THE OXIDATION OF CARBON-14-LABELED COMPOUNDS IN MICE WITH AND WITHOUT THE FACTOR FOR SPONTANEOUS MAMMARY TUMORS

By

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FOR SPONTANEOUS MAMMARY TUMORS

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CHAPTER I

INTRODUCTION

The purpose of this work was to study the metabolism of carbon-14-labeled glucose in C3H mice with and without the factor for mammary tumor (33). This method could lead to a method for identifying individual C3H mice with the factor. Determination of the effect of drugs on the metabolic rates of carbon-14-labeled glucose in C3H/HeJ mice might be a useful test for screening compounds as anti-cancer agents.

The apparatus used in this study for collection of respiratory carbon dioxide from an intact animal is similar to the Roth metabolic cage (37), a useful pharmacologic tool for studying *in vivo* metabolism rates. The apparatus has two parts: (a) a small chamber with an inflow of air that is free of carbon dioxide and an outflow of air containing radioactive carbon dioxide and (b) a system for trapping and measuring this carbon-14 dioxide.

The carbon-14-labeled glucose and citric acid used in this research are oxidized and excreted as respiratory carbon-14 dioxide (Figure 1). Citric acid is an intermediate in the tricarboxylic acid cycle (TCA). Glucose can be oxidized via the hexose monophosphate pathway (HMP) and the Embden-Meyerhoff pathway (EMP) to form pyruvate, which is converted to lactate or to acetyl CoA. Acetyl CoA is a precursor of the TCA. Alteration of the metabolism by a pharmacologic agent is reflected in a change in the amount of carbon-14 dioxide in the expired air.

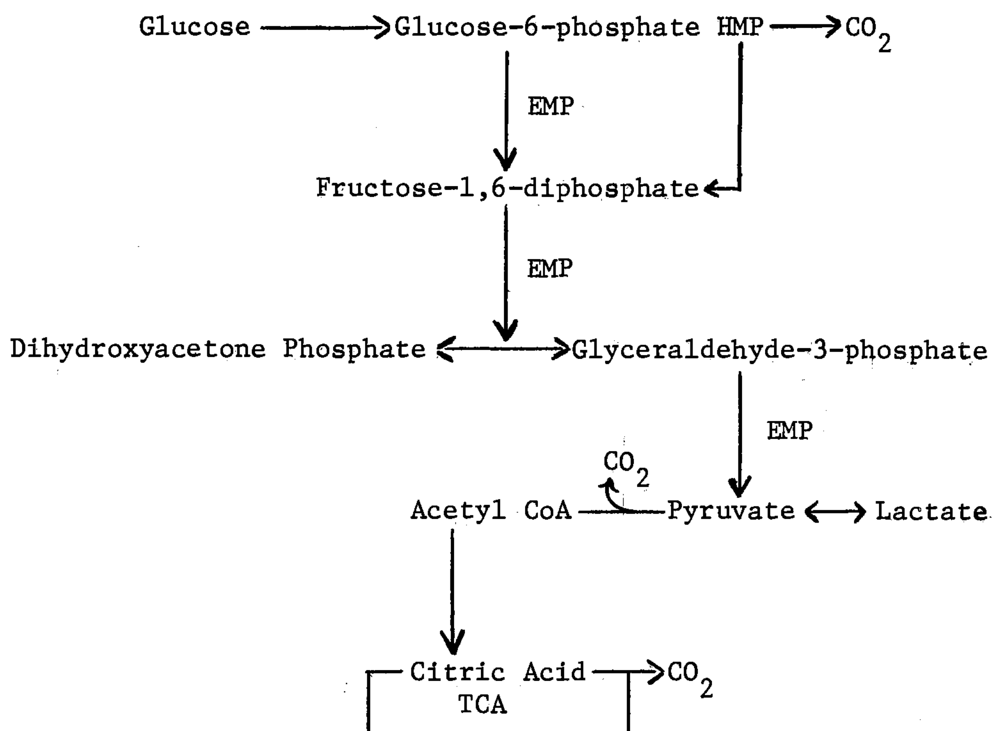


Figure 1. Oxidation of Carbon-14-Labeled Substrates to Carbon-14 Dioxide by Major Metabolic Pathways

Three of the four anticancer drugs used in this work have been or will be clinically tested as anticancer drugs: depo-testosterone cypionate (DTC); 2-oxo-3-ethoxybutyraldehyde bis(thiosemicarbazone) (KTS) (27), and 3-hydroxy-2-pyridinecarbaldehyde thiosemicarbazone (HEPTS) (8). The fourth, polyacrylic acid (PAA), has shown significant activity against the intramuscular Walker sarcoma of the rat (18).

The trapping solution for carbon-14 dioxide is an organic quaternary ammonium base the excess of which can be titrated with a standard solution of hydrochloric acid to determine carbon dioxide quantitatively; the trapping solution can be counted in a liquid scintillation counter to determine radioactivity.

CHAPTER II

HISTORICAL

Warburg postulated in 1926 that respiration is impaired and glycolysis is increased in tumor cells (42). He assumed that glycolysis is necessary to replace the energy-yielding steps in the respiratory pathway. Although the detailed mechanism of cancer [metabolic] imbalance remains unknown, its reality is not questioned. In particular, the imbalance between respiration and glycolysis first emphasized by Warburg remains the foundation of biochemical investigations in cancer.

Dickens and Weil-Malherber found that both aerobic and anaerobic glycolysis is a property of tumors (7). Lepage found that tumor tissue was metabolized glucose differently from normal tissues (23). Burk and coworkers at the National Cancer Institute in 1956 and 1967 found evidence for impaired respiration and increased anaerobic processes in tumor tissues. Cancer cells descended _in vitro_ from a single normal cell were _in vivo_ more malignant, the higher the "fermentation" rate (8). Wenner and Weinhouse (44) in 1956) also found _in vitro_ that cancer had both anaerobic and aerobic glycolytic properties using carbon-14-labeled glucose. Hilf and Lerner (17) did _in vitro_ metabolic studies in 1969 with mammary cancer which supported these earlier postulates.

Glucose-1-^{14}C injected intraperitoneally into ascites-bearing mice was found to be converted entirely to lactate by the tumor cells before any significant amount reached the blood stream (16). Okita presented

metabolic data (32a, 33) which indicated a decrease in TCA activity.

Carbon-14-labeled compounds have been used in many recent pharmacological studies since carbon-14 can be counted with liquid scintillation counters and other common instruments. Liquid scintillation counting is useful with β-emitting isotopes such as carbon-14 because of its high efficiency and relative freedom from self-absorption.

Tolbert (15) and Okita (33) have studied the *in vivo* metabolic rates associated with cancer by means of sophisticated instrumentation for the continuous monitoring of respiratory carbon-14 dioxide. Tolbert and associates (40) and Okita (24) developed similar instruments about the same time. Tolbert used a carbon dioxide monitor employing infrared absorption, an ion chamber to detect respired carbon-14, and a ratio analyzer for determining specific activity. Okita used an air-collection system, a ventilation meter, a carbon dioxide infrared analyzer, and a 4π Geiger-Müller flow counter.

Carbon-14 can also be collected and counted by the barium carbonate precipitation technique. This procedure usually has a very low counting efficiency and requires time for sample preparation. The most convenient method for determining carbon-14 activity is a liquid scintillation spectrometer (13) for which the sample preparation is relatively easy and rapid.

Passman, Radin and Cooper (34) found that carbon dioxide could be dissolved in toluene containing a high molecular weight quaternary ammonium hydroxide such as the hydroxide of hyamine (13), which is one of the favorite carbon dioxide absorbers for biological investigations. Frederickson and Ono (11) improved on Passman's procedure by direct trapping and scintillation counting rather than barium carbonate precipi-

tation and subsequent combustion to carbon-14 dioxide for trapping.

Several solutions have been used for radioactive carbon dioxide collection and subsequent scintillation counting. Primene (a mixture of amines with a molecular weight of 191) in methanol (36), ethanolamine in ethylene glycol monmethyl ether (19), and a thixotropic scintillator gel suspending agent (30) have been used. All of these methods use toluene-based scintillation solutions. They give higher efficiency and are less subject to the problems of chemiluminescence.

A recent and helpful contribution to biological tracer studies was the development of solubilizer (14) which is a product of Nuclear of Chicago, consisting of a toluene-soluble quaternary ammonium base.

Data from the study of the oxidation of carbon-14 labeled substrates to carbon-14 dioxide can be used in the evaluation of the major metabolic pathways: (1) glycolysis, referred to as the Embden-Meyerhoff pathway (EMP) in Figure 2; (2) the pentose shunt, referred to as the hexose monophosphate pathway (HMP) in Figure 3; and (3) the Krebs cycle, referred to as the tricarboxylic cycle (TCA) in Figure 4. All three of these are energy-yielding processes (25).

Labeled glucose has been extensively used for elucidating the pathways of catabolism (20, 38, 21). Most researchers have postulated that the EMP yields equal amounts of carbon-14 dioxide from glucose-1-^{14}C or glucose-6-^{14}C. The HMP yields one mole of carbon-14 dioxide for each glucose-1-^{14}C metabolized by this pathway and no carbon-14 dioxide from glucose-6-^{14}C (Equation D, Figure 3).

