THE OXIDATION OF GLUCOSE AND ACETATE BY SACCHAROMYCES CEREVISIAE[1]

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There is still no general agreement concerning the major pathway of oxidation of carbon compounds by yeasts. For example, Weinhouse and Millington (1947) and Weinhouse *et al.* (1948), using bakers' yeast, have presented evidence indicating that glucose is degraded to acetate which is oxidized further by way of the tricarboxylic acid cycle. Krebs *et al.* (1952), on the other hand, have suggested that the tricarboxylic acid cycle serves the purpose of supplying intermediates for the synthesis of cell material and is not a primary route of oxidation in yeast.

Some support has been given recently to this latter view by work with other organisms. Roberts and Abelson (1953), working with *Escherichia coli*, have calculated that only twenty-five per cent of the carbon entering the tricarboxylic acid cycle is converted to carbon dioxide and that the carbon dioxide output of the cycle accounts for only two per cent of the total carbon dioxide produced from glucose. In addition to this, Wiame and Bourgeois (1953) have described a variant of *Bacillus subtilis* that uses tricarboxylic acid cycle intermediates in the mobilization of glutamic acid nitrogen for growth, although these intermediates cannot be oxidized. It would appear, then, that the main function of the tricarboxylic acid cycle in these organisms is to supply not energy but intermediates for growth.

An alternative pathway for the oxidation of glucose by yeast has been postulated (Horecker *et al.*, 1953). Lipmann (1936) and Dickens (1936) suggested that glucose may be oxidized by way of 6-phosphogluconic acid. This possibility has been investigated in more detail by Cohen and Scott (1950), Horecker (1950), and Horecker and Smyrniotis (1951). Horecker *et al.* (1953) showed that a seven-carbon intermediate, sedoheptulose, is involved in the further metabolism of 6-phosphogluconate and suggested a cyclic mechanism for glucose oxidation entirely independent of the tricarboxylic acid cycle. Gilvarg (1952), however, in studies with glucose-1-C^{14} has shown that this mechanism cannot be a major pathway of glucose oxidation in yeast.

Experiments with *Saccharomyces cerevisiae*, to be described below, show that different oxidative patterns may be operative in yeast, depending upon the stage during the growth cycle at which the cells have been harvested. In the case of cells harvested in the stationary or late logarithmic phases, the data are consistent with the assumption that glucose is degraded by way of the Embden-Meyerhof-Parnas pathway to alcohol, which is oxidized then more or less completely by way of the tricarboxylic acid cycle. Cells harvested earlier, however, appear to oxidize glucose by some mechanism in which neither the tricarboxylic acid cycle nor the hexose monophosphate shunt is primarily involved.

MATERIALS AND METHODS

Saccharomyces cerevisiae, strain LK2G12, was used throughout this investigation. The organism was grown in a medium of the following composition: 1 per cent glucose, 0.1 per cent ammonium chloride, 0.1 per cent yeast extract (Difco) and M/10 KH_2PO_4. Incubation was at 30 C with air vigorously bubbled through the culture. Growth was followed by turbidity measurements, using a Klett-Summerson colorimeter. Stock cultures of the organism were kept on slants of the above composition with 1.5 per cent agar added. Oxygen uptake was measured in a conventional Warburg apparatus at 30 C, with air as the gas phase.

Cell-free extracts were obtained by grinding with alumina (McIlwain, 1948). After centrifugation at 10,000 rpm in a Sorvall centrifuge for twenty minutes, the protein content of the clarified extracts was measured colorimetrically

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by the method of Lowry *et al.* (1951), using crystalline ovalbumin as a standard.

Acetate-1-C^{14} was synthesized from $BaC^{14}O_3$ by the method described by Calvin *et al.* (1949).

3,4-C^{14}-glucose[2] was prepared by the method of Zilversmit *et al.* (1948) and was purified by paper chromatography according to the methods devised by Putman and Hassid (1952).

