STUDIES ON THE CONVERSION OF CARBON-14 DIOXIDE INTO GLUTAMIC ACID IN *NICOTIANA RUSTICA L.* AND ON THE HORMONAL CONTROL OF PYRUVATE-2-^{14}C METABOLISM IN RAT LIVER

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TABLE OF CONTENTS

PART I

Chapter		Page
I.	INTRODUCTION	2
II.	EXPERIMENTAL	8
	A. Isolation Procedures	8
	B. Counting Techniques	9
	C. Degradation Procedures	10
III.	RESULTS AND DISCUSSION	11
IV.	SUMMARY	20
A SELECTED BIBLIOGRAPHY		21

PART II

Chapter		Page
I.	INTRODUCTION	24
II.	EXPERIMENTAL	30
	A. Isolation and Assay of Blood Glucose	30
	B. Animal Experiments	32
	1. Changes in Blood Glucose Levels with Glucagon Administration	32
	2. Isotope Experiments	33
	C. Counting Techniques and Degradation Procedures	33
III.	RESULTS AND DISCUSSION	34
IV.	SUMMARY	44
A SELECTED BIBLIOGRAPHY		45
APPENDIX A		48

LIST OF TABLES

PART I

Table | Page

I. Labeling in Leaf Amino Acids of <u>Nicotiana</u> <u>Rustica</u> <u>L</u>. after Exposure to $^{14}CO_2$. 12

II. Derivation of Glutamate Carbon Atoms 14

PART II

I. Incorporation of Pyruvate-2-^{14}C into Blood Blucose of Intact Rats 38

II. Labeling Patterns in Liver Glutamate after Pyruvate-2-^{14}C Injection. 40

LIST OF ILLUSTRATIONS

PART I

Figure | Page

1. Glutamate-Symmetrical Intermediate Pathway for Pyrrolidine Ring Biosynthesis. 3
2. Glutamate Labeling Patterns from $^{14}CO_2$ 16
3. Possible Pyrrolidine Ring Labeling Patterns. 19

PART II

1. Blood Glucose Levels after Glucagon Administration as a Function of Time. 35
2. Blood Glucose Response to Varying Levels of Glucagon 36

PART I

STUDIES ON THE CONVERSION OF CARBON-14 DIOXIDE INTO GLUTAMIC ACID IN *NICOTIANA RUSTICA* L.

PART I

CHAPTER I

INTRODUCTION

Prior to 1964, information pertaining to the pathways of synthesis of alkaloids in tobacco plants had been obtained almost exclusively through potential precursor feeding (a general term for administering different compounds, not including CO_2). Supported by the results of experiments with ornithine-2-^{14}C (1,2), putrescine-1,4-^{14}C (3), and glutamic acid-2-^{14}C (4), Leete (5), in 1956, proposed the glutamate-symmetrical intermediate hypothesis which has been the generally accepted concept for the biosynthesis of the pyrrolidine ring of nicotine (Figure 1). Cyclization of the δ-semialdehyde (I), which can be obtained from either glutamic acid or ornithine, yields Δ^1-pyrroline-5-carboxylic acid (II) which decarboxylates forming the symmetrical anion (III). Incorporation of glutamate-5-^{14}C into this anion would then result in equal labeling of the pyrrolidine ring of nicotine at the C-2' and C-5' positions. Alternatively, ornithine can be decarboxylated to putrescine which can then cyclize to (IV); however, the incorporation of putrescine is only 25% that of ornithine (6).

Leete's hypothesis (5) has also received support from results obtained from the administration of 2-, 3-, and 4-carbon precursors such as acetate, glycerol, propionate and aspartate. When acetate-1-^{14}C was administered hydroponically to intact <u>Nicotiana</u> <u>rustica</u> and the plants

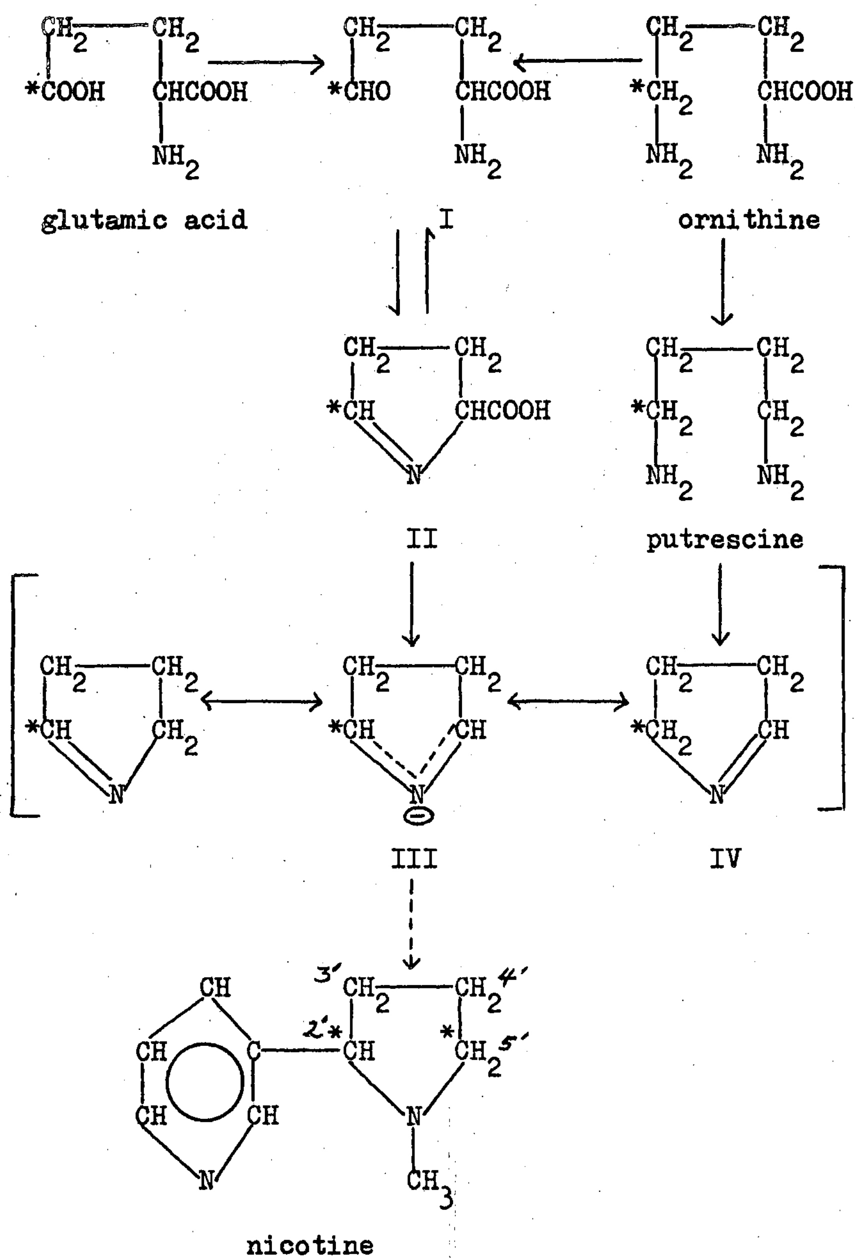

Figure 1. Glutamate-Symmetrical Intermediate Pathway for Pyrrolidine Ring Biosynthesis

were allowed to metabolize the isotope for 168 hours, the C-2' and C-5' positions of the pyrrolidine ring of the nicotine contained equivalent amounts of ^{14}C, and the total ^{14}C in these two carbons represented 95% of the ^{14}C in the pyrrolidine ring (7). Reducing the metabolism period to 3 and 6 hours did not alter the percentage of ^{14}C in C-2' (C-5' was not isolated). The administration of propionate-3-^{14}C resulted in labeling patterns identical to those found with acetate-1-^{14}C, indicating that tobacco plants metabolize propionate via acetate, probably along the β-oxidation pathway reported by Giovanelli and Stumpf (8). Similarly, glycerol-2-^{14}C administration yielded 38% of the ^{14}C located in carbon 2 of the pyrrolidine ring after 168 hours; shortening the incorporation time to 2 hours increased the amount of ^{14}C in C-2' to 50%. Wu <u>et</u> <u>al</u>.(7) assumed an equal percentage of radioactivity in C-5'. These results would be compatible with the metabolism of glycerol-2-^{14}C through the glycolytic pathway to pyruvate-2-^{14}C and to acetate-1-^{14}C. Administering pyruvate-2-^{14}C has also been shown to yield nicotine in which 40-50% of the activity of the pyrrolidine ring was present at C-2' (9).

Four carbon intermediates such as aspartic acid-3-^{14}C, fumarate-2-^{14}C and succinate-2-^{14}C were incorporated over extended periods into nicotine with about 25% of the activity in the pyrrolidine ring at C-2' as would be expected by the operation of the tricarboxylic acid cycle (7,9).

