The Utilization by Yeasts of Acids of the Tricarboxylic Acid Cycle

By J. A. BARNETT

Low Temperature Research Station, Cambridge

AND H. L. KORNBERG

*M.R.C. Cell Metabolism Research Unit, Department of
Biochemistry, University of Oxford*

(Received 7 January 1960)

SUMMARY

Growth and respiration on intermediates of the tricarboxylic acid cycle as sole sources of carbon were studied with a number of different yeasts. The incorporation of [1:2-¹⁴C]acetate by acetate-respiring cells was studied with yeasts capable of utilizing exogenously supplied cycle intermediates (K+) and also with those unable to do so (K-). Enzyme systems were examined by using cell-free extracts. It was concluded that the difference between K+ and K- yeasts was not one of a major metabolic pathway, but probably of the permeability of the intact cells. Evidence was found for the operation of the tricarboxylic acid cycle in yeasts under differing growth conditions, and for the operation of the glyoxylate cycle in yeasts growing on acetate.

INTRODUCTION

Various workers have tested the ability of yeasts to utilize acids of the tricarboxylic acid (TCA) cycle. For example, Leberle & Will (1910) indicated that four strains of *Candida mycoderma* were probably capable of utilizing succinic and malic acids for growth. Okunuki (1931) reported that species of the genus *Rhodotorula* and the genus *Sporobolomyces* seemed able to use citric, succinic and malic acids. In these experiments peptone was present in the growth medium in addition to the organic acid being tested. In 1948, Wickerham & Burton reported that many yeasts could grow with intermediates of the TCA cycle as sole sources of carbon, utilizing citric, succinic, fumaric and malic acids. Wickerham (1951) described 15 species of the genus *Hansenula*; strains of 12 of these species were able to grow on citric or succinic acids. Testing with citrate, succinate and malate, Kudriavzev (1954) found that 36 out of 68 species of ascosporogenous yeasts utilized at least succinic acid. Such intermediates of the TCA cycle are now used in routine identification by the Yeast Division of the Centraalbureau voor Schimmelcultures (CBS). Yeasts may thus be divided into two groups: 'K+', those capable of using as sole carbon source for growth or respiration one or more intermediates of the TCA cycle; 'K-', those yeasts unable to do so.

METHODS

Media. Yeasts were grown in the carbon-free defined medium (A₂) of Barnett & Ingram (1955), to which was added respectively, glucose (A₂G), succinate (A₂S), acetate (A₂Ac) or citrate (A₂Cit.) to final concentrations of 1% (w/v). In addition, Difco Bacto Y-M broth and agar were used where indicated.

Organisms. Stock cultures were maintained at $+1^{\circ}$ on slopes of Y-M agar and subcultured every 6 months (cf. Kirsop, 1954). The yeasts were incubated at 25° .

The yeasts came from the following collections: The Yeast Division of the Centraalbureau voor Schimmelcultures, Delft, Holland (CBS); The National Collection of Yeast Cultures, Great Britain (NCYC); The American Type Culture Collection (ATCC); The North Regional Research Laboratories, Peoria, Illinois, U.S.A. (NRRL); The Carlsberg Laboratory, Copenhagen, Denmark (CARL); The Division of Bacteriology and Dairy Research, Department of Agriculture, Ottawa, Canada (DBDR); The Low Temperature Station, Cambridge, England (LTS).

The strain of *Saccharomyces drosophilarum*, CBS 2896, was received from Professor V. I. Kudriavzev (Institute of Microbiology, Academy of Sciences of the U.S.S.R.) as *Zygofabospora krasilnikovii* (see Kudriavzev, 1954) and deposited with the CBS.

Growth of yeasts for inocula and suspensions

For growth experiments. For each growth experiment, a fresh Y-M agar slope was inoculated from the stock culture. After incubation for 2 days, the yeasts were washed off the slope with medium A_2 and the entire crop used to inoculate a Roux bottle containing about 100 ml. of A_2G agar. The cultures were harvested after incubation for 2 days.

For manometric experiments. For each manometric experiment a 2-day culture from a Y-M agar slope was used to inoculate a 1 l. Fernbach culture flask, containing 200 ml. medium. These flasks were shaken (at *c.* 2 cycles/sec.; 5 cm. travel) for *c.* 24 hr. at 25° .

For isotope experiments. The suspension of yeast cells was in each case prepared exactly as for manometry; but four Fernbach flask cultures were necessary for each experiment.

For preparation of cell-free extracts. The several grammes of yeast needed in each experiment were grown in a Hoover washing machine adapted for this purpose. The machine consisted of a stainless steel vessel (80 l. capacity) with a mechanical stirrer, and a hole in the bottom of the tank through which sterile air was pumped. A metal top and gasket prevented contamination from the air; heat produced metabolically (Winzler & Baumberger, 1938) was prevented from increasing the temperature of the medium above that of the thermostatically controlled room by means of a cooling coil. The yeast was grown for 24 hr. in media A_2Ac , A_2Cit , or A_2S . The crop was harvested with a Sharples Laboratory Super-centrifuge, washed in 10 l. of 0.9% (w/v) potassium chloride, re-centrifuged and stored at -15° .

Growth experiments

The methods used were based on those described by Barnett & Ingram (1955).

Auxanography. Each culture was harvested into 10 ml. medium A_2 and transferred to a '1 oz.' (25 ml.) screw-capped bottle by means of a sterile pipette. The concentration of cells was checked roughly with a nephelometer and adjusted aseptically to *c.* 5×10^8 cells/ml. Of this suspension, 0.5 ml. was added to a tube containing 10 ml. melted A_2 agar medium which had been cooled to 48° . The contents of the tube were poured into a Petri dish (9 cm. diameter) after quick but thorough mixing. The surface of the agar was dried by incubating the dishes, open

and inverted, at 37° for 1.5 hr. The carbon sources were placed on the surface with a platinum spoon in 2–5 mg. quantities, not more than three test-substances/plate. The auxanograms were incubated and examined every 2 days for a week.

T-tubes. The inocula were prepared exactly as for auxanograms. The suspension of cells was added to 400 ml. A₂ medium contained in a sterile 500 ml. Quickfit interchangeable round flask with a flat bottom and short neck, stoppered with a gauze-covered cotton wool plug. This was then attached to a sterile automatic burette, a modification of the inoculating apparatus described by Barnes (1950), for distributing 10 ml. inoculated medium into each T-tube. When only a few tests were to be made on any one strain of yeast, smaller quantities were prepared and delivered with 10 ml. sterile pipettes.

The carbon-sources were distributed with a sterile pipette to each T-tube to a final concentration of *c.* 1 %. They were made up and stored as 10 % (w/v) solutions at pH 5.0 and sterilized through a membrane filter. (This apparatus was Model Stefi 4 of Membranfilter Gesellschaft, Göttingen, Germany, and the bacteria-proof membrane their Spezial-Membranfilter Co 5. For distributing the sterile solutions, the filter was set up as recommended for a Seitz filter by Mackie & McCartney, 1956).

