METABOLISM OF GLUCOSE-U-C^{14} BY BONE

A Thesis

Presented to

the Faculty of Graduate Studies

University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Helen Katherine Wedel

Department of Oral Biology

May 1964

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Abstract. The metabolism of glucose-U-C^{14} was studied in vitro by incubating calvarial preparations from one- to two-day old Long Evans rats with varying levels of this substrate. Incubations, generally lasting for two hours, were carried out at 37°C. in air with calcium-free phosphate- or THAM-buffered medium (pH 7.4). Carbon dioxide, either metabolic alone, or total after addition of acid to the tissue, was collected, chemically determined by manometric procedures, and assayed for radioactivity. Glucose and lactate were obtained from the medium after incubation by elution with a linear HCl gradient from Dowex-1-Cl anion exchange resin columns. Glucose was determined by the glucose oxidase-3,3'-dimethoxybenzidine (o-dianisidine) procedure, and lactate by the DPN-lactic dehydrogenase procedure. Like carbon dioxide, these compounds were assayed for radioactivity: all activity determinations in these experiments were carried out either with a thin window gas flow tube or by liquid scintillation. In some of the experiments, after incubation, the skulls were extracted with various solvents so as to determine the distribution of activity in different components of the tissue. Paper chromatography and radioautography were used to identify and estimate the quantity of radioactivity in these components of the tissue, and in metabolic intermediates found in the medium after incubation.

Two preliminary experiments, in which calvaria were incubated with glucose-U-C^{14} in phosphate-buffered medium (pH 7.4) for two hours, were performed. From the oxygen uptake of 14.34 μmoles/gram wet weight/hour,

and the carbon dioxide production of 16.73 μmoles/gram wet weight/2 hours, an R.Q. of 0.58 was determined. The inverse relation between the specific activity and production of lactic acid indicated varying degrees of endogenous dilution. On the basis of previous work by Dowse et al, 1963, it was likely that glycogen was the source of this dilution. The specific activities of carbon dioxide and lactate were considerably lower than that of glucose, but the specific activity of the CO_2 was much like that of the lactate. Therefore, much more dilution was occurring in the glycolytic cycle than between pyruvate and the decarboxylation steps of the TCA cycle. In one instance the specific activity of the CO_2 actually exceeded that of the lactic acid: this would be possible if the hexose monophosphate shunt were more active in this case.

In experiment 2 calvaria were incubated as in the preliminary experiments. The quantity of glucose utilized and its utilization rate were determined. Glucose uptake was constant, as was the fraction of utilized activity in lactate and CO_2. The specific activity and production of lactate were not individually constant, but were inversely related to each other. From the above results it could be seen that, at a given phosphate level, the conversion of glucose-C^{14} to lactate-C^{14} was unaffected by the varying degrees of endogenous dilution which were occurring.

In experiments 7, 8, and 10, calvaria were incubated with varying concentrations of glucose-U-C^{14} for two hours in air. Phosphate and THAM buffers (pH 7.4) were used in experiments 7 and 8, and 10 respectively. The specific activity of the glucose in the medium after incubation was lower than that of the original specific activity, and the effect was

more marked in phosphate than in THAM buffer. Within a given experiment, in which the phosphate level was constant, the specific activity of the lactate rose with increasing levels of glucose in the medium. Hence, at a given phosphate concentration, the production of lactate-C^{14} from glucose-C^{14} was directly dependent on the glucose concentration. With similar initial glucose, and therefore similar glucose-1-phosphate concentrations, on the basis of specific activities, more dilution of the lactate occurred in phosphate than in THAM buffer. The above results supported the hypothesis already developed, that endogenous dilution was occurring, and that glycogen was likely responsible for this dilution. The hydrolysis of glycogen is directly dependent on the ratio of $\frac{\text{inorganic phosphate}}{\text{glucose-1-phosphate}}$.

The average percentage of utilized activity in the lactate formed in phosphate buffer (experiment 8) was 29% as compared to 40% for that formed in THAM buffer (experiment 10). In both experiments, 2-4% of the utilized activity was recovered in the CO_2. Up to 58% of the utilized activity was calculated to be in compounds other than glucose and lactate in the medium after incubation in experiment 8, and up to 34% in experiment 10. 14-38% of the total counts utilized were calculated to be in the tissue of both experiments.

Paper chromatography and radioautography were used to identify and estimate the amount of activity in various components of calvaria. The ethanol extract of tissue incubated with glucose-U-C^{14} in phosphate buffer (pH 7.4) for 2 hours in experiment 3 contained labeled phosphorylated glycolytic intermediates, amino acids, and organic acids. Glutamic

and lactic acids were more heavily labeled than any of the other metabolic intermediates. In experiment 5 radioautograms were prepared from ethanol extracts of skulls incubated with glucose-U-C^{14} in phosphate buffer (pH 7.4) for 15 minutes, 30 minutes, 1 hour, 2 hours, and 3 hours respectively. After 15 minutes there was more activity in the phosphorylated glycolytic intermediates than in the amino acids, but after 3 hours, there was a much greater quantity of activity in amino acids such as glutamate than in the phosphorylated intermediates. This showed that, with time, much of the activity from the glucose had moved on through the glycolytic and tricarboxylic acid cycles to form labeled glutamic acid, which is used in protein synthesis.

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CHAPTER I

INTRODUCTION

Little comprehensive work has been done on carbohydrate metabolism in calvaria. Investigators in this field have generally preferred to use metaphyseal bone preparations (Borle, Nichols, and Nichols, 1960a, 1960b; Lekan, Laskin, and Engel, 1960; Cohn and Forscher, 1961, 1962a, 1962b; Vaes and Nichols, 1961, 1962a, 1962b). However, a biochemical study of calvaria from newborn rats was carried out by Dowse et al, 1963. The metabolic behaviour of calvaria was found to be qualitatively similar to that of metaphyseal preparations of bone. In the present report an attempt was made to elucidate further the metabolic behaviour of calvaria by incubating the tissue with glucose-U-C^{14} in vitro and tracing the products of metabolism.

I. PURPOSE OF THE STUDY

The metabolism of glucose-U-C^{14} by calvaria in different buffers at 37°C. in air was investigated. The object of the study was to (1) determine, by the use of paper chromatography, the distribution of radioactivity in the components of the medium after incubation and in various extracts of the skulls after incubation of the latter with glucose-U-C^{14}; (2) determine the utilization of oxygen, and the production and specific activity of metabolic CO_2 during the incubation of calvarial preparations; (3) determine the utilization of glucose by

calvaria and to assess the specific activity of the glucose in the medium before and after incubation; (4) assess the production and specific activity of lactic acid formed during the incubation of calvarial preparations; (5) determine the fate of glucose-U-C^{14} when calvarial preparations were incubated in media containing different levels of substrate and different buffers.

From the information obtained on the above parameters in normal calvaria, it should be possible to study more intelligently the effect of parathyroid extract on the metabolism of glucose-U-C^{14} by calvaria.

II. RELATION OF THE STUDY TO CARBOHYDRATE METABOLISM IN BONE

Although much work has been done on the effects of parathyroid hormone on carbohydrate metabolism in metaphyseal bone preparations, the results have not always been too meaningful, due to an inadequate understanding of carbohydrate metabolism in normal metaphyseal bone preparations. Cohn and Forscher (1962b) incubated epiphyseal-metaphyseal slices from the femurs of control and parathyroid extract-treated rabbits with glucose-U-C^{14}, glucose-1-C^{14}, and glucose-6-C^{14}. They found an increase in the total activity incorporated into CO_2, but did not know whether this increase was due to net changes of carbon dioxide or simply to increases in specific activity. In the present investigation information was to be obtained on the specific activity of metabolic CO_2 produced by calvaria from normal rats.

Experiments were to be performed to determine the percentage of utilized activity which would be present in various intermediates

formed by incubating calvaria from normal animals with glucose-U-C^{14}. To our knowledge, no data has yet appeared on the fate of glucose-U-C^{14} upon being incubated with calvaria. An adequate representation of the distribution of label in the skulls, CO_2, and components of the medium after incubation of control calvarial preparations was to be obtained. This would help to create a more complete picture of carbohydrate metabolism in normal calvaria. The ground-work would then be laid for a future investigation of the effects of parathyroid hormone on glucose-U-C^{14} metabolism in calvaria.

III. LIMITATIONS OF THE INVESTIGATION

The calvarium, containing a large number of cells, is a ready-made tissue slice which can be readily extracted from newborn rats. According to Gaillard, 1959, the chief advantages of this tissue are convenience, reproducibility, and responsiveness to parathyroid hormone in vitro. However, it is emphasized that since the present study is conducted on calvaria, the findings may or may not apply to bone cells in general.

Firschein (1962), discussing the parathyroid hormone and carbohydrate metabolism in bone, compares results obtained from in vivo and in vitro studies. The in vivo data shows that bone is constantly synthesizing and releasing citrate into the circulation, and that parathyroid hormone stimulates this process. Since bone mineral can remove citrate from the medium by phosphate exchange and binding to the crystals, the bone slice would not be very suitable for studying the effect of

parathyroid hormone upon citrate metabolism. However, Firschein mentions several workers (Laskin and Engel, 1956; Lekan et al, 1960; Ranney, 1960; Hekkelman, 1961; Neuman and Dowse, 1961; Vaes and Nichols, 1961) who, by using in vitro systems, have nevertheless been able to demonstrate an effect of parathyroid hormone upon citrate metabolism. The in vivo studies of Firschein et al, 1959, have shown that parathyroid hormone also stimulates the formation and release of lactic acid by bone. These findings were confirmed by means of the in vitro bone slice system (Borle et al, 1960b; Neuman and Dowse, 1961; Schartum and Nichols, 1961). However, Firschein (1962) mentions that the evidence from in vivo studies indicates that citrate is capable of causing bone dissolution, whereas, in vitro, there seems to be no correlation between the amount of citrate (or lactate) produced by bone and demineralization. He suggests that the limitations of the in vitro bone slice system may be responsible for this discrepancy. Since the present report on glucose-U-C^{14} metabolism by calvaria is based on results obtained from an in vitro system, it is necessary to keep this in mind when reading this study.

IV. DEFINITIONS OF TERMS USED

Calvaria and skulls. These terms are used interchangeably in this report and represent that fraction of the cranium consisting of the greater part of the parietal bones and that portion of the frontal bone above the eye sockets.

Cpm. This is an abbreviation which stands for counts per minute.

Dpm. This is the abbreviation used for disintegrations per minute. When liquid scintillation was used to assess the radioactivity of samples, correction of the actual efficiency of the system to 100% was made by the channels ratio method. Absolute activity determinations made in this way are designated as dpm in this report.

Q_{O_2}. This represents the rate of oxygen consumption.

R.Q. This stands for respiratory quotient, and is calculated by finding the ratio between the rate of carbon dioxide production and oxygen consumption.

S.A. This is used to represent the specific activity of a substance.

TCA. This shorthand notation stands for trichloroacetic acid.

THAM-buffered medium. THAM refers to Tris(hydroxymethyl)aminomethane, a substance sometimes used instead of phosphate buffer in the medium of incubation flasks.

μmole and μM. These two forms are used interchangeably throughout the thesis to stand for micromole, the former in the text and the latter, because of shortage of space, in the tables and figures. Attention is drawn especially to the use of the symbol uM in this sense as it is somewhat at variance with commonly accepted practice.

V. ORGANIZATION OF THE REST OF THE REPORT

In Chapter II there is a discussion of previous investigations of problems similar to the one reviewed in the present report. Chapter III gives an account of the methods of procedure and experimental techniques used to investigate the present problem. Paper chromatography and radioautography were used to separate and identify various metabolic intermediates formed from glucose-U-C^{14} by calvarial preparations: this is described in Chapter IV. Two preliminary experiments designed to yield general information concerning glucose-U-C^{14} metabolism by calvaria are described in Cahpter V. In Chapter VI there is an account of an experiment in which the quantity of glucose utilized by calvaria was determined, and the percentage of total counts utilized present in various end-products was calculated. Chapter VII describes the effect of different substrate levels and medium phosphate concentration upon glycolysis in calvaria. In Chapter VIII there is a description of the effect of different substrate levels and medium phosphate concentration upon the distribution of activity in various components of the rat calvarium. The last chapter, Chapter IX, summarizes the developments of previous chapters and states the more important conclusions of the whole study.

CHAPTER II

HISTORY OF THE LITERATURE

Chapter II contains a brief résumé of the literature related to the present problem. Since bone is not too suitable for biochemical metabolic studies, progress in the study of this tissue has been slow. In the past, numerous scientists have used metaphyseal preparations of bone, although these are seriously contaminated with marrow cells. In the present study, glucose-U-C^{14} metabolism by calvaria from one- to two-day old rats is studied. Until the present time, little comprehensive work has been done on calvaria.

I. METABOLISM OF BONE CELLS

Borle et al (1960a) examined the metabolic behavior of bone cells in vitro by using slices from the metaphyses of the long bones of adult mice. They found that bone cells utilized oxygen slowly as compared to other tissues such as liver, but consumed glucose at a brisk rate. This indicated that the major portion of the substrate consumed passed through the glycolytic pathways, and that only a small percentage was left to go through the tricarboxylic acid cycle or for synthetic processes. The chief end product of bone cell metabolism was lactic acid, even under aerobic conditions, and the citric acid produced was less than 1/70 of the amount of lactate formed.

In another investigation, Borle et al (1960b) incubated metaphyses of mice pretreated with parathyroid extract to study the metabolic pattern

of resorption, and metaphyses of the long bones in male mice treated with estrogenic hormones to study the metabolism of accretion. Control samples were used as well. No significant differences were found between the rates of oxygen consumption determined for the three groups of animals. This indicated that tricarboxylic acid cycle function and presumably the rate of citric acid production were not greatly altered during the processes of resorption and accretion in bone.

The metabolic study of bone in vitro begun earlier by Borle et al (1960a and 1960b) was continued by Vaes and Nichols (1961). They incubated metaphyseal bone preparations from adult male mice treated with parathyroid extract and estradiol respectively. The production of lactate from pyruvate and oxaloacetate by bone from normal mice was about 100-fold that of citrate. When normal bone cells were incubated with high levels of citrate, the rate of citrate oxidation was higher than the rate of citrate production.

Vaes and Nichols (1962b) used metaphyseal preparations from rats and mice to study the in vitro metabolism of glycine labeled with C^{14} in the carboxyl carbon. They found that although small amounts of glycine were decarboxylated, much larger amounts were incorporated intact into the organic matrix of bone from 2-month-old rats. Larger rates were found in young than in older animals. This indicated that the formation of bone is more rapid in young than in older animals. Hence, it is to be expected that calvaria from newborn rats would be metabolically very active. The rate of glycine incorporation into the organic matrix of incubated bone samples was found to depend also on the presence of various hormones.

Vaes and Nichols demonstrated the importance of endocrine factors in the control of bone matrix formation.

In a series of experiments in which epiphyseal and metaphyseal bone preparations from 6-week-old rabbits were incubated in vitro with glucose-1-C^{14}, glucose-6-C^{14}, glucose-U-C^{14}, pyruvate-2-C^{14}, and lactate-2-C^{14}, Cohn and Forscher (1962a) studied the aerobic metabolism of glucose by bone. They found that under their experimental conditions the major end product of glucose metabolism was lactic acid. The specific activity of the lactate, compared with the initial glucose specific activity, was of almost theoretical value. This indicated that all of the lactic acid arose from the added glucose. From data on the differences in formation of $C^{14}O_2$ from glucose-6-C^{14} and glucose-1-C^{14}, and on the degradation of lactate, it was determined that about 15% of the lactate devolved from the pentose cycle, and the rest, from the Embden-Meyerhof pathway. Because of the large accumulation of lactate and the relatively small production of $C^{14}O_2$, Cohn and Forscher concluded that the role of the Krebs cycle in the metabolism of glucose by the bone slices appeared to be minor.

In another series of experiments, Cohn and Forscher (1962b) studied the metabolism of labeled glucose by weanling rabbits treated with parathyroid extract. Epiphyseal-metaphyseal slices from the distal end of femurs and minces of soft tissue were incubated with glucose-U-C^{14}, glucose-6-C^{14}, and glucose-1-C^{14}. In these experiments there was not a 2:1 relationship between the μ moles of lactate formed and the μ moles of glucose consumed. In an earlier paper (Cohn and Forscher, 1962a), it

appeared that all of the lactic acid produced arose from the added glucose.

A comprehensive biochemical study of calvaria from newborn rats was carried out by Dowse et al, 1963. In terms of its chemical composition, the tissue was found to be intermediate between soft tissue and mature bone. Its metabolic behavior was qualitatively similar to that of metaphyseal preparations in that it showed excess reserve succinate dehydrogenation capacity, low oxygen consumption, high glycolytic capacity, and a high rate of aerobic glycolysis (incomplete Pasteur effect). According to this report, there would seem to be no reason to doubt the existence of the TCA cycle in bone. It was found to be present from succinate to citrate in calvaria, and citrate can be further metabolized.

II. VARYING INITIAL GLUCOSE LEVELS

Activity incorporated into CO_2. Cohn and Forscher (1961) used epiphyseal and metaphyseal slices from weanling rabbits to study the metabolism of osseous tissue. They found a greater increase in the total activity incorporated into CO_2 at lower substrate levels than at higher levels. Since no data was given on the specific activity of CO_2, it was not clear whether this increase was due to a rise in specific activity, or in the chemical quantity of CO_2.

Production of lactate. Borle et al (1960a) found that the quantity of lactate produced by metaphyseal preparations from mice depended

on the presence of substrate. Much greater quantities of lactate were produced when glucose was present than when it was absent.

Calvarial preparations were incubated with varying levels of glucose by Dowse et al (1963). They found that lactate production increased rapidly with increasing concentrations of glucose up to about 4 millimolar. Beyond this level of glucose, lactate production increased more slowly.

III. RESULTS OBTAINED WITH DIFFERENT BUFFERS

Borle et al (1960a) found that the quantity of lactate produced by metaphyseal preparations from mice depended on the kind of buffer used. With glucose and oxygen present, 30% more lactate was produced in bicarbonate buffer than in phosphate buffer.

Epiphyseal and metaphyseal bone preparations from 6-week-old rabbits were incubated in vitro with various substrates by Cohn and Forscher (1962a) to investigate the aerobic metabolism of glucose by bone. Due to the similarity of $C^{14}O_2$ production in THAM and bicarbonate buffers, they felt that most of the CO_2 evolved from Krebs' cycle activity. Bicarbonate buffer promoted lactate formation when compared to THAM buffer, and they suggested that this was due to the specific effect of HCO_3^- or CO_2.

Calvarial preparations were incubated with phosphate and bicarbonate buffers by Dowse et al (1963). Bicarbonate ion was at a 25 millimolar concentration, and phosphate at a 1.2 millimolar concentration in the bicarbonate-buffered medium. Bicarbonate ion was absent,

and phosphate was at a 16 millimolar concentration in the phosphate-buffered medium. Lactic acid production was determined after 30 and 60-minute incubations. Under aerobic conditions, considerably more lactate was produced in phosphate than in bicarbonate medium. Addition of bicarbonate suppressed the phosphate effect. When phosphate at 16 millimolar and bicarbonate at 25 millimolar concentrations were present in the same medium, the quantity of lactate produced was almost the same as that found in the standard bicarbonate-buffered medium.

