THE CONTRIBUTION OF ALTERNATE PATHWAYS OF GLUCOSE METABOLISM TO BIOSYNTHESIS IN BAKERS' YEAST

by

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TO MY WIFE

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INTRODUCTION

Pathways of Glucose Metabolism

The classical pathway

The classical route followed by glucose from its entrance into the cell until its ultimate complete combustion to CO_2 and H_2O is known as the glycolytic-tricarboxylic acid (TCA) pathway.

This sequence of some 19 reactions was constructed by the efforts of a great number of investigators during a period of over 30 years beginning about 1905. For a large proportion of the organisms studied this pathway has been shown to be the principal source of cellular energy, and of carbon skeletons for biosynthesis; particularly of amino acids.

The alternate pathways

That at least one other pathway of glucose utilization existed was known in the late 1930's from the work of Dickens (19), Warburg (41, 42), and Lipmann (33) on the so-called "direct oxidative" pathway, but the work received little attention. Since at the time of this early work the means were not at hand to make a rapid

evaluation of the occurrence and importance of the alternate pathway in a particular organism the general feeling was, apparently, that the oxidative pathway was a laboratory curiosity of limited occurrence among organisms generally, and quantitatively of little significance in its contribution to glucose utilization.

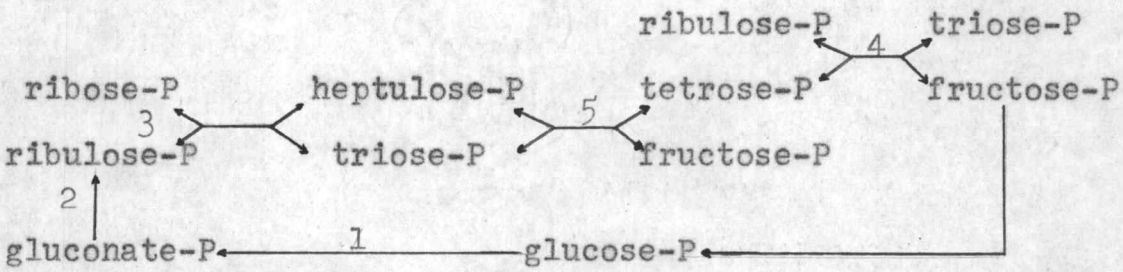
It had been shown by the work of Dickens et al. that yeast contained enzymes capable of oxidizing glucose-6 phosphate to 6-phosphogluconic acid which then yielded one mole of CO_2 and one mole of pentose phosphate. The pentose phosphate could be fermented to yield one mole each of ethanol, CO_2, and a two carbon compound analogous to glycolaldehyde. That these reactions were of a cyclic nature was indicated by the observation by Dische (20) that blood haemolysates decompose ribose from adenosine, causing phosphorus to be consumed and giving products which appeared to include triose and a hexose diphosphate.

Beginning about 1948 there was a renewal of interest in this earlier work. By this time the availability of carbon-14 and the improvement in enzymatic techniques combined to yield rapid progress toward the elucidation of the occurrence and mechanism of this alternate pathway.

This pathway was demonstrated to occur in Escherichia coli, liver, blood haemolysates, and plants and a variety of intermediates were isolated among which were

hexose mono-, and hexose diphosphate; heptulose mono- and di-phosphates, and ribulose phosphate. Horecker and Smyrniotis (26) isolated the enzyme which synthesized sedoheptulose from ribulose-5 phosphate and gave it the name transketolase. Later these same workers isolated the enzyme transaldolase which synthesizes fructose-phosphate from sedoheptulose and glyceraldehyde-3 phosphate, and demonstrated that the "direct" oxidation of glucose was, in fact, a new, complete cyclic pathway. This "pentose cycle" has been demonstrated in liver (5), spinach (3), acetic acid bacteria (14), pea aphid (34), and wheat smut fungi (35).

The pentose cycle in its present form is shown diagrammatically as:



The enzymes involved and their required co-factors are shown in the accompanying table.

reaction no.	enzyme name	co-factors
1	glucose-6 phosphate dehydrogenase	TPN
2	gluconate-6 phosphate dehydrogenase	TPN
3	pentose phosphate isomerase	-
4	transketolase	TPP and Mg ion
5	transaldolase	none

At the present time "direct" oxidation of glucose, at least to the pentose stage, has been shown to occur in varying degrees in a number of microorganisms. It has been demonstrated to occur appreciably in the yeasts Toropsilus utilis and Saccharomyces cerevisiae (9), in E. coli (16), in the mold Penicillium digitatum (36), as well as in several species of Streptomyces (15) and in fruits (13). The "direct" pathway appears to be the dominant route for glucose utilization in bacteria of the Pseudomonas and Leuconostoc groups (38), and in Penicillium chrysogenum (18). In animal tissue, bone marrow and testis (6), rat mammary gland (1), retina (31, 32), and adrenal (30), have thus far been shown to have a high proportion of the "direct" pathway activity.

Still another oxidative pathway has been shown to occur in Pseudomonas saccharophila when glucose is utilized under aerobic conditions (21). In this organism the disappearance of one mole of glucose phosphate is

accompanied by the appearance of two moles of pyruvate as in the glycolytic or Embden-Meyerhof pathway. However, by isolating and degrading pyruvate formed by cells metabolizing glucose-1-C^{14} it can be shown (23) that carbon 1 of glucose forms the carboxyl carbon of one of the pyruvate molecules. The proposed mechanism involves a cleavage between carbons 3 and 4, of phosphogluconic acid formed from the oxidation of glucose-6 phosphate as in the pentose cycle. This route is sometimes referred to as the Entner-Doudoroff pathway.

Function of the alternative pathways. With the wide occurrence of the pentose cycle and its variations clearly established some consideration must be given to the function of this pathway in the cell.

In the broadest sense such a pathway must function to provide either energy, or biosynthesis, or both to varying degrees. Evidence bearing on these points will be discussed in the following paragraphs.

Considering that the direct oxidative pathway may function as a source of energy it is natural to compare the amount of energy which may be available with that provided by the conventional glycolytic scheme. Since no direct studies have been made on the comparative energetics of the alternative pathways little may be said on this question except on a speculative basis. From such a

viewpoint the alternate pathways would appear to be energetically equivalent. In the combustion of one mole of pyruvate to CO_2 and H_2O via the TCA cycle 5 atoms of oxygen are consumed. Based on the studies of Ochoa and others (37, 27) this is equivalent to the production of 15 pyrophosphate bonds. A similar estimation may be made for the equivalent combustion via the pentose pathway.

From the work of Lehninger and Smith (30) it is known that the P/O ratio (number of phosphate bonds formed/ number of atoms of oxygen consumed) for the oxidation of DPNH is approximately 3. Since the two dehydrogenases of the pentose pathway are either TPN or DPN linked, oxidation of the first three carbons of glucose could yield 18 moles of ATP, for a net release of energy at least equal to that of the TCA cycle.

The possible role of the oxidative pathway in biosynthesis is founded on more direct evidence than that cited above concerning its contribution to the cellular energy requirements.

Some experimental evidence is available to indicate that sedoheptulose formed via the pentose pathway may be an intermediate in the biosynthesis of aromatic amino acids. Gilvarg and Bloch (22) on allowing yeast to grow on glucose-1-C^{14} found a high incorporation of activity into phenylalanine and tyrosine. The specific activities

were such that a rather direct conversion was suggested. Most of the activity was either in carbon 3 of the side chain or carbons 2 and 6 of the ring. It was suggested that this situation might arise from the formation of a 7 carbon compound from a tetrose and a triose with subsequent cyclization. Similar studies of the biosynthesis of shikimic acid by E. coli mutants (39) have shown that when the mutant was grown on glucose-3,4-C^{14} the distribution of the activity in shikimic acid was such as to indicate that two or more fragments of the hexose chain must combine in the biosynthesis, and in such a manner that carbon 6 is better utilized than carbon 1.

Evidence for the participation of pentose cycle ribose in the formation of ribose nucleic acid (RNA) has been provided from two types of experiments performed by Cohen (16). In one experiment E. coli was grown on glucose-1-C^{14} and the ribose of RNA isolated. Such ribose showed a specific activity of 20-30% that of the glucose-1-C^{14}. This indicated that this pentose must have arisen from a pathway involving preferential loss of carbon 1 of glucose.

In another experiment on E. coli the radiochemical yield[1] of CO_2 from glucose-1-C^{14} for normal growing cells

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[1] The radiochemical yield of $C^{14}O_2$ is defined as the per cent of the activity initially present recovered as $C^{14}O_2$.

was compared with that for cells infected with the bacterial virus T2. In the normal cells the ratio of ribose nucleic acid to deoxyribose nucleic acid (RNA/DNA) was from 3:1 to 5:1. In the virus infected cells no RNA synthesis took place while DNA synthesis was stimulated fourfold. In the normal cell from 16% to 38% of the glucose was metabolized via the "direct oxidative" pathway while in the infected cells the contribution of the "direct" pathway dropped to a value of from 6% to 29%. Cohen points out that these differences are real and reproducible; and are of the same order of magnitude as the shift in the amounts of ribose and deoxyribose produced. That this is not due to inhibition by the virus of one of the steps of the oxidative pathway is shown by the fact that virus and DNA synthesis may be forced through this path when gluconate, ribose, arabinose, or guanosine, are utilized as sole carbon sources. It would appear from these data that while a major function of the "direct" pathway in some organisms may be the furnishing of ribose for RNA synthesis this is not the only function. Cohen's results show that even under conditions in which no RNA synthesis is taking place the preferential decarboxylation of carbon 1 of glucose still continues at an appreciable rate (6-29%).

Another experiment in which a high requirement of

the cell for RNA synthesis may be correlated with an appreciable proportion of "direct oxidation" of glucose is that of Agranoff et al. (2) on the ratio of the radiochemical yield of $C^{14}O_2$ from glucose-1-C^{14} to the radiochemical yield of $C^{14}O_2$ from glucose-6-C^{14}; hereafter referred to as the C_1/C_6 ratio.[2] The data of Agranoff on the C_1/C_6 ratio is compared in the tabulation below on the ratio of the turnover rate of RNA to the turnover rate of DNA for the same tissues (12).

tissue (rat)	C_1/C_6	turnover ratio RNA/DNA
normal liver	3.33	5.18
regenerating liver	2.1	1.27
fetal liver	2.5	about unity

Similar results reported by Bloom (6) are suggestive of a correlation between the need for a high rate of RNA synthesis and appreciable pathway activity.

tissue (rabbit)	C_1/C_6	turnover ratio RNA/DNA
spleen	2.13	3 (11)
testis	3.28	high[3]

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[2] A C_1/C_6 ratio greater than one is generally regarded as indicating the presence of the oxidative pathway. See p. 12.