The T-tubes were shaken (*c.* 2 cyc./sec., 5 cm. travel) at 25°, readings being taken with an E.E.L. nephelometer at 0, 2, 4 and 6 days. The nephelometer used was set to give a reading of 52 on the scale with the solid standard provided.

Manometric experiments

For measurements of the oxidation of acetate and of intermediates of the TCA cycle, the centrifuged yeast was suspended in 0.1 M-potassium phosphate buffer (pH 5.9) and re-centrifuged. The washed yeast was re-suspended in 0.1 M-phosphate buffer, pH 5.9, to a final concentration equivalent to *c.* 5 mg. dry wt./ml. This suspension was placed in a Fernbach flask and shaken again at 25° for 1 hr. in order to decrease the rate of endogenous respiration. The rates of oxygen uptake on addition of substrates were measured with Warburg manometers. Flasks (approx. vol. 20 ml.) with single side arms contained 2 ml. yeast suspension in the main compartment, and 25 μ mole of the substrates in 0.1 ml. water in the side arms. Strips of Whatman no. 40 filter paper and 0.2 ml. of 2 N-KOH were placed in the centre wells. The gas phase in the manometers was air, and they were equilibrated at 25° for 10–20 min., and the contents of the side arms mixed with those of the main compartments. Readings were taken at 10 min. intervals and recorded.

Incorporation of [¹⁴C] acetate by whole yeast cells

The methods used were based on those described by Kornberg (1958).

Yeast cells which had been centrifuged after growth in Fernbach culture flasks were washed, and re-suspended in 0.9 % (w/v) KCl at a concentration equiv. *c.* 10 mg. dry wt./ml. About 50 ml. of the suspension were shaken in a Fernbach flask at 25° for about 1 hr. to lower the rate of endogenous respiration.

Of this suspension, 7 ml. were added to 7 ml. of 0.2 M-phosphate buffer (pH 5.9) in the 100 ml. conical Quickfit flask of an automatic pipette. (These pipettes, delivering 1 ± 0.1 ml., can be obtained from W. G. Flaig and Sons Ltd., 39 Waterloo

Road, London, N.W. 2.) The pipette flask was partly immersed in a Warburg bath and shaken at 25°.

Two Warburg manometers, operating in the same bath, were used to follow, simultaneously, the rates of respiration of the yeast, one with and the other without substrate. The Warburg flasks (approx. vol. 20 ml.), with single side arms, contained 1 ml. yeast suspension and 1 ml. 0.2 M-phosphate buffer (pH 5.9) in the main compartments; and one flask had 25 μ mole sodium acetate in 0.1 ml. of water in the side arm. Strips of Whatman no. 40 filter paper and 0.2 ml. of 2N-KOH were placed in the centre wells. The gas phase in the manometers was air, and they were equilibrated for about 20 min. at 25°. Readings were taken at 5 min. intervals and recorded.

The contents of the Warburg flask side arm were mixed with those of the main compartment, and at the same moment 175 μ mole of sodium acetate in 0.5 ml. water were added to the pipette flask and the contents well mixed.

When the rate of acetate oxidation was steady (usually after about 20 min.) the automatic pipette was removed from the bath, 0.5 ml. [1:2-¹⁴C] acetate solution (prepared by mixing equal portions of [1-¹⁴C] and [2-¹⁴C]acetate, and containing 18.5 μ mole of acetate, 100 μ c., and giving 6.5×10^6 counts/min. under the conditions of radio-assay used) was added and the top of the pipette replaced. After rapid manual swirling of the contents to ensure good mixing, a 1 ml. sample was pipetted into a centrifuge tube standing in ice. At intervals after adding the radioactive acetate further samples were removed and the time was recorded to the nearest second. Each sample was centrifuged immediately at *c.* 20,000 *g* at -3° for *c.* 30 sec. and the supernatant fluids discarded. (These supernatant fluids did not contain a significant quantity of ethanol-soluble and non-volatile radioactive material. This procedure eliminated interference from radiation decomposition products; the latter, forming 0.05–0.1 % of the total radioactivity of acetate (cf. Kornberg, 1958), evidently did not penetrate the cells.)

Preparation of aqueous ethanolic extracts. To the packed cells was rapidly added 1 ml. of absolute ethanol; the suspension was stirred well and re-centrifuged. The clear supernatant solutions were poured into thick-walled Pyrex tubes and the precipitates were resuspended, each in 1 ml. 20 % (v/v) ethanol in water. After re-centrifuging the corresponding supernatant solutions were combined and evaporated to dryness at *c.* 60° under a stream of nitrogen. The dry material was resuspended in 0.5 ml. of 20 % (v/v) ethanol in water, and samples thereof (0.1 or 0.2 ml.) were analysed by chromatography and radioautography as previously described (Kornberg, 1958).

Experiments with cell-free extracts

Preparation of extracts. Frozen cells were crushed in a Hughes Press (Hughes, 1951) without using abrasive. The crushed yeast was mixed with 9 vol. of 0.9 % (w/v) KCl and homogenized in a glass homogenizer (Umbreit, Burris & Stauffer, 1949). The homogenate was centrifuged at *c.* 20,000 *g* at -3° for 5 min., and the supernatant solution used for the experiment.

The acetic thiokinase activity of the supernatant solutions was measured by the method of Jones & Lipmann (1955).

Malate synthetase activity. In early experiments this was measured as the quantity

of isotope incorporated into malate when cell extracts were incubated with [^{14}C]acetate, MgCl_2 , glutathione, coenzyme A, adenosine triphosphate (ATP) and glyoxylate (Kornberg & Madsen, 1958; see below). In later experiments, the activity of the enzyme was measured by the optical procedure of Dixon & Kornberg (1959).

Isocitratase activity was determined by the assay of glyoxylate (Friedemann & Haugen, 1948) formed by the cleavage of *isocitrate* (see Kornberg & Madsen, 1957), the identity of the keto compound being confirmed by chromatography of the 2:4-dinitrophenylhydrazones (El Hawary & Thompson, 1958). The development of more direct assay procedures (Dixon & Kornberg, 1959) allowed quantitative determinations to be made during later parts of this work.