Dowse et al used the CO_2 buffer technique suggested by Pardee and developed by Krebs (Umbreit, 1957) to determine the Q_{O_2}. No difference in this parameter was found in bicarbonate and phosphate-buffered medium. Use of Warburg's "indirect" method yielded values for the Q_{O_2} which were 3 times as high in the bicarbonate as in the phosphate-buffered medium. No explanation for the above discrepancies was suggested, although a variable CO_2 retention by the tissue is one possibility.

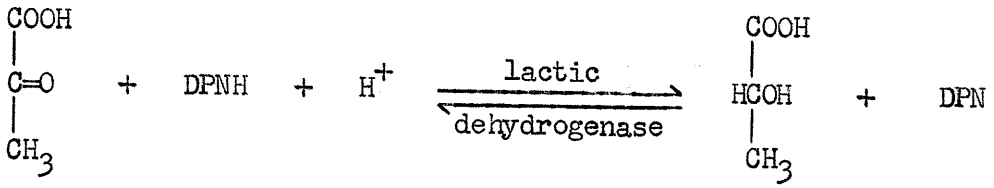
CHAPTER III

GENERAL METHODS EMPLOYED

Chapter III gives an account of the methods utilized in the experiments described in this report. The first part of the chapter deals with the analytical methods for determination of the chemical amounts of lactate and glucose. Two methods for assessing the quantity of radioactivity in C^{14}-labeled substances are then described. This is followed by a section on the materials used and incubation procedures. Finally, a description of the separation of radioactive components from the medium after incubation by anion exchange chromatography is given.

I. DETERMINATION OF LACTIC ACID

Principle of the method. When lactic acid is oxidized, it forms pyruvic acid. Lactic dehydrogenase, a widely distributed enzyme, catalyzes this oxidation of lactic acid with DPN.



Pyruvic acid — Lactic acid

The above diagram was taken from White et al, 1959. The absorbance of DPNH at a wavelength of 366 mμ is measured in a Beckman DU spectrophotometer. The more DPNH that is formed, the greater is the optical density.

In turn, the amount of DPNH depends on the quantity of lactic acid present. Hence, when a sample of lactic acid solution is allowed to react with buffer, DPN, and lactic dehydrogenase, a measure of the optical density of the resulting solution (after two hours at room temperature) is a direct indication of the amount of lactic acid there was in the original solution.

Experimental procedure. The lactic dehydrogenase-DPN method used was based on the work of Cohen and Noell (1960) and Horn and Bruns (1956). The microassay method developed by Cohen and Noell was used. A stock lactate solution, 3 millimolar-zinc lactate, or 6 millimolar-L-lactate, was made up. Standards consisted of 10 and 25 µl of stock solution, made up to the volume of the largest sample. Blanks were set up just like standards, except that they contained water instead of lactate samples.

25-200 µl of the neutralized samples to be assayed were pipetted into 10 X 75 mm test tubes. The volumes in all tubes were adjusted with water to that of the largest sample. 500 µl of glycine-NaOH buffer, pH 10.5, containing semicarbazide-hydrochloride, were then added to each tube. This was followed by 100 µl of a solution of DPN.$4H_2O$/tube. Last of all, 25 µl of a solution of lactic dehydrogenase were added to each tube. The enzyme solution used was made by dilution of the concentrated solution 1:4 with 6% $NaHCO_3$.

The tubes were sealed with parafilm, shaken, (as one unit) and allowed to stand at room temperature for at least two hours. There was

no visible color development. The absorbance of the samples was read in microcuvettes at 366 mμ against the blank sample. In order to exclude CO_2, and thereby prevent pH change, it was necessary to seal the blank cuvette with parafilm. All test tubes were sealed with parafilm until the contents were read on the Beckman DU spectrophotometer.

Materials used. The following materials were used in the lactic acid method:

L(+)-Lactic Acid:[1] zinc lactate. The water of crystallization was 12.9%.

Glycine-NaOH Buffer: pH 10.5. The buffer was prepared by mixing a 6% solution of semicarbazide with 0.25 molar-glycine.

β-Diphosphopyridine-Nucleotide:[2] assay, 98%. It was corrected for $4H_2O$ per mole when prepared. Cozymase. From yeast. The solution used contained 15 mg DPN.$4H_2O$/ml.

Lactic Dehydrogenase:[3] type II. Crystalline from muscle. Substantially free of pyruvate kinase. $(NH_4)_2SO_4$ suspension. 10 mg/ml.

Accuracy of the method. In a series of 21 separate analyses the mean difference between duplicate determinations of standard

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[1]The zinc lactate was prepared by Pfanstiehl Laboratories, Inc., Waukegan, Illinois.

[2,3]The DPN and enzyme were obtained from the Sigma Chemical Company, St. Louis 18, Missouri, U.S.A.

samples of lactic acid was 0.149 μg ± 0.031 μg (S.E.) at the 5.3 μg level and 0.134 μg ± 0.022 μg (S.E.) at the 13.3 μg level.

II. GLUCOSE OXIDASE METHOD FOR DETERMINATION OF GLUCOSE

Principle of the method. According to Kingsley and Getchell (1960), Müller (1928) first demonstrated that β-glucose oxidase promoted the oxidation of glucose by molecular oxygen to gluconic acid. Then Franke and Lorenz (1937) discovered that hydrogen peroxide is simultaneously formed in this reaction. This H_2O_2 will quantitatively oxidize certain amino substituted dibenzene derivatives to form colored compounds. O-tolidine and o-dianisidine have been used by most workers and the overall reactions represented by the equations

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

$$H_2O_2 + \begin{matrix}\text{o-dianisidine} \\ \text{o-tolidine}\end{matrix} \xrightarrow{\text{peroxidase}} \text{color}$$

have formed the basis of a large number of methods, differing slightly in detail. McComb and Yushok (1958) discovered that the addition of concentrated sulfuric acid changed the color of the o-dianisidine reaction mixture from amber to a deep pink color that was stable for several hours. It is the absorption of this pink color which is read on the Beckman DU spectrophotometer. The intensity of the color is directly proportional to the quantity of H_2O_2 formed and, therefore, to the amount of glucose present in the samples.

Procedure. Glucose was determined by a glucose oxidase-3,3'-dimethoxybenzidine (o-dianisidine) method modified from that of Kingsley and Getchell (1960). Samples of 25-200 µl were pipetted into 10 X 75 mm test tubes. Duplicate standards of 5, 10, 25, and 50 µl of a solution containing glucose at a concentration of 0.4 µg/µl were also put into appropriate test tubes. The volumes in all tubes were adjusted to 200 µl with distilled water. Blank tubes contained 200 µl of water instead of glucose sample.

To each tube were added 800 µl of the buffer-reagent solution, and then 100 µl of glucose oxidase solution. All samples were thoroughly mixed, covered with parafilm, and incubated at 37°C. for 60 minutes (the time was not too critical: it could range from 45 to 75 minutes. However, the time should be the same for all tubes).

At the end of the incubation period, 400 µl of 37.4% sulfuric acid were added to each tube. The tubes were cooled, and the contents thoroughly mixed on a vortex mixer. The absorbance of each solution was read in a microcuvette against the blank sample in a Beckman DU spectrophotometer. The samples were read at a wavelength of 530 mµ.

Materials used. The following materials were used in the glucose oxidase method for determination of glucose:

Glucose Oxidase:[4] crude; contains catalase.

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[4]The glucose oxidase was obtained from the Sigma Chemical Company, St. Louis 18, Missouri, U.S.A.

Glucose:[5] dextrose. Reagent, A.C.S. Anhydrous powder.

Peroxidase:[6] horseradish Type I. There were approximately 70 (20 sec) Purpurogallin Units/mg.

O-Dianisidine:[7] 3,3'-Dimethoxybenzidine. 0.250 gm were dissolved in 25 ml of methyl alcohol.

Peroxidase Buffer Reagent: 0.8409 gm of Na_2HPO_4 and 1.1194 gm of KH_2PO_4 were dissolved in distilled water. 5.625 mg of peroxidase were added to the above solution, which was then adjusted to 500 ml. 5.625 ml of o-dianisidine solution were added to the top of the 500 ml.

Accuracy of the method. In a series of 14 separate analyses the mean difference between duplicate determinations of standard samples of glucose was 0.055 µg ± 0.011 µg (S.E.) at the 4.0 µg level and 0.167 µg ± 0.043 µg (S.E.) at the 20 µg level.

III. DETERMINATION OF THE TOTAL ACTIVITY OF C^{14}-LABELED COMPOUNDS BY OXIDATION WITH POTASSIUM PERSULFATE

Principle. The persulfate oxidation as described by Osburn and Werkman (1932) can be used for water-soluble compounds only. This

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[5,7]The glucose and o-dianisidine were obtained from Matheson, Coleman, & Bell, Division of the Matheson Company, Inc. Norwood (Cincinnati), Ohio; East Rutherford, N.J., U.S.A.

[6]The peroxidase was obtained from the Sigma Chemical Company, St. Louis 18, Missouri, U.S.A.

method has been adopted by Weinhouse (1949) and Anthony and Long (1952) for C^{14} assay. Volatile as well as nonvolatile compounds may be oxidized by this method, as long as the compounds are water-soluble (Katz, Abraham, and Baker, 1954).

This reaction can be described by the following equation

$$H_2O + K_2S_2O_8 \longrightarrow 2KHSO_4 + 1/2\ O_2$$

obtained from an article by Abraham and Hassid (1957).

Experimental procedure. The oxidation method used was based on the work of Abraham and Hassid (1957). About 500 to 600 mg of solid potassium persulfate were placed into the main compartment of the flask. Then the sample (which should yield from 10 to 80 mg of $BaCO_3$) and water were added to the main compartment to give a volume of 5 to 15 ml. After the flask had been swirled to partially dissolve the persulfate, the mixture was acidified with a few drops of dilute H_2SO_4. 1 ml of 4% silver nitrate solution was then added to the mixture. Enough CO_2-free NaOH to absorb the CO_2 formed during the oxidation was delivered into the center well.

After the flask had been closed with a rubber stopper, it was evacuated by insertion into the stopper of a 22-gage needle connected to a vacuum line. The flask was placed into a water bath at 40 to 50°C. and brought to about 70°C. in 15 to 20 minutes. It was left at 70-75°C. for approximately 30 minutes (i.e., until the mixture was clear). To ensure complete CO_2 absorption, the flask was left in the water bath for

an additional 15 to 20 minutes.

After the flask had cooled to room temperature, the vacuum was released by insertion through the rubber stopper of a hypodermic needle connected to a soda-lime tube. The flask was opened and the contents of the center well were rapidly and quantitatively transferred, by means of suction, into an Erlenmeyer flask containing 1 ml of saturated barium chloride solution.

The above procedure was used for the combustion of C^{14}-lactate and glucose-U-C^{14}.

Preparation of $BaC^{14}O_3$ precipitates. The $BaC^{14}O_3$ precipitates obtained after addition of $Na_2C^{14}O_3$ to a saturated $BaCl_2$ solution were transferred to weighed 25 mm MF® Millipore filters, type HA or RA, by means of Pasteur pipettes. These Millipore filters had been thoroughly dried in a desiccator before use, and were mounted in a Millipore filter holder[8] before application of the radioactive precipitate.

After addition of $BaC^{14}O_3$ to the Millipore filters, they were dried in a desiccator for at least 12 hours, and then reweighed. The completeness of oxidation was checked by these weights of barium carbonate, since enough carrier carbohydrate such as lactate was added to the radioactive sample before oxidation to yield 10 mg of $BaCO_3$ or 2 mg/cm^2 on the planchets used. The chemical amount of $BaCO_3$ obtained

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[8]This was obtained from the Millipore Filter Corporation, Bedford, Massachusetts, U.S.A.

from the radioactive sample was negligible. Samples of CO_2 in NaOH did not have to be oxidized and were precipitated directly as $BaCO_3$. Again, enough carrier was added to yield about 10 mg of $BaCO_3$.

Counting of $BaC^{14}O_3$ precipitates. The radioactive $BaCO_3$ samples on Millipore filters were put into stainless steel planchets and held down with brass rings especially constructed for this purpose. A thin window gas flow tube was used to count the samples. Since there was a certain amount of self-absorption of radioactivity by the samples themselves, the cpm obtained were corrected to infinite thinness. This self-absorption correction took into account the weight of $BaCO_3$. The amount of self-absorption is given by the quotient I/I_o and may be represented by the following equation taken from Aronoff (1956):

$$\frac{I}{I_o} = \frac{1 - e^{-0.29L}}{0.29L}$$

where I is the observed activity

I_o is the activity at infinite thinness

L is the weight of $BaCO_3$ per unit area (mg/cm^2).

IV. DETERMINATION OF THE ACTIVITY OF C^{14}-LABELED COMPOUNDS BY LIQUID SCINTILLATION

Liquid scintillation counting made possible the rapid determination of radioactivity in toluene-soluble compounds. A Nuclear-Chicago three channel liquid scintillation unit was used. The solvent employed was "Liquifluor" diluted 1:25, a toluene-based solvent system

containing 4 gm "PPO" [2,5-Diphenyloxazole] and 50 mg "POPOP" [1,4-bis-2-(5-phenyloxazolyl)-benzene] per litre. The channels ratio counting technique was used to determine the actual efficiency in homogeneous counting systems of toluene or toluene-methanol and enable absolute activity to be calculated.

By definition, liquid scintillation counting is a method of detecting radioactivity by means of a solution of fluors and a photomultiplier tube. The energy of the primary particle emitted by the radioactive sample is converted to light by the scintillation solution and the phototube responds to this light energy by producing a charge pulse which can be amplified and counted by a scaling circuit.

The channels ratio counting technique is based on the fact that when there is quenching, the height of the pulses produced by beta particles is, on the average decreased. As a result, the spectrum shifts towards a lower energy (Figure 1). Hence, when the pulse height spectrum of an isotope is properly divided into two counting channels, the ratio of the count rates in the two channels will change in a unique and directly related manner with the amount of quenching and, therefore, with the counting efficiency for the isotope. Because of this correlation between the count rate ratio and counting efficiency it is possible to construct a quench correction curve such as that in Figure 2, by counting a series of standard samples of known activity and varying degrees of quench. Once the quench correction curve is plotted, efficiencies for samples of unknown quench can be read directly from the curve as soon as the channels ratio for each is calculated. Normally a quench correction

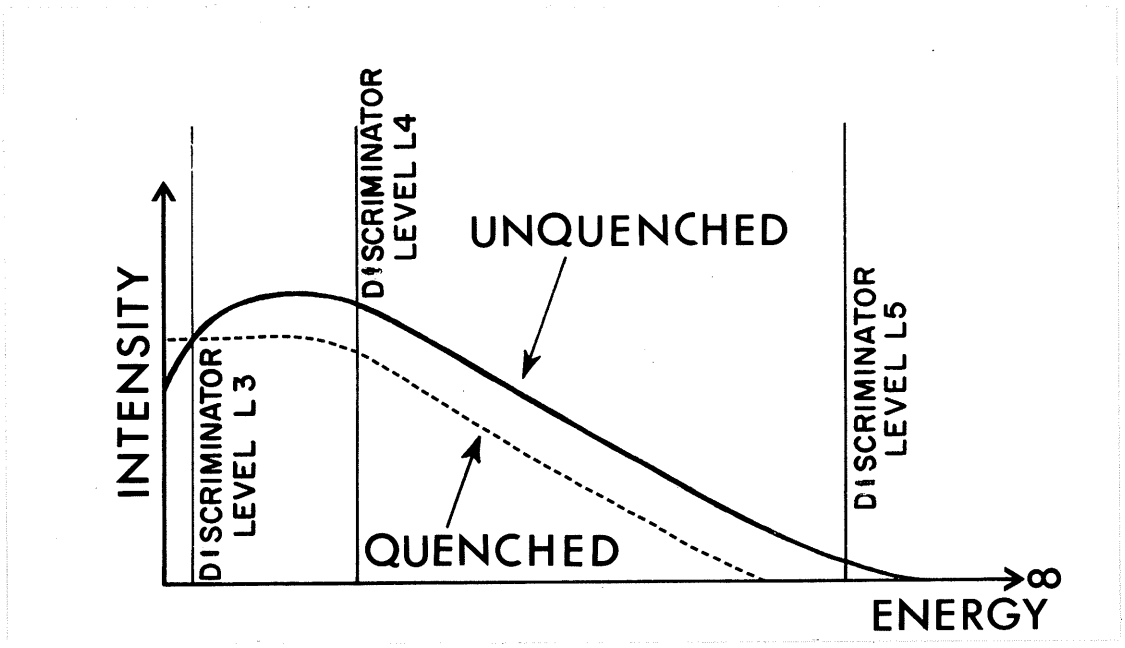

Figure 1. Effect of quenching on the energy spectrum of an isotope.

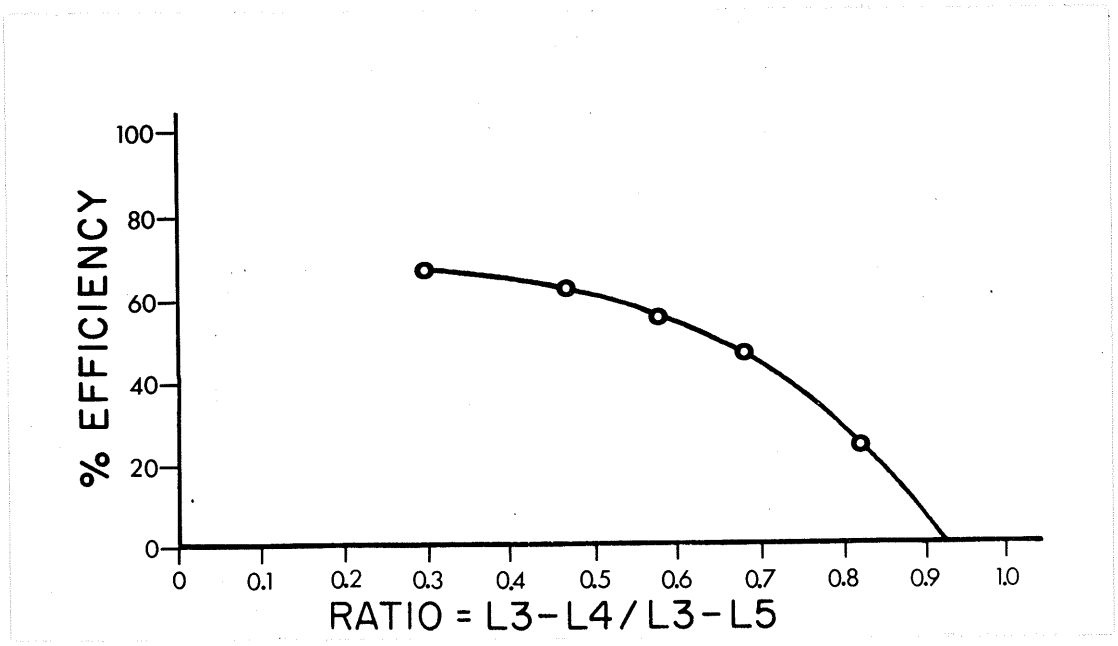

Figure 2. Quench correction curve. Relationship between efficiency and ratio of counting in two channels.

curve plotted for one type of sample with a certain quenching agent can be used for many types of samples of the same isotope with different quenching agents.

