SOME ASPECTS OF THE METABOLISM OF D- AND L-LACTIC ACID-2-C^{14} IN RATS

by

NILO FRANCIS INCIARDI

Bachelor of Science
Oklahoma State University
Stillwater, Oklahoma
1948

Master of Science
Oklahoma State University
Stillwater, Oklahoma
1949

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Thesis Approved:

Roger E. Koeppe
Thesis Adviser

Robert K. Gholson

Dean of the Graduate School

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CHAPTER I

INTRODUCTION

The metabolic fate of lactic acid in animal tissue has not as yet been completely elucidated. In 1929 Cori (1, 2) showed that both d- and l-lactic acid[1] were metabolized in rat tissue, but l-lactic acid was metabolized more slowly. He isolated the liver glycogen from a number of rats after 24 hours of fasting to determine the amount of glycogen found there normally. Then he fed separate groups of rats d-, l-, and r-sodium lactate. After 3 hours the rats were decapitated and the liver glycogen of each rat was isolated. The results were averaged and the glycogen normally found in the liver after 24 hours of fasting was subtracted. About 4 times as much liver glycogen was isolated from rats fed d-lactate as from rats fed l-lactate with the value for r-lactate being in between. About 30 per cent of the l-lactate was lost through the kidneys into the urine. The "Cori Cycle" shows that d-lactate in the blood goes to the liver where it is

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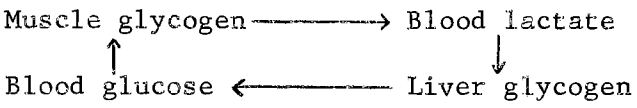
[1]The d- and l-prefixes originally referred to the dextro (to the right) or levulo (to the left) rotation of polarized light. The prefix r- indicated a racemic mixture. Presently the assymetry of the hexoses is based upon the position of the hydroxyl group on the highest numbered assymetric carbon when compared to glyceraldehyde so that the hydroxyl group of the "D" form is on the right and that of the "L" form is on the left. This is followed by a (+) to indicate that the direction of rotation of polarized light is to the right or a (-) to indicate rotation to the left.

converted to glucose as follows:



The Cori Cycle

The glucose is carried by the blood to the muscle where it is converted to muscle glycogen. Muscular contraction causes the breakdown of glycogen to d-lactate and the cycle repeats itself.

Isotopic labeling techniques have been of great importance in the determination of metabolic pathways. These techniques have been used extensively in studies on lactic acid metabolism. Of prime interest to us is the equilibration of the glycogenic pathway of lactic acid with the "dicarboxylic acid shuttle" (Figure 1) of the TCA cycle prior to the formation of glycogen. Lorber et al. (3) have shown that lactate-2-C^{14} fe to rats is incorporated into liver glycogen with the radioactive isotope appearing in carbons 1, 2, 5, and 6 of the glucose molecule. Less than 16 per cent goes to glucose directly. The randomization of the isotope is probably accomplished by a glycogenic pathway involving the dicarboxylic acids. Pyruvate is converted to malate (4, 5) and thence to fumarate (6, 7). Fumarate is chemically and biologically symmetrical so that when reversal takes place and oxaloacetate is converted to phosphoenolpyruvate, the labeling will be in the 2 and 3 carbons of the molecule. Incorporation of this phosphoenolpyruvate into glucose through the Embden-Meyerhof pathway labels the glucose in carbons 1, 2, 5, and 6. Gould et al. (8) compared the rates of conversion of pyruvate-2-C^{14} and $C^{14}O_2$ to glycogen. Their results indicated that pyruvate is equilibrated more rapidly with carbon dioxide than it is converted to glycogen. Hastings and coworkers (9) and Marks and Horecker (10) found that in

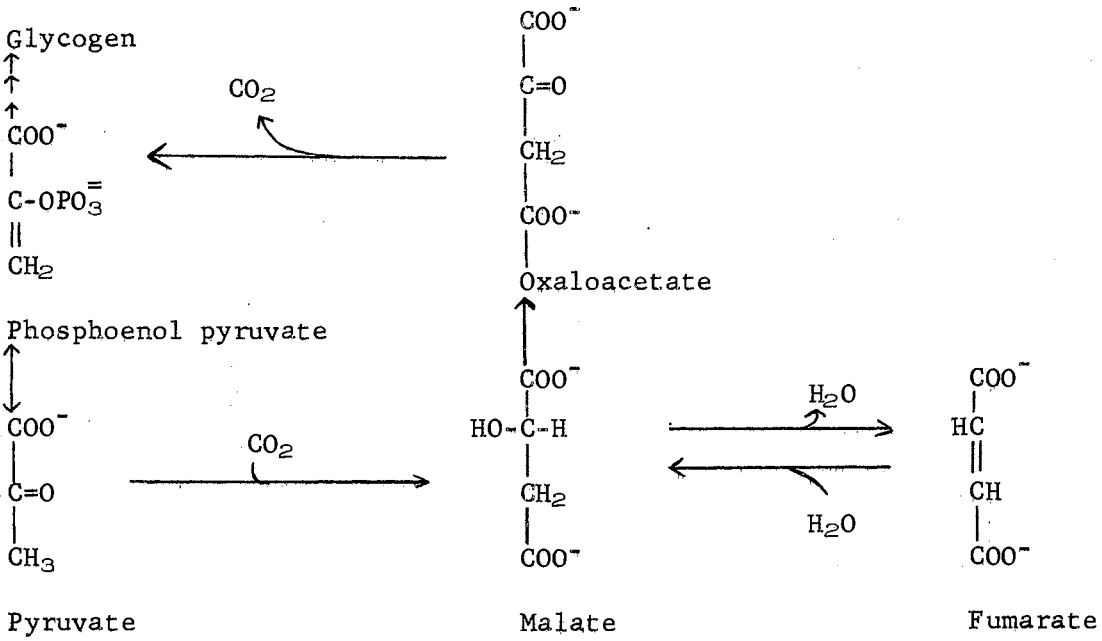


Figure 1. The dicarboxylic acid shuttle.

contrast to fasted rats, when fed rats were given pyruvate-C^{14} or $C^{14}O_2$, there was assymetry in the glucose molecule when the specific activity of carbons 1 to 3 were compared with that of carbons 4 to 6. A possible explanation for these results is that in the fed rat there is an adequate supply of the 3 carbon pool and unlabeled dihydroxyacetonephosphate is available for the fructoaldolase step. However in the fasted rat, some of the labeled glyceraldehyde-3-phosphate must be converted by isomerase to dihydroxyacetonephosphate in order that fructose-1,6-diphosphate may be formed. Koeppe et al. (11) have shown that pyruvate metabolism in the liver fluctuates more and proceeds via oxaloacetate to a greater extent, than in the carcass. Also, fasting seems to reduce the conversion of pyruvate to acetyl-CoA in the liver.

Hoberman and D'Adamo (12) showed that about 11 per cent as much D(-) lactate as L(+) lactate is incorporated into glucose. They also pointed out that their work showed that twice as much C^{14} was utilized in glycogen synthesis in the liver after equilibration with fumarate than by the direct phosphorylation of pyruvate. Then using malate-3-C^{14}, an intermediate between pyruvate and fumarate in the "dicarboxylic acid shuttle," they found identical labeling in carbons 5 and 6 of glucose (13). This finding supports the idea that randomization does occur in the "dicarboxylic acid shuttle."

A number of workers (14, 15, 16) have shown that labeled carbon dioxide is incorporated into rat liver glycogen in vivo and in vitro. Labeled carbon dioxide is combined with pyruvate to form malate through the action of malic enzyme. Then, after equilibration with fumarate, the reaction goes forward with labeling in the carboxyl group of phosphoenolpyruvate. This eventually becomes carbons 3 and 4 of glucose.