[3] The ratio RNA/DNA for this tissue is 2.6 (24), and it has been shown that the rate of DNA turnover is high (17). Thus a high requirement for RNA synthesis is inferred.

Estimation of the quantitative importance of the alternate pathways. Once the oxidative pathway had been shown to exist in a variety of organisms, and evidence of its possible role in biosynthesis began to accumulate, the next logical development was to attempt to devise means by which the contribution of this pathway to carbohydrate metabolism might be established.

The fact that the availability of carbon-14 greatly simplified the task of evaluating the importance of the oxidative pathway was mentioned earlier. A number of suggestions have been made and equations proposed to permit evaluation of the over-all contribution of the pentose pathway to glucose metabolism but, in general, the methods are based either upon the radiochemical yields of $C^{14}O_2$ from glucose labeled in different carbons or on the extent and location of labeling in a chosen intermediary metabolite when an isotopic substrate is metabolized.

An example of the latter method is that recently used by Blumenthal *et al.* (9) to determine the contribution of the pentose pathway to glucose utilization in *S. cerevisiae* and *T. utilis*. In this method the organism was allowed to metabolize glucose-1-C^{14} and glucose-U-C^{14} (glucose uniformly labeled on all carbons), and the specific activity of acetate isolated from the cells grown on the different substrates was compared. If the

specific activity of acetate is compared with that of the glucose-1-C^{14} used, on the basis of activity (cpm) per millimole (mM) of carbon then the acetate should have a specific activity of 1.5 times that of the glucose-1-C^{14} if all the glucose is metabolized via glycolysis. If all the glucose-1-C^{14} is utilized by means of the oxidative pathway the specific activity of the acetate isolated should be zero since all the active carbon will have been removed as CO_2. In practice the maximum specific activity of the acetate would be less than 1.5 times that of the glucose-1-C^{14} due to the formation of unlabeled acetate from endogenous glucose. If glucose-U-C^{14} is metabolized the acetate formed would have a specific activity equal to that of the glucose-U-C^{14} if there were no dilution of the acetate from the labeled glucose by non-isotopic acetate. If dilution does take place the extent of this dilution may be determined and a correction applied to the data for the contribution from endogenous glucose.

In this paper a quantity called the relative specific activity (RSA) was defined as the ratio of the specific activity of acetate to the specific activity of glucose, times 100. The corrected specific activity was then defined as the ratio:

$$\frac{\text{observed RSA for acetate from glucose-1-}C^{14}}{\text{observed RSA for acetate from glucose-U-}C^{14}},$$

assuming that two carbon units were being produced only

from the glycolytic-TCA, or the direct oxidative pathways. The final relation used to calculate the per cent of glucose metabolized via the glycolytic pathway was:

% glucose via E.M. = corrected RSA x 100/1.5.

The method first mentioned based on the radiochemical yields of $C^{14}O_2$ is relatively simple and widely used since it is readily adaptable to Warburg studies. The basic idea is briefly as follows.

If glucose is completely oxidized to CO_2 via glycolysis and the citric acid cycle the CO_2 formed first is from carbons 3 and 4 of glucose which became the carboxyl carbons of pyruvate (glycolysis). At the next turn of the TCA cycle carbons 2 and 5 of glucose appear together as CO_2, followed finally by CO_2 from carbons 1 and 6. If glucose-1-C^{14} is metabolized by an organism in which the pentose pathway is appreciable, some of the radioactive carbon of glucose-1-C^{14} will be removed as $C^{14}O_2$ long before activity from glucose-6-C^{14} begins to appear as respired CO_2. Therefore at any given time the proportion of the initial activity recovered as $C^{14}O_2$ will be higher for cells using glucose-1-C^{14} than it will be for cells using glucose-6-C^{14}. This proportion of the initial activity recovered is the radiochemical yield, as previously defined. If glucose is exclusively burned via the glycolytic-TCA pathway, $C^{14}O_2$ from carbons 1 and

6 of glucose will appear simultaneously and in equal quantity so that the C_1/C_6 ratio (defined earlier) will be unity. Estimates of the relative importance of the pathways of glucose metabolism made on this basis are responsible for most of the data available on the comparative biochemistry of these alternate pathways. The fundamental limitation of this method is that it is cumulative. The basis for such a statement will be clearer when data for a typical time course study with glucose-1-, glucose-2-, and glucose-6-C^{14} have been presented but it is clear that a C_1/C_6 ratio determined at the end of a given interval gives a measure, not of the relative contribution of the alternate pathways at the time the sample was taken, but of the over-all relative contributions of the alternative paths for the entire interval. Such information is obviously very useful, but only if some indication is given regarding the conditions to which the ratio applies such as the duration of the experiment or, preferably, the amount of glucose utilized at the point for which the ratio is given. Such necessary qualifying information has often not been given in conjunction with experimental results. The use of the C_1/C_6 ratio has the additional disadvantage that it is not directly usable for a calculation of the proportional contributions of the alternate pathways.

Since the radiochemical yield of $C^{14}O_2$ is a convenient quantity to measure in manometric work, a number of papers have been published on methods for using radiochemical yields of $C^{14}O_2$ for quantitative estimation of the occurrence of the alternate pathways.

The original equations of Bloom et al. (7) were derived on the assumptions that only carbon 1 of glucose contributed to CO_2via the oxidative pathway, and that the conversion of pentose to a triose plus a two carbon fragment did not take place. The equations derived on this basis require measurement of the radiochemical yields of $C^{14}O_2$ from lactate-1-, lactate-2-, lactate-3-, glucose-1-, and glucose-U-C^{14}. From these equations Bloom et al. calculated that at least 75% of the glucose catabolized by rat liver slices went by way of the oxidative pathway.

Katz et al. (28) pointed out an error in the derivation of Bloom and called attention to the fact that the high concentrations of lactate and acetate used by Bloom et al. depressed glycolysis. From their own equations Katz et al. calculated that 20% or less of glucose utilized by rat liver slices was metabolized via the oxidative route.

Abraham et al. (1) attempted to apply the equations of Katz to results obtained with rat mammary gland but found that in this tissue the assumption that only carbon

1 of glucose contributed to CO_2 by the oxidative route was untenable since twice as much isotopic fatty acid was recovered from glucose-6-C^{14} as from glucose-1-C^{14}, indicating that the pentose formed by preferential decarboxylation of glucose must be further metabolized. Thus for such tissues the equations of Katz could not be used.

A recent report by Bloom et al. (8) included derivations of two new expressions, based in part on those originally presented. One expression required use of lactate-1-, lactate-3, and glucose-1-C^{14} while the other required the use of all three of the above substrates in addition to glucose-6-, gluconate-1-, and gluconate-6-C^{14}. The results from both equations were in good agreement, and indicated that about 50% of the glucose metabolized by rat liver slices followed the pentose pathway.

The latest paper by Katz et al. (29) has extended their equations so that the fraction of glucose metabolized via the oxidative pathway may be calculated from a single equation which includes terms accounting for CO_2 produced by both pathways. They conclude that the fraction of CO_2 produced glycolytically is from 83-98% for rat liver slices but note that if carbons other than carbon-1 contribute to CO_2 in the reactions of the oxidative pathway the extent of this pathway will be greater than that calculated.

The Present Studies

The course of the research described in this thesis was influenced by the following factors.

1. The great majority of studies made previously on yeast and other organisms, with the intent of evaluating the contribution of the alternate pathways had been made on resting cells. Thus, only the contribution of these various pathways to respiration was being determined. In view of the interest in a possible biosynthetic role of the oxidative pathway it seemed desirable to repeat previous studies in a medium which would permit cell growth.

2. In order to make such determinations more nearly comparable and meaningful it was felt that the conditions should be standardized as nearly as possible.

3. Although the use of selective inhibitors had contributed greatly to the elucidation of the classical scheme of glucose degradation and to the discovery of the existance of alternate pathways, little work had been done on changing the C_1/C_6 ratio by this means.

4. Beevers and Gibbs (4) had reported the results of an experiment in which they determined the C_1/C_6 ratio at intervals of 3, 5, and 11 hours; and found that the C_1/C_6 ratio decreased during this period from 2.53 to 0.38.

Consideration of these facters led to the use of two types of experiments. The first type utilized manometric methods to determine the C_1/C_6 ratio in a growth medium under specified conditions. Since iodoacetate is a known respiratory inhibitor for yeast (5) its effect on the C_1/C_6 ratio was investigated in one series of experiments. Since indoleacetic acid had been shown to increase oxygen consumption in plants (10) and in liver and kidney tissue (43), its ability to change the oxygen consumption and the C_1/C_6 ratio in yeast was studied in a second series of experiments. In addition to these studies the differences in the incorporation of activity into amino acids from ribose-1-, acetate-1-, and acetate-2-C^{14} were investigated using Warburg techniques.

The second type of experiments performed were time course studies, both of the changing patterns of the activity of respiratory CO_2, and of the incorporation of activity into cellular components for glucose-1-, glucose-2-, and glucose-6-C^{14}. This technique made it possible to circumvent the restriction to cumulative data imposed by traditional Warburg methods. In addition the ease of following the level of glucose in the cell suspensions under investigation made it possible to complete the overall picture of the role of the pentose pathway in yeast by continuing the experiments far beyond the time of depletion of glucose from the medium.

EXPERIMENTAL AND RESULTS

Growth of the Yeast

Fifty ml. of a malt extract medium,[4] previously steamed for 15 minutes, was inoculated with a small loop of yeast cells from a malt slant.[5] This inoculum was incubated 16 hours at 30° C., and then used to inoculate the remaining 950 ml. of the steamed malt medium. The second incubation period also lasted 16 hours at 30° C. During this time the suspension was stirred at 200 r.p.m., and the pH occasionally adjusted to 4.0 to 4.5 by the addition of NH_4OH.

At the completion of the second incubation period the suspension was centrifuged, and the yeast washed twice with cold distilled water. Ten grams of the wet cells were transferred to 1 liter of a salts medium.[6] After 4 hours incubation at 30° C. (pH 4.0 to 4.5) the glucose was exhausted from the medium. At this time the suspension was centrifuged and washed as before. The

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[4] Two hundred gm. Blue Ribbon malt extract, 2 gm. KH_2PO_4, 1 gm. urea, tap water to make 1 liter.

[5] Ten gm. Blue Ribbon malt extract, 0.2 gm. KH_2PO_4, 1.5 gm. agar, tap water to 100 ml.; pH 4.8.

