[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE HOUDRY PROCESS CORPORATION]

Acetate Metabolism in Yeast, Studied with Isotopic Carbon[1]

BY SIDNEY WEINHOUSE AND RUTH H. MILLINGTON

Recent studies have established the tricarboxylic acid cycle, originally formulated by Krebs[2] to account for pyruvate oxidation in animal tissues, as a mechanism of wide scope in the oxidative metabolism, not only of carbohydrate, but of fatty acids and at least some of the amino acids.[3] As presently envisioned by us, the process starts by the condensation of oxalacetate with an acetyl group to yield a 6-carbon tricarboxylic acid, whose subsequent breakdown through the steps of α-ketoglutarate, succinate, fumarate and malate results in the complete oxidation of the acetyl group and regeneration of oxalacetate to carry on the cycle.[3,4]

Inasmuch as the broad outlines of this cyclic process were developed from studies with animal tissues, it was of interest whether oxidative metabolism of microörganisms proceeds by a similar pathway. To gain more definite information concerning the oxidative pathways of microörganisms, a study was made of the metabolism in yeast of acetate labeled with carbon 13. Yeast is especially suitable for such an investigation, since acetate oxidation occurs rapidly without addition of other substrates; endogenous metabolism is virtually nil; and during acetate oxidation sufficient amounts of the tricarboxylic acid, citric acid, accumulate to allow its isolation and chemical degradation.[5,6] The results of this study, particularly the amount and distribution of C^{13} in the citric acid, were in accord with the conception that the tricarboxylic acids are intermediates of a major metabolic pathway in yeast.

Results

Effect of Barium and Magnesium Ions on Citrate Formation.—Wieland and Sonderhoff[5] first observed the accumulation of citrate during oxidation of barium acetate by brewers' yeast, in amounts approximately 10% of the acetate utilized. Virtanen and Sundman[4] subsequently found, in a study of the effect of metal ions on acetate oxidation, that citrate accumulation was maximal with barium and magnesium acetate. Before proceeding with the isotopic experiments, a comparison was made of acetate utilization and citrate formation with sodium, barium and magnesium acetate. Fleischmann bakers' yeast (3.50 g. corresponding to 1.06 g. dry weight) was suspended in 100 ml. of distilled water and shaken in oxygen at 25° for sixteen hours to deplete the cells of endogenous nutrients.[5] The respective salts, 10 ml. of a 2 N solution, were then added and the flasks again shaken at 25° in a stream of oxygen. Aliquots were withdrawn at various intervals for citrate determination. A parallel set of experiments was made for determination of acetate utilization, the unutilized acetate being recovered by centrifugation and steam distillation of the centrifugate.

No particular differences were observed in the utilization of the three salts. After a short induction period, oxidation of acetate proceeded linearly until about three-fourths was metabolized in sixteen hours; thereafter, the rate gradually fell off, but oxidation was over 95% complete in twenty-four hours. As shown in Table I, considerable difference was noted in the accumulation of citrate; it was highest with magnesium acetate, accounting for about 20% of the acetate utilized in eight hours; it was lower with barium acetate, and lowest with sodium acetate.[7] With all three salts, citrate accumulation was maximal in about eight to fourteen hours, at which time one-half to two-thirds of the acetate was metabolized.

TABLE I

CITRATE FORMATION FROM SODIUM, MAGNESIUM AND BARIUM ACETATES

Values are in mg. of citric acid

Substrate, acetate	Time, hr. 2	5	8	14	24
Magnesium	10.5	50.0	112	119	102
Barium	6.4	31.0	62.7	71.4	33.6
Sodium	6.3	12.7	15.4	8.0	6.3

Oxidation of Isotopic Acetate.—The results of experiments on the oxidation of magnesium and barium acetate in which the acetate carboxyl carbon was tagged with excess C^{13} are given in Table II. A total of 13.5 mM. of the isotopic magnesium acetate was metabolized, corresponding to 75% of the amount added; with barium acetate, 25.4 mM. of acetate was utilized, corresponding to 63% of the amount added. In each experiment, the recovered acetate had almost the same C^{13} content as the original isotopic substrate, indicating there was little if any endogenous acetate

(1) This work was carried out under sponsorship by the Sun Oil Company. Part has been presented before the Division of Biological Chemistry of the American Chemical Society at Atlantic City, April 15, 1947.