Acetate-2-^{14}C, and precursors of acetate-2-^{14}C such as glycerol-1,3-^{14}C, propionate-2-^{14}C and alanine-3-^{14}C , have produced labeling patterns which are difficult to explain without invoking experimental error. After the incorporation of acetate-2-^{14}C into <u>Nicotiana</u> <u>rustica</u>

plants over a period of 168 hours, carbon 2 of the pyrrolidine ring contained 14% of the ^{14}C in the pyrrolidine ring, presumably leaving by difference 72% in carbons 3 and 4; however, reducing the incorporation period to 2 hours increased the amount of ^{14}C in C-2' to 20%, leaving 60% in carbons 3 and 4 (7). A shorter incorporation period, however, should reduce the percentage of ^{14}C in carbons 2 and 5 by reducing the number of turns of the tricarboxylic acid cycle and thus reducing the amount of randomization occurring.

In addition, other precursors gave labeling patterns in nicotine inconsistent with those predicted by the tricarboxylic acid cycle and Leete's hypothesis. Among these precursors are: succinate-1-^{14}C and citrate-1-^{14}C which should result in about 50% of the activity in the pyrrolidine ring at C-2', as with acetate-1-^{14}C; however, with succinate-1-^{14}C, only 15% of the activity was located at C-2' of the pyrrolidine ring (9). With citrate-1-^{14}C only 3% of the activity was located at C-2' (9). Also, glucose-1-^{14}C and glucose-6-^{14}C, which, via the glycolytic pathway, should both yield acetate-2-^{14}C, in fact, produced 19% and 4%, respectively, at carbon 2 of the pyrrolidine ring (9).

These discrepancies (7,9) described above have not been resolved although it has been theorized (10) that the pathways which led to the above labeling patterns are aberrant pathways stimulated by deviations from the natural growth conditions of the plant. Rapoport _et al_. (11) have studied the differences found in the labeling patterns of the pyrrolidine ring of nicotine with potential precursor feedings and with exposures to normal levels of $^{14}CO_2$. Working with relatively short time exposures (2 to 12 hours) of _Nicotiana glutinosa_ to $^{14}CO_2$, they found a very low percentage of ^{14}C, varying from 0.3% to 3.8%, located in C-2'

of the pyrrolidine ring, a result which does not conform to the glutamate-symmetrical intermediate hypothesis, using known glutamate biosyntheses (11). The incorporation of $^{14}CO_2$ via the carbon reduction cycle would result in glucose-6-phosphate uniformly labeled in all carbons after ten minutes; with shorter time periods, carbons 3 and 4 would contain equal activity and relatively more activity than carbons 1, 2, 5, and 6, also equally labeled (12). This would yield, after ten minutes, pyruvate and acetate equally labeled in all carbons, and would result in equal amounts of activity in all carbons of the pyrrolidine ring of nicotine; however, a low percentage of activity at C-2' implies a low percentage at C-5', if a glutamate-symmetrical intermediate is accepted, leaving the remainder, about 94%, divided between positions 3 and 4 of the pyrrolidine ring. Further degradations were done (10) to determine the ^{14}C present in C-2' and C-3' and, by difference, C-4' and C-5'. These analyses yielded about 2% of the total nicotine activity in C-2' and in C-3' with C-4'+C-5' containing 13%. Assuming C-2' = C-5', C-4' would then contain almost five times as much activity as any of the other carbons of the pyrrolidine ring, a result which could not support the glutamate-symmetrical intermediate hypothesis. The species difference between Rapoport's work with CO_2 and <u>Nicotiana glutinosa</u> and the earlier feeding experiments with ornithine-2-^{14}C and <u>Nicotiana rustica</u> were nullified by feeding ornithine-2-^{14}C to <u>N. glutinosa</u> plants and finding an equal quantity of label at C-2' and C-5' of the pyrrolidine ring of nicotine (10). The most significant conclusion drawn by Rapoport and his group (10) from these degradations is that the labeling pattern in the pyrrolidine ring obtained from precursor feedings via a symmetrical intermediate could result from a minor or aberrant

pathway of nicotine biosynthesis, since the $^{14}CO_2$ exposures are representative of normal growth conditions.

Furthermore, no known pathways of glutamate biosynthesis from CO_2 could explain such a labeling pattern. In fact, much speculation had been voiced by Rapoport and his group (6) concerning the biosynthetic pathways of glutamic acid, even to the point of proposing a hypothetical pathway of glutamate biosynthesis to account for a glutamate labeled primarily in carbon 3 involving the condensation of equally labeled glycolic acid and acetate via malate synthetase to form malate which then participates, without randomization, in all the tricarboxylic acid cycle reactions yielding α-ketoglutarate labeled only in position 3. Their more recent degradations, however, showing the greatest percentage of activity to be at C-4' ruled out the above possibility. The problem of PART I of this thesis is concerned with the determination of the labeling patterns in the glutamic acid of tobacco plants following $^{14}CO_2$ exposure since no experimental work had been done along these lines in this system.

CHAPTER II

EXPERIMENTAL

A. Isolation Procedures

Pots containing 3 to 5 Nicotiana rustica L. plants, 1 month old, were prepared and kept in growth chambers under constant conditions (12 hour day, 86°F, 100% humidity) for at least a week prior to these experiments. A biosynthetic chamber was used of approximately 2 liters in volume, fitted with a sidearm in which the radioisotope was placed and a socket for a Geiger-Muller tube. A Geiger-Muller tube connected to a count-rate meter with a recorder monitored the level of isotope present in the chamber. The plants, one pot per experiment, were exposed to 100 μc of ^{14}C-labeled carbon dioxide released from 0.326 mg of labeled sodium bicarbonate in water (400 μc/ml; 25.8 mc/mmole, purchased as a dry powder from Nuclear-Chicago) by the addition of 1 ml of 12 N H_2SO_4. This introduced 0.095 ml of $^{14}CO_2$, an increase of 0.005% above the atmospheric level of 0.03%. A very slight vacuum was drawn in the chamber and an equilibration period of 21 minutes and 5 minutes (for the long and short illumination periods, respectively) was allowed for the isotope to diffuse through the chamber. All of the above procedures were done in the dark and in the early morning, 6:00 A.M. to 8:00 A.M., since the plants had become accustomed to light illumination beginning in the growth chambers at around these times. The plants were then illuminated for either 18 or 3 minutes with 2 Sylvania "Gro-Lux" lamps

positioned vertically on either side of the biosynthetic chamber and 1 Westinghouse 150V Reflector Spot fastened above the chamber. At the end of the illumination period, the chamber was swept in the dark with room air for 33 minutes and 6 minutes (for the long and short illumination periods, respectively) into 1 N NaOH scrubbers. The plants were then removed from the chamber and the leaves were placed in liquid nitrogen within one minute of the time of removal from the chamber. The free amino acids were extracted from the leaves by the method of Zelitch (13). Glutamic and aspartic acids were isolated from the crude extracts by ion-exchange chromatography using a 1.5 x 20 cm Dowex 1x8, acetate column as described by O'Neal and Koeppe (14). A ninhydrin assay was used to determine the amount of amino acid present (15). Averages of 1.6 and 1.4 μmoles per g of leaf tissue of glutamate and aspartate, respectively, were obtained.

B. Counting Techniques

All liquid samples, i.e., the crude extracts of the leaves and the amino acid fractions eluted from the column, were counted using a Tri-Carb Series 3000 Liquid Scintillation Spectrometer. Bray's solution was used as a scintillation fluid (16). Background under these conditions averages 25 cpm with an efficiency of about 53% as determined by correlation with the wet combustion of the diluted, crystallized samples. Samples were counted for a sufficient time such that 99% of the samples had an error of less than $\pm$1%.

All glutamic and aspartic acid samples were diluted with nonradioactive amino acid to a workable specific activity (10 to 20 mμc/mmole) in such quantity as to allow complete degradation (at least 2.5 mmoles). Weighed amounts of these crystalline samples were combusted in a wet

combustion-vibrating reed apparatus (17) to determine the specific activity of the sample. Recrystallization was continued until a constant specific activity was obtained.

C. Degradation Procedures

The degradation scheme for glutamic acid was carried out by the method of Mosbach *et al*. (18) as modified by Koeppe and Hill (19). Aspartic acid was decarboxylated with ninhydrin in the wet combustion-vibrating reed apparatus (17).

CHAPTER III

RESULTS AND DISCUSSION

The significant features of the experimental results (Table I) are:

A. The labeling of C-4 and C-5 were nearly equal, as were C-2 and C-3;

B. The labeling of C-4 + C-5 was always much larger than C-2 + C-3;

C. The labeling of C-1 was always higher than C-2 or C-3;

D. Shortening the exposure time greatly increased the percent labeling in C-4 and C-5 at the expense of the other three carbons of glutamate, and increased the percent of radioactivity incorporated into the carboxyl carbons of aspartate;

E. The ratio of the specific activity of glutamate to the specific activity of aspartate increased as the exposure time increased.