[6] Eighteen gm. glucose, $(NH_4)_2SO_4$ 2.5 gm., NaCl 2 gm., KH_2PO_4 2 gm., $MgSO_4 \cdot 7H_2O$ 250 mg., $CaCl_2 \cdot 2H_2O$ 250 mg., H_3BO_3 1 mg., $ZnSO_4$ 1 mg., $MnSO_4 \cdot 4H_2O$ 1 mg., $FeCl_3$ 1 mg., $TlCl_3$ 0.5 mg., $CuSO_4 \cdot 5H_2O$ 0.1 mg., KI 0.1 mg., Bacto-yeast extract 100 mg., distilled water to make 1 liter.

desired amount of cells was then resuspended in the salts medium, or in a phosphate buffer medium of pH 5.0, as the experiment required.

Warburg Experiments

Experiment W-1

In this experiment 4 large Warburg vessels were used. Each vessel contained 27.3 ml. of a homogenous suspension of yeast in the salts medium, at a concentration of 4 mg./ml. of yeast (dry weight). This cell concentration was used throughout the experiments described in this thesis. To the suspension were added 250 micromoles of glucose containing 1 microcurie of radioactivity. In this experiment the C_1/C_6 ratio in salts medium was compared to the C_1/C_6 ratio in the same medium containing a high concentration of indoleacetic acid. The 4 flasks were divided into 2 sets. One pair of flasks contained the salts medium, while the other set contained, in addition, 100 micrograms of indoleacetic acid (IAA) per cup. One flask in each set contained glucose-1-C^{14}, the other contained glucose-6-C^{14}. Each flask contained 1.5 ml. of 6N HCl in the side arm, and 1.2 ml. of 5N, CO_2-free, NaOH in the center well.

The flasks were allowed to equilibrate in the water bath at 30° C. for 10 minutes after which the manometers

were closed and readings taken of the change in manometer level every 10 minutes. This was continued until the desired level of oxygen consumption had been reached, at which time respiration was stopped by tipping in the acid from the side arm. The vessels were left in the water bath for an additional 20-30 minutes.

After the flasks were removed from the manometers, aliquots of the cell suspensions and of the center well solutions were withdrawn. The cells were centrifuged, washed and dried. The aliquots from the center wells were diluted with water and the carbonate was precipitated as $BaCO_3$ by the addition to the center well solutions of 10 ml. of a precipitation mixture, 1 M in $BaCl_2$ and 1 M in NH_4Cl. When small flasks were used a standard volume of Na_2CO_3 solution was added to each aliquot to serve as a carrier. The $BaCO_3$ was plated on aluminum planchets by the centrifugation technique and the plates assayed for radioactivity on an end window Geiger-Muller counter. Corrections for background and for self-absorption of the $BaCO_3$ were applied in the conventional manner.

From the total activity of these samples and the amount of radioactivity initially present, the radiochemical yields were computed, and from this the C_1/C_6 ratios calculated. The results of this experiment are given in Table 1, flask numbers 1 to 4.

Experiment W-2

In this investigation the C_1/C_6 ratio in the salts medium was compared to that in a phosphate buffer medium. Four large Warburg vessels were used. These were divided into two parts containing glucose-1-C^{14} or glucose-6-C^{14} in either salts medium or buffer. The glucose concentration was 300 micromoles with a total activity of 0.1 microcurie. The cell concentration was the same as in the preceding experiment. The results are shown in Table 1, flasks numbers 5 to 8.

Experiment W-3

This study utilized 8 small Warburg vessels and was designed to establish the concentration of indoleacetic acid required to appreciably affect the respiration of the yeast cells. Each flask contained 2.0 ml. of yeast suspension in salts medium and 10 micromoles of glucose. Each flask contained 0.2 ml. of either 5N NaOH, CO_2-free, or 0.2 ml. of distilled water in the center well, and 0.3 ml. of 6 N HCl in the side arm. Each flask contained from 1 to 50 micrograms of indoleacetic acid (IAA). The results are shown in Table 2.

Experiment W-4

This experiment was made using yeast that had been grown for experiment W-3 and refrigerated overnight. Concentrations of cells, glucose and side arm contents were

the same as in the preceding experiment. All center wells contained NaOH. The C_1/C_6 ratios of cells utilizing glucose alone were compared to the C_1/C_6 ratio for cells utilizing glucose in the presence of IAA (50 micrograms per flask) or iodoacetic acid (1.5×10^{-3} molar). The level of radioactivity was 0.1 microcurie per flask. Table 3 gives the results.

Experiment W-5

This experiment was performed to compare the C_1/C_6 ratio of cells using glucose in a salts medium with the same ratio in the presence of iodoacetatic acid (IOA), and with the C_1/C_6 ratio in phosphate buffer. Substrate and IAA concentrations were the same as in the preceding experiment. The concentration of IOA was reduced one tenth. The initial radioactivity was 0.2 microcurie and the volume of NaOH in the center wells was increased to 0.4 ml. The data are presented in Table 4.

Experiment W-6

The incorporation of ribose-1, acetate-1, and acetate-2-C^{14} into yeast amino acids was studied in this experiment. The level of radioactivity was 2.0 microcurie per flask. Fifteen micromoles of ribose and 10 moles of acetate were used with cells in salts medium at the usual concentration of 4 mg. of dry yeast/ml. (2.0 ml. per flask). These cells had been grown for the

preceding experiment and refrigerated for about 12 hours before use. In each case the experiment was continued until the oxygen consumption had fallen to a low and constant value.

The cells in this experiment were centrifuged, washed, and dried as previously described. After drying, the cells were weighed and sealed into Pyrex test tubes with 1 ml. of 6 N HCl and hydrolyzed by autoclaving for 10 hours at 15 p.s.i. The hydrolyzates were filtered to remove the humin, then dried in vacuo over P_2O_5 and KOH pellets. After drying, the hydrolyzates were diluted with distilled water to the desired concentrations. These hydrolyzates were then subjected to one dimensional chromatography on paper, in two different solvent systems, butanol, acetic acid, and water, 4:1:5 (BAW), and secondary butanol-ammonia.[7]

Radioautographs were prepared by stapling or taping a finished chromatogram to a sheet of Eastman no-screen x-ray film and taping the 2 sheets firmly to a piece of heavy cardboard or wallboard. This procedure was carried out in a darkroom under an x-ray "safelight". Before

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[7] The secondary butanol solvent is prepared by thoroughly agitating 120 ml. of peroxide free secondary butanol with 40 ml. of 3% ammonia. The aqueous phase is drained off and used to saturate the chromatography chamber while the butanol phase is used for development of the chromatogram.

exposure to light the films were covered with black paper and then with several layers of aluminum foil and stored in a black cloth bag. The x-ray film required approximately 10^6 beta particles per square centimeter to give a good spot; therefore, the exposure time was varied accordingly. After exposure was complete the films were developed in the conventional manner. The information obtained from these radioautographs, as well as from radioautographs prepared in experiments described below is discussed under the heading "Incorporation Data" in the final section of this thesis.

The results of the Warburg experiments are summarized in Table 5.

Time Course Studies

The apparatus used in these experiments is shown in Figures 1 and 2. The complete system is shown in Figure 1, while Figure 2 gives the details of the reaction flask. This system was designed to permit the yeast suspension in the reaction flask to be kept in constant and vigorous agitation by the influx of dry, CO_2-free, gas from the gas scrubbing train. The respiratory CO_2 produced by the organism was swept out of the reaction flask by the stream of gas and was collected in the trap which was filled with 10 ml. of CO_2-free NaOH (about 1 N) and 20

ml. of CO_2-free distilled water. Usually 2 or 3 drops of octanol were added to the trap solution to prevent violent foaming. Periodically samples of the medium and yeast were taken through the sampling tube (Figure 2) by applying mild suction to a filter flask containing a 4 inch test tube and attached to the sampling tube by means of the rubber stopper shown in the figure.

When desired, substrates were added to the reaction flask through a vial seal in the center neck of the flask. Since such seals proved to be somewhat unreliable under prolonged vacuum, they were usually replaced by a small rubber stopper.

In a typical experiment the yeast suspensions were pipetted aseptically into the reaction flasks by removing the center fitting, then the vacuum applied and adjusted to give the desired degree of agitation and the suspensions aerated 10 or 15 minutes prior to the addition of substrate. The substrate was added through the center well, the trap solution renewed, and an initial medium and cell sample taken immediately after thorough mixing had taken place. Samples of the medium were usually removed every hour until the glucose had disappeared from the medium. Trap solutions were replaced every half hour until several hours after disappearance of the glucose, after which they were changed hourly. The time required