Incorporation of [^{14}C]acetate by cell-free extracts. The methods used for studying the incorporation of labelled acetate by cell-free extracts were modified from those described by Kornberg & Madsen (1958). The incubation mixture contained 100 μmole potassium phosphate (pH 7.5), 10 μmole reduced glutathione, 10 μmole MgCl_2 , 10 μmole ATP, 0.08 μmole coenzyme A (Sigma Chemical Co., St Louis, Missouri, U.S.A.), 0.1 ml. of crushed yeast extract, 1.9 μmole $\text{CH}_3^{14}\text{COONa}$ (containing 10 μc . ^{14}C ; giving 6.5×10^5 counts/min.), 10 μmole unlabelled potassium acetate. The quantities of other unlabelled substrates, added as indicated, were as follows: 10 μmole sodium oxaloacetate; 10 μmole sodium glyoxylate; 40 μmole DL + allo *isocitrate* (potassium salt); 20 μmole sodium pyruvate; 0.4×10^{-3} μmole diphosphopyridine nucleotide. Water was added to 1 ml. After incubation at 30° for 30 min., the reaction in each tube was stopped by adding 3 ml. hot absolute ethanol. The 75% (v/v) ethanolic aqueous suspensions were centrifuged at *c.* 250 g, the precipitates washed with 1 ml. 20% (v/v) ethanol in water and the combined supernatant solutions evaporated to dryness at *c.* 60° under a stream of nitrogen. The chromatographic-autoradiographic analysis was carried out as described by Kornberg (1958).

Protein estimation. The protein content of each yeast extract was estimated by nephelometric-turbidity measurements of trichloro-acetic acid precipitates (Stadtman, Novelli & Lipmann, 1951). A standard curve was obtained by using Armour's bovine serum albumin as a standard. For the estimations, 0.2 ml. of the yeast extract was added to 1.8 ml. water; to this 3 ml. of 5% (w/v) trichloro-acetic acid was added, and the turbidity measured.

RESULTS

Growth experiments

The results for the K + yeasts are summarized in Table 1. The yeasts listed below were found unable to grow on citrate, fumarate, malate or succinate as sole sources of carbon. The response to acetate, where tested, is indicated in brackets: [+] growth, [-] no growth. *Kloeckera apiculata* (LTS 5); *Saccharomyces acidifaciens* (CBS 749, DEDR 427, ATCC 8766, CBS 685, NRRL Y54, ATCC 2602, CBS 1085); *S. bailii* (CBS 680 [+]); *S. bisporus* (CBS 702 [+]); *S. cerevisiae* (NCYC 77 [+], NCYC 281 [+], NCYC 176 [+], CARL 53, LTS 4); *S. cerevisiae* var. *ellipsoideus* (NCYC 93 [+], NCYC 94 [+], CBS 818 [-]); *S. delbrueckii* var. *mongolicus* (CBS 705); *S. fermentati* (NCYC 161); *S. mellis* (CBS 736, CBS 684, DBDR Z7, CBS 787, CBS 742, DBDR M1); *S. pastori* (CBS 704); *S. rosei* (CBS 728); *S. rouxii* (LTS 16,

LTS 17, LTS 18, CBS 710, CBS 781, CBS 782, ATCC 2623, CBS 682, CBS 687, CBS 688, CBS 686, DBDR Z5, CBS 788, CBS 741, ATCC 4898, DBDR Y139, ATCC 2619, DBDR Z1); *S. rouxii* var. *polymorphus* (CBS 711, DBDR Z3, NCYC 170, DBDR Z4, DBDR 58, CBS 678, CBS 706, CBS 784, ATCC 2624, DBDR Z2, CBS 727, CBS 713, CBS 726, ATCC 2617). These data suggest the following two generalizations. First, K+ yeasts (capable of utilizing TCA cycle intermediates) include those tested which Wickerham (1955; Wickerham & Burton, 1956a, b)

Table 1. *Results of growth tests on TCA cycle intermediates as substrates: K+ yeasts*

Auxanograms. For each auxanogram, a suspension of c. 2.5×10^8 yeast cells in 0.5 ml. of water was added to a tube containing 10 ml. melted agar medium without carbon source, at 48°. After mixing, the medium was poured into a Petri dish, cooled, and the surface dried in an incubator. Test carbon sources were placed on the agar surface of each plate, which was incubated and examined every 2 days for a week. *T-tubes.* Each T-tube contained initially 10 ml. of carbon-free liquid medium, 1 % (w/v) of carbon source, yeast cells to give an initial nephelometer reading ('zeroed' at 52) of c. 10. The T-tubes were shaken at 25° and nephelometer readings taken at 0, 2, 4 and 6 days. *Symbols.* The letter 'A' indicates that the auxanographic method was used: in all other cases T-tubes were employed. C = citrate; F = fumarate; M = malate; S = succinate; Ac = acetate. - < 10, + 10-20, ++ > 20 (difference between initial and final nephelometer reading). The auxanographic responses are given in terms of + (growth) and - (no growth). nt = not tested.

CBS name	Strains		Substrate				
			C	F	M	S	Ac
<i>Candida krusei</i>	CBS 2066		++	++	++	++	++
<i>C. utilis</i>	NCYC 359	A	+	+	+	+	nt
<i>Debaryomyces hansenii</i>	LTS 1		++	++	++	++	-
<i>Hansenula anomala</i>	CBS 1981, CBS 2576, CBS 2577		++	++	++	++	++
<i>H. suaveolens</i>	NCYC 57		-	++	++	++	++
<i>Pichia membranaefaciens</i>	NCYC 54, CBS 240	A	-	+	-	+	nt
<i>Rhodotorula flava</i>	NCYC 69		-	++	+	+	-
<i>Saccharomyces drosophilae</i>	CBS 2896		++	++	++	++	++
<i>S. fermentati</i>	CBS 748	A	-	+	-	+	nt
			-	-	+	++	-
<i>S. florentinus</i>	CBS 746, NCYC 172	A	-	+	-	+	nt
<i>S. lactis</i>	CBS 739		-	+	-	+	nt
			-	-	-	++	++
	CBS 683	A	-	+	-	+	nt
			-	-	++	++	nt
	CBS 743	A	-	+	-	+	nt
			-	-	-	+	++
<i>S. marzianus</i>	NCYC 111		+	+	+	++	++
	NCYC 243	A	-	+	-	+	nt
			-	+	++	++	++
	CBS 745	A	-	+	-	+	nt
			+	-	+	+	nt
	CBS 712	A	+	+	+	+	nt
			++	+	++	++	nt
<i>S. pini</i>	CBS 744		++	+	+	++	-

placed in the group 'Dekkeromyces' or Kudriavzev (1954) in the group 'Fabospora' (and 'ZygoFabospora'); these were *Saccharomyces drosophilae*, *S. lactis*, and *S. marxianus*. Secondly, K-yeasts include *S. acidifaciens*, *S. cerevisiae* and some of the *Saccharomyces* most able to tolerate high concentrations of sugar, such as *S. mellis* and *S. rouxii* (e.g. Ingram, 1958). In these experiments, all the K+ yeasts were capable of using succinate; of the other TCA cycle intermediates, citrate was used least often. A minority of K+ and K- strains did not grow on acetate.