Cook and Lorber (17) showed that glucose-1-C^{14} went into the glycogen of rat liver and muscle 80 to 90 per cent intact. This would indicate that once glucose is formed, it generally goes directly into glycogen rather than through some alternate pathway. However, Marks and Feigelson (18) injected glucose-2-C^{14} into the rat intraperitoneally and found a significant amount of labeling in carbon 1 and some in carbons 3 to 6 of glucose from liver glycogen. However, in glucose from muscle glycogen all of the labeling was in carbon 2. Siu and Wood (19) also showed that glucose-2-C^{14} is subject to randomization in the liver and attribute it to an alternate pathway, known as the pentose shunt (20), through glucose-6-phosphate. The results of Stetten et al. (21) also indicate that there exists a pathway other than Embden-Meyerhof for glucose degradation. This is probably the pentose shunt. Marks and Feigelson (22), again using glucose-2-C^{14} in the rat, found predominantly carbon 1 labeling in ribose of RNA. When labeled sodium bicarbonate was used it resulted in glucose-3,4-C^{14} labeling and ribose-2,3-C^{14} labeling, again indicating a carbon 1 decarboxylation of 6-phosphogluconolactone through the pentose shunt.

Another metabolic pathway that should be considered for lactic acid metabolism is that involving methylglyoxal. Brin and Olson (23) and Brin et al. (24) showed that both L(+)- and D(-)-lactic acids are oxidized by heart, liver, and brain tissue. However, D(-)-lactate was oxidized 20 to 33 per cent as fast in the heart and liver and 5 to 10 per cent as fast in the brain as was L(+)-lactate. It was suggested that either a racemase enzyme or a lactic dehydrogenase enzyme specific for D(-)-lactic acid was operating, or that an alternate pathway through non-reducing intermediates was present. Brin et al. (25)

later found that thiamine deficiency causes a decrease in the transketolase step of the pentose shunt with an accumulation of pentose to 3 times normal values.

It is well known (26, 27) that thiamine deficient animals accumulate methylglyoxal. Van Eys et al. (26) found that D(-)-lactic acid was excreted in the urine by both thiamine deficient and normal animals but that the D(-)-lactate to L(+)-lactate ratio was greater in deficient animals. The urinary pyruvate to lactate ratio increased and the pyruvate to L(+)-lactate ratio increased considerably.

Miller and Olson (28) observed, in experiments with heart muscle slices using pyruvate and lactate as substrates in an anaerobic system, that 0.1 M fluoride completely inhibited the utilization of pyruvate while lactate utilization was unimpaired. Since fluoride inhibits the enolase reaction in the Embden-Meyerhof glycolytic pathway, they postulated that lactate could be metabolized by an alternate pathway not involving its conversion to pyruvate. Miller et al. (29) suggested that the lactate pathway might involve lactaldehyde, acetol, and 1,2-propanediol either in the free or phosphorylated form. Shull and Miller (30) state that it has been shown that these intermediates are glycogenic in the alternate anaerobic lactate pathway. DL-Lactaldehyde was used in these experiments. Preliminary investigation indicates that both rat and mouse liver contain an enzyme system which in the presence of NAD catalyzes the oxidation of acetol. Although the product of this reaction has not been identified, the oxidation of the primary alcohol group in acetol by the removal of two hydrogens would produce methylglyoxal. Methylglyoxal through the enzymic action of the glyoxalases yields D(-)-lactate.

Horecker et al. (31) have reported that when pyruvate-2-C^{14} was incubated with rat liver and rat diaphragm in vitro, the glucose from liver glycogen showed labeling predominantly in carbons 1, 2, 5, and 6 while glucose from the muscle glycogen showed labeling primarily in carbons 2 and 5. This would indicate that muscle is capable of converting lactate (via pyruvate) to glucose and glycogen without randomization of carbons 2 and 3, and that muscle is not entirely dependent upon the liver for glucose.

The objective of this investigation was to further elucidate the metabolism in vivo of D(-)- and L(+)-lactic acid in rats. Since Cori (1, 2) has shown that D(-)- and L(+)-lactic acids are both glycogenic in the rat in vivo and Horecker et al. (31) have shown that pyruvate-2-C^{14} is glycogenic in vitro in both muscle and liver, it should be possible to administer D(-)- or L(+)-lactate-2-C^{14} to the rat and from the labeling patterns found in liver and muscle glycogen, postulate the probable pathways of metabolism. This investigation attempted to show in vivo whether glycogen is formed directly in the muscle without randomization from labeled D(-)- and L(+)-lactic acid converted directly to muscle glycogen or indirectly via the liver and blood glucose.

The approach used was to administer glucose to the fasted rats followed in a short time by D(-)- or L(+)-lactate-2-C^{14}. The glucose will stimulate glycogenesis in the liver and muscle so that when labeled lactate is added a larger amount will be assimilated than if lactate is added without starving and administering of glucose. After an interval of time to allow assimilation of lactate to take place the animals were sacrificed and the liver and muscle glycogen and blood glucose were

isolated. Two periods of time were used to determine whether a shorter period would show a different labeling pattern than the longer period.

CHAPTER II

EXPERIMENTAL

A. Synthesis of Compounds Used

Labeled L- and D-lactate-2-C^{14} are not available commercially. Therefore these compounds were prepared by the enzymatic reduction of pyruvate-2-C^{14}, using enzymes specific for each isomer. L-Lactate-2-C^{14} was prepared with rabbit muscle lactic dehydrogenase obtained from the Worthington Biochemical Corporation and prepared by the method of Racker (32). NADH was obtained from the California Corporation for Biochemical Research and pyruvate-2-C^{14} from the Nuclear-Chicago Corporation. The following incubation mixture was used:

Sodium pyruvate-2-C^{14}	0.05 mM	1 ml
NADH	0.18 mM	150 mg
L(+)-lactic dehydrogenase	50 units	1 ml
0.03 M phosphate buffer pH 7.5		3 ml

The mixture was allowed to react for 2 hours at 25° C although there was enough enzyme present to cause the reaction to go to completion in 1 minute. A unit of enzyme activity is defined as that amount which causes an initial rate of oxidation of NADH of 1 micromole per minute.

Since lactic dehydrogenase specific for D-lactate was not available commercially, it was necessary to prepare it. Lactobacillus leichmannii (ATCC 4797), which produces D-lactate, was selected as a source of

D-lactic dehydrogenase. The bacteria were grown according to the method of Brin et al. (33). A test tube containing 10 ml of media was inoculated and incubated at 37° C for 36 hours. A stab culture was started in agar media and the remainder was used to inoculate 100 ml of liquid media. These were again incubated for 36 hours, after which the stab culture was stored in the refrigerator at 4° C and the 100 ml culture was used to inoculate 1 liter of media. In order to perpetuate the culture every 1 to 2 weeks the stab culture was used to inoculate a 10 ml tube of liquid media which, in turn, was used to inoculate a fresh tube of agar media. After incubation the agar tube was again stored at 4° C in the refrigerator. The 1 liter culture was transferred to a final culture of 10 liters and incubated. The bacteria were harvested with a Sharples centrifuge. At this point the bacteria can be stored for 1 to 2 hours at 4° C or overnight frozen in 0.1 M phosphate buffer pH 6.3.

The extraction and purification of the enzyme was done by the method of Yamada et al. (35) and the enzyme further purified by precipitation with ammonium sulfate. All precipitates were separated by centrifugation, dissolved in 0.1 M phosphate buffer of pH 6.3, and assayed for specific and total activities. The ammonium sulfate fractions collected were 0 to 50, 50 to 60, 60 to 70, and 70 to 80 per cent. The 60 to 70 per cent fraction showed the greatest activity and was used in the synthesis of D(-)-lactate. All work was done in the cold room and all enzyme preparations were stored at -20° C. Table I shows the results of the purification of the D-lactic dehydrogenase.