Points A and B indicate that an equally labeled 2-carbon source, such as acetate, is the main precursor of carbons 4 and 5 of glutamic acid. If such is the case, the smaller amount of activity equally distributed between C-2 and C-3 of glutamate would be the natural result of the randomization of C-4 and C-5 through succinate and fumarate by each turn of the tricarboxylic acid cycle. C-1 would also acquire activity by this procedure. Point D then emphasizes the above statement since shortening the exposure time would reduce the amount of randomization that would occur, thereby increasing the percentage of activity in C-4

TABLE I

LABELING IN LEAF AMINO ACIDS OF NICOTIANA RUSTICA L. AFTER EXPOSURE TO $^{14}CO_2$

Amino Acid	Light Exposure Time - 3 min.				Light Exposure Time - 18 min.			
	μcuries/ mmole	per cent of total	μcuries/ mmole	per cent of total	μcuries/ mmole	per cent of total	μcuries/ mmole	per cent of total
Glutamic acid								
Total	1.76		0.54		14.3		39.1	
Carbon 1, COOH		9.1*		>20.0*		30.4		32.0
Carbon 2, $CHNH_2$		1.9		< 1.0		7.1		10.3
Carbon 3, CH_2		2.3		< 1.0		7.6		11.0
Carbon 4, CH_2		40.8		38.0		25.3		21.1
Carbon 5, COOH		45.8		40.4		27.4		21.8
Aspartic acid								
Total	3.86		1.62		15.4		58.5	
Carbon 1 + 4, COOH		79.9		92.4		75.8		70.6

*by difference

and C-5 at the expense of the other three carbons of glutamate. The high ratio of activity in C-1 to C-2 + C-3 in glutamate and the high carboxyl labeling in aspartate, both decreasing somewhat with time, can be explained by continuous $^{14}CO_2$ fixation into oxalacetate and a somewhat slower formation of symmetrically labeled pyruvate via the carbon reduction cycle. Point E, indicating that the specific activity of the glutamate increases relative to the specific activity of the aspartate, may reflect a difference in the turnover rates of the two metabolic pools.

These data are not compatible with the following routes of glutamate biosynthesis (Table II):

A. The glyoxylate-malate proposal by Alworth _et al_. (6);

B. The reductive reversal of the tricarboxylic acid cycle postulated to occur in _Chlorobium thiosulfatophilum_ (20);

C. The reversal of glutamate fermentation in _Clostridium tetanomorphum_ (21,22) and in _Acetobacter suboxydans_ (23);

D. The synthesis of glutamate in _Clostridium kluyveri_ (24).

The formation of equally labeled acetate can be explained by the carbon reduction pathway (25) and, assuming that the glyoxylate formed in _Nicotiana rustica_ as a result of $^{14}CO_2$ fixation is rapidly labeled, then Table II shows that none of the above glutamate biosyntheses will give a labeling pattern such that C-4 = C-5, C-2 = C-3, and C-4 and C-5 will contain more activity than C-2 and C-3. Pathway A would produce a glutamate labeled mainly in C-3; B would yield C-3 = C-4 as would C and D.

The incorporation of equally labeled acetate obtained from the carbon reduction cycle into glutamate via the normal operation of the

TABLE II

DERIVATION OF GLUTAMATE CARBON ATOMS

Pathway	Carbon Atoms				
	C-1	C-2	C-3	C-4	C-5
Glyoxylate-malate proposal (6)	a-1[a]	a-2[b]	g[c]	a-2	a-1
Chlorobium thiosulfatophilum (20)	CO_2[d]	CO_2	p-2,3[e]	p-2,3	CO_2
The citramalate pathway (21-23)	a-1	a-2	p-3	p-2	CO_2
Clostridium kluyveri (24)	a-1	a-2	a-1	a-2	CO_2

[a] a-1 = acetate, C-1

[b] a-2 = acetate, C-2

[c] g = glyoxylate

[d] CO_2 = carbon derived from CO_2 fixation such as pyruvate C-1 and oxalacetate C-1 and C-4

[e] p-2,3 = pyruvate, C-2 and C-3

tricarboxylic acid cycle, along with the fixation of $^{14}CO_2$ to form oxalacetate, would yield the labeling pattern observed (Figure 2). Referring to the work of Calvin and Bassham (25) on the carbon reduction cycle, with $^{14}CO_2$ as a radioactive carbon source, after a 5.4 second exposure, the carbons of fructose have the following labeling: C-1, 3%; C-2, 3%; C-3, 43%; C-4, 42%; C-5, 3%; C-6, 3%. After a 10 minute exposure, glucose-6-phosphate has the following labeling pattern: C-1, 17%; C-2, 16%; C-3, 19%; C-4, 22%; C-5, 13%; C-6, 13% (12). By Embden-Meyerhof glycolysis, carbons 3 and 4 become C-1 of pyruvate which is subsequently lost by decarboxylation to acetate. Carbons 1 and 6 of the hexose become carbon 2 of acetate, and carbons 2 and 5 become carbon 1 of acetate. Thus, after a metabolism period of 10 minutes, the acetate entering the tricarboxylic acid cycle should be equally labeled.

According to Calvin and Bassham (25), the carboxylation by which phosphoglyceric acid is formed does not require cofactors derived from light energy, but the reaction by which phosphoglyceric acid is reduced to triose phosphate is a reaction which does require such cofactors. The reservoirs of these cofactors are presumed to be so small that the supply is exhausted very soon after the light is turned off. Thus, the reaction by which phosphoglyceric acid is reduced would stop soon after the light is turned off, but the reaction by which phosphoglyceric acid is formed (the carboxydismutase system) would continue until the supply of CO_2 acceptor is exhausted. The shortest metabolism period used in these experiments is 3 minutes in the light and 6 minutes in the dark, a reaction period which possibly would not yield equally labeled acetate via the carbon reduction pathway (it may be, of course, that the equal distribution of ^{14}C among the carbons of the hexose can be achieved in

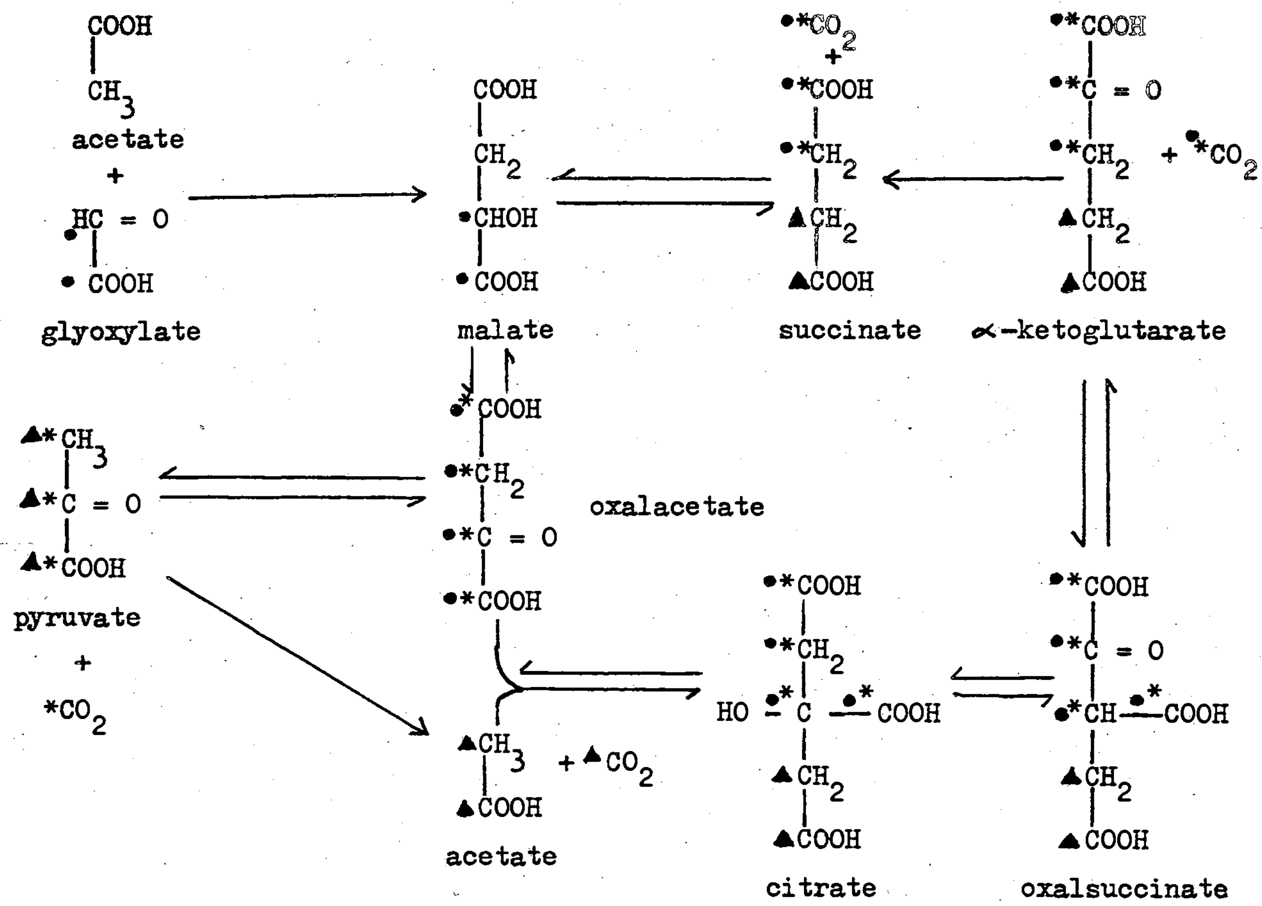

Figure 2. Glutamate Labeling Patterns from $^{14}CO_2$

shorter time periods than 10 minutes).

