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Oxidation of Glucose by Yeast, Studied with Isotopic Carbon[1]

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Recent studies have shown that the oxidation of acetate by yeast[2-4] occurs by way of the tricarboxylic acid cycle, the same mechanism involved in the oxidation of such intermediates of fat and carbohydrate metabolism as acetate,[5] acetoacetate[6,7] and pyruvate[8] in animal tissues.

These findings emphasize the role of acetic acid (or a functional derivative thereof) as a common intermediate of fat and carbohydrate metabolism in animals, and suggest that this substance may also function as an intermediate in the oxidation of carbohydrate by yeast. To test this possibility, bakers' yeast was allowed to oxidize glucose in the presence of isotopic acetate. Inasmuch as bakers' yeast, when depleted of its food reserves by aeration, has a negligible endogenous respiration, and since isotopic acetate during its oxidation is not diluted appreciably in its isotope content, the extent of the isotopic dilution of acetate during the simultaneous oxidation of non-isotopic glucose can be used as a measure of acetate formation from glucose. Three possibilities were recognized: (1) If glucose is oxidized *via* acetate as intermediary, the isotopic content of the acetate will be lowered, and any components of the tricarboxylic acid cycle accumulating will have a lower isotope content than when acetate alone is oxidized. (2) If glucose is oxidized *via* the tricarboxylic acid cycle, but without the intermediate formation of acetate, no dilution of the acetate will occur, but the isotope content of components of the cycle will be lowered. (3) If glucose does not form acetate and is not oxidized *via* the tricarboxylic acid cycle, neither the acetate nor the components of the cycle will be lowered in their isotope content. The results of this study provided definite evidence for the first possibility; namely, the conversion of glucose to acetate, and thence to components of the tricarboxylic acid cycle, is a major oxidative pathway of carbohydrates in yeast.

Experimental Results

The isotopic magnesium acetate, labeled in the carboxyl carbon, and isotopic sodium bicarbonate were prepared as described previously.[2] *Saccharomyces cerevisiae* (ordinary Fleischmann's bakers' yeast) was suspended in distilled water, an aliquot was removed for determination of the dry weight; a volume of the suspension was then taken corresponding to 2.0 g. dry weight and diluted to 200 ml. After sixteen hours of aeration to deplete the cells of reserve nutrients, the substrates were added and the flasks shaken for seven hours at 25° while a stream of oxygen was passed through the flasks into a bead tower containing carbon dioxide-free sodium hydroxide for absorption of the respiratory carbon dioxide. Except as noted below the procedures for isolation of products were the same as described previously.[2] The *Torulopsis* yeast was transferred from malt extract agar subcultures to the glucose-salts medium of Pavcek, Petersen and Elvehjem[9] and grown according to their procedure for forty-eight hours at 25°, after which the cells were centrifuged and washed thoroughly with water. Before an experiment, the cells were suspended in distilled water, and after depletion of endogenous substrates by aeration treated exactly as described for *Saccharomyces*.

Ethanol and Acetic Acid.—After centrifugation of the cells, the combined supernate and washings were strongly acidified and distilled with steam. The distillate was neutralized and redistilled, thereby recovering the ethanol in the distillate. This was converted, by two hours reflux with excess of potassium permanganate, to acetic acid, which was recovered by steam-distillation and conversion to the silver salt. Tests with isotopic acetate and non-isotopic ethanol, in amounts of the order involved in these experiments, revealed that a complete separation is made by this procedure. The recovery of alcohol as acetate is 90%; the recorded values are corrected accordingly. The acetate was recovered from the alkaline residue of the ethanol distillation by acidification, steam distillation, and neutralization of the distillate.