The enzymic assays were done spectrophotometrically on a model 14

TABLE I

PURIFICATION OF D(-)-LACTIC DEHYDROGENASE

Sample	Total Protein	Specific Activity*	Total Activity
Crude Protein	84	3.1	250
0 - 50%	40	2.2	88
50 - 60%	7.0	12.4	87
60 - 70%	7.8	26.2	204
70 - 80%	8.8	0.45	4

*oxidation of DPNH in μm/min/mg

Cary recording spectrophotometer by the method of Neilands (35) modified by using a 0.03 M phosphate buffer at the pH optimum of the particular enzyme. The assay was followed by the change in absorbancy at 340 mμ as NADH was oxidized to NAD^+.

Protein was determined by the Folin-Cicolteau method (36).

The pH optimum of each enzyme was determined by using the enzyme assay system but varying the pH of the phosphate buffer at intervals of 0.5 pH units from pH 6.0 to 9.0. The pH optimums were found to be 7.0 for D-lactic dehydrogenase and 7.5 for L-lactic dehydrogenase (Figure 2). The latter value is in agreement with that reported by Racker (32).

D-Lactic acid was synthesized by the method described above for L-lactic acid except that D-lactic dehydrogenase and a pH 7.0 phosphate buffer were used.

The reaction mixtures containing the labeled lactic acids were evaporated to a small volume under a stream of air at room temperature. These were then brought to pH 1.0 or less with 25 per cent sulfuric acid and taken up with dry Celite previously prepared for column chromatography. The sample was transferred to a Celite column and 100 ml of CB-5 (see Part B) was passed through the column to remove any unreacted pyruvic acid-2-C^{14}. This was followed by 125 ml of CB-10 to elute the lactic acid-2-C^{14}. Elution was accomplished at the rate of 0.5 to 1.0 ml per minute with 5 ml fractions being collected in an automatic fraction collector. The fractions were titrated with 0.01 N sodium hydroxide to a pH 7.5 endpoint using a Beckman zeromatic pH meter. One lambda samples from each tube were transferred to planchets and assayed for radioactivity in a gas flow Geiger counter. The results of the titrations and radioactivity determinations are shown in Figures 3 and 4. The titration and

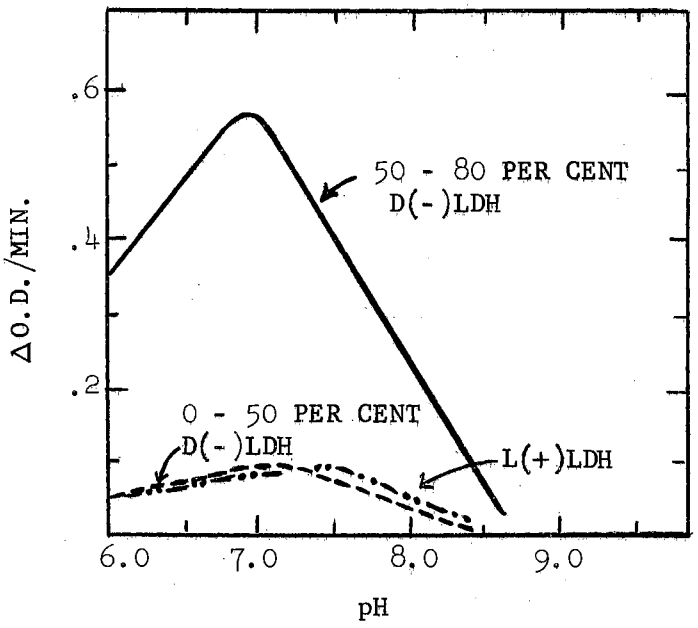


Figure 2. pH optimums for two fractions of D(-)LDH and L(+)LDH in the reaction from lactic acid to pyruvic acid.

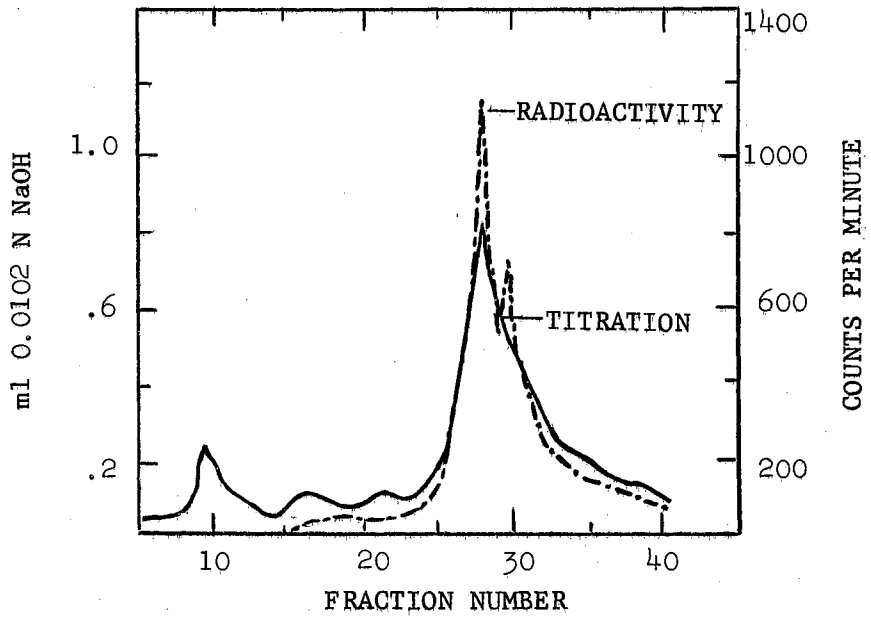


Figure 3. Titration and radioactivity plot for the enzymically prepared L(+)-lactic acid.

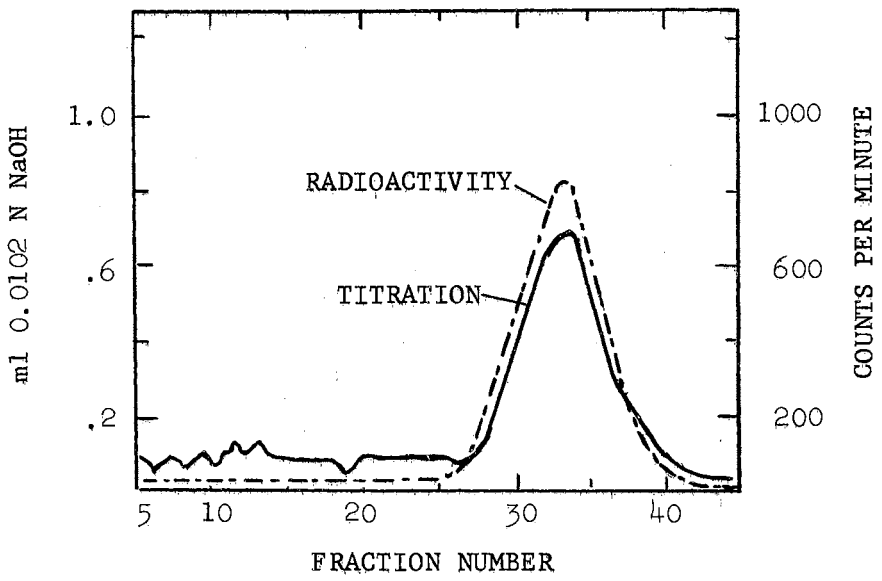


Figure 4. Titration and radioactivity curve for enzymically prepared D(-)-lactic acid.

radioactivity peaks were nearly coincident for both lactic acid isomers.

