8	3	6	3						
Glucagon (pg/ml)	83	121	149	140	141	137						
SEM ±	8	40	51	34	40	32						
<b>Glutamine (N = 5)</b>												
Glucose (mg/100 ml)	87	87	88	92	96¶	95						
SEM ±	2	4	4	1	5	5						
Insulin (μU/ml)	8	10	11	13‡	11	13						
SEM ±	1	2	2	1	1	2						
Glucagon (pg/ml)	91	110	135‡	162	153†	161						
SEM ±	10	20	27	37	30	36						
<b>Glycine (N = 4)</b>												
Glucose (mg/100 ml)	87	89	91	95	95	93						
SEM ±	2	3	5	4	3	3						
Insulin (μU/ml)	7	17	10	8‡	7	10						
SEM ±	1	4	2	1	2	2						
Glucagon (pg/ml)	158	212	337	330	199	161						
SEM ±	23	53	94	70	29	25						
<b>Histidine (N = 4)</b>												
Glucose (mg/100 ml)	87	90	90	91	93	94						
SEM ±	1	2	1	1	3	2						
Insulin (μU/ml)	9	9	10	9	10	10						
SEM ±	1	1	1	1	2	1						
Glucagon (pg/ml)	101	119	131	133	139	125						
SEM ±	13	24	34	26	25	32						
<b>Isoleucine (N = 4)</b>												
Glucose (mg/100 ml)	79	77	77	76	73	76						
SEM ±	2	4	2	4	3	4						
Insulin (μU/ml)	10	21	21†	14‡	13	11						
SEM ±	1	6	2	2	4	1						
Glucagon (pg/ml)	149	110	118	131	126	121						
SEM ±	16	18	14	16	11	15						
<b>Leucine (N = 5)</b>												
Glucose (mg/100 ml)	101	105	102	103	91	96						
SEM ±	3	7	7	6	5	5						
<b>Insulin (μU/ml)</b>												
Insulin (μU/ml)	11	21¶	25	30**	15	19‡						
SEM ±	1	4	7	5	1	3						
<b>Glucagon (pg/ml)</b>												
Glucagon (pg/ml)	59	60	54	51	43**	60						
SEM ±	8	19	17	19	17	18						
<b>Lysine (N = 4)</b>												
Glucose (mg/100 ml)	75	79	81	79	75	75						
SEM ±	2	3	5	2	5	3						
Insulin (μU/ml)	4	10	7	5	4	4						
SEM ±	1	2	1	1	1	1						
Glucagon (pg/ml)	107	147	154	161	109	105						
SEM ±	29	78	60	78	52	40						
<b>Methionine (N = 4)</b>												
Glucose (mg/100 ml)	82	85	83	87	85	89						
SEM ±	3	6	6	5	5	5						
Insulin (μU/ml)	8	10	15	12‡	9	10						
SEM ±	1	2	6	1	2	3						
Glucagon (pg/ml)	115	162	193	139†	125	119						
SEM ±	24	58	92	48	49	38						
<b>Ornithine (N = 4)</b>												
Glucose (mg/100 ml)	86	96**	97¶	93	98	97						
SEM ±	2	5	3	4	5	7						
Insulin (μU/ml)	10	18	14	10	11	9						
SEM ±	1	5	3	1	1	1						
Glucagon (pg/ml)	100	142	145	154	149	135						
SEM ±	15	42	43	54	47	50						
<b>Phenylalanine (N = 4)</b>												
Glucose (mg/100 ml)	85	97†	96¶	99**	90	89**						
SEM ±	3	11	7	7	5	6						
Insulin (μU/ml)	8	25¶	13	14	7	7						
SEM ±	1	2	1	3	1	1						
Glucagon (pg/ml)	83	229	293	170‡	118	105¶						
SEM ±	14	45	100	34	23	25						
<b>Proline (N = 4)</b>												
Glucose (mg/100 ml)	81	84	82	84**	84	84						
SEM ±	3	4	7	4	5	4						
Insulin (μU/ml)	8	16	10	12†	9	10						
SEM ±	1	4	2	2	1	1						
Glucagon (pg/ml)	128	160‡	163†	171	157	162						
SEM ±	16	34	38	38	32	36						
<b>Serine (N = 4)</b>												
Glucose (mg/100 ml)	84	89	91	92	89	91						
SEM ±	2	2	2	1	5	4						
Insulin (μU/ml)	13	26	12	11	9	7						
SEM ±	4	5	2	2	1	2						
Glucagon (pg/ml)	167	246†	304	290†	239	176						
SEM ±	23	53	77	53	75	35						
<b>Threonine (N = 4)</b>												
Glucose (mg/100 ml)	79	78	87†	87	86†	86‡						
SEM ±	2	2	2	4	3	2						
Insulin (μU/ml)	9	15	11	13	11	10						
SEM ±	1	3	2	4	5	3						
Glucagon (pg/ml)	80	137	159†	173**	133†	102						
SEM ±	10	32	34	29	31	30						
<b>Tryptophan (N = 4)</b>												
Glucose (mg/100 ml)	85	109¶	108¶	100¶	97¶	98†						
SEM ±	2	4	3	2	2	6						
Insulin (μU/ml)	10	56	27†	16	11	9						
SEM ±	1	15	5	3	1	1						
Glucagon (pg/ml)	172	353‡	275**	218†	194	183						
SEM ±	22	61	48	40	38	43						
<b>Valine (N = 4)</b>												
Glucose (mg/100 ml)	83	85	85	86	87	86						
SEM ±	3	5	5	5	6	6						
Insulin (μU/ml)	10	13	10	10	8	11						
SEM ±	1	2	3	1	1	2						
Glucagon (pg/ml)	109	119	114	121	122	115						
SEM ±	12	23	29	23	19	17						
<b>Pyruvate (N = 4)</b>												
Glucose (mg/100 ml)	77	73	77	79	79	81						
SEM ±	2											

