The Pathway of Oxidation of Acetate in Baker's Yeast

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Lynen & Neciullah (1939), Lynen (1942, 1943), Weinhouse & Millington (1947) and Martius & Lynen (1950) have put forward the view that the tricarboxylic acid cycle represents the main pathway of oxidation of acetate in yeast cells. This hypothesis was based on the observation that the intermediary stages of the cycle can occur in yeast preparations and that the oxidation of isotopic acetate leads to the formation of isotopically labelled citrate and succinate, the isotope being in the positions expected on the basis of the hypothesis. The validity of the evidence has been questioned (Krebs, 1949, 1950) because of the very low rate at which some of the intermediates of the tricarboxylic acid cycle are metabolized.

Isotope experiments and other results reported in this paper show that acetate can be oxidized in yeast cells through a mechanism other than the tricarboxylic acid cycle, or a dicarboxylic acid cycle such as postulated by Foster *et al.* (1949). At the same time the occurrence of the component reactions of the tricarboxylic acid cycle in yeast cells is confirmed. Reasons are put forward in support of the view that in yeast cells the reactions of the tricarboxylic acid cycle serve to supply intermediates for synthetic processes but may not primarily be concerned with the production of energy.

Permeability barriers, and the inability to prepare respiring yeast extracts, present obstacles to experiments on the intermediary metabolism of yeast cells. In intact cells the rate of penetration of the di- and tri-carboxylic acids is negligible compared with the rate of respiration, but Dixon & Atkins (1913) and Lynen (1939) have shown that the permeability barriers can be overcome by exposing the cells to the temperature of liquid air or liquid nitrogen. Although cold-treatment somewhat reduces the activity of the oxidative enzymes, a considerable proportion of the oxidizing activity remains if the cold-treatment is carried out under suitable controlled conditions. The first part of this paper is concerned with the study of the properties of cold-treated cells, the second with the application of the isotope technique to the study of oxidative processes in such cells.

METHODS AND RESULTS

The great majority of the experiments was carried out on baker's yeast supplied by the Standard Yeast Co. Ltd., but some were repeated with pure cultures of Fleischmann's yeast. This was grown at 25° in Roux bottles on medium containing 10 ml. yeast extract (prepared by heating baker's yeast with an equal volume of water to 100° for 30 min. and filtering), 1 g. glucose, 2 g. casein hydrolysate ('Pronutrin') and 2 g. agar in 100 ml. The gas exchange was measured manometrically in conical Warburg manometers at 25° unless otherwise stated. In all aerobic experiments the gas space of the manometer vessels contained O₂ and the centre well 0.2 ml. 2N-NaOH to absorb CO₂. Solid CO₂, henceforth referred to as 'dry ice', was used in most experiments for cold-treatment.

Oxidation of various substrates by frozen cells. Baker's yeast which had been stored in the refrigerator was crumbled into small pieces and thoroughly mixed, and left in contact for 20 min., with an excess of dry ice. On thawing, the yeast became liquid; it was suspended in 40 parts of 0.133M-phosphate buffer, pH 6.5. Each manometer vessel contained 3 ml. yeast suspension in the main compartment and 1 ml. 0.1M-substrate solution in the side arm. The substrate was added after an equilibration period of 10 min. The O₂ uptake for the periods 40–60 min. and 80–100 min. after the addition of substrate is shown in Table 1, together with comparative data for yeast not exposed to dry ice. In the untreated cells, acetate, ethanol, glucose, D- and L-lactate and pyruvate were readily oxidized. There was considerable blank respiration which was not appreciably increased by the ten other substrates tested. Cold-treatment and washing had no effect on the rate of the blank control. Acetate, ethanol, glucose, D- and L-lactate and pyruvate all increased the O₂ uptake of treated cells, but the increase (for the 80–100 min. period) was not more than 25% of that found in the fresh cells. Succinate which had no effect in the untreated cells markedly increased the O₂ consumption in the frozen cells. There was no definite effect of the other nine substrates. Thus, whilst the oxidation of the substrates which are generally oxidized in yeasts is slower in the cold-treated cells, succinate, though not oxidized

in untreated cells, becomes oxidizable after cold-treatment.

Dialysis for 16 hr. against running tap water had no major effect on the cold-treated yeast. The blank respiration did not appreciably decrease, nor were the rates of oxidation of acetate and succinate affected. On storage in the refrigerator the oxidative capacity fell, e.g. 50 % in 48 hr.

Table 1. *Oxidation of various substrates by dry-ice-treated baker's yeast*

(For experimental conditions see text.)

Substrate added	O_2 absorbed by 4 ml. suspension (40 mg. fresh wt.) (μ l.)			
	Untreated cells		Dry-ice-treated cells	
	40-60 min.	80-100 min.	40-60 min.	80-100 min.
None	61	43	69	31
Acetate	468	492	119	140
Ethanol	581	651	148	165
Glucose	638	576	172	183
D-Lactate	328	242	132	97
L-Lactate	324	211	187	123
Pyruvate	267	310	107	96
Butyrate	68	51	76	37
Formate	77	42	66	37
Glutamate	66	44	65	29
α -Ketoglutarate	62	44	77	34
L-Aspartate	62	43	75	36
Citrate	59	44	69	32
Fumarate	57	41	53	23
Tartrate	60	43	68	32
Succinate	61	45	83	55

Demonstration of increased permeability. That the non-oxidation of fumarate and citrate in cold-treated cells is not due to permeability barriers is shown by the rapid formation of L-malate and iso-citrate on addition of fumarate and citrate.

To test fumarase activity, dry-ice-treated yeast (10 g.) was twice washed with 100 ml. water and the sediment suspended in a volume of 100 ml. A sample (10 ml.) of this suspension, 15 ml. 0.1 M-fumarate, 0.5 ml. M-NaHCO₃ and 4.5 ml. water were mixed and saturated with 5% CO₂ + 95% N₂ (v/v) at 25°. Samples of 5 ml. were removed at intervals for polarimetric analysis. They were mixed in quick succession with 1.25 ml. acetic acid, 6 ml. water, 2.5 ml. M-sodium citrate and 11.25 ml. 29% (w/v) ammonium molybdate. After filtration, readings were taken in 2 dm. tubes (see Krebs & Eggleston, 1943).

Time (min.)	Reading (α_D^{17})	L-Malic acid (mg./ml. incubated solution)
0	0	0
15	+0.78	1.25
30	+1.34	2.34

No measurable amount of malate was formed in untreated cells. Washing removed blank rotations, but did not appreciably affect the fumarase activity. The supernatant was inactive. The fumarase activity

was thus very high, compared with other metabolic processes, Q_{malate} being about 75 μ l./mg. dry wt./hr. under the test conditions.

To test aconitase activity the washed, dry-ice-treated cells were suspended in an equal volume of water and 8 ml. of the suspension were mixed with 10 ml. M-sodium citrate, 6 ml. water and 1 ml. M-NaHCO₃ and saturated at 25° with 5% CO₂ in 95% N₂ (v/v). For the polarimetry (see Eggleston & Krebs, 1949) 8 ml. samples were used, the concentration of all the reagents being the same as in the malate determination. A little charcoal was required to obtain a clear filtrate. Readings were as follows:

Time (min.)	Readings (α_D^{17})	isoCitric acid (mg./ml. incubated solution)
20	-0.73	1.46
40	-1.14	2.37

It follows from these data that $Q_{\text{isocitrate}}$ was about 16 μ l./mg. dry wt./hr. under test conditions. Again washing with water did not reduce the enzyme activity. The absence of enzyme activity in the washing fluid shows that fumarate and citrate reacted within the cell. There is therefore no doubt that these di- and tri-carboxylic acids penetrate into the frozen cells. There is no evidence that monocarboxylic acids cannot penetrate into untreated yeast cells.

Fumarase has previously been demonstrated in extracts of brewer's yeast by Jacobsohn (1931) and aconitase in extracts of baker's yeast by Weinhouse & Millington (1947) and by Foulkes (1951). Thus under certain conditions the two enzymes can be extracted from the cells.