B. Chromatographic Procedures

1. Column Partition Chromatography

The technique of Phares et al. (37) was used for the purification of acetic acid and the separation and purification of lactic acid from pyruvic acid. Chromatographic columns (1 x 38 cm) were packed with Celite by the addition of a slurry (which had previously been mixed by grinding 100 gm of Celite with 50 ml of 0.5 N sulfuric acid using mortar and pestle) suspended in a 10 per cent solution of acetone in n-hexane. A 2 cm layer of anhydrous sodium sulfate was packed on top of the Celite to remove any traces of moisture. Prior to use the column was equilibrated by passing over the column 50 ml of the first eluant to be used. Chloroform-butanol solutions equilibrated with 0.5 N H_2SO_4 were used as eluants i.e., CB-1 means 1 per cent butanol in chloroform.

2. Charcoal Column Chromatography

Hydrolysis of glycogen, if complete, results in the destruction of a portion of the glucose formed. At the point of maximum glucose yield there will be a mixture of some decomposition products and some products of incomplete hydrolysis, mainly maltose. The contaminants were removed by the method of Whistler and Durso (38) and Whistler (39) with some modifications.

Nuchar C-190, plus 30 mesh (supplied by the West Virginia Pulp and Paper Co.) was prepared by removing the fine particles with several washings. This was poured as a slurry into a glass column (2.5 x 23 cm), and treated with 1 N hydrochloric acid followed by washing with deionized water until chloride negative as shown by testing with silver nitrate. Elution was done with 5 per cent ethanol

at the rate of 0.5 to 1.0 ml per minute. This procedure will separate monosaccharides from di- and trisaccharides. Glucose comes through between 60 and 260 ml when eluted with 5 per cent ethanol.

3. Ion Exchange Chromatography

Lactic acid is found in the blood of the rat and is extracted with the glucose. Since lactate is one of the products obtained during the degradation of glucose, blood lactate must be removed before degrading blood glucose. Dowex 1 x 8 (50 to 100 mesh, Cl^- form) was treated with 3 N sodium acetate until chloride negative. Then the resin was washed with deionized water until the pH of the wash water coming out was the same as that going into the column.

The blood filtrate sample, usually about 10 ml, was put on the column (1 x 3 cm) and washed with about 25 ml of deionized water. The anthrone test was used qualitatively to determine when the glucose had passed through. This treatment was shown to quantitatively remove lactate.

In order to remove sulfate ions from the glucose solution after the hydrolysis of glycogen, Amberlite CG-45 (type 1), a weak cation exchange resin, was used. Although it is received from the manufacturer in the freebase form, 1 N sodium hydroxide was passed through the resin. The resin was then washed with deionized water until the pH of the water eluted was the same as that of the water being added.

4. Paper Chromatography

Before the glucose samples were degraded the purity was determined through the use of paper chromatography (41). The tubes from the charcoal column which contained glucose were detected quali-

tatively by the anthrone test (40). Every fifth tube was spotted on Whatman No. 1 filter paper. Fifty gamma samples of standard dextrose and maltose were also spotted for comparison. The chromatogram was placed in jars containing n-butanol-methyl cellosolve-water (2:1:1) and allowed to develop for about 8 hours. After drying the spots were developed by spraying with 0.5 N potassium hydroxide in methanol followed by 0.2 per cent silver nitrate solution in acetone. The chromatogram can then be fixed by rinsing in a 10 per cent solution of sodium thiosulfate.

C. Purity and Radioactivity of D(-)- and L(+)-Lactate-2-C^{14}

Zinc lactate was prepared according to the method of Brin et al. (33). Samples of L(+)-calcium lactate and of D(-)-calcium lactate were each passed over Dowex 50 in the hydrogen form and the columns washed with water to yield 2 mmoles of D(-)- or L(+)-lactic acid. To each of the lactic acid samples was added 0.10 ml of labeled lactate as indicated below.

	Labeled Isomer Added	
Unlabeled Isomer	L(+)-Lactic Acid	D(-)-Lactic Acid
D(-)-Lactic Acid	0.10 ml	0.10 ml
L(+)-Lactic Acid	0.10 ml	0.10 ml

The zinc lactate crystals were prepared from each sample and radioactivity determined after the second and third crystallizations. The results are shown in Table II.

By this procedure the total radioactivity and the purity of each isomer was determined. The radioactivity remaining after repeated crystallization of a mixture of the labeled isomer and its enantiomorph indicates the maximum contamination of the labeled compound by

TABLE II

RESULTS OF RADIOACTIVITY DETERMINATIONS OF ZINC LACTATE AFTER THE NUMBER OF RECRYSTALLIZATIONS INDICATED TO DETERMINE PURITY

Sample	Radioactivity			
	Crystallizations			
	First Run		Second Run	Total Activity μc per ml
	2	3	5	
L(+) and L(+)*	969	968		9.7
L(+) and D(-)*	190	95		
D(-) and D(-)*	935	1000	976	9.7
D(-) and L(-)*	113	49.5	19.8	

its enantiomorph (42). On the other hand, when the labeled isomer was diluted with carrier, there was no loss of specific activity. This again showed the absence of impure optical isomer which in this case would have formed a racemic mixture and resulted in loss of activity. This process also gave the total activity of each optical isomer synthesized.

D. Animal Experiments

Albino rats of Holtzman origin were used in all experiments. All rats were fasted 48 hours except Rat 189 which was fasted 72 hours. Ether was used to anesthetize all the animals except Rat 193 which was anesthetized with nembutal. Two gm of glucose in 2 ml of water was administered by stomach tube and 30 minutes later the lactate-2-C^{14} was administered, depending on the experiment, (1) intraperitoneally, (2) by heart puncture, and (3) through the canulated carotid artery. These details are indicated in Table III. The rats were placed in a metabolism chamber and expired CO_2 was trapped in 1 N sodium hydroxide as room air was slowly drawn through the chamber. Rat 182 was decapitated an hour after the injection of labeled lactic and the remaining rats 30 minutes after injection.

E. Isolation of Glycogen and Blood Glucose

1. Liver Glycogen (43)

The liver was immediately removed, weighed, cut into small pieces and extracted with 2.5 volumes of cold 10 per cent trichloroacetic acid in a Waring Blender. After centrifugation the supernatant portions were combined, 1.5 volumes of 95 per cent ethanol added, and glycogen allowed to precipitate for 48 hours at 4° C. After centrifugation the precipitated glycogen was washed twice with 95 per cent ethanol

TABLE III

TREATMENT OF RATS IN EACH EXPERIMENT

Rat No.	Hours Fasted	Method of Administration	Compound Administered	Time From Administration to Killing Hours
182	48	Intraperitoneally	L(+)	1
185	48	Intraperitoneally	D(-)	1/2
190	48	Heart Puncture	L(+)	1/2
193	48	Canulated Carotid Artery	D(-)	1/2

and once with ether. This was then put into a vacuum dessicator to dry over phosphorous pentoxide for 24 hours.

2. Muscle Glycogen (44)

After the removal of the liver the carcass was skinned and eviscerated. The head, paws and tail were removed and the carcass was cut into small pieces. For each 70 to 80 gm of carcass, 300 ml of 30 per cent potassium hydroxide was added. The mixture was digested for 8 hours over a steam bath, filtered through glass wool, and treated with 1.5 volumes of 95 per cent ethanol. Glycogen was allowed to precipitate for 48 hours at 4° C. After centrifugation the precipitate was extracted twice with 10 per cent trichloroacetic acid and glycogen precipitated and washed as described for liver glycogen.

3. Blood Glucose

The blood glucose was extracted by the method of Busch, Hurlbert and Potter (45) using perchloric acid to precipitate the protein. One ml of blood was combined with 1 ml of 0.66 N perchloric acid giving a solution of approximately 0.33 N perchloric acid. If the solution was too thick 1 ml of 0.33 N perchloric acid was added. After centrifugation the precipitated protein was again treated with 1 to 2 ml of 0.33 N perchloric acid for a second extraction. This was centrifuged again and the two supernatant fluids combined. The perchlorate ion was precipitated by neutralizing with potassium hydroxide. Potassium perchlorate was removed by centrifugation after refrigerating overnight.