Channels ratio counting of quenched C^{14} samples only. In this procedure the channels ratio technique is used to count quenched C^{14} samples. The quench correction ratio, counting windows, and channels used were these:

$$\text{RATIO 1} = \frac{\text{L3-L4}}{\text{L3-L5}} = \frac{\text{CHANNEL 3}}{\text{CHANNEL 2}}$$

Total Counting Window = L3-L5.

The "Calibration Procedure for Counting Samples of One Isotope Only" (Nuclear-Chicago Liquid Scintillation Manual for Liquid Scintillation Systems 724 and 725) was used to set the data and gate operating voltages at the balance point, or optimum value, where maximum count rate was obtained in a total counting window defined by a lower level discriminator (L3) and an upper level discriminator (L5). The voltages at the three discriminator levels used were 0.5, 2.3, and 7.9 respectively.

The total counting window was split into two overlapping windows on two separated channels for quench correction using the channels ratio technique as follows. The Channel 3 selector was set at L3-L4; the Channel 2 selector was set at L3-L5. The Level 4 discriminator control was set so as to obtain a value as close as possible to 0.3 for the R1 ratio, since the ratio of 0.3 for an unquenched C^{14} sample has been found to produce the best possible efficiency versus ratio

curve for the majority of C^{14} counting (Nuclear-Chicago Liquid Scintillation Manual).

The graph in Figure 2, page 23, of efficiency versus the L3-L4/L3-L5 ratio for quenched standards was plotted by determining the efficiency of counting for the toluene-C^{14} standard obtained from Nuclear-Chicago. When the value for Rl was adjusted to 0.3, the efficiency obtained for the standard was 67%. Efficiency was calculated by dividing the recorded counts/minute for the standard by the disintegrations/minute for the standard, or:

$$\text{EFFICIENCY} = \frac{\text{CPM}}{\text{SAMPLE DPM}} .$$

Hence, the values obtained above (Rl=0.3: efficiency=67%) were used to plot the highest point on a quench correction curve. The rest of the curve was obtained from the one in the Nuclear-Chicago Liquid Scintillation Manual. Every point on this newly-constructed curve was 11 units below the corresponding point on the curve in the Manual, since the efficiency of counting of the C^{14} standard with an Rl value of 0.3 on the latter curve was 78% as opposed to 67% on the former curve. The efficiency versus ratio curve was used to determine the counting efficiency for unknown quenched C^{14} samples once the L3-L4/L3-L5 ratio of these samples was determined.

V. MATERIALS USED AND INCUBATION PROCEDURES

Experiments were done on the calvaria of one- to two-day old Long-Evans rats. Previous work had been done on calvaria: they were used to show histological effects of parathyroid hormone (Gaillard, 1959),

resorption in tissue culture (Goldhaber, 1960), the cellular role in maintaining calcium levels above those due to passive solubility only (Raisz, Au, and Tepperman, 1961), and the metabolic effects in bone resulting from purified parathyroid hormone added in vitro (Dowse et al, 1963).

Most of the biochemical studies of metabolism in bone have been carried out with sliced or crushed metaphyses, as discussed in some detail in Chapter II, although these are contaminated with marrow cells. In the calvarium there is little contamination with marrow cells, and the cell population is high for bone. The following five general cell types predominate morphologically: osteoblasts, osteocytes, osteoclasts, cartilage cells, and preosseous mesenchymal cells (Dowse et al, 1963). This tissue can be obtained readily with a minimum of cut or damaged cells. According to Gaillard, 1959, the main advantages of the calvarium are convenience, reproducibility, and responsiveness to parathyroid hormone in vitro. Since future experiments on the effect of parathyroid hormone on glucose metabolism by calvaria are planned, it is wise to work with a tissue responsive to parathyroid hormone. The results in this report are based on glucose-U-C^{14} metabolism by calvaria, and may, or may not, apply to bone cells in general.

Method of preparation. One- to two-day old Long-Evans rats of either sex from a colony maintained in the Faculty of Dentistry were used.

The rats were decapitated and the head grasped firmly by inserting

one limb of a pair of forceps through the occipital cavity. The skin was peeled back to reveal the skull. The thin layer of loose connective tissue which covers the bone was removed and a piece of the skull comprising the major fraction of the parietal bones and that portion of the frontal bone superior to the eye sockets was cut out with a scalpel.

The calvaria thus removed were inverted and any connective tissue adherent to the inner surface removed. They were then divided down the midline and the sagittal suture region cut away. This latter section of the parietal bones and the occipital bone were excluded from the tissue to be incubated as histological study showed that a variable amount of cartilage was present in these regions of the skull.

After removal from the rats, the calvarial preparations were placed in weighed beakers containing ice-cold incubation medium. The tissue was randomized among flasks. Wet weight was used as the basis of reference. According to Dowse et al, 1963, this was found to be at least as reliable as dry weight, noncollagen nitrogen, or deoxyribonucleic acid.

Incubation procedures. The bone tissue was incubated in air in calcium-free phosphate or THAM-buffered medium (pH 7.4) in a water bath at 37°C. The ionic composition of the medium is shown in Table I. Varying concentrations of glucose-U-C^{14} were used in the medium of different experiments. Furthermore, the specific activity of the radioactive glucose in the medium before incubation varied in different experiments due to varying dilutions of the labeled glucose with carrier glucose.

TABLE I

IONIC COMPOSITION OF PHOSPHATE- AND THAM-BUFFERED MEDIA IN μM/ML

	Na^+	K^+	Mg^{++}	Cl^-	$SO_4^=$	P
Phosphate-Buffered Medium	146.8	5.70	1.425	152.6	1.425	15.60
THAM-Buffered Medium	146.8	5.70	1.425	152.6	1.425	1.29

The carbon dioxide given off during the experiment was collected in carbon dioxide-free NaOH in the center well of the flask. During the incubation, the flask was continuously shaken in a water bath.

The period of incubation was generally two hours: any variation is indicated in the description of the individual experiment. Two methods were used to stop the incubations; either the flask was placed on ice and the calvaria removed from the medium immediately, or acid was tipped from a side-arm into the main compartment of the flask. The skulls were rinsed with ice-cold 0.154 M NaCl, and then frozen. The $Na_2C^{14}O_3$ from the center well was removed by suction and stored in the cold until it was subsequently assayed as $BaC^{14}O_3$ in a thin-window gas flow counter or directly, after absorption on paper, in a liquid scintillation system. Whatever was left in the main compartment after removal of the calvaria was removed and frozen until it could be analyzed further. Part of this medium obtained after incubation was subjected to anion exchange chromatography, part was analyzed chemically for glucose and lactate, and part was assessed for radioactivity. Aliquots were also used for the preparation of radioautograms. Details of the analytical procedures used are given subsequently.

VI. SEPARATION OF RADIOACTIVE COMPONENTS FROM THE MEDIUM AFTER INCUBATION BY ANION EXCHANGE CHROMATOGRAPHY

Samples of medium following incubation were pipetted onto Dowex-1-Cl resin columns, 1 X 7 cm in size. The anion exchange resin, 1-X4, 200-400 mesh, was prepared for use by being washed alternately

with 2 N NaOH and 2 N NaCl. This recycling was carried out twice with each solution. Between additions of the OH^- and Cl^- ions, the resin was thoroughly rinsed with distilled water.

A linear HCl gradient, from 0-0.02 N over 400 ml, after the manner of Kinoshita, Masurat and Helfant (1955), was used to elute the radioactive components of the medium. A micropump from Buchler Instruments was used to pump the liquid through the column. The contents of the first six tubes were collected by elution with water only: the labeled glucose was present in the first few fractions. Each tube contained approximately 4-ml fractions. C^{14}-lactic acid was usually found in approximately tubes 25-30. The contents of tubes containing radioactive glucose or lactate were lyophilized, and then made up to a relatively small volume, so that aliquots could be assessed for chemical quantity and radioactivity. Paper chromatography and radioautography of these aliquots was also carried out.

CHAPTER IV

PAPER CHROMATOGRAPHY

Paper chromatography, a technique for effecting the separation of closely related substances, was used to separate C^{14}-labeled products of metabolism found in the media after incubation, and various labeled compounds present in the alcohol extracts of calvaria after incubation. For convenience, the position of a substance on a chromatogram is specified by its "R_f", which is defined as the distance the material has moved from the original point of application divided by the distance the solvent front has travelled from that point (Smith, 1960).

Preparation of the papers. Whatman #4 paper was generally used, but on occasion Whatman #1 paper was used as well. Prior to use, the paper was washed for 2 hours in a 1% oxalic acid solution, to enable it to bind heavy metals and thus prevent phosphate esters from sticking on the origin (Moses, 1960). It was then rinsed 10X with distilled water over a period of two days. This was done in a porcelain tray, with a false perforated bottom on which to place the paper. The sheets were then thoroughly dried at room temperature.

For two-dimensional chromatography, the papers were cut 10 in. square to fit into a stainless steel frame especially constructed to hold paper chromatograms. Holes were punched into the paper at each corner, so that the chromatograms would fit onto the steel rods in each frame.

Description of the solvent systems used. Ascending paper chromatography was used in all cases. The chromatograms were put into glass tanks closed by heavy glass plates. In two-dimensional chromatography, the papers were first allowed to run in a phenol-water solvent, prepared by mixing 100 g of phenol with 39 ml of distilled water. After the sheets had been thoroughly dried overnight in a fume hood (an air fan at room temperature was used to hasten the initial phases of the drying process), they were developed in a second solvent system, n-butanol-propionic acid-water, in a direction at right angles to the first run. This solvent was used second because it is impossible to remove all traces of propionic acid by drying the papers at room temperature. Two solutions, A and B, were required to make up this second solvent. Solution A contained 919 ml of n-butanol and 81 ml of distilled water, whereas solution B contained 469 ml of propionic acid and 531 ml of distilled water. Immediately before use, equal volumes of solutions A and B were mixed (Moses, 1960). Having been developed in the second solvent, the paper chromatograms were removed from the tank and thoroughly dried in a current of air at room temperature. They were now ready to be treated with staining reagents.

Standards used. Standard solutions of amino and organic acids, and of glucose were prepared. The concentration of the amino acid solutions was 50 mg/10 ml, and that of the organic acid solutions was 200 mg/10 ml. The latter two solutions were made up in 10% isopropanol. The glucose solution was prepared at a concentration of 1 g/100 ml of a 0.5% solution of isopropanol.

Location reagents. A 0.2% solution of ninhydrin in acetone (w/v) was used to spray paper chromatograms containing amino acids. The solution was either obtained commercially or prepared in the laboratory. Having been sprayed, the papers were hung up at room temperature, or, after the acetone had evaporated, they were heated for 2-3 minutes in an oven at 105°C. Most of the amino acids yielded purple colors with ninhydrin.

A solution of acridine containing 0.1 g in 100 ml of 99.5% ethanol was prepared (Nordmann and Nordmann, 1960). Chromatograms containing organic acids were rapidly drawn through the reagent and then laid flat on a sheet of filter paper. Pale yellow spots on a white background appeared for acidic compounds. Under ultra-violet light lactic acid fluoresced dark blue. Most of the other organic acids exhibited a green-yellowish fluorescence.

Aniline-hydrogenphthalate reagent was prepared as follows:

0.9 ml of aniline and

1.66 g of phthalic acid were dissolved in

100.0 ml of acetone.

The papers were dipped through the reagent (Kawerau, 1960), and then heated for 15 minutes at 100°C. Glucose reacted to form a brown spot on the chromatogram. The color was stable for many months.

Identification of standard amino acids. 10 µl of a mixture of nine standard amino acids were applied at the origin of a paper chromatogram in two 5-µl quantities. The paper was thoroughly dried between

addition of the 5-µl aliquots. 5.556 µg of each amino acid were applied to the chromatogram. Having been thoroughly dried in air, the sheet was subjected to two-dimensional chromatography as outlined earlier in this chapter. It was then sprayed with ninhydrin solution and allowed to dry at room temperature. Most of the amino acids chromatographed turned a purple color, but tryptophan, proline, and hydroxy-proline did not. Tryptophan was grayish in color, proline was yellowish-gray, and hydroxy-proline was pale yellow. The nine amino acids separated are shown in Figure 3. The corresponding identification key for these amino acids is given in Figure 4, page 36. Since hydroxy-proline was pale yellow, it did not show up in the photograph in Figure 3.

Exposure of paper chromatograms to X-ray film. Paper chromatograms containing radioactive substances were exposed to X-ray film in the dark. The type of film used was Ilford Ltd. X-ray film, with an Ilfex safety base. The papers were allowed to remain in contact with the film for varying periods of time, sometimes up to several months. Thereafter the X-ray film was developed to show up darkened areas on the film. These areas of blackening corresponded with active areas on the paper.

The radioautograms obtained were very useful. From the "R_f" values determinable from the X-ray film it was possible to identify labeled substances. These could be compared to stained amino acids or organic acids on the corresponding paper chromatogram. From the intensity of the darkened area on a film, the quantity of label in a particular compound could be estimated.

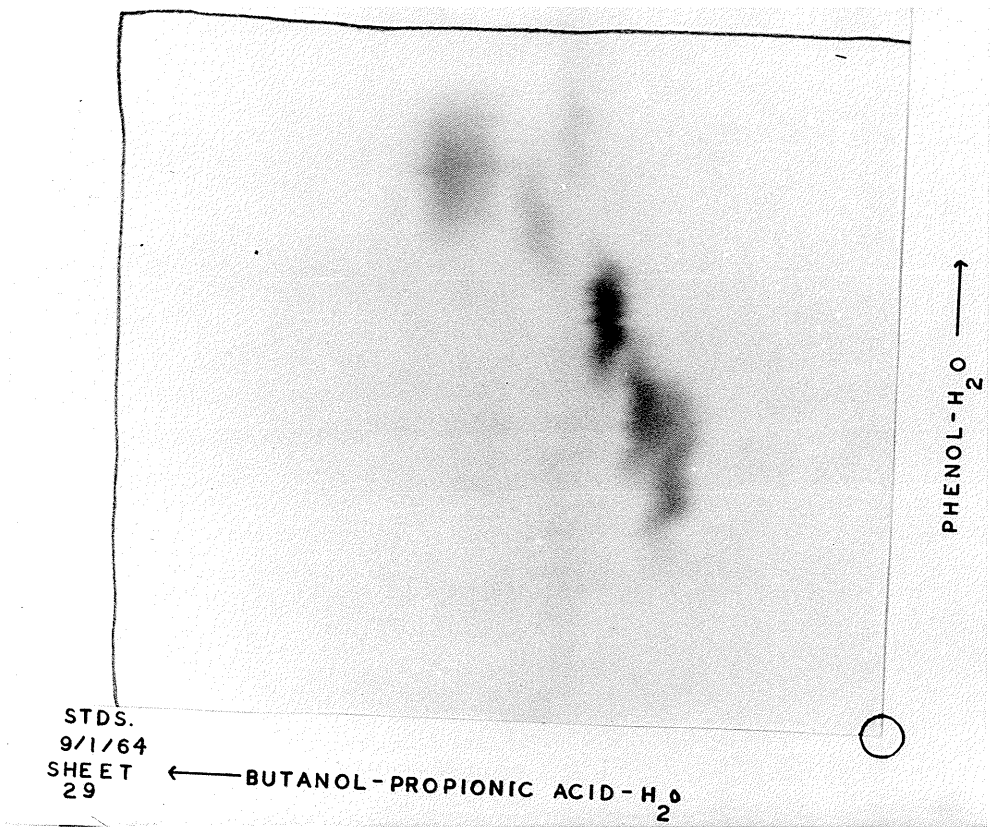

Figure 3. Two-dimensional paper chromatogram of standard amino acids. Sprayed with ninhydrin. 5.5 μg of each amino acid chromatographed. For identification of spots see Figure 4.

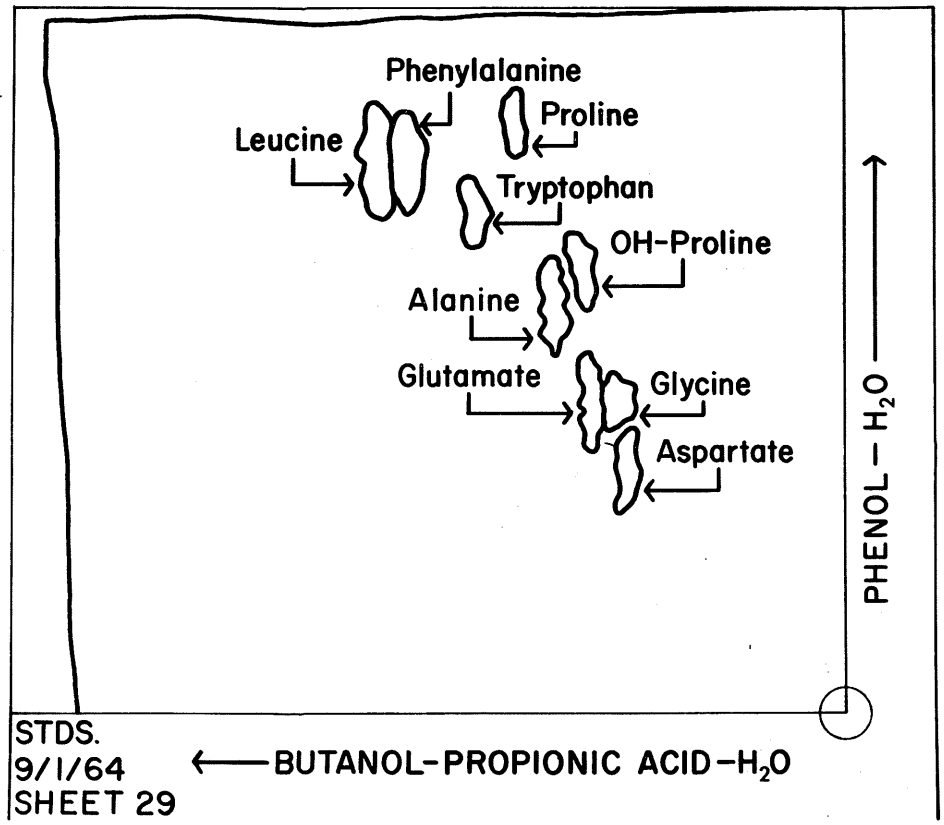

Figure 4. Identification key for Figure 3. Since OH-proline was pale yellow, it did not show up on the photograph in Figure 3.

Results of radioautography in experiment 3. After incubating for two hours in a phosphate-buffered solution containing glucose-U-C^{14}, skulls in experiment 3 were removed and extracted with ethanol (Chapter V, page 47). Aliquots of the alcohol extract were chromatographed, and the resultant chromatograms were exposed to X-ray films as described in the previous section.

A typical radioautogram is shown in Figure 5, page 39. Glucose and lactate were heavily labeled, and there was a fair amount of label in the amino acids, aspartate, glutamate, and alanine. Serine was not quite as heavily labeled as the amino acids already mentioned. Glucose-6-phosphate was more heavily labeled than fructose-6-phosphate or 3-phosphoglyceric acid. This indicated that the total amount of radioactivity in the glucose-6-phosphate pool was greater than that in the fructose-6-phosphate or 3-phosphoglyceric acid pools. A trace of activity was detected in citric and malic acids, intermediates of the TCA cycle.