(2) Krebs, "Advances in Enzymology and Related Subjects," **3**, 191 (1943).

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

(4) Weinhouse, Medes and Floyd, *J. Biol. Chem.*, **166**, 691 (1946).

(5) Wieland and Sonderhoff, *Ann.*, **499**, 213 (1932).

(6) Virtanen and Sundman, *Biochem. Z.*, **313**, 236 (1942).

(7) The effects of these ions on citrate accumulation remain unknown. Though citrate is not oxidized appreciably by intact yeast, Lynen (*Ann.*, **539**, 1 (1939)) has shown this to be due to impermeability of the cell membrane, since citrate oxidation occurs rapidly in yeast whose cells are ruptured by immersion in liquid air. We have observed identical rates of disappearance of sodium and magnesium citrate aerobically in liquid nitrogen-treated yeast; hence, the enhancement of citrate accumulation by magnesium ions cannot be attributed to their effect on citrate oxidation.

TABLE II

C^{13} DISTRIBUTION IN PRODUCTS OF ACETATE OXIDATION BY YEAST

C^{13} values are in atom per cent. excess.

	Expt. 1 Isotopic Mg acetate		Expt. 2 Isotopic Ba acetate		Expt. 3 Non-isotopic Mg acetate + isotopic $NaHCO_3$	
	mM.	C^{13} Excess	mM.	C^{13} Excess	mM.	C^{13} Excess
Acetate added	18.03	2.77	40.0	2.75	19.10	0.00
Acetate recovered	4.57	2.62	14.65	2.61	6.25	.01
Acetate utilized	13.46	..	25.35	..	12.85	..
$NaHCO_3$ added	0	..	0	..	5.00	7.57
CO_2 recovered	15.5	3.11	19.5	3.00	15.04	2.06
Lipids	109.1[a]	0.74	184.4[a]	0.99	86.0[a]	0.00
Cell residue	846[a]	.10	....	.21	...	.01
Citrate	0.75	1.94	0.91	2.02	0.52	.01

[a] Milligrams.

formation. That endogenous respiration was negligible is shown by the high content of C^{13} in the respiratory carbon dioxide; indeed, its C^{13} content in each experiment was actually slightly higher than the over-all acetate C^{13}. This curious finding will be discussed later.

C^{13} in Cell Components.—An interesting finding was the high content of C^{13} in the cell lipids. On the assumption that such substances were formed by multiple condensation of acetyl groups,[8] it can be calculated that one-fourth to one-third of the lipids isolated were newly synthesized during the seven-hour period.

The distribution of excess C^{13} among the various lipid fractions isolated from the barium acetate experiment is shown in Table III. The fatty acids had a higher C^{13} excess than the unsaponifiable matter, and the saturated fatty acids about 50% more C^{13} than the unsaturated fatty acids. These results are in agreement with the concept, developed by previous investigators[9-11] of a rapid lipid synthesis from acetate by yeast. A small C^{13} excess was also found in the cell residue after lipid extraction. More complete data on the C^{13} distribution in the cell components is being reserved for a later report:

TABLE III

C^{13} DISTRIBUTION IN LIPID FRACTIONS

	Weight, mg.	C^{13} excess
Total lipids	184.4	0.99
Unsaponifiable matter	28.5	.49
Fatty acids	122.9	1.12
Saturated	...	1.44
Unsaturated	...	0.97

C^{13} in Citric Acid.—In the experiment with magnesium acetate, 0.75 mM. of citrate was formed, corresponding to 17% of the acetate utilized. With barium acetate, 0.91 mM. of citrate accumulated, equivalent to 11% of the utilized acetate. The C^{13} excesses in the respective experiments were 1.94 and 2.02%; or 70 and 73% respectively, of the acetate C^{13} excess.