If triose phosphates are considered, the EMP yields a mole of triose phosphate from the C-1 or C-6 position (Equation E, Figure 2) and HMP yields triose phosphate from glucose-6-^{14}C (Equation E, Figure 3). Of

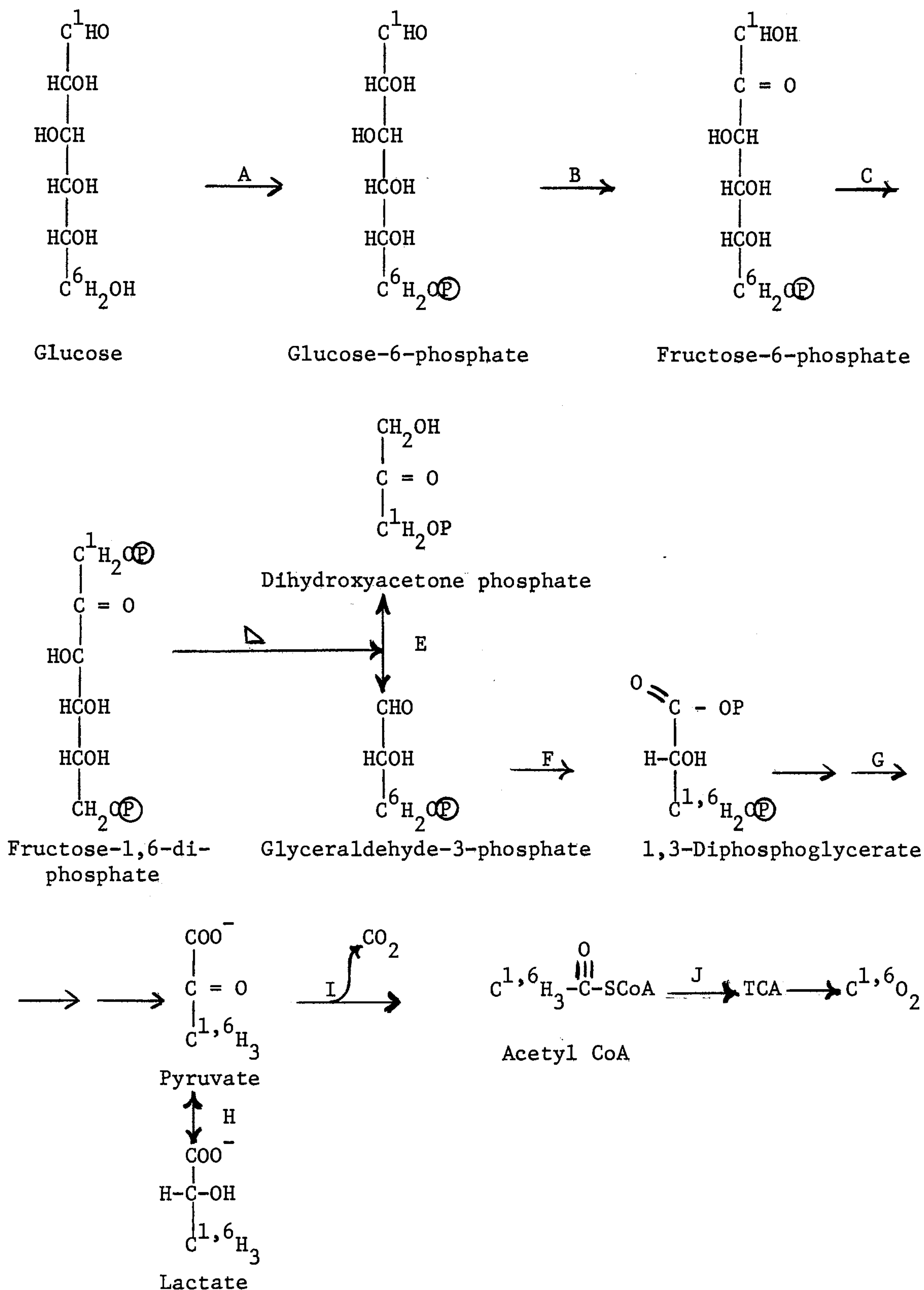
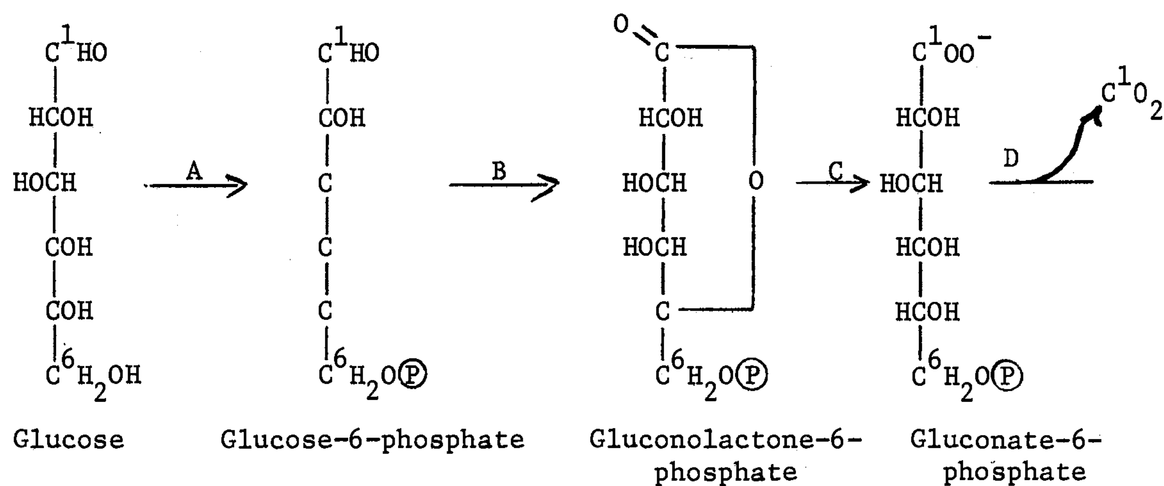


Figure 2. Embden-Meyerhoff Pathway Showing C-1 and C-6 Positions of Labeled Glucose Eliminated as Carbon-14 Dioxide.



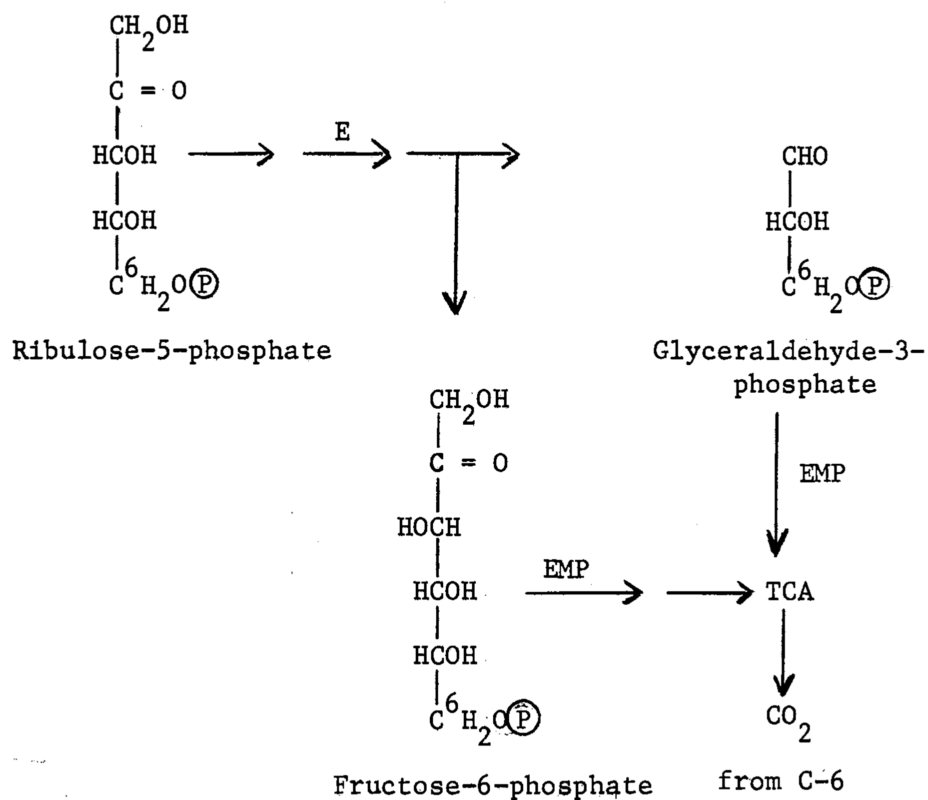


Figure 3. Hexose Monophosphate Pathway Showing Elimination of C-1 Position of Labeled Glupose as Carbon-14 Dioxide and C-6 Position by Formation of Glyceraldehyde-3-Phosphate for Ultimate Elimination of C^6O_2 in TCA

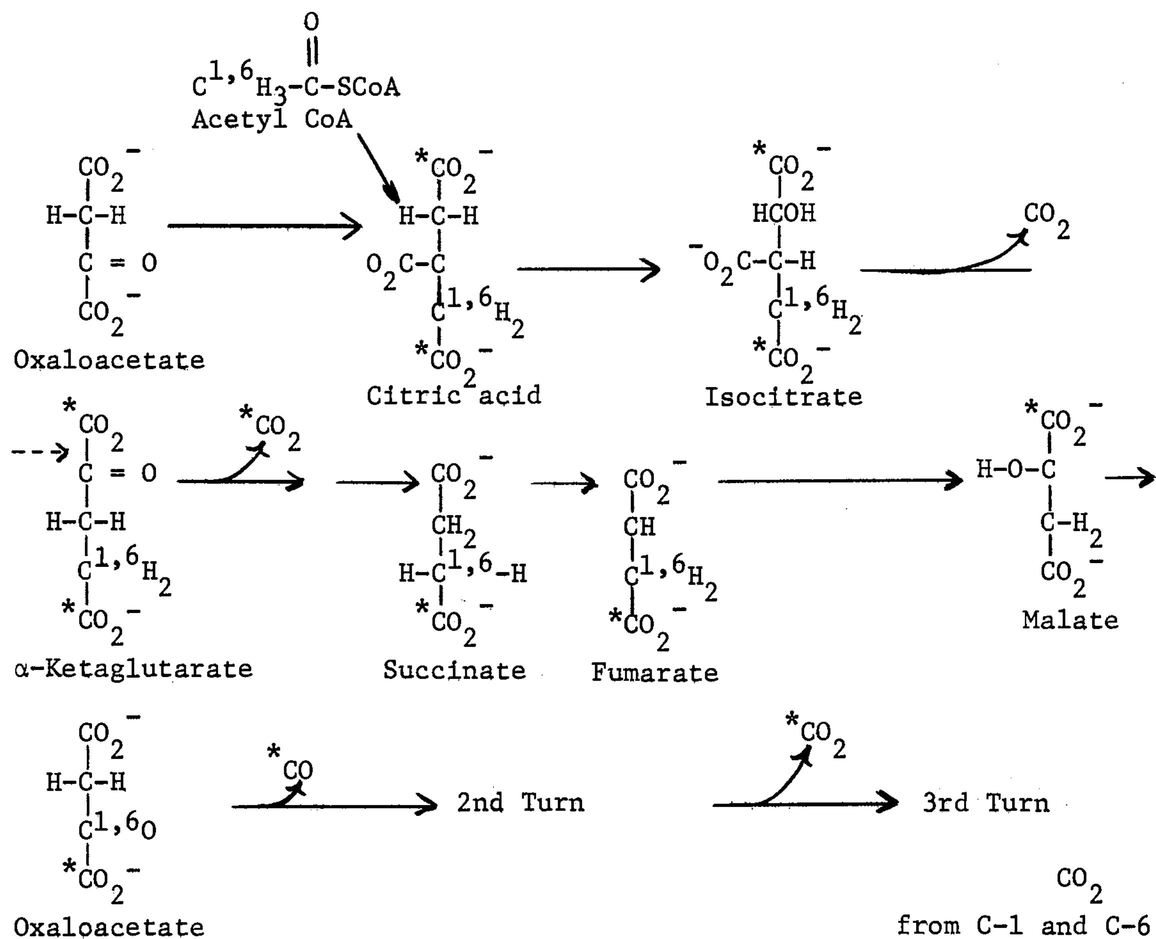


Figure 4. Tricarboxylic Acid Pathway Showing Elimination of C-1 and C-5 of Labeled Citric Acid as Carbon-14 Dioxide and Also Following the C-1 and C-6 Positions of Labeled Glucose Into This Pathway

course, the triose phosphate formed from these two processes may go into the TCA (Figure 3) where carbon-14 dioxide is given off.