Fig. 1. Oxidation of tricarboxylic acid cycle intermediates by *Saccharomyces marxianus* (CBS 712) grown on (a) acetate, (b) citrate, and (c) succinate as the sole source of carbon. Manometer flasks contained: washed yeast in 2 ml. of 0.1 M-phosphate buffer (pH 5.9) in the main compartments; 25 μmole substrate (as indicated) in 0.1 ml. water in the side bulbs; 0.2 ml. of 2 N-KOH with filter paper in each centre well. Incubated in air at 25°; substrates added at zero time.

Manometric experiments

The results of the measurements of the oxidation of TCA cycle intermediates are summarized in Table 2. Curves showing the course of oxidation in certain cases are given in Fig. 1. These experiments showed that a number of yeasts of different genera were capable of respiring acetate and intermediates of the TCA cycle at rates comparable to that at which they oxidized glucose. Yeasts grown on acetate tended to respire TCA cycle intermediates only very slowly, as compared with those cultivated on succinate. Two strains, *Saccharomyces cerevisiae* (NCYC 231) and

S. acidifaciens (CBS 749), which did not grow on TCA cycle intermediates, were also unable to respire citrate, succinate or fumarate.

Thus, generally, yeasts able to grow on TCA cycle intermediates could respire them too, and conversely. A case of special interest, therefore, is the behaviour of *Saccharomyces drosophilarum*, CBS 2896, which had been grown on citrate as sole carbon source. These citrate-grown cells, unlike those of *S. marxianus*, CBS 712, not only respired other TCA cycle intermediates particularly slowly, but seemed unable to respire citrate itself (Table 2). When washed citrate-grown cells were resuspended in citrate medium of the kind in which they had been grown, the rate of respiration was indistinguishable from that of the negative-control cells to which no substrate had been added. However, a growing 12 hr. culture, concentrated by centrifugation

Table 2. *Rates of respiration on TCA cycle intermediates as substrates*

Manometer flasks contained washed yeast cells in 2 ml. of 0.1 M-phosphate buffer, pH 5.9, in the main compartments; 25 μ mole of substrates (as indicated) in 0.1 ml. of water in the side bulbs; and 0.2 ml. of 2 N-KOH with filter paper in each centre well. Incubated in air at 25°. The figures for Q_{O_2} have not been corrected for endogenous respiration. 24 hr. cultures were used, unless otherwise indicated. Abbreviations: G = glucose; Ac = acetate; C = citrate; S = succinate; F = fumarate; M = malate; END = no carbon substrate.

Yeast	Growth medium	Q_{O_2} : Uptake of oxygen (μ l./mg. dry wt. of yeast/hr.)						
		G	Ac	C	S	F	M	END
<i>Saccharomyces drosophilarum</i> CBS 2896	Y-M	174	110	15	44	7	28	5
		148	51	12	68	8	8	6
	A ₂ glucose	38	.	4	11	4	2	3
	A ₂ acetate	74	66	11	3	4	.	4
		128	130	41	24	17	.	15
	A ₂ citrate	69	61	9	14	9	.	6
		86	48	7	17	.	12	7
	A ₂ succinate	103	.	10	173	58	107	4
		136	67	17	77	34	.	12
	<i>S. marxianus</i> CBS 712	Y-M	111	.	14	14	14	14
104			.	.	19	15	15	13
A ₂ glucose		112	.	.	29	10	49	4
		78	.	23	14	9	3	4
A ₂ acetate		125	125	10	13	7	.	5
		88	87	25	11	.	20	7
A ₂ citrate (48 hr.)		116	.	91	98	45	46	5
A ₂ succinate		150	155	134	166	158	120	7
		140	.	133	134	128	112	9
<i>Candida krusei</i> CBS 2066		Y-M	64	76	17	30	13	17
	A ₂ succinate	11	4	2	24	10	.	2
	A ₂ acetate	33	.	5	10	5	.	6
<i>Hansenula anomala</i> CBS 1981	Y-M	97	78	43	31	15	15	9
	A ₂ acetate (48 hr.)	13	24	2	1	1	.	2
	A ₂ succinate	31	10	30	26	17	.	6
<i>Saccharomyces cerevisiae</i> NCYC 231	A ₂ acetate	102	77	4	3	3	.	3
<i>S. acidifaciens</i> CBS 749	A ₂ acetate	90	78	4	6	5	.	6

to equiv. 2.7 mg. dry wt. yeast/ml. A_2 Cit. medium (but the cells not washed, and the medium not changed), gave a Q_{O_2} of 150 and an R.Q. of 1.7. A comparable situation was reported by Cabelli (1955) for *Klebsiella pneumoniae*. It is possible that washing the cells impaired their ability to oxidize citrate through the removal of a necessary co-factor.

Incorporation of [1:2- ^{14}C]acetate by whole cells

In six experiments, the incorporation of ^{14}C from labelled acetate by whole cells was studied. Of these experiments, two were carried out with succinate-grown *Saccharomyces drosophilarum* (CBS 2896), one with this yeast grown on acetate, one with it grown on citrate, and one each with *S. cerevisiae* (NCYC 281) and *S. acidifaciens* (CBS 749) grown on acetate. The results of experiments performed under comparable conditions were similar; those obtained with *S. drosophilarum* are shown in Fig. 2. After addition of labelled acetate to the respiring yeast, ^{14}C was rapidly and linearly incorporated into the fractions soluble in aqueous ethanol. At the earliest times, isotope appeared only in intermediates of the TCA cycle and in amino acids derived from them. The labelling patterns (Fig. 2a) observed with the yeast grown on succinate (and citrate) are consistent with those expected from the operation of the TCA cycle. This is not so for the acetate-grown yeasts (Fig. 2b). In particular, the proportions of isotope present in succinate and malate indicate that, at the earliest times, ^{14}C from acetate entered malate without passing through the stage of succinate. Whilst, during the later part of the experiment, succinate contained significantly more ^{14}C than did malate (indicating that the 'pool size' of succinate was greater than that of malate), the succinate in the earliest samples contributed less to the total radioactivity than did malate. The ratio ^{14}C incorporated into malate: ^{14}C incorporated into succinate rose with time in the succinate-grown yeast, which is consistent with the expected formation of labelled malate from labelled succinate (Table 3), but decreased with time in acetate-grown cells. This indicates that, in the acetate-grown cells, labelled succinate could not have been the sole precursor of labelled malate, but that pathways must operate whereby ^{14}C from acetate can enter malate without passing first through succinate.

These results, which are similar to those reported for *Pseudomonas fluorescens* (Kornberg, 1958), offer presumptive evidence that acetate is utilized by the acetate-grown yeast via the concerted action of the tricarboxylic acid and glyoxylate cycles (see Kornberg & Krebs, 1957), but that this latter cycle does not operate in yeasts grown on succinate.

Experiments with cell-free extracts

Measurements of acetate-activation. The results of assaying the activity of acetic thiokinase in extracts of the yeasts are shown in Table 4. The extracts from *Saccharomyces drosophilarum* (CBS 2896) grown on acetate or succinate, and *S. acidifaciens* (CBS 749) and *S. cerevisiae* (NCYC 281) grown on acetate were each capable of forming about 2 μ mole acethydroxamic acid/mg. protein/hr. from acetate under the test conditions of Jones & Lipmann (1955).