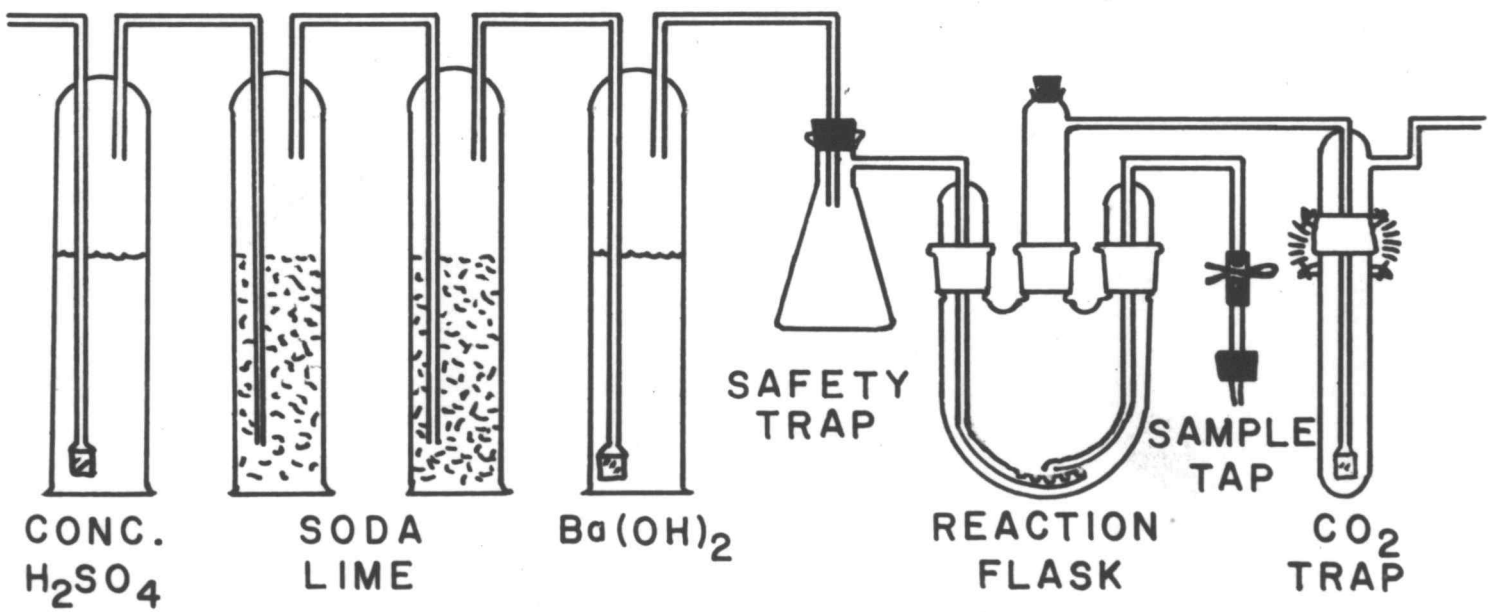
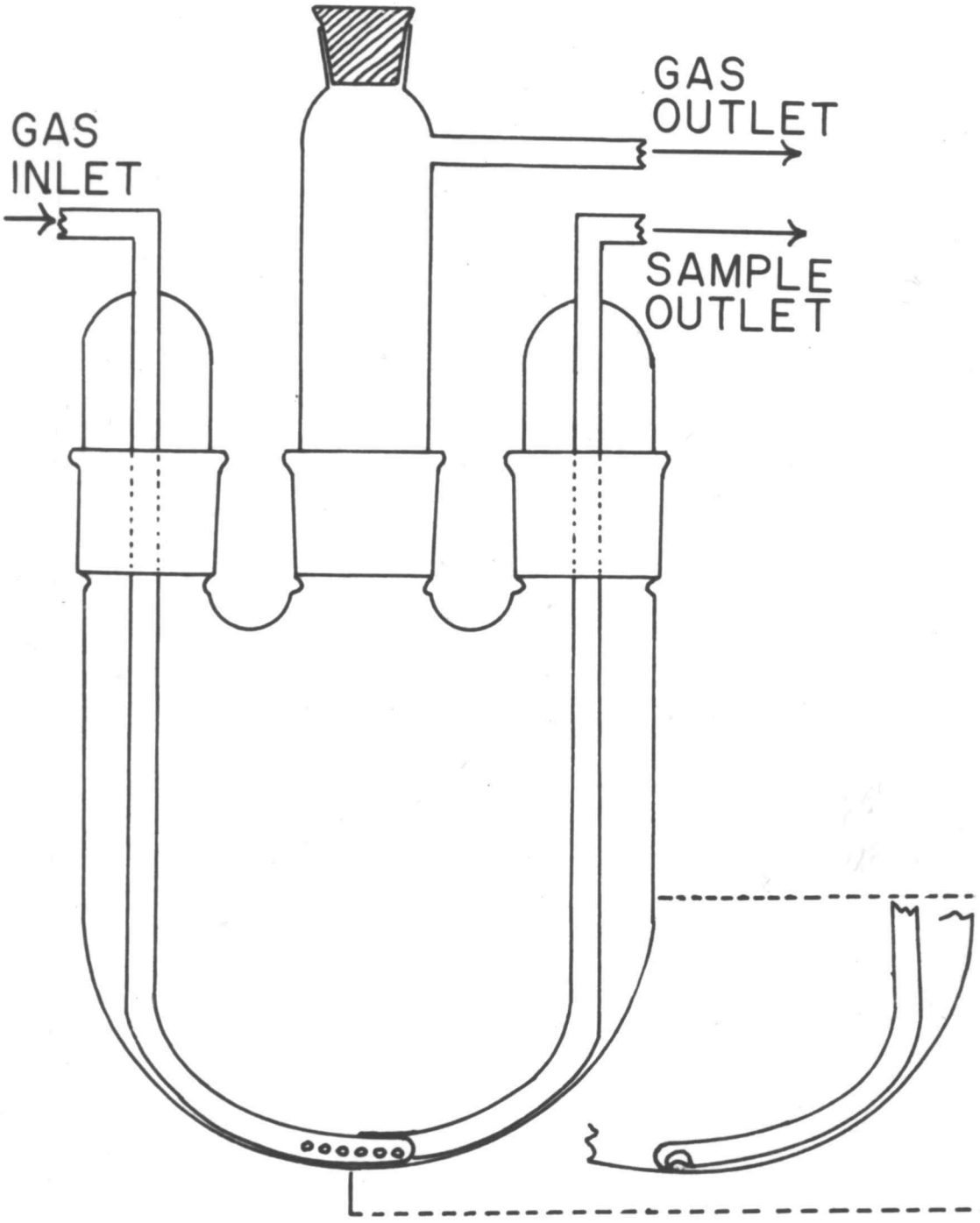

CONC.
H_2SO_4
SODA
LIME
$Ba(OH)_2$
SAFETY
TRAP
REACTION
FLASK
SAMPLE
TAP
CO_2
TRAP

FIGURE 1

FIGURE 2



to replace a trap solution was 20 to 30 seconds.

The medium and cell samples were immediately centrifuged after addition of acid to kill the cells. The cells were washed once with water and then dried while the medium was assayed for glucose by counting an aliquot with an end-window counter, or by the Shaffer-Somogyi-Hartmann method.

The CO_2 samples were precipitated as $BaCO_3$ as previously described except that no carrier was required.

In the experiments to be described glucose-1-, glucose-6-, and glucose-2-C^{14} were commonly used. The data obtained by radiochemical assay of the respiratory $C^{14}O_2$ for the varieties of glucose used could be presented in several ways.

Comparison could be made on the basis of specific activities (counts per minute per mg. of $BaCO_3$), or on a per cent specific activity basis (see below). In addition the results could be given in terms of the radiochemical yield. The specific activity was determined by simply counting the $BaCO_3$ sample, correcting for background and self-absorption and dividing the final figure for the counts per minute (c.p.m.) by the weight of $BaCO_3$ counted. One hundred per cent specific activity was calculated by dividing the total initial activity by the amount of glucose initially provided, expressed as $BaCO_3$, and the measured specific activity divided by this value is the

per cent specific activity. Calculation of the radiochemical yield was made by dividing the total activity (c.p.m.) of a sample by the amount of activity initially present. Such data, plotted vs. time, are shown in Figure 3 for a typical time course study. In addition a carbon recovery curve is shown. The values on this latter curve were calculated by dividing the weight of $BaCO_3$ of each sample by the weight of glucose supplied, expressed as $BaCO_3$. Figure 3 also shows a glucose depletion curve.

The results of the 4 time course studies to be described below will be discussed in relation to the typical results shown in Figure 3.

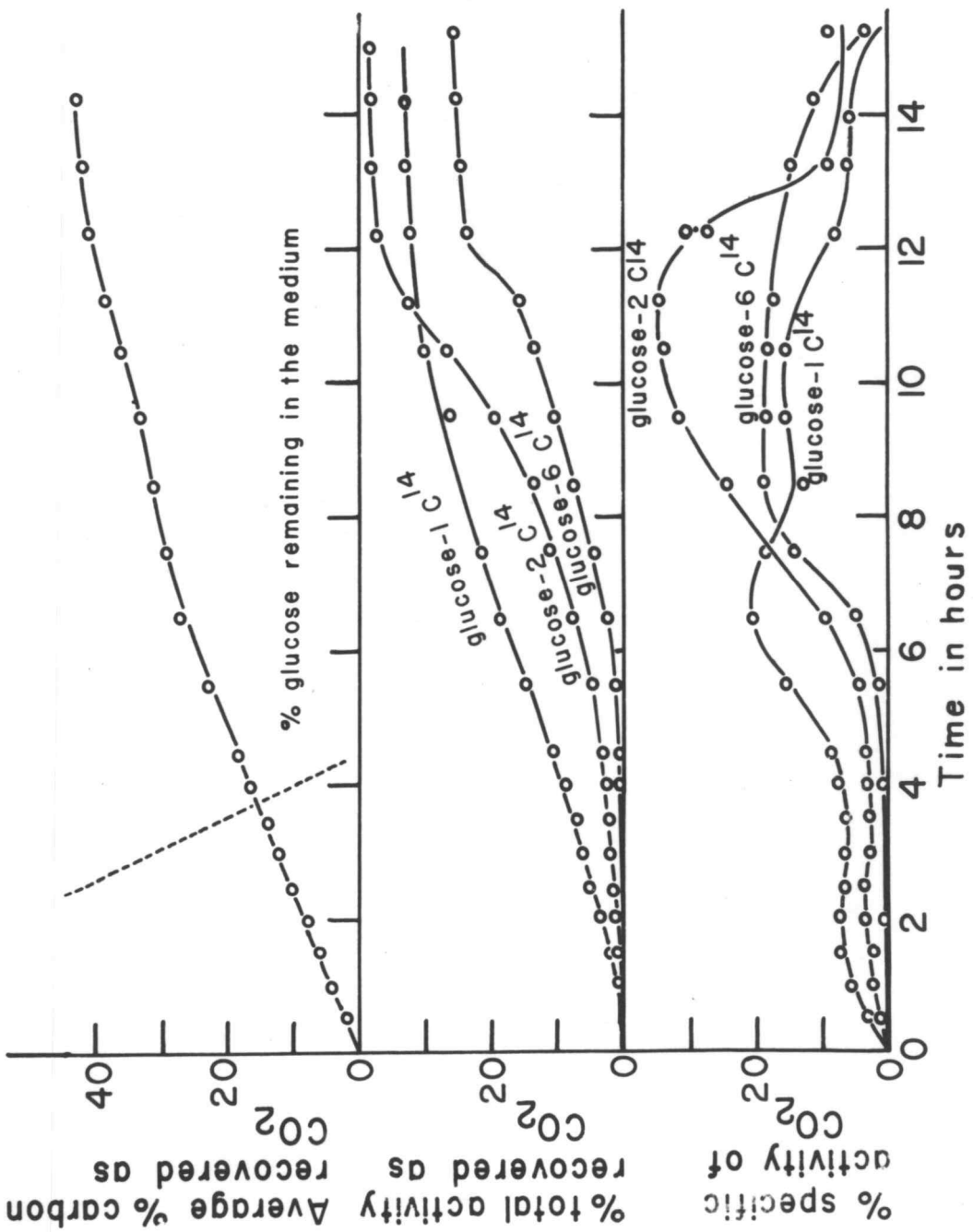
Experiment TC-1

This experiment was made before the apparatus shown in Figure 2 had been developed. In this experiment a similar flask was employed, but rubber stoppers were used in place of the ground joints shown in Figure 2. This experiment was performed at 30° C. with oxygen as the sweeping gas.