The key to these pathways is the ultimate fate of pyruvate. Pyruvate formed from labeled glucose in mammals has several metabolic fates shown in Figure 5 (26). An important feature to consider here is the increased formation of lactate since cancer is associated with anaerobic and aerobic glycolysis (5, 7) and the deactivation of the TCA (2).

Lactate is one of the rare dead ends in a mammalian metabolic path. Lactate can only return to the metabolic path by oxidation to pyruvic acid. The _in vitro_ studies by Wenner and Weinhouse (44) showed that glucose-1-^{14}C and glucose-6-^{14}C were equally incorporated into lactic acid. They calculated that from 77 to 94% of the total glucose going to carbon dioxide arose via the EMP and TCA.

Since the spontaneous mammary tumor is hormone-dependent (3), one would expect mammary tumors to react favorably to hormonal therapy. Okita (33) showed that the _in vivo_ metabolic rate of carbon dioxide elimination in tumor-bearing mice was altered by the administration of testosterone. Testosterone propionate has been shown to have an effect on mammary tumors (17) induced by 7,12-dimethylbenzanthrene. Testosterone is used clinically in the treatment of breat cancer (34). Based on Okita's findings, anticancer drugs cush as testosterone can alter the metabolism of the of the tumor-bearing C3H/HeJ mice to that of control animals.

Another testosterone ester, used for treatment of inoperable breast cancer, is the 17B-cyclopentylpropionate of testosterone (I); depo-testosterone cypionate (DTC). It is similar to other testosterone esters in physiologic activity. 2-Oxo-3-ethoxybutyraldehyde bis(thiosemicar-

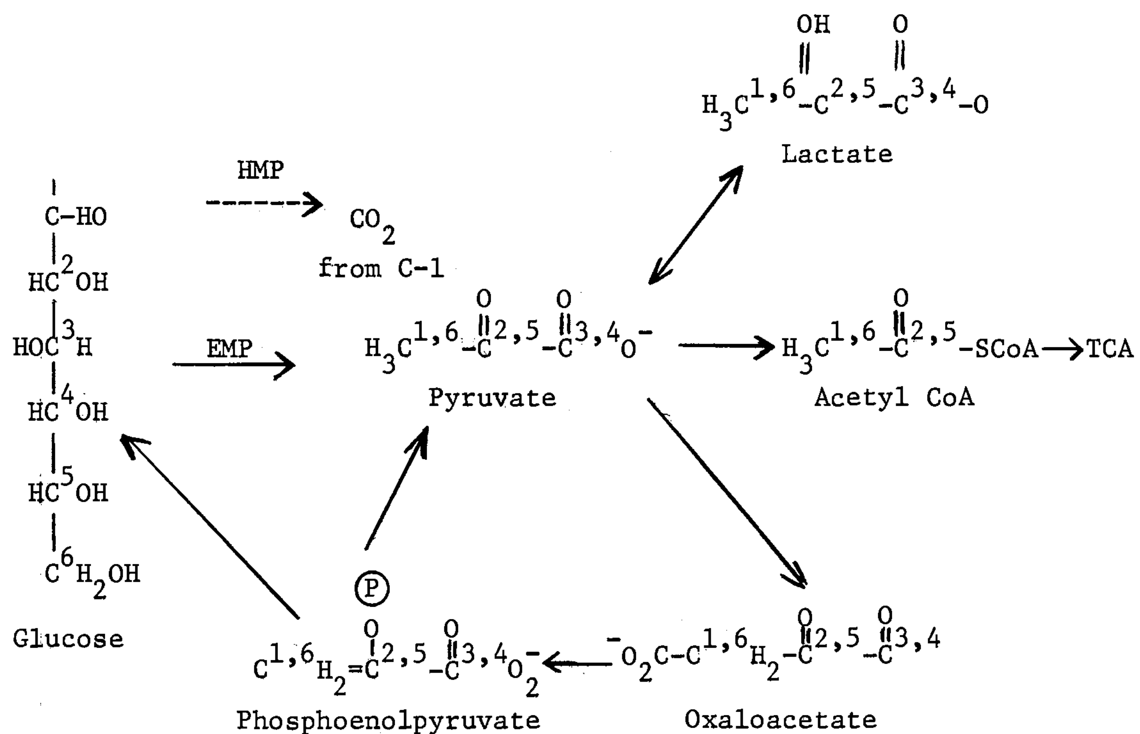


Figure 5. Fate of Pyruvate

I

bazone) (KTS) (II) was found to have significant activity in leukemia

II

L1210 of mice (9). KTS was later found by French to have antitumor activity against sarcoma 180 in mice (10). Petering and associates (35) found KTS to have antitumor activity in rodent tumors. Mihich and Nichol reported significant physiological activity for KTS in the spontaneous mammary tumor system A of C3H mice (27).

Another thiosemicarbazone found to have significant pharmacologic activity in several types of cancer is 3-hydroxy-2-pyridinecarbaldehyde thiosemicarbazone (HFPTS) (III). French and Blanz reported activity of

III

III against leukemia L1210, sarcoma 180, Lewis lung carcinoma and adenocarcinoma 755 of mice (8).

Another compound of interest to this work is polyacrylic acid (IV) (PAA) (18) which was effective in the treatment of intramuscular Walker sarcoma of rats in tests conducted by the Cancer Chemotherapy National Service Center. It is a high molecular weight polymer now awaiting preclinical testing.

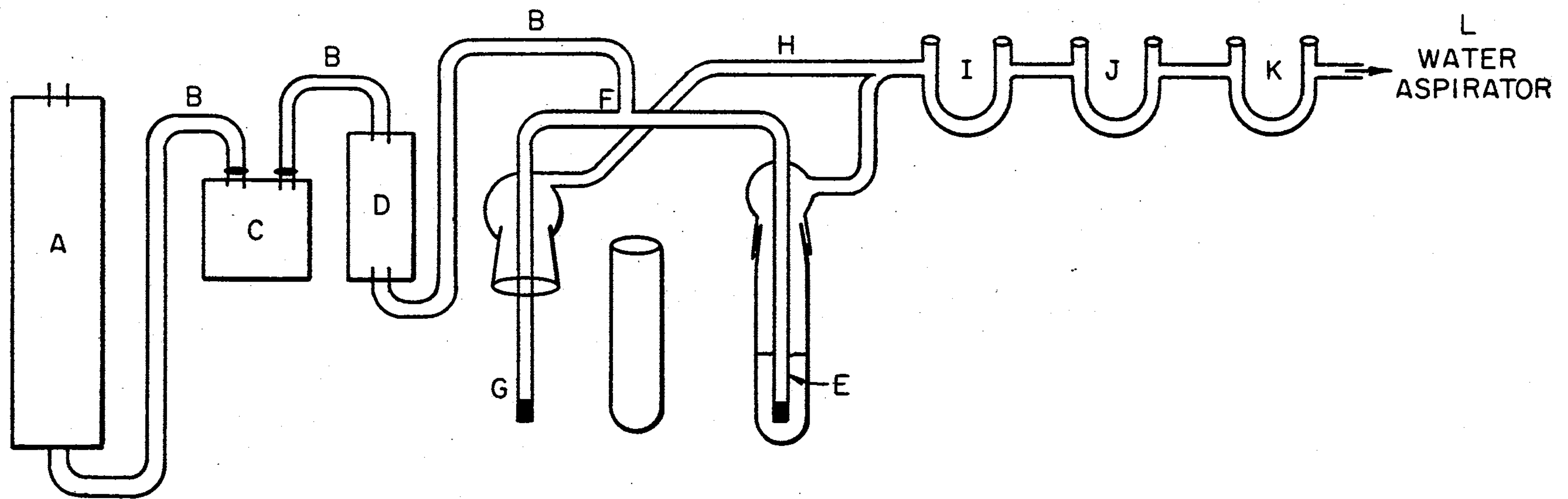
$$\left[-CH_2-CH(CO_2H)- \right]$$ IV

CHAPTER III

EXPERIMENTAL

Apparatus

The metabolic carbon dioxide collection apparatus used in these experiments is shown in Sketch 1. Column A contains Drierite over solid sodium hydroxide pellets for the absorption of atmospheric carbon dioxide and moisture. Tygon tubing (B) is used for air-flow to and from the animal chamber (C). The chamber is a glass beaker of approximately 100 ml with a two-hole rubber stopper for entrance of carbon-dioxide-free air and exit of respiratory carbon dioxide. A drying tube (D) with magnesium perchlorate to absorb the moisture from the expired air is inserted between the chamber and trapping solution (E). The two-armed carbon dioxide collection device (F) with a three-way stopcock allows continuous gas collection. Each arm is capped with a fritted dispersion bulb (G) to insure maximum surface contact of respiratory carbon dioxide with the NCS solution. The carbon dioxide collection device is connected to a water aspirator (L) by vacuum tubing (H) which gives an adjustable air-flow or approximately 60 ml/min ± 10 ml/min to the system. Three U-tubes containing Ascarite (J) and magnesium perchlorate (K) and (I) are inserted between the trapping solution and aspirator to allow a gravimetric determination of carbon dioxide which is not absorbed in the organic base.