Variations in freezing procedure. The effects of freezing are not due to exposure to low temperature itself, but depend on the rate at which cooling takes place. If cells are gradually cooled by transfer first to a refrigerator (to 2°), then to a deep freeze (about -10°) and, when frozen solid, to dry ice, cells are obtained which on thawing are almost indistinguishable from untreated cells. Cooling in dry ice affects yeast cells only if it occurs rapidly, and the effects of freezing are the greater the more sudden the temperature drop near freezing point.

The oxidation of acetate and succinate by samples of yeast exposed to different treatments is shown in Table 2. Sample 1 was the untreated control. Sample 2, before mixing with dry ice, was kept for 2 hr. in a refrigerator (about +2°) and transferred to a deep freeze (-10°) where it was left for 4 hr. Sample 3 was kept for 3 hr. at room temperature and sample 4 was kept overnight in the refrigerator before dry-ice treatment. Samples 2-4 in addition were kept for 20 min. with an excess of dry ice, allowed to thaw and suspended in 39 vol. 0.1 M-phosphate buffer, pH 6.5, like the untreated sample. The manometer cups contained 3 ml. yeast suspension and 1 ml. 0.1 M-substrate solution. It will be

seen (Table 2) that transfer from room temperature of dry ice destroys almost completely the oxidation of acetate whilst it enhances that of succinate. Transfer from +2° to dry ice partly preserves the oxidation of acetate and increases the oxidation of succinate.

Table 2. *Effect of cooling procedure on the properties of frozen yeast cells*

(For treatment of samples see text; the O₂-uptake figures refer to the 80–100 min. period after addition of substrate.)

O₂ absorbed by 4 ml. yeast suspensions in 20 min. (μl.)

Sample no.	Blank	Acetate	Succinate
1	44	458	48
2	59	388	62
3	66	75	113
4	66	211	97

In further experiments samples of yeast were kept for 1 hr. at 60, 50, 40 and 30°, respectively, before the dry-ice treatment, washed with 10 vol. of water and suspended in 19 vol. 0.1M-phosphate buffer, pH 6.5. None of these samples oxidized acetate at an appreciable rate. The blank respiration and the oxidation of succinate are shown in Table 3. Exposure to 60° destroys most of the oxidizing capacity. Exposure to 50° abolishes the blank respiration almost completely but leaves the activity of the succinic-oxidase system. Exposure to 40 or 30° reduced the blank and increased the rate of oxidation of succinate. Table 3 further shows that the

Table 3. *Effect of prewarming of yeast cells before dry-ice treatment*

(Baker's yeast was warmed for 60 min. to the temperature specified. O₂ uptake by 4 ml. yeast cells containing 150 mg. fresh wt. Period 40–60 min. after addition of substrate; substrate concentration 0.020M.)

Kept 1 hr. at	O ₂ absorbed in 20 min. (μl.)			
	Heated only		Heated and frozen	
	Blank	Succinate	Blank	Succinate
60°	5	11	2	10
50°	170	199	3	100
40°	191	198	51	126
30°	156	161	112	163

effects observed at 50, 40 and 30° are not due to the heat treatment only, but mainly to the effect of solid CO₂ on the pre-warmed material.

The enzyme system responsible for the oxidation of succinate thus differs from those dealing with the oxidation of acetate. Cells preheated to 50° and then frozen in dry ice lend themselves with advantage to the study of the oxidation of succinate

owing to the inactivity of most of the other oxidizing systems.

Observations on the oxidation of succinate. Yeast cells, preheated to 50°, treated with dry ice, washed once with 10 vol. of water and suspended in 0.1M-phosphate buffer, pH 6.5, were used for the following experiments, unless otherwise stated. Of the substrates listed in Table 1, only succinate and D- and L-lactate were appreciably oxidized, the rate of oxidation of all three substances being about the same. Citrate caused a slight increase, amounting to about 50 % of the small blank. The effect of the succinate concentration on the rate of oxidation is shown by the following figures representing the amount of O₂ absorbed in 20 min. by 4 ml. yeast suspension containing 150 mg. fresh weight:

Succinate concentration (M)	O ₂ absorbed 40–60 min. after addition of substrate (μl.)
0.125	149
0.0625	139
0.03125	123
0.015625	113
0.00781	71

The oxidation of succinate was inhibited by 0.002M-hydrogen cyanide and by saturated octanol. No CO₂ was formed. For each molecule of O₂ used, 2 molecules of succinate disappeared and 2 molecules of fumarate + L-malate were formed. The product of oxidation was determined manometrically with *Lactobacillus arabinosus* (Korkes & Ochoa, 1948; Nossal, 1952). Reducing the pretreatment period from 60 to 30 min. made no difference, whilst heating for 120 min. slightly lowered the rate of succinate oxidation.

Lynen (1943) has already found that the oxidation of succinate in yeast is competitively inhibited by malonate. This was confirmed by the observation that the inhibition depends on the succinate concentration; for example, the inhibition by 0.0025M-malonate was 70 % at 0.0625M-succinate and 96 % at 0.00625M-succinate. Whilst in mammalian muscle tissue a 50 % inhibition is found when the concentration ratio [succinate]/[malonate] is about 10 (Krebs & Johnson, 1948), the yeast enzyme showed a 50 % inhibition when the ratio was about 60, as illustrated by the experiment recorded in Table 4. The relations between enzyme activity and the concentrations of substrate and a competitive inhibitor may be expressed by the formula

$$\frac{1-n}{n} \times \frac{[\text{substrate}]}{[\text{inhibitor}]} = K,$$

where n is $\frac{\text{reaction rate with inhibitor}}{\text{reaction rate without inhibitor}}$ and the brackets indicate concentrations (Krebs & Johnson, 1948). This formula holds provided that virtually all

Table 4. *Inhibition of yeast succinic dehydrogenase by malonate*

(Baker's yeast heated to 50° for 60 min.; cooled by dry ice for 16 hr. Washed once with water. Suspended in 19 vol. 0.1 M-phosphate buffer, pH 6.5. Per vessel 3 ml. yeast suspension, 1 ml. substrate-inhibitor solution. For significance of n and K see text.)

Substrate concentration (M)		O ₂ absorbed in 60 min. (μl.)		n	K
Succinate	Malonate	Observed	Corrected for blank		
0	0	15	—	—	—
0.0625	0	254	239	—	—
0.0625	0.005	57	42	0.176	59
0.0625	0.0025	81	66	0.276	65
0.0625	0.00125	117	102	0.426	68
0.0625	0.000625	167	152	0.636	57
0	0.005	13	—	—	—

the enzyme is present either in combination with the substrate or with the inhibitor, but is not present in an uncombined form. K represents the ratio $\frac{\text{substrate}}{\text{inhibitor}}$ giving 50% inhibition. It will be seen from Table 4 that K is about 60. The relative affinity of succinic dehydrogenase for malonate is thus considerably higher in yeast than in animal tissue.

The effect of pH on the oxidation of succinate is shown in Table 5. The pH values were measured electrometrically and were found to agree with those calculated from the pK_H values (pK_H for triethanolamine = 7.77; for diethanolamine = 8.88; Hall & Sprinkle, 1932). There is a broad optimum between pH 7.4 and 8.5. Preliminary experiments showed that di- and tri-ethanolamine as such do not affect the O₂ uptake as there was no difference in phosphate buffer and di- and tri-ethanolamine buffers at pH 7.8.

Ethanolamine buffers are generally useful in enzyme experiments because of their high solubility and the absence of inhibiting effects; together with monoethanolamine (pK_H 9.44) they cover the pH range between 7 and 10. Stock solutions of the bases can be conveniently freed of bound CO₂ by boiling them in an open beaker until the volume is reduced to about half.

