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Further Observations on Factors Influencing the Utilization of Citrate by Yeast

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Evidence presented previously (Foulkes, 1951, 1953) showed oxidation of citrate by particulate enzyme systems from *Saccharomyces cerevisiae* to be activated by Mg^{2+} ions and a dialysable factor, citrate oxidation factor (COF), present in boiled yeast juice; this factor behaved as a weak base toward anionic exchange resins and its nature has now been further investigated.

METHODS

Preparation of aqueous extracts of yeast (A). Extracts were prepared as follows: weighed quantities (300 g.) of fresh whole yeast (Distillers' Co. Ltd., 20–21 St James Square, S.W. 1) washed twice with 0·85% (w/v) saline, were suspended in water (1:1) and heated to boiling for 5 min. The debris was removed by centrifuging at 200 *g* and washed once with water. The combined supernatant and washings were concentrated at pH 7 by freeze-drying, in the presence of phosphate buffer (50 ml., 0·1 M, $Na_2HPO_4 + KH_2PO_4$, 13:7). Change of pH during concentration of unbuffered preparations reduced the activity of the product in stimulating citrate oxidation. The active concentrate (*A*) was a yellow gum containing uracil, citric acid, nucleotides, amino acids and ammonium salts; and for the purposes of assay it was dissolved in 20 ml. of water.

Preparation of yeast enzymes. Washed yeast, 5–6 g., was fragmented by being shaken with 0·9% (w/v) KCl (4 ml.) as described by Foulkes (1951). The cellular debris was separated by centrifuging at 1200 *g*. The enzyme preparation was free from citrates and ammonium salts after dialysis in cellophan for 4·5 hr.

Assay of factors stimulating citrate oxidation. The same assay system was employed as described by Foulkes (1953) except that $MgCl_2$ was replaced by $MgSO_4$ (0·1 ml., 0·5%, w/v). Incubation was continued for 40 min. at 37° and the pH value determined before the addition of trichloroacetic acid or ethanol.

Analytical methods. The centrifuged denatured incubation mixture (0·2 ml. or 0·4 ml. as required) was used for citrate analysis (Pucher, Sherman & Vickery, 1936; Buffa & Peters, 1950). The ammonium ion concentration was determined by distillation of the liberated ammonia at pH 8·6.

RESULTS

Activity of aqueous extracts of yeast

Preliminary experiments confirmed that the enzyme preparation was able to oxidize citrate (6 μmoles/40 min.) and that this value was increased (8·9 μmoles/40 min.) by the addition of the aqueous extract *A* described above and by the fractionated product (*V*.280) described by Foulkes (1951, 1953). Chromatographic analysis showed that the stimulatory concentrate *A* contained several amino acids, uracil, nucleotides and ammonium salts. The influence of the following compounds on citrate oxidation was therefore examined severally: L-glutamic acid, L-glutamine, L-asparagine, DL-alanine, nicotinamide (slight activity), glycine, L-tyrosine, DL-aspartic acid, D-glucosamine, urea (inactive). The only compounds in our experiments

significantly accelerating the disappearance of citrate were ammonium salts, diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN). In contrast to Foulkes's results, it was not found necessary to add adenosine triphosphate (ATP) to the test system.

Effect of added ammonium salts on citrate oxidation

Addition of 30 μmoles ammonium salts (sulphate, chloride, carbonate or sulphamate) resulted in the complete utilization of citrate (10 μmoles) by the enzyme system. The effect of added ammonia at two levels of concentration on citrate oxidation is shown in Table 1. From this it appears that, between pH 6·5 and 7·1, a quantitative relationship exists between the amount of ammonia added and the increase in the amount of citrate metabolized. High concentrations of ammonium ion (0·15 M), however, inhibit the oxidation of citrate (Table 1).

Hydroxylamine and methylamine hydrochlorides did not stimulate citrate oxidation in comparable concentrations under conditions in which ammonium salts were active.

Table 1. *Effect of ammonia on citrate oxidation by disintegrated yeast cells*

For details see text. 10 μmoles of citrate were present initially. In Expt. 1 the control preparation metabolized 7·3 μmoles citrate, and in Expt. 2, 6·3 μmoles.

Ammonia added (μmoles)	Final pH after incubation	Increase in citrate metabolism due to NH_3 (μmoles)
	Expt. 1	
None	6·42	0·0
10·0	7·04	—
4·0	6·88	2·5
2·0	6·48	2·0
1·0	6·42	1·1
0·4	6·58	0·5
	Expt. 2	
—	6·85	0·0
300	6·98	0·2
60	6·90	3·5
15	7·02	3·6
3	7·02	2·2

Ammonium content of yeast and yeast extracts

The active extracts so far examined contained ammonium salts in the following concentrations: yeast extract *A* 0·0145 N, an earlier fractionated preparation (*V.* 280) 0·060 N and an active preparation *B* made according to the directions of Foulkes (1953), 0·88 N-NH_4^+. Yeast cells (D.C.L.) contained 20 mg./100 g. (dry weight) of ammonium ion.