Some of the later samples, after being diluted with reagent grade glucose, were passed over a Dowex 1 column in the acetate form (as described earlier) to remove blood lactate.

Blood glucose determinations were made by the Nelson (46) method.

F. Degradation of Glycogen and Blood Glucose

1. Hydrolysis of Glycogen

Each isolated sample of glycogen, suitably diluted with purified rat liver glycogen, was hydrolyzed for 5 hours with 1 N sulfuric acid in a steam bath. To each 500 mg of glycogen was added 12.5 ml of 1 N sulfuric acid. The hydrolysis is best carried out in a test tube, with a marble over the opening, immersed in the steam bath.

The sulfate ions were removed by means of an IR-45 ion exchange column (1 x 15 cm) in the free base form. When all of the glucose had passed through, as determined by the anthrone test (39), the volume was reduced by evaporation under a stream of air on a steam hot plate. The glucose was then added to a charcoal column for purification (See Part B). The fractions containing glucose were combined and evaporated to a small volume by a jet of air at 60° to 70° C.

2. Chemical Degradation of Glucose

The glucose from muscle and liver glycogen of Rat 182 was degraded chemically. Carbons 1, 2, and 6 of glucose can be isolated by this method. The degradation scheme is summarized as shown by Hobbs (47).

The chromatographically purified glucose (Rat 182) was oxidized to potassium gluconate by the method of Moore and Link (48). Resublimed iodine (1.57 gm) and redistilled methanol (27.8 ml) were placed in a 125 ml Erlenmeyer flask. Glucose (714 mg) was dissolved in a minimum volume of water and transferred to the flask using a little methanol to rinse out the beaker. The flask was placed in a

40° C water bath and stirred magnetically. To this mixture 22.6 ml of 4 per cent potassium hydroxide in methanol was added dropwise over a period of 10 minutes from a separatory funnel fitted with a drying tube. Then a little more 4 per cent potassium hydroxide was added to bring the mixture to a light straw color or colorless.

After standing overnight at 5° C the precipitated potassium gluconate was obtained by filtration, washed with methanol and dried over phosphorous pentoxide. The yield was 700 mg, of which 50 mg was set aside for radioactivity determination and the remainder was used to prepare glucobenzimidazole by the method of Moore and Link (48). In a 20 ml test tube were placed 650 mg of potassium gluconate, 550 mg of o-phenylenediamine hydrochloride, 0.5 ml of concentrated hydrochloric acid, 0.15 ml of 85 per cent phosphoric acid, 2.5 ml of water, and 1.25 ml of ethanol. This mixture was heated in an oil bath to boiling. Condensed water vapor was wiped from the sides of the test tube as it was found to inhibit the reaction. The temperature rose to 140° C in about 1 hour and was maintained there for an additional hour.

After cooling, a little water and activated charcoal were added. The mixture was heated to boiling and filtered hot. Concentrated ammonium hydroxide was added until the pH was above 7.0 and the mixture was allowed to cool and crystallize at 4° C overnight. The crystals were filtered cold, washed with a little cold water and dissolved in 5 to 7 ml of hot water. This solution was boiled with charcoal, filtered while hot, and allowed to crystallize at 4° C for 3 hours. The precipitate was removed by filtration and partially dried. The yield was about 230 mg of which 30 mg was set aside for

radioactivity assay.

The preparation of benzimidazolealdehyde was done by the method of Heubner et al. (49). Glucobenzimidazole (200 mg) was dissolved in 50 ml of water and 1.7 gm of sodium metaperiodate was added. After standing at room temperature for 30 minutes, 5 ml of saturated sodium bicarbonate was added and the solution refrigerated for 30 minutes. The precipitated benzimidazolealdehyde was filtered, washed with water and partially dried at 40° C for 1 hour. The material was not allowed to dry completely in order to facilitate the formation of a smooth suspension in the next step. The benzimidazolealdehyde was oxidized to benzimidazolecarboxylic acid using the technique of Bistrzycke and Przeworski (50). The moist aldehyde was suspended as smoothly as possible in 10 ml of 0.1 N sodium hydroxide. The reduced surface area due to large particles was found to hinder complete oxidation. While the solution was stirred magnetically, 5 per cent potassium permanganate was added slowly until the purple color persisted. The mixture was acidified with sulfuric acid and heated over steam for 15 minutes to complete oxidation. The solution was decolorized with sodium bisulfite. Benzimidazolecarboxylic acid crystallized when the solution was cooled. The acid was dissolved in alkali and recrystallized by acidification of the heated solution, followed by cooling. After taking a sample for radioactivity assay, representing carbons 1 and 2, about 69 mg was left for decarboxylation.

Referring back to Heubner et al. (49) for the decarboxylation step, the benzimidazolecarboxylic acid was placed in a pear-shaped flask with provision for sweeping the released carbon dioxide with carbon dioxide free air into about 5 ml of 1 N sodium hydroxide using 1 trap

of the Schmidt apparatus (51). The flask was heated carefully with a microburner. When the reaction was completed and the carbon dioxide removed, the pear-shaped flask was attached to a standard taper joint fitted with a stopcock. The apparatus was evacuated using a vacuum pump and the stopcock closed. When the flask containing the benzimidazole was heated the material sublimed to the walls of the tube. This was then removed for combustion and determination of radioactivity in carbon 1 of the original glucose.

For the isolation of carbon 6 of glucose, the filtrate obtained during the filtration of benzimidazolealdehyde was used. It was caught in a container with sufficient barium acetate to neutralize the acids present. This mixture was filtered. The filtrate contained formaldehyde from the oxidation by periodate. This was removed by distillation to a small volume 3 times. A solution of 2,4-dinitrophenylhydrazine in sulfuric acid was added to the combined chilled distillates until no more precipitate formed. After refrigeration overnight at 4° C the 2,4-dinitrophenylhydrazone derivative of formaldehyde was filtered and recrystallized from hot aqueous ethanol followed by recrystallization from hot aqueous methyl cellosolve. This material was then assayed for radioactivity by the wet combustion method.

3. Bacterial Degradation of Glucose

This method was used in all the experiments except those on Rat 182. Leuconostoc mesenteroides (strain 39) procured from the American Type Culture Collection (No. 12291) was started and maintained according to the method of Wood et al. (52). The ampule in which the bacteria were received was broken, a small amount was transferred

aseptically to 10 ml of prepared media (52) and the remainder evacuated and sealed in another ampule. This was stored at -20° C. The inoculated media were incubated at 30° C for 24 hours under nitrogen by running nitrogen into the test tube and stoppering to exclude oxygen. These bacteria were used to inoculate the agar media (52) and also to inoculate the growing media. The agar media should be renewed each 1 to 2 weeks by inoculating liquid media, as above, and then inoculating another tube of agar media.

The media in which the bacteria were grown to make an enzyme preparation to degrade glucose was deficient in salts solution (52) until the final inoculation. This final medium contained salts solution. The object was, evidently, to produce a hardy strain of bacteria on deficient media which would produce a large quantity of the glucose degrading enzymes when the complete medium was used (53). These cells were harvested in a Sharples centrifuge and lyophilized. Horecker *et al.* (54) reported that they could be stored in this condition up to 6 months at -20° C. However, the second large preparation of lyophilized cells failed to show sufficient activity and had to be discarded. Rather than go through another lengthy preparation at this stage, live cells grown in 1 liter of complete media were used (55). A 1 per cent inoculum of rapidly growing cells were harvested by centrifugation, washed with distilled water and used immediately.