Without ATP and NADPH (26), the metabolism of phosphoglyceric acid (PGA) proceeds predominantly by the following alternate routes (25):

$$\text{3-PGA-1-}^{14}\text{C} \longrightarrow \text{2-PGA-1-}^{14}\text{C} \longrightarrow \text{phosphoenolpyruvate-1-}^{14}\text{C}$$

$$\downarrow \leftarrow {}^{14}CO_2$$

$$\text{glutamate-1-}^{14}\text{C} \dashleftarrow \text{oxalacetate-1,4-}^{14}\text{C}$$

The greater percentage of activity in C-1 of glutamate as compared to C-2 and C-3 following $^{14}CO_2$ exposures could be a result of the dark reaction of phosphoglyceric acid-1-^{14}C leading to glutamate-1-^{14}C. The label in C-1 of glutamate would be derived from the fixed $^{14}CO_2$ rather than from the phosphoglyceric acid-1-^{14}C.

Other biosynthetic pathways which could account for the labeling patterns observed utilize glycolate as a precursor. The rapid formation of symmetrically labeled glycolate, a reaction known to occur in tobacco plants (13), and its subsequent metabolism via glyoxylate, glycine, serine, and pyruvate yield almost uniformly labeled pyruvate and, therefore, acetate (27). The randomization of C-4 and C-5 of glutamate derived from acetate would produce labeling in C-2 and C-3 as observed. In addition, Sekizawa _et al_. (28) have studied an enzymatic condensation of glyoxylate with oxalacetate in _Acetobacter suboxydans_ which could explain these data by the conversion of symmetrically labeled glycolate to glutamate via glyoxylate, oxalmalate, α-hydroxy, γ-ketoglutarate and α-ketoglutarate. The first three carbons of oxalacetate, formed from pyruvate derived from the carbon reduction cycle and $^{14}CO_2$, would be labeled more slowly than glyoxylate, thus accounting for the observed increase with time of C-2+C-3/C-4+C-5. The formation of α-ketoglutarate from glyoxylate and pyruvate via γ-hydroxy, α-ketoglutarate is another

possibility and would account for C-1 containing more activity than C-2 or C-3. Some evidence for the formation of γ-hydroxy, α-ketoglutarate in plants has been recorded (29,30).

In a recent paper containing an improved nicotine degradation method, Rapoport and his group (31) found carbons 4 and 5 of the pyrrolidine ring to contain almost equal amounts of label. C-2' consistently contained low levels of incorporation, i.e., 1.3% to 3.3% as compared to 13% for C-4' + C-5'. C-3' was variable, sometimes equaling C-2' and at times equaling C-4', depending upon the metabolic conditions. These results led them to indicate an involvement of glycolate.

Recent work by Kisaki _et al_. (32) has indicated an involvement of γ-methylaminobutyraldehyde as a precursor of the pyrrolidine ring and the methyl group of nicotine. This finding implies a new pathway with a new set of precursors for the biosynthesis of the pyrrolidine ring of nicotine.

While the work of this thesis cannot be directly applied to the problem of nicotine biosynthesis[1], some conclusions regarding possible labeling patterns can be drawn. If glutamate is an intermediate in pyrrolidine ring biosynthesis from $^{14}CO_2$, and if a symmetrical intermediate is involved as has been proposed in the conversion of glutamate to the pyrrolidine ring, all carbons in the pyrrolidine ring should be equally labeled. If no symmetrical intermediate exists, then the predicted labeling would be C-2' = C-3' and C-4' = C-5' (Figure 3). Low specific activity in the nicotine isolated from $^{14}CO_2$-exposed plants and low

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[1]Cooperative efforts with Dr. R. U. Byerrum and Horst Zelke of Michigan State University are now being made to correlate the nicotine and glutamic acid labeling patterns in _Nicotiana_ plants exposed to $^{14}CO_2$ and fed potential precursors.

yields in the steps of nicotine degradations have hampered accuracy in this area, and improvements are constantly being sought. If, indeed, $^{14}CO_2$ is found, with improved nicotine degradation schemes, to give unsymmetrical labeling in the pyrrolidine ring, then a pathway of pyrrolidine ring biosynthesis involving neither glutamate nor a glutamate-symmetrical intermediate must be invoked.

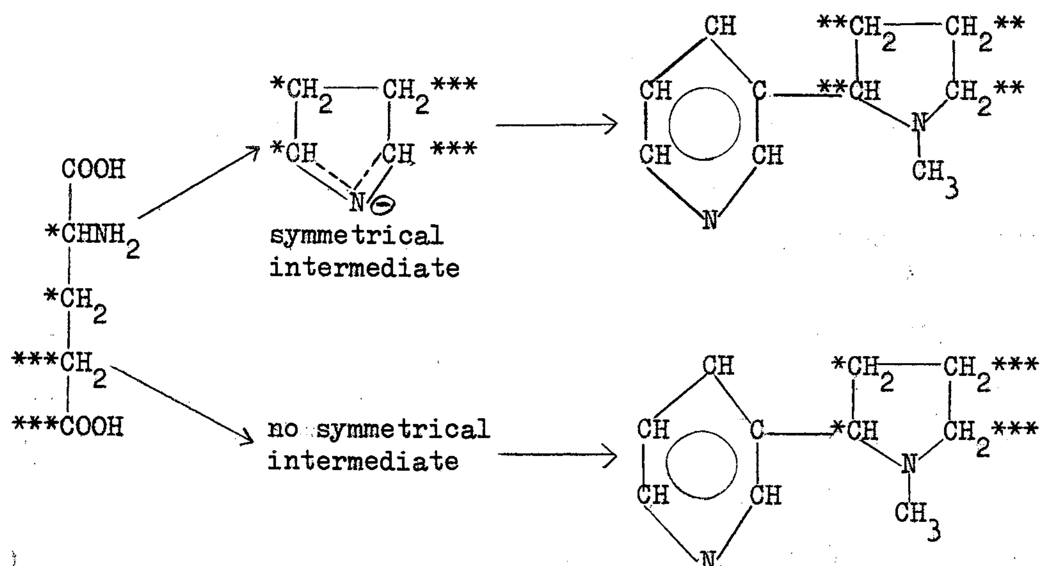

Figure 3. Possible Pyrrolidine Ring Labeling Patterns

CHAPTER IV

SUMMARY

When one month old Nicotiana rustica L. plants were exposed to $^{14}CO_2$ for 3 and 18 minutes in the light, degradations of the free glutamic acid isolated from the leaves produced the following results:

1. The labeling of C-4 and C-5 were nearly equal, as were C-2 and C-3;

2. The labeling of C-4 + C-5 was always much larger than C-2 + C-3;

3. The labeling of C-1 was always higher than C-2 or C-3;

4. Shortening the exposure time greatly increased the per cent labeling in C-4 and C-5 at the expense of the other three carbons of glutamate.

These data can be explained by the normal operation of the carbon reduction and tricarboxylic acid cycles and indicate that if the pyrrolidine ring of nicotine is formed from a symmetrical intermediate derived from glutamic acid via Δ^1-pyrroline-5-carboxylic acid, then all the carbons in the pyrrolidine ring should be equally labeled. If, however, no symmetrical intermediate exists in this pathway, formation of the pyrrolidine ring from glutamic acid should yield C-2' = C-3' and C-4' = C-5'.

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PART II

STUDIES ON THE HORMONAL CONTROL OF PYRUVATE-2-^{14}C METABOLISM IN RAT LIVER

PART II

CHAPTER I

INTRODUCTION

In the mammal, the liver and the kidney cortex are the main sites where amino acids and other precursors can be converted to carbohydrate. Although the enzymatic sequences involved in gluconeogenesis have been well documented, the control of this very important biosynthetic pathway is not well understood despite voluminous material on the subject (1-3). Depression of gluconeogenesis apparently occurs during ingestion of carbohydrate and with an insufficient supply of adrenocorticosteroids. Enhancement of the pathway follows fasting and diabetic conditions and the administration of adrenal cortical hormones. The magnitude of the effects of the above conditions and their site of action or mechanism have yet to be elucidated.