Addition of the partially fractioned extracts (*V.* 280 and *B*) to the enzyme system stimulated the uptake of citrate in the same way as an equivalent amount of an ammonium salt (Table 2). The unfractionated extract *A*, however, was considerably more active in this respect than could be accounted for on the basis of its content of ammonium salts (Table 2), and this is believed to be due to the presence of TPN and DPN believed to be present in the extract on the basis of ultraviolet absorption and precipitation with silver nitrate.

Action of nitrous acid and silver ions on unfractionated yeast extract A

Extract *A* (1 ml.) was mixed with KH_2PO_4 (1 ml., 0·1 N), the pH was adjusted to 5·2 with acetic acid and 0·2 ml. sodium nitrite (0·75 M) was added. After 3 hr., 1 ml. of the resulting solution was treated with 0·2 ml. urea (0·17 M) and allowed to

Table 2. *Comparison of activities of crude boiled-yeast extracts with those of equivalent amounts of ammonia on citrate metabolism*

Details of enzyme preparation given in text. 20 μmoles of citrate were present initially in Expt. 1, and 30 μmoles in Expt. 2.

Additions	Ammonia (μmoles)	Citrate metabolized (μmoles)	Amount of citrate metabolized above control
	Expt. 1		
None	—	12·6	—
0·2 ml. yeast extract *A**	2·9	23·8	11·2
0·4 ml. yeast extract *A**	5·8	30·2	17·6
0·2 ml. 0·0146 N-NH_4Cl	2·9	15·4	2·9
0·4 ml. 0·0146 N-NH_4Cl	5·8	17·5	5·0
	Expt. 2		
None	—	14·4	—
0·2 ml. extract *B* (10 times diluted)	17·6	23·3	8·9
0·2 ml. 0·084 N-NH_4Cl	16·8	23·3	8·9
0·2 ml. yeast extract *V.* 280	12·0	21·7	7·3
0·2 ml. 0·063 N-NH_4Cl	12·6	22·0	7·6

* Containing 32·5 μmoles of citrate/ml. taken into account in computing the total amount of citrate metabolized.

stand for 2½ hr. The pH values of this and the remaining nitrite-treated solution were adjusted to 7·2 by addition to each of 0·8 ml. Na_2HPO_4 (0·1 M) and solid K_2CO_3. Deamination of extract *A* resulted in a decrease (Table 3) in the stimulatory action on citrate oxidation, equivalent to the amount of ammonium salt present (3·2 μmoles). Under these conditions nicotinamide and its derivatives are not deaminated (Schlenk, Hellström & von Euler, 1938). Previous reports (Foulkes, 1953) that no reduction in activity of yeast extracts follows after deamination with nitrous acid, may be attributed to the use of ammonium sulphamate to remove unchanged nitrous acid.

Addition of silver nitrate to yeast extract *A* resulted in precipitation of DPN and TPN. The resulting solution (freed from silver ions by addition of 1 ml. 1·53 N-KCl) contained ammonium salts (3·6 μmoles) and its activity in stimulating citrate oxidation (3·5 μmoles) was accountable on that basis (Table 4).

Formation of glutamic acid

Estimation of the residual ammonia concentration in the assay system, both in the presence and absence of semicarbazide, showed that the disappearance of ammonia was equivalent to the increased quantity of citrate oxidized. In the presence of ammonia, glutamic acid was produced. This was shown by paper chromatographic analysis (*n*-butanol–acetic acid–water; 4:1:5, by vol.) of samples of the incubation mixtures denatured with ethanol. The spots developed with ninhydrin were excised and eluted with water (1 ml.) and the coloration was measured on a Spekker absorptiometer at 570 mμ. (Table 5).

Effect of other activators and inhibitors

ATP was found to increase only slightly the oxidation of citrate by dialysed enzyme preparations, but the possibility that the ATP added was a source of ammonium ion, either by contamination or deamination, cannot be excluded. Ethylenediaminetetraacetate (used as the sodium salt) and 8-hydroxyquinoline were without effect on the activity of boiled yeast preparations. Both semicarbazide (see Table 5) and cyanide ions inhibited the ammonia-stimulated citrate oxidation.

As has been described for other systems (Beinert *et al.* 1953) addition of sodium salts or substitution of potassium chloride by sodium chloride resulted in a reduction of the amount of citrate oxidation, both with and without added ammonium ion.