Citrate was determined by the method of Weil-Malherbe and Bone (1949) and acetyl-coenzyme A by the method of Lipmann and Tuttle (1945).

Acetate activating enzyme and condensing enzyme activities were measured according to the methods of Novelli and Lipmann (1950). Aconitase and fumarase activities were measured according to the methods of Racker (1950).

Glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase activities were measured by the rate of reduction of triphosphopyridine nucleotide (TPN) in the following system: 0.1 ml substrate (M/10), 0.3 ml triphosphopyridine nucleotide (1 mg per ml, 65 per cent pure), and tris buffer (pH 8) to a total volume of 3.0 ml. The increase in optical density at 340 mμ was measured with a Beckman DU model spectrophotometer.

All radioactivity measurements were carried out with a Tracerlab Autoscaler, using a thin window Geiger tube. In the experiments with radioactive acetate, the isolated lipids were plated on copper disks and counted, with no correction made for self-absorption. In the respiratory experiments with labeled glucose, the carbon dioxide was absorbed in NaOH placed in the center well of a Warburg vessel. This absorbed carbon dioxide was converted subsequently to barium carbonate. The observed activity of the barium carbonate was corrected for self-absorption by the method described by Calvin *et al.* (1949).

RESULTS

Oxidations by "young" and "old" cells. It has been shown (Novelli and Lipmann, 1950) that fully grown cultures of *S. cerevisiae*, strain LK2G12, can oxidize glucose and acetate rapidly. Considerable difference is observed, however, between the oxidative abilities of cells harvested during the early logarithmic phase of growth ("young" cells) and cells harvested during the late logarithmic or stationary phases ("old" cells). Figure 1 shows a comparison of glucose, ethanol, and acetate oxidation by these two kinds of cells. "Old" cells are seen to oxidize the three substrates at essentially the same rapid rate. "Young" cells, however, oxidize ethanol and acetate only very slowly although glucose is oxidized at a rate comparable to that of "old" cells.

An obvious explanation for this inability of "young" cells to oxidize acetate is that such cells are impermeable to this substrate. To test this possibility, two different approaches were used.

Experiments with C^{14}-acetate. Acetate, labeled in the carboxyl group with C^{14}, was given to suspensions of "young" and "old" cells that were actively metabolizing glucose. After aeration for two hours at room temperature, the cells were harvested, washed twice with distilled water, and resuspended in 1 N alcoholic KOH. This suspension was saponified on a boiling water bath for 1½ hours. The residue was acidified with sulfuric acid and extracted with three 20 ml portions of petroleum ether. The combined ether extracts were evaporated to dryness, redissolved in 15 ml of petroleum ether, and washed twice with M/10 acetic acid and three times with distilled water. The ether solution was again evaporated to dryness, and the residue was dissolved in 5 ml of chloroform. Aliquots of this chloroform solution, containing the cellular fatty acids and nonsaponifiable material, were plated on copper disks, and the radioactivity was determined. The amount of C^{14} present in the isolated lipids was used as a measure of the permeability of the cells to acetate. The results (table 1) show that acetate is incorporated equally readily into lipids by "young" and "old" cells.

Studies with permeable preparations. Treatment of "old" cells with dry ice, according to the technique of Krebs *et al.* (1952), yielded preparations with oxidative properties given in table 2. Since succinate is oxidized by the treated cells, but not by intact cells, it is apparent that these "old" cells have been rendered permeable. Furthermore, it may be seen that the treated cells oxidize both glucose and acetate.