There is much support for the proposal (4) that steroid hormones affect the enzyme-forming system so as to increase the amount of certain enzymes (5-13). Presumably the glucocorticoid hormones affect the enzymes involved in amino acid degradation and gluconeogenesis. Of particular interest here, phosphoenolpyruvate carboxykinase (EC 4.1.1.32), a pacemaker in gluconeogenesis and the enzyme responsible for converting oxalacetate to phosphoenolpyruvate, has been shown to respond to adrenocortical hormone administration (14-16). In adrenalectomized rats, cortisone and hydrocortisone, at a dose of 10 mg per day for 5 days,

increased the activity of this enzyme 2.5 fold (16). This enhanced activity is apparently the result of new enzyme synthesis since it can be abolished entirely by puromycin or actinomycin D (14,17). Two findings, however, are in disagreement: one, the increased activity of phosphoenolpyruvate carboxykinase is discernible only long after the early physiological effect of the adrenal corticoids on glucose formation is known to occur, and, two, Ray et al. (17) have found that hydrocortisone stimulated liver glycogen deposition in normal and adrenalectomized rats even though enzyme induction was prevented by actinomycin D. There must be, then, primary effects other than enzyme induction. In addition to the above, adrenalectomized rats when fasted exhibit the same increase in phosphoenolpyruvate carboxykinase as do normal rats, indicating that the adrenal corticoids are not necessary for the enhancement of activity (16). Ray et al. (17) concluded that the adrenocortical hormones are neither the exclusive inducers nor do they act permissively with respect to enhancement caused by fasting; it is more probable that the hormones have some other effect which is amplified through a variety of metabolic influences until finally enzyme synthesis is increased. Other investigators have reached the same conclusion (18-20).

Other treatments have included fasting for 48 hours, which increases phosphoenolpyruvate carboxykinase activity 3 fold above the activity of a normal fed animal (16). It is particularly interesting that refeeding a mixed diet restores the level to normal while refeeding diets containing fat, casein, vitamins and minerals but no carbohydrate result in even further enhancement of the enzyme's activity (16). Adding sucrose back to this synthetic diet serves to restore the enzyme activity

to normal. Glucose or glycerol alone given orally also result in the same effect, but serine, alanine and 2-deoxyglucose do not. From these data it could be reasoned that glucose or glucose derivatives serve to repress the phosphoenolpyruvate carboxykinase system; however, this must not be the case, since in the diabetic rat, the abundant supply of glucose is not effective in reducing the activity of the enzyme. In fact, in alloxan-diabetic rats, the enzyme levels are 6 fold that of normal (16). Temporary diabetes produced by mannoheptulose also increases the enzyme levels rapidly, to 3 fold that of a normal rat after 4 hours; pancreatectomy results in levels 2.5 fold that of normal. Insulin administration rapidly depresses the increased levels of this enzyme in the alloxan-diabetic animal although it has little or no effect on the normal animal. Lardy _et al_.(16) feel that the more likely action is that the metabolite which depresses phosphoenolpyruvate carboxykinase activity is formed from glucose but only in the presence of insulin.

It should be noted that a coincident pacemaker step along with the conversion of oxalacetate to phosphoenolpyruvate is the carboxylation of pyruvate to oxalacetate by pyruvate carboxylase (EC 6.4.1.1), an enzyme known to be under the feedback control of acetyl CoA (1); however, while phosphoenolpyruvate carboxykinase activity is influenced greatly by glucocorticoid administration, in fasting and in diabetes, pyruvate carboxylase in the mitochondria in unaffected (1,21). These findings have been challenged by Henning _et al_. (22) who have found pyruvate carboxylase activity in the cytoplasm in sufficient quantity to cover the gluconeogenic capacity of rat liver; their data shows this enzyme to be activated by starvation and alloxan diabetes although the

stimulation is relatively low compared to that of phosphoenolpyruvate carboxykinase.

Extensive work has been done previously in attempts to correlate various animal treatments and conditions with the labeling patterns in glutamic acid in hope of using this method to demonstrate a shift in pyruvate metabolism (23, 24). This compound has been shown to be useful in studying the metabolism of carboxyl and bicarbonate compounds in intact rats (25). Labeling patterns in glutamate may be indicative of the relative amounts of pyruvate converted to acetate and to oxalacetate since acetyl CoA is the precursor of carbons 4 and 5 of glutamate (via α-ketoglutarate) and oxalacetate is the precursor of carbons 1, 2, and 3 of glutamate. It is well established in mammalian systems that acetate-1-^{14}C will label only C-1 and C-5 of glutamic acid (23,26-28). Thus, if the decarboxylation of pyruvate is the predominant metabolic reaction, a large portion of the labeling from pyruvate-2-^{14}C will be found in C-5 of glutamate. If, however, gluconeogenesis is accelerated and pyruvate is mainly carboxylated to oxalacetate, relatively little activity will be found in C-5 of glutamate from pyruvate-2-^{14}C and most of this activity will be found at positions 2 and 3 after randomization in fumarate. Koeppe et al. (23) have shown that in a normal fed rat given pyruvate-2-^{14}C, about 38% of the total activity in the liver glutamic acid is located in carbon 5. On the other hand, fasting for 48 hours reduced this percentage to about 3%, reflecting the shift in pyruvate metabolism. The mechanism of this shift and its precise control have not yet been explained.

The administration of hydrocortisone and 9-α-fluoroprednisolone, both adrenocorticoids, to normal fed rats followed by injection of pyru-

vate-2-^{14}C 4 and 24 hours later, resulted in 35% of the total activity in the liver glutamate located at carbon 5, results which are similar to those obtained in a normal fed control (24). Alloxan diabetes of short duration (8 to 14 days) reduced the percentage of total activity located at C-5 to 5%. Prolonging the alloxan diabetes to 6 weeks, however, returned the labeling pattern to a normal 35% in C-5 (23). This difference is yet to be explained. Feeding a high fat diet in order to stimulate pyruvate carboxylase through increased levels of acetyl CoA resulted in 13% in C-5 (24). Severe thiamine deficiency which should impair the decarboxylation of pyruvate to acetyl CoA did not alter the relative amounts of pyruvate metabolized via acetyl CoA compared to the amounts metabolized via oxalacetate as indicated by the labeling patterns in glutamate from a thiamine deficient animal and a normal control (24).

In addition to the above treatments, one which became of interest concerned the effect of glucagon on the metabolism of pyruvate. Perhaps the most striking physiological effect of glucagon administration to a well-fed animal is the very rapid rise in blood sugar (29). It is generally accepted that this abrupt elevation is due to the activation of liver phosphorylase, an effect mediated by adenosine-3',5'-phosphate (cyclic AMP) which is formed by the interaction of glucagon with the membrane-bound adenylate cyclase system (30). Glucagon not only stimulates glycogenolysis, but it has also been found to increase the rate of glucose synthesis from 3-carbon precursors (31-33). While this mechanism has not been clearly defined, glucagon has recently been shown to have a direct lipolytic action on liver which may help to explain its gluconeogenic activity (34). Increased hepatic lipolysis with the concurrent enhanced availability of free fatty acids results in a rise in

fatty acyl CoA and acetyl CoA levels. Elevation of acetyl CoA levels serve to activate pyruvate carboxylase and thus perhaps stimulate gluconeogenesis. Also, Lardy and his group (14) have previously found elevations in phosphoenolpyruvate kinase activity after glucagon administration. A purpose of PART II of this thesis is to test the magnitude of this effect as reflected in the labeling patterns in liver glutamate following glucagon and pyruvate-2-^{14}C injection.

Further work done by the author in cooperation with Dr. S. K. Meghal concerning mannoheptulose is also contained in this thesis. Mannoheptulose is a seven carbon sugar found in large concentrations in the avacado, which has been shown to cause severe hyperglycemia of several hours duration in the rat, and to cause a diabetes-like syndrome, characterized by hyperglycemia, glucosuria and an increase in ketone bodies (35). It has been postulated that these phenomena resulting from mannoheptulose administration are brought about by the impairment of glucose utilization and by an increase in glucose production (36). Further, mannoheptulose is thought to exert its effect by blocking the release of insulin from the β-cells of the pancreas (14). Since Shrago _et al_.(14) have reported increases in phosphoenolpyruvate carboxykinase of 2 fold after mannoheptulose administration, it was also of interest to determine the effect of this compound on pyruvate metabolism as reflected in the labeling of C-5 of glutamic acid.