DISCUSSION

While it has been confirmed that the complex mixture of substances present in boiled yeast extracts stimulates the oxidation of citric acid by yeast enzymes, the major effect is due to the DPN or TPN and ammonia. The residual activity (approx. 25 %) after precipitation of nucleotides with silver ions and the activity of fractions previously described has been traced to the influence

Table 3. *Effect of deamination with nitrous acid on activity of boiled yeast extracts*

20 μmoles of citrate were added initially. Extract *A* (diluted fourfold) contained 8·1 μmoles of citrate. The control preparation metabolized 6·8 μmoles of citrate.

Additions	Ammonia present (μmoles)	Amount citrate metabolized above control (μmoles)
1·0 ml. diluted yeast extract *A*	3·6	11·6
1·0 ml. diluted yeast extract *A* + NO_2^-	0·0	5·7
1·0 ml. diluted yeast extract + NO_2^- + urea	0·0	8·2

Table 4. *Effect of precipitation with silver ions on activity of boiled yeast extracts*

20 μmoles of citrate added initially. The control metabolized 6·0 μmoles of citrate. The yeast extract *A* contained 4·0 μmoles of citrate/ml.

Additions	Amount citrate metabolized above control (μmoles)
1·0 ml. diluted yeast extract *A*	15·2
1·0 ml. diluted yeast extract treated with $AgNO_3$ + KCl	3·5
1·0 ml. water treated with $AgNO_3$ + KCl	0·7

Table 5. *Correlation of citrate metabolism, ammonia uptake and formation of glutamic acid*

Details of the enzyme preparation in text. 60 μmoles of citrate were present initially.

Additions	Glutamic acid (R_F 0·22) Spekker units	Initial NH_3 conc. (μmoles)	Amount ammonia utilized (μmoles)	Total citrate metabolized	Increase in citrate metabolism due to NH_3 (μmoles)
—	0·39	0	0	29·5	0
NH_4Cl (0·3 ml., 0·165 M)	0·88	93	32·6	59·7	30·2
NH_4Cl (0·3 ml., 0·165 M) + semicarbazide HCl (0·2 ml., 0·25 M)	0·54	93	12·8	42·5	13·0
Semicarbazide HCl (0·2 ml., 0·25 M)	—	0	0	31·4	—

of ammonium salts present. These results, however, do not preclude the possibility that other substances may stimulate the system. It is realized that several observations reported in this communication are at variance with those in a previous paper from this laboratory (Foulkes, 1953). Many of these receive an explanation in the fact that ammonia proves active only between certain limits of concentration. The apparent failure to inactivate with nitrous acid was due to the addition of ammonium sulphamate after deamination.

In the test system ammonia is actively removed in amounts equivalent to the increase in citrate disappearance and glutamic acid is formed. This is consistent with the suggestion (Krebs, 1952; Krebs, Gurin & Eggleston, 1952) that the presence of tricarboxylic acids in yeast is related to the supply of intermediates for synthetic processes. In yeast, two pathways for the conversion of isocitric acid into α-oxoglutarate have been recognized (Kornberg & Pricer, 1951) and the DPN-activated reductive amination of the latter to glutamic acid is well established (von Euler, Adler & Eriksen, 1937; Adler, Günther & Everett, 1938).

It is of interest that, under our experimental conditions at least, the relevant enzymes are associated with cell particles (cf. Nossal, 1954).

SUMMARY

1. The oxidation of citrate in particulate yeast preparations is stimulated by boiled yeast extract (citrate oxidation factor). This action has been shown to be partly due to ammonium salts in whole boiled extract, and entirely due to ammonium salts in fractions of boiled extract described previously (Foulkes, 1953).

2. Increased oxidation is accompanied by the combination of considerable amounts of ammonia, chiefly to form glutamic acid.

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Differential Fractionation of Hydrogen Isotopes in Liver and Mammary Gland

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The validity of the use of isotopes for tracer purposes depends on the assumption that molecules of normal and abnormal isotopic composition behave qualitatively and quantitatively in the same way. The possibility, however, that they might not do so because of mass differences received much attention after the discovery of deuterium in 1932. Thus Eyring & Sherman (1933) predicted on theoretical grounds that deuterium might react only half as fast as hydrogen in chemical reactions at ordinary temperatures, while Wheeler & Pease (1936) found that the rate of homogeneous reaction of hydrogen with ethylene at 500° was 2·5 times that of deuterium. More recently, differences in the rates of reaction of carbon isotopes have been observed (see review by Ropp, 1952).

Early work on the effect of isotopes in biological processes was devoted mainly to examination of the gross physiological effects of heavy water on the intact organism (see review by Barbour, 1937). There is much contradictory evidence, but in general it may be concluded that concentrations of heavy