The first experiment of Table I presents data obtained in the simultaneous oxidation of glucose and isotopic magnesium acetate by bakers' yeast over a seven-hour period at 25°. Although most of the glucose disappeared, there was an apparent utilization of only 5.7 mm. of acetate. This is in marked contrast to previous experiments with acetate as sole substrate,[2] in which two to three times this amount of acetate was utilized under the same conditions. However, the lowering of the C^{13} excess from an initial value of 3.17 atom per cent. to 2.34% in the recovered acetate indicates that a considerable quantity of non-isotopic

TABLE I

OXIDATION OF GLUCOSE AND C^{13}-COOH-TAGGED ACETATE BY YEAST

	Experiment 1 *Saccharomyces cerevisiae* Aerobic		Experiment 2 *Saccharomyces cerevisiae* Anaerobic		Experiment 3 *Torulopsis Utilis* Aerobic	
	mM.	C^{13} Excess	mM.	C^{13} Excess	mM.	C^{13} Excess
Glucose, start	20.00		20.00		20.00	
Glucose, end	2.08		0.15		2.67	
Acetate, start	21.3	3.17	26.8	3.42	9.51	3.51
Acetate, end	15.6	2.34	25.6	3.17	6.01	1.23
Ethanol	9.41	0.00	23.6	0.00	2.94	0.02
Respiratory CO_2	39.8	0.68	31.5	.10	52.7	0.61
Citric acid	0.34	1.00	0.07	.00	0.20	..
Succinic acid	1.51	0.98	0.60	.87	.....	..
Cell lipids	181.5[a]	0.82	115.9[a]	.24	211.3[a]	0.44
Cell residue	2160[a]	0.07	2155[a]	.00	2240[a]	0.05

[a] Milligrams.

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(2) Weinhouse and Millington, THIS JOURNAL, **69**, 3089 (1947).

(3) Lynen, *Ann.*, **544**, 40 (1943).

(4) Lynen and Neciullah, *ibid.*, **541**, 203 (1939).

(5) Weinhouse, Medes, Floyd and Noda, *J. Biol. Chem.*, **161**, 745 (1945).

(6) Buchanan, Sakami, Gurin and Wilson, *ibid.*, **159**, 695 (1945).

(7) Weinhouse, Medes and Floyd, *ibid.*, **166**, 691 (1946).

(8) Wood, *Physiol. Rev.*, **26**, 198 (1946).

(9) Pavcek, Petersen and Elvehjem, *Ind. Eng. Chem.*, **29**, 536 (1937).

acetate had been formed; hence the recovered acetate represents a balance between formation and utilization. Of the 15.6 mM. of acetate recovered, $(3.17 - 2.34) \times 100/3.17 = 26\%$ or 4.1 mM. was non-isotopic, hence derived from glucose carbon. To gain a better indication of the actual quantity of acetate derived from glucose, a previously described[10] equation was used, which takes into account the non-isotopic acetate metabolized concurrently with the isotopic acetate. Calculation by this method gives a value of 5.6 mM. acetate derived from glucose.

Anaerobically (experiment 2, Table I) an isotopic dilution of acetate was observed, indicating some acetate "turnover" under these conditions, but the quantity was considerably lower than aerobically, being of the order of 2 mM.

To determine whether the formation of acetate from glucose is restricted to the highly fermentative species similar experiments were made with *Torulopsis utilis*.[11] As shown in experiment 3 of Table I an isotopic dilution of the acetate of similar magnitude was observed corresponding to a formation of 7.9 mM. of acetate from glucose. In these experiments with combined substrates an aerobic fermentation was observed under conditions in which neither acetate nor glucose alone produced any alcohol whatever. Aerobic alcohol formation was found also with *Torulopsis* yeast, though in smaller quantity than with *Saccharomyces*. This effect of acetate remains unexplained; however the absence of C^{13} in the alcohol indicates it must have been derived exclusively from glucose carbon by a pathway involving neither acetate nor substances in equilibrium with or derived therefrom.

Respiratory Carbon Dioxide.—Of the 39.8 mM. of carbon dioxide obtained in experiment 1, the C^{13} excess of 0.68% indicates that $0.68 \times 39.8/3.17 = 8.5$ mM. was derived by the complete oxidation of 4.25 mM. of the isotopic acetate, and $39.8 - 8.5 = 31.3$ came from glucose. From the quantity of alcohol formed, 9.4 mM. of carbon dioxide arose by fermentation; and from the quantity of non-isotopic acetate present, 4.1 mM. of carbon dioxide must have arisen by decarboxylation in the formation of acetate. Thus, $31.5 - 13.5 = 18.0$ mM. of carbon dioxide represents the complete oxidation of $18.0/6 = 3.0$ mM. of glucose. We can now calculate that of the 17.9 mM. of glucose utilized, 3.0 was completely oxidized to carbon dioxide, 2.05 was recovered as 4.1 mM. of acetate, and 4.7 was fermented to 9.4 mM. of alcohol.