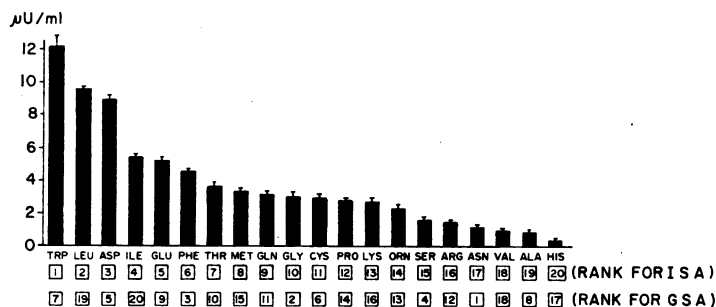


FIGURE 2 Insulin-stimulating activity (ISA) of 1 mmole/kg of 20 L-amino acids in the dog in decreasing order of activity.

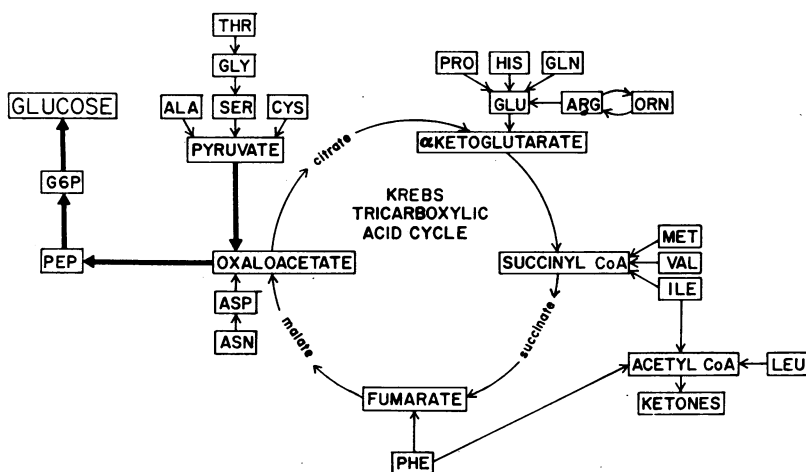


FIGURE 3 Entry points of carbon skeletons of amino acids into gluconeogenic and ketogenic pathways (14, 15).