The yeast was grown as previously described. The amount of yeast used in each flask was 720 mg. (dry weight), in 200 ml. of salts medium. The glucose level was 5 millimoles per flask, with a radioactivity of approximately 5.5 microcuries.

In this experiment the yeast suspension was swept

FIGURE 3


% glucose remaining in the medium
Average % carbon recovered as CO2
% total activity recovered as CO2
% specific activity of CO2
glucose-1 C14
glucose-2 C14
glucose-6 C14
Time in hours
0
2
4
6
8
10
12
14
20
40

with oxygen for five minutes before the glucose solution was added by hypodermic syringe through a vial seal.

In comparison with Figure 3, which represents data taken at 21° C., the data of experiment TC-1 (Table 6) show that the effect of the higher temperature is to increase the rate of utilization of glucose although the proportional contributions of the alternate pathways of glucose utilization do not change appreciably: in experiment TC-1 the duration of the initial flat portion of the per cent specific activity curves was reduced to 2 hours (in comparison with Figure 3), at which time glucose disappeared from the medium. At that point the value of the radiochemical yield (per cent total activity recovered as $C^{14}O_2$) from glucose-1-C^{14} was 14.5 per cent. The final levels of the radiochemical yields and the amount of carbon recovery shown in Figure 3 were reached in experiment TC-1 after 9½ hours.

About 5 mg. of each of the periodically removed yeast samples were hydrolyzed as previously described and chromatogramed in BAW, 4:1:5, and in 80% phenol. After the finished chromatograms were dried, radioautographs were prepared as previously described.

Experiment TC-2

This investigation was carried out using the apparatus pictured in Figure 2. The experiment was designed to

duplicate the previous study with the isotope concentration raised to 10 microcuries in order that radioautographs of the cell hydrolyzates would develop more quickly. The cells were grown as usual and 180 mg. (dry weight) in 200 ml. of salts medium were added to each flask. The glucose concentration was 4.5 millimoles per flask. The experiment was carried out at 30° C., and the glucose disappeared from the medium in about 4 hours. The experiment was terminated after 6 hours and the cells dried, hydrolyzed, chromatographed and radioautographed as before. The per cent specific activity (p.s.a.) curves were plotted for glucose-1-, glucose-2-, and glucose-6-C^{14} and were in good agreement with those of the previous experiment.

The data taken for the respiratory CO_2 are tabulated in Table 7.

Experiment TC-3

In this experiment 100 mg. of cells and 4.6 millimoles of glucose were added to each of 7 flasks at room temperature (about 21° C. throughout the experiment) and swept with air. Each flask contained 200 ml. of medium.

One set of 3 flasks contained glucose-1-, glucose-2-, or glucose-6-C^{14} in the salts medium, while another pair of flasks contained glucose-1-, and glucose-6-C^{14} in phosphate buffer medium. The remaining pair of flasks

contained cells in salts medium and glucose but no isotope. After 3½ hours glucose-1-, and glucose-6-C^{14} were added to these latter flasks. The $C^{14}O_2$ data for this series of experiments are presented in Tables 8 through 10. Glucose remained in the salts medium for about 7 hours.

The CO_2 curves for glucose-1-, -2-, and -6-C^{14} in salts medium were, as expected, essentially the same as those already shown (Figure 3) for these experimental conditions. Likewise, the results were similar in the pair of flasks to which the addition of isotopic glucose had been delayed.

The data obtained from the respiratory CO_2 of the cells utilizing glucose in buffer medium were in agreement with the results obtained manometrically. The p.s.a. curve for glucose-1-C^{14} showed an initial rapid rise, as in salts medium, followed by a period of gradual rise which extended throughout the duration of this experiment. For glucose-1-C^{14} the p.s.a. curve was the same as in previous experiments. The glucose-6-C^{14}curve rose appreciably higher than for the corresponding curve in salts medium, but remained below the glucose-1-C^{14} curve. The result, in terms of radiochemical yields, was that the C_1/C_6 ratio was nearly unity, while glucose was still present in the medium and rose slowly thereafter. The C_1/C_6 ratio was always much lower than the ratio

in salts medium. In these studies glucose was present in the flasks containing salts medium for about 7 hours. The experiment was continued for 6 hours beyond the point of disappearance of glucose from the medium. For the 3 flasks containing the phosphate buffer, medium glucose was still present in the medium at the time of termination of the experiment.

Experiment TC-4

In experiment TC-4, 1.67 millimoles of glucose was added to each of 6 flasks containing yeast in the salts medium. To 3 flasks about 5 microcurie of glucose-1-, glucose-2-, or glucose-6-C^{14} were added at the beginning of the experiment. The remaining 3 flasks contained glucose but no isotope initially.

The yeast used in this study differed from that used in the previous experiments in that the 4 hour growth on glucose in the salts medium was omitted. The cells were added to the reaction flasks after centrifugation from the malt extract medium. Bromcresol green (1 or 2 drops of 0.04% solution) was added to the medium as an internal indicator. The labeled glucose was to be added to the second set of flasks when a change in the indicator color showed that the pH of the medium had dropped below the optimum range (4.0 to 4.5). It was found, however, that the glucose

concentration was too low to cause a detectable pH change in the highly buffered salt medium. In order to investigate the effect of subnormal pH on the CO_2 evolution, a sufficient quantity of acid was added to the second set of flasks to cause a change in the indicator color (pH 3.8). This was done at 4½ hours after the beginning of the experiment. At this time radioactive glucose was added to the second set of flasks and enough non-isotopic glucose to raise the glucose concentration in the medium to its value at the start of the experiment.

The effect of a sudden drop in pH was to give per cent specific activity curves strikingly similar in the early phase (glucose still present in the medium) to those previously obtained in the phosphate buffer medium, that is, there was an appreciable contribution to the isotopic CO_2 from glucose-6-C^{14} in contrast to the situation in salts medium in which practically no $C^{14}O_2$ appeared from glucose-6-C^{14} until after glucose had disappeared from the medium.

The data obtained for the 3 flasks which had contained isotopic glucose initially were as shown in Figure 3. The results obtained in the flasks in which the pH had been dropped are also represented by Figure 3 for the time subsequent to disappearance of glucose from the medium.

The CO_2 data for this series are tabulated in Tables 11 and 12.

TABLE 1

A COMPARISON OF THE C_1/C_6 RATIOS IN SALTS MEDIUM, WITH AND WITHOUT INDOLEACETIC ACID, AND IN PHOSPHATE BUFFER

Flask No.	Medium	Position of Labeled Carbon	Additions to the Medium	% Oxygen Consumption	C_1/C_6
1	Salts	1	-	34	3.54
2	Salts	6	-	34	
3	Salts	1	IAA	32	2.65
4	Salts	6	IAA	32	
5	Salts	1	-	-	1.15
6	Salts	6	-	43	
7	Buffer	1	-	43	0.58
8	Buffer	6	-	-	

Each flask contained 109 mg. yeast, 250 micromoles of glucose. The concentration of IAA was 0.1 mg. per flask.

Flasks 1 to 4 contained 1 microcurie of radioactivity.

Flasks 5 to 8 contained 0.1 microcurie of radioactivity.

Some of the radioactivity from flask number 7 was accidently lost.

TABLE 2

OXYGEN CONSUMPTION AND CO_2 EVOLUTION OF YEAST UTILIZING GLUCOSE IN SALTS MEDIUM CONTAINING VARYING LEVELS OF INDOLEACETIC ACID (IAA)

Flask No.	Additions to the Medium	Concentration of IAA (Microgram)	% Oxygen Consumption	% CO_2 Evolution
9	IAA	1	-	70
10	IAA	1	92	-
11	IAA	5	-	54
12	IAA	5	74	-
13	IAA	10	-	67
14	IAA	10	100	-
15	IAA	50	-	78
16	IAA	50	100	-

Each flask contained 8.0 mg. yeast, and 10 micromoles of glucose.

TABLE 3

THE C_1/C_6 RATIOS IN SALTS MEDIUM, WITH AND WITHOUT INDOLEACETIC ACID (IAA), OR IODOACETATE (IOA)

Flask No.	Position of Labeled Carbon	Additions to the Medium	% Oxygen Consumption	C_1/C_6
17	1	-	44.8	3.12
18	6	-	40.2	
19	1	-	-	2.49
20	6	-	-	
21	1	IAA	25.7	1.26
22	6	IAA	32.2	
23	1	IAA	-	1.18
24	6	IAA	32.6	
25	1	IOA	2.0	0.945
26	6	IOA	2.0	

Each flask contained 8 mg. yeast, 10 micromoles of glucose (0.1 microcurie). The concentration of IAA was 50 mg. per flask, and the concentration of IOA was 1.5×10^{-3} M.

TABLE 4

THE C_1/C_6 RATIOS IN SALTS MEDIUM, WITH AND WITHOUT INDOLEACETIC ACID (IAA), OR IODOACETATE (IOA), AND IN PHOSPHATE BUFFER

Flask No.	Medium	Position of Labeled Carbon	Additions to the Medium	% Oxygen Consumption	C_1/C_6
27	Salts	1	-	31	3.61
28	Salts	6	-	38.8	
29	Salts	1	IAA	36.6	1.81
30	Salts	6	IAA	39.7	
31	Salts	1	IOA	41.8	1.45
32	Salts	6	IOA	42.8	
33	Buffer	1	-	38.6	1.70
34	Buffer	6	-	33.6	

Each flask contained 8 mg. yeast, 10 micromoles of glucose (0.1 microcurie). The concentration of IAA was 50 mg. per flask, and the concentration of IOA was 1.5×10^{-4} M.