Sketch 1

METABOLIC CARBON DIOXIDE COLLECTION APPARATUS

Animals

The mice in this research were inbred C3H/HeJ and C3HeB/FeJ strains purchased from Jackson Laboratories, Bar Harbor, Maine. The C3H/HeJ mice have a high susceptibility (> 95% incidence) to spontaneous mammary tumor (7). The C3HeB/FeJ mice which have a low incidence of mammary tumor were used as controls in the experiments. The mice were maintained on a Rockland Mouse/Rat Pellet diet and given tap water for drinking. They were not fasted prior to the experiments. The mice were kept in a room at 25°, which was the temperature at which all experiments were conducted. The C3H/HeJ mice and the C3HeB/FeJ mice were of two age groups: 3½ months and 2 months. No difference in the *in vivo* rates of oxidation of labeled substrates could be attributed to age. The mice in both groups weighed from twenty to thirty grams.

All carbon-14 labeled substances and drugs were administered by intraperitoneal injections (IP).

Chemicals

Ascarite (Arthur H. Thomas Co.) Lot #8358, 8 to 20 mesh was used.

Bromophenol Blue (Fisher Scientific). Reagent grade was used.

Citric-1,5-^{14}C (Mallinckrodt-Nuclear). Lot #2336, specific activity 8.18 mC/mmole; activity 0.5 mC, was used as received and stored under refrigeration at 2°.

Depo-Testosterone Cypionate (Upjohn). 17β-Cyclopentylpropionate of testosterone in cottonseed oil. The 100 mg/cc solution was diluted to 30 ml with isotonic saline solution and stored under refrigeration.

Dextrose (Baker). Reagent grade was used without further purification.

2,5-Diphenyloxazole (Pilot or Packard). Scintillation grade was used as received.

Drierite (Fisher Scientific). Reagent grade, 6-mesh, was used.

Ethyl Alcohol (U.S. Industrial). Technical grade was used without further purification.

Glucose-1-^{14}C (Mallinckrodt-Nuclear). Lot #2534, specific activity 8.8 mC/mmole; activity 0.3 mC, was used as received and stored under refrigeration.

Glucose-6-^{14}C (Mallinckrodt-Nuclear). Lot #1726, specific activity 8.8 mC/mmole; activity 0.3 mC, was used as received and stored under refrigeration.

Hydrochloric Acid (Fisher Scientific). Reagent grade 0.10 N was used as received.

3-Hydroxy-2-pyridinecarbaldehyde thiosemicarbazone (HFPTS) was obtained from Frederic A. French, Mount Zion Hospital, Palo Alto, California, and was used as received.

2-Oxo-3-ethoxybutyraldehyde bis(thiosemicarbazone) (KTS) (Nutritional Biochemicals Corporation). Reagent grade was used as received.

Magnesium perchlorate (Fisher Scientific). Anhydrous reagent grade was used.

Methyl orange (Fisher Scientific). Reagent grade was used.

NCS Solubilizer (Amersham/Searle). Reagent grade 0.6 N solution in toluene was diluted to 0.1 N with toluene prior to use and stored under refrigeration.

1,4-Bis[2-(5-phenyloxazolyl)]benzene (Arapahoe). Scintillation grade was used as received.

Phenolphthalein (Fisher Scientific). Reagent grade indicator was used as received.

Polyacrylic Acid (Nalco). Very high molecular weight ($\sim 10^7$) polymer was used as received.

Polyoxyethylene sorbitan monostearate (Atlas). Tween 60 was diluted and used as a 20% solution in doubly distilled water.

Sodium chloride (Fisher Scientific). Reagent grade was used as received.

Sodium hydroxide (W. H. Curtin). USP pellets were used as received.

Toluene (Fisher Scientific). Reagent grade was used. It was stored over solid sodium and carefully decanted at time of use.

Instrumentation

Beckman Research pH Meter. The potentiometric titrations were performed using this instrument. The samples were titrated at a constant temperature of 25° in a glass cup around which was a water jacket; water was circulated through a Hoke heating unit and a Brinkman Thermocool cooling unit. Stirring was accomplished by use of a Teflon-coated magnetic stirring bar and a magnetic stirrer.

Tri-Carb Model 3320 Automatic Liquid Scintillation Spectrometer. This instrument had a 200-sample capacity and counted at a controlled temperature of 9°. It used a high-performance Bialkali photomultiplier tube. It also had a Packard Model 515 Digital Printer which recorded the sample number, counting time, and gross (or net) counts for each channel on a 2¼" paper strip.

Preliminary Experiments

The radioactivity of each compound was checked by a method similar to that employed by Waterbury and Jaffee (43). The 0.2-ml aliquot was dissolved in 10 ml of NCS solubilizer (0.1 N). A 1-ml aliquot of this solution was counted by liquid scintillation to determine activity. The channels ratio method was used for determination of liquid scintillation counting efficiency (1). A standard quench set (Nuclear of Chicago) was used for establishing quench curves for appropriate corresponding efficiency (1). A standard quench set (Nuclear of Chicago) was used for establishing quench curves for appropriate corresponding efficiency determinations. All carbon-14 samples on the Tri-Carb 3320 were in the 86 to 90% efficiency range.

To insure that all carbon dioxide or carbon-14 dioxide was being absorbed by the trapping agent, three U-tubes (see Figure 6) filled with Ascarite and magnesium perchlorate were placed in the line leading to the water aspirator. Gravimetric determination showed that 99.7 percent of all expired carbon dioxide was being absorbed by the trapping solution. Gravimetric determination of carbon dioxide without collection in the organic base confirmed that out titration method using a colorimetric end-point was satisfactory for this work. Either method gave approximately the same yield of carbon dioxide which was dependent on the activity of the mouse.

A white solid that formed when the organic base came in contact with water was dissolved when ethyl alcohol was added. As a result, the first end-point (phenolphthalein, red → colorless) was readily detectable. The second end-point (Methyl orange, yellow → red) was concealed as the color went from yellow → tan. Other indicators tried in the pH range of 3.1 to 4.4 were bromophenol blue (3.0 to 4.6) and bromocresol green (3.8 to 5.4). Neither of these gave satisfactory end-points. With Vogel (41) as a reference, it was found that a 3:1 mixture of methyl orange to bromophenol blue as indicator solution gave a sharp (green → yellow) end-point. Potentiometric titrations confirmed that the two colorimetric endpoints were correct for the conversion of carbonate to bicarbonate and bicarbonate to carbonic acid.

General Experimental Procedure

A freshly prepared trapping solution of carbonate-free 0.1 N NCS solubilizer is used for each experiment. This solution is quantitatively transferred with a 10-ml volumetric pipette to seven sealed collection

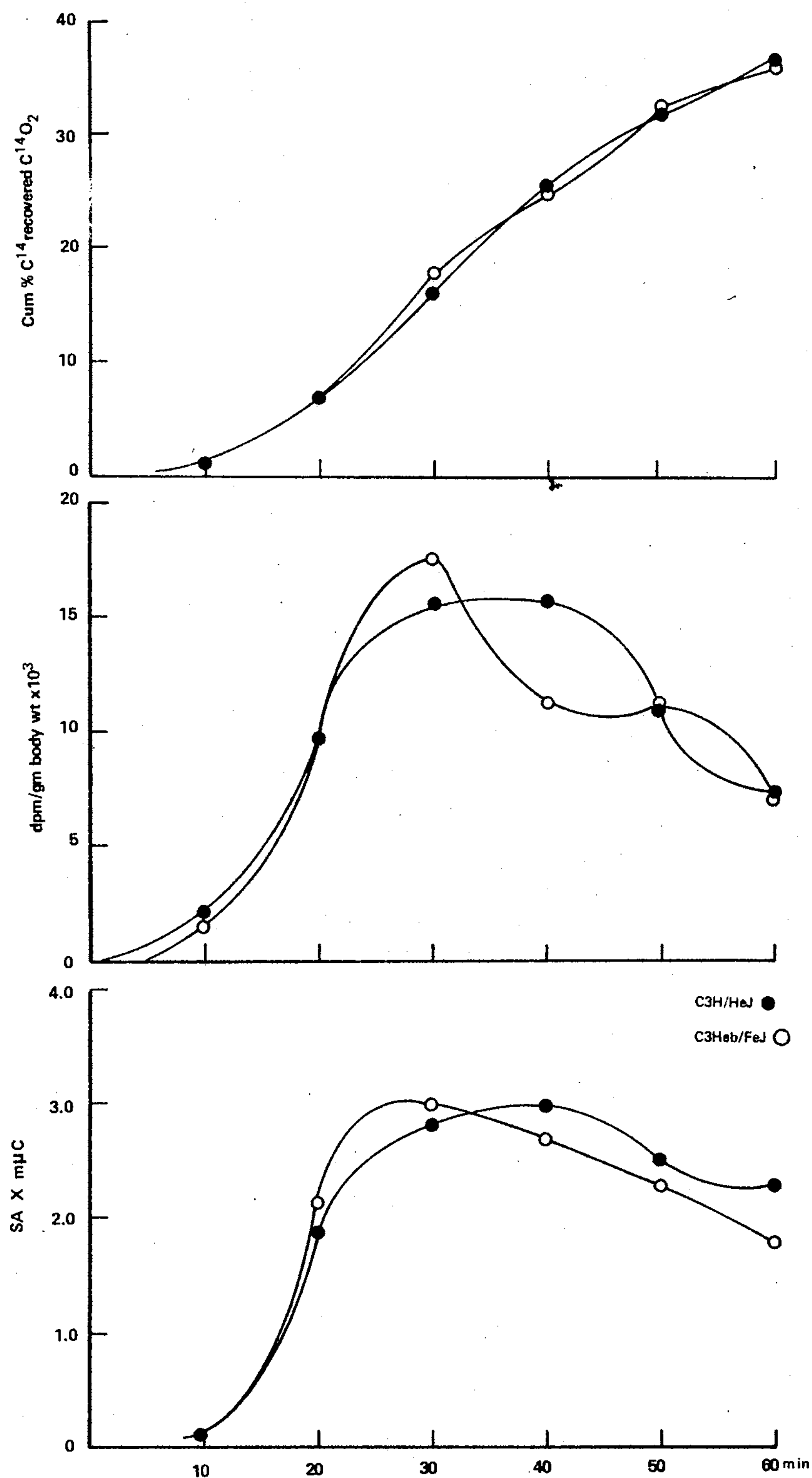


fig. 6

IN VIVO METABOLISM CITRIC–1, 5–^{14}C

tubes. The seventh is used as a blank.

The mouse is weighed prior to IP injection of the labeled substrate. The injection of the mouse and placement in the chamber starts the carbon dioxide collection period. The flow rate is maintained at 60 ml per minute by adjustment with the water aspirator. Collection periods are 10 minutes for each solution unless otherwise noted. The three-way stopcock permits control of the air flow and continuous collection of expired carbon dioxide. After sixty minutes the carbon dioxide collection is stopped. The mouse is removed from the metabolism chamber and weighed.