enol pyruvate. According to Felig,<sup>7</sup> this group of amino acids, primarily alanine, probably gives rise to between 75 and 90% of amino acid-derived glucose. Carbon skeletons of two other amino acids, asparagine and its derivative, aspartate, may enter the Krebs cycle as oxaloacetate and pass directly into the gluconeogenic pathway via phosphoenol pyruvate. All of the other gluconeogenic amino acids enter at distal points; half of the carbon skeleton of phenylalanine enters the Krebs cycle as fumarate, those of isoleucine, methionine and valine enter as succinyl CoA, and those of arginine and ornithine, histidine, proline, glutamine, and glutamate enter as  $\alpha$ -ketoglutarate. One amino acid, namely leucine, is nongluconeogenic and enters the Krebs cycle as acetyl CoA, while the entry point of lysine is unknown (15). Tryptophan generates alanine (14, 15).

In Table II the GSA's of these groups of amino acids are compared with an index of their in vivo contribution to gluconeogenesis, namely, the basal splanchnic exchange rates in man, as recently reported by Felig and

Wahren (16). All of the amino acids which enter as pyruvate, and which, according to Felig and Wahren (16), have a positive splanchnic exchange rate which totals more than 100  $\mu$ moles/min, had a mean GSA of 70  $\mu$ g/ml ( $SEM \pm 7$ ). This is significantly greater ( $P < 0.005$ ) than that of the amino acids entering as succinyl CoA,

TABLE II  
Entry Points of the Carbon Skeletons of the Amino Acids

	Pyruvate	$\alpha$ -Ketoglutarate	Succinyl CoA
GSA ( $\mu$ g/ml)	70 ( $\pm 7$ )	42 ( $\pm 4$ )	14 ( $\pm 9$ )
Mean $\pm SEM$	$P < 0.01$ (between Pyruvate and $\alpha$ -Ketoglutarate) $P < 0.005$ (between Pyruvate and Succinyl CoA)		
Splanchnic exchange* ( $\mu$ moles/min)	102	18	3
ISA ( $\mu$ U/ml)	2.5 ( $\pm 0.5$ )	2.6 ( $\pm 0.7$ )	3.2 ( $\pm 1.3$ )
Mean $\pm SEM$			
I/G	0.85 ( $\pm 0.2$ )	1.34 ( $\pm 0.3$ )	43.8 ( $\pm 41$ )
Mean $\pm SEM$			

<sup>7</sup> Personal communication.

\* From Felig and Wahren (16).

which had a GSA totaling 14 pg/ml ( $SEM \pm 9$ ) and those entering as  $\alpha$ -ketoglutarate, which had a GSA totaling 42 pg/ml ( $SEM \pm 4$ ). Asparagine and aspartate had GSA's in the range of the pyruvigenic group, but their splanchnic exchange rates are unknown.

Neither the ISA's nor the molar insulin:glucagon ratio<sup>8</sup> of the three groups of amino acids differed significantly from one another, although the group entering as pyruvate had the lowest ISA and the lowest insulin:glucagon ratio.

*Glucagon-stimulating activity of pyruvate.* In order to determine if pyruvate itself was responsible for the increased GSA of the pyruvigenic amino acids, 1 mmole/kg of pyruvate was infused in a manner identical to that used for the amino acids. No increase in glucagon was observed (Table I).

*Glucagon-stimulating activity and amino acid R-chain structure.* To determine if the GSA of amino acids is the consequence of a common structural feature, the chemical structure of their R-groups was examined in relation to GSA. No clear-cut relationship between the structure or the size of the R-group and ability to stimulate glucagon was observed.