In the case of "young" cells (table 3), succinate was oxidized to a limited extent by both intact and treated cells. Thus, the utilization of suc-

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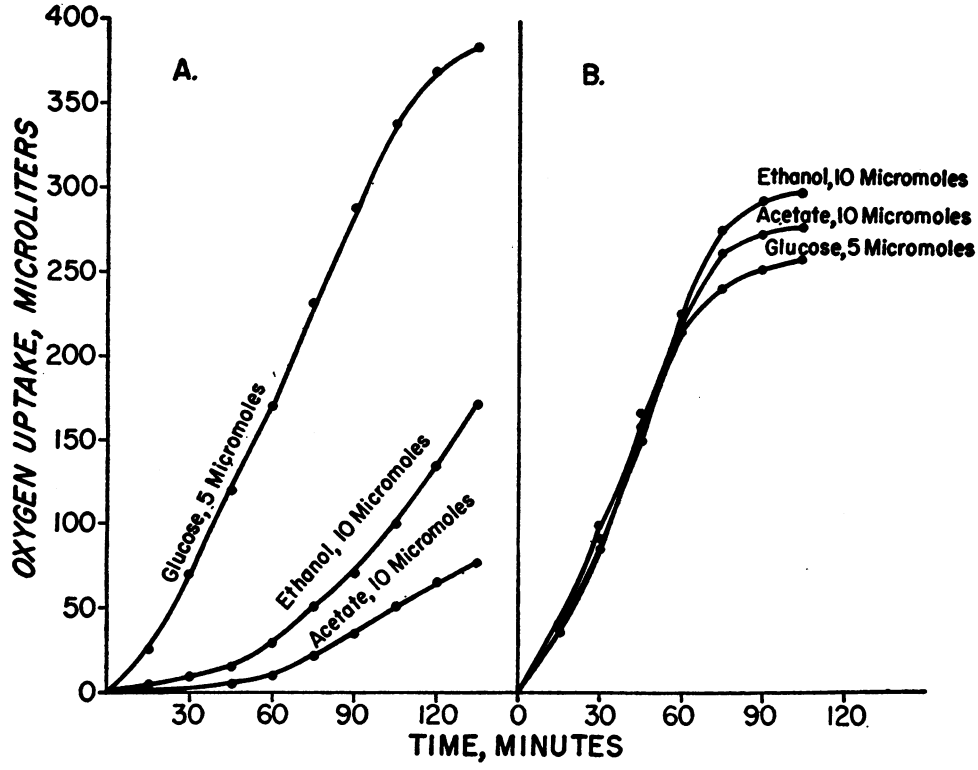

Figure 1. Oxidation of glucose, alcohol, and acetate by "young" and "old" cells of *Saccharomyces cerevisiae.*

A. Cells (4.3 mg dry weight) six hours old; B. Cells (4.0 mg dry weight) twenty-four hours old. Endogenous values subtracted from each curve.

TABLE 1

The incorporation of 1-C^{14}-acetate by "young" and "old" cells

AGE	GROWTH PHASE	Q_{O_2} ON ACETATE	LIPIDS*
hr			
6	early log	8.6	6.8×10^3
23	late log	65.4	4.7×10^3

* Counts per minute per mg cells.

Added acetate: 5.5×10^6 counts per minute. Controls, with acetate added after KOH, showed no labeling in lipid fraction.

cinate by treated cells could not be used as an indication of permeability. Therefore, in order to test whether "young" cells had been made permeable by the freeze-thaw treatment, the ability of the treated "young" cells to oxidize hexosephosphate esters was determined. It is clear from table 3 that intact cells do not oxidize these substrates. Their rapid oxidation by treated cells, however, indicates that the permeability barrier has been removed. The behavior of permeable "young" cells with respect to glucose and acetate, on the other hand, is essentially the same as that of intact cells.

On the basis of these experiments, using radioactive acetate and permeable preparations, it must be concluded that impermeability to acetate cannot explain the decreased ability of "young" cells to oxidize this substrate.