The incubation mixture for the degradation of glucose contained 60 ml of 0.05 M phosphate buffer, pH 6.0, about 1 millimole of the glucose sample and 2 to 3 gm (wet weight) of the bacteria cells. The mixture was incubated at 30° C for 2 hours under a flow of nitrogen. Then it was made acid with 25 per cent sulfuric acid (pH 1.0 or less)

and swept vigorously with nitrogen for 30 minutes. The carbon dioxide was trapped in 1 N sodium hydroxide after passing it through a trap containing 1 N sulfuric acid (Figure 5). The products of the degradation are lactic acid, ethanol, and carbon dioxide. Figure 6 outlines the complete degradation procedure.

4. Chemical Degradation of Lactic Acid and Acetic Acid

The reaction mixture of the bacterial degradation of glucose was made basic with sodium hydroxide, centrifuged to remove the bacterial residue, and then distilled to a small volume to remove the ethanol. The ethanol was oxidized to acetic acid by heating for 2 hours at 90° C with 0.5 gm of potassium dichromate in 4 N sulfuric acid. The reaction mixture was steam distilled and the distillate titrated with 0.1 N sodium hydroxide to determine the yield of acetic acid. Then it was evaporated to a small volume and the pH was brought to 1.0 or less with 25 per cent sulfuric acid. The sample was adsorbed on some prepared Celite and transferred to a Celite column. The column was eluted with 100 ml of CB-1 followed by 100 ml of CB-5. Fractions of 5 ml each were collected with an automatic fraction collector and titrated with 0.1 N sodium hydroxide. The pure acetate was recovered between tubes 13 and 22. The tubes containing acetic acid were combined in a separatory funnel, the aqueous portion reduced in volume on a rotary evaporator, and dried overnight in a small pear-shaped flask at 110° C in preparation for the Schmidt degradation.

In the Schmidt reaction (56) for the degradation of acetic acid, 0.6 ml of 100 per cent sulfuric acid per millimole of sodium acetate was added and heated briefly to dissolve the acetate. After 15 minutes of chilling in an ice bath 100 mg of sodium azide per milli-

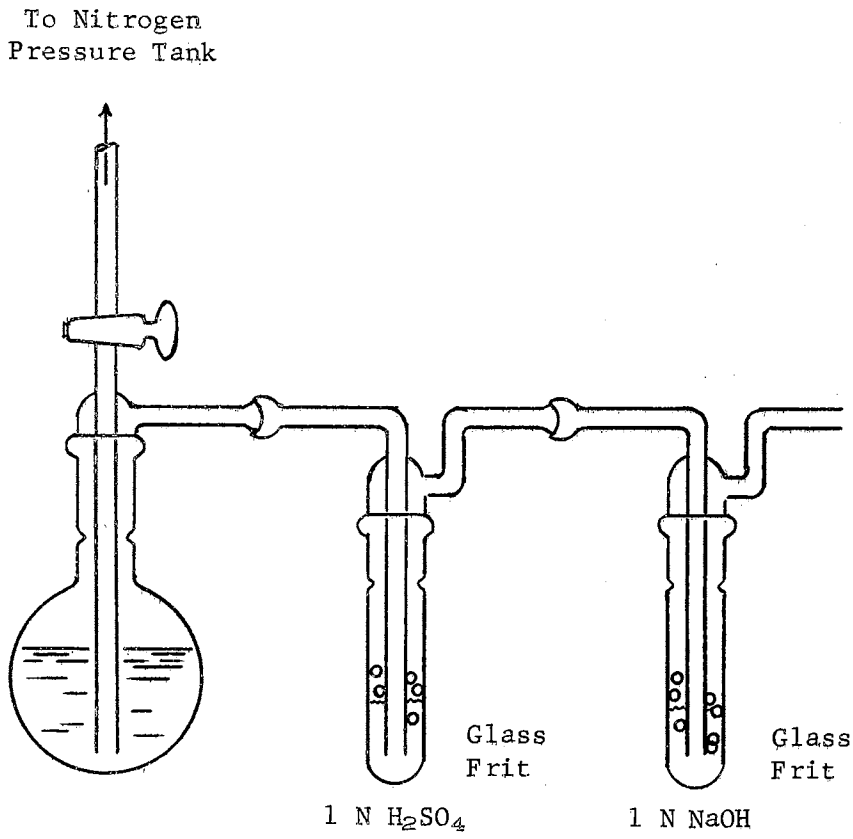


Figure 5. Apparatus used for bacterial degradation of glucose and oxidation of lactic acid.

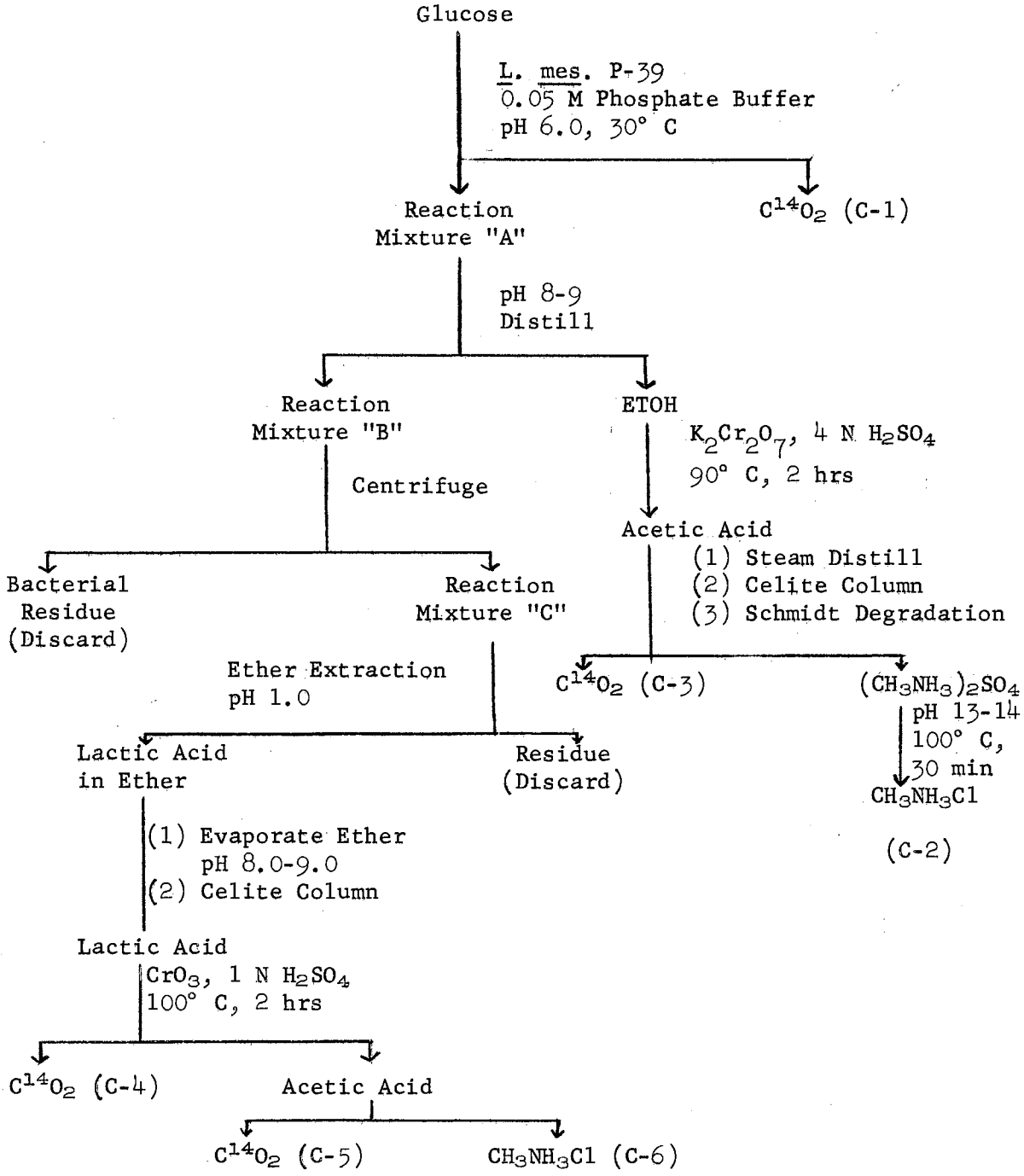


Figure 6. Bacterial degradation procedure for glucose.

mole of sodium acetate was added to the flask and the system closed. The aspirator was turned on and when the system was determined to be free of leaks the temperature of the water bath was raised to 40° C. Then over a period of 1 hour the temperature was slowly raised to 70° C after which the temperature was raised rapidly to 100° C and swept under vacuum with carbon dioxide free air for 1 hour. The released carbon dioxide was caught in a trap containing 5 ml of 1 N sodium hydroxide after passing through a scrubber trap containing 5 per cent potassium permanganate in 0.5 N sulfuric acid. The residue in the reaction flask, which contained methylamine sulfate, was made basic and swept with room air at a temperature of 95° C directly into a trap containing about 5 ml of 1 N hydrochloric acid.