Measurements of isocitratase activity. Measurements of the rate of glyoxylate production from isocitrate in extracts from the same yeasts are shown in Table 5. *Saccharomyces drosophilarum* (CBS 2896) grown on acetate as sole carbon source

Fig. 2. Incorporation of ^{14}C from $[1-2-^{14}\text{C}]$ acetate into the ethanol-soluble fractions of *Saccharomyces drosophilarum* (CBS 2896) respiring acetate: (a) succinate-grown, (b) acetate-grown. A solution of $^{14}\text{CH}_3\text{CO}_2\text{Na}$, containing $18.5 \mu\text{mole}$ acetate and $100 \mu\text{C.}$ of ^{14}C , was added at zero time to 14 ml. of a suspension of the succinate- or acetate-grown yeast, respiring in an automatic pipette on 12.5 mM -sodium acetate. Samples (approx. 1 ml.) were pipetted at known times into tubes standing in ice. The precipitates were each treated with 1 ml. of absolute ethanol, and each precipitate washed with 1 ml. of 20% (v/v) aqueous ethanol. The corresponding supernatant solutions were combined and evaporated to dryness under N_2 , resuspended in 20% (v/v) aqueous ethanol and analysed by two-dimensional paper chromatography. The radioactive compounds were located by radioautography, and the ^{14}C content was determined by direct radioassay of the radioactive areas with a mica end-window β -counter tube. Each point on the graphs represents the mean of two chromatograms.

Table 3. Change with time of the ratio of the amount of isotope in succinate to that in malate for respiring suspensions of acetate- and succinate-grown *Saccharomyces drosophilum* (CBS 2896) incubated with sodium [1:2- ^{14}C]acetate

Conditions were similar to those described in Fig. 2.

Carbon source for growth of suspensions	Time (sec.)	Ratio:
		$\frac{^{14}\text{C incorporated into malate}}{^{14}\text{C incorporated into succinate}}$
Succinate	9	1.0
	24	1.1
	120	0.81
	300	1.3
	787	1.6
Acetate	10	1.2
	30	1.0
	60	0.82
	120	0.78
	360	0.94
	660	0.84

Table 4. Acetic thiokinase activity of cell-free extracts

Each tube contained 350 μmole potassium phosphate (pH 7.5), 35 μmole MgCl_2 , 36 μmole reduced glutathione, 170 μmole KF, 85 μmole ATP, 700 μmole hydroxylamine, 70 μmole potassium acetate, 0.28 μmole coenzyme A, 0.5 ml. of yeast extract, and water to 3.5 ml. Incubation was at 30° for 30 min., 1 ml. samples pipetted into 1.5 ml. of FeCl_3 reagent, and the acethydroxamate measured with 1 cm. glass cells in an Unicam sp. 600 at 540 $\text{m}\mu$.

Yeast	Carbon source for growth of cells	Activity of extract (μmoles acethydroxamic acid/mg. protein/hr.)
<i>Saccharomyces drosophilum</i> (CBS 2896)	Acetate	2.4
	Succinate	2.4
	Citrate	0.2
<i>S. acidifaciens</i> (CBS 749)	Acetate	2.4
<i>S. cerevisiae</i> (NCYC 231)	Acetate	2.2

readily formed glyoxylate from *isocitrate*, the specific activity of the enzyme in the crude cell extract was 15.5 μmole glyoxylate formed/mg. soluble protein/hr., as estimated by the method of Dixon & Kornberg (1959). The corresponding specific activity for extracts of succinate-grown cells was 0.56, for those grown on citrate 0.55. Acetate-grown *S. acidifaciens* (CBS 749) and *S. cerevisiae* (NCYC 231) gave an activity of a half and a quarter, respectively, of that of acetate-grown *S. drosophilum*.

These differences in the measured rates of glyoxylate formation from *isocitrate* by extracts obtained from *Saccharomyces drosophilum* grown on succinate, citrate or acetate as carbon sources, were also strikingly confirmed by visual examination of the 2:4-dinitrophenylhydrazones of the keto acids formed under these conditions (Fig. 3). All the extracts from *S. drosophilum* produced, from added *isocitrate*, α -ketoglutarate and a small quantity of pyruvate; only the extract of the acetate-grown yeast, however, catalysed in addition the formation of appreciable amounts of glyoxylate.

Measurements of malate synthetase activity. Extracts from *Saccharomyces drosophilum* (CBS 2896) grown on acetate had a malate synthetase activity under the conditions of assay of 16.8 μ mole acetyl CoA split/mg. soluble protein/hr. The corresponding figures for extracts of the yeast grown on succinate and citrate were 4.3 and 4.2 (Table 5).

Table 5. *Isocitratase and malate synthetase in extracts of Saccharomyces drosophilum (CBS 2896)*

Extracts were prepared by crushing the yeast, grown on acetate, succinate or citrate as sole carbon sources, in a Hughes press, and homogenizing the crushed material with equal volumes of 0.1 M-potassium phosphate, pH 7.5. To each ml. of these suspensions were added 0.1 ml. of 2% protamine sulphate. The precipitates were removed by centrifugation. The *isocitratase activity* of the clear supernatant solution was estimated as the rate of change of optical density at 324 m μ when the extracts were incubated with 200 μ mole potassium phosphate, pH 6.9, 6 μ mole cysteine hydrochloride, 20 μ mole magnesium chloride, 5 μ mole phenylhydrazine hydrochloride, 5 μ mole potassium L₄-*iso*-citrate and water to 3 ml. *Malate synthetase* was determined by the rate of decrease of optical density at 232 m μ resulting from the enzymic removal of acetyl coenzyme A in the presence of glyoxylate (Dixon & Kornberg, 1959). The ratios *A* and *B* were calculated from the data in Table 6, ratio *C* from those in this table.

Carbon source for growth	Protein concn. of original extract (mg./ml.)	Specific activity (μ mole reacting/mg. protein/hr.) of		Ratio		
		<i>iso</i> Citratase	Malate synthetase	<i>A</i>	<i>B</i>	<i>C</i>
				Condensing enzyme Malate synthetase	Condensing enzyme <i>iso</i> Citratase	Malate synthetase <i>iso</i> Citratase
Acetate	18	15.5	16.8	0.85	1.13	1.08
Succinate	14.8	0.56	4.3	2.47	34.8	7.7
Citrate	28	0.55	4.2	2.30	33.2	7.6

Incorporation of [¹⁴C]acetate. The results of experiments on the reactions of [¹⁴C]acetate in extracts are given in Table 6. The assay showed that extracts of the yeast grown under all conditions promoted the incorporation of major amounts of label from [¹⁴C]acetate into citrate, in the presence of oxaloacetate, and into malate, in the presence of glyoxylate. However, the incorporation into malate, in the presence of *isocitrate*, was much less in the succinate and citrate-grown yeast than in that grown on acetate.