Analysis for enzymes of the tricarboxylic acid cycle. From the results on the study of acetate incorporation, it seemed likely that acetate can be activated to acetyl-coenzyme A by "young" cells once it has entered the cell. The poor rate of respiration on acetate, then, did not seem to be a result of a deficiency of the acetate activating enzyme of Lipmann *et al.* (1952). This assumption was confirmed by analyzing for acetyl-coenzyme A formation in extracts of "young" and "old" cells. Indeed, extracts from "young" cells always

TABLE 2

Oxidation of glucose, acetate, and succinate by intact and permeable "old" cells

SUBSTRATE	O_2 UPTAKE IN μL AFTER 155 MINUTES*	
	Intact cells	Treated cells
5 μM glucose	141	88
10 μM acetate	252	83
10 μM succinate	4	243

* Endogenous subtracted.

The cells were grown for 42 hours. Each vessel contained either 4.5 mg cells in 1 ml M/10 phosphate buffer (pH 7.2) or 1 ml of the freeze-thaw preparation diluted 50 per cent with M/10 phosphate buffer (pH 7.2), 1 ml aqueous solution of substrate, and 0.2 ml 20 per cent KOH in the center well.

TABLE 3

Oxidation of glucose, acetate, succinate, and hexosephosphate esters by intact and permeable "young" cells

EXPERIMENT	SUBSTRATE	O_2 UPTAKE IN μL AFTER 90 MINUTES*	
		Intact cells	Treated cells
1	5 μM glucose	195	108
	10 μM acetate	11	4
	10 μM succinate	57	41
2	5 μM glucose	225	194
	10 μM acetate	9	8
	10 μM fructose-6-phosphate	15	390
	10 μM hexosediphosphate	38	234

* Endogenous subtracted.

Experiments 1 and 2 represent different cultures, each grown for 9 hours. Each flask contained either 4.8 mg of cells (experiment 1) or 5.3 mg cells (experiment 2) suspended in 1 ml M/10 phosphate buffer (pH 7.2) or 1 ml of treated cells diluted 50 per cent with M/10 phosphate buffer (pH 7.2), 1 ml aqueous solution of substrate, and 0.2 ml 20 per cent KOH in the center well.

appeared to have a greater activity in this respect (table 4).

The next reaction involved in the conventional tricarboxylic acid cycle is the condensation of acetyl-coenzyme A and oxalacetate to form citrate. The enzyme catalyzing this reaction has been shown to be present in this strain of yeast by Novelli and Lipmann (1950). Its presence was confirmed in extracts from "old" cells, but similar preparations from "young" cells consistently showed a lower activity of this enzyme (table 5). Analyses for aconitase and fumarase showed correspondingly low activities in extracts from "young" cells (table 6).

These findings support the contention that acetate is oxidized by way of the tricarboxylic acid cycle in "old" cells. The impaired ability of

TABLE 4

Acetate activating enzyme in extracts of "young" and "old" cells

EXPERIMENT	AGE	ACETYL-COENZYME A*
1	10 hours ("young")	1.10
	76 hours ("old")	0.59
2	9 hours ("young")	0.79
	47 hours ("old")	0.29

* μM formed per mg protein in 90 minutes.

TABLE 5

Condensing enzyme activity in extracts of "young" and "old" cells

EXPERIMENT	AGE	CITRATE*
1	10 hours ("young")	0.29
	76 hours ("old")	0.97
2	6 hours ("young")	0.17
	23 hours ("old")	0.43

* μM formed per mg protein in 90 minutes.

TABLE 6

Aconitase and fumarase activities in extracts of "young" and "old" cells

EXTRACT	ACONITASE*	FUMARASE*
"young"	0.006	0.038
"old"	0.049	0.120

* Increase in optical density per minute per mg protein, at 240 mμ. Each tube contained 0.1 ml of M/10 aqueous solution of citric acid (for aconitase) or DL-malic acid (for fumarase), 0.4 ml of extract and 0.05 M tris buffer (pH 8.0) to a total volume of 3.09 ml.

"young" cells to oxidize acetate may, therefore, be the result of a deficiency of at least some of the tricarboxylic acid cycle enzymes.