Carbon Dioxide Assimilation by Yeast.—The third experiment shown in Table I, in which non-isotopic magnesium acetate was oxidized by yeast in the presence of isotopic sodium bicarbonate having a C^{13} excess of 7.57%, indicates that carbon dioxide assimilation does not occur to an appreciable extent under the conditions of these experiments. No significant excess of C^{13} was found in any of the products isolated; the excess of 0.01% in the citrate being within the 0.02% error of the mass-spectrographic measurements.

C^{13} Distribution among Citrate Carbons.—The citrates, isolated from the 3 experiments recorded in Table I, were submitted to a chemical degradation procedure described previously.[4] The C^{13} values obtained for the primary and tertiary carboxyls and the three non-carboxyl carbons are compared in Table IV with the values for the acetate carboxyl and the respiratory carbon dioxide. In adjoining columns are given the actual values and those calculated on the basis of 100% C^{13} in the acetate carboxyl. The citrate formed from magnesium acetate had a C^{13} content 35% of that of the acetate carboxyl, distributed in such a way that the primary carboxyls had 80% and the tertiary carboxyl 57% of the acetate carboxyl C^{13} excess. There was no excess C^{13} in the 3 non-carboxyl carbons. The citrate formed from barium acetate gave results in close agreement, the corresponding values being 2% higher. Degradation of the citrate formed in the presence of isotopic bicarbonate indicates that assimilation of carbon dioxide may be involved in citrate formation, but the values are too low to permit any interpretation. We can only conclude that intermediates of the conversion of acetate to citrate are not in rapid equilibrium with carbon dioxide or bicarbonate.

TABLE IV

DISTRIBUTION OF C^{13} IN CARBON ATOMS OF CITRATE COMPARED WITH ACETATE CARBOXYL AND RESPIRATORY CARBON DIOXIDE

The experiment numbers refer to the corresponding experiments in Table II.

	Expt. 1 Isotopic Mg acetate		Expt. 2 Isotopic Ba acetate		Expt. 3 Non-isotopic Mg acetate + isotopic $NaHCO_3$	
	C^{13} excess	Acetate COOH, %	C^{13} excess	Acetate COOH, %	C^{13} excess	HCO_3^- (2.06), %
Acetate carboxyl	5.54	100	5.50	100	0.00	0
Citrate over-all	1.94	35	2.02	37	.01	0.5
Tertiary carboxyl	3.16	57	3.27	59	.06	3
Primary carboxyls	4.42	80	4.49	82	.02	1
Non-carboxyl carbon	0.00	0	0.00	0	.00	0
Respiratory CO_2	3.11	56	3.00	55	2.06	100

Experimental

Isotopic magnesium and barium acetates were prepared by acidification of sodium acetate prepared as described

(8) Rittenberg and Bloch, *J. Biol. Chem.*, **154**, 311 (1944).
(9) Smedley-MacLean and Hoffert, *Biochem. J.*, **20**, 343 (1926).
(10) MacLeod and Smedley-MacLean, *ibid.*, **32**, 1571 (1938).
(11) Sonderhoff and Thomas, *Ann.*, **530**, 195 (1937).

previously,[4] followed by distillation and neutralization of the distillate with the respective hydroxides. Isotopic sodium bicarbonate was made by absorption of carbon dioxide in a 1 N solution of carbon dioxide-free sodium hydroxide. Using an excess of carbon dioxide in a closed system, its absorption was followed by means of thymol blue indicator. The bicarbonate was isolated by precipitation with two volumes of alcohol, filtration, and successive washing with alcohol and ether.