Each 10-ml solution containing trapped carbon-14 dioxide is divided into three solutions. A 5-ml aliquot is transferred using a volumetric pipette to a 50-ml Erlenmeyer flask which is capped off. It is diluted to 25-ml with ethyl alcohol. Two drops of phenolphthalein indicator are added to the solution and this is titrated with 0.1 N HCl to the first end point (pink → colorless). Two drops of a methyl orange: bromophenol blue solution in the ratio of 3:1 are added and the titration is continued to the second end point (green → yellow). The seventh 10-ml aliquot is treated accordingly to determine if a correction for absorbed atmospheric carbon dioxide is required.

Two 1-ml aliquots are transferred with a volumetric pipette to two 5-dram scintillation vials. This aliquot is diluted to 20 ml with a scintillation cocktail composed of 0.1 grams 1,4-bis[2-(5-phenyloxazolyl)] benzene and 4.0 grams diphenyloxazole per liter of toluene for subsequent counting in a liquid scintillation spectrometer. The blank solution in the seventh tube is treated accordingly for background and correction in the radioactivity determination.

Calculations are then made to determine the specific radioactivity

(SA) of respiratory carbon dioxide, the cumulative percentage of carbon-14 converted to carbon-14 dioxide, and disintegrations per minute per gram body weight. The ratio of total carbon-14 dioxide from glucose-6-^{14}C and glucose-1-^{14}C was used to study the effects of anticancer drugs.

The specific radioactivity of carbon dioxide is determined for each 10-min collection period by the equation

$$SA = {}^{14}CO_2/CO_2,$$

and has the units of microcuries per mole (μC/mole).

The disintegration per minute per gram body weight is calculated from the disintegrations per minute for the appropriate time period divided by body weight in grams. The body weight was taken as an average of the mouse weight at time zero and at 60 min.

The cumulative percentage of carbon-14 converted to carbon-14 dioxide is determined by the addition of the percentage of radioactivity collected for each 10-min period. The results of this addition give the total percentage of radioactivity recovered in the form of carbon-14 dioxide.

The G-6/G-1 ratio is determined by using the percentage radioactivity recovered by injection of glucose-6-^{14}C and of glucose-1-^{14}C.

These data are plotted as a function of time for citric-1,5-^{14}C acid, glucose-1-^{14}C and glucose-6-^{14}C. The G-6/G-1 ratio is used to correlate the effect of anticancer drugs on the rates of oxidation in C3H/HeJ mice with those in C3HeB/FeJ control mice.

In Vivo Oxidation of Citric-1,5-^{14}C Acid

The metabolism of citric-1,5-^{14}C acid, a key intermediate of the Krebs cycle (Figure 2), was studied by the procedure as outlined. The citric acid was prepared for injection by dilution of 0.8 ml of labeled

citric acid (17.6 mC/mmole, activity 0.5 mC) to 25 ml with isotonic saline. Each mouse receiving citric-1,5-^{14}C acid was injected with 0.2 ml of an isotonic saline solution containing 2.0 μC of radioactivity. Intraperitoneal injections (IP) were administered to five C3H/HeJ mice and to five C3HeB/FeJ mice at time zero. Each was placed in the metabolic carbon dioxide collection apparatus and carbon-14 dioxide was collected for ten-minutes periods with continuous trapping of the respiratory carbon dioxide up to sixty minutes. The results of this experiment are plotted in Figure 6. The data for this experiment are in the Appendix.

Because of the small difference in the results from the two kinds of mice, experiments were repeated with the labeled citric acid in the 10-min to 30-min range. Three mice of each type were used in this experiment. The expired carbon dioxide was trapped for ten minutes and then for successive periods of five minutes up to thirty minutes. Two additional precancerous mice were subjected to IP injection and carbon-14 was collected for 30 minutes. The data are plotted in Figure 7. The data are in the Appendix.

<u>In Vivo Oxidation of Carbon-14-Labeled Glucose</u>

Glucose-1-^{14}C (activity 0.3 mC) and glucose-6-^{14}C (activity 0.3 mC) were prepared for Ip injection by the method of Minard (32). The labeled glucose was diluted with unlabeled glucose in 25 ml of isotonic saline so that each mouse receiving glucose-1-^{14}C and glucose-6-^{14}C was injected with 0.2 ml of an isotonic saline solution containing 2.4 μC of radioactivity and 0.87 mg of D-glucose. This solution was kept frozen between experiments. It was kept under refrigeration at 2° when being

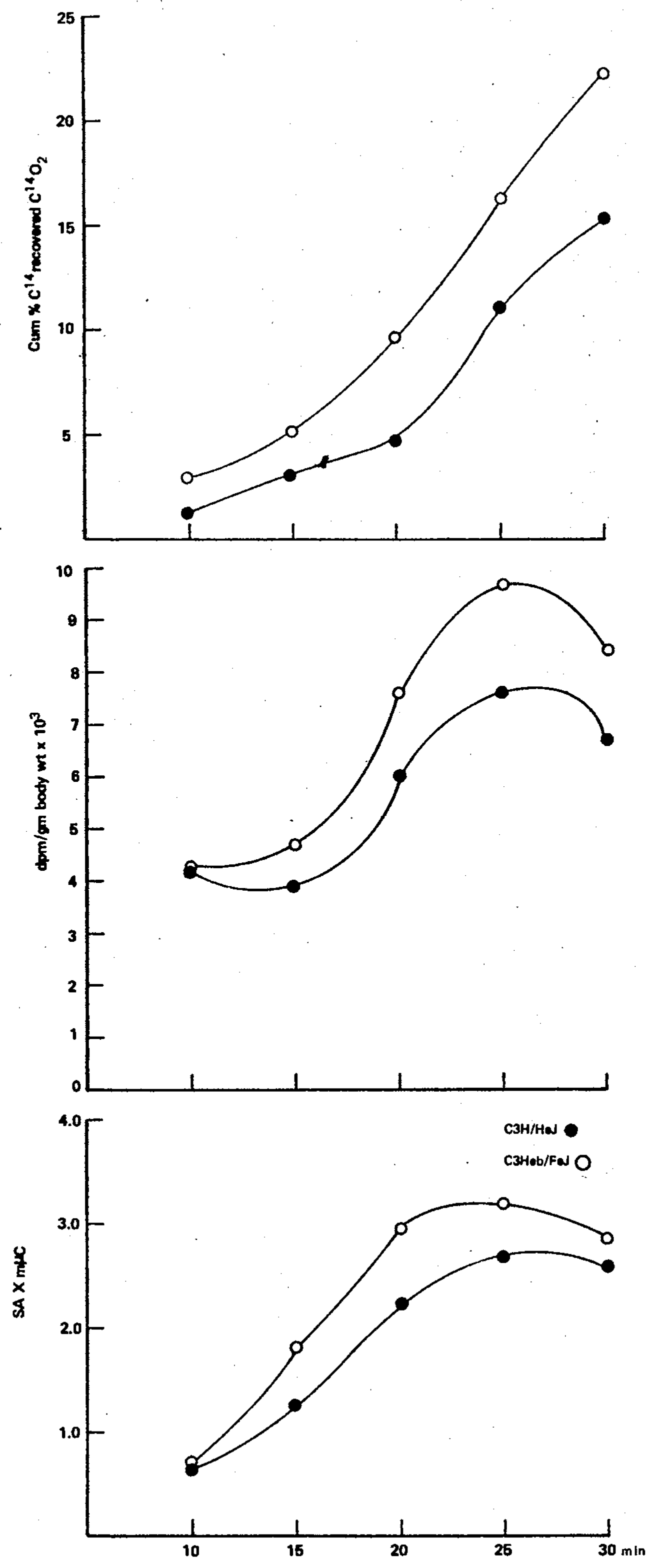


fig. 7

IN VIVO METABOLISM CITRIC-1,5-^{14}C

used.

Glucose-1-^{14}C experiments were carried out using the general procedure. Six C3HeB/FeJ mice and six C3H/HeJ mice were used for establishing the oxidation rate of glucose by continuous collection of respiratory carbon dioxide. Glucose-6-^{14}C experiments duplicated the techniques and procedures for glucose-1-^{14}C. The results of these experiments are plotted in Figure 8 and 9 and the data used in obtaining these figures is given in the Appendix.

From the results of these experiments, G-6/G-1 determined from cumulative percent of carbon-14 converted to carbon-14 dioxide can be established for C3HeB/FeJ and C3H/HeJ animals (Table I) and used for comparison in later experiments.

TABLE I

OXIDATION OF GLUCOSE-6-^{14}C AND GLUCOSE-1-^{14}C DURING ONE HOUR

	Yield of CO_2 From G-6, %	Yield of CO_2 From G-1, %	$\frac{G-6}{G-1}$
C3HeB/FeJ	32.7	38.8	0.84
C3H/HeJ	40.0	42.7	0.94

In Vivo Oxidation of Glucose-1-^{14}C and Glucose-6-^{14}C After Treatment With Depo-testosterone Cypionate

The general procedure was used in this investigation on the effects of a hormone on the oxidation of glucose-1-^{14}C and glucose-6-^{14}C to respiratory carbon-14 dioxide. The drug was prepared by diluting 0.5 cc of DTC solution to 13.3 ml with isotonic saline. The dose for each C3H/HeJ mouse was adjusted to 0.3 mg per gram of body weight (33). The