TABLE 5

A SUMMARY OF THE WARBURG DATA ON THE C_1/C_6 RATIOS IN SALTS MEDIUM, WITH AND WITHOUT INDOLEACETIC ACID (IAA) OR IODOACETATE (IOA), AND IN PHOSPHATE BUFFER

Medium	Number of Experiments	Additions to the Medium	% Oxygen Consumption	C_1/C_6
Salts	4	-	37	3.2
Salts	4	IAA	33	1.7
Salts	1	IOA	42	1.4
Buffer	2	-	36	1.7

TABLE 6

THE $C^{14}O_2$ DATA FROM YEAST METABOLIZING ISOTOPIC GLUCOSE (5 millimoles) IN SALTS MEDIUM AT 30° C. UNDER OXYGEN

Glucose-1-C^{14} (5.48 microcuries)

Time-Hours from Zero	Specific Activity (c.p.m./mg. $BaCO_3$	% Specific Activity	% Total Activity	% Carbon Recovered
½	61.1	7.96	2.5	5.2
1	65.5	8.60	6.1	12.3
1½	88.8	11.6	10.9	19.0
2	99.0	12.9	15.0	24.3
2½	114.	14.9	17.2	26.8
3	128.	16.7	18.7	28.3
3½	121.	15.8	20.0	29.7
4	106.	13.8	21.0	30.8
4½	89.7	11.7	21.8	31.9
5½	113.	14.7	23.9	34.4
6½	87.7	11.4	26.8	36.6
7½	86.6	11.2	28.0	38.4
8½	84.8	11.1	29.4	40.5
9½	83.2	10.9	30.8	42.8

TABLE 6 (Continued)

Glucose-2-C^{14} (5.50 microcuries)

Time-Hours from Zero	Specific Activity (c.p.m./mg. $BaCO_3$	% Specific Activity	% Total Activity	% Carbon Recovered
½	25	3.2	1.0	5.1
1	27.8	3.6	2.4	11.6
1½	33.6	4.4	3.9	17.3
2	47.3	6.1	5.9	22.8
2½	78.5	10.2	7.4	25.2
3	132	17.1	9.1	26.9
3½	158	20.5	11.2	28.6
4	169	21.9	13.2	30.1
4½	171	22.2	15.6	31.8
5½	192	24.9	21.5	35.8
6½	205	26.6	28.0	40.0
7½	200	26.0	33.7	43.6
8½	170	22.1	34.4	45.1
9½	155	20.1	35.4	46.0

TABLE 6 (Continued)

Glucose-6-C^{14} (5.53 microcuries)

Time-Hours from Zero	Specific Activity (c.p.m./mg. $BaCO_3$	% Specific Activity	% Total Activity	% Carbon Recovered
½	2.14	0.28	0.081	4.8
1	4.29	0.55	0.31	11.9
1½	6.56	0.85	0.67	19.0
2	9.15	1.18	0.94	22.7
2½	32.6	4.22	1.45	24.7
3	75.5	9.75	2.06	35.2
3½	105	13.6	3.5	37.0
4	146	18.9	6.5	38.5
4½	164	21.2	8.5	40.0
5½	157	20.3	11.9	42.8
6½	152	19.7	14.7	50.2
7½	116	15.0	17.9	53.7
8½	136	17.6	21.4	57.1
9½	93	12.0	23.6	60.3

TABLE 7

THE $C^{14}O_2$ DATA FROM YEAST METABOLIZING ISOTOPIC GLUCOSE (4.5 millimoles) IN SALTS MEDIUM AT 30° C. IN AIR

Glucose-1-C^{14} (10.1 microcuries)

Time-Hours from Zero	Specific Activity (c.p.m./mg. $BaCO_3$)	% Specific Activity
½	283	17.9
1	288	18.0
1½	294	18.6
2	292	18.5
2½	314	19.9
3	380	24.0
3½	476	30.1
4	630	39.4
4½	625	39.6
5	531	33.6
5½	506	32.0
6	481	30.4

TABLE 7 (Continued)

Glucose-2-C^{14} (10.0 microcuries)

Time-Hours from Zero	Specific Activity (c.p.m./mg. $BaCO_3$)	% Specific Activity
½	280	18.1
1	219	14.1
1½	247	15.9
2	258	16.7
2½	285	18.4
3	307	19.8
3½	270	17.4
4	485	31.3
4½	735	47.4
5	675	43.6
5½	854	55.1
6	865	55.8

TABLE 7 (Continued)

Glucose-6-C^{14} (10.1 microcuries)

Time-Hours from Zero	Specific Activity (c.p.m./mg. $BaCO_3$)	% Specific Activity
½	63.7	4.00
1	12.9	0.80
1½	20.1	1.27
2	22.0	1.40
2½	55.2	3.20
3	130	8.30
3½	296	18.8
4	527	33.4
4½	474	30.0
5	493	31.2
5½	493	31.2
6	334	21.2

TABLE 8

THE $C^{14}O_2$ DATA FROM YEAST METABOLIZING ISOTOPIC GLUCOSE (4.6 millimoles) IN SALTS MEDIUM AT 21° C.

Glucose-1-C^{14} (1.0 microcurie)

Time-Hours from Zero	Specific Activity (c.p.m./mg. $BaCO_3$	% Specific Activity	% Total Activity	% Carbon Recovered
½	4.52	8.3	0.34	0.9
1	6.08	11.1	0.85	2.2
1½	5.32	9.74	1.95	3.7
2	4.40	8.04	2.00	5.7
2½	4.77	8.7	2.77	7.7
3	5.37	9.8	3.78	10.0
3½	5.62	10.2	4.94	12.7
4	4.75	8.65	5.84	15.1
4½	6.08	11.1	7.00	17.5
5½	7.28	13.3	8.36	19.9
6½	8.50	15.5	9.9	22.2
7½	11.7	21.3	11.6	24.0
8½	18.4	33.6	14.1	25.7
9½	20.5	37.4	15.8	26.8
10½	15.3	27.9	17.2	27.9
11¼	16.4	29.9	19.0	28.2
12¼	15.1	27.5	19.9	30.0
13¼	15.5	28.3	21.4	30.6

TABLE 8 (Continued)

Glucose-2-C^{14} (0.64 microcurie)

Time-Hours from Zero	Specific Activity (c.p.m./mg. $BaCO_3$)	% Specific Activity	% Total Activity	% Carbon Recovered
½	1.52	3.2	-	1.1
1	1.86	3.92	0.55	2.53
1½	1.2	2.5	0.77	4.04
2	1.15	2.4	1.02	5.7
2½	1.69	3.6	1.45	7.7
3	-	-	-	10.1
3½	-	-	-	12.7
4	-	-	-	15.3
4½	2.08	4.4	3.9	18.1
5½	2.38	5.0	4.7	21.0
6½	2.61	5.5	5.7	23.5
7½	3.98	8.4	6.6	25.4
8½	8.75	18.5	8.5	27.2
9½	11.1	34.0	10.4	28.1
10½	20.0	42.0	12.9	29.1
11¼	19.9	42.0	16.0	30.3
12¼	24.4	52.0	19.1	31.4
13¼	24.8	52.4	22.5	32.4
14¼	25.2	53.3	26.2	33.6

TABLE 8 (Continued)

Glucose-6-C^{14} (1.0 microcurie)

Time-Hours from Zero	Specific Activity (c.p.m./mg. $BaCO_3$)	% Specific Activity	% Total Activity	% Carbon Recovered
½	0.307	0.39	.036	1.55
1	0.119	0.15	.052	2.94
1½	0.152	0.20	.073	4.64
2	0.149	0.20	.098	6.84
2½	0.29	0.39	.15	9.15
3	0.67	0.86	.28	11.7
3½	0.34	0.43	.35	14.5
4	0.48	0.62	.45	17.1
4½	-	-	-	19.8
5½	1.14	1.46	.68	22.3
6½	1.65	2.12	.97	24.7
7½	3.17	4.06	1.38	26.4
8½	10.35	13.3	2.61	27.9
9½	17.9	22.9	3.98	29.0
10½	16.8	21.5	5.44	30.1
11¼	15.7	20.1	7.15	31.5
12¼	15.9	20.4	8.09	32.3
13¼	15.5	19.9	9.46	33.4

TABLE 9

THE $C^{14}O_2$ DATA FROM YEAST TO WHICH ISOTOPIC GLUCOSE WAS ADDED 3½ HOURS AFTER INITIAL ADDITION OF UNLABELED GLUCOSE (4.6 millimoles) WITH THE CELLS IN SALTS MEDIUM AT 21° C.

Glucose-1-C^{14} (1.0 microcurie)

Time-Hours from Zero	Specific Activity (c.p.m./mg. $BaCO_3$)	% Specific Activity	% Total Activity
½	2.31	3.06	0.69
1	4.21	5.64	1.53
1½	4.91	6.51	2.66
2	4.72	6.26	3.78
2½	5.91	7.85	5.02
3	5.83	7.74	6.28
3½	6.95	9.22	7.50
4	7.50	9.90	9.00
4½	-	-	-
5½	-	-	-
6½	-	-	-
7½	11.0	14.6	13.5
8½	13.5	17.9	14.6
9½	16.7	22.2	15.4
10½	13.7	18.2	16.7
11¼	11.5	15.3	17.5

The % carbon recovered at termination of this experiment was 33.2%.

TABLE 9 (Continued)

Glucose-6-C^{14} (1.0 microcurie)

Time-Hours from Zero	Specific Activity (c.p.m./mg. $BaCO_3$)	% Specific Activity	% Total Activity
½	0.463	0.500	-
1	0.290	0.400	-
1½	0.330	0.420	-
2	-	-	-
2½	0.417	0.530	0.400
3	0.540	0.690	-
3½	0.650	0.850	-
4	0.680	0.870	0.800
4½	-	-	-
5½	-	-	-
6½	2.40	3.08	1.73
7½	3.73	4.78	2.17
8½	8.60	11.0	2.70
9½	12.4	15.9	3.94
10½	12.5	15.9	4.94

The % carbon recovered at termination of this experiment was 35.3.

TABLE 10

THE $C^{14}O_2$ DATA FROM YEAST METABOLIZING ISOTOPIC GLUCOSE (4.6 millimoles) IN PHOSPHATE BUFFER MEDIUM AT 21° C.