In similar fashion, we can calculate that of the 52.7 mM. of respiratory carbon dioxide obtained in experiment 3 with *Torulopsis* yeast 9.15 mM. came from oxidation of 4.6 mM. of isotopic acetate, 2.9 mM. arose by fermentation, and 3.9 mM. arose in the formation of 3.9 mM. of non-isotopic acetate, leaving 36.7 mM. of carbon dioxide derived by complete oxidation of $36.7/6 = 6.1$ mM. of glucose.

In the anaerobic experiment the presence of 0.10% of C^{13} excess represents the oxidation of 0.46 mM. of isotopic acetate. That this slow anaerobic oxidation of acetate is brought about in some manner by the presence of glucose is indicated by the fact that in unpublished experiments no acetate oxidation was observed anaerobically in the absence of glucose.

Succinate and Citrate.—After removal of volatile substances, the residual solution was extracted continuously for seventy-two hours with ether, the extract was taken up in water and the silver salts precipitated, dried, weighed, then decomposed with hydrogen sulfide. To separate succinic from citric acid, advantage was taken of their widely different solubilities in ether. The mixture of acids, consisting mainly of succinic and citric, with small amounts of malic, was made to 10 ml. in water, and extracted six times successively with 50-ml. portions of ether. The ether extracts were then combined, evaporated, made again to 10 ml. in water and again extracted six times with ether. From our experimentally determined partition constants we calculated that this process extracts 87% of the succinic acid but only 0.2% of the citric and 3% of the malic. One precipitation through the barium salt usually sufficed to give pure succinic acid, melting between 178 and 184°.

The aqueous residues from these extractions were combined, evaporated to about 5 ml., and the citrate recovered as the quinidine salt.[6] This was recrystallized and submitted to the degradation procedure previously outlined for determination of the C^{13} distribution.[6] To obtain the C^{13} content of the succinate carboxyls, the barium salt was heated at 500° for several hours. One of the carboxyl carbons is thereby converted to barium carbonate, the carbon dioxide of which was recovered by acidification.

In previous experiments with acetate as sole substrate[2] the only member of the tricarboxylic acid cycle which accumulated in appreciable quantity was citric acid. With glucose alone only succinate accumulated. With glucose and acetate together, both citrate and succinate accumulated though the relative amounts of each varied from one experiment to another. If glucose and acetate followed independent metabolic pathways it would be expected that the citrate would be isotopic and the succinate non-isotopic. The fact, as shown in Table I, that the citrate and succinate had C^{13} excesses of similar magnitude points to the participation of both acetate and glucose carbon in the formation of these substances. From considerations outlined in the previous paper[2] the citrate would be expected to have 70 per cent. of the acetate C^{13} content, and the succinate to have 57 per cent., whereas, the percentages were 43 and 42, respectively. Also, the succinate should have had about 80% of the citrate C^{13} content whereas their observed C^{13} contents were about equal. The distribution of C^{13} in these substances, however, was found to be virtually the same as when acetate alone was employed. Of the 1.00% over-all C^{13} excess in the citrate, there was 2.33% in the primary carboxyls, 1.42 in the tertiary COOH and none in the 3 non-COOH carbons. In the succinate all of the excess C^{13} was in the carboxyl carbons.

In view of the complexities introduced by the use of two substrates, better agreement with the results of experiments with acetate alone may be unwarranted. At least two other reactions besides those of the tricarboxylic acid cycle may be involved in the formation of succinate; namely, dehydrogenative coupling of acetate (equation 1)

$$2CH_3C^{13}OOH \xrightarrow{-2H} HOOC^{13}CH_2CH_2C^{13}OOH \quad (1)$$

and assimilation of isotopic carbon dioxide *via* the Wood and Werkman reaction[8] (equation 2)

$$C^{13}O_2 + CH_3COCOOH \longrightarrow HOOC^{13}CH_2COCOOH \xrightarrow{4H} HOOC^{13}CH_2CH_2COOH \quad (2)$$

Unfortunately no means are available for testing the occurrence of reaction 1 directly in these experiments since succinate does not accumulate in the absence of glucose.