Oxidation of Isotopic Magnesium Acetate.—Fleischmann bakers' yeast, 3.50 g. corresponding to a dry weight of 1.06 g., was suspended in 100 ml. of distilled water and shaken in oxygen at 25° for approximately sixteen hours to deplete the cells of endogenous nutrients. Ten ml. of an approximately 2 N solution of isotopic magnesium acetate was added, the flask was connected to a bead tower containing an excess of carbon dioxide-free sodium hydroxide; and while shaking mechanically, a slow stream of oxygen was passed through the flask and into the bead tower. After seven hours at 25°, the solution was acidified to 1 N with 50% sulfuric acid; and shaking continued fifteen minutes to liberate any bound carbon dioxide. The contents of the bead tower were washed down and carbon dioxide precipitated as barium carbonate by the addition of barium chloride. The precipitate was filtered, washed, dried, and weighed; these steps being made in a nitrogen atmosphere to avoid contamination by atmospheric carbon dioxide.

After standing overnight[12] the acidified solution was centrifuged and the cells washed twice with 25-ml. portions of water. After addition of 50 g. of $MgSO_4 \cdot 7H_2O$ the combined centrifugate and washings was steam-distilled and the acetic acid neutralized with standard alkali, reacidified and redistilled. The distillate was neutralized, evaporated to a small volume, and the acetate finally isolated as the silver salt by precipitation with silver nitrate.

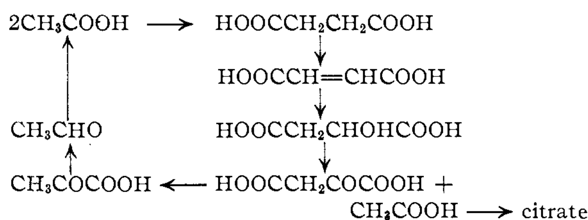
Isolation of Citrate.—The residue of the steam distillation contained, by colorimetric assay, 145 mg. of citric acid. It was extracted continuously with ether for seventy-two hours and the extract evaporated and taken up in water. An excess of silver nitrate was added, the solution neutralized with ammonium hydroxide, and the precipitated silver salts filtered, washed thoroughly, and dried—yield 266.5 mg. These were suspended in water, decomposed by hydrogen sulfide, and the filtrate evaporated to a volume of 7 ml. To the hot solution sufficient quinidine was added to bring the pH to 6.5; after cooling, the turbid solution was filtered and then seeded with a crystal of quinidine citrate. After standing forty-eight hours at room temperature, the quinidine citrate was filtered, washed with two 1-ml. portions of water and dried in a high vacuum. There was obtained 177 mg. of quinidine citrate, m. p. 129–131°. A portion of this salt was degraded by the procedure previously described[4]; the remainder was converted to the silver salt for determination of the over-all C^{13} content.

Isolation of Lipids.—The washed cells were extracted successively for twenty-four hour periods with alcohol and ether; the combined extracts were evaporated, and the residue taken up in petroleum ether. After saponification with 2 N alcoholic potassium hydroxide, separation was made into unsaponifiable and fatty acid fractions. The latter was then submitted to the lead salt procedure for separation of the saturated fatty acids.[13]

The other two experiments were made in exactly the same way, except that in the barium acetate experiment double the quantities of yeast and acetate were used.

Discussion

The formation of citrate during acetate oxidation by yeast was considered by Wieland and Sonderhoff[5] to be a side-reaction on the acetate oxidation pathway of Thunberg and Knoop[2] as outlined.



Two observations specifically exclude this mechanism. First, the citrate should have had the same C^{13} content as the acetate; whereas, it had only 70%. Second, the isotopic acetate should have been diluted by "metabolic" non-isotopic acetate arising from the two central oxalacetate carbons, whereas such dilution was not observed.

Krebs has considered the possibility of the occurrence of the tricarboxylic acid cycle in yeast[2] and concluded, on the basis of the apparent absence of the dehydrogenases for the various components of the cycle and the absence of fumarase and aconitase,[14] that tricarboxylic acids are not involved in the oxidative processes of yeast. Recently this possibility has been reopened by Lynen and Neciullah,[15] who indicated that the failure of yeast to dehydrogenate the di- and tri-carboxylic acids was due to impermeability of the cell membrane. Using yeast, whose cell structure was destroyed by freezing in liquid air, they demonstrated the rapid dehydrogenation of citrate, α-ketoglutarate, succinate, and malate. Lynen also demonstrated the inhibition of acetate oxidation by malonate[16] and has concluded that acetate is oxidized in yeast by the tricarboxylic acid cycle, pointing out that the experiments of Sonderhoff and Thomas[11] are in accord with this hypothesis. These authors found that the oxidation of trideuterioacetate by yeast resulted in the accumulation of isotopic succinate and citrate, with a higher D content in the latter than in the former, a result inexplicable on the basis of the oxidation scheme of Wieland and Sonderhoff described above.