Glucose-1-C^{14} (1.0 microcurie)

Time-Hours from Zero	Specific Activity (c.p.m./mg. $BaCO_3$)	% Specific Activity	% Total Activity	% Carbon Recovered
½	3.95	5.24	0.12	1.17
1	5.17	6.86	0.28	2.45
1½	5.92	7.86	0.46	3.68
2	6.08	8.06	0.65	4.98
2½	6.67	8.85	0.86	6.21
3	6.57	8.71	1.06	7.44
3½	6.43	8.52	1.26	8.66
4	5.62	7.45	1.43	9.75
4½	5.62	7.45	1.61	11.2
5½	6.57	8.71	1.80	12.1
6½	6.82	9.05	1.99	13.0
7½	7.43	9.85	2.2	14.0
8½	7.03	10.1	2.47	15.6
9½	3.88	-	3.53	17.3
10½	7.93	10.5	4.48	18.8
11½	8.48	11.2	5.59	20.3
12½	-	-	-	21.0
13½	9.76	13.0	6.64	22.4

TABLE 10 (Continued)

Glucose-6-C^{14} (1.0 microcurie)

Time-Hours from Zero	Specific Activity (c.p.m./mg. $BaCO_3$)	% Specific Activity	% Total Activity	% Carbon Recovered
½	0.505	0.65	-	1.28
1	0.740	0.95	-	2.16
1½	1.13	1.45	0.22	3.42
2	1.93	2.47	0.40	4.74
2½	1.51	1.94	0.56	6.04
3	1.72	2.2	0.74	7.31
3½	2.34	3.0	0.97	8.62
4	2.72	3.49	1.22	9.85
4½	2.17	2.78	1.41	11.0
5½	2.81	3.6	1.69	12.2
6½	2.59	3.32	1.92	13.4
7½	2.95	3.88	2.15	14.4
8½	3.50	4.48	2.65	16.3
9½	-	-	-	18.1
10½	3.54	4.54	3.08	19.6
11¼	3.82	4.90	3.54	21.3
12¼	-	-	-	22.0
13¼	4.78	6.13	4.00	23.3

TABLE 11

A COMPARISON OF THE $C^{14}O_2$ FROM YEAST METABOLIZING ISOTOPIC GLUCOSE (1.67 millimoles) IN SALTS MEDIUM AT 21° C. UNDER AIR

Glucose-1-C^{14} (0.55 microcurie)

Time-Hours from Zero	Specific Activity (c.p.m./mg. $BaCO_3$)	% Specific Activity	% Total Activity	% Carbon Recovered
½	5.08	3.68	0.63	2.3
1½	8.52	6.17	1.40	4.0
2	10.35	7.49	2.72	6.4
2½	9.42	7.17	3.97	8.8
3	8.65	6.26	5.28	11.5
3½	9.47	6.85	6.36	13.8
4	8.70	6.30	6.99	15.0
4½	10.6	7.62	8.80	18.2
5½	11.8	8.55	10.6	21.0
6½	21.5	15.6	14.8	24.8
7½	28.2	20.4	18.8	27.8
8½	25.4	18.4	21.5	29.5
9½	17.8	12.9	22.2	30.4
10½	21.7	15.7	26.0	33.6
11¼	21.7	15.3	30.4	37.5
12¼	40.2	29.1	32.2	38.8
13¼	11.2	8.1	32.7	39.4
14½	8.6	6.2	32.9	39.8
15¼	9.0	6.5	33.0	45.0

TABLE 11 (Continued)

Glucose-2-C^{14} (0.60 microcurie)

Time-Hours from Zero	Specific Activity (c.p.m./mg. $BaCO_3$)	% Specific Activity	% Total Activity	% Carbon Recovered
½	1.90	1.27	0.18	1.8
1	3.72	2.25	0.40	3.2
1½	3.75	2.53	0.80	5.2
2	4.34	3.56	1.19	7.0
2½	4.08	3.34	1.62	9.2
3	4.27	2.88	1.95	10.7
3½	4.34	2.93	2.15	11.6
4	4.49	3.03	2.72	14.2
4½	4.67	3.14	3.33	16.9
5½	6.74	4.37	4.80	21.5
6½	14.7	9.90	7.75	25.5
7½	26.9	18.1	11.0	28.0
8½	36.0	24.3	13.5	29.4
9½	47.2	31.9	19.5	32.0
10½	50.2	33.8	26.8	34.9
11¼	51.0	34.4	32.8	37.4
12¼	45.3	30.6	37.5	39.4
13¼	13.8	9.20	38.0	40.0
14¼	8.95	6.00	38.0	40.3
15¼	14.4	9.90	38.6	41.0

TABLE 11 (Continued)

Glucose-6-C^{14} (0.62 microcurie)

Time-Hours from Zero	Specific Activity (c.p.m./mg. $BaCO_3$)	% Specific Activity	% Total Activity	% Carbon Recovered
½	0.280	0.220	-	2.0
1½	0.714	0.570	0.075	3.5
2	1.17	0.930	0.179	5.4
2½	0.750	0.600	0.253	7.4
3	1.36	1.08	0.400	9.7
3½	0.710	0.570	0.470	11.9
4	3.66	2.90	0.610	12.6
4½	1.00	0.790	0.750	15.6
5½	1.34	1.08	0.920	18.3
6½	1.66	1.32	1.26	22.2
7½	6.50	5.16	2.60	27.1
8½	18.4	14.6	4.80	29.6
9½	23.8	18.9	7.70	32.2
10½	23.3	18.5	10.4	34.7
11¼	22.7	18.0	13.5	37.6
12¼	21.6	17.2	15.7	39.7
13¼	34.5	27.4	24.2	43.7
14¼	18.8	14.9	24.9	44.4
15¼	14.4	11.4	25.2	44.8

TABLE 12

THE $C^{14}O_2$ DATA FROM YEAST TO WHICH ISOTOPIC GLUCOSE (1.67 millimoles) WAS ADDED 4¼ HOURS AFTER INITIAL ADDITION OF UNLABELED GLUCOSE WITH CELLS IN SALTS MEDIUM, IN AIR AT 21° C. AND pH 3.8 OR BELOW

Glucose-1-C^{14} (0.55 microcurie)

Time-Hours from Zero	Specific Activity (c.p.m./mg. $BaCO_3$)	% Specific Activity	% Total Activity	% Carbon Recovered
½	5.32	4.75	0.610	2.10
1½	7.75	6.92	2.20	5.50
2	7.98	7.13	3.60	9.50
2½	8.00	7.15	5.40	12.8
3	8.48	7.60	7.40	16.8
3½	9.22	8.24	8.80	21.9
4	10.2	9.10	10.5	23.9
4½	11.0	9.80	12.7	27.4
5	11.4	10.2	14.2	31.2
5½	16.8	15.0	15.7	33.6
6¼	15.8	14.1	17.3	35.8
7¼	13.8	12.3	19.4	37.2
8¼	12.8	10.8	22.1	40.1
9¼	9.50	8.50	23.8	43.2
10¼	10.5	9.40	24.9	44.6
11¼	7.70	6.90	26.8	47.5
12¼	10.2	9.20	27.6	50.5
13¼	11.7	19.1	27.9	51.5
14¼	8.31	7.40	-	52.0

TABLE 12 (Continued)

Glucose-2-C^{14} (0.60 microcurie)

Time-Hours from Zero	Specific Activity (c.p.m./mg. $BaCO_3$	% Specific Activity	% Total Activity	% Carbon Recovered
½	1.09	0.890	-	1.80
1	3.03	2.50	0.610	5.30
1½	2.99	2.45	1.18	9.10
2	3.98	3.26	1.81	12.3
2½	3.49	2.89	2.56	16.6
3	2.79	2.29	3.35	22.3
3½	4.85	3.97	4.04	25.2
4	6.16	5.05	5.09	28.6
4½	-	-	-	-
5	9.28	7.60	6.22	31.1
5½	13.2	10.8	7.06	32.4
6¼	21.6	17.7	9.16	34.3
7¼	27.4	22.4	13.6	37.6
8¼	31.0	25.7	18.6	40.8
9¼	27.6	22.6	21.8	43.2
10¼	28.4	23.3	26.5	46.5
11¼	29.8	24.4	30.8	49.5
12¼	28.6	23.5	35.8	52.9
13¼	14.2	11.6	36.4	54.5
14¼	10.7	8.78	37.0	54.6

TABLE 12 (Continued)

Glucose-6-C^{14} (0.62 microcurie)

Time-Hours from Zero	Specific Activity (c.p.m./mg. $BaCO_3$	% Specific Activity	% Total Activity	% Carbon Recovered
½	10.3	8.16	1.50	3.12
1½	-	-	-	7.00
2	7.66	6.09	3.10	10.5
2½	7.58	6.02	4.46	14.0
3	5.97	4.74	5.82	18.7
3½	6.27	4.98	7.35	21.7
4	7.57	6.01	8.50	26.0
4½	7.28	6.18	9.90	29.5
5	9.57	7.60	12.0	33.7
5½	12.4	9.85	13.2	35.6
6¼	18.1	14.4	14.6	37.0
7¼	18.8	14.9	16.6	39.1
8¼	17.8	14.1	20.0	42.8
9¼	16.3	12.9	22.9	46.2
10¼	19.9	15.8	25.0	51.6
11¼	16.7	13.3	27.7	53.0
12¼	12.5	9.9	28.3	57.0
13¼	8.10	6.4	30.0	58.8
14¼	20.9	10.4	31.9	59.5

DISCUSSION AND SUMMARY

The Respiratory CO_2 Data

The Warburg studies

As shown in Table 1, yeast allowed to utilize glucose aerobically in a salts medium yielded an average C_1/C_6 ratio, for four experiments, of 3.14. To facilitate comparison each of these experiments was terminated at an average oxygen consumption of 39%, calculated on the basis of the oxygen theoretically required for complete combustion of the glucose. This value of the C_1/C_6 ratio clearly indicates an appreciable contribution to the respired CO_2 from the alternate pathways of glucose utilization. The existence of a high C_1/C_6 ratio in the early phases of glucose dissimilation was further confirmed by the time course studies.

Also, as shown in Table 1, for 4 experiments, the average value of the C_1/C_6 ratio was 1.73 when indoleacetic acid (IAA) was included in the salts medium. It is interesting to note that, while the radiochemical yields of CO_2 from both glucose-1-C^{14} and glucose-6-C^{14} decreased in the presence of IAA, the decreased C_1/C_6 ratio was exclusively due to the depression of the radiochemical yield of CO_2 from glucose-1-C^{14}. This is rather surprising in view of the report cited (43), that IAA

decreased glycolysis and increased oxygen consumption in animal tissues. The present result suggests that, in yeast, IAA may specifically suppress one or more of the reactions of the alternate pathways of glucose utilization.

In the experiments in which yeast utilized glucose in a phosphate buffer medium the C_1/C_6 ratio of radiochemical yields was appreciably lower, for the same oxygen consumption, than for experiments conducted in the salts medium. The C_1/C_6 ratio of 1.76 (Table 1) obtained for buffer medium is in reasonably good agreement with that reported by Beevers and Gibbs (4). In their experiment a C_1/C_6 ratio of 2.0 was obtained for yeast which had utilized glucose in a phosphate buffer to the point at which 10-20% of the glucose initially added had been respired.

The time course studies

The cumulative, or radiochemical yield curve (per cent total activity), clearly indicates the difficulty encountered in Warburg studies. In such studies the C_1/C_6 ratios are taken as indicating the proportional contribution of the alternative pathways of glucose metabolism to the respiratory CO_2. That the conditions (oxygen consumption, or time) for which the C_1/C_6 is given must be specified is apparent from this curve in

which the glucose-1-, and glucose-6-C^{14} curves diverge rapidly in the early phase, then run parallel for a long period and finally converge slowly. It is instructive to note that in a growth medium the radiochemical yield of $C^{14}O_2$ from glucose-1-C^{14} is always higher than that from glucose-6-C^{14} suggesting that carbon 6 of glucose contributes to biosynthesis to a higher degree than does carbon 1, so that the 6-carbon tends to be conserved. These results are in agreement with those of Heath and Koffler for P. chrysogenum.