Effect of dry-ice treatment on anaerobic reactions of baker's yeast. Dixon & Atkins (1913) have already shown that liquid-air-treated brewer's yeast can ferment sugars, and Lynen (1939) found that liquid-air-treated brewer's yeast, in contrast to fresh yeast (see also Smythe, 1938; Barron, Ardao & Hearon, 1950), readily decarboxylates pyruvate. Data on the anaerobic CO₂ production by baker's yeast exposed to dry ice are shown in Table 6. The capacity to ferment glucose decreased on cold-treatment, whilst the rate of CO₂ evolution from pyruvate and α -ketoglutarate increased. All effects were more marked when the yeast was preheated to 30 or 50° before the exposure to dry ice. In sample 4

(Table 6) (preheating to 50°) glucose was not fermented, and this type of preparation may be used as a carboxylase for the determination of pyruvic acid in place of Lebedev juice from brewer's yeast

Table 5. *pH-Activity curve of the oxidation of succinate by cold-treated baker's yeasts*

(Yeast kept 60 min. at 50°, then treated with dry-ice. Washed once with water, resuspended in 19 vol. 0.1 M-buffer. Each vessel contained 3 ml. yeast suspension in the main compartment and 1 ml. 0.1 M-sodium succinate in the side arm; temp., 25°; gas, O₂. The data refer to O₂ absorbed for 10–40 min. period after addition of substrate. 'Salt' in the case of phosphate refers to Na₂HPO₄, and in the case of the amines to the hydrochlorides.)

Buffer	Ratio of salt to acid or base	pH	O ₂ absorbed in 30 min. (μl.)
Acetate	4:1	5.3	18
Acetate	10:1	5.7	32
Phosphate	1:10	5.8	43
Phosphate	1:4	6.2	59
Phosphate	1:1	6.8	85
Phosphate	4:1	7.4	125
Phosphate	10:1	7.8	128
Triethanolamine	1:4	8.4	126
Diethanolamine	2:1	8.5	115
Diethanolamine	1:1	8.8	92
Diethanolamine	1:4	9.4	62

Table 6. *Anaerobic CO₂ production of cold-treated yeast*

(Baker's yeast suspended in 40 vol. 0.1 M-KH₂PO₄; 0.04 M-substrate; gas, N₂. Sample 1: untreated yeast; sample 2: transferred from refrigerator to dry ice; sample 3: left 60 min. at 30° before treated in the dry ice; sample 4: kept 60 min. at 50° before treated in the dry ice.)

Substrate	CO ₂ formed in 30 min. (μl.)			
	Sample 1	Sample 2	Sample 3	Sample 4
None	8	8	2	2
Glucose	1860	730	368	4
Pyruvate	15	244	422	120
α -Ketoglutarate	10	21	30	10

(Krebs & Johnson, 1937). A sample (1 ml.) of a yeast suspension in 0.1M-acetate buffer, pH 4.5, containing 0.5 g. yeast decomposed 0.1 ml. 0.2M-pyruvate in 0.05M-acetate buffer, pH 4.5, within 40 min. at 25°.

Effect of malonate on the oxidation of acetate

As in mammalian tissues, malonate can be used in frozen yeast cells to test the participation of succinic dehydrogenase in the oxidation of acetate. For this test cells are required which are capable of oxidizing both succinate and acetate. Yeast transferred from the refrigerator to dry ice and, after washing, suspended in phosphate buffer, pH 6.5, was therefore used. Further details of a representative experiment are recorded in Table 7. The yeast used had a considerable blank respiration; for the interpretation of the results this has been subtracted from the values observed on addition of substrates. Whilst the oxidation of succinate was almost completely inhibited by malonate there was no effect on the oxidation of acetate or on the blank respiration. The effect on the oxidation of succinate indicates that malonate freely penetrated into the cells. The results lead to the conclusion that under the test conditions succinate could not be an intermediate in the oxidation of acetate or the blank respiration, unless the improbable assumption is made that the oxidation of succinate and acetate takes place at different sites and that malonate has access to the site of succinate oxidation, but not to that of acetate oxidation.

These experiments are in apparent contradiction to recent results of Barron *et al.* (1950) who found an inhibition by malonic acid of the oxidation of pyruvic acid in acid media (pH 2.8), and also to those of Lynen (1943) who observed a malonic acid inhibition of the oxidation of acetic acid in acid media. The experiment recorded in Table 8 confirms the findings of Barron *et al.* and Lynen, including the high rate of oxidation of pyruvic and acetic acids in the acid medium. It is noteworthy that relatively high concentrations (0.0125M) of malonic acid are required; there was no inhibition at 0.001M-malonic acid. Added fumaric, succinic and citric acids are not appreciably oxidized by cells suspended in the acid medium, nor do these substrates affect the malonic acid inhibition of pyruvic or acetic acid oxidation. Lynen (1943), in contrast, found a partial reversal by fumarate of the malonate inhibition of acetic acid oxidation.

A further study of the malonic acid effect showed that its characteristics are very different from those of the inhibition of succinic dehydrogenase. Firstly, the inhibition is irreversible. After incubation for 1 hr. the cells were centrifuged, washed once in phosphate buffer, and resuspended in the original volume. The rate of respiration with pyruvic acid

Table 7. *Effect of malonate on the oxidation of acetate*

(Dry-ice-treated baker's yeast (2 g.) shaken 5 min. in 80 ml. 0.125M-phosphate buffer, pH 6.5; centrifuged, resuspended in 40 ml. buffer. Per cup 3 ml. $\equiv$ 150 mg. fresh cells. Added substrate and malonate were contained in 1 ml.; temp., 25°; gas, O_2 . The concentrations refer to final values.

Substrate(s) added ...	Arrangement and O_2 consumption					
	Succinate (0.025M)	Succinate + malonate (0.025M)	Succinate + malonate (0.005M)	Succinate (0.025M) + malonate (0.001M)	Malonate (0.025M)	Acetate (0.025M)
Absorbed O_2 (μ l.):						Acetate (0.025M) + malonate (0.005M)
20-30 min.	316	414	334	335	318	577
60-100 min.	132	210	137	128	139	596
Malonate inhibition (%) during time 60-100 min.	—	—	90*	100*	—	9†
						620
						646
						2†

* Percentage of O_2 uptake due to addition of substrate.

† Percentage of total O_2 uptake.

was the same as before washing with pyruvic and malonic acids. Secondly, no accumulation of succinic acid in the presence of malonic acid could be demonstrated, in contrast to the findings of Barron *et al.* (1950) and of Lynen (1943). The experimental conditions were similar to those described by Barron *et al.* (1950) (see especially Table IV, p. 220) but 0.1 M-phosphoric acid buffer, pH 2.2, consisting of one part of phosphoric acid and two parts of potassium dihydrogen phosphate was used as a medium. The suspension contained 180 mg. fresh yeast in 4 ml. The concentration of pyruvic acid was

Table 8. *Malonate inhibition of yeast respiration in acid medium*

(Yeast suspension (3 ml.): 1.5 g. fresh baker's yeast in 100 ml. buffer (20 ml. 0.1 M- H_3PO_4 , 80 ml. 0.1 M- KH_2PO_4 , pH 2.5). 1 ml. substrate and/or inhibitor solution. Substrates added as free acids to final concentration 0.0125 M; temp., 25°; gas, O_2 .)

Substrates added	O_2 absorbed in 30 min. ($\mu\text{l.}$)
None	29
Pyruvic acid	515
Acetic acid	333
Citric acid	30
Succinic acid	35
Fumaric acid	33
Pyruvic and malonic acids	95
Acetic and malonic acids	46
Acetic, malonic and fumaric acids	49
Pyruvic and fumaric acids	510
Pyruvic, malonic and fumaric acids	90
Glucose	520
Glucose, malonic acid	140
Glucose, malonic and fumaric acids	158

0.025 M, of malonic acid 0.0125 M. The maximum Q_{O_2} in this medium in the presence of pyruvate was 80 $\mu\text{l.}/\text{mg. dry wt.}/\text{hr.}$ After 110 min. incubation the total O_2 consumption with pyruvic acid was 4120 $\mu\text{l.}$, and in the presence of pyruvic acid and malonic acid 1430 $\mu\text{l.}$ The solutions were deproteinized with tungstic acid and the filtrate was treated with acid permanganate (see Krebs & Eggleston, 1948). No appreciable quantities of succinic acid were found, whilst succinic acid added to the yeast suspensions and incubated for the same period was almost quantitatively recovered. It is, however, confirmed that under the conditions used by Lynen (1943, Table 4, p. 54) succinate is formed aerobically in the presence of fumaric, acetic and malonic acids. Lynen used very high yeast concentrations (16.7% wet wt. in the incubated suspensions) and the rate of respiration was very low in his experiment (Q_{O_2} about -6). No appreciable amounts of succinate were found in more dilute yeast suspensions, e.g. in the experiment recorded in Table 8.