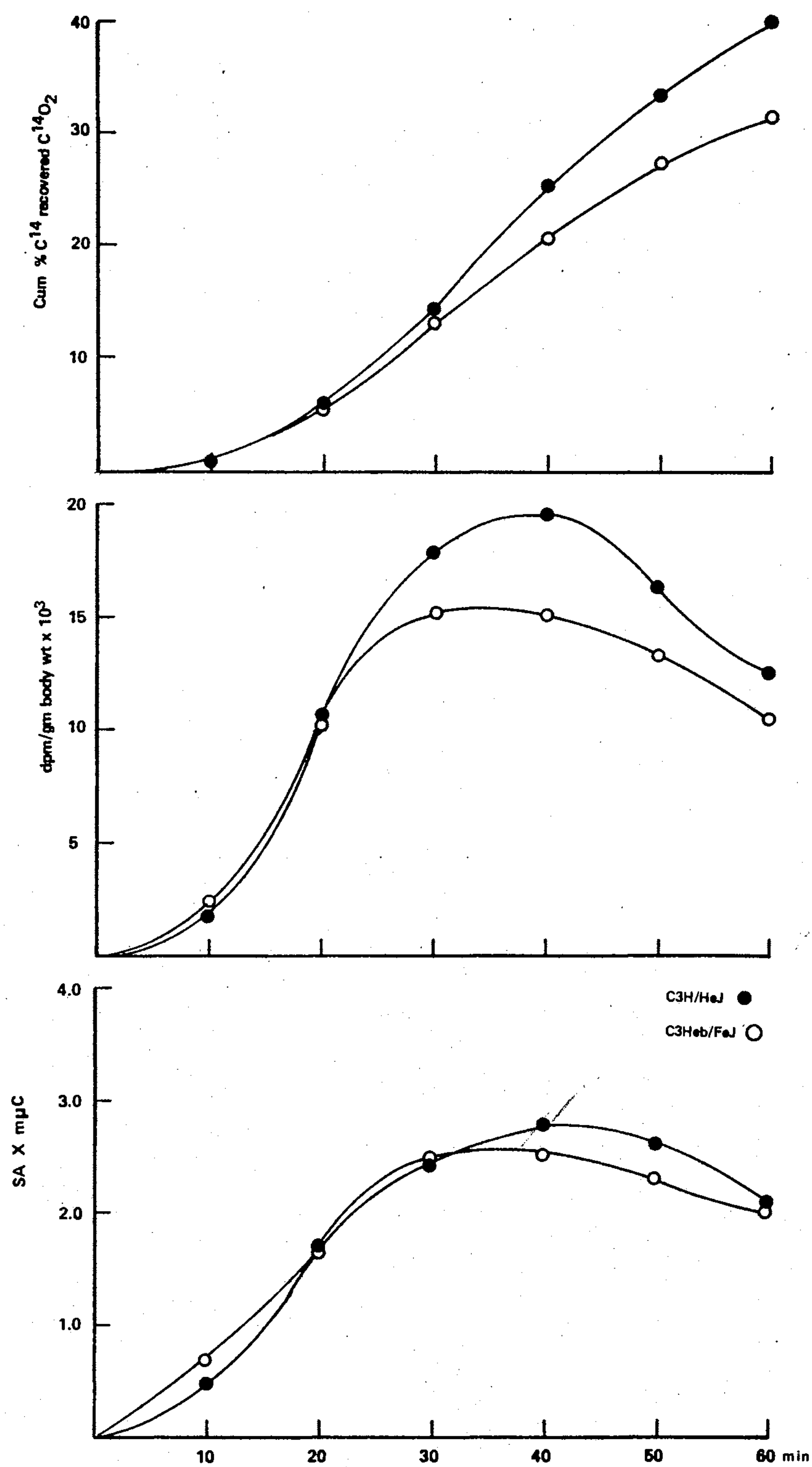


fig. 8
IN VIVO METABOLISM of GLUCOSE – 6 –^{14}C

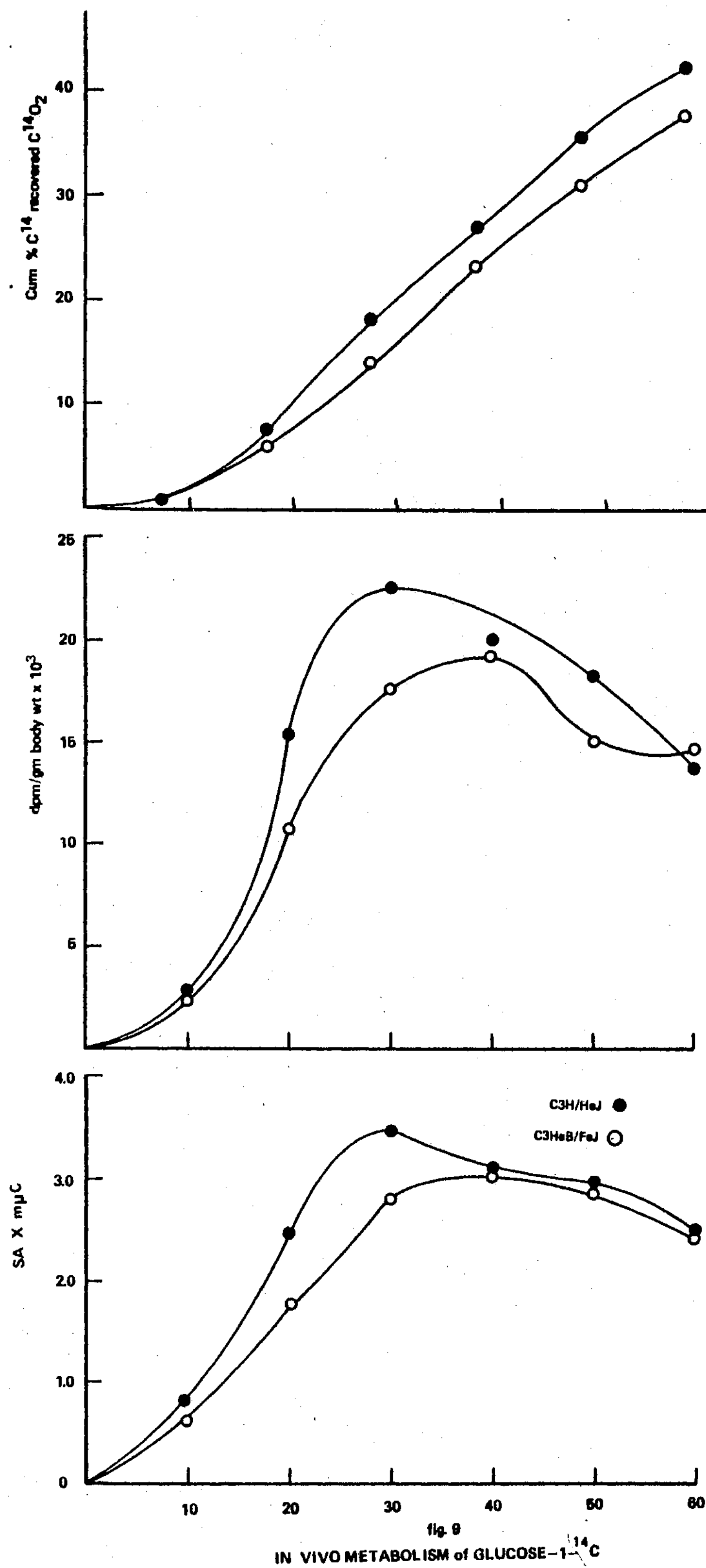


fig. 9
IN VIVO METABOLISM of GLUCOSE-1-^{14}C

drug was kept under refrigeration at all times. IP inoculations were given two hours prior to injecting labeled glucose. Six C3H/HeJ mice (3 each for glucose-1-^{14}C and glucose-6-^{14}C) were used for experimental work. Two additional mice were used with glucose-1-^{14}C to measure the rate of carbon-14 dioxide elimination. The data for these experiments are shown in the Appendix and results are summarized in Table II.

TABLE II

OXIDATION OF GLUCOSE-6-^{14}C AND GLUCOSE-1-^{14}C AFTER INJECTION OF DEPO-TESTOSTERONE CYPIONATE

	Yield of CO_2 From C-6, %	Yield of CO_2 From C-1, %	$\frac{G-6}{G-1}$
C3HeB/FeJ	32.7	38.8	0.84
C3H/HeJ	40.0	42.7	0.94
C3H/HeJ + DTC	28.6	36.3	0.79

<u>In Vivo Oxidation of Glucose-1-^{14}C and Glucose-6-^{14}C After Treatment With 2-Oxo-3ethoxybutyraldehyde Bis(thiosemicarbazone)</u>

KTS was prepared for injection by the method of Petering, Buskirk and Underwood (35). The KTS was ground with 95 percent ethanol containing a drop or two of 20 percent Tween 60 solution and diluted with 19 volumes of isotonic saline. It was stored under refrigeration at 2°. The dose for each mouse was adjusted to 50 mg/kg per day (27).

The drug was given at 6:00 a.m. for three consecutive days. On the third day, glucose-1-^{14}C was administered at 8:00 a.m. to the first C3H/HeJ mouse, 9:15 a.m. to the second and 10:30 a.m. to the third mouse for subsequent carbon dioxide collection. Glucose-6-^{14}C was administered eight hours later, at 4:00 p.m., 5:15 p.m., and 6:30 p.m., respectively,

and carbon dioxide was trapped in the organic base. The data from this experiment are in the Appendix and the values of G-6/G-1 are listed in Table III.

The third day was the last day for KTS injection. On the fifth day, the injection of glucose-1-^{14}C and glucose-6-^{14}C were repeated on the same time schedule. These data are shown in the Appendix and the results are summarized in Table III.

Because of an apparatus failure, an additional C3H/HeJ mouse was treated with KTS. Glucose was injected on the fifth day for experimental analysis. The results of this trial are included in Table III and in the Appendix.

TABLE III

OXIDATION OF GLUCOSE-6-^{14}C AND GLUCOSE-1-^{14}C AFTER INJECTION OF 2-OXO-3-ETHOXYBUTYRALDEHYDE BIS(THIOSEMICARBAZONE)

	Yield of CO_2 From G-6, %	Yield of CO_2 From G-1, %	G-6/G-1
C3HeB/FeJ	32.7	38.8	0.84
C3H/HeJ	40.0	42.7	0.94
C3H/HeJ + KTS (3)	36.6	31.8	1.15
	34.8	35.2	0.99
	31.4	34.7	0.90
C3H/HeJ + KTS (5)	36.6	50.6	0.72
	26.2	48.2	0.54
	33.2	45.9	0.72

(3) Three days, (5) five days

In Vivo Oxidation of Glucose-1-^{14}C and Glucose-6-^{14}C After Treatment With 3-Hydroxy-2-pyridinecarbaldehyde Thiosemicarbazone

The drug was prepared for administration to C3H/HeJ mice by the method of Petering and associates (35). It was ground with 95 percent

ethanol containing a drop or two of 20 percent Tween 60 and diluted with 19 volumes of isotonic saline. It was stored under refrigeration at 2°. The dose for each mouse was adjusted to 71 mg/kg per day (8).