CHAPTER II

EXPERIMENTAL

A. Isolation and Assay of Blood Glucose

An attempt was made to determine blood glucose specific activity after isotope injection using the method of Friedmann *et al.* (37); Amberlite MB3 mixed-bed resin was put in the sodium bicarbonate form by slurrying it with 6 volumes of 0.9 N sodium bicarbonate until all the carbon dioxide was evolved; this slurrying process was repeated twice. A column, 1.9 x 30 cm, was packed and washed with 2 liters of 0.9 N sodium bicarbonate. The resin was then stored in 0.9 N sodium bicarbonate and refrigerated until needed. Small columns, 0.8 x 20 cm, were then packed and washed with water until the conductivity of the effluent was within 10 times that of the wash water. The purpose of such a column was to remove possible radioactive ionized metabolites, particulary the injected pyruvate, from the blood glucose sample. Preliminary tests with unlabeled glucose and pyruvate-2-^{14}C indicated that some radioactive compound was passing through the column and was being eluted in the same fractions as the glucose. Fractions taken off the column (1 ml each) were counted in scintillation vials containing 15 ml Bray's solution (38) and 0.5 ml water in the Tri-Carb Series 3000 Liquid Scintillation Spectrometer. Analyses showed 0.8% of the isotope placed on the column was recovered from fractions 3-10. Paper chromatography in n-butanol: 95% ethanol: 0.5 M NH_4OH (7:1:2) on Whatman No. 1 of the dinitrophenyl-

hydrazine derivatives of the pyruvate sample used (one which was stored lyophilized in 0.9% NaCl under nitrogen for 1 year) yielded 1.7% of the total pyruvate activity present in parapyruvate and 1.0% present at the origin of the chromatogram (unidentified). It was felt that perhaps one of these contaminants was passing through the column. Correspondance with Dr. Sidney Weinhouse (January 10, 1967) revealed that they had encountered the same difficulty, but they felt that the maximum degree of contamination was low with respect to the conversion that they were measuring. This would be valid provided we were measuring differences in incorporation into glucose comparable to those between fed and fasted animals which vary from 1.0 μc/mmole in a fed rat to 28.4 μc/mmole in a fasted rat (Table I); however, the change in the amount incorporated after the administration of glucagon is not as pronounced, and since a high degree of accuracy was desired, a modification of the osazone derivative method of Steele _et al._ (39) was adopted. The modification consisted of making the glucosazone from the glucose sample taken off the MB3 column rather than making the derivative directly from the blood filtrate. For comparison purposes, specific activities were still determined directly from the column glucose sample by the following procedure: a measured quantity of blood, usually 1 to 2 ml, was deproteinized with 10% $ZnSO_4$ and 0.5 N NaOH according to the method of Somogyi (40), and 5 ml aliquots were passed through a column of MB3 resin as described above. The eluate plus water washings was made to 10.0 ml with water, and 0.5 ml aliquots were used for radioactivity measurements in the Tri-Carb Series 3000 Liquid Scintillation Spectrometer as described for the amino acids in PART I. Another sample was used for glucose determination with the Glucostat (Worthington Biochemical Company) using

reaction conditions of 37° for 30 minutes. The remainder of the sample was used to make a glucosazone derivative which was then combusted and counted in the wet combustion-vibrating reed apparatus referenced in PART I. Specific activities of blood glucose determined by the glucosazone method ranged from 14% to 38% lower than those determined directly from the column glucose sample. A chromatogram of a glucose sample off the column run in n-butanol: acetic acid: water (4:1:5) on Whatman No. 1 yielded activity at the glucose position and at the origin (unidentified).

B. Animal Experiments

1. Changes in Blood Glucose Levels with Glucagon Administration

Solutions of 1 mg/ml were prepared by dissolving a weighed amount (1 mg at a time) of crystalline glucagon (lot no. 126B-0440, Sigma Chemical Company) in water at pH 2.5 with HCl. These solutions were kept at 5°, and the glucagon remains in solution with maximum stability under these conditions for 1 year (41). Addition of a solution 0.05 M in KCl, however, would rapidly and irreversibly denature it. A blood dilution pipet was calibrated to contain 0.044 ml of blood and was used whenever small blood samples were needed. In order to determine the variation in blood glucose levels after glucagon administration, 3 young male Holtzmann rats (175-250 g) were used. A stock Purina diet and water were fed *ad libitum*. All animals were used in the early morning to insure a fed condition. The rats were injected intraperitoneally with varying amounts of the glucagon solution, and blood samples were taken from nicks in a tail vein onto a heparinized slide at five minute intervals for 40 minutes. A device employed to immobilize the animals while the samples were being taken consisted essentially of 2 hinged sponges, between which the rats were restrained, mounted on a small platform (42).

The blood samples were deproteinized according to the microtechnique of Somogyi (40), and Glucostat assays were run in duplicate on each sample to determine the amount of glucose present. Each rat served as its own control, the same procedure being used after the intraperitoneal injection of a solution of water at pH 2.5 with HCl.

2. Isotope Experiments

Young male albino rats, as above, were used (203-290 g) and were maintained under the same conditions unless specified, as in Table I where fasting conditions were imposed for 48 hours upon two animals. Glucagon (2 mg/kg; 1 mg/ml) was given intraperitoneally 15 minutes and 3 hours prior to the injection, also intraperitoneal, of sodium pyruvate-2-^{14}C in 0.9% NaCl (10 mc/mmole, purchased lyophilized from Nuclear-Chicago). D-mannoheptulose, a gift from Dr. Nelson Richtmyer, National Institutes of Health, Bethesda, Maryland, was given subcutaneously (600 mg per rat in water) followed by sodium pyruvate-2-^{14}C, injected intraperitoneally, 1 and 4 hours later. The animals were allowed to metabolize the isotope for 12 minutes. They were then stunned and their throats cut; the blood was collected in a beaker treated with a very small amount of 10% potassium oxalate and was deproteinized within 5 minutes of exsanguination as described in Part A of CHAPTER II. The liver was removed, blotted on filter paper and frozen in liquid nitrogen. The free glutamic and aspartic acids were isolated as described by O'Neal and Koeppe (43). The rats were considered fed if their stomachs still contained food upon post-mortem examination.

C. Counting Techniques and Degradation Procedures

The counting techniques and degradation procedures for the amino acids are identical to those described and referenced in PART I.

CHAPTER III

RESULTS AND DISCUSSION

Figure 1 demonstrates the rapid rise in blood glucose levels after the intraperitoneal injection of glucagon (1 mg/kg). In all animals a fairly constant maximum level was achieved at about 30 minutes after injection, although the initial response appears to be characteristic of each animal. Other workers have also noted this 30 minutes period necessary for maximum effect after glucagon administration (34,44). Increasing the dose to 2 mg/kg resulted in a linear increase in the blood sugar level with no significant alteration in the length of time in which the peak level of blood glucose was obtained (Figure 2). The rate of fall of the glucose level after its peak rise was much more rapid with the increased dose. It is interesting to note that the stress placed upon each of the rats by restraining them every 5 minutes for a period of 40 minutes in an apparatus specifically designed for tail vein injection in order to remove a tail vein blood sample elicited a rise in blood sugar of 33 $\pm$ 13%. Such stress probably boosted the peak blood sugar levels and undoubtedly affected the initial slope of the curves.

The occurrence of enhanced gluconeogenesis after glucagon administration to rats <u>in vivo</u> has been demonstrated by the incorporation of increased amounts of ^{14}C-bicarbonate into plasma glucose (45). Glucagon has been shown to produce a five fold increase of ^{14}C-glucose production after 30 minutes (46). As can be seen in Table I, fasted rats incor-