If citrate represents an intermediate stage in the oxidation of acetate, either as a direct component of the tricarboxylic acid cycle, or as a side-reaction product of some other C_6 acid which is the direct participant in the cycle, then the C^{13} distribution among the carbons of the isotopic citrate must satisfy the following criteria. First, the distribution must be in accord with the formation of citrate from an acetyl group and a C_4-dicarboxylic acid, the latter having an equal distribution of C^{13}

(12) It was found that nearly all of the citrate is intracellular, its complete removal from the yeast requiring at least several hours treatment with 1 N acid.

(13) Hilditch, "The Chemical Constitution of Natural Fats," New York, N. Y., 1941, p. 370.

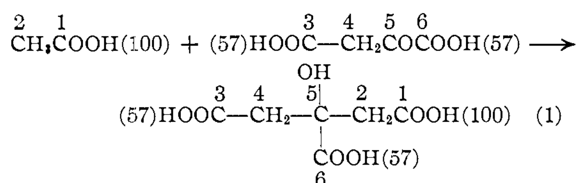
(14) We have found that yeast contains aconitase which is extracted readily with $M/150$ phosphate solution at pH 7 from cells previously frozen by immersion in liquid nitrogen. With *cis*-aconitate as substrate, the calculated $Q_{citrate}^{25°}$ is around 3–5, as compared with values of 6 to 120 at 40° reported for animal tissues (Johnson, *Biochem. J.*, **33**, 1046 (1939)).

(15) Lynen and Neciullah, *Ann.*, **541**, 203 (1939).

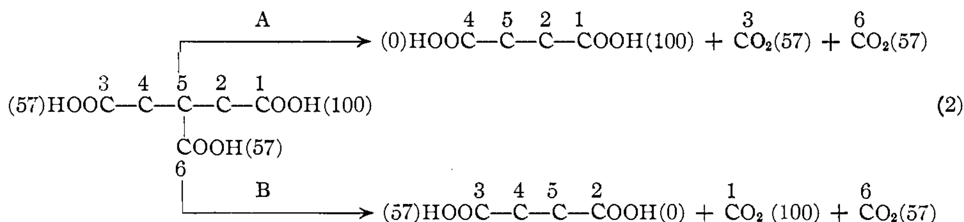
(16) Lynen, *Ann.*, **554**, 40 (1943).

in its two carboxyl carbons (since it must have passed through the symmetrical succinate stage). Second, the distribution must account for the formation, through α-ketoglutarate, of succinate having the same C^{13} content and distribution as the C_4 acid undergoing condensation with acetate (since the C_6 acids, according to the cycle, are simultaneously derived from and are the source of the C_4 acids). Third, the successive decarboxylations involved in the breakdown of the C_6 to the C_4 acids must yield carbon dioxide with the observed C^{13} distribution.

Formation of Isotopic Citrate.[17]—Though there was no appreciable accumulation of C_4 dicarboxylic acids in these experiments,[18] it is easy to surmise what their C^{13} content should have been. It is evident that one of the carboxyls of the C_4 acids is represented by the citrate tertiary carboxyl and hence had a C^{13} content of 57%. Of the two citrate primary carboxyls, having an observed average C^{13} content of 80%, one represents the acetate carboxyl with 100% C^{13}; hence the other, representing the second carboxyl of the C_4 acids had 60% C^{13}, a value sufficiently close to 57 to justify the assumption of an equal C^{13} content in both carboxyls. The observed distribution of C^{13} among the citrate carboxyls is thus in accord with the formation of citrate by condensation of acetate with oxalacetate having a C^{13} content approximately 57% of the acetate carboxyl, distributed equally between the two carboxyls (equation 1).