For example, glycine, with an R-group consisting only of a hydrogen atom, ranked second in GSA, while phenylalanine with its large benzene ring ranked third. One seemingly definite structure-function relationship was observed, however. Amino acids with branched aliphatic R-chains, valine, leucine, and isoleucine, were devoid of GSA. In fact, isoleucine, and perhaps leucine as well, appeared to cause a modest reduction in glucagon concentration.

## DISCUSSION

These studies reveal that in the supraphysiologic doses employed in this study, most of the amino acids tested have substantial GSA in the dog. Of 20 amino acids tested, only three, valine, isoleucine, and leucine failed to stimulate glucagon secretion. It seems probable, then, that the glucagon response to the ingestion of protein is influenced by most of the amino acids absorbed.

Glucagon is widely regarded as a potent gluconeogenic hormone which may play a central role in the regulation of exogenous gluconeogenesis. It is, therefore, of interest that glucagon secretion is stimulated most strongly by those amino acids which contribute the most to amino acid-derived gluconeogenesis; alanine and the other amino acids which enter the gluconeogenic pathway as pyruvate and which, according to Felig,<sup>9</sup> generate 75–90% of amino acid-derived glucose, have significantly greater glucagon-stimulating activity than amino acids

<sup>8</sup> Molar ratio of insulin:glucagon = ( $\mu$ U/ml)/(pg/ml)  $\times$  23.3.

<sup>9</sup> Personal communication.

which enter as succinyl CoA or  $\alpha$ -ketoglutarate. Only asparagine and aspartate, which enter as oxaloacetate, and phenylalanine, which enters as fumarate, have GSA comparable to the "pyruvigenic" group, but their relative contribution to gluconeogenesis is undetermined. Leucine, the only exclusively nongluconeogenic amino acid tested, was devoid of GSA, confirming the report of Pek, Fajans, Floyd, Knopf, and Conn (1).

Although it should be kept in mind that these results reflect relative molar potency in a supraphysiologic concentration range, and do not necessarily indicate relative physiologic potency, they are compatible with a system of feedback regulation of amino acid disposition by the amino acid concentration. However, the fate of an amino acid depends not only upon the glucagon response, but also upon the insulin response; an increase in insulin, a potent antigluconeogenic hormone, would reduce gluconeogenic activity and spare amino acids for incorporation into protein. The ISA and insulin:glucagon ratios of the most gluconeogenic amino acids which enter as pyruvate were lower than the other groups, but not significantly so. The complexity of the hormonal response to protein-induced hyperaminoacidemia is apparent, if one considers the variation in amino acid composition of the ingested proteins, the varying catabolic rates of each amino acid, the influence of simultaneously absorbed carbohydrate and fat, the influence of the various gut hormones, and the conditioning of the islet cell responses by antecedent diet (17). Only by simulating the postprandial level of each amino acid, could information of physiologic significance be obtained.

But these results may provide other potentially significant information. First, the fact that pyruvate itself is devoid of GSA indicates either that the marked activity of the pyruvate precursor amino acids does not involve their metabolism to pyruvate, or, that pyruvate is not readily admitted to the alpha cell. The recent report of Fajans et al. (18), indicating that a nonmetabolizable analog of arginine has GSA would favor the former hypothesis.

The fact that virtually all nitrogenous precursors of glucose, but not pyruvate, lactate,<sup>10</sup> glycerol,<sup>10</sup> or fructose<sup>1</sup> cause a prompt and substantial increase in glucagon secretion and thus would enhance their own catabolism to glucose may have physiological implications in terms of the over-all economy of amino acids, the most precious of the precursors of glucose. It may be that the powerful GSA of certain nitrogenous precursors of glucose increases conversion of amino acids to glucose during periods of amino acid abundance, such as after a large protein meal, but, at other times, when glucagon levels are unstimulated, more expendable non-nitrogenous precursors would be used preferentially for gluconeogenesis.

<sup>10</sup> Unpublished observations.

In starvation, for example, glucagon rises only slightly and transiently (19) and returns to near-normal levels soon thereafter (20), when amino acid conservation becomes evident.

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