TABLE II

GLUCOSE METABOLISM BY *Saccharomyces cerevisiae* IN PRESENCE OF ISOTOPIC BICARBONATE

	Aerobic		Anaerobic	
	mM.	C^{13} Excess	mM.	C^{13} Excess
Glucose, start	10.00		10.00	
Glucose, end	0.26		0.46	
$NaHCO_3$, start	5.00	6.46	5.00	6.46
CO_2, end	22.8	1.26	18.9	1.57
Acetate	1.57	0.01	0.54	0.02
Ethanol	0.00	..	11.1	.00
Citric acid	.04	..	0.03	
Succinic acid	*Ca.* .5	0.02	0.3	.09
Cell lipids	101.7[a]	.00	61.9[a]	.00
Cell residue	1352[a]	.00	1012	.00

[a] Milligrams.

(10) Medes, Floyd and Weinhouse, *J. Biol. Chem.*, **162**, 1 (1946).

(11) Kindly supplied by Dr. C. N. Frey of the Fleischmann Laboratories.

However the occurrence of carbon dioxide assimilation was tested by experiments with non-isotopic glucose in the presence of isotopic carbon dioxide. Although with acetate alone, $C^{13}O_2$ is not assimilated appreciably[2] it was conceivable that this reaction would be favored in the presence of pyruvate resulting from glucose dissimilation. Kleinzeller[12] found an appreciable accumulation of succinate during glucose oxidation only when bicarbonate was present, attributing its formation to the Wood and Werkman reaction. As shown in Table II, there is a definite assimilation of carbon dioxide, particularly under anaerobic conditions, but the low content of C^{13} incorporated in the succinate indicates that this reaction is of minor significance quantitatively. As expected, all of the C^{13} was found in the carboxyl carbons of succinate; none of the other substances isolated had any isotope excess.

Cell Components.—As found previously,[2] there was a rapid synthesis of lipids in these experiments, occurring anaerobically as well as aerobically, though to a much smaller extent in the former than in the latter condition. There was also a small incorporation of C^{13} in the lipid-free cell residue, presumably a result of incorporation of acetate carbon into cell carbohydrates, as found in similar experiments by White and Werkman.[13] Though no attempt was made to account quantitatively for all of the carbon, the increase in the dry weight of the cells indicates there was carbohydrate synthesis corresponding to about 30% of the glucose utilized. It is plausible that some acetate carbon would appear in the cell carbohydrate by way of components of the tricarboxylic acid cycle in a manner similar to the formation of glycogen from acetate in animal tissues.[8]

Discussion

Acetate Formation from Glucose.—From the data of experiment 1 of Table I we calculated that 3.0 mM. of glucose was completely oxidized to carbon dioxide and another 2.05 mM. was utilized for the formation of 4.1 mM. of acetate. Assuming that each glucose molecule can yield 2 molecules of acetate a maximum of 10.1 mM. of acetate could have been formed. From the isotopic dilution we calculated that 5.6 mM. of acetate had been formed from glucose. Thus 5.6/10.1 = 55% of the glucose oxidized can be accounted for by conversion to acetate. In experiment 3 of Table I (*Torulopsis* yeast) 16.1 mM. of acetate could have been formed by the quantity of glucose utilized for conversion to acetate and complete oxidation, whereas from the isotopic dilution of the acetate 7.9 mM. was calculated to have been formed, thus accounting for 7.9/16.1 = 49%.

Actually these figures should be considered minimal, for in the calculation of acetate formation by isotopic dilution it is assumed there was complete equilibration between the added isotopic acetate and the non-isotopic acetate formed intra-cellularly from glucose. Such a condition seems quite unlikely in these experiments with intact yeast cells; thus it is reasonable to assume that at least a major portion, if not all, of the glucose had passed through the intermediate stage of acetate on its pathway to complete oxidation. The results of these experiments are typical of a total of six experiments of this type; four with *Saccharomyces* and two with *Torulopsis*. In every experiment at least 50% of the glucose which had undergone complete oxidation or was converted to acetate could be accounted for by acetate formation on the basis of the isotope dilution.