DISCUSSION

The ability of K⁺ yeasts to use succinate. A survey of the literature suggests that most K⁺ yeasts can use succinic acid: that is, few yeasts seem unable to use succinate, but able to grow on citrate, fumarate or malate. Exceptions have been reported for *Pichia farinosa*, a strain of a *Torulopsis* species (Beech, 1957), *Torulopsis pseudaria* (Zsolt, 1958), *Rhodotorula crocea* (Shifrine & Phaff, 1956) and *Saccharomycopsis guttulata* (Shifrine & Phaff, 1958). All the K⁺ yeasts studied for the present work (Table 1) utilized succinate. However, the degree of agreement in the literature is more surprising than any disagreement. Apart from expected differences between strains, the answers obtained seem to depend greatly on the methods used, as is indicated by the results for *Saccharomyces fermentati*, *S. lactis*, and *S. marxianus* shown in Table 1. None the less previously published work supports the tentative generalizations already made about the 'Dekkeromyces' group and *Saccharomyces*

mellis and *S. rouxii* (cf. Albritton, 1953; Barnett & Ingram, 1955; Beech, 1957; Hedrick & Burke, 1951; Miller & Phaff, 1958; Nickerson & Carroll, 1945; Niehaus, 1932; Phaff & Knapp, 1956; Phaff, Miller & Shifrine, 1956; Shehata, Mrak & Phaff, 1955; Shifrine & Phaff, 1956; Skinner & Huxley, 1956; Teunisson, 1954; Wiles, 1954).

Fig. 3. Chromatogram of the 2:4-dinitrophenylhydrazones of the keto acids produced by extracts of *Saccharomyces drosophilarum* (CBS 2896) incubated alone and with isocitrate. Cells grown on acetate (A), citrate (B) or succinate (C) as sole carbon sources.

The characterization of Saccharomyces cerevisiae. Of great interest are the yeasts classified as *S. cerevisiae*, *S. carlsbergensis* and similar organisms. They are of considerable industrial importance and there is much biochemical literature on the terminal respiratory mechanisms of *S. cerevisiae*, usually as baker's yeast. There is much confusion about the biochemical characteristics of *S. cerevisiae*, and it is worth reviewing the facts at some length here. Baker's yeast is almost always *S. cerevisiae* (White, 1954; Nickerson, 1957; Pyke, 1958). Brewer's yeast is commonly so, though lager yeasts may be classified as *S. carlsbergensis* (Guilliermond, 1920; Lodder & Kreger-van Rij, 1952; Thorne, 1957). The data given earlier agree with those of Kudriavzev (1954) in showing that *S. cerevisiae* is unable to grow on exogenous intermediates of the TCA cycle. This is also in agreement with observations on baker's and brewer's yeasts by other workers (e.g. Brandt, 1945; Krebs,

Table 6. *Reactions of [¹⁴C]acetate in extracts of yeasts*

Extracts were prepared by crushing the yeast, grown on either acetate, succinate or citrate, in a Hughes Press and homogenizing the crushed material with 9 vol. of 0.9% KCl. The suspensions were centrifuged at 15,000 g and 0.1 ml. of the supernatant solutions incubated with potassium phosphate, MgCl₂, ATP, coenzyme A, glutathione, [¹⁴C]acetate and additional substrates as indicated, at 30° for 30 min. At this time, 3 ml. hot absolute ethanol was added to each tube, and the ethanol soluble fractions were subsequently analysed by chromatography and autoradiography.

	Carbon source for growth of yeast	Protein concentration of original extract (mg./ml.)	Substrate added	Radioactivity (counts/min.) incorporated into	
				Malate	Citrate
<i>Saccharomyces drosophilarum</i> (CBS 2896)	Acetate	5.46	None	40	20
			Oxaloacetate	60	159,000
			isoCitrate	140,000	30
			Glyoxylate	187,000	10
	Succinate	7.27	None	20	10
			Oxaloacetate	40	160,000
			isoCitrate	4,660	60
			Glyoxylate	64,800	60
	Citrate	9.05	None	20	10
			Oxaloacetate	60	89,100
			isoCitrate	2,690	30
			Glyoxylate	38,800	30
<i>S. acidifaciens</i> (CBS 749)	Acetate	2.41	None	70	40
			Oxaloacetate	650	189,000
			isoCitrate	165,000	70
			Glyoxylate	194,000	40
<i>S. cerevisiae</i> (NCYC 231)	Acetate	1.92	None	60	20
			Oxaloacetate	180	105,000
			isoCitrate	122,000	90
			Glyoxylate	151,000	40

1949; Barron, Ardao & Hearon, 1951; Slonimski, 1953; Krebs, 1954; Singer, Massey & Kearney, 1957). An exception is the report by Krebs (1935) of baker's yeast respiring α -ketoglutarate. Kilkenny & Hinshelwood (1951) reported that five strains of *S. cerevisiae* (designated A, B, C, D, E) were able to grow on all the acids of the TCA cycle, except for strain C which did not grow on aconitic, citric, α -ketoglutaric or oxaloacetic acids. Probably none of these five was in fact a strain of *S. cerevisiae* (Sir C. Hinshelwood, personal communication 1957). They were identified later at the Yeast Division of the CBS (Dr W. Ch. Slooff, personal communication 1957) as three strains of *Hansenula anomala* (A, B and D), one of *Candida krusei* (C) and one (E) of which the identity is in doubt. It would be more useful to regard *S. cerevisiae* as a K- yeast, and all strains capable of metabolizing exogenous TCA cycle intermediates would thus be excluded from this species. This would agree with the exclusion of *Debaryomyces mandshuricus* Naganishi, by Kudriavzev (1954) and of *Zygosaccharomyces fermentati* by both Kudriavzev (1954) and Phaff, Miller & Shifrine (1956). These two were classified as *S. cerevisiae* by Lodder & Kreger-van Rij (1952).

The tricarboxylic acid cycle in yeasts. The growth studies described here, and those

of previous workers, show that yeasts can be classified as K+ or K- by their ability to utilize for growth intermediates of the TCA cycle, but this classification does not imply that K- yeasts lack this cycle. Many K+ yeasts cannot utilize all the cycle intermediates. Manometric data also differentiate K+ and K- yeasts, but the oxidation of intermediates is variable and depends on the conditions of growth. Moreover, K+ yeasts grown on acetate tend to have the respiratory behaviour of K- yeasts.