Areas containing radioactive compounds were cut out of duplicate chromatograms, one of which is that in Figure 5, page 39. They were then put into liquid scintillation vials and counted. From Table II, it can be seen that results were reproducible for a certain substance from one chromatogram to another, except for lactic acid. The activity found in this substance on one sheet was approximately half that found on the other sheet. The second solvent, n-butanol-propionic acid-water, was an acidic one. The most likely explanation for the poor agreement between the lactic acid figures was unequal loss of this compound, which is volatile in the undissociated form, during drying of the chromatograms.

TABLE II

DISTRIBUTION OF ACTIVITY IN METABOLIC INTERMEDIATES PRESENT IN AQUEOUS ETHANOL EXTRACTS OF CALVARIA IN EXPERIMENT 3

Spot Number	Identity[a]	cpm Sheet #4	cpm Sheet #6[b]
0	Origin	112	118
2	Glucose-6-phosphate	189	183
3	3-Phosphoglyceric	146	139
4	Fructose-6-phosphate	117	120
5	Citric	27	28
6	Malic	38	29
7	Aspartic	133	139
8	Serine	129	124
9	Glutamic	781	701
10	Glucose	2238	2009
11	Lactic	455	968
12	Alanine	141	137

For experimental details see Table IV, page 49.
Chromatographed on Whatman #4 in phenol-H_2O:n-butanol-propionic acid-H_2O.

[a]See Figure 5.

[b]Sheet #6 and Sheet #4 duplicate chromatograms.

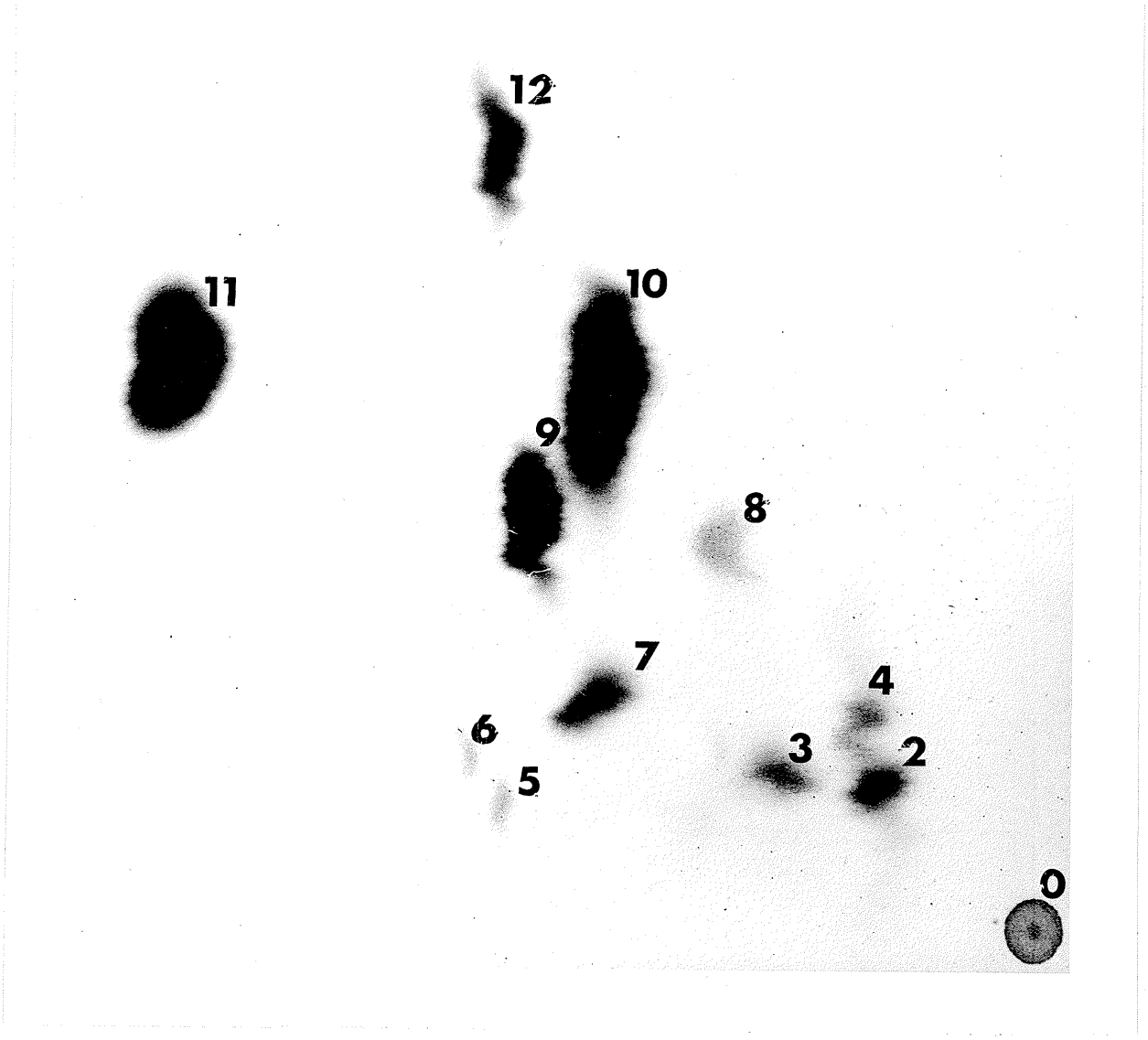

Figure 5. Reproduction of radioautogram of 2-dimensional chromatogram of alcohol extract of calvaria incubated for 2 hours with glucose-U-C^{14}. Experiment 3. For key to numbers see Table II.

The relatively large amount of label in lactic acid supported the concept of an active aerobic glycolysis in calvaria (Dowse et al, 1963). Since citrate and malate were also labeled, this indicated the existence of the tricarboxylic acid cycle in bone. Further experiments were done to elucidate the incorporation of C^{14} into the above intermediates with time. In experiment 5, radioautograms were prepared from alcohol extracts of skulls removed from incubation media at varying times.

Preparation of radioautograms in experiment 5. The purpose of experiment 5 was to follow the incorporation of C^{14} into skulls with time. A total of twenty-one calvaria in one flask were allowed to metabolize in phosphate-buffered medium (pH 7.4) containing glucose-U-C^{14}. The amount of tissue removed after varying periods of incubation is indicated in Table III.

Immediately after withdrawal, the tissue was rinsed with cold 0.154 molar saline, blotted dry, put onto a filtering funnel, and extracted with ethanol. 2 ml of boiling 80% ethanol, boiling 20% ethanol, and boiling water respectively were used to extract the tissue for successive 30-minute periods. After each extraction, the skulls and extracting solvent were centrifuged, and the supernatants saved. The rinse water was pooled with the previous alcohol extracts to yield one alcohol extract. The above procedure was carried out for all five groups of tissue shown in Table III.

The pooled alcohol extracts were frozen, and subsequently lyophilized. Aliquots were subjected to two-dimensional paper chromatography. The chromatograms were exposed to X-ray film for 70 days.

TABLE III

QUANTITY AND TIME OF INCUBATION OF TISSUE FOR ALCOHOL EXTRACTION, EXPERIMENT 5

Extraction Number	Time of Incubation	Number of Calvaria Removed from the Flask
I.	15 minutes	9 halves
II.	30 minutes	10 halves
III.	1 hour	8 halves
IV.	2 hours	7 halves
V.	3 hours	8 halves

2 ml phosphate-buffered medium. Incubation in air.
Initial glucose concentration 1.105 μM/ml.
Initial glucose specific activity 1.58×10^6 cpm/μM.

Results of radioautography in experiment 5. There was a steady progression of label into metabolic intermediates found in the alcohol extract of skulls incubated for varying periods of time with glucose-U-C^{14}. Radioautograms were prepared from alcohol extracts of skulls incubated for 15 minutes, 30 minutes, 1 hour, 2 hours, and 3 hours respectively.

After 15 minutes of incubation, a fair amount of label had been incorporated into lactic acid. However, glucose was by far the most heavily labeled substance in the alcohol extract. The amino acids glutamic and aspartic, closely linked to the TCA cycle, were distinctly labeled. The spot for alanine was barely perceptible. A small amount of activity had been incorporated into citric and malic acids: this demonstrated the existence of the TCA cycle in rat calvaria. There was more activity in the phosphorylated intermediates near the origin than in the amino acids; i.e., the spots for glucose-6-phosphate and 3-phosphoglyceric acid were darker than the spots corresponding to aspartic and glutamic acids.

Fructose-6-phosphate yielded a much lighter spot than glucose-6-phosphate. This could be due to unequal pool sizes of these two substances. Kahana et al (1960) measured the equilibrium concentrations of glucose-6-phosphate and fructose-6-phosphate. Equilibrium mixtures of the two phosphates were obtained by using rabbit muscle isomerase. They found that at 38°, 30°, and 20° ratios of glucose-6-phosphate to fructose-6-phosphate were 3.06, 3.36, and 3.85 respectively. In a later paper, Lowry et al (1964) describe substrate levels in mouse

brain before and after periods of ischemia. Again, the ratio of glucose-6-phosphate to fructose-6-phosphate was >1. In fact, it exceeded the expected value of 3:1 obtained by Kahana et al (1960). They suggest that this might indicate that equilibrium is not quite maintained, although there is a possibility that the true fructose-6-phosphate values were somewhat higher than the observed levels. Hess (1963) studied the steady-state concentrations of glycolytic intermediates formed by Ehrlich ascites tumor cells. Under endogenous conditions the ratio of glucose-6-phosphate to fructose-6-phosphate was approximately 2, and under conditions of glucose saturation, 4.38. Both brain and Ehrlich ascites tumor cells exhibit an active aerobic glycolysis. The above reports show that glucose-6-phosphate and fructose-6-phosphate, intermediates of glycolysis, are present in a ratio of approximately 3:1 in these tissues. Since calvaria have been shown to exhibit an active aerobic glycolysis (Dowse et al, 1963), it is reasonable to assume that the glucose-6-phosphate pool in this tissue would be larger than the fructose-6-phosphate pool.

The radioautogram prepared from the alcohol extract of skulls incubated for 3 hours was very different from the one described above. Lactic as well as glutamic acid were approximately as heavily labeled as glucose. This indicated active participation of the glycolytic and tricarboxylic acid cycles in the metabolism of glucose-U-C^{14} by calvaria. Aspartic acid was more heavily labeled than in the earlier radioautogram: since it is reversibly linked to fumaric and oxalacetic acids in the TCA cycle, this indicated that more activity was being incorporated into TCA

cycle intermediates with time. The spot for alanine had also become darker. Since this amino acid undergoes a reversible reaction with pyruvic acid, it appeared that a greater percentage of the original activity present in the glucose appeared in the compounds of the glycolytic cycle with time. Citric and malic acids also yielded darker spots. Therefore, a greater quantity of radioactivity had been incorporated into intermediates of the TCA cycle after 3 hours than after 15 minutes. After 3 hours, there was also a much greater quantity of activity in glutamic acid than in the phosphorylated intermediates. This suggested, that, with time, much of the activity from the glucose had moved on through the glycolytic and tricarboxylic acid cycles to form labeled glutamic acid, which is used in protein synthesis. It appeared that at the glucose level used (1.105 μmoles/ml), rat calvaria were synthesizing glutamic acid, and, therefore, protein. This concept was further supported by the fact that proline, formed from glutamic acid, had also become labeled after a 3-hour incubation period.

After 3 hours, some activity had also been incorporated into the amino acid, serine. No spot for serine was detectable after the 15-minute incubation. Since serine is formed by a three-step process from 3-phosphoglyceric acid, which is largely converted to 2-phosphoglyceric acid, it is to be expected that a relatively small fraction of the total activity would be incorporated into serine. Since serine is an intermediate in the formation of the nucleotide inosine-monophosphate, important in the synthesis of the nucleic acids RNA and DNA, it is conceivable that, in this system, a small amount of radioactivity could be found in RNA and DNA.

CHAPTER V

PRELIMINARY EXPERIMENTS

Two preliminary experiments were performed in order to yield general information concerning glucose-U-C^{14} metabolism by calvarial preparations. This chapter gives an account of the procedures carried out in experiments 1 and 3, the results obtained, and the conclusions drawn from these results.

I. MATERIAL AND INCUBATION PROCEDURE

In experiment 1, approximately 100 mg of bone were placed in each of six Warburg flasks. Two of the flasks were controls and contained 2 ml of substrate-free phosphate medium (pH 7.4) in the main compartment and 0.5 ml of 17.5% trichloroacetic acid in the side-arm of each. The four experimental flasks contained 1.5 ml of medium (pH 7.4) and 0.3 ml of 17.5% TCA each. Glucose-U-C^{14} (1.96×10^4 cpm/μmole) was present in the medium of the four experimental flasks. In the center well of each of the six flasks were 250 μl of N NaOH.

Forty-two rats were randomized, and divided into three groups of fourteen each. Flasks 1 and 2 contained seven skulls or fourteen halves each: alternating halves, i.e., right or left, were put into flasks 1 and 2 respectively. Similarly, flasks 3 and 4, and 5 and 6 contained fourteen skulls per pair.

Incubation was carried out in a Warburg apparatus at 37°C. It was stopped at time zero in the control flasks and after 2 hours in the

experimental flasks by the addition of trichloroacetic acid from the side-arm. CO_2 evolved was absorbed in the alkali in the center well. Recording of the O_2 uptake during incubation made it possible to determine the Q_{O_2}.

After the incubation, the contents of the center well in each flask were transferred by suction to a 5-ml volumetric flask. The volume was adjusted to 5 ml, and later suitable aliquots were removed for determination of the CO_2 content on a Warburg apparatus. From the difference in CO_2 content of samples from experimental and control flasks, it was possible to calculate the metabolic CO_2 production. Aliquots were also removed for activity determinations.

The TCA supernatants obtained after incubation were frozen, as were the skulls.

The second preliminary experiment, experiment 3, was carried out for two main reasons. Information was to be obtained on the presence of label in intermediates identified by paper chromatographic techniques. Also, it was to be determined in a preliminary way if the specific activity of the metabolic CO_2 could be assessed more accurately by collecting and assaying the CO_2 produced during the incubation only rather than the total after addition of acid.

In experiment 3, 270 mg of tissue were used. To accommodate this quantity of tissue, a larger flask was required than in experiment 1. Hence, a 50-ml Erlenmeyer flask with a fused-in center well was used. 4.0 ml of phosphate medium (pH 7.4) of the usual ionic composition were put into the main compartment of the flask: glucose-U-C^{14} was present

at a specific activity of 1.30×10^6 cpm/μmole. The center well of the flask contained 0.4 ml of 2 N NaOH. The incubation was carried out in a shaking water bath at 37°C.

After two hours, the flask was removed from the water bath, and the contents of the center well were transferred to a 5-ml volumetric flask as in experiment 1. As before, aliquots were used to determine chemical quantity and radioactivity of CO_2. Since no acid was added to the incubation flask, the figure for the amount of CO_2 liberated was obtained from CO_2 evolved from tissue and medium during the two-hour incubation period only.

The contents of the main compartment of the flask and the tissue were then rapidly transferred to a funnel. The liquid was removed by suction. A few ml of water were used to wash the tissue, which was then extracted for 1 hour periods successively with 5 ml of boiling 80% ethanol, 2 ml of boiling 20% ethanol, and 2 ml of boiling water. After each extraction, the skulls and extracting solvent were centrifuged. In each case, the supernatants were saved and pooled with previous ones to form one alcohol extract. After lyophilization of this alcohol extract, small aliquots were subjected to two-dimensional paper chromatography (Chapter IV, page 32).

After extraction as above, the tissue remaining was decalcified by dialysis against ethylenediaminetetraacetic acid (EDTA). It was then washed by dialysis against water. The material remaining was dried on a planchet, weighed, and counted.

As in experiment 1, the medium obtained after incubation was

frozen. Lactic acid determinations were subsequently carried out on this medium and on the neutralized protein-free trichloroacetic acid extract from experiment 1. In order to obtain the specific activity of the lactate in the media of both experiments 1 and 3, it was necessary to separate the lactate from the other radioactive components of the medium. This was done by putting a sample of medium onto a Dowex-1-Cl anion exchange column, and eluting the sample by using a linear HCl gradient as described elsewhere (Chapter III, page 30). Some of the lactic acid obtained in this way was oxidized, and some was analyzed chemically. In experiment 1, 5 mg of carrier lactic acid were added to each sample of radioactive medium before it was put onto the resin column. No carrier was added to the medium in experiment 3.

II. EXPERIMENTAL RESULTS AND DISCUSSION

The weight of tissue, volume of medium, and initial quantity of glucose per flask in experiments 1 and 3 are given in Table IV. Various parameters for lactic acid, whether determined experimentally or calculated, are also included in this table.

Lactic acid production and specific activity. Although the production of lactic acid per gram wet weight of tissue was quite variable, an inverse correlation between this and the specific activity of the lactic acid was present so that the fraction of the total original activity which was present in the lactic acid formed was much more constant.

TABLE IV

QUANTITY AND SPECIFIC ACTIVITY OF LACTIC ACID PRODUCED FROM GLUCOSE-U-C^{14} DURING INCUBATION OF CALVARIAL PREPARATIONS

	Experiment 1				Experiment 3
Flask Number	1	4	9	7	1
Wet Weight of Tissue (mg)	107	109	105	100	270
Volume of Medium (ml)	1.5	1.5	1.5	1.5	3.75
Initial Glucose (μM)	1.67	1.67	1.67	1.67	4.16
S.A. Glucose (cpm/μM)	1.96×10^4	1.96×10^4	1.96×10^4	1.96×10^4	1.30×10^6
Total Counts Original (cpm)	3.34×10^4	3.34×10^4	3.34×10^4	3.34×10^4	5.41×10^6
Lactic Acid Produced (μM)	1.42	0.95	1.17	1.09	2.30
Production Rate (μM/gm/2 hours)	13.2	8.7	11.1	10.8	8.5
S.A. Lactate (cpm/μM)	1.61×10^3	3.44×10^3	2.13×10^3	2.85×10^3	2.25×10^5
Total Counts in Lactate (cpm)	2.28×10^3	3.26×10^3	2.49×10^3	3.08×10^3	5.18×10^5
% of Total Counts Original	6.8	9.8	7.5	9.2	9.6

Phosphate-buffered medium. Initial glucose concentration 1.11 μM/ml. Incubation time 2 hours in air.

The ratio of the specific activities (in cpm/μatom of carbon) of glucose and lactic acid ranged from 3:1 to 6:1. This indicated a dilution of the glucose to lactic acid pathway by an endogenous substrate. It has been shown previously that glycogen present in the tissue can produce considerable quantities of lactic acid under conditions where no external substrate is added (Dowse et al, 1963). At the level of glucose used in these experiments (1.11 μmoles/ml), there could be varying degrees of dilution by endogenous substrate. This it is thought is probably glycogen.

Metabolic CO_2 production. Two possible methods of determining metabolic CO_2 production were considered. It was decided that a differential manometric approach would be unsatisfactory because of the labile nature of the large quantity of CO_2 demonstrated by Borle et al (1960a) in metaphyseal bone preparations. That the CO_2 in calvaria is labile is clear also from the large release of CO_2 found in experiment 3 during incubation (Table VI, page 53). The method chosen, therefore, was one in which the difference between the total CO_2 content of tissue and medium in experimental and control flasks was determined.