The lactic acid residue remaining after the removal of the ethanol was made acid with sulfuric acid and continuously extracted with ether for 24 hours. The ether was evaporated under a stream of air leaving a small amount of liquid residue. The sample was then titrated with 0.1 N sodium hydroxide to check the amount of lactate recovered. Titration when about 10 ml remains will prevent loss of the sample if it is inadvertently allowed to go dry. The remainder of the evaporation was done on a steam hot plate under a stream of air. The lactate was made acid (pH 1.0 or less), taken up with Celite, as described earlier, and transferred to a Celite column. The sample was eluted with 100 ml of CB-5 followed by 125 ml of CB-10. The pure lactate was recovered between tubes 29 and 44. Each fraction was titrated with 0.1 N sodium hydroxide. The fractions containing lactic acid were combined and evaporated to small volume of about 10 to 15 ml.

The lactic acid was oxidized to acetic acid by the method

described by Hiatt et al. (54). The lactic acid in about 50 ml of a solution made 1 N with sulfuric acid was combined with 200 mg of chromium trioxide. The flask was attached to the apparatus used for the bacterial degradation of glucose using 2 traps; one contained 5 ml of 1 N sulfuric acid to catch any acetic acid that distilled over and the second contained 5 ml of 1 N sodium hydroxide to trap the carbon dioxide which was released. The mixture was heated in a water bath at 100° C for 3 hours while sweeping with nitrogen under pressure. The acetic acid formed was isolated by steam distillation, purified over Celite, and degraded as described above.

G. Radioactivity Determinations

Carbon analyses involved manometric measurement of carbon dioxide (57) and carbon 14 assays were done with a vibrating reed electrometer (58). Carbon dioxide was measured directly by treating the alkaline solution with phosphoric acid. The methylamine, which was trapped as the hydrochloride salt, was dried on a steam plate under a stream of air and oxidized to carbon dioxide by the wet combustion method of Van Slyke (55).

CHAPTER III

RESULTS AND CONCLUSIONS

The experimental results are shown in Table IV. The muscle and liver glycogen from Rat 182, which was administered L(+)-lactate-2-C^{14} intraperitoneally, were degraded chemically and the specific activity of carbons 1, 2, and 6 were determined. The degree of randomization can be calculated from carbons 1 and 2 or 5 and 6. If no randomization occurred, lactate labeled in carbon 2 would produce labeling only in carbons 2 and 5 of glucose (Figure 7). To calculate the degree of randomization for Rat 182 twice the activity of carbon 1 was divided by the total activity of carbons 1 and 2 (14). In subsequent animals the specific activities of all of the carbons were determined, and there the calculation was twice the specific activity of carbons 1 and 6 divided by the sum of the specific activities of carbons 1, 2, 5, and 6.

By this procedure the randomization of labeling in Rat 182 was found to be 96 per cent in the liver glycogen and 79 per cent in the muscle glycogen. If the lactate was converted to glucose only in the liver, the labeling in carbons 1, 2, 5, and 6 should be highly randomized (3). However, if lactate was converted to glycogen in the muscle, the labeling would be expected to be primarily in carbons 2 and 5 with little randomization (31).

Rat 185 was given D(-)-lactate-2-C^{14} intraperitoneally. The

TABLE IV

EXPERIMENTAL RESULTS OF RADIOACTIVITY DETERMINATIONS OF GLUCOSE FROM HYDROLYZED GLYCOGEN AND OF EACH CARBON FROM GLUCOSE DEGRADATIONS

Rat No. and Treatment	Glycogen Source	Specific Activity in mμc							Per Cent Randomized
		Glucose	Carbon Number						
			1	2	3	4	5	6	
Rat 182	Muscle	523	83.4	129				98.3	79
L(+)-Lactate	Liver	2075	382	412				449	96
Rat 185	Muscle	51	7.9	14.7	2.1				71
D(-)-Lactate	Liver	Lost							
Rat 190	Muscle	43.7	(a) 0.74	2.56	0.34	2.0	110	*	45
L(+)-Lactate			(b) 0+	3.4	1.53	5.5	4.9	1.6	32
	Liver	31.9	5.5	10.66		1.32	9.8	3.10	59
	Blood	(a) 1877**	(a) 57.7	90.8	38.1	63.6	633**	58.7	92
		(b) 694	(b) 90.0	85.1	43.4	64.7	240*		
Rat 193	Muscle	40.2	(a) 1.64	11.98	0.89				22
D(-)-Lactate			(b) 2.45	12.5	1.2	0.88	21.7	1.48	
	Liver	None Recovered							
	Blood	1203	(a) 209	177	71				100
			(b) 327	303	162	155	3090**	228	