Mechanism of Acetate Formation.—Although no direct evidence is presented for the pathway of this conversion it seems most reasonable to assume this occurs *via* the glycolysis scheme of Embden, Meyerhof and Parnas,[14] the end-product of which, pyruvate, is known to yield acetate under many circumstances.[15]

The fermentation and oxidation of glucose may be represented by 2 separate pathways stemming from pyruvate as shown in equation 3.

$$CH_3COCOOH \xrightarrow{O_2} \begin{cases} CO_2 + CH_3COOH \xrightarrow{O_2} CO_2 \\ CO_2 + CH_3CHO \xrightarrow{H_2} CH_3CH_2OH \end{cases} \quad (3)$$

It seems more likely, however, that in yeast at least, the fermentative and oxidation pathways diverge at the acetaldehyde level as indicated by equation 4.

$$CH_3COCOOH \longrightarrow CO_2 + CH_3CHO \begin{cases} \xrightarrow{O} CH_3COOH \xrightarrow{O} CO_2 \\ \xrightarrow{H} CH_3CH_2OH \end{cases} \quad (4)$$

This possibility has already been suggested by Pickett and Clifton,[16] based among other considerations on their finding of an accumulation of a bisulfite-binding substance, presumably acetaldehyde, during glucose and pyruvate oxidation by yeast in the presence of bisulfite. Further evidence for the participation of acetate in carbohydrate metabolism of yeast was brought forward recently by Novelli and Lipmann[17] in connection with their studies of coenzyme A, a pantothenic acid derivative known to be concerned with carbohydrate metabolism. They found that coenzyme A-deficient yeast oxidized acetate at a lower rate than normal yeast and that ethanol oxidation by Co A-deficient yeast resulted in accumulation of acetate.

Though many details of carbohydrate oxidation require further investigation the data reported in this paper provides additional support for the growing body of evidence that acetate is a key intermediate in carbohydrate metabolism and that C_2–C_4 condensation is a major pathway of cellular respiration.

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(12) Kleinzeller, *Biochem. J.*, **35**, 495 (1941).
(13) White and Werkman, *Arch. Biochem.*, **13**, 11 (1947).
(14) Werkman and Wood, *Botan. Rev.*, **8**, 1 (1942).
(15) Bloch, *Physiol. Rev.*, **27**, 574 (1947).
(16) Pickett and Clifton, *J. Cell. Comp. Physiol.*, **22**, 147 (1943).
(17) Novelli and Lipmann, *J. Biol. Chem.*, **171**, 833 (1948).

Summary

Experiments on the oxidation of glucose by yeast in the presence of isotopic acetate or bicarbonate lead to the following conclusions.

At least half and probably much more of glucose undergoing complete oxidation passes through the intermediate stage of acetate.

The tricarboxylic acid cycle is a common pathway for the complete oxidation of both glucose and acetate.

Intermediates of the fermentation process in yeast are not in equilibrium with acetate.

Assimilation of carbon dioxide occurs but is of minor importance in the formation of succinate from glucose.

The data are in accord with the postulate that fermentation and oxidation of glucose diverge at acetaldehyde.

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Spectral Changes of Some Dyes in Soluble Silicate Solutions

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The spectral changes of the dyes pinacyanol chloride, toluidine blue O and Rhodamine 6G in sodium silicate solutions were reported recently.[1] The present paper reports the effect of sodium silicates on the spectra of eleven additional dyes and the influence of two potassium silicates and of seven sodium silicates of different SiO_2/Na_2O ratios, on the absorption spectrum of 1×10^{-5} M pinacyanol chloride. The influence of sodium chloride, sulfate, and orthophosphate on the spectrum of pinacyanol chloride in water and in a silicate solution (0.00598 M $Na_2O \cdot 3.33$ SiO_2) is also studied.

Experimental

Compositions of the soluble silicates used are summarized in Table I. The sodium oxide content was determined by titration with standardized hydrochloric acid to the methyl orange endpoint. Silica was determined gravimetrically. The remainder of each silicate is almost entirely water. Impurities were usually less than 0.1%. The orthosilicate, sesquisilicate, and metasilicate are white free flowing solids; the others are aqueous solutions. "Kasil #1," "E" and "Star" are clear and transparent.