Since the values for metabolic CO_2 production were obtained from figures differing by only about 10%, there was an inherent error in this method which limited the reliability of the estimation of this parameter in experiment 1. From Table V it can be seen that the average value for metabolic CO_2 production in three flasks in experiment 1 was 16.73 μmoles/gram wet weight/2 hours. When this is compared to the average Q_{O_2} determined of 14.34 μmoles/gram wet weight/hour, an R. Q. of 0.58 is obtained.

TABLE V

OXYGEN UPTAKE, CARBON DIOXIDE PRODUCTION, AND RESPIRATORY QUOTIENT OF CALVARIAL PREPARATIONS IN EXPERIMENT 1

Flask Number	O_2 Uptake (μM/gm/hour)	Total CO_2 Found After 2-Hour Incubation (μg/mg)	(μM/gm)	Metabolic CO_2 Production (μM/gm/2 hours)	Respiratory Quotient
1	13.52	10.49[a]	238.4		
4	12.71	8.45	192.0	19.3	
9	16.69	8.11	184.3	11.6	0.58[c]
7	14.44	8.45	192.0	19.3	
Control		7.53	172.7[b]		
Control		7.66			

About 100 mg tissue incubated in 1.5 ml phosphate-buffered medium. Incubation time 2 hours in air. Initial glucose concentration 1.11 μM/ml.

[a]Sample contaminated with atmospheric CO_2. Not used in estimation of metabolic CO_2.

[b]Mean value. Acid added to controls at zero time.

[c]Mean CO_2 production/hour divided by mean O_2 uptake/hour.

Since the R. Q. obtained has a rather low value, further study would be necessary to assess the reliability of this method for determining metabolic CO_2 production. The problem was not pursued further in the present work.

Metabolic CO_2 specific activity. The specific activity of the metabolic CO_2 in experiment 1 (Table VI) ranged from 13.8-27.5% of that of the glucose (to calculate these percentages, the specific activities of both CO_2 and glucose in terms of cpm/μatom of carbon were used). This indicated that a large part of the CO_2 was produced elsewhere than in the hexose monophosphate shunt. It also indicated that most of the metabolic CO_2 arose from sources other than the glucose added. As mentioned earlier in this chapter (page 50), it is likely that at the glucose level used in this experiment (1.11 μmoles/ml) there are varying degrees of dilution by endogenous substrate, which is probably glycogen.

The specific activity of the metabolic CO_2 in experiment 1 (Table VI) varied from 39.1-126.8% of that of the lactic acid (as above, these percentages were calculated by using specific activities expressed as cpm/μatom of carbon). From these results it appeared that the system was probably not at equilibrium after a 2-hour incubation period. It is likely that after a longer period of incubation the specific activity of the CO_2 would be very close to that of the lactic acid, or even exceed it, as it did in one instance above. In the case where the specific activity of the CO_2 was greater than that of the lactate, it is obvious

TABLE VI

ACTIVITY INCORPORATED INTO CARBON DIOXIDE DURING INCUBATION OF CALVARIAL PREPARATIONS

Flask Number	Experiment 1				Experiment 3
	1	4	9	7	1
Total CO_2 Found (μM)	25.3[a]	21.0	19.4	19.3	24.5[b]
S.A. Total CO_2 (cpm/μM)	33.0	44.9	56.3	55.0	4.93×10^3
Total Counts in CO_2 (cpm)	8.3×10^2	9.4×10^2	10.9×10^2	10.6×10^2	1.21×10^5
% of Total Counts Original	2.5	2.8	3.3	3.2	2.2
Metabolic CO_2 Production (μM/gm/2 hours)		19.3	11.6	19.3	
Metabolic CO_2 Production (μM in 2 hours)		2.1	1.2	1.9	
S.A. Metabolic CO_2 (cpm/μM)		4.5×10^2	9.0×10^2	5.6×10^2	

Phosphate-buffered medium. Initial glucose concentration 1.11 μM/ml. Incubation time 2 hours in air.

[a]Sample contaminated with atmospheric CO_2. Not used in estimation of metabolic CO_2.

[b]No acid added in experiment 3. Figure represents CO_2 evolved from tissue and medium during 2-hour incubation only.

that the lactate was diluted more than the CO_2: this could happen if some of the CO_2 were formed in the hexose monophosphate shunt.

CO_2 production and specific activity in experiment 3. A surprisingly large amount of CO_2, amounting to some 50% of the total CO_2 estimated to be present in the tissue, was evolved during the incubation in this experiment. This is evidence of the markedly labile nature of the CO_2 present in such preparations of bone. It is interesting to note that Borle et al (1960a) produced evidence for the reverse phenomenon, i.e., a considerable net uptake of CO_2 during incubation in bicarbonate medium of metaphyseal preparations from mice.

In two later papers, Vaes and Nichols (1962a and 1962b) assume, somewhat surprisingly, that exchange of metabolically produced CO_2 with the carbonate in the mineral of similar preparations will be minimal on the basis of earlier work by Buchanan and Nakao (1952). The studies of Buchanan and Nakao, however, were concerned with in vivo turnover rates of CO_2 in whole bones measured over extended periods of time and probably have little relevance to the in vitro conditions of the present work and that of Vaes and Nichols. In a further recent paper, without reference to any supportive evidence, and, therefore, presumably based upon the same in vivo data of Buchanan and Nakao referred to in their earlier papers, Vaes and Nichols (1963) assume that the specific activity of the bone mineral CO_2 was zero. They state that this assumption was made because exchange of bone mineral CO_2 with the medium CO_2 seems to proceed slowly and is incomplete over the short periods of incubation used.

Data obtained by Buchanan and Nakao (1952) indicated that even

slight growth stimulated the turnover of bone carbonate. They studied the turnover rates of bone carbonate in rats and mice of various ages. Since the calvarium used in the present study was a rapidly growing tissue, it is likely that the turnover of bone carbonate in this tissue preparation was quite high. Buchanan and Nakao (1958) studied the loss of bone carbonate from powdered rabbit bone in phosphate buffer (pH 7.4). After an interval of 3 hours, they found that approximately 25% of the bone carbonate had been lost. There was a progressive loss of 45% of the carbonate during the first 2 days and little thereafter. From this it appeared that approximately half of the bone carbonate was quite labile.

Borle et al (1960a) demonstrated a net uptake of CO_2 during incubation in bicarbonate medium of metaphyseal preparations from mice: Buchanan and Nakao (1958) were able to show a net loss of CO_2 from powdered rabbit bone in phosphate buffer. Hence, a greater incorporation of $C^{14}O_2$ into bone mineral might be expected in bicarbonate than in phosphate buffer. The total activity present in the CO_2 collected in experiment 3 was equivalent to 2.2% of the original activity present as glucose-U-C^{14}, only slightly lower than the corresponding values obtained in experiment 1 (Table VI, page 53). This shows that little activity was incorporated as carbonate into the calvarial preparations during incubation in phosphate-buffered medium.

Data is, therefore, available on the net uptake and net loss of CO_2 by various bone preparations during incubation in vitro. Experiments such as those of Buchanan and Nakao noted above have supplied data on the

rate of exchange of bone carbonate under in vivo conditions. There is, however, no quantitative information on the amount of CO_2 exchange occurring during the incubation of particular bone preparations in vitro.

Results of paper chromatography in experiment 3. From the radioautograms of the two-dimensional paper chromatograms of the tissue extract (Figure 5, page 39), it could be seen that label was present in approximately equal amount in alanine, aspartic acid, and glutamic acid. Lactic acid, of course, was heavily labeled and faint spots corresponding to some of the phosphorylated glycolytic intermediates were present. Slight traces of activity could be detected in the positions which would be occupied by a few of the Krebs Cycle intermediates, but these could not be visualized chemically. Since many of these acids combine with bone mineral, their concentration in an alcohol:water extract may be very low. Cohn and Forscher (1962a) found less than 1% of the utilized activity present in Krebs Cycle intermediates separated on silicic acid from a perchloric acid extract of metaphyseal preparations incubated with glucose-U-C^{14} under conditions similar to the present ones. After extraction and decalcification, the residual tissue in experiment 3 counted at 1×10^4 cpm when directly plated. It is reasonable to assume that this material is largely protein.

Summary. From the results of experiments 1 and 3 it was possible to reach certain conclusions. Lactic acid production and specific activity were not constant but were inversely related to each other. This indicated endogenous dilution, possibly by glycogen. The estimated

metabolic CO_2 production by the tissue of 16.73 µmoles/gram wet weight/ 2 hours indicated a low R. Q. of 0.58. The markedly lower specific activity of the metabolic CO_2 compared to that of glucose indicated that endogenous dilution was occurring. Evidence indicating that the CO_2 content of the tissue was very labile, was obtained. In the amino acids aspartate and glutamate, closely linked to the TCA cycle, there was found to be radioactivity, as well as in the insoluble residue after decalcification. Hence, active synthesis of high molecular weight components was still continuing.

Future experiments were planned in order to determine the quantity of glucose consumed and its rate of consumption per flask. From the total number of counts utilized, it would be possible to calculate the percentage of utilized activity present in lactic acid, CO_2, and other products of glucose metabolism. This would be essential in estimating the incidence of various pathways of glucose metabolism.

CHAPTER VI

GLUCOSE UTILIZED BY CALVARIA DURING INCUBATION

The preliminary experiments had supplied some information on the lactic acid production, oxygen uptake, and carbon dioxide production of the cells in a calvarial preparation. Information concerning the specific activity of the lactic acid, "total" carbon dioxide, and metabolic carbon dioxide formed during the incubation had also been obtained. However, no data was available on the amount of glucose utilized, or its utilization rate.

In experiment 2 an attempt was made to elucidate further the metabolism of glucose-U-C^{14} by calvaria. An analysis of the glucose in the medium before and after incubation was carried out. From this it was possible to calculate the glucose utilized and its rate of utilization in any particular flask during incubation.

I. EXPERIMENTAL PROCEDURE

Two 50-ml Erlenmeyer flasks with fused-in center wells were used in experiment 2. Phosphate buffer (pH 7.4) of the usual ionic composition was used: it contained glucose-U-C^{14} with a specific activity of 1.30 X 10^5 cpm/μmole at a concentration of 1.11 μmoles/ml. In flask 1, there were 10.0 ml of medium, and in flask 2, 7.5 ml of medium (Table VII). A total of fifty calvaria were used in flask 1; in flask 2, there were forty-five. In both flasks, 0.5 ml of sodium hydroxide were present in the center well to absorb carbon dioxide.

TABLE VII

UTILIZATION OF GLUCOSE-U-C^{14} BY CALVARIA, EXPERIMENT 2

Flask Number	1	2
Wet Weight of Tissue (mg)	789	589
Volume of Medium (ml)	10.0	7.5
Initial Glucose (μM)	11.1	8.33
Glucose Utilized (μM)	4.21	3.19
Utilization Rate (μM/gm/2 hours)	5.33	5.33
Total Counts Original (cpm)	1.44×10^6	1.08×10^6
Total Counts Utilized (cpm)	5.48×10^5	4.15×10^5

Phosphate-buffered medium. Incubation time 2 hours in air.
Initial glucose concentration 1.11 μM/ml.
Glucose specific activity 1.30×10^5 cpm/μM.

After the flasks had been incubating for two hours, trichloroacetic acid was added to the main compartment by means of a needle and syringe. The needle projected through a stopper into the flask, and had polythene tubing at the bottom. This tubing fitted into the flask so that it was just above the liquid level. After the TCA had been administered, the needle was removed from the stopper. During the two-hour incubation period, the needle was plugged with a wad of parafilm. 2.5 ml of 15% TCA were added to flask 1, and 1.875 ml to flask 2. Each flask was then left to shake for another hour at 37°C.

After removal of the flasks from the water bath, the contents of the center well were transferred to a 5-ml volumetric flask. The liquid in the volumetric flask was made up to the mark with boiled water. Aliquots were removed from this volumetric flask and analyzed for chemical quantity and radioactivity of CO_2.

The neutralized protein-free trichloroacetic acid extracts and calvaria obtained after incubation were frozen. Some of the extract was subsequently analyzed for glucose and lactic acid, and some was put onto anion exchange resin columns. No carrier lactate was added to the samples put onto Dowex-1-Cl columns. The specific activity of lactic acid eluted from the columns was determined. The fractions from flask 1 containing lactic acid were lyophilized and aliquots chromatographed on Whatman #1 paper. The buffered phenol solvent A of McFarren (1951) was used. Glutamic acid was identified in the earlier fractions and its specific activity determined.

II. RESULTS AND DISCUSSION

Recovery of C^{14}. Only 36% of the radioactivity of the glucose-U-C^{14} metabolized was recovered in experiment 2 in the intermediates isolated. About 28% was present in the lactic acid formed, 7% in the CO_2 and 1% in the glutamic acid. Similar results were obtained in experiments 1 and 3 as to the fraction of activity originally present in the medium which appeared in the lactic acid and CO_2. If the assumption was made that the glucose uptake was similar in all experiments, therefore, about two-thirds of the label metabolized was not yet accounted for. The radioautograms prepared in experiment 3 indicated the fate of a part of this. Alanine, aspartic acid and glutamic acid were all labeled in approximately equal amount. Faint spots corresponding to some of the phosphorylated glycolytic intermediates were present. Cohn and Forscher (1962a) recovered about 60% of the utilized activity in lactic and pyruvic acids and about 6% in the CO_2. They incubated metaphyseal preparations of bone with glucose-U-C^{14} under conditions similar to the present ones.

Lactic acid production and specific activity. As in experiments 1 and 3, the production of lactic acid per gram of tissue was quite variable although an inverse correlation between this and the specific activity of the lactic acid was present. Despite a two-fold difference in the net lactic acid production a very similar fraction, approximately 28% of the activity utilized, was present in the lactic acid of both flasks in experiment 2 (Table VIII). Since the ratios of the specific activities (the

TABLE VIII

INCORPORATION OF CARBON-14 INTO LACTIC ACID
BY CALVARIA, EXPERIMENT 2

Flask Number	1	2
Lactic Acid Produced (μM)	6.20	2.42
Production Rate (μM/gm/2 hours)	7.90	4.11
S.A. Lactic Acid (cpm/μM)	2.61×10^4	4.68×10^4
Total Counts in Lactic Acid (cpm)	1.62×10^5	1.13×10^5
% of Total Counts Utilized	29.6	27.2
% of Total Counts Original	11.2	10.5

See Table VII for experimental details.

specific activities used for the calculations were expressed in terms of cpm/μatom of carbon) of glucose and lactic acid were approximately 2.5 and 1.4, this indicated a dilution of the glucose to lactic acid pathway by an endogenous substrate. As discussed in Chapter V, this endogenous substrate is likely glycogen. Because of the marked variation in lactic acid production noted above, yet the apparent constancy of the glucose uptake (Table VII, page 59), it is argued that the concentration of glucose selected has been such as to allow varying degrees of dilution by endogenous substrate. Cohn and Forscher (1962a) found the specific activity of the lactic acid produced by metaphyseal bone preparations incubated with glucose-U-C^{14} (at a concentration almost identical to that used in the present study) in bicarbonate buffer to be the same as that of the glucose. This indicated that all of the lactate arose from the added glucose, and, therefore, that there was no endogenous dilution of the lactate. They also found that approximately 1 μmole of lactate appeared for each μmole of glucose which disappeared. This suggested that other products besides lactate were being formed from the glucose. In a later paper, Cohn and Forscher (1962b) report results which show that in the metaphyseal bone preparation they utilized, there was considerable variation in the ratio between the lactate formed (in μmoles) and the glucose consumed (in μmoles). From figures given in Table I of their paper (unfortunately means of 8 or 9 incubation flasks with no measure of the variance), it can be calculated that when the initial substrate concentration was 0.5 μmoles/ml, the ratio of lactate formed (in μmoles) to glucose consumed (in μmoles) ranged from

0.88 to 1.54. When the initial substrate concentration was 4.0 μmoles/ml, the ratio of lactate formed to glucose consumed varied from 1.32 to 1.78. This variation in lactate production at a given glucose level could have been due to varying degrees of endogenous dilution by glycogen. Although no data directly concerning the glycogen content of their preparations is available, Laskin and Engel (1956) demonstrated glycogen in similar preparations from adult rabbits. Since Cohn and Forscher (1962b) do not report the specific activity of the lactic acid formed, it is impossible to decide whether or not the observed variation in lactate production was due to endogenous dilution by glycogen.

Radioactivity in CO_2. Although the total counts utilized in flasks 1 and 2 varied by about 25%, the total counts in the CO_2 also varied by about the same amount, so that the percentage of total counts utilized present in the CO_2 was almost identical in the two flasks (Table IX). The percentage of total original counts in the CO_2 was of the same order as that in experiments 1 and 3. Hence, it was reasonable to assume, as on page 61, that at this glucose level about 7% of the total counts utilized appeared in the CO_2.

Summary. Approximately 28% of the utilized activity was recovered in the lactic acid, 7% in the CO_2, and 1% in the glutamic acid. This indicated a more complex fate for substrate glucose than appeared to be the case from earlier non-tracer studies. Glucose uptake appeared to be constant as did the fraction of utilized activity appearing in lactic acid and CO_2. As found in experiments 1 and 3, lactic acid

TABLE IX

INCORPORATION OF CARBON-14 INTO CARBON DIOXIDE
BY CALVARIA, EXPERIMENT 2

Flask Number	1	2
Total CO_2 Found (μM)	130.0	90.9
Total CO_2 Found (μM/gm)	164.7	154.5
S.A. Total CO_2 (cpm/μM)	2.81×10^2	2.99×10^2
Total Counts in CO_2 (cpm)	3.65×10^4	2.73×10^4
% of Total Counts Utilized	6.7	6.6
% of Total Counts Original	2.5	2.5

See Table VII, page 59, for experimental details.

production and specific activity were not constant but were inversely related to each other. This indicated endogenous dilution, possibly by glycogen.

CHAPTER VII

THE EFFECT OF DIFFERENT SUBSTRATE LEVELS AND MEDIUM PHOSPHATE CONCENTRATION UPON GLYCOLYSIS IN CALVARIA

In the experiments performed up to this time, the initial glucose level used was always 1.11 μmoles/ml. To observe the effects of changing the glucose level in the medium a series of experiments in which the glucose concentration varied from 0.400 to 2.189 μmoles/ml was planned and the results of three of these are reported and discussed in this and the following chapter.

In an earlier preliminary experiment, the specific activity of the glucose separated by ion exchange from the medium after incubation was determined and found to be lower than the initial specific activity, presumably due to the release of glucose into the medium by the tissue. The most likely source of the glucose released by the tissue was glycogen, the rate of hydrolysis of which by phosphorylase is dependent upon the ratio of the concentrations of glucose-1-phosphate and inorganic phosphate. The effects of variation in the initial glucose concentration and in the medium phosphate content upon the change in glucose specific activity were, therefore, investigated.

I. DESCRIPTION OF THE EXPERIMENTS PERFORMED

Contents of the flasks. Phosphate-buffered medium (pH 7.4) of the usual ionic composition was used in experiments 7 and 8; THAM-buffered medium (pH 7.4) was used in experiment 10. Warburg flasks with two side-

arms each served as incubation flasks. 0.4 ml of 2 N NaOH were put into the center well and 1.0 ml of medium into the main compartment of each flask. In experiment 7, the numbers from 1-40 were arranged in random order, and every set of four numbers assigned to flasks 1-4 respectively. Hence, every flask contained 10 calvaria picked out randomly. The same randomization procedure was used for assigning rats to flasks in experiments 8 and 10.