Thirdly, the inhibition by malonic acid is not specific; similar effects are given by oxalic acid,

equal concentrations of oxalic and malonic acids being about equally effective. In contrast, yeast succinic dehydrogenase under the conditions specified in Table 4 was not inhibited by 0.01 M-oxalate. Fourthly, the inhibition by malonic acid was independent of the concentration of added substrates (succinic, pyruvic or acetic acids) and was thus not competitive.

In view of these findings the malonic acid inhibition of fresh yeast respiration in a strongly acid medium cannot be taken as evidence of a participation of succinic dehydrogenase. The mechanism of this inhibition remains to be elucidated.

It is remarkable that fresh yeast cells show a normal rate of respiration in a medium of pH 2.1. Measurement of the intracellular pH indicates that this was not affected by the acidity of the medium. For the measurement of the intracellular pH the yeast suspension after incubation with pyruvic acid was centrifuged in a plastic centrifuge tube, the supernatant was poured off and the residue washed once in a small volume of water. This was decanted and the tube placed in dry ice, to remove permeability barriers. After thawing, the pH was measured electrometrically in the liquefied material. A pH value of 5.7 was obtained.

Experiments with [^{14}C] acetate

Isotopic acetate together with non-isotopic succinate, fumarate, malate, or α -ketoglutarate, was added to yeast cells which had been made permeable to di- and tri-carboxylic acids by cold-treatment. Yeast was transferred from the refrigerator (+2°) to dry ice, washed twice with water and suspended in 24 vol. of 0.1 M-phosphate buffer, pH 6.5. In order to provide sufficient material for the isolation and quantitative determination of intermediates, large conical Warburg vessels of about 120 ml. capacity were used (see Krebs & Eggleston, 1945). In most experiments the flask contained 20 ml. yeast suspension and 5 ml. additional substrate solution. The centre well contained 1 ml. CO_2 -free 2N-sodium hydroxide and filter paper. In most experiments the O_2 uptake during the period of incubation was measured. The term 'extra O_2 ' in the following tables refers to the additional O_2 consumption caused by the substrate. At the end of the experiment the filter paper in the centre well was removed, rinsed with water and, together with the alkali solution, transferred to a centrifuge tube containing a solution of barium hydroxide. The barium carbonate was centrifuged, washed and dried for the radioactivity assay.

Isotopic material. Carboxyl-labelled sodium acetate was prepared from non-radioactive methyl iodide and radioactive CO_2 by the Grignard reaction according to Sakami, Evans & Gurin (1947). The radioactive acetate was diluted with carrier acetate to give 1580 counts/min./mg. carbon.

Methyl-labelled sodium acetate was obtained from Tracerlab Inc., Boston. After dilution with carrier acetate it gave 921 counts/min./mg. carbon.

Radioactivity measurements. All measurements were made with a mica end-window counter on samples of BaCO_3 obtained by wet oxidation of the organic substances according to Van Slyke & Folch (1940). Plates of BaCO_3 were prepared by grinding the BaCO_3 with acetone, pouring the resulting suspension quickly on to an aluminium disk fitted with a stainless-steel cylinder and evaporating the acetone by radiation from a lamp placed above the cylinder. The weight of the BaCO_3 layer was determined and corrections for self-absorption were applied in order to obtain the activity at infinite thickness (20 mg. $\text{BaCO}_3/\text{cm}^2$) using the correction table of Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949). The counting time was sufficiently long so that the standard error was no greater than 5%.

precipitated with a solution of HgNO_3 (10% (w/v)) in 5% (v/v) HNO_3 (Stotz, 1937). The precipitate was centrifuged and reprecipitated by dissolving it in 15% (v/v) HNO_3 at 100° , adding 2 vol. of water and the HgNO_3 reagent. A nearly colourless precipitate, obtained on chilling, was washed in water, suspended in water and treated with H_2S . The colourless filtrate was evaporated to 1–2 ml. and chilled. Crystals of fumaric acid appeared which, after two recrystallizations from hot water and drying *in vacuo*, sublimed at 200° and melted at $285\text{--}290^\circ$.

For the isolation of malic acid the filtrates of the HgNO_3 precipitation were continuously extracted with ether for 5 hr. in the Kutscher-Staudel apparatus. The ether was evaporated and the residue taken up in a small volume of water. The solution was acidified with HNO_3 to pH 1, mixed with 1 ml. 10% (w/v) AgNO_3 and brought to pH 6 with 0.5N-ammonia. The silver salts were centrifuged off, re-

Table 9. *Simultaneous oxidation of $\text{CH}_3\text{-}^{14}\text{COOH}$ and non-isotopic succinate*

(For general conditions see text; incubation 110 min.; flask 4 was duplicated to supply sufficient material.)

		Arrangement and O_2 consumption			
Flask	...	1	2	3	4
Substrate	...	None	Succinate (0.02M)	$\text{CH}_3\text{-COOH}$ (0.02M)	$\text{CH}_3\text{-}^{14}\text{COOH}$ (0.02M) + succinate (0.02M)
O_2 absorbed ($\mu\text{l.}$)		5 600	11 110	13 570	18 120
Extra O_2 ($\mu\text{l.}$)		—	+ 5 510	+ 7 970	+ 12 520

Substrate changes and isotope data in flask 4

	Amount (m-moles)	Specific activity (counts/min./mg. C)
Acetic acid, initial	0.75	1580
Acetic acid, final	0.44	1572
CO_2 , final	—	627
Fumaric acid	—	6
Malic acid	—	3
Succinic acid, initial	0.50	0
Succinic acid, final	Nil	—

Simultaneous oxidation of carboxyl-labelled acetate and succinate. The arrangement of the experiment and data on the O_2 consumption are shown in Table 9. It will be seen that both succinate and acetate increased the O_2 consumption and that the effects were additive. At the end of the incubation period the contents of the duplicate flasks 4 were combined. The determination of succinate in a 3 ml. sample showed that all added succinate had been oxidized. The remaining suspension was deproteinized with 5 ml. 15% HPO_3 , and acetic acid was separated by steam distillation. The distillate was titrated with standard alkali and evaporated to dryness. The residue was repeatedly extracted with 3 ml. boiling 95% (v/v) ethanol. The combined extract was evaporated to a small volume and sodium acetate precipitated with ether. The precipitate was centrifuged, dried, dissolved in a little water and acidified to pH 1 with HNO_3 . 10% (w/v) AgNO_3 (1 ml.) was added and the pH was adjusted to 5–6 with 0.5N-ammonia. Silver acetate was centrifuged off, washed three times with a little cold water, once with ethanol, once with ether, and dried *in vacuo*. The specimen obtained served for the measurement of radioactivity.

The non-volatile residue was acidified with HNO_3 to a final concentration of 5% (v/v) and fumaric acid was pre-

dissolved in 0.1N- HNO_3 and reprecipitated with NH_4OH at pH 6. The precipitate obtained was treated in aqueous suspension with H_2S and filtered off. The filtrate was evaporated to dryness, the residue dissolved in about 2 ml. warm ethanol and neutralized by the addition of solid brucine. On chilling, dibrucine malate separated out. It was recrystallized three times from a small quantity of boiling ethanol; m.p. and mixed m.p. $207\text{--}208^\circ$. The dibrucine salt was dissolved in a little water, made alkaline with 2N- NaOH and the suspension was extracted three times with ether to remove brucine. The aqueous residue was acidified to pH 1 with HNO_3 and silver malate was obtained by addition of AgNO_3 and adjustment of pH to 6. The silver salt was centrifuged and reprecipitated from dil. HNO_3 . It was finally washed with water and dried *in vacuo* for radioactivity measurements.

The results of the radioactivity measurements are shown in Table 9. The specific activity of the recovered acetate was the same as that of the added acetate showing that no new acetate had been formed. The CO_2 formed was radioactive, showing that the acetate removed had been oxidized to CO_2 .

Some of the added succinate was recovered as fumarate and malate, both acids being inactive.

The experiment proves that fumarate and malate cannot have been intermediates in the oxidation of acetate. Any fumarate and malate that might have arisen from acetate by the di- or tri-carboxylic acid cycles would have been radioactive and would have mixed with the fumarate and malate arising from succinate. The recovered fumarate and malate would therefore have been radioactive. The inactivity of the acids cannot have been due to permeability barriers because the substrate arose from succinate within the cells.