On the mechanism of glucose oxidation by "young" cells. Data presented above (figure 1) show that "young" cells oxidize glucose well beyond the level of acetate although neither ethanol nor acetate is oxidized to any great extent. In some cases, the oxygen consumed may account for as much as 75 per cent of the glucose added. The most plausible explanation for this seemed to be that glucose might be oxidized by way of the hexose monophosphate shunt. If this were the case, one might expect to find a difference in the activities of the enzymes, glucose-6-phosphate dehydrogenase, and 6-phosphogluconic acid dehydrogenase, in "young" and "old" cells, since inverse differences had already been found in certain of the tricarboxylic acid cycle enzymes. The activities of these enzymes, however, were found to be essentially the same in extracts of "young" and "old" cells.

The possibility that the hexose monophosphate pathway is operative was investigated further by the use of glucose, labeled in the 3 and 4 positions with C^{14}. If such a mechanism for the oxidation of glucose were involved, the carbon dioxide released during the initial stages of oxidation would arise from carbon 1 and therefore would be unlabeled. On the other hand, if glucose were oxidized subsequent to glycolysis, one would expect the carbon dioxide to be highly labeled. As seen in table 7, with cells utilizing glucose under anaerobic conditions, all of the carbon dioxide produced arises from carbons 3 and 4.

TABLE 7

Utilization of 3,4-C^{14}-glucose by "young" cells

	μM	SPECIFIC ACTIVITY*
Glucose added	55	1,810 (of 3-
CO_2 formed anaerobically	111	1,810 and
CO_2 formed aerobically†	110	1,390 4-
CO_2 formed aerobically† with 5×10^{-3} M fluoroacetate added	102	1,720 carbons)

* Counts per minute per mg carbon.

† In the experiments carried out in air, the reaction was stopped after enough oxygen had been taken up to take the added glucose to the level of acetate.

Similarly, under aerobic conditions, almost all of the carbon dioxide produced during the initial stages of oxidation arises also from the 3- and 4-carbon atoms. The small amount of dilution observed in this latter case may be the result of a partial further oxidation of the glucose molecule or of nonisotopic carbon dioxide arising from endogenous reserves. That the further oxidation of glucose contributes to this dilution is shown by the effect of the addition of fluoroacetate which inhibits glucose oxidation past the oxidation level of acetate. Under these conditions, the specific activity of the carbon dioxide produced approached that produced under anaerobic conditions.

These results are incompatible with the concept of oxidation by way of a hexose monophosphate shunt mechanism and, with respect to the strain of yeast used in this study, are a confirmation of the findings of Gilvarg (1952) that this does not represent a major pathway of oxidation in yeast.

DISCUSSION

Differences in oxidative abilities of young and old cells have been described, from time to time, in other organisms. Cochrane and Peck (1953) ascribed the inability of young cultures of *Streptomyces coelicolor* to oxidize acetate to a permeability barrier although no definitive evidence was presented.

Powelson *et al.* (1947) and Goldschmidt and Powelson (1953) have studied a similar inability of young cells of *Micrococcus pyogenes* var. *aureus* to oxidize acetate. It was concluded that the acetate oxidizing system in this organism is adaptive and that certain constituents of the growth medium inhibit this adaptation. There was some indication that glucose was the interfering substance.

The inhibition of acetate oxidation by glucose also has been reported by Umbarger (1953*a*,*b*), working with *E. coli*.

Preliminary experiments with our yeast indicate that in this case also glucose concentration of the growth medium may be a determining factor in the ability of the cells to oxidize acetate. Certain differences are apparent, however, in the case of the yeast studied here. Acetate oxidation is not sparked by the addition of succinate, as with the other systems mentioned. In addition, the oxidation of glucose by cells

incapable of rapid acetate oxidation does not lead to an accumulation of acetate as in *Micrococcus* but proceeds almost to completion.