Four flasks were used in experiment 7. The glucose levels were 0.547, 1.094, 1.641, and 2.189 μmoles/ml respectively, and the initial specific activity of the glucose was 5.16×10^6 dpm/μmole (Table X, page 70). All radioactivity determinations in experiments 7, 8, and 10 were done by liquid scintillation, unless indicated otherwise.

In experiment 8, four flasks were also used. In flasks 1 and 2 the glucose concentration was 0.40 μmoles/ml, and in flasks 3 and 4, 1.601 μmoles/ml. The initial specific activity of the glucose was 4.99×10^6 dpm/μmole (Table X, page 70).

Three sets of duplicate incubation flasks were used in experiment 10: flasks 1 and 2 contained 0.574 μmoles/ml of glucose each, flasks 3 and 4, 1.149 μmoles/ml, and flasks 5 and 6, 1.723 μmoles/ml. In this experiment the initial specific activity of the glucose was lower than that in experiments 7 and 8: it was 2.88×10^6 dpm/μmole (Table X, page 70).

Experimental procedure. The following procedure was carried out for every flask in experiments 7, 8, and 10. After a two-hour incubation

at 37°. in air the flask was removed from the shaking water bath and put onto ice. The contents of the center well were immediately transferred to a 5-ml volumetric flask by suction: the volume was adjusted to 5 ml with boiled water. Aliquots were later assessed for radioactivity.

The tissue was then removed from the medium as rapidly as possible, rinsed with 1 ml of 0.154 molar NaCl solution, and frozen. The medium obtained after incubation was frozen, and subsequently analyzed for glucose, lactate, and total C^{14} content. From each flask, 500 µl of medium after incubation were put onto a Dowex-1-Cl anion exchange column. No carrier glucose or lactate was added to any of the samples. Chemical and radioactivity determinations were done on the glucose and lactate eluted from the column.

In experiment 7, the contents of individual tubes containing labeled glucose and lactate were lyophilized. Aliquots from each tube were then subjected to chemical and radioactivity determinations. In experiments 8 and 10, radioactive glucose samples eluted from a certain column were pooled, put into a 100-ml round or pear-shaped flask, lyophilized, and dissolved in a small quantity of water for subsequent analysis. The same procedure was carried out with the lactate samples.

II. RESULTS AND DISCUSSIONS

Change in the specific activity of glucose during incubation. The effects of varying initial glucose concentration and medium composition on the final specific activity of the medium glucose in experiments 7, 8, and 10 are shown in Table X. As the initial glucose concentration

TABLE X

CHANGE IN MEDIUM GLUCOSE SPECIFIC ACTIVITY DURING INCUBATION OF CALVARIAL PREPARATIONS

Medium	Initial Glucose Concentration (μM/ml)	Initial Glucose Specific Activity (dpm/μM)	Final Glucose Specific Activity (dpm/μM)	% Decrease in Specific Activity
Phosphate Buffer[a]	0.547	5.16×10^6	3.11×10^6	40
	1.094		4.09	21
	1.641		4.57	11
	2.189		4.65	10
Phosphate Buffer[b]	0.400	4.99×10^6	3.53	29
	0.400		3.35	33
	1.601		4.04	19
	1.601		4.15	17
THAM Buffer[c]	0.574	2.88×10^6	2.61	9
	0.574		2.85	1
	1.149		2.57	11
	1.149		2.68	7
	1.723		2.56	11
	1.723		2.80	3

Approximately 100 mg tissue incubated in 1 ml medium. Incubation time 2 hours in air.

[a,b,c]Experiments 7, 8, and 10 respectively.

increased in experiment 7, the final specific activity of the glucose in the medium rose also. However, the final specific activity seemed to level off as higher initial glucose concentrations were used, i.e., there was a smaller difference between the final glucose specific activity in flasks 3 and 4 than between that in flasks 1 and 2. The specific activity of the glucose in the medium after incubation appeared to be approaching the initial glucose specific activity asymptotically with increasing glucose concentration.

In experiment 8, in which duplicate flasks were used, the specific activity of the glucose in the medium after incubation, like that in experiment 7, was considerably higher in the flasks with a higher initial glucose concentration. The final glucose specific activities in duplicate flasks were in close agreement at each glucose concentration.

In experiment 10 there was no significant variation in the final specific activity of the glucose between flasks with different initial glucose concentrations. Furthermore, the final glucose specific activity was almost identical with that of the initial glucose specific activity. As shown in Table X, page 70, the greatest percentage decrease in specific activity was 11%, as compared to 40% and 33% for experiments 7 and 8 respectively. In Figure 6, there is a comparison of the final specific activity to the initial specific activity of medium glucose in experiments 7, 8, and 10.

The above results indicated that there was less dilution of the labeled glucose in experiment 10 than in experiments 7 or 8. Furthermore, when THAM buffer was used, there was little dilution of glucose-U-C^{14} at

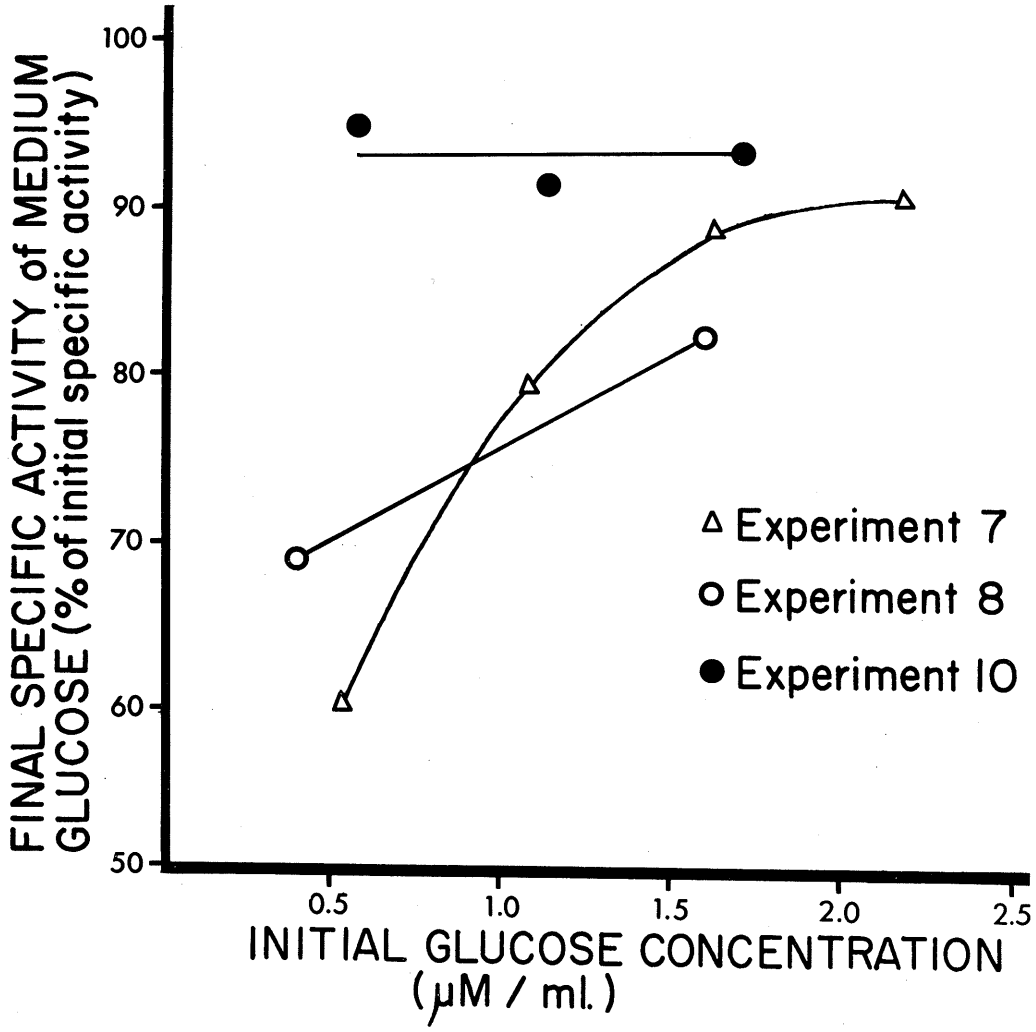

Figure 6. Comparison of final specific activity to initial specific activity of glucose with varying glucose concentrations in phosphate (experiments 7 and 8) and THAM-buffered medium (experiment 10). Approximately 100 mg tissue incubated in 1 ml medium. Incubation time 2 hours in air.

any glucose level. Because of the difference between the initial and final glucose specific activities it was possible to calculate the glucose uptake from isotope dilution. The following formula was used to calculate the quantity of glucose taken up by the tissue:

$$\frac{G_I A_I - G_F A_F}{A_I} = G_U,$$

where G_I represents the glucose concentration in μmoles/ml in the medium before incubation

A_I represents the initial specific activity of the glucose

G_F represents the glucose concentration in μmoles/ml in the medium after incubation

A_F represents the final specific activity of the glucose

and G_U represents the glucose uptake calculated from isotope dilution.

The values obtained from the above formula were actually minimal values since it was assumed that the specific activity of the glucose taken up by the tissue was equal to that of the initial specific activity. It would, in actual fact, have been somewhat lower than the initial specific activity.

Table XI compares the glucose uptake calculated from isotope dilution to that found by chemical analysis. The difference between the two represents the minimal quantity of glucose released by the tissue. It can readily be seen that much smaller quantities of glucose were

TABLE XI

CALCULATION OF CORRECTED VALUES FOR GLUCOSE UPTAKE AND RELEASE BY TISSUE DURING INCUBATION OF CALVARIAL PREPARATIONS

Initial Glucose Concentration (μM/ml)	Wet Weight of tissue (mg)	Glucose Uptake (μM) from isotope dilution	Glucose Uptake (μM) from chemical analysis	Glucose Released By Tissue (μM)	Glucose Uptake Rate (μM/gm/2 hrs) from isotope dilution	Glucose Uptake Rate (μM/gm/2 hrs) from chemical analysis	% "Error"
0.547[a]	146.2	0.372	0.257	0.115	2.54	1.76	31
1.094	103.3	0.624	0.502	0.122	6.04	4.86	20
1.641	114.8	0.663	0.539	0.124	5.78	4.70	19
2.189	126.8	0.663	0.495	0.168	5.23	3.90	25
0.400[b]	80.8	0.150	0.047	0.103	1.85	0.58	69
0.400	95.5	0.200	0.104	0.096	2.09	1.09	48
1.601	85.7	0.575	0.334	0.241	6.71	3.90	42
1.601	75.3	0.511	0.290	0.221	6.79	3.85	43
0.574[c]	75.7	0.185	0.146	0.039	2.44	1.93	21
0.574	91.1	0.199	0.196	0.003	2.18	2.15	1
1.149	84.3	0.385	0.293	0.092	4.56	3.48	24
1.149	79.9	0.353	0.293	0.060	4.42	3.67	17
1.723	76.1	0.691	0.562	0.129	9.08	7.39	19
1.723	58.6	0.494	0.461	0.031	8.43	7.87	7

Incubation time 2 hours in air.

[a,b,c]Experiments 7, 8, and 10 respectively. Phosphate buffer, experiments 7 and 8; THAM buffer, experiment 10.

released by the tissue in experiment 10 than in experiments 7 and 8. Since the major difference between experiments 7 and 8, and 10 lay in the phosphate level of the medium, variation in this parameter could be largely responsible for the observed differences in endogenous dilution of the glucose. The most likely source of the glucose released by the tissue was glycogen.

The rate of hydrolysis of glycogen by phosphorylase is dependent upon the ratio of the concentrations of glucose-1-phosphate and inorganic phosphate. The reaction of glycogen with inorganic phosphate is readily reversible, and can be represented as follows:

$$[C_6H_{10}O_5]_n + H_3PO_4 \rightleftharpoons [C_6H_{10}O_5]_{n-1} + \text{glucose-1-}PO_4$$

According to Conn and Stumpf (1963), the equilibrium is independent of the polysaccharide concentration, provided a certain minimum concentration is exceeded. Hence, at any given pH the equilibrium constant depends on the relative concentrations of glucose-1-phosphate and inorganic phosphate.

$$K_{eq} = \frac{[C_6H_{10}O_5]_{n-1}\ [\text{glucose-1-}PO_4]}{[C_6H_{10}O_5]_n\ [H_3PO_4]}$$

$$= \frac{[\text{glucose-1-}PO_4]}{[H_3PO_4]}$$

$$= 0.3$$

(pH 7.0)

In the THAM-buffered medium used in experiment 10, the inorganic phosphate level was low, approximately 1.3 millimolar. Hence, the system was out of equilibrium, and glycogen synthesis was favored. Very small quantities of glucose were released by the tissue. In the phosphate-buffered medium used in experiments 7 and 8, on the other hand, the inorganic phosphate level at 15.6 millimolar, was considerably higher and glycogenolysis was favored. Much larger quantities of glucose were released by the tissue in experiments 7 and 8 than in experiment 10.

Glucose utilization rate. As shown in Table XI, page 74, when the glucose uptake rate was calculated on the basis of figures obtained from isotope dilution rather than from chemical analysis, different values were obtained for the utilization rates. In some cases the rates calculated on the basis of chemical analysis were in considerable error, as indicated in the table.

The utilization rate of glucose seemed to reach a steady state in phosphate medium after an initial glucose concentration of 1 millimolar had been reached in experiment 7. However, there was a steady increase in the utilization rate of glucose with increasing glucose concentrations in the flasks in experiment 10. Figure 7 shows the relationship between glucose uptake rate calculated on the basis of isotope dilution, and initial glucose concentration in experiments 7, 8, and 10.

Production rate and specific activity of lactic acid produced in experiments 7, 8, and 10. As shown in Table XII, page 78, the production rate of lactic acid by calvarial preparations in phosphate-buffered medium

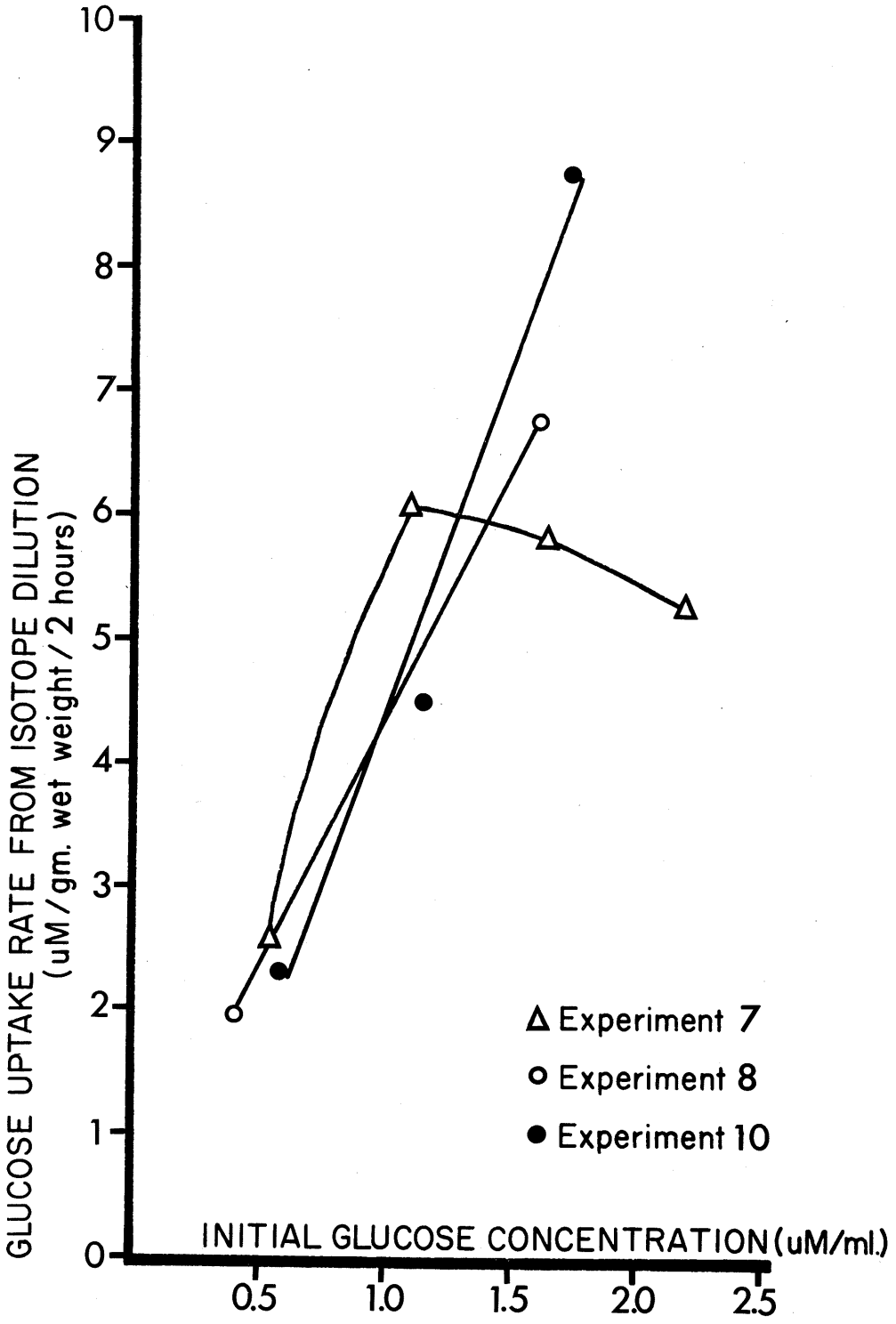

Figure 7. Relationship between glucose uptake rate calculated on the basis of isotope dilution, and initial glucose concentration in phosphate (experiments 7 and 8) and THAM-buffered medium (experiment 10). Approximately 100 mg tissue incubated in 1 ml medium. Incubation time 2 hours in air.

TABLE XII

EFFECT OF GLUCOSE CONCENTRATION AND MEDIUM COMPOSITION ON PRODUCTION AND SPECIFIC ACTIVITY OF LACTATE DURING INCUBATION OF CALVARIAL PREPARATIONS

Medium	Initial Glucose Concentration (μM/ml)	Initial Glucose Specific Activity (dpm/μM)	Lactate Produced (μM)	Production Rate (μM/gm/2 hours)	Lactate Specific Activity (dpm/μM)
Phosphate Buffer[a]	0.547	5.16×10^6	1.624	11.11	0.370×10^6
	1.094		1.476	14.29	0.890
	1.641		0.558	4.86	1.172
	2.189		1.877	14.80	1.389
Phosphate Buffer[b]	0.400	4.99×10^6	0.616	7.62	0.390
	0.400		0.997	10.44	0.383
	1.601		0.892	10.41	0.813
	1.601		0.591	7.85	0.877
THAM Buffer[c]	0.574	2.88×10^6	0.473	6.25	0.412
	0.574		0.560	6.15	0.413
	1.149		0.666	7.90	0.660
	1.149		0.622	7.78	0.630
	1.723		0.924	12.14	0.936
	1.723		0.667	11.38	0.935

Approximately 100 mg tissue incubated in 1 ml medium. Incubation time 2 hours in air.