These findings can be explained by the existence of permeability barriers, which prevent free access of externally added substrates to intracellular enzyme systems. All the component reactions of the TCA cycle have been shown to occur in extracts of acetate-grown yeasts (for review, see Kornberg, 1959*a*), and the cycle has been shown to be of major importance in acetate oxidation by baker's yeast, a typical K- yeast (De Moss & Swim, 1957). Comparison of the patterns of isotope incorporation from [1:2-¹⁴C]acetate by a K+ yeast (*Saccharomyces drosophilorum*) with those of K- yeasts (*S. cerevisiae*, *S. acidifaciens*) support this view. Although the procedure used in this work does not permit quantitative estimates to be made of the rate of incorporation of isotope from acetate, the distribution of radioactivity among the labelled components of each sample fairly reflects the time course of acetate incorporation. It is therefore significant that isotope from acetate was incorporated at early times only into intermediates of the TCA cycle and into amino acids (such as aspartate and glutamate) directly derived from them, and that these labelled compounds are found both in K+ yeasts grown on succinate or citrate (which presumably metabolize these materials and acetate via the TCA cycle) and in acetate-grown K- yeasts. Metabolism of acetate via the TCA cycle would result in an isotope content of succinate initially higher than that of malate: this was found to be the case (Table 3). Only in acetate-grown yeasts was this criterion not obeyed, and malate acquired isotope from acetate without passing through the stage of succinate. This indicated the operation of the glyoxylate cycle (Kornberg & Krebs, 1957) under these conditions, in addition to that of the TCA cycle.

The glyoxylate cycle in yeasts. The key enzymes of the glyoxylate cycle are *isocitratase* (Smith & Gunsalus, 1955; Olson, 1954) and *malate synthetase* (Wong & Ajl, 1956), both of which have been shown to be present in extracts of baker's yeast (Olson, 1959; Kornberg, 1959*b*). The experiments reported in this paper confirm these findings. Extracts of acetate-grown yeasts, supplemented with necessary co-factors, catalysed the incorporation of isotope from acetate into malate if incubated with glyoxylate or *isocitrate* (Table 6), and contained high activities of malate synthetase and *isocitratase* (Table 5). Extracts of yeasts grown on succinate or citrate contained malate synthetase at *c.* 25% and *isocitratase* at *c.* 3% of the specific activities observed with acetate-grown yeasts. This indicates that the operation of the glyoxylate cycle in yeasts may be controlled by influencing the intracellular activity of *isocitratase*, as has also been found with *Pseudomonas ovalis* (Kornberg & Lund, 1959) and *Micrococcus denitrificans* (Kornberg, Collins & Bigley, 1960).

We are grateful to Professor Sir Hans Krebs, F.R.S., for his encouragement and interest, to Miss Patricia Lund for expert technical assistance, and to Dr H. B. Vickery (Connecticut Agricultural Experiment Station, New Haven, U.S.A.) for

generous gifts of L₂-isocitrate. The work of one of us (H.L.K.) was supported, in part, by the Office of Scientific Research of the Air Research Development Command of the U.S. Air Force, through its European Office, under Contract No. AF 61 (514)-1180, and by the Rockefeller Foundation.

REFERENCES

- ALBRITTON, E. C. (1953). *Standard Values in Nutrition and Metabolism*. Dayton: McGregor and Werner.
- BARNES, E. M. (1950). *An investigation of antibacterial substances produced by micro-organisms*. D.Phil. Thesis, University of Oxford.
- BARNETT, J. A. & INGRAM, M. (1955). Technique in the study of yeast assimilation reactions. *J. appl. Bact.* **18**, 131.
- BARRON, E. S. G., ARDAO, M. I. & HEARON, M. (1951). Regulatory mechanisms of cellular respiration. III. Enzyme distribution in the cell. Its influence on the metabolism of pyruvic acid by bakers' yeast. *J. gen. Physiol.* **34**, 211.
- BEECH, F. W. (1957). *The incidence and classification of cider yeasts*. Ph.D. Thesis, University of Bristol.
- BRANDT, K. M. (1945). The metabolic effect and the binding of carbon dioxide in baker's yeast. *Acta physiol. scand.* **10**, suppl. XXX.
- CABELLI, V. J. (1955). The tricarboxylic acid cycle in the oxidative and synthetic metabolism of *Klebsiella pneumoniae*. *J. Bact.* **70**, 23.
- DE MOSS, J. A. & SWIM, H. E. (1957). Quantitative aspects of the tricarboxylic acid cycle in baker's yeast. *J. Bact.* **74**, 445.
- DIXON, G. H. & KORNBERG, H. L. (1959). Assay methods for key enzymes of the glyoxylate cycle. *Biochem. J.* **72**, 3P.
- EL HAWARY, M. F. S. & THOMPSON, R. H. S. (1953). Separation and estimation of blood keto acids by paper chromatography. *Biochem. J.* **53**, 340.
- FRIEDEMANN, T. E. & HAUGEN, G. E. (1943). Pyruvic acid. II. The determination of keto acids in blood and urine. *J. biol. Chem.* **147**, 415.
- GUILLIERMOND, A. (1920). *The Yeasts*. New York: Wiley.
- HEDRICK, L. R. & BURKE, G. C. (1951). Two new yeasts from Hawaiian fruit flies. *Mycopathologia*, **6**, 92.
- HUGHES, D. E. (1951). A press for disrupting bacteria and other micro-organisms. *Brit. J. exp. Path.* **32**, 97.
- INGRAM, M. (1958). Yeasts in food spoilage. In COOK, A. H.: *The Chemistry and Biology of Yeasts*. New York: Academic Press Inc.
- JONES, M. E. & LIPMANN, F. (1955). Aceto-CoA-kinase. In COLOWICK, S. P. & KAPLAN, N. O. *Methods in Enzymology*, vol. 1. New York: Academic Press Inc.
- KILKENNY, B. C. & HINSHELWOOD, C. (1951). The utilization of carbon sources by certain yeast strains. *Proc. roy. Soc. B*, **138**, 375.
- KIRSOP, B. (1954). The National Collection of Yeast Cultures. *J. Inst. Brew.* **60**, 210.
- KORNBERG, H. L. (1958). The metabolism of C₂ compounds in micro-organisms. 1. The incorporation of [2-¹⁴C]acetate by *Pseudomonas fluorescens*, and by a *Corynebacterium*, grown on ammonium acetate. *Biochem. J.* **68**, 535.
- KORNBERG, H. L. (1959a). Aspects of terminal respiration in micro-organisms. *Annu. Rev. Microbiol.* **13**, 49.
- KORNBERG, H. L. (1959b). Synthesis of cell constituents from acetate via the glyoxylate cycle. *Proc. IVth int. Congr. Biochem.* **13**, 25.
- KORNBERG, H. L., COLLINS, J. F. & (in part) BIGLEY, D. (1960). The influence of growth substrates on metabolic pathways in *Micrococcus denitrificans*. *Biochim. biophys. Acta*, **39**, 13.
- KORNBERG, H. L. & KREBS, H. A. (1957). Synthesis of cell constituents from C₂-units by a modified tricarboxylic acid cycle. *Nature, Lond.* **179**, 988.
- KORNBERG, H. L. & LUND, P. (1959). Influence of growth substrates on levels of glyoxylate cycle enzymes in *Pseudomonas ovalis* Chester. *Biochem. J.* **72**, 33P.