Three C3H/HeJ mice were injected IP daily for three consecutive days at 6:00 a.m. with HFPTS. Glucose-1-^{14}C was administered IP at 8:00 a.m., 9:15 a.m., and 10:30 a.m. and the respired carbon dioxide for trapping was assayed. Eight hours after the glucose-1-^{14}C experiment, glucose-6-^{14}C was injected and the carbon dioxide was collected. The rates of metabolism of these compounds are shown in the Appendix and the G-6/G-1 ratio is shown in Table IV with respect to C3HeB/FeJ and C3H/HeJ animals.

The same three mice were subjected to glucose-1-^{14}C and glucose-6-14 C *in vivo* metabolism tests on the fifth day using an identical time schedule to that of the third day. The data from this experiment for the individual mice are shown in the Appendix and the results are shown in Table IV.

TABLE IV

OXIDATION OF GLUCOSE-6-^{14}C AND GLUCOSE-1-^{14}C AFTER INJECTION OF 3-HYDROXY-2-PYRIDINECARBALDEHYDE THIOSEMICARBAZONE

	Yield of CO_2 From G-1, %	Yield of CO_2 From G-6, %	$\frac{G-6}{G-1}$
C3HeB/FeJ	32.7	38.8	0.84
C3H/HeJ	40.0	42.7	0.94
C3H/HeJ + HFPTS (3)	29.6	29.8	0.99
	29.0	34.5	0.84
	42.0	49.6	0.84
C3H/HeJ + HFPTS (5)	27.0	25.6	1.05
	31.6	25.2	1.25
	28.4	37.2	0.76

(3) three days, (5) five days

In Vivo Oxidation of Glucose-1-^{14}C and Glucose-6-^{14}C After Treatment With Polyacrylic Acid

Polyacrylic acid was prepared for injection by dissolving 5.0 mg in 20 ml isotonic saline solution. The dose for each mouse was adjusted to 2.5 mg/kg per day (18). It was stored under refrigeration at 2°.

Three C3H/HeJ mice were injected daily for three consecutive days at 6:00 a.m. No additional injections with PAA were made. On the third day, glucose-1-^{14}C was injected IP at 8:00 a.m., 9:15 a.m., and 10:30 a.m. Respired carbon-14 dioxide was collected for successive 10-minute periods up to one hour. Eight hours later glucose-6-^{14}C was administered. The results of these experiments are exhibited in the Appendix and the G-6/G-1 ratio for each mouse is shown in Table V. This experiment was duplicated on the fifth day. The results of these experiments are shown in the Appendix and Table V shows the G-6/G-1 ratio for C3HeB/FeJ and C3H/HeJ mice.

TABLE V

OXIDATION OF GLUCOSE-6-^{14}C AND GLUCOSE-1-^{14}C AFTER INJECTION OF POLYACRYLIC ACID

	Yield of CO_2 From G-1, %	Yield of CO_2 From G-6, %	G-6/G-1
C3HeB/FeJ	32.7	38.8	0.84
C3H/HeJ	40.0	42.7	0.94
C3H/HeJ + PAA (3)	41.1	43.6	0.94
	30.9	38.4	0.80
	37.0	38.4	0.96
C3H/HeJ + PAA (5)	28.4	57.0	0.49
	26.5	30.2	0.88
	43.5	42.8	1.02

(3) three days, (5) five days

CHAPTER V

RESULTS AND DISCUSSIONS

The results of this research, summarized in Table V, indicate a difference in the rates of metabolism of carbon-14-labeled glucose by C3H/HeJ mice with the factor for spontaneous mammary tumors and by C3HeB/FeJ mice without the factor. The metabolism of the C3H/HeJ mice can be associated with a increase in glycolysis and a decrease in the tricarboxylic acid cycle activity. Depo-testosterone cypionate, an ester of testosterone, altered the rate of metabolism of C3H/HeJ mice to that of C3HeB/FeJ mice. 2-Oxo-3-ethoxybutyraldehyde bis(thiosemicarbazone) appeared to alter the *in vivo* rate of metabolism of mice with the mammary tumor factor to that of mice without the factor. 3-Hydroxy-2-pyridinecarbaldehyde thiosemicarbazone and polyacrylic acid had smaller effects on the *in vivo* oxidation of labeled glucose in C3H/HeJ mice.

The apparatus used in this research is an economical tool for metabolism studies but lacks precision. Okita (33) found a G-6/G-1 ratio of 0.66 for C3HeB/FeJ compared to 0.84 in these experiments. He found the G-6/G-1 ration for C3H/HeJ mice to be 1.04, comparable to the value of 0.94 in these experiments. Although the differences in the G-6/G-1 ratios found by Okita are larger, the magnitudes of these ratios found in this work are similar to his.

C3H/HeJ mice can be distinguished from C3HeB/FeJ mice (Table XVI)

by use of the method and apparatus used in this research. This method might aid in the detection of a precancerous state prior to the appearance of malignant growth.

The decreased rate of metabolism of citric-1,5-^{14}C acid in C3H/HeJ mice compared to that in C3HeB/FeJ mice indicates decreased Krebs cycle activity. The G-6/G-1 ratio in C3H/HeJ mice compared to that in C3HeB/FeJ animals (0.94 versus 0.84) indicates an increase in glycolysis. The formation of lactate is a dead end in metabolic pathways. Therefore, whether glucose is labeled in the C-1 or C-6 position, the rate of elimination of carbon-14 dioxide should be about the same in the C3H/HeJ mice with the factor. The lactate formed from glycolysis must be reoxidized to pyruvate and eventually eliminated via the Krebs cycle. Since tumor tissue can utilize both aerobic and anerobic glycolysis (7), one would expect a slight increase in the C-1 elimination because of the hexose monophosphate pathway activity (44). These results confirm these expectation although the data of Okita (33) do not.

One would expect a greater difference in glucose-1-^{14}C and glucose-6-^{14}C in C3HeB/FeJ mice because of the normal oxidative pathways, the Krebs cycle and the hexose monophosphate pathway, which contribute to the metabolism of the glucose-1-^{14}C. The Krebs cycle is the major means of glucose-6-^{14}C elimination.

Hormonal treatment of mammary tumors (17) (31) is an accepted clinical procedure. Depo-testosterone cypinate altered the oxidation of labeled glucose in precancerous C3H/HeJ mice to that of C3HeB/FeJ mice. 2-Oxo-3-ethoxybutyraldehyde bis(thiosemicarbazone) caused the metabolic elimination of carbon dioxide from precancerous C3H/HeJ mice to be altered toward that of the C3HeB/FeJ controls on the fifth day

TABLE VI

OXIDATION OF GLUCOSE-1-^{14}C AND GLUCOSE-6-^{14}C DURING 1 HOUR

	Yield of CO_2 From G-6, %	Yield of CO_2 From G-1, %	$\frac{G-6}{G-1}$
C3HeB/FeJ Control	32.7 ± 1.9	38.8 ± 2.7	0.84
C3H/HeJ Precancerous	40.0 ± 0.5	42.7 ± 1.6	0.94
C3H/HeJ + DTC	28.6 ± 1.9	36.3 ± 2.3	0.79
C3H/HeJ + KTS (3 Days)	36.6	31.8	1.15
	34.8	35.2	0.99
	31.4	34.7	0.90
C3H/HeJ + KTS (5 Days)	36.6	50.6	0.72
	26.2	48.2	0.54
	33.2	45.9	0.72
C3H/HeJ + HFPTS (3 Days)	29.6	29.8	0.99
	29.0	34.5	0.84
	42.0	49.6	0.84
C3H/HeJ + HFPTS (5 Days)	27.0	25.6	1.05
	31.6	25.2	1.25
	28.4	37.2	0.76
C3H/HeJ + PAA (3 Days)	41.1	43.6	0.94
	30.9	38.4	0.80
	37.0	38.4	0.96
C3H/HeJ + PAA (5 Days)	28.4	57.0	0.49
	26.5	30.2	0.88
	43.5	42.8	1.02

although not on the third day. The 3-hydroxy-2-pyridinecarbaldehyde thiosemicarbazone and polyacrylic acid were less effective in altering metabolism of the precancerous C3H/HeJ mice. Additional experiments should be made with more mice in order to improve the statistical treatment. In particular the C3HeB/FeJ mice should be treated with the same compounds to determine their response.

The analysis of variance was performed according to Steel and Torrie (30) to evaluate the significance of the data from these experiments. The analysis of variance (AOV) for the in vivo metabolism of labeled glucose (Table VII) was a 2 x 2 factorial design with two levels for each factor. The C3H/HeJ and C3HeB/FeJ mice were the two levels for facter A. The glucose samples labeled in the one position and in the six position were the levels for factor B.

TABLE VII

ANALYSIS OF VARIANCE FOR IN VIVO OXIDATION OF GLUCOSE-6-^{14}C AND GLUCOSE-1-^{14}C IN UNTREATED C4H/HeJ AND C3HeB/FeJ MICE

Source	dF	SS	MS	F	0.05
Treatments	3	264.02			
A	1	155.13	155.13	23.6	4.49**
B	1	94.61	94.61	14.4	4.49**
AB	1	14.28	14.28	2.18	4.49
Error	16	104.95	6.56		
Total	19				

**Main effects are highly significant.

The AOV for the effect of depo-testosterone cypinate is shown in

Table VIII. It is a one-way classification since the experiment deviated from the others in mode of administration of the drug.

TABLE VIII

ANALYSIS OF VARIANCE FOR IN VIVO OXIDATION OF GLUCOSE-6-^{14}C AND GLUCOSE-1-^{14}C IN C3H/HeJ MICE AFTER TREATMENT WITH DEPO-TESTOSTERONE CYPIONATE

Source	dF	SS	MS	F	0.05
A	1	1778.07	1778.07	222	6.61**

**Difference among positions is highly significant.

The AOV for 2-oxo-3ethoxybutyraldehyde bis(thiosemicarbazone), 3-hydroxy-2-pyridinecarbaldehyde thiosemicarbzaone, and polyacrylic acid were performed on results obtained in three days (Table VIII) and five days (Table X).

TABLE IX

ANALYSIS OF VARIANCE FOR IN VIVO OXIDATION OF GLUCOSE-6-^{14}C GLUCOSE-1-^{14}C IN C3H/HeJ MICE AFTER TREATMENT WITH 2-OXO-3-ETHOXYBUTYRALDEHYDE BIS(THIOSEMICARBAZONE), 3-HYDROXY-2-PYRIDINECARBALDEHYDE THIOSEMICARBAZONE, AND POLYACRYLIC ACID AT THREE DAYS

Source	dF	SS	MS	F	0.05
Treatments	5	103.68			
A	1	30.94	30.94	0.90	4.75
B	2	52.19	26.10	0.76	3.89
AB	2	20.55	10.28	0.30	3.89
Total	17				

No significant difference among levels of either factor.