[a,b,c]Experiments 7, 8, and 10 respectively.

was quite variable, and bore no direct relationship to the initial glucose concentration in the medium. In THAM-buffered medium, however, the production rate of lactic acid increased steadily with increasing levels of glucose in the medium.

As the initial glucose level rose in the flasks of experiment 7, there was a linear increase in the specific activity of the lactic acid produced. This can be seen in Table XII, page 78.

Similar results were obtained in experiment 8. With increasing initial glucose concentrations, there was a linear increase in the specific activity of the lactic acid produced. Despite a variable production rate of lactate in duplicate flasks, the specific activity was almost identical. Hence, whatever was responsible for the variable rate of lactate production was affecting the formation of unlabeled lactate in the same way as the labeled.

As in the phosphate-buffered medium, the specific activity of the lactate produced increased linearly in THAM-buffered medium with increasing initial glucose concentrations. This can be seen in Figure 8. Again, the values for duplicate flasks agreed well. Unlike the situation in experiments 7 and 8, the production rate of lactate was almost identical for duplicate flasks in experiment 10. Hence, in a pair of flasks in this experiment similar production rates of lactate gave rise to lactate with similar specific activities.

Table XIII, page 81, shows the percentages of total lactic acid produced coming from uniformly labeled glucose. These values were calculated by finding the ratio between the specific activities of lactate

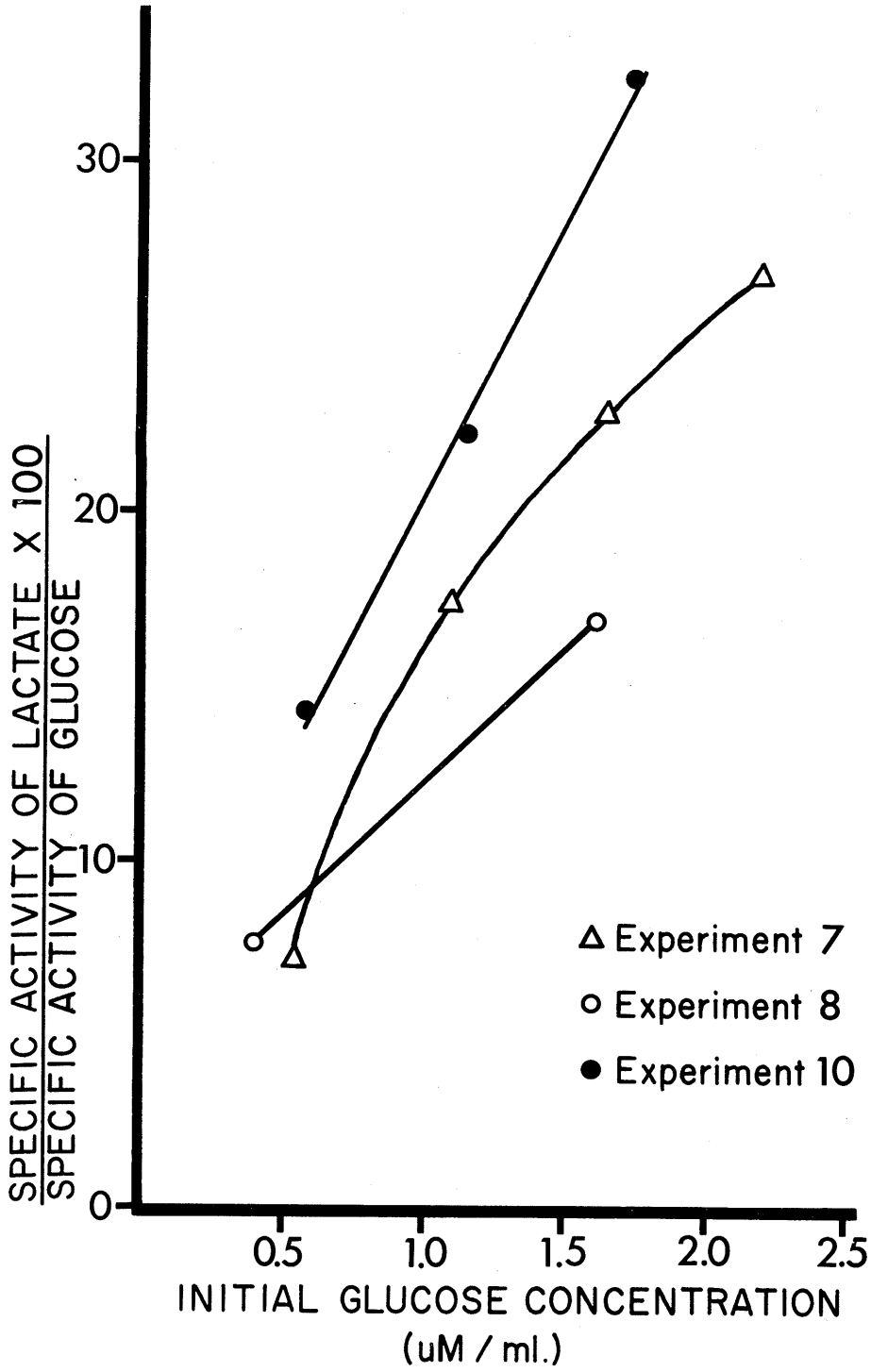

Figure 8. Ratios of the specific activities of lactate and glucose with varying glucose concentrations in phosphate (experiments 7 and 8) and THAM-buffered medium (experiment 10). Approximately 100 mg tissue incubated in 1 ml medium. Incubation time 2 hours in air.

TABLE XIII

ORIGIN OF LACTATE PRODUCED DURING INCUBATION OF CALVARIAL PREPARATIONS

Medium	Initial Glucose Concentration (μM/ml)	% of Total Lactate Produced		Production Rate (μM/gm/2 hours)	
		from[d] Glucose-U-C^{14}	from Other Sources	Lactate from Glucose-U-C^{14}	Lactate from Other Sources
Phosphate Buffer[a]	0.547	14.4	85.6	1.60	9.51
	1.094	34.4	65.6	4.92	9.37
	1.641	45.5	54.8	2.21	2.65
	2.189	53.8	46.2	7.96	6.83
Phosphate Buffer[b]	0.400	15.6	84.4	1.19	6.44
	0.400	15.2	84.8	1.59	8.81
	1.601	32.5	67.5	3.38	7.02
	1.601	35.2	64.8	2.76	5.20
THAM Buffer[c]	0.574	28.8	71.3	1.79	4.45
	0.574	28.5	71.4	1.75	4.39
	1.149	45.6	54.4	3.61	4.29
	1.149	43.7	56.3	3.41	4.38
	1.723	64.9	35.1	7.89	3.94
	1.723	64.8	35.2	7.37	3.69

Approximately 100 mg tissue incubated in 1 ml medium. Incubation time 2 hours in air.

[a,b,c]Experiments 7, 8, and 10 respectively.

[d]Calculated by using formula, % of total lactate from glucose-U-C^{14} = $\frac{\text{S.A. lactate (cpm/μM)}}{\text{S.A. glucose (cpm/μM)}} \times 200$

and glucose, and multiplying by 200, as indicated in the following formula:

$$\% \text{ of total lactate from glucose-U-}C^{14} = \frac{\text{S.A. lactate (cpm/}\mu\text{mole)}}{\text{S.A. glucose (cpm/}\mu\text{mole)}} \times 200$$

Since the percentage of lactate produced from uniformly labeled glucose increased with increasing glucose levels, this indicated less dilution of the lactate. As mentioned earlier in this chapter (page 75), the most likely source of dilution is glycogen, the rate of hydrolysis of which by phosphorylase depends upon the ratio of the concentrations of glucose-1-phosphate and inorganic phosphate.

In experiment 8, the phosphate level was identical in the medium of all flasks studied. However, the glucose level in flasks 3 and 4 was four times as high as that in flasks 1 and 2. Hence, it is reasonable to assume that the glucose-1-phosphate pool would be greater in flasks 3 and 4 than in 1 and 2. As a result, the ratio of glucose-1-phosphate to inorganic phosphate would be higher in flasks 3 and 4 than in 1 and 2, and less glycogenolysis would be expected to occur in the former flasks than in the latter (page 75). This concept was supported by the fact that averages of 15.4 and 33.9% of the total lactic acid produced came from glucose-U-C^{14} in flasks 1 and 2, and 3 and 4 respectively. A rise in the percentage of lactate produced from uniformly labeled glucose with increasing glucose levels occurred also in experiments 7 and 10, and could be explained on the same basis. Furthermore, Madsen (1961) made the observation that UDP-glucose acts as a competitive inhibitor of bacterial and muscle phosphorylase, with about the same affinity for the

enzyme as glucose-1-phosphate. Hence, an increase in the concentration of UDP-glucose *in vivo* not only would stimulate glycogen synthesis but might also inhibit its degradation. The same effect may occur in calvaria *in vitro*. Since UDP-glucose is directly linked to glucose by a reversible reaction, it stands to reason that the concentration of UDP-glucose would increase with increasing glucose concentrations in the medium of incubation flasks.

When flasks with similar initial glucose levels but different phosphate levels were compared (Table XIII, page 81), it could be seen that more dilution of the lactate was occurring in flasks containing phosphate buffer than in those containing THAM buffer. This supported the argument presented on page 76, that glycogenolysis is favored when the inorganic phosphate level is high, or when the ratio of glucose-1-phosphate to inorganic phosphate is lowered. Hence, if the glucose-1-phosphate pool is constant in size, an increase in the level of inorganic phosphate will promote glycogenolysis, and glycogen synthesis will likely be depressed. This is supported by the work of Cabib (1963), who noted that in the instance of glycogen synthetase the phosphorylated enzyme is the inactive form.

Summary. When calvaria were incubated in phosphate-buffered medium (pH 7.4) with glucose-U-C^{14}, the specific activity of the glucose in the medium after incubation decreased to as little as 60% of the original specific activity. In THAM-buffered medium (pH 7.4), the lowest specific activity of the glucose was 89% of the original. These results indicated

that endogenous dilution of the glucose was occurring, and on the basis of previous work, glycogen was the most likely source of this dilution. They also indicated that a greater percentage of the glucose was being diluted in phosphate than in THAM-buffered medium. That more glycogenolysis occurred in phosphate than in THAM-buffered medium with similar glucose, and, therefore, similar glucose-1-phosphate levels, was indicated by the fact that, on the basis of specific activity determinations of lactate, more dilution of the lactate was occurring in flasks containing phosphate buffer. Within a given experiment, in which the phosphate level was the same in each flask, a smaller percentage of the lactate was formed directly from glucose-U-C^{14} in the flasks with a lower initial glucose concentration. Thus, whenever the ratio of glucose-1-phosphate to inorganic phosphate was lowered, more glycogenolysis occurred.

CHAPTER VIII

THE EFFECT OF DIFFERENT SUBSTRATE LEVELS AND MEDIUM PHOSPHATE CONCENTRATION UPON THE DISTRIBUTION OF ACTIVITY IN VARIOUS COMPONENTS OF THE RAT CALVARIUM

The first part of the results in this chapter deals with the fate of glucose-U-C^{14} in experiments 8 and 10 during incubation of calvarial preparations. The percentages of utilized activity in CO_2, various components of the medium, and the tissue from flasks with different levels of glucose and different buffers, are given. To determine the distribution of activity in various components of the rat calvarium, it was necessary to extract the tissue with different solvents. Information obtained by counting samples of these tissue extracts is discussed in the latter part of this chapter.

Details of the general experimental procedures in experiments 8 and 10 are given in Chapter VII, pages 67-69. The steps employed in the extraction of the tissue in these experiments are given in the following section of this chapter.

I. METHODS USED

Extraction of tissue. The frozen skulls from experiments 8 and 10 were lyophilized. The skulls from each flask were kept in a separate test tube, i.e., the tissue was not pooled. After lyophilization approximately half of the dried material in each flask was put into another set of test tubes. The dry weight of the tissue from each flask was obtained.

The tissue in one set of tubes (designated the "a" group), was extracted with KOH, washed, dried, and counted. That in the second set of tubes (the "b" group) was extracted successively with ethanol, petroleum ether, trichloroacetic acid, and KOH.

The "a" group was dealt with as follows: to each of the ten tubes in this group were added 3 ml of 30% KOH. Each sample was then heated in a water bath at 80-90°C. for 1 hour. All samples were frequently shaken during the heating period. After they had cooled to room temperature, the tubes were centrifuged at 1,500 rpm for twenty minutes. The supernatant (KOH extract) was removed from each tube, and the precipitate was washed twice with 2 ml of distilled water, dried, and transferred to a planchet. A thin window gas flow tube was used to count the radioactivity in each precipitate.

50 µl aliquots were removed from the supernatant or KOH extract obtained by the above procedure from each of the ten samples. These aliquots were assayed for total C^{14} content. Part of the supernatant from each sample was then treated with methanol to cause precipitation of the glycogen. To 1 ml of supernatant were added 250 µl of a solution containing 5 mg of carrier glycogen, and 4 ml of anhydrous methanol. The mixture was heated to 40°C., cooled in the refrigerator overnight, and centrifuged. The precipitates of glycogen were further purified twice by reprecipitation with 4 ml of anhydrous methanol from solution in 1 ml of distilled water. The glycogen thus obtained was put onto planchets, dried, and counted with a thin window gas flow tube.

The "b" group was dealt with as follows: to each of the ten tubes

in this group were added, for successive 15-minute periods, 1 ml of boiling 60% ethanol, 1 ml of boiling 60% ethanol, and 1 ml of boiling water. All tubes were shaken in the rack every 5 minutes during extraction. The extracts were pooled. Then 1 more ml of boiling water was added to each tube and allowed to sit for 1 hour. The contents of the tubes containing the pooled alcohol extracts, and the ones containing the final ml of rinse-water, were lyophilized. Aliquots were assayed for radioactivity.

Having been extracted with ethanol, the skulls were lyophilized for a second time. 2-ml quantities of petroleum ether were added to each tube, allowed to sit for half an hour, and poured off. The process was repeated for the same length of time and with the same volume of petroleum ether. These extracts were pooled, and called PE I (petroleum ether extraction I). Another 2 ml of petroleum ether were added to each tube for a third time. This extract was kept apart from PE I, and called PE III. Aliquots from each extraction were assayed for radioactivity.

1 ml of 3% TCA was added to each tube containing skulls already subjected to alcohol and petroleum ether extractions. The tubes were placed in the refrigerator and shaken at several intervals during the next few days. The TCA was then poured off into other tubes, and frozen. Aliquots of the TCA solution were subsequently counted.

After the TCA extraction, the tissue in each tube was rinsed with 5 ml of distilled water, which was decanted and discarded. The skulls were dried on Whatman #1 paper, and put into appropriately labeled test tubes. 3 ml of 30% KOH were added to each tube. All of the samples were

then heated in a water bath at 80-90°C. for 1 hour. The tubes were shaken approximately every 15 minutes during heating, and allowed to remain in the water bath as it cooled. Although the major part of the skulls dissolved completely in the KOH, very small particles could still be seen in the liquid. Aliquots of the KOH extract were assayed for radioactivity.

II. RESULTS AND DISCUSSION

Distribution of radioactivity among various products formed from glucose-U-C^{14} by calvarial preparations. The percentage of total counts utilized which was found in lactate varied to quite an extent in phosphate-buffered medium. As can be seen in Table XIV, much more uniform values were obtained in THAM-buffered medium. In the latter medium, also, values for duplicate flasks were closer than those in phosphate medium.

When all the flasks in experiments 8 and 10 are examined, it can be seen that the average percentage of utilized activity in the lactate formed in phosphate-buffered medium (28.95%) is lower than that in the THAM-buffered medium (40.40%). The percentage of utilized activity in CO_2 was fairly similar for all flasks of experiments 8 and 10. However, in both buffer systems, there was a slight decrease in the percentage found in CO_2 from flasks with higher initial glucose concentrations.

A greater fraction of utilized activity was calculated to be in compounds other than glucose and lactate in the medium in experiment 8 than in experiment 10, up to 58% of the utilized activity in the phosphate-buffered medium of experiment 8, and a maximum of only 34% in the THAM-buffered medium

TABLE XIV

FATE OF GLUCOSE-U-C^{14} DURING INCUBATION OF CALVARIAL PREPARATIONS

	Initial Glucose Concentration (μM/ml)	% Distribution of Utilized Glucose-U-C^{14}			
		Lactate	CO_2	Medium other than Glucose and Lactate	Tissue[c]
Phosphate Buffer[a]	0.400	32.0	3.7	29.3	35.0
	0.400	38.0	3.1	26.9	31.9
	1.601	25.4	1.6	58.1	14.4
	1.601	20.4	1.6	54.7	23.4
THAM Buffer[b]	0.574	36.6	3.4	34.1	24.9
	0.574	40.3	4.1	16.8	38.0
	1.149	39.6	2.7	30.8	25.0
	1.149	38.6	3.1	32.0	24.4
	1.723	43.5	2.1	27.4	20.6
	1.723	43.8	2.3	20.3	32.4

Approximately 100 mg tissue incubated in 1 ml medium. Incubation time 2 hours in air.

[a,b]Experiments 8 and 10 respectively.

[c]Calculated values.

of experiment 10. Table XIV shows these results. To calculate these values, the activity in the medium glucose and lactate was subtracted from the total activity in the medium after incubation.

The calculated percentage of total counts utilized to appear in the tissue varied between 14.4 and 38.0% in experiments 8 and 10. These values were obtained by determining the radioactivity in aliquots of medium before and after incubation. The loss in activity from the medium represented that going into carbon dioxide and tissue. Since the activity in the carbon dioxide given off was determined, it was possible to calculate the percentage of utilized activity in the tissue.

Total activity found in the KOH extractable tissue of the "a" group. A negligible amount of activity was detected in the solid residue of tissue left after extraction with KOH. Only 0.03-0.09% of the total counts utilized were recovered in this solid residue.

Much greater quantities of radioactivity were found in the KOH extract. From 8.2-19.1% of the counts utilized were recovered in the KOH extractable tissue of experiments 8 and 10 (Table XVI, page 94). On the whole, values for duplicate flasks agreed quite well, although the agreement was better for the flasks in THAM-buffered than in phosphate-buffered medium. There was less activity in the KOH extract of skulls incubated with a high than with a lower initial glucose concentration in the medium. This effect was more marked in phosphate than in THAM-buffered medium.

Glycogen was obtained by addition of methanol to aliquots of the KOH extracts. Very small quantities of radioactivity were found in the

precipitates of glycogen: only 0.01-0.07% of the total counts utilized were recovered in this compound.