TABLE I

COMPOSITION OF THE SOLUBLE SILICATES

Name[a]	Mol. formula	Mol. wt.[b]	Percentages Na_2O	Percentages SiO_2
Na orthosilicate	Na_4SiO_4	185.4	61.35	29.40
Metso 99	$Na_2O \cdot 0.590SiO_2$	148.2	36.12	24.62
Metso crystals	$Na_2SiO_3 \cdot 5H_2O$	122.4	29.24	28.20
BW	$Na_2O \cdot 1.71SiO_2$	164.8	19.35	32.09
Star	$Na_2O \cdot 2.62SiO_2$	219.6	10.34	26.29
E	$Na_2O \cdot 3.33SiO_2$	262.0	8.72	28.13
S	$Na_2O \cdot 3.95SiO_2$	291.7	6.58	25.16
Kasil 1	$K_2O \cdot 3.79SiO_2$	321.6	7.77[c]	18.72
Kasil 6	$K_2O \cdot 3.27SiO_2$	290.8	12.82[c]	26.76

[a] Philadelphia Quartz Co. trade names (except Na orthosilicate which was a product of the Dow Chemical Co.). [b] Molecular weights determined on the basis of Na_2O and SiO_2 determinations. Theoretical percentages for pure "orthosilicate" 184.0 and for pure sodium metasilicate 122.05. [c] K_2O for Kasils.

(1) R. C. Merrill, R. W. Spencer and R. Getty, THIS JOURNAL, 70, 2460 (1948).

"BW," "S" and "Kasil #6" are opalescent due mainly to a small amount of impurities. The sodium hydroxide, chloride, sulfate and orthophosphate were J. T. Baker reagent grade chemicals.

All of the dyes were commercial products and used without further purification. Crystal violet (C. I. 681), basic fuchsin (C. I. 677), malachite green, phenosafranin (C. I. 840), quinaldine red, and pinacyanol chloride were purchased from the Eastman Kodak Co. Nile blue A (C. I. 913), thionin (C. I. 920) and niagara sky blue 6B (C. I. 518) were obtained from Eimer and Amend. The safranine O (C. I. 841) and toluidine blue O were obtained from A. H. Thomas and the methylene blue from Merck. Most of the dyes were manufactured by the National Aniline Division of the Allied Chemical and Dye Corp. According to the supplier the total dye content of basic fuchsin was 91%, of crystal violet 93%, nile blue A 86%, thionin 86%, safranine O 94%, and toluidine blue O 66%. The molar concentrations are given on the basis of actual dye content assuming the remaining dyes were pure.

The absorption spectra were obtained at room temperature (~22°) with a General Electric Co. recording spectrophotometer. Molar extinction coefficients, ϵ_m, were calculated from the transmission curves using the equation $\epsilon_m = 1/cd \log_{10} I_0/I$, where c is the molar concentration of dye, d the width of the cell (1.00 cm.), and I_0 and I the intensity of the incident and transmitted light. Stock solutions of the dyes and silicates were mixed immediately before the transmission curves were determined in order to avoid or minimize possible errors due to fading of some of the dyes. Basic fuchsin, malachite green, nile blue A and crystal violet fade in less than one-half hour at pH's above 12 so that their absorption spectra in 0.020M sodium metasilicate and 0.0158M sodium hydroxide were not determined. Any fading of the other dyes during our experiments was considered negligible.

Results

Absorption spectra of the thiazine dyes, methylene blue, toluidine blue O and thionin in solutions of the more siliceous silicates, $Na_2O \cdot 3.33SiO_2$ and $Na_2O \cdot 3.95SiO_2$, show pronounced differences from those for the same concentration of dye in water or in dilute sodium hydroxide solutions of the same pH as the silicate solutions. The molar absorption curve of 1×10^{-5} M methylene blue in water shows the α band maximum at 662 mμ with ϵ_m = 67,600 and indications of the β band at 615 mμ where ϵ_m = 37,200 (Fig. 1). The absorption curve is practically the same for this concentration of methylene blue in 0.0158 M so-