The fact that glucose is oxidized well beyond the level of acetate by "young" cells and that neither acetate nor ethanol is oxidized appreciably by these cells shows that these substances cannot be obligatory intermediates in glucose oxidation. It might be argued that "young" cells, oxidizing glucose in the Warburg vessel, adapt during glucose oxidation to the oxidation of acetate. Certain observations, however, make this appear unlikely. First of all, with both glucose and acetate present in the vessel, oxygen is taken up rapidly for a period, and then the rate declines to that of the endogenous. (This "break" in rate coincides with that observed when an equivalent amount of glucose alone is present in the vessel.) Subsequent to this, after a lag period of about 30 minutes, rapid oxidation (presumably of acetate) ensues once more. Secondly, during vigorous glucose oxidation, if cells are removed from the vessel just prior to the exhaustion of glucose and are washed and given acetate as the substrate, oxygen uptake now is negligible. Both of these observations indicate that the ability to oxidize acetate does not develop during glucose oxidation. On the contrary, they support the idea that the capacity to oxidize acetate is suppressed until glucose is no longer available.

In "old" cells, which oxidize acetate rapidly, certain tricarboxylic acid cycle enzymes appear to be much more active than in "young" cells. Thus, the inability of "young" cells to oxidize acetate may be a result of deficiencies in the tricarboxylic acid cycle. It seems highly unlikely, then, that glucose can be oxidized by this pathway in "young" cells.

Experiments with labeled glucose have indicated that the 3- and 4-carbons of glucose are converted to carbon dioxide, with very little carbon from the other positions in the glucose molecule appearing as carbon dioxide during the initial stages of oxidation. These observations lead to the conclusion that glucose probably is metabolized by way of the Embden-Meyerhof-Parnas pathway to pyruvate, the latter being degraded to carbon dioxide and some two-carbon fragment which may be oxidized further.

Since the above experiments indicate that oxidation of glucose by "young" cells proceeds subsequent to glycolysis, it is of considerable interest that neither ethanol nor acetate is oxidized under these conditions. Furthermore, from experiments with crude extracts, it is clear that these cells are capable of producing acetyl-coenzyme A from acetate. It is suggested therefore on these bases, and in view of the impairment of the tricarboxylic acid cycle, that glucose oxidation in "young" cells occurs by degradation to carbon dioxide and some two-carbon compound, not in equilibrium with ethanol, acetate, or acetyl-coenzyme A. The nature of this compound and its further metabolism are, at this time, unknown. It is possible, of course, that the two-carbon compound is not itself oxidized further, but rather that it condensed with other such fragments to yield ultimately a new molecule of hexose. Then the latter may be degraded through pyruvate to carbon dioxide and another pair of two-carbon compounds.

SUMMARY

Cells of *Saccharomyces cerevisiae* harvested in the late logarithmic or stationary phases of growth oxidize glucose, ethanol, and acetate rapidly, while cells harvested in the early logarithmic phase oxidize only glucose appreciably.

The inability of young cells to oxidize acetate cannot be explained on the basis of impermeability to this substrate but may be the result of a deficiency of certain tricarboxylic acid cycle enzymes.

The oxidation of glucose by young cells proceeds well past the oxidation level of acetate. Evidence is presented concerning the mechanism of glucose oxidation by young cells, and the possibility of the existence of a new pathway is discussed.

REFERENCES

CALVIN, M., HEIDELBERGER, C., REID, J. C., TOLBERT, B. M., AND YANKWICH, P. E. 1949 *Isotopic carbon: Techniques in its measurement and chemical manipulation.* John Wiley and Sons, Inc., New York, N. Y.

COCHRANE, V. W., AND PECK, H. D., JR. 1953 The metabolism of species of *Streptomyces*. VI. Tricarboxylic acid cycle reactions in *Streptomyces coelicolor*. J. Bact., **65**, 37–44.

COHEN, S. S., AND SCOTT, D. B. M. 1950 Formation of pentose phosphate from 6-phosphogluconate. Science, **111**, 543–544.

DICKENS, F. 1936 Mechanism of carbohydrate oxidation. Nature, **138**, 1057.

GILVARG, C. 1952 Utilization of glucose-1-C^{14} by yeast. J. Biol. Chem., **199**, 57–64.