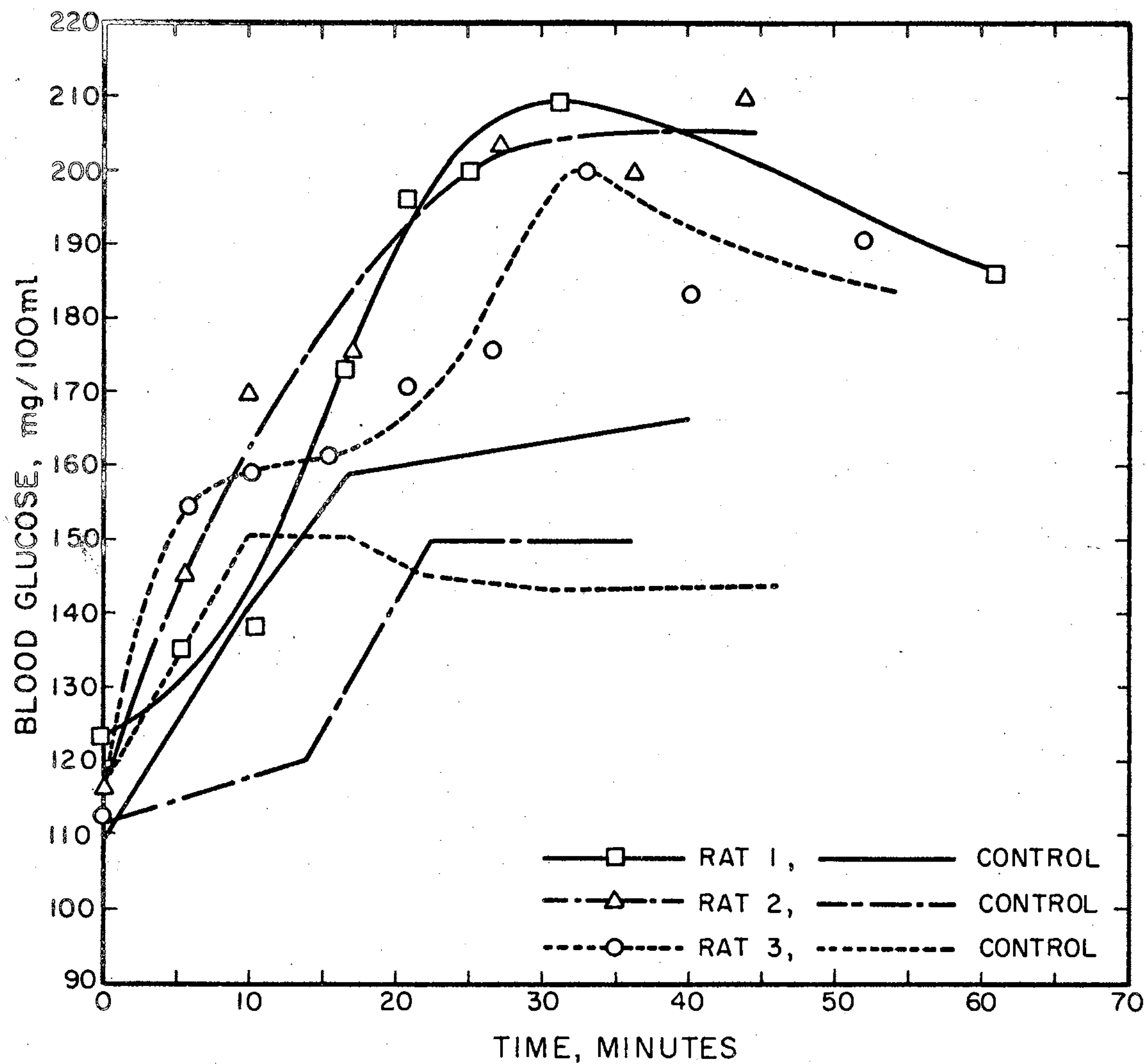
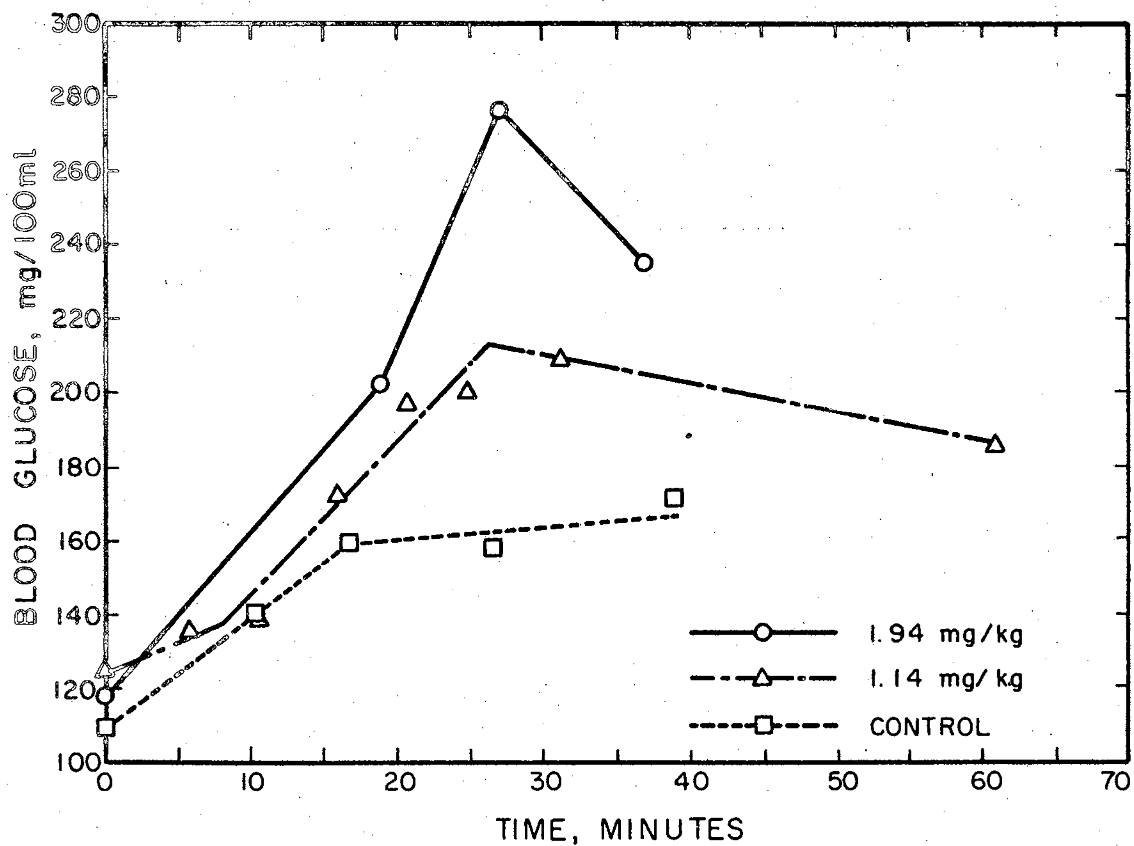
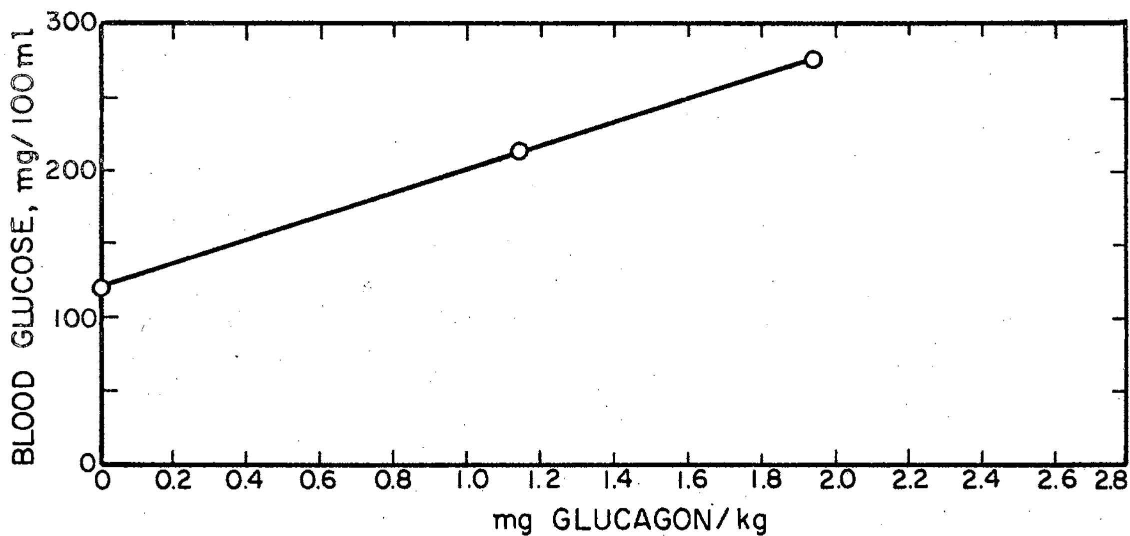


Figure 1. Blood Glucose Levels after Glucagon Administration as a Function of Time

Rats were given glucagon (1 mg/kg) intraperitoneally, and blood samples were taken from a tail vein at 5 minute intervals as described in the text. Each rat served as its own control.



A. Blood Glucose Levels after Glucagon Administration as a Function of Time



B. Blood Glucose Levels as a Function of Dosage

Figure 2. Blood Glucose Response to Varying Levels of Glucagon

porate greatly increased amounts of pyruvate-2-^{14}C as compared to fed rats. The blood glucose specific activity should then be indicative of the amount of pyruvate that is converted to glucose and thus should correlate with the percentage of labeling in C-5 of glutamate. In using hyperglycemic agents, the total amount of incorporation into glucose should be considered since the increased rate of glucose production may result in dilution of the isotope due to an increased glucose pool size.

As is shown in Table I, the total incorporation of activity into glucose in 100 ml of blood from pyruvate-2-^{14}C in a fasted rat was about 10 times the incorporation into the blood glucose of a fed rat. Administering glucagon 15 minutes before the injection of the isotope increased the amount of incorporation somewhat, while 3 hours after glucagon treatment, the total incorporation of activity into the glucose in 100 ml of blood resembled that of a normal fed control. Mannoheptulose administration had an immediate effect as well as a long-lasting effect; the amounts of incorporation after 1 and 4 hours were similar and more than doubled the total incorporation into blood glucose obtained with glucagon. The fact that mannoheptulose retains its effectiveness in this respect even after 4 hours may indicate not only an inhibition of insulin release from the pancreas but also an impairment of insulin synthesis by this compound as has been proposed by Moskalewski (47).

By allowing the rats to metabolize the isotope for a duration of 12 minutes, 15 minutes after the injection of glucagon, it was hoped to determine whether the rapid production of new glucose at this point was a result of gluconeogenesis, which would yield glutamate with a very low per cent labeling in C-5, or whether the new glucose was derived from glycogenolysis, which would result in high labeling in C-5 of glutamate.