The cumulative activity curve also shows that activity from the metabolism of glucose-6-C^{14} does not begin to appear in the respiratory CO_2 until after the glucose has disappeared from the medium, regardless of the level of glucose administered to the medium initially. However, this is true only in salts medium. In phosphate buffer, activity from glucose-6-C^{14} appears in the earliest CO_2 samples in appreciable quantities. This indicates that the alternate pathways operate to the same degree in a buffer medium as in a growth medium, the difference being that only in a growth medium is carbon 6 conserved to a larger extent than carbon 1.

Another important feature of the radiochemical yield curve is that from it the contribution of the alternate pathways may be readily calculated. Since in the early

phase the radiochemical yield of $C^{14}O_2$ from glucose-6-C^{14} is negligible, the contribution of the alternate pathways, on a percentage basis, is consequently given directly by the radiochemical yield from glucose-1-C^{14} at this point. Thus, for a typical experiment (Figure 3), the maximum contribution of the alternate pathways is approximately 15%. This figure is in general agreement with that given by other investigators (9). For later phases in which appreciable activity appears in CO_2 from glucose-6-C^{14} the radiochemical yield curve from glucose-1-C^{14} probably represents the sum of the contributions of both the glycolytic-citric acid cycle, and the alternate pathways. The glucose-6-C^{14} curve represents more nearly the conversion of carbon 6 of glucose to CO_2 via triose and the citric acid route. As a corollary of this conclusion it may be inferred that the proportion of glucose metabolized via the alternate pathways remains nearly constant for a considerable period since the radiochemical yield curves for both glucose-1-, and glucose-6-C^{14} remain essentially parallel. This method of calculation is simpler and more rapid than the other types of analysis which have been suggested.

The data on the specific activity of $C^{14}O_2$ collected over an interval may be plotted directly against time, or it may be plotted on a percentage basis. The latter is

more convenient for comparing the results of different experiments. These percentage specific activity curves exhibit some striking properties.

On the basis of the glycolysis-citric acid pattern carbons 3 and 4 of glucose should appear first as CO_2 followed in turn by carbons 2 and 5, and finally by carbons 1 and 6. In all the time course studies made the maximum specific activity of CO_2 from the 3 labeled carbons appeared in the order: 1, 6, 2. The initial rise of the glucose-1-C^{14} curve is to be expected if preferential oxidative decarboxylation of carbon 1 of glucose is taking place. The rise in per cent specific activity (p.s.a.) of CO_2 from glucose-6-C^{14} which is accompanied by a corresponding slight rise in the p.s.a. curve from glucose-1-C^{14} is also expected on the basis of glycolysis-Krebs cycle activity. The fact that the peak of the glucose-6-C^{14} curve is lower than that of glucose-2-C^{14} but occurs earlier than the glucose-2-C^{14} peak is in accordance with the findings of Wang *et al.* (40) who showed that under the conditions of these experiments pyruvate was incorporated via the Wood-Werkman reaction to the extent of 70%. This being the case a relatively smaller portion of carbon from carbon 2 of glucose will appear as the carboxyl carbon of acetate and should appear as CO_2 sooner than that of carbon 6. The greater

proportion of carbon 2 will appear as the alpha carbon of a 4 carbon acid. When such an acid is metabolized via the TCA cycle, carbon 2 of glucose behaves the same as carbon 6. This interpretation explains why the p.s.a. curve of glucose-2-C^{14} has a higher peak value than that of glucose-6-C^{14} but reaches its peak value later. See page 71.

In respect to the portion of glucose (15%) metabolized via the alternate pathway it is of interest to note that the pentose formed in this fashion probably was not further utilized by way of the pentose cycle to yield CO_2 from glucose-2-C^{14}. This is evidenced by the fact that the peak of the p.s.a. curve from glucose-2-C^{14} occurs appreciably later than that of glucose-1-C^{14}, in addition to the fact that when the specific activity of CO_2 from glucose-1-C^{14} is a maximum the specific activity of CO_2 from glucose 6 is still very low. This observation, coupled with the finding that the proportion of pentose pathway is essentially constant, strongly suggests that, in yeast under conditions of growth, the oxidative pathway functions primarily as a source of pentose which is a key biosynthetic intermediate.

It may also be noted that the relative contribution of the alternate, and Embden-Meyerhof pathways to glucose dissimilation is essentially the same in all experiments performed in synthetic medium, irrespective of whether the experiment was carried out at 21° or 30°, whether

under oxygen or air; and whether or not the yeast had passed through a four hour "pre-growth phase" in salts medium prior to the tracer experiment. Furthermore, the proportion of the two pathways appears to be independent of the pH of the medium under normal conditions. This conclusion was also reached by Blumenthal *et al.* (9). However, in one experiment, in which the medium was suddenly made strongly acid, the results obtained were analogous to those found in buffer medium in which the p.s.a. curve for glucose-1-C^{14} remained high while the corresponding curve for glucose-6-C^{14} was much higher in the early phase than in previous experiments, though still below the glucose-1-C^{14} curve. This led to a decreased C_1/C_6 ratio. This indicates that the sudden drop in pH probably had caused marked reduction in biosynthetic function.

The Incorporation Studies

Examination of the radioautographs made from chromatograms of the yeast hydrolyzates from various experiments reveal an essentially uniform picture of the incorporation of radioactivity from labeled glucose samples into the various amino acids. The observations will be discussed in connection with the various amino acids.

Glutamic acid

This amino acid contained the major portion of the glucose radioactivity incorporated into protein during the first half of experiment TC-2, and a similar pattern was found in other cases. This is in accord with the view that glutamic acid accumulates when carbon sources are utilized by yeast, and thus serves as a storehouse of key biosynthetic units. In later stages the heavy labeling of glutamic acid is gradually transferred to other amino acids.

Lysine

This compound is also observed to have an appreciable fraction of the incorporated radioactivity in the earliest samples. This is not surprising inasmuch as glutamic acid is regarded as a key intermediate in lysine biosynthesis. This result suggests that 4 carbon acids are not important precursors of the lysine molecule.

It was also observed that, although depletion of endogenous carbon caused a marked drop in the amount of isotope present in glutamic acid, the activity of lysine continued at its initial high level throughout the duration of the experiment. This indicates that the equilibria of the reactions leading to lysine from glutamic acid

are strongly in favor of lysine formation.

Aspartic acid

Aspartic acid was found to be only faintly labeled in these experiments. Thus, aspartic acid apparently does not function as a "storehouse" which differentiates the function of aspartic from that of glutamic acid. This result may be due to the fact that the aspartate-oxalacetate system is in a rapid equilibrium with respiratory processes.

Tyrosine

In these experiments tyrosine was found to be only slightly labeled in comparison with the other amino acids discussed above. The biosynthesis of the aromatic amino acids thus seems closely related to TCA cycle intermediates rather than intact glucose or related sugars.

Phenylalanine

The results found for phenylalanine were essentially the same as those for tyrosine.

Proline

Though proline has been considered as closely related to glutamic acid this compound was not labeled from

any of the substrates used until the later samples. This is probably due to the rate of the biosynthetic reactions leading to proline being relatively slow.

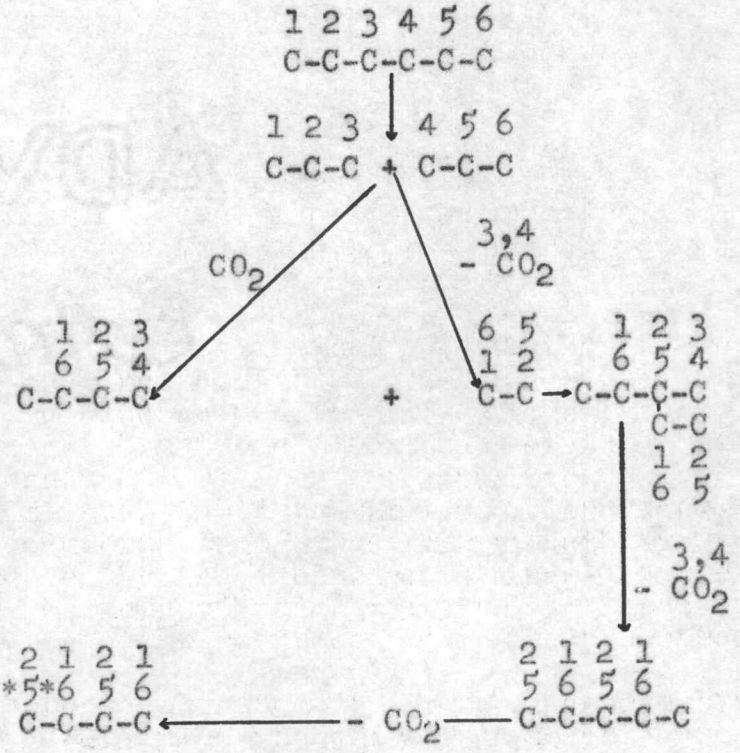
Alanine

This amino acid was the only one in which a difference in isotope concentration could be distinguished for the different labeled sugars used as substrates. In the early samples alanine was unlabeled when glucose-1-C^{14} was the substrate while alanine from either glucose-2-, or glucose-6-C^{14} was faintly labeled in the earliest samples. This difference disappeared in the later samples. This indicates that the observed preferential oxidation of carbon 1 of glucose is not due to the operation of the Entner-Douderoff pathway. If this pathway were operative, pyruvate, and consequently alanine, would be equally labeled irrespective of the position of the labeled carbon in the glucose metabolized. That this is not observed indicates that the preferential oxidation of carbon 1 of glucose takes place via the pentose pathway.

Radioactivity from ribose-1-C^{14} was observed to be incorporated into lysine, glutamic acid, alanine, and a single spot representing aspartic acid, glycine, and serine. This indicates that ribose can be converted to Krebs cycle intermediates by way of either a C_2-C_3

cleavage or by some other unknown pathways. The radioautographs from yeast which had utilized acetate-1-, and acetate-2-C^{14} did not give a sufficiently clear picture to allow additional conclusions to be drawn concerning the fate of the ribose fragments.

Figure 4



* From this 4 carbon acid, carbons 1 and 6, or 2 and 5, have an equal chance of appearing as respiratory CO_2 on the next turn of the citric acid cycle.

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