GOLDSCHMIDT, M. C., AND POWELSON, D. M. 1953 Effect of the culture medium on the oxidation of acetate by *Micrococcus pyogenes* var. *aureus*. Arch. Biochem. and Biophys., **46**, 154–163.

HORECKER, B. L. 1950 Phosphogluconic acid metabolism. Federation Proc., **9**, 185–186.

HORECKER, B. L., AND SMYRNIOTIS, P. Z. 1951 Phosphogluconic acid dehydrogenase from yeast. J. Biol. Chem., **193**, 371–381.

HORECKER, B. L., SMYRNIOTIS, P. Z., AND KLENOW, H. 1953 Role of sedoheptulose phosphate in carbohydrate metabolism. Federation Proc., **12**, 219–220.

KREBS, H. A., GURIN, S., AND EGGLESTON, L. V. 1952 The pathway of oxidation of acetate in baker's yeast. Biochem. J. (London), **51**, 614–628.

LIPMANN, F. 1936 Fermentation of phosphogluconic acid. Nature, **138**, 588.

LIPMANN, F., AND TUTTLE, L. C. 1945 A specific micromethod for the determination of acyl phosphates. J. Biol. Chem., **159**, 21–28.

LIPMANN, F., JONES, M. E., BLACK, S., AND FLYNN, R. M. 1952 Enzymatic phosphorylation of coenzyme A by adenosine triphosphate. J. Am. Chem. Soc., **74**, 2384.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem., **193**, 265–275.

MCILWAIN, H. 1948 Preparation of cell-free bacterial extracts with powdered alumina. J. Gen. Microbiol., **2**, 288–291.

NOVELLI, G. D., AND LIPMANN, F. 1950 The catalytic function of coenzyme A in citric acid synthesis. J. Biol. Chem., **182**, 213–228.

POWELSON, D. M., WILSON, P. W., AND BURRIS, R. H. 1947 Oxidation of glucose glycerol and acetate by *Staphylococcus aureus*. Biochem. J. (London), **41**, 486–491.

PUTMAN, E. W., AND HASSID, W. Z. 1952 Isolation and purification of radioactive sugars by means of paper chromatography. J. Biol. Chem., **196**, 749–752.

RACKER, E. 1950 Spectrophotometric measurements of the enzymatic formation of fumaric and cis-aconitic acids. Biochim. et Biophys. Acta, **4**, 211–214.

ROBERTS, R. B., AND ABELSON, P. H. 1953 The role of the tricarboxylic acid cycle in amino acid synthesis in *E. coli*. Science, **117**, 471.

UMBARGER, H. E. 1953*a* Acetate oxidation in a mutant strain of *E. coli*. Federation Proc., **12**, 282.

UMBARGER, H. E. 1953*b* Influence of glucose on the growth factor requirement of a mutant of *Escherichia coli*. J. Bact., **66**, 328–332.

WEIL-MALHERBE, H., AND BONE, A. D. 1949 The microestimation of citric acid. Biochem. J. (London), **45**, 377–381.

WEINHOUSE, S., AND MILLINGTON, R. H. 1947 Acetate metabolism in yeast studied with isotopic carbon. J. Am. Chem. Soc., **69**, 3089–3093.

WEINHOUSE, S., MILLINGTON, R. H., AND LEWIS, K. F. 1948 Oxidation of glucose by yeast, studied with isotopic carbon. J. Am. Chem. Soc., **70**, 3680–3683.

WIAME, J. M., AND BOURGEOIS, S. 1953 Effect of citric acid on the growth of a variant of *Bacillus subtilis*. Nature, **172**, 310–311.

ZILVERSMIT, D. B., CHAIKOFF, I. L., FELLER, D. D., AND MASORO, E. J. 1948 Oxidation of glucose labeled with radioactive carbon by normal and alloxan-diabetic rats. J. Biol. Chem., **176**, 389–400.