As all the added succinate had been removed, a shorter period of incubation was chosen in a second experiment with the same substrates. Because of the shorter incubation period, less acetate was added. Details of the experiment are given in Table 10. The quantitative manometric succinate determination at the end of the incubation showed that about half

of the added quantity had been removed. No succinate was found in flasks 1 and 3.

Succinic acid was recovered from the residue of the de-proteinized filtrate after the removal of acetic acid by steam distillation. The residue was concentrated *in vacuo* to 40 ml. and continuously extracted with ether for 90 min. After evaporation of ether the residue was dissolved in 4 ml. hot ethyl acetate. The solution was filtered and the filtrate concentrated to 1 ml. by a current of dry air. The precipitate obtained on chilling was recrystallized twice from ether; m.p. and mixed m.p. 190°.

Whilst the CO₂ was radioactive the recovered succinate was inactive (Table 10). Thus succinate cannot have been formed by the oxidation of acetate.

Simultaneous oxidation of methyl-labelled acetate and succinate. The general procedure was the same as in the preceding experiments. Details are given in Table 11. It will be seen that 18% of the added acetate and 78% of the added succinate were removed. The CO₂ formed was radioactive, whereas

Table 10. *Simultaneous oxidation of CH₃.¹⁴COOH and non-isotopic succinate*

(Incubation 57 min.; for other conditions see Table 9.)

Arrangement and O ₂ consumption				
Flask ...	...	...	...	...
Substrate	...	...	...	...
	1	2	3	4
	None	Succinate (0.02 M)	CH ₃ .COOH (0.0092 M)	Succinate (0.02 M) + CH ₃ . ¹⁴ COOH (0.0092 M)
O ₂ used by 25 ml. suspension (μl.)	3860	4560	6210	7280
Extra O ₂ (μl.)	—	+700	+2350	+3420

Substrate changes and isotope data in flask 4

	Amount (m-moles)	Specific activity (counts/min./mg. C)
Acetic acid, initial	0.23	1580
Acetic acid, final	0.12	—
Succinic acid, initial	0.50	0
Succinic acid, final	0.29	3
CO ₂ , final	—	485

Table 11. *Simultaneous oxidation of ¹⁴CH₃.COOH and succinate*

(For general conditions see text; incubation 80 min.)

Arrangement and O ₂ consumption				
Flask ...	...	...	...	...
Substrate	...	...	...	...
	1	2	3	4
	None	Succinate (0.02 M)	CH ₃ .COOH (0.02 M)	¹⁴ CH ₃ .COOH (0.02 M) + succinate (0.02 M)
O ₂ used by 25 ml. suspension (μl.) ...	2400	4360	4290	6190
Extra O ₂ (μl.) ...	—	+1960	+1890	+3790

Substrate changes and isotope data in flask 4

	Amount (m-moles)	Specific activity (counts/min./mg. C)
Acetic acid, initial	0.50	921
Acetic acid, final	0.41	—
Succinic acid, initial	0.50	0
Succinic acid, final	0.11	0.4
CO ₂ , final	0.27	188

the recovered succinate was inactive. The experiment thus confirms those with carboxyl-labelled acetate.

Simultaneous oxidation of carboxyl-labelled acetate and α -ketoglutarate. Isotope data on an experiment with α -ketoglutarate are shown in Table 12.

After steam distillation of the deproteinized yeast filtrate the solution containing the non-volatile substances was concentrated *in vacuo* to about 25 ml. and continuously extracted with ether for 2 hr. The ether was evaporated to dryness, the residue taken up in 2 ml. water, and the α -ketoglutaric acid semicarbazone precipitated by the addition of solid semicarbazide hydrochloride. After chilling, the crystalline material was centrifuged and recrystallized three times from hot water; m.p. and mixed m.p. 200°.

Table 12. *Simultaneous oxidation of $\text{CH}_3\text{-}^{14}\text{COOH}$ and α -ketoglutarate*

(For general conditions see text; incubation 120 min.; 25 μ l. yeast suspension.)

Substance	Amount (m-moles)	Specific activity (counts/min./ mg. C)
Acetic acid, initial	0.50	1580
Acetic acid, final	0.40	—
α -Ketoglutaric acid, initial	0.50	0
α -Ketoglutaric acid, final	—	0.6
CO_2 , final	0.27	700

α -Ketoglutarate did not contain ^{14}C (Table 12) and is thus excluded as an intermediate in the oxidation of acetate.

Simultaneous oxidation of formate and doubly labelled acetate. A mixture of carboxyl- and methyl-labelled acetate and fresh yeast (not treated with dry ice) were used; otherwise the experimental conditions were similar to those of the preceding experiments. To isolate formic acid the deproteinized filtrate was steam distilled. The distillate was treated with HgSO_4 in H_2SO_4 solution and the CO_2 evolved from formic acid was collected as BaCO_3 . Acetic acid was recovered by steam distillation from the solution after the removal of formic acid and isolated as the Ag salt as previously described.

Formic acid was found to be inactive (Table 13).

Table 13. *Simultaneous oxidation of $^{14}\text{CH}_3\text{-}^{14}\text{COOH}$ and formate*

(For general conditions see text; incubation 60 min.; 25 ml. yeast suspension.)

Substance	Amount (m-moles)	Specific activity (counts/min./ mg. C)
Acetic acid initial	0.20	1250
Formic acid, final	—	4
CO_2 , final	—	488

Simultaneous oxidation of glyoxylic acid and methyl-labelled acetate. Dry-ice-treated yeast was used. At the end of the incubation period of 60 min. the suspension was mixed with 2 vol. of ethanol and centrifuged after storage in

the cold. The clear supernatant was concentrated *in vacuo* to a few ml., freed of insoluble material by centrifugation and mixed with solid semicarbazide hydrochloride.

The colourless crystals of glyoxylic acid semicarbazone did not contain ^{14}C .

Simultaneous oxidation of acetoacetate and carboxyl-labelled acetate. The incubated yeast suspension was treated with 5 ml. 20% (w/v) CuSO_4 and 5 ml. 10% (w/v) $\text{Ca}(\text{OH})_2$ suspension. The filtrate was acidified to pH 3 and aerated for 15 min. at room temperature. Denigès mercuric sulphate reagent was added and the CO_2 formed on heating under reflux and derived from the carboxyl group of acetoacetate was collected. The mercury-acetone precipitate was centrifuged, redissolved in 2N-HCl and the acetone was distilled into fresh Denigès reagent. The distillate was refluxed with Denigès reagent and the precipitate was centrifuged, washed and dried.

Both the CO_2 and the acetone derived from the acetoacetate were inactive (Table 14).

Table 14. *Simultaneous oxidation of $\text{CH}_3\text{-}^{14}\text{COOH}$ and acetoacetate*

(For general conditions see text; incubation 60 min.; 25 ml. suspension fresh yeast.)

Substance	Amount (m-moles)	Specific activity (counts/min./ mg. C)
Acetic acid, initial	0.20	1580
Acetoacetic acid	0.20	—
CO_2 derived from acetoacetic acid	—	2
Acetone derived from acetoacetic acid	—	0
CO_2 , final	—	831

Homogeneity of cold-treated yeast

Dr Sidney Weinhouse has personally pointed out to the authors that the results of the simultaneous oxidation experiments, as well as those on the malonate inhibition, would be inconclusive if suspensions of cold-treated yeast cells contained two different types of cells: (1) normal cells capable of oxidizing acetate and incapable of oxidizing added succinate, this incapacity being due to a permeability barrier; (2) 'damaged' or 'dead' cells incapable of oxidizing acetate and capable of oxidizing added succinate; the prototype of such damaged cells are those heated to 50° and then exposed to dry ice as described earlier in this paper. If this kind of inhomogeneity existed, the oxidation of succinate and acetate in the cold-treated yeast suspension would occur at different sites and the results reported in this paper could not be regarded as evidence against the participation of the dicarboxylic acids in the oxidation of acetate.

Attempts to detect two types of cells by vital staining with methylene blue or triphenyltetrazolium chloride remained inconclusive, but the following two experiments meet the criticism.