Breakdown of Isotopic Citrate.—A 6-carbon acid having the observed C^{13} distribution and the citrate carbon skeleton can yield succinate and CO_2 by the 2 following pathways



By pathway A succinate is formed from carbon atoms 4, 5, 2, 1 and thus would have an average C^{13} content of 50% in its carboxyl carbons, and the carbon dioxide, arising from carbons 3 and 6, would have 57% C^{13}. By pathway B succinate arises from carbon atoms 3, 4, 5, 2 and would have $57/2 = 29\%$ C^{13} in its carboxyl carbons; and the carbon dioxide coming from carbons 1 and 6, would have $(100 + 57)/2 = 79\%$ C^{13}. If pathways A and B are followed to equal extents as would occur if the 6-carbon acid were citrate itself, the succinate would have 40% C^{13} in its carboxyl carbons, and the carbon dioxide would have 68% C^{13}. A comparison of these possibilities with the observed value of the respiratory carbon dioxide and the derived value of the C_4 acids shown in Table V, indicates that the data are in best agreement with pathway A. Although citric acid as such is thus excluded, the observed C^{13} of the respiratory carbon dioxide is well accounted for on the assumption that the condensation product is an unsymmetrical substance having the citrate carbon skeleton which breaks down in such fashion that the metabolic carbon dioxide arises from the carboxyls of the C_4 acid moiety; and hence the newly formed succinate molecules carry intact an acetyl moiety from the previous condensation.

TABLE V

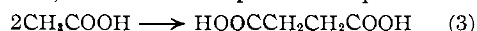
COMPARISON OF OBSERVED C^{13} VALUES AND THOSE CALCULATED ON THE BASIS OF MECHANISMS A AND B

Values are in per cent. of acetate carboxyl group.

	Observed	Mechanism A	B	A + B equally
Succinate carboxyls	57	50	29	40
Respiratory CO_2	55	57	79	68

Direct Formation of C_4 Acids from Acetate.—It is evident from equation 2 that any C_4 acids provided by the cycle can have in their carboxyl groups only 50% of the C^{13} content of the acetate carboxyl. To account for the formation of C_4 acids having 57% C^{13} in their carboxyls, it is necessary to assume that a supplementary mechanism for their formation exists, independent of the cycle. The existence of such a reaction may be surmised also from other considerations. If the only source of C_4 acids is the C_6 acids, then the removal of a significant quantity of the latter in the form of citrate should slow down or stop the oxidation of acetate; actually, however, oxidation of acetate proceeds almost linearly to completion.

One such reaction, namely, the dehydrogenative coupling of two acetyl groups to yield succinate (equation 3) has often been postulated particularly



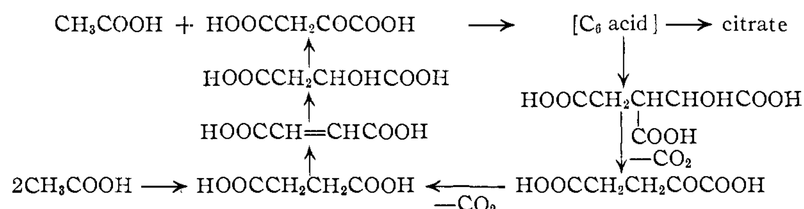
$$2CH_3COOH \longrightarrow HOOCCH_2CH_2COOH \quad (3)$$

(17) The data used for the following discussion are from the isotopic magnesium acetate experiment.

(18) Contrary to previous observations,[5,11] we never observed the accumulation of significant quantities of succinate (or any acid other than citrate) during acetate oxidation. Kleinzeller (*Biochem. J.*, **35**, 495 (1941)) also reported the absence of succinate in similar experiments.

in connection with the Thunberg–Knoop oxidation scheme described above; and has recently been suggested by Slade and Werkman to account for the formation of isotopic succinate from C^{13} tagged acetate during glucose fermentation by *Aerobacter*.[19] The occurrence of this reaction to a small extent simultaneous with the reactions of the tricarboxylic acid cycle would account very well for the presence of 57% C^{13} in the carboxyl carbons of the C_4 acids.