*low carbon yield
**high due to lactate not removed

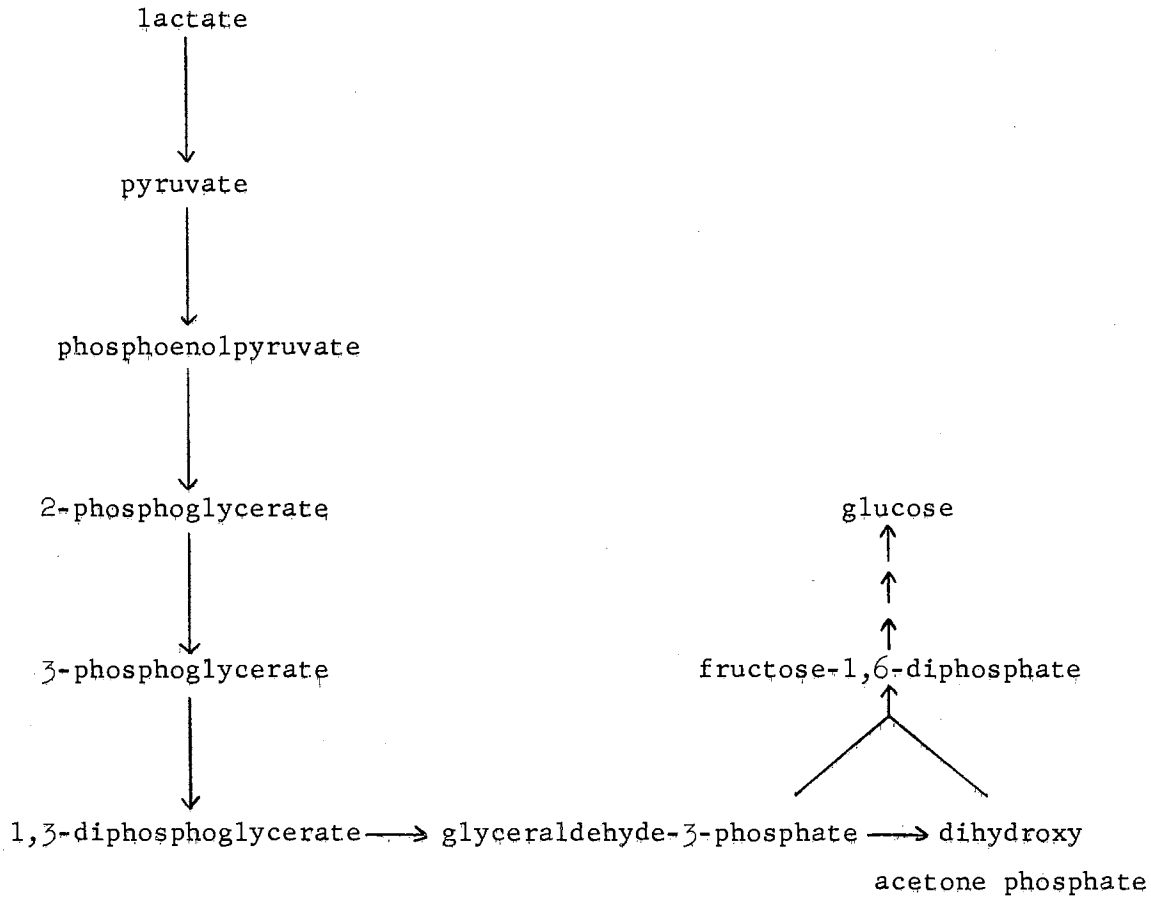


Figure 7. Glucose formation from 2 moles of lactate.

randomization of labeling in muscle glycogen was 71 per cent. Unfortunately the liver glycogen from Rat 185 was lost. Although the extent of randomization was similar to that observed in muscle glycogen of Rat 182, there was only 10 per cent as much radioactivity in the muscle glycogen of Rat 185 as in that of Rat 182. This observation agrees with the work of Hoberman and D'Adamo (12) and indicates that L(+)-lactate is a better glycogen precursor than D(-)-lactate.

In an effort to obtain muscle glycogen in which the labeling was less randomized, the lactate was administered by heart puncture and intracarotid injection (Table IV) and the time of the experiment was reduced to 30 minutes. Changing the site of administration permits the lactate to go directly to the muscle. This should decrease the randomization of labeling in muscle glycogen. Shortening the duration of the experiment might reduce the randomization in muscle glycogen by diminishing the conversion of lactate to randomized blood glucose in the liver.

This proved to be the case in Rat 190 and 193. Rat 190 was administered L(+)-lactate-2-C^{14} by heart puncture and Rat 193 was administered D(-)-lactate-2-C^{14} by means of a canula in the carotid artery. These different methods of administering the labeled lactate to the rat were tried in order to determine the most convenient one.

It should be noted here that the specific activities of the liver and muscle glycogen were very low in the 30 minute animals and that the yield of liver glycogen from Rat 190 was very small and from Rat 193 was zero. Because of these low activities, accurate C^{14} determinations on each carbon were difficult. Two degradations of glucose from the muscle glycogen of Rat 190 were carried out and the degree of

randomization was found to be about 35 per cent from the combined results of carbons 1 and 2 of the first degradation and carbons 1, 2, 5, and 6 of the second degradation. The liver glycogen was 59 per cent randomized as calculated from carbons 1, 2, 5, and 6 of one complete degradation. This percentage is not as high as the values of the one hour animals but is significantly greater than the randomization in the muscle glycogen. Two degradations of glucose from muscle glycogen of Rat 193 were performed and 22 per cent randomization was found from the combined results of carbons 1 and 2 of the first degradation and carbons 1, 2, 5, and 6 of the second degradation.

The blood glucose of Rat 190 and 193 had a very high specific activity compared to that of the liver and muscle glycogen and the degree of randomization was found to be 91 and 100 per cent respectively (Table IV). The high level of randomization indicates that the blood glucose is formed from the administered lactate. This also supports the view that the initially unrandomized glycogen formed from lactate in the muscle is rapidly diluted with randomized labeling from blood glucose formed in the liver.

The finding of only slight randomization of C^{14} in the muscle glycogen of Rats 190 and 193 strongly suggests that some blood lactate was converted to muscle glycogen. Apparently, when labeled D- or L-lactate was given by intravascular injection and the experimental time was reduced, the incorporation of C^{14} into muscle glycogen from blood glucose synthesized in the liver was diminished. Thus the masking effect of randomization due to glucogenesis in the liver is decreased, permitting the isolation of muscle glycogen in which the C^{14} was slightly randomized.

Glutamic acid was isolated from the muscle protein of Rats 190 and 193 (59). Degradation of these samples showed that only a small amount of C^{14} was located in carbon 4. This finding is also in accord with the proposal that blood lactate does penetrate muscle tissue and is metabolized by it. However, the fact that it was difficult to get substantial labeling, unrandomized, into muscle glycogen from labeled lactate, supports the idea that blood glucose is a much better precursor than is blood lactate. It seems likely that the reason that only small amounts of lactate are converted to muscle glycogen is not because lactate does not penetrate muscle but because of a slow reversal of glycolysis in muscle.

The results presented support *in vivo* the observation *in vitro* of Hiatt *et al.* (31) that little randomization occurs during muscle glycogen synthesis from pyruvate. The finding that D(-)-lactate-2-C^{14} gave randomizations similar to those of L(+)-lactate-2-C^{14} is in agreement with the proposal (23, 24) that mammalian tissue converts D(-)-lactate to pyruvate.

CHAPTER IV

SUMMARY

When 48 hour fasted rats were given D(-)- or L(+)-lactate-2-C^{14} 30 minutes after receiving 2 gm of glucose by stomach tube, degradation of muscle and liver glycogen and blood glucose gave the following results:

1. Rat 182, given L(+)-lactate-2-C^{14} intraperitoneally and sacrificed after 1 hour, showed slightly less randomization of labeling in muscle glycogen than in liver glycogen. Rat 185, given D(-)-lactate-2-C^{14} intraperitoneally and sacrificed after 1 hour, had a randomization pattern for muscle glycogen similar to Rat 182 but with only 10 per cent as much activity. The liver glycogen was lost.

2. Rat 190, given L(+)-lactate-2-C^{14} by heart puncture and sacrificed after 30 minutes, showed much less randomization of labeling in muscle glycogen than did the 1 hour animal. The randomization of labeling in the liver glycogen was much greater than that of the muscle glycogen. The yield of liver glycogen was very low.

3. Rat 193, given D(-)-lactate-2-C^{14} by intracarotid injection and sacrificed after 30 minutes, showed a labeling pattern similar to that of Rat 190 in muscle glycogen. No liver glycogen was recovered.

4. The labeling in blood glucose of Rat 190 and 193 was highly randomized and the specific activity was very high.

5. Labeling in carbon 4 of muscle glutamic acid from Rats 190

and 193 was low.

These results indicate that muscle tissue is capable of metabolizing lactate directly but that blood glucose is the preferred precursor of muscle glycogen.

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VITA

Nilo Francis Inciardi

Candidate for the Degree of

Master of Science

Thesis: SOME ASPECTS OF THE METABOLISM OF D- AND L-LACTIC ACID-2-C^{14} IN RATS

Major Field: Chemistry (Biochemistry)

Biographical:

Personal Data: Born in Cicero, Illinois, December 21, 1918, the son of Henry and Jennie Inciardi.

Education: Graduated from J. Sterling Morton High School, Cicero, Illinois, in 1936; graduated from J. Sterling Morton Junior College, in 1940; received the Bachelor of Science degree with a major in Horticulture from Oklahoma State University, Stillwater, Oklahoma, in 1948; received the Master of Science degree in Horticulture from Oklahoma State University, Stillwater, Oklahoma, in 1949; completed the requirements for the Master of Science degree in Biochemistry in January, 1963.

Professional experience: Owned and operated the Inciardi Greenhouses, Stillwater, Oklahoma, from 1949 to 1963.

Professional and Honorary Societies: Alpha Zeta and American Chemical Society