Percentage distribution of the recovered utilized activity in various extracts of the tissue in the "b" group. Table XV shows the percentages of the recovered utilized activity found in the various extracts of calvaria. The aqueous ethanol and KOH extracts of skulls incubated in phosphate-buffered medium contained approximately equal quantities of activity. A total of 83-86% of the recovered activity in these skulls was found in the ethanol and KOH extracts. With the exception of one flask, all aqueous ethanol extracts of calvaria incubated in THAM-buffered medium contained approximately 62-67% of the recovered activity in these skulls. Between 10-26% was found in the KOH extract. In both buffer systems, the incorporation of activity into the ethanol and KOH-extractable components of the tissue was independent of the initial glucose concentrations used. In THAM-buffered medium, a greater percentage of the utilized activity was found in the aqueous ethanol extracts of the tissue than in phosphate medium, but a smaller percentage was found in the KOH extracts. Aqueous ethanol extracts of calvaria contain amino acids, organic acids, phosphorylated glycolytic intermediates, and perhaps small peptides, whereas KOH extracts contain dissolved protein from the skulls. Hence, for skulls incubated with glucose-U-C^{14} in phosphate buffer, most of the activity incorporated into the skulls was incorporated into small molecular weight compounds and protein, whereas for those incubated in THAM buffer, most of the activity was incorporated into small molecular

TABLE XV

PERCENTAGE DISTRIBUTION OF THE RECOVERED UTILIZED ACTIVITY IN VARIOUS EXTRACTS OF CALVARIA

	Initial Glucose Concentration (μM/ml)	% of the Recovered Utilized Activity in Various Fractions				
		Aqueous Ethanol Extract	Petroleum Ether I[a]	Petroleum Ether III	TCA Extract	KOH Extract
Phosphate Buffer[b]	0.400	52.50	1.14	0.07	12.81	33.47
	0.400	40.33	1.65	0.18	13.99	43.85
	1.601	45.64	1.78	0.09	14.71	37.78
	1.601	44.14	1.90	0.13	12.70	41.12
THAM Buffer[c]	0.574	64.10	1.12	0.10	24.58	10.11
	0.574	42.90	2.06	0.11	28.87	26.07
	1.149	61.98	1.70	0.14	21.76	14.42
	1.149	63.89	2.16	0.17	21.16	12.62
	1.723	65.96	2.47	0.15	13.76	17.66
	1.723	67.10	3.42	0.26	15.35	13.87

Approximately 100 mg tissue incubated in 1 ml medium. Incubation time 2 hours in air.

[a]For explanation of petroleum ether extracts see page 87.

[b,c]Experiments 8 and 10 respectively.

weight compounds, and relatively little into protein. This suggests that higher levels of inorganic phosphate in the incubation medium tend to cause an increase in the percentage of activity incorporated into the protein of calvaria.

Relatively small quantities of the recovered activity, approximately 1-2%, and 1-4%, were found in the petroleum ether extracts of the skulls incubated in phosphate and THAM buffers respectively. The trichloroacetic acid extracts of skulls incubated in phosphate buffer contained approximately 13-15% of the activity recovered in these skulls, at both glucose levels studied. For skulls incubated in THAM buffer, approximately 27% at the lowest glucose level, and approximately 15% at the highest glucose level studied, were found in the TCA extracts. This indicated that for skulls incubated in THAM-buffered medium, a greater percentage of activity was being incorporated into peptides and polysaccharides at low than at higher initial glucose concentrations.

A comparison of the calculated and experimentally determined percentages of utilized activity in calvaria. The amounts of carbon-14 recovered from the tissue samples by the extraction procedures used in group "a" and group "b" are compared in Table XVI to the amounts calculated to be present in the tissue, both being expressed as a percentage of the glucose-U-C^{14} utilized. If the calculated values, based on direct assay of the medium, are assumed to be accurate, the recoveries by the different extraction procedures are seen to be incomplete, being 40-60% in group "a" and 12-30% in group "b". As all tissue fractions

TABLE XVI

A COMPARISON OF THE CALCULATED AND EXPERIMENTALLY DETERMINED PERCENTAGES OF UTILIZED ACTIVITY IN CALVARIA

	Initial Glucose Concentration (μM/ml)	Calculated Total Activity in Tissue (% of counts utilized)	Total Activity Found in KOH Extractable Tissue (% of counts utilized) Group "a"	Sum of Activity Found in Ethanol, Ether, TCA, and KOH Extractions (% of counts utilized) Group "b"
Phosphate Buffer[a]	0.400	35.0	14.5	10.5
	0.400	31.9	19.1	9.1
	1.601	14.4	8.2	3.9
	1.601	23.4	10.0	3.3
THAM Buffer[b]	0.574	24.9	14.9	6.6
	0.574	38.0	15.3	8.6
	1.149	25.0	12.8	4.0
	1.149	24.4	12.2	4.9
	1.723	20.6	10.1	3.9
	1.723	32.4	12.0	4.0

Approximately 100 mg tissue incubated in 1 ml medium. Incubation time 2 hours in air.

[a,b]Experiments 8 and 10 respectively.

were assayed the poor recovery is presumably due to experimental loss during the subsequent handling of the tissue. This is borne out by the fact that the percentage recovery in group "b", which was serially extracted with a number of solvents, is less than that in group "a", where a single extraction with KOH was carried out. Agreement between duplicates was, on the whole, good. Therefore, although the distribution pattern shown in Table XV, page 92, is presumably weighted in favor of the earlier fractions, meaningful comparisons can still be made between the fate of glucose-U-C^{14} in phosphate and in THAM-buffered media.

Summary. The average percentage of utilized activity in the lactate formed in phosphate-buffered medium (experiment 8) was 28.95% as compared to 40.40% for that formed in the THAM-buffered medium (experiment 10). The percentage of utilized activity in CO_2 was fairly similar for all flasks of experiments 8 and 10, although there was a slight decrease in the percentage found in CO_2 from flasks with higher initial glucose concentrations. Up to 58% of the utilized activity was calculated to be in compounds other than glucose and lactate in the medium after incubation in experiment 8, and up to 34% in experiment 10. The calculated percentage of total counts utilized to appear in the tissue varied between 14.4 and 38.0% in experiments 8 and 10.

When the skulls were directly extracted with KOH, from 8.2-19.1% of the counts utilized were recovered in the KOH extractable tissue of experiments 8 and 10. A negligible amount of activity remained in the solid residue of tissue after extraction with KOH.

A portion of the skulls in experiments 8 and 10 were extracted

with a series of solvents to determine the distribution of activity in various components of the rat calvarium. The aqueous ethanol and KOH extracts of skulls incubated in phosphate-buffered medium contained approximately equal quantities of activity: a total of 83-86% of the recovered activity in these skulls was found in these extracts at both glucose levels studied. Hence, it appeared that for skulls incubated with glucose-U-C^{14} in phosphate buffer, most of the activity incorporated into the skulls was incorporated into small molecular weight compounds and protein. Only 1-2% of the recovered utilized activity was found in the petroleum ether extracts of these skulls, and approximately 13-15% in the trichloroacetic acid extracts. The latter extracts contained peptides and polysaccharides.

Except for one flask, all aqueous ethanol extracts of calvaria incubated in THAM-buffered medium contained approximately 62-67% of the recovered activity in these skulls. Between 10-26% of the recovered activity was found in the KOH extract. Only from 1-4% of the recovered activity of the skulls was detected in lipid material. At the lowest glucose level studied, approximately 27%, and at the highest glucose level studied, approximately 15% of the recovered activity in the skulls was found in the TCA extracts. Therefore, at lower glucose levels a greater percentage of activity was being incorporated into the polysaccharides of skulls incubated in THAM medium than at higher glucose levels.

When KOH was directly added to the skulls in experiments 8 and 10, approximately half of the calculated activity was actually recovered

in the KOH extract. A total of 12.35-30.00% of the calculated activity in the tissue was recovered in aqueous ethanol, petroleum ether, TCA, and KOH extracts.

CHAPTER IX

SUMMARY AND CONCLUSIONS

The *in vitro* metabolism of glucose-U-C^{14} by calvaria was studied in the present report. Calvaria from one- to two-day old Long-Evans rats were incubated in air in calcium-free phosphate or THAM-buffered medium (pH 7.4) in a water bath at 37°C. The duration of incubation was generally two hours. Varying concentrations of glucose-U-C^{14} were used in the medium of different experiments.

Glucose was determined by a glucose oxidase-3,3'-dimethoxybenzidine (o-dianisidine) method. The lactic dehydrogenase-DPN method developed by Horn and Bruns (1956) and Cohen and Noell (1960) was used to determine the quantity of lactic acid. Two methods were used to assess the C^{14} content of radioactive samples. The potassium persulfate oxidation described by Abraham and Hassid (1957) was used for radioactivity determinations up to experiment 7. Labeled samples were oxidized to $C^{14}O_2$, which was collected in CO_2-free NaOH to form $Na_2C^{14}O_3$. The radioactive sodium carbonate was then precipitated with $BaCl_2$ to form $BaC^{14}O_3$, which was plated, and counted with a thin window gas flow tube. All radioactivity determinations in experiments 7, 8, and 10 were done by liquid scintillation, unless indicated otherwise. Homogeneous counting systems of toluene or toluene-methanol were used.

Separation of the medium obtained after incubation into its radioactive components was achieved by pipetting a sample of the medium onto a Dowex-1-Cl resin column and eluting it with HCl, over a range of 0-0.02 N.

Paper chromatography and radioautography were used to identify and estimate the amount of radioactivity in various labeled products of glucose-U-C^{14} metabolism.

Two preliminary experiments in which calvaria were incubated in phosphate-buffered medium (pH 7.4) for two hours in the presence of glucose-U-C^{14} were performed in order to furnish some general information on glucose-U-C^{14} metabolism by calvarial preparations. The oxygen uptake and CO_2 production of the tissue were determined on a Warburg apparatus. From the oxygen uptake of 14.34 µmoles/gram wet weight/hour, and the metabolic CO_2 production of 16.73 µmoles/gram wet weight/2 hours, an R.Q. of 0.58 was calculated. Since the R.Q. obtained had a rather low value, further study would be necessary to assess the reliability of the method used for determining metabolic CO_2 production. The problem was not pursued further in the present work.

The specific activities of both the CO_2 and lactic acid produced were considerably lower than that of the original glucose-U-C^{14}. This is evidence for dilution of the activity by unlabeled intermediates of endogenous origin during the metabolism of the glucose-U-C^{14}. The difference between the specific activities of the CO_2 and lactic acid was, by comparison, much less, that of the former varying between 39-127% that of the latter. These data show that much less endogenous dilution occurred between pyruvate and the decarboxylation steps in the TCA cycle than in glycolysis. It is even possible that dilution of the label after pyruvate was negligible, the lower specific activity of CO_2 relative to that of lactic acid being due simply to a slower approach to an equilibrium

specific activity of the intermediates in the TCA cycle.

The specific activity of the CO_2 actually exceeded that of the lactate in one instance. Hence, the lactate was diluted more than the CO_2. This would be possible if the hexose monophosphate shunt had been more active in this case. The origin of the endogenous dilution could be glycogen which Dowse et al (1963) have shown is present in calvarial preparations and can produce considerable quantities of lactic acid under conditions where no external substrate is present.

Further study of the metabolism of uniformly labeled glucose by calvaria in phosphate-buffered medium (pH 7.4) was carried out in experiment 2. Analysis of the glucose in the medium before and after incubation made it possible to calculate the quantity of glucose utilized and its rate of utilization in any particular flask during incubation. This enabled a more detailed analysis of the problem of dilution noted earlier to be carried out. The uptake of glucose was found to be constant as was the fraction of the utilized activity appearing in lactic acid and CO_2. However, the production rate and specific activity of the lactic acid produced were not individually constant but were rather inversely related to one another in any given flask. These facts lead to the conclusion that although the amount of endogenous dilution occurring varied between flasks, the uptake of glucose-C^{14} and the metabolism of this to lactate-C^{14} was independent of such variation.

Approximately 28% of the utilized activity was recovered in the lactic acid, 7% in the CO_2, and 1% in the glutamic acid. This indicated a more complex fate for substrate glucose than appeared to be the case

from earlier non-tracer studies: almost two-thirds of the label metabolized was not yet accounted for. Radioautograms prepared from the alcohol extracts of calvaria in experiment 3 indicated the fate of a part of this. Alanine, aspartic acid, and glutamic acid were all quite heavily labeled. Faint spots corresponding to some of the phosphorylated glycolytic intermediates were present, and traces of activity were detected in citric and malic acids, intermediates of the TCA cycle.

Incorporation of label into intermediates of glucose metabolism with time was studied in experiment 5. Skulls were removed from phosphate-buffered medium (pH 7.4) containing glucose-U-C^{14} after 15 minutes, 30 minutes, 1 hour, 2 hours, and 3 hours of incubation respectively. Two-dimensional paper chromatography of aliquots of the alcohol extracts of these skulls was carried out. From the radioautograms of these paper chromatograms it could be seen that there was a steady progression of label into the metabolic intermediates formed. After 15 minutes of incubation, lactate was fairly heavily labeled, but glucose yielded a much darker spot. The phosphorylated glycolytic intermediates contained more activity than the amino acids. Darkened areas corresponding to citric and malic acids were barely perceptible. After 3 hours of incubation, lactic as well as glutamic acid had become approximately as heavily labeled as glucose. This indicated active participation of the glycolytic and tricarboxylic acid cycles in the metabolism of glucose-U-C^{14} by calvaria. Aspartic, citric, and malic acids yielded darker spots than those in the earlier radioautogram: a greater quantity of radioactivity had been incorporated into intermediates of the TCA cycle after 3 hours than

after 15 minutes. There was a much greater quantity of activity in glutamic acid than in the phosphorylated glycolytic intermediates after 3 hours. This showed that, with time, much of the activity from the glucose had moved on through the glycolytic and tricarboxylic acid cycles to form labeled glutamic acid. Since glutamic acid is used in protein synthesis, it was quite likely that at the glucose level used (1.105 μmoles/ml), rat calvaria were synthesizing protein.

In experiments 7, 8, and 10, the effect of different substrate levels and medium phosphate concentration upon glucose-U-C^{14} metabolism by calvaria was studied. In a given experiment, with the same phosphate concentration in each flask, the production of lactate-C^{14} from glucose-C^{14} was directly dependent on the glucose concentration. The specific activity of the glucose in the medium after incubation was found to be lower than the initial specific activity, the decrease being greater in phosphate-buffered medium than in THAM-buffered medium. In flasks with similar glucose, and therefore similar glucose-1-phosphate levels the specific activity of the lactate produced was also lower in phosphate than in THAM-buffered medium. Thus the production of unlabeled glucose and lactate was dependent upon the medium phosphate concentration. Allowing for the variability which occurred in the phosphate-buffered medium, the production of unlabeled lactate was independent of the glucose concentration. From the above results it can be seen that whenever the ratio of the concentrations of glucose-1-phosphate to inorganic phosphate was lowered more dilution of the glucose and lactate occurred. As developed more fully in Chapter VII, this finding is consistent with the hypothesis

that the major source of the endogenous dilution occurring was glycogen, the hydrolysis of which is directly dependent upon the ratio

$$\frac{\text{inorganic phosphate}}{\text{glucose-1-phosphate}}.$$

The specific activity of the intermediates of glycolysis and therefore of all subsequent metabolic intermediates will be modifiable by the presence of glycogen in the tissue to an extent dependent upon its rate of hydrolysis. This will be more pronounced at low glucose and/or high inorganic phosphate levels. There is no data to indicate the effect of varying glycogen levels upon the rate of hydrolysis, but according to Conn and Stumpf, 1963, the equilibrium of the phosphorylase reaction is independent of the polysaccharide concentration, provided a certain minimum concentration is exceeded.

It is of interest to relate the present work to some of the recent findings on the action of parathyroid hormone. Effects of this hormone on various aspects of cellular and subcellular metabolism which have been reported to date are many, and as yet interrelationships between many of them are unclear. However, a considerable amount of evidence exists showing stimulation of phosphate uptake by intact beef heart mitochondria (Brierley, Bachmann, and Green, 1962) and by rat liver mitochondria (Sallis, DeLuca, and Rasmussen, 1963a and 1963b), of P^{32} incorporation into the organic phosphates of bone (calvarium) in rats (Egawa and Neuman, 1963), and of phosphate accumulation and transport by the rat intestine (Borle, Keutmann, and Neuman, 1963).

Inorganic phosphate occupies an important position as a control

mechanism in glycolysis and has been shown in the present work to increase the rate of breakdown of glycogen to pyruvate but not apparently to affect the rate of uptake of glucose. In earlier work with identical tissue preparations (Dowse et al, 1963) it was demonstrated that lactate accumulation was greater in phosphate-buffered medium than in bicarbonate medium, that lactate accumulation was increased by the addition of parathyroid hormone to a greater degree in bicarbonate medium than in phosphate and that the effect of the hormone was present to the same degree over a range of medium glucose concentration from 0 to 17 millimolar. If this accumulation of lactate were due to an increase in lactate production, both glycogen breakdown and glucose uptake must have been stimulated by the hormone, an effect which may well have been a sequence to an increased intracellular phosphate concentration. Data on glucose uptake and lactate specific activity under such conditions would enable a more positive conclusion to be made on this point. It is significant, that the effect of the hormone on lactate was absent in anaerobiosis, for the stimulation of the mitochondrial uptake of phosphate by the hormone is also abolished under anaerobic conditions.

In order to get meaningful results on the effects of parathyroid hormone on the total metabolism of glucose-U-C^{14} by calvaria, it is necessary to have a clear picture of the metabolism of glucose-U-C^{14} by normal calvaria, so that adequate comparisons can be made. For this reason, a quantitative study of the utilization of glucose-U-C^{14} by normal calvaria was made. The overall fate of glucose-U-C^{14} during incubation of calvarial preparations in phosphate (experiment 8) and THAM-buffered medium

(experiment 10) was determined. In the former medium, the average percentage of the utilized activity in the lactate was 28.95%, and in the latter medium, 40.40%. The percentage of utilized activity in CO_2 was fairly similar for all flasks of experiments 8 and 10, but was slightly lower with higher initial glucose concentrations. Up to 58% of the utilized activity in experiment 8 and 34% in experiment 10 was calculated to be in compounds other than glucose and lactate in the medium after incubation. The calculated percentage of total counts utilized to appear in the tissue ranged from 14.4-38.0% in these experiments.

When skulls from experiments 8 and 10 were directly extracted with KOH (group "a"), 8.2-19.1% of the counts utilized were recovered in the extract, and a negligible amount in the solid residue.

A portion of the skulls from experiments 8 and 10 (group "b") were extracted with a series of solvents. The aqueous ethanol and KOH extracts of skulls incubated in phosphate-buffered medium contained approximately equal quantities of activity: 83-86% of the recovered activity in these skulls was found in these extracts. With one exception, the aqueous ethanol extracts of calvaria incubated in THAM-buffered medium contained approximately 62-67% of the recovered activity in these skulls. Between 10-26% was found in the KOH extract. Hence, in phosphate buffer, most of the activity incorporated into the skulls was incorporated into small molecular weight compounds and protein, and in THAM buffer, most of it was incorporated into small molecular weight compounds. Only 1-2% and 1-4% of the recovered utilized activity was found in the petroleum ether extracts of skulls from experiments 8 and 10 respectively. Approximately

13-15% of the recovered utilized activity was found in the TCA extracts (containing polysaccharides) of skulls from experiment 8, and 27% at the lowest glucose level and 15% at the highest glucose level studied in experiment 10.

40-60% of the calculated activity in the skulls was recovered in the extracts of the "a" group, and 12-30% in the extracts of the "b" group of experiments 8 and 10. As all tissue fractions were assayed the poor recovery was presumably due to experimental loss during the subsequent handling of the tissue. This was supported by the fact that the percentage recovery in group "b", which was serially extracted with a number of solvents, was less than that in group "a", where a single extraction with KOH was carried out.

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