- KORNBERG, H. L. & MADSEN, N. B. (1957). Synthesis of C₄-dicarboxylic acids from acetate by a 'glyoxylate bypass' of the tricarboxylic acid cycle. *Biochim. biophys. Acta*, **24**, 651.
- KORNBERG, H. L. & MADSEN, N. B. (1958). The metabolism of C₂ compounds in micro-organisms. 3. Synthesis of malate from acetate via the glyoxylate cycle. *Biochem. J.* **68**, 549.
- KREBS, H. A. (1935). Metabolism of amino-acids. III. Deamination of amino acids. *Biochem. J.* **29**, 1620.
- KREBS, H. A. (1949). The occurrence of the tricarboxylic acid cycle in yeast. *Abstr. 1st int. Congr. Biochem.* p. 836.
- KREBS, H. A. (1954). The tricarboxylic acid cycle. In GREENBERG, D. M.: *Chemical Pathways of Metabolism*. New York: Academic Press Inc.
- KUDRIAVZEV, V. I. (1954). *The Systematics of Yeasts*. Moscow: Academy of Sciences.
- LEBERLE, H. & WILL, H. (1910). Beiträge zur Kenntnis der Gattung Mycoderma. *Zbl. Bakt.* (2 Abt.), **28**, 1.
- LODDER, J. & KREGER-VAN RIJ, N. J. W. (1952). *The Yeasts. A Taxonomic Study*. Amsterdam: North Holland Publishing Co.
- MACKIE, T. J. & MCCARTNEY, J. E. (1956). *Handbook of Practical Bacteriology*, 9th ed. Edinburgh: E. and S. Livingstone.
- MILLER, M. W. & PHAFF, H. J. (1958). A comparative study of the apiculate yeasts. *Mycopathologia*, **10**, 113.
- NICKERSON, W. J. (1957). Cytology and cytochemistry of baker's yeast. In ROMAN, W.: *Yeasts*. The Hague: Junk.
- NICKERSON, W. J. & CARROLL, W. R. (1945). On the metabolism of *Zygosaccharomyces*. *Arch. Biochem.* **7**, 257.
- NIEHAUS, C. J. G. (1932). Untersuchungen über Apiculatushefen. *Zbl. Bakt.* (2 Abt.), **87**, 97.
- OKUNUKI, K. (1931). Beiträge zur Kenntnis der rosafarbigen Sprosspilze. *Jap. J. Bot.* **5**, 285.
- OLSON, J. A. (1954). The *d*-isocitric lyase system: the formation of glyoxylic and succinic acids from *d*-isocitric acid. *Nature, Lond.*, **174**, 695.
- OLSON, J. A. (1959). The purification and properties of yeast isocitric lyase. *J. biol. Chem.* **234**, 5.
- PHAFF, H. J. & KNAPP, E. P. (1956). The taxonomy of yeasts found in exudates of certain trees and other natural breeding sites of some species of *Drosophila*. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **22**, 117.
- PHAFF, H. J., MILLER, M. W. & SHIFRINE, M. (1956). The taxonomy of yeasts isolated from *Drosophila* in the Yosemite region of California. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **22**, 145.
- PYKE, M. (1958). The technology of yeast. In COOK, A. H.: *The Chemistry and Biology of Yeasts*. New York: Academic Press Inc.
- SHEHATA, A. M. E. T., MRAK, E. M. & PHAFF, H. J. (1955). Yeasts isolated from *Drosophila* and from their suspected feeding places in Southern and Central California. *Mycologia*, **47**, 799.
- SHIFRINE, M. & PHAFF, H. J. (1956). The association of yeasts with certain bark beetles. *Mycologia*, **48**, 41.
- SHIFRINE, M. & PHAFF, H. J. (1958). On the isolation, ecology and taxonomy of *Saccharomycopsis guttulata*. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **24**, 198.
- SINGER, T. P., MASSEY, V. & KEARNEY, E. B. (1957). Studies on succinic dehydrogenase. V. Isolation and properties of the dehydrogenase from baker's yeast. *Arch. Biochem.* **69**, 405.
- SKINNER, C. E. & HUXLEY, M. J. (1956). *Rhodotorula glutinis*. *Mycologia*, **48**, 371.
- SLONIMSKI, P. (1953). Recherches sur la formation des enzymes respiratoires chez la levure. *Actualités biochim.* **17**, 1.
- SMITH, R. A. & GUNSALUS, I. C. (1955). Distribution and formation of isocitratase. *Nature, Lond.* **175**, 774.

- STADTMANN, E. R., NOVELLI, G. D. & LIPMANN, F. (1951). Coenzyme A function in and acetyl transfer by the phosphotransacetylase system. *J. biol. Chem.* **191**, 365.
- TEUNISSEN, D. J. (1954). Yeasts from freshly combined rough rice stored in a sealed bin. *Appl. Microbiol.* **2**, 215.
- THORNE, R. S. W. (1957). Brewer's yeasts. In ROMAN, W.: *Yeasts*. The Hague: Junk.
- UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1949). *Manometric Techniques and Tissue Metabolism*, 2nd ed. Minneapolis: Burgess Publishing Co.
- WHITE, J. (1954). *Yeast Technology*. London: Chapman and Hall.
- WICKERHAM, L. J. (1951). Taxonomy of yeasts. *Tech. Bull. U.S. Dep. Agric.* no. 1029.
- WICKERHAM, L. J. (1955). New materials and procedures for genetic studies of yeasts. *Nature, Lond.* **176**, 22.
- WICKERHAM, L. J. & BURTON, K. A. (1948). Carbon assimilation tests for the classification of yeasts. *J. Bact.* **56**, 363.
- WICKERHAM, L. J. & BURTON, K. A. (1956*a*). Hybridization studies involving *Saccharomyces lactis* and *Zygosaccharomyces ashbyi*. *J. Bact.* **71**, 290.
- WICKERHAM, L. J. & BURTON, K. A. (1956*b*). Hybridization studies involving *Saccharomyces fragilis* and *Zygosaccharomyces dobzhanskii*. *J. Bact.* **71**, 296.
- WILES, A. E. (1954). The wild yeast problem as a facet of brewing technology. *Wallerstein Labs Commun.* **17**, 259.
- WINZLER, R. J. & BAUMBERGER, J. P. (1938). The degradation of energy in the metabolism of yeast cells. *J. cell. comp. Physiol.* **12**, 183.
- WONG, D. T. O. & AJL, S. J. (1956). Conversion of acetate and glyoxylate to malate. *J. Amer. chem. Soc.* **78**, 3230.
- ZSOLT, J. (1958). *Torulopsis pseudoceria* nov.spec., a new yeast from soil. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **24**, 210.