In the first experiment the specific activity of intra- and extra-cellular succinic acid after incubation of fresh yeast cells with $^{14}\text{CH}_3\text{COOH}$ was measured. The type of baker's yeast used contained 2–3 mg. intracellular succinic acid/g. fresh weight. Some of this was lost to the medium on incubation and in order to obtain enough cell material for the isolation of succinic acid a relatively high concentration of cells was used. The temperature was kept low as this makes it easier to saturate the concentrated cell suspension with O_2 .

Fresh baker's yeast (2.5 g.) was suspended in 22.5 ml. of medium containing methyl-labelled acetate buffer (0.0316 M), pH 5.0, and unlabelled succinate (0.00236 M), and shaken in large conical Warburg vessels for 100 min. in O_2 at 8°. Preliminary experiments had shown that under these conditions the intracellular concentration of succinate remains above that of the medium. Duplicate flasks were set up in order to provide sufficient material for the isolation of succinic acid. The respiratory CO_2 was absorbed by 2N NaOH contained in the centre well.

On completion of the incubation period the cell suspension was ice-cooled and centrifuged. The supernatant was decanted and stored at -14° until it could be analysed. The sediment was diluted with water to 20 ml. and deproteinized with 5 ml. 15% HPO_3 . The precipitate was discarded. The decanted supernatant medium and the deproteinized extract of the cells were treated in the same way. Acetic acid was distilled off at pH 2 and titrated with NaOH in the distillate. Succinic acid was extracted from the residue with ether, dissolved in water and determined in one-fifth of the volume. The remaining succinate was diluted with carrier succinic acid before further recrystallizations. A sample (5.3 mg.) of succinic acid obtained from the cells was diluted with 15.4 mg. carrier, and 18.3 mg. carrier were added to 7.1 mg. obtained from the medium. In all other respects the procedures were as before. The relevant data are given in Table 15.

About half of the added acetate was removed during incubation. The respiratory CO_2 was radioactive, but its specific activity was only about 3% of that of the acetate. Differences between the specific activities of CO_2 and acetic acid may be partly due to the high blank respiration which was 77.5% of that of the sample containing acetate. It is also probable that the unlabelled carboxyl carbon of acetate yields CO_2 more rapidly than the methyl carbon. If acetate were oxidized through the tri-carboxylic acid cycle the first two turns of the cycle would yield four unlabelled and no labelled CO_2 molecules. Only during the third turn would labelled CO_2 begin to appear. Similar relations may hold for other intermediary mechanisms.

The intracellular succinate was highly radioactive, the specific activity being about one-quarter of that of the acetate. This result is in accordance with that of Lynen (1947) who found deuteriosuccinate on incubation of baker's yeast with fumarate and deuterioacetate. But the most significant finding was the radioactivity of the extracellular succinic acid

which was almost twice that of the respiratory CO_2 . It shows that fresh cells are in fact not completely impermeable to succinate and can discharge the substance into the medium. This is also borne out by the increase in the concentration of succinate in the external medium. The differences in the specific activity of the extra- and intra-cellular succinic acid, however, indicate that the permeability is restricted.

Table 15. *Specific activity of intra- and extra-cellular succinic acid after incubation of fresh yeast with $^{14}\text{CH}_3\text{COOH}$*

(For general conditions see text; the data refer to 50 ml. suspension.)

Arrangement and O_2 consumption			
Flask no. ...	...	1	2
Substrate ...	...	Succinate (0.00236 M)	Succinate (0.00236 M) + acetate (0.0316 M)
O_2 absorbed ($\mu\text{l.}$)		14 610	18 870
Substrate changes and isotope data in flask 2			
		Amount (m-moles)	Specific activity (counts/ min./mg. C)
Acetic acid, initial		1.59	6195
Acetic acid, final		0.930	—
CO_2 , final		—	193
Succinic acid, initial, intracellular		0.110	0
Succinic acid, initial, extracellular		0.118	0
Succinic acid, initial, total		0.228	—
Succinic acid, final, intracellular		0.049	1520
Succinic acid, final, extracellular		0.256	344
Succinic acid, final, total		0.305	—

Before the significance of these results is further considered it should be mentioned that the experiment was repeated with washed cold-treated cells.

The conditions were the same except that the initial succinate concentration was higher and equal to that of acetate. This was necessary because of the rapid oxidation of succinate in cold-treated cells; it also favoured the trapping of labelled succinate. The experiment differed from that recorded in Table 11 in respect to pH, temperature and especially a much higher specific activity of the added acetate (6195 counts/min./mg. C) which again favoured the detection of a formation of succinate from acetate. Cells and medium were not separated for the analysis.

The specific activity of the respiratory CO_2 was found to be 1920 counts/min./mg. C and that of the succinic acid isolated at the end of incubation was 34. This value is well above the limits of error, but it is far too low to justify the conclusion that an appreciable part of the oxidized acetate had passed through the stage of succinate. It should be recalled that if mixing between the succinate formed as an intermediate and that present in the medium is

complete the specific activity of succinate must be higher than that of the respiratory CO_2 .

The conclusion may be drawn from these experiments that cold-treated yeast contained no appreciable quantities of 'intact' cells behaving like fresh yeast; otherwise radioactive succinate would have been found under the test conditions. They also show that cold-treatment almost completely destroys the mechanism whereby succinic acid is produced from acetate, whilst that responsible for the oxidation of acetate to CO_2 remains.

The second experiment was designed to assess quantitatively the proportion of cells in a dry-ice-treated sample which, like fresh cells, possesses a permeability barrier for dicarboxylic acids. It is based on the fact that fresh cells maintain a high concentration of L-malate within the cells when shaken in aqueous media containing no L-malate, whilst 'damaged' cells no longer maintain concentration gradients.

Fresh cells and cold-treated cells (2.5 g.) were vigorously shaken for 15 min. at 10° in 47.5 ml. of water. A measured sample of this suspension (48 ml.) was then quickly ice-cooled and centrifuged. After decanting, the volume of the supernatant was measured and reduced on a steam bath to about one-quarter. The volume of the sediment was available by difference and the amounts of cells and supernatant in the residue were calculated assuming the specific gravity to be 1.090. The total residue was diluted with water to a volume of 10 ml. and heated for 15 min. to 100° . Malate was determined in 2.5 ml. samples of this suspension by the method of Nossal (1952) and in the concentrated supernatant.

The results are shown in Table 16. Fresh cells contained 8.44 μ moles malate/g. after shaking.

Table 16. Comparison of the distribution of L-malate between cells and medium in fresh and dry-ice-treated baker's yeast

(The data refer to 2.5 g. of yeast suspended in 47.5 ml. of water. For further details see text.)

Treatment of cells	L-Malic acid found (μ moles)			
	Cells		Supernatant	
	In total	Per g.	In total	Per ml.
Untreated	21.1	8.44	1.98	0.042
Dry-ice-treated	3.61	1.44	23.8	0.50

Only about 10% of the intracellular malate had been lost to the medium and the concentration in the medium was about $\frac{1}{500}$ th of that in the cells. The total malate content of the suspension of cold-treated cells was higher than that of the fresh cells, because cold-treated cells oxidize intracellular succinate to fumarate and malate, but over 85% of the malate was present in the medium. The concentration in the cells was still a little higher than in the medium, and on the assumption that the excess

found in the cells was due to an admixture of 'undamaged' cells it can be calculated that 7% of the cells were undamaged. This is a maximum value because some of the intracellular malate may have been formed by oxidation of succinate during and after centrifugation. Even ice cooling and rapid manipulation in the analysis of the residue cannot completely eliminate this source of error. It is noteworthy that succinate leaves fresh cells much more readily than L-malate. Under identical conditions about half the intracellular succinate was lost in the medium.

DISCUSSION

Terminal oxidation mechanisms in yeast. Two independent sets of observations provide evidence against the view that the tricarboxylic acid cycle or a dicarboxylic acid cycle is the main terminal oxidation mechanism in dry-ice-treated yeast. The fact that malonate has no effect on the oxidation of acetate under conditions where it blocks oxidation of succinate alone is proof against the participation of succinate in the oxidation of acetate. The simultaneous-oxidation experiments with isotopic acetate and non-isotopic additional substrate are also proof of the non-participation of succinate, fumarate, malate or α -ketoglutarate, as well as of glyoxalate or formate, as intermediates in the oxidation of acetate.