TABLE X

ANALYSIS OF VARIANCE FOR IN VIVO OXIDATION OF GLUCOSE-6-^{14}C AND GLUCOSE-1-^{14}C IN C3H/HeJ MICE AFTER TREATMENT WITH 2-OXO-3-ETHOXYBUTYRALDEHYDE BIS(THIOSEMICARBAZONE), 3-HYDROXY-2-PYRIDINECARBALDEHYDE THIOSEMICARBAZONE, AND POLY-ACRYLIC ACID AT FIVE DAYS

Source	dF	SS	MS	F	0.05
Treatments	5	965.52			
A	1	363.60	363.60	6.23	4.75*
B	2	410.09	205.05	3.52	3.89
AB	2	191.83	95.9	1.64	3.89
Error	12	699.87	58.32		
Total	17				

*Difference among positions is significant.

Aov for simple effects of these three drugs are in Table XI.

TABLE XI

ANALYSIS OF VARIANCE OF SIMPLE EFFECTS IN VIVO OXIDATION OF GLUCOSE-6-^{14}C AND GLUCOSE-1-^{14}C FOR C3H/HeJ MICE AFTER TREATMENT WITH 2-OXO-3-ETHOXYBUTYRALDEHYDE BIS(THIOSEMICARBAZONE, 3-HYDROXY-2-PYRIDINE-CARBALDEHYDE THIOSEMICARBAZONE, AND POLYACRYLIC ACID AT FIVE DAYS

Treatment Comparison	dF	MS	F
G-1 and G-6 with KTS	1	388.82	4.75**
G-1 and G-6 with HFPTS	1	0.625	4.75
G-1 and G-6 with PAA	1	166.43	4.75**
KTS, HFPTS and PAA with G-1	2	278.61	3.89**
KTS, HFPTS and PAA with G-6	2	63.65	3.89**

**All simple effects are highly significant except G-1 and G-6 with HFPTS.

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APPENDIX

APPENDIX

CUMULATIVE RECOVERY OF CARBON-14 IN RESPIRED CARBON DIOXIDE, %

Treatment	Compound Injected	Mouse Type	Mouse No.	Time, min					
				10	20	30	40	50	60
None	Citric-1,5-^{14}C	C3HeB/FeJ	F03	0.5	7.9	21.7	26.9	36.4	37.4
			F04	1.8	7.0	17.0	25.1	33.2	38.6
			F05	1.5	7.9	19.0	24.4	29.7	34.4
			F06	.8	5.4	14.1	26.7	32.0	38.3
			F07	.3	6.4	17.8	22.1	30.0	32.9
			Average	1.0	6.9	17.9	25.0	32.3	36.3
None	Citric-1,5-^{14}C	C3H/HeJ	H05	1.5	8.5	15.5	24.1	32.7	36.6
			H06	0.6	6.1	14.1	25.1	30.6	35.6
			H07	1.4	8.0	19.2	29.8	35.1	37.6
			H08	1.4	4.1	11.6	19.5	27.3	35.7
			H09	1.3	7.3	20.1	29.2	33.9	37.0
			Average	1.2	6.8	16.1	25.5	31.9	36.5
None	Citric-1,5-^{14}C	C3HeB/FeJ	F09	0.7	2.4	5.0	14.1	19.9	
			F10	1.9	4.6	8.0	13.4	19.5	
			F11	3.9	9.4	16.3	21.2	26.3	
			Average	2.1	5.5	9.8	16.2	21.9	
None	Citric-1,5-^{14}C	C3H/HeJ	H10	.6	1.2	6.4	12.0	14.7	
			H11	.1	.1	7.0	9.9	11.7	
			H12	2.8	4.9	6.9	11.7	18.9	
			Average	1.1	2.1	6.8	11.2	15.2 ± 1.7*	
None	Glucose-6-^{14}C	C3HeB/FeJ	F19	0.9	4.8	12.1	18.7	26.6	31.3
			F20	0.1	7.6	16.5	25.0	31.7	36.8
			F21	0.4	2.8	11.7	18.1	26.2	32.4
			F22	0.7	4.0	10.9	20.3	27.1	33.5
			F23	2.4	10.0	17.2	22.2	26.3	29.7
			F24[a]	2.1	9.8	19.3	30.2	38.1	45.9
			Average	0.9	5.8	13.7	20.9	27.6	32.7 ± 1.9

APPENDIX (Continued)

Treatment	Compound Injected	Mouse Type	Mouse No.	Time, min 10	20	30	40	50	60
None	Glucose-6-^{14}C	C3H/HeJ	H19	1.0	7.0	15.5	24.7	33.2	41.0
			H20	.3	2.8	10.5	22.5	30.2	38.3
			H21	.7	2.9	11.1	21.1	30.4	37.7
			H22	1.2	9.0	18.8	28.5	36.8	41.7*
			H23	1.5	11.6	21.9	30.7	37.2	41.3
			H24[a]	.4	5.6	12.6	18.3	23.4	27.9
			Average	0.9	6.1	14.7	25.5	33.6	40.0 ± 0.5
None	Glucose-1-^{14}C	C3HeB/FeJ	F13	1.0	5.2	14.1	23.3	*29.7→	35.9+
			F14	1.0	7.0	14.5	24.1	32.6	41.5
			F15	.8	5.6	15.3	24.9	32.3	40.6
			F16	1.6	8.2	14.6	26.2	34.5	40.9
			F17	1.8	6.7	15.4	22.5	28.4	35.0
			F18	1.0	5.5	12.7	20.0	25.0	32.1
			Average	1.2	6.5	14.8	24.2	31.9	38.8
None	Glucose-1-^{14}C	C3H/HeJ	H13	.7	5.1	14.0	22.9	31.7	38.4
			H14	1.1	9.1	21.3	29.0	38.0	43.8
			H15	1.4	8.3	20.0	27.8	36.6	42.7
			H16	.9	10.5	19.1	30.5	37.1	43.6
			H17	1.3	7.8	18.7	28.50	37.8	44.8
			H18[a]	1.5	6.0	16.0	22.3	27.4	32.4
			Average	1.1	8.2	18.6	27.7	36.2	42.7±1.6
DTC	Glucose-6-^{14}C		H18	1.2	3.7	10.6	17.0	24.5	31.4
			H19	.9	3.8	8.9	15.3	21.1	26.1
			H20	.8	3.4	9.2	14.9	20.9	28.3
			Average						
DTC	Glucose-1-^{14}C		H13	.4	4.2	10.1	18.6	26.1	32.6
			H14	1.0	5.1	15.4	22.8	32.0	37.8
			H15	.6	6.6	14.9	*	27.0	35.4
			H16	1.5	9.1	19.3	28.6	33.4	39.3

APPENDIX (Continued)

Treatment	Compound Injected	Mouse Type	Mouse No.	Time, min					
				10	20	30	40	50	60
			H17[a]	2.0	8.5	18.9	30.6	39.5	47.8
			Average						
KTS	Glucose-6-^{14}C		H21	.06	5.5	14.6	21.6	29.6	36.6
			H22	1.4	6.9	14.7	23.1	29.6	34.8
			H23	1.1	5.7	14.0	20.7	26.1	31.4
			Average						
KTS	Glucose-1-^{14}C		H21	*	*	13.8	20.7	25.7	31.8
			H22	0.8	4.6	11.6	20.5	28.2	35.2
			H23	1.5	7.0	15.6	23.9	29.5	34.7
			Average						
KTS	Glucose-1-^{14}C		H21	1.6	13.2	26.4	36.4	44.0	50.6
			H22	2.4	10.6	19.9	31.2	42.5	48.2
			H23[a]	1.1					
			H24	0.8	7.8	18.4	31.0	39.6	45.9
			Average						
KTS	Glucose-6-^{14}C		H21	1.5	*12.7	20.4	25.7	32.0	36.6
			H22	1.3	6.7	11.7	17.2	22.3	26.2
			H23[a]	3.3	15.0	23.6	31.1	36.1	40.9
			H24	0.9	5.6	11.1	19.8	26.1	33.2
			Average						
HFPTS	Glucose-6-^{14}C		H25	0.6	5.4	10.5	17.8	23.9	29.6
			H26	1.1	4.8	10.8	17.4	23.6	29.0
			H27	0.6	7.6	15.1	26.2	35.3	42.0
			Average						
HFPTS	Glucose-1-^{14}C		H25	0.7	4.8	10.9	17.4	24.4	29.8
			H26	0.8	4.3	10.0	18.2	26.0	34.5
			H27	1.1	8.8	20.9	32.7	41.9	49.6
			Average						

APPENDIX (Continued)

Treatment	Compound Injected	Mouse Type	Mouse No.	Time, min 10	20	30	40	50	60
HFPTS	Glucose-6-^{14}C		H25	.5	4.9	10.0	16.2	21.2	27.0
			H26	1.0	7.3	14.1	22.4	27.1	31.6
			H27	.5	6.1	13.2	19.4	25.0	28.4
			Average						
HFPTS	Glucose-1-^{14}C		H25	.5	4.7	10.1	16.3	20.7	25.6
			H26	.9	4.2	9.8	16.1	21.7	25.2
			H27	2.7	13.5	22.1	29.3	34.1	37.2
			Average						
PAA	Glucose-6-^{14}C		H28	1.1	9.6	19.3	27.8	35.5	41.1
			H29	1.3	8.8	15.0	22.2	26.5	30.9
			H30	1.5	9.1	18.6	25.0	31.2	37.0
			Average						
PAA	Glucose-1-^{14}C		H28	1.1	10.2	20.2	32.3	38.1	43.6
			H29	0.9	8.5	13.3	25.2	32.3	38.4
			H30	1.0	6.0	14.1	23.1	30.6	38.4
			Average						
PAA	Glucose-6-^{14}C		H28	0.6	3.7	10.7	18.5	24.9	28.4
			H29	1.0	7.3	11.6	17.0	21.8	26.5
			H30	1.0	10.1	22.3	30.4	37.8	43.5
			Average						
PAA	Glucose-1-^{14}C		H28	1.9	13.8	27.4	40.3	51.0	57.0
			H29	4.3	10.1	15.1	20.4	25.4	30.2
			H30	1.7	9.4	16.2	27.9	35.2	42.8
			Average						

VITA

2

Charles Harvey Moore

Candidate for the Degree of

Master of Science

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