TABLE I

INCORPORATION OF PYRUVATE-2-^{14}C INTO BLOOD GLUCOSE OF INTACT RATS

Rat Number	Treatment, Time	Amount of Pyruvate-2-^{14}C Injected, μc	Blood Glucose mg/100ml	Blood Glucose Spec. Act., μc/mmole	Blood Glucose μc/100ml
324	Fed	11.0	104	1.0	0.58
326	Fed	25.0	99	2.2	1.21
325	Fasted, 48 hours	21.0	90	27.6	13.79
327	Fasted, 48 hours	21.0	66	28.4	10.41
328	Glucagon, 15 minutes	21.0	230	1.3	1.66
329	Glucagon, 15 minutes	22.0	113	2.7	1.68
330	Glucagon, 3 hours	21.5	109	2.3	1.39
335	Glucagon, 3 hours	21.0	99	2.1	1.16
331	Mannoheptulose, 1 hour	22.8	339	2.1	4.00
332	Mannoheptulose, 4 hours	22.8	134	5.4	4.02

Any degree of cooperation between these pathways should moderate the results between these two extremes. Further work was also done on rats given glucagon 3 hours prior to pyruvate-2-^{14}C injection since Shrago et al. (14) have found a 2.5 fold increase of phosphoenolpyruvate carboxykinase activity above normal 3 hours after giving glucagon. If this enzyme is indeed pertinent to the control of gluconeogenesis and is relatively more active, then a shift in labeling from high C-5 to lower C-5 in glutamate would be observed. A summary of the results is given in Table II. The labeling patterns found in both the 15 minutes and 3 hours experiments resemble those of normal fed rats. This would seem to indicate the predominance of glycogenolysis over gluconeogenesis in the effect of glucagon; however, it can be assumed that a slight increase in gluconeogenesis occurred as shown in the incorporation of more activity into blood glucose 15 minutes after glucagon administration. The high labeling in C-5 of glutamate probably indicates that this method utilizing glutamate labeling patterns is not sensitive enough to reflect small changes in the relative routes of pyruvate metabolism.

Mannoheptulose, on the other hand, exerts a profound effect upon the labeling in glutamate. The per cent labeling found in C-5 was 8%, both 1 and 4 hours after its administration. The effect upon pyruvate metabolism is clearly seen after 1 hour although the rise in phosphoenolpyruvate carboxykinase activity is less than the rise in activity following glucagon administration after 3 hours, which did not alter the amount of labeling in C-5 of glutamate as compared to a normal fed rat. Predictably, these data indicate that factors other than changes in phosphoenolpyruvate carboxykinase activity are responsible for the differences in the labeling of C-5 of glutamate and implicate insulin in the

TABLE II

LABELING PATTERNS IN LIVER GLUTAMATE AFTER PYRUVATE-2-^{14}C INJECTION

Rat Number	Treatment, Time	Amount of Pyruvate-2-^{14}C Injected, μc	Distribution of ^{14}C in Glutamate				
			Spec.Act., μc/mmole	Per Cent of Activity			
				C-1	C-2+C-3	C-4	C-5
324	Fed*	11.0	3.1	18	34	1.9	47
326	Fed*	25.0	3.7	17			34
325	Fasted,* 48 hours	21.0	11.2	7	94	0.2	2
327	Fasted,* 48 hours	21.0	3.3	6	88	0.2	2
328	Glucagon, 15 minutes	21.0	6.2	11	26	0.8	45
329	Glucagon, 15 minutes	22.0	3.0		63		37
330	Glucagon, 3 hours	21.5	2.9	17	42	3.1	39
335	Glucagon, 3 hours	21.0	6.7		64		32
331	Mannoheptulose, 1 hour	22.8	6.2		87		8
332	Mannoheptulose, 4 hours	22.8	5.2		91		9

* S. K. Meghal, personal communication.

control of this pathway.

In two glucagon-treated animals the percentage of activity in C-4 was also determined. Work by Greenbaum et al. (48) has shown that the $NAD^+/NADH$ ratio in the liver of glucagon-treated rats rose from the control value of 3.6 to 5.0. The response of the $NAD^+/NADH$ ratio to various treatments is significant since, while liver mitochondria carboxylate pyruvate to form oxalacetate, the latter does not diffuse through the membrane, and, instead, must be either converted to citrate, transaminated to form aspartate, or reduced by NADH to malate, all of which can diffuse from the mitochondria. In gluconeogenesis, malate in the cytoplasm must be oxidized by NAD^+, and the oxalacetate produced converted to phosphoenolpyruvate. In the cytosol[1] the ratio $NAD^+/NADH$ would be lowered by the relative conversion of more malate to oxalacetate; conversely, if more oxalacetate is reduced to malate, prior to the conversion of this malate to oxalacetate and then to citrate in the mitochondria, the ratio $NAD^+/NADH$ would be increased. In the cytosol of a fed rat the concentration of NAD^+ is almost 9 times that of NADH; in a rat fasted for 24 hours, however, the ratio $NAD^+/NADH$ drops to 4.5, indicating a shift to the oxidation of malate to oxalacetate in preparation for the conversion of oxalacetate to phosphoenolpyruvate and then to glucose (49). If pyruvate-2-^{14}C were metabolized via oxalacetate, malate, and randomized in fumarate in the tricarboxylic acid cycle of the mitochondria and then converted back to phosphoenolpyruvate via malate and oxalacetate in the cytosol, the cytoplasmic phosphoenolpyruvate would be labeled in both carbon 2 and carbon 3. Metabolism of this

[1]Cytosol is defined (49) as the cytoplasm minus the mitochondria and endoplasmic reticulum components.

intermediate to pyruvate and then to acetate would result in acetate labeled in both C-1 and C-2 with the amount of labeling in C-2 being proportional to the amount of pyruvate traversing such a pathway. Since C-2 of acetate is the precursor of C-4 of glutamate, the amount of activity in C-4 of glutamate as compared to the amount in C-5 may reflect this metabolic route, although C-2 of oxalacetate also labels C-4 of glutamate after one turn of the tricarboxylic acid cycle. The relative dominance of this pathway could either control or be controlled by the ratio of NAD+ to NADH since the greater the amount of oxidizing power available, the more malate can be converted to oxalacetate, and, conversely, the more malate converted to oxalacetate, the higher the concentration of NADH. The data presented here indicate no significant change from the results with a normal fed rat, which has about 2% in C-4 of glutamate as compared to about 35% in C-5 (23), indicating that, after glucagon treatment, the formation of doubly labeled acetyl CoA derived from oxalacetate that had equilibrated with fumarate is not accelerated.

It seems more likely, then, as indicated by the data presented here, that the primary source of the new glucose produced by the injection of glucagon is the breakdown of glycogen rather than increased gluconeogenesis. While gluconeogenesis may be very slightly accelerated, this increase is too small to be detected by a reduction in the amount of radioactivity incorporated into C-5 of glutamate from pyruvate-2-^{14}C.

Since the blockage of insulin release by mannoheptulose has been well documented (14), the effect of mannoheptulose is probably best defined as a lack of insulin, which results in a diabetic state with all the concurrent symptoms, including severe hyperglycemia. Although the effect of mannoheptulose is not permanent, the duration of the syndrome

following a single injection suggests a more severe effect such as impairment of insulin synthesis.

CHAPTER IV

SUMMARY

When glucagon and mannoheptulose were given to normal fed rats prior to the injection of pyruvate-2-^{14}C, the following results were obtained:

1. The hyperglycemic effect of one intraperitoneal injection of glucagon was immediate, and blood glucose reached a peak level in about 30 minutes after injection;

2. Glucagon did not alter the labeling pattern found in the free liver glutamic acid either 15 minutes or 3 hours after injection as compared to a normal fed control;

3. Mannoheptulose reduced the per cent of activity in C-5 of free liver glutamic acid about four fold as compared to a normal fed rat, both 1 hour and 4 hours after injection;

4. Mannoheptulose more than doubled the amount of pyruvate-2-^{14}C incorporated into blood glucose as compared to a normal fed control, while glucagon only very slightly increased the amount of isotope incorporated into blood glucose over that of a normal fed rat.

These data indicate that the primary source of the new glucose produced after glucagon injection is glycogenolysis rather than increased gluconeogenesis, and that, based upon comparisons with other studies, the effect of mannoheptulose is probably best described as insulin insufficiency.

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APPENDIX A

BLOOD GLUCOSE LEVELS PRIOR TO TERMINATION OF EXPERIMENTS

Rat Number	Rat Weight	Treatment, Time	Blood Glucose, mg/100ml Pre-injection	15 min.	30 min.
328	203	Glucagon, 15 minutes	116	168	
329	211	Glucagon, 15 minutes	116	149	
330	209	Glucagon, 3 hours	130		146
335	204	Glucagon, 3 hours	123	150	141
331	260	Mannoheptulose, 1 hour	135		
332	290	Mannoheptulose, 4 hours	109		

VITA

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Master of Science

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