Over-all Scheme for Acetate Oxidation in Yeast.—The conclusions derived from these experiments are summarized in the following outline which may be considered to represent the "steady state" of acetate oxidation in yeast.



The chemical form in which acetate enters the cycle is still in doubt as is the identity of the C_6 condensation product. Various possibilities have already been discussed.[3,4]

The remarkable similarity to the analogous processes of animal tissues is immediately apparent. One feature, constituting one of the principal modifications of the original Krebs cycle, is the intermediary formation of an unsymmetrical C_6 acid rather than citrate. This was already deduced for animal tissues from the key observations: (a) the presence of isotopic carbon in the carboxyl adjacent to the keto group of α-ketoglutarate formed during the oxidation of pyruvate by pigeon liver in the presence of isotopic bicarbonate[2,3]; (b) the presence of isotopic carbon mainly in the δ position of α-ketoglutarate formed during the oxidation of isotopic acetate[20] and acetoacetate[21] by kidney. The fact that this conclusion was derived for yeast entirely independently of the data on animal tissues emphasizes not only the broad scope of the tricarboxylic acid cycle but also the correctness of this oxidative mechanism.

Acknowledgment.—We acknowledge with thanks the support and interest of the Sun Oil Company in this work.

Summary

The oxidation of carboxyl-C^{13}-tagged magnesium and barium acetate by bakers' yeast led to the accumulation of citrate and carbon dioxide, whose C^{13} content and distribution were in accord with the conception that the tricarboxylic acid cycle is a major oxidative process in yeast. The data indicate, first, that an unsymmetrical C_6 acid rather than citrate is the direct participant in the cycle; second, that not all of the C_4 acids are formed from the cycle, but that another independent mechanism exists for their formation from acetate; third, that under the conditions of these experiments intermediates in the conversion of acetate to citrate are not in rapid equilibrium with carbon dioxide or bicarbonate.

The rapid synthesis of cellular lipids from acetate was confirmed.

(19) Slade and Werkman, *Arch. Biochem.*, **2**, 97 (1943).

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

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

PHILADELPHIA, PENNA. RECEIVED JUNE 21, 1947

[CONTRIBUTION FROM SOCONY-VACUUM LABORATORIES (A DIVISION OF SOCONY-VACUUM OIL CO., INC.) RESEARCH AND DEVELOPMENT DEPARTMENT]

Acylation Studies in the Thiophene and Furan Series. IV. Strong Inorganic Oxyacids as Catalysts

BY HOWARD D. HARTOUGH AND ALVIN I. KOSAK[1]

The authors reported previously that iodine and hydriodic acid,[2] zinc chloride[3] and silicate compositions such as montmorillonite clays and synthetic silica–metal oxide gels[4] promote the acylation of thiophene and furan with anhydrides and acyl halides.

Strong inorganic oxyacids such as phosphoric acids, sulfuric acid, and fluo-acids of phosphorus, sulfur and boron have now been found to catalyze the acylation of thiophene and furan with acyl anhydrides and halides. Orthophosphoric acid is the most efficient catalyst and produced the least side reactions of any of the acids used. It was chosen, therefore, for the bulk of this work and was used exclusively in the examples described in the experimental part. However, all the inorganic oxyacids containing fluorine, sulfur, or phosphorus tested, having at least one ionizable hydrogen and an ionization constant greater than 1×10^{-2} for the first hydrogen ion are effective catalysts for the acylation of thiophene and furan. Phosphorous acid (50%), pyrophosphoric acid, fluo-

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(2) Hartough and Kosak, THIS JOURNAL, **68**, 2639 (1946).

(3) Hartough and Kosak, *ibid.*, **69**, 1012 (1947).

(4) Hartough, Kosak and Sardella, *ibid.*, **69**, 1014 (1947).