The failure to find C_2 or C_1 compounds that fulfil the requirements of intermediates in the oxidation of acetate suggests that acetate, in order to undergo oxidation, must enter some kind of condensation reaction. One of the possibilities which suggests itself, considering the relative stability of acetyl-coenzyme A (Lynen & Reichert, 1951), is the oxidation of acetate in the form of acetylcoenzyme A, the intermediate stages being glycollyl-, glyoxalyl-, oxalyl- and formyl-coenzyme A. That coenzyme A is required for the oxidation of acetate in yeast is suggested by the observation of Novelli & Lipmann (1947) that the rate of oxidation of acetate is reduced in coenzyme A-deficient yeast cells.

Occurrence of the component reactions of the tricarboxylic acid cycle. Qualitatively all the component reactions of the tricarboxylic acid cycle have been shown to occur in yeast cells. The relevant facts may be summarized as follows.

(1) Citric acid can be formed by yeast cells when magnesium or barium acetate is added (Wieland & Sonderhoff, 1932; Virtanen & Sundman, 1942; Weinhouse & Millington, 1947). Citric acid is formed in extracts from yeast cells when acetate and oxaloacetate are present (Novelli & Lipmann, 1950).

(2) Citric acid can be converted to α -ketoglutaric acid by preparations of baker's yeast and of pure cultures of *Saccharomyces cerevisiae* (Foulkes, 1951; Kornberg & Pricer, 1951).

(3) Preparations of yeast cells can oxidize succinate to fumarate and malate to oxaloacetate under certain conditions, as was first shown by Lynen (1943).

(4) Yeast extracts contain fumarase (Jacobsohn, 1931) and aconitase (Weinhouse & Millington, 1947; Racker, 1950; Foulkes, 1951).

Thus all the enzyme systems required for the tricarboxylic acid cycle are present in yeast cells, but with the exception of fumarase and aconitase the activities of the enzymes are low in comparison with the rate of acetate oxidation, the slowest reaction being the oxidation of malate to oxaloacetate.

The role of the tricarboxylic acid cycle in yeast cells. If the cycle is not the mechanism by which energy is produced from acetate, but if the enzyme systems responsible for its component reactions nevertheless occur in the cell, the problem of the physiological significance of the cycle presents itself. It is relevant with regard to this problem that in addition to the energy-giving mechanism, there is another major set of chemical changes in rapidly growing organisms, namely, the synthetic processes connected with growth. As far as the turnover of carbon is concerned both types of reactions can be of the same order of magnitude (see Clifton, 1946). The observation that resting yeast cells accumulate succinate when glucose is available, whilst growing cells do not (Kleinzeller, 1941) and that growing cells, in contrast to resting cells, can utilize added succinate, fumarate and malate at low pH values (Nossal, 1951) supports the assumption that the tricarboxylic acid cycle is related to growth in yeast cells. The findings of Ehrensvärd, Reio, Saluste & Stjernholm (1951), who studied the synthesis of amino-acids in *Torulopsis utilis* grown on a medium in which $^{13}\text{CH}_3\cdot^{14}\text{COOH}$ was the only source of carbon, are in accordance with the view that the cycle supplies carbon skeletons for the synthesis of organic substances. At the same time these authors come to the conclusion that formation of citrate by condensation of acetate with oxaloacetate cannot be the main entrance of acetate into intermediary metabolism.

There are numerous observations, briefly summarized in the following section, which support the view that in many other (though not in all) micro-organisms the tricarboxylic acid cycle supplies not primarily energy but intermediates.

The role of the tricarboxylic acid cycle in other micro-organisms

Escherichia coli. *Esch. coli* fails to grow on media in which citrate is the sole source of carbon (Koser, 1923); yet if citrate is present in the medium together with glucose and a source of nitrogen, citrate is utilized (Lominski, Conway, Harper & Rennie, 1947). These observations may be inter-

preted as indicating that citrate cannot serve as a source of energy but may be used by growing cells for other purposes. The occurrence of the tricarboxylic acid cycle in *Esch. coli* is also made improbable by simultaneous-oxidation experiments of Ajl & Kamen (1951). Addition of labelled acetate and unlabelled succinate, α -ketoglutarate, fumarate, malate and oxaloacetate led to the incorporation of labelled carbon in the C_4 dicarboxylic acids, but not in α -ketoglutarate. Citrate was not tested. Whilst these data argue against the oxidation of acetate through the tricarboxylic acid cycle they permit the assumption of a dicarboxylic acid cycle of the oxidation of acetate. Experiments of Cutinelli, Ehrensvärd, Reio, Saluste & Stjernholm (1951) analogous to those quoted for *Torulopsis* (Ehrensvärd *et al.* 1951) directly support the assumption that the synthesis of the carbon skeleton of amino-acids, e.g. glutamic acid, aspartic acid and alanine, involves the reactions of the tricarboxylic acid cycle. Moreover, some of the necessary enzymes, namely those responsible for the synthesis of citric acid from oxaloacetate and acetate (Novelli & Lipmann, 1950; Stern & Ochoa, 1949), have been demonstrated.

Aerobacter aerogenes. Simultaneous-oxidation experiments with ^{14}C -labelled acetate and non-labelled intermediates of the tricarboxylic acid cycle on *A. aerogenes* grown on a citrate-containing medium showed labelling in all intermediates of the tricarboxylic acid cycle including citrate and α -ketoglutarate (Ajl & Wong, 1951). These findings suggest the occurrence of the tricarboxylic acid cycle in this organism, but further observations indicate that a second mechanism is concerned with acetate oxidation. The specific activity of the respiratory CO_2 was much higher than that of any carbon atom of citrate, α -ketoglutarate or succinate. This might be explained by one of two assumptions: either the added intermediates were not in equilibrium with the intracellular substances, or there is a second mechanism of acetate oxidation, in which the above intermediates do not take part. Other findings of Ajl & Wong (1951) are in favour of the second alternative. In the presence of an intermediate of the tricarboxylic acid cycle, the acetate recovered at the end of the incubation period had a lower specific activity than the starting material, and when inactive acetate but radioactive intermediates of the cycle were added the acetate became active on incubation. Thus the oxidation of the di- and tri-carboxylic acids leads to the formation of acetate, which is difficult to reconcile with the tricarboxylic acid cycle, but is expected for the dicarboxylic acid cycle.

'Simultaneous-adaptation' experiments (Stanier, 1947) likewise argue against the participation of citrate in the main oxidation mechanism. Citrate-adapted cells are also adapted to oxidize acetate, but

the reverse is not true; acetate-adapted cells do not oxidize citrate rapidly.

Micrococcus lysodeikticus. Simultaneous-oxidation experiments by Ajl, Kamen, Ranson & Wong (1951) indicate that in this organism α -ketoglutarate and the C_4 dicarboxylic acids participate equally as intermediates in the oxidation of acetate. This is in agreement with the assumption that here the tricarboxylic acid cycle is the main mechanism of acetate oxidation (see also Saz & Krampitz, 1951).

Neurospora crassa. A number of mutant strains of *Neurospora* require succinic acid or related compounds for growth (Lewis, 1948). Succinate can be replaced by fumarate, glutamate, α -ketoglutarate, aspartate, malate and to some extent acetate. Lewis (1948) suggests that the results can be interpreted on the assumption that the tricarboxylic acid cycle operates in *Neurospora*. But it is necessary to make a second assumption, namely that the cycle can be blocked without producing a lethal effect. This means that *Neurospora* must be able to obtain energy from reactions other than the tricarboxylic acid cycle and is in agreement with the idea that in *Neurospora* the cycle is not concerned with energy supply but only with the production of intermediates for synthesis.

Aerobacter agilis. The occurrence of a tricarboxylic acid cycle in *Aerobacter agilis* has been questioned by Karlsson & Barker (1948) on the grounds of 'simultaneous-adaptation' experiments. This organism can oxidize α -ketoglutarate, succinate, fumarate, malate, pyruvate and acetate, and all the enzymes, with the exception of the acetate enzyme, are adaptive. But addition of pyruvate or acetate does not cause adaptation to α -ketoglutarate, and the authors conclude that α -ketoglutarate is, therefore, not an intermediate in the oxidation of acetate. The oxidation of fumarate is likewise slow in cells grown on pyruvate or acetate. Fumarate is therefore also unlikely to be an intermediate.

Multiple functions of enzymic mechanisms

That the chemical constituents of living matter may serve multiple purposes has long been recognized; amino-acids, for instance, are not only constituents of proteins but also precursors of hormones such as adrenaline and thyroxine. The concept that enzymic mechanisms also might serve more than one purpose is supported by general considerations concerning the evolution of biochemical mechanisms. Examples illustrating this are the mechanisms disposing of surplus ammonia in higher organisms, namely the urea synthesis in mammals and amphibians, and uric acid synthesis in birds and non-chelonian reptiles.

Already at primitive levels of evolution, that of unicellular organisms, enzyme systems synthesizing amino-acids and purine bases are present. The

former include a system synthesizing arginine, and according to the genetic studies of Srb & Horowitz (1944) and Volcani & Snell (1948), ornithine and citrulline are intermediates in the synthesis of arginine in *Neurospora* and various lactic acid bacteria. The liver of mammals and amphibians utilizes the same type of enzyme systems for the synthesis of urea by coupling it with arginase, which is also present in some micro-organisms. It has been stated that the ornithine cycle occurs in *Neurospora* but, while it is correct to say that the necessary enzyme systems are present, the statement that the cycle takes place makes little sense from the biological viewpoint, since *Neurospora* does not produce urea as an end product of nitrogen metabolism. Ammonia is not toxic to *Neurospora* within wide limits and there is therefore no need for a detoxication of ammonia. The situation is more appropriately described by the statement that *Neurospora* is equipped with the basic enzyme systems required for the synthesis of amino-acids as well as with those needed for the hydrolytic breakdown of cell constituents and that the urea-forming system of the mammalian liver has evolved from these basic mechanisms.

As for the evolution of the uric acid-synthesizing system of birds and reptiles, it is of importance that all animals are capable of synthesizing the purine bases which they require for the synthesis of nucleic acids and nucleotides from small organic molecules and ammonium salts. Many, perhaps all, also possess xanthine oxidase which converts purine bases into uric acid and thereby removes surplus purine bases. Birds form uric acid via the purine bases from the same starting materials—glycine, formate, ammonia, carbon dioxide (Sonne, Buchanan & Delluva, 1948; Buchanan, Sonne & Delluva, 1948)—which other phyla (Heinrich & Wilson, 1950; Shemin & Rittenberg, 1947), and even yeast (Abrams, Hammarsten & Shemin, 1948), use in the synthesis of nucleic acids. By concentrating the same, or very similar enzymes, to a much higher level of activity at special sites, they can employ them for the disposal of all surplus ammonia.

It is the object of these considerations to show that it is not a far-fetched idea to ascribe to the tricarboxylic acid cycle two different functions. Nor are there difficulties in assuming that in some organisms it is the tricarboxylic acid cycle and in others a dicarboxylic acid cycle or still another mechanism which in the course of evolution has developed into the main energy-giving process. Lower organisms which like yeast can grow on acetate as the sole major carbon source are in any case bound to produce a C_4 dicarboxylic acid before the tricarboxylic acid cycle could operate. The mechanism by which a C_4 acid is formed, possibly a dicarboxylic acid cycle, might well have evolved

to become in some species the main energy-producing mechanism. It is also feasible that two mechanisms may occur in the same species side by side, the relative preponderance varying according to environmental conditions. In fact adaptation to new sources of energy may be the adjustment to a second function of a mechanism already present.

SUMMARY

1. The properties of baker's yeast made permeable to di- and tri-carboxylic acids by exposure to dry ice are studied. The effects of cooling on permeability were found to depend on the rate of cooling. Gradual cooling to a temperature of dry ice has no major effect. Sudden transfer from 20–40° to dry ice almost completely abolishes the capacity to oxidize acetate whilst it increases that for oxidizing succinate. Transfer from +2° to dry ice partly preserves the capacity to oxidize acetate, ethanol and glucose. Yeast cells heated for 1 hr. to 50° and then transferred to dry ice have a low blank respiration and do not oxidize acetate, ethanol or glucose but attack succinate and D- and L-lactate.

2. Dry-ice-treated cells are permeable to fumarate, malate and citrate as demonstrated by their high fumarase and aconitase activity. Permeability for succinate is shown by the oxidation of this substance in the cells. The enzymes concerned are all attached to the cells and do not appear in solution.

3. The oxidation of succinate is competitively inhibited by malonate. The inhibition is 50 % when the concentration ratio [succinate]/[malonate] is about 60.

4. The oxidation of acetate by dry-ice-treated yeast is not inhibited by malonate under conditions where the oxidation of succinate is inhibited. This result excludes succinate as an intermediate in the oxidation of acetate. Apparent discrepancies between these results and those of Barron *et al.* (1950) and Lynen (1943) are discussed.

5. Simultaneous oxidation experiments with [¹⁴C]acetate and non-labelled second substrates exclude succinate, fumarate, malate, α -ketoglutarate, glyoxylate, formate and acetoacetate as intermediates in the oxidation of acetate in dry-ice-treated yeast.

6. Thus, whilst the tricarboxylic acid cycle cannot be the main mechanism of oxidation of acetate in yeast, the component reactions of the cycle can nevertheless occur in yeast cells. Evidence is presented which suggests that the component reactions of the cycle serve to supply intermediates for organic syntheses.

7. A number of observations are discussed which support the view that in many other micro-organisms the reactions of the tricarboxylic acid cycle are primarily concerned with the supply of intermediates, rather than of energy.

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Displacement Chromatography on Synthetic Ion-exchange Resins

8. A SYSTEMATIC METHOD FOR THE SEPARATION OF AMINO-ACIDS

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In this paper it is intended to combine an account of recent work with a survey of the results described in some previous communications in this series (Partridge & Westall, 1949; Partridge, 1949*a*, *b*; Partridge & Brimley, 1951*a*, *b*) in such a way as to present a systematic procedure for the isolation of amino-acids from the hydrolysis products of proteins or from extracts of biological materials. In addition, the results obtained from model experiments with the hydrolysis products of two representative proteins are given; the proteins chosen were egg albumin and a protein fraction extracted from baker's yeast.

The experiments with egg albumin were carried out on what may be regarded as a large laboratory scale, and, in the model experiment described, a batch of 280 g. of the protein was used for hydrolysis. As large quantities of protein were required for these experiments it was convenient to use a commercial grade of egg albumin, but since this product contained considerable quantities of carbohydrate, significant losses of some of the amino-acids were to be expected, due to humin formation during the course of the hydrolysis. It should be emphasized that displacement chromatograms, unlike elution chromatograms, are very flexible as regards the scale on which the experiments are carried out; the amount of material which a column of given dimensions can handle is necessarily much larger with displacement development than it is with elution development, but the most important factor limiting an increase in the scale of an elution chromatogram is the disturbance due to uneven

fronts, which is always encountered when broad columns are used. With displacement chromatograms this disturbance may be very largely eliminated by the use of a multiple column, in which the filtration tubes are connected as a series of units of diminishing diameter, and using this device there is no reason to anticipate difficulty in increasing the scale to meet requirements for large quantities of pure bases or amino-acids. In the second model experiment, that with yeast protein, the scale was reduced to approximately one-thirtieth. In this case the weight of the individual amino-acids isolated (from 10 g. of protein) was from 0.1 to 1.0 g., and the scale may be regarded as near the minimum for convenient crystallization and recovery of the less abundant amino-acids.

An important application of any simple method for the isolation of amino-acids from mixtures would lie in the possibility of preparing isotopically labelled amino-acids by a biosynthetic process. It has been suggested (cf. Burris, 1947) that a suitable source of amino-acids labelled with ^{15}N would be the protein from yeast grown with ^{15}N ammonium salts as the source of nitrogen. The experiment with yeast protein described here was primarily intended as an exploratory step in this direction.

In presenting the systematic procedure it should be emphasized that no single scheme can be the most effective for all mixtures, and various modifications must be made to suit individual requirements. With this in mind, the two examples given show considerable variations in detail, some of which were designed to suit the difference in scale.