MORPHOLOGICAL AND BIOCHEMICAL EFFECTS OF FREEZING ON YEAST CELLS*

by

I. A. HANSEN AND P. M. NOSSAL

Department of Biochemistry, University of Adelaide (South Australia)

In an attempt to overcome the permeability problems associated with the study of yeast metabolism, many workers have subjected the cells to temperature shock, usually by dropping crumbled yeast into liquid air or liquid nitrogen, or by freezing with solid CO_2. Subsequent thawing yields a viscous mixture which was believed by some workers to contain enzymes liberated through disintegration of cells (CONWAY AND DOWNEY[1]; LYNEN AND NECIULLAH[2]; WIELAND *et al.*[3]). Since aldehyde dehydrogenase can be prepared from the supernatant of liquid-air frozen yeast[4], some disintegration must occur. Other workers (*e.g.* KREBS *et al.*[5]) have realized that after dry-ice treatment, at least, most of the cells are still intact. They have therefore taken the view that freezing merely increases the permeability to such compounds as di- and tricarboxylic acids, and that results obtained with frozen cells may be used to deduce the normal mechanism of yeast respiration.

The present work shows that freezing, besides increasing permeability, causes extensive changes in the morphology and enzymic structure of the cells.

MATERIALS AND METHODS

CO_2-frozen cells: In preparation for all the experiments, unless otherwise stated, finely crumbled fresh commercial bakers' yeast (stored in a humidor at 2°) was incubated at 30° for 60 min in a stoppered flask to avoid desiccation. At the end of this period, the yeast was intimately mixed with excess powdered solid CO_2 (dry ice) and the CO_2 evaporated either 4 h at 1° or 16 h at −12°. In specified cases, the cells were washed twice with 19 vols. 0.1 *M* phosphate buffer pH 6.5.

Cell-free extracts: A mixture consisting of 2 g wet weight yeast, 10 g Ballotini glass beads No. 12, and 10 ml 0.9% KCl was shaken for 10 sec at 2° in the disintegrator previously described[6]**. The resulting mixture was centrifuged at 1000 *g* for 10 min to remove glass and cell debris. The first supernatant, *i.e.* the cell-free whole extract (*W*), was centrifuged at 15,000 *g* for 30 min and the second supernatant (*S*) decanted. The residue (*R*) was washed once (to the original volume) with 0.9% KCl and, after resedimentation at 15,000 *g* and removal of the washings (*RW*), was resuspended in half the original volume of 0.9% KCl.

Dialysis: The fractions *W* and *S* were dialysed at 2° in cellophane bags against a large volume of 0.9% KCl for 20–24 h with magnetic stirring.

Comparative runs: In all the experiments in which a comparison was made between fresh and CO_2-frozen cells, both were always from the same batch of yeast. CO_2-frozen cells kept longer than

* This work was supported by a grant (to I.A.H.) from the University of Adelaide. Partial support was also given by the National Health and Medical Research Council of Australia and the Rockefeller Foundation.

** The ultra-rapid shaker may now be obtained from the Shandon Scientific Co., Cromwell Place, London S.W.7.

References p. 512.

4 h were stored at −12°. Disintegration and all other operations were carried out concurrently and under the same conditions with the two types of cells.

Thunberg experiments: Evacuated Thunberg tubes contained 0.02 *M* Na-K phosphate buffer pH 7.0, 0.1 *M* substrate and 0.3 μmole methylene blue. The time for 90% decolorization at 38° was used in calculating Q_{MB} (μl methylene blue decolorized/mg dry wt/h).

Fumarase and aconitase measurements: The method of RACKER[7] was used. Specific activities were calculated as previously described[8], and the same experimental conditions were adhered to.

Dry weights were determined as previously described. Correction was made for the presence of KCl or buffer.

Coenzymes: Diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) were laboratory preparations. DPN was 47% pure and contained no TPN. TPN was 10% pure and contained 9% DPN. The total DPN + TPN content was assayed by formation of the cyanide complex[9]. Adenosine-5′-phosphate (A-5-P) was a commercial sample (Light & Co. Ltd. Colnbrook, Bucks., England).

RESULTS

Morphological changes caused by freezing

The fresh yeast cell presents a clear picture in which one or two quite large vacuoles are visible under phase-contrast illumination (Fig. 1). The vacuole is surrounded at its periphery by granules, which appear refractile under phase-contrast and dark-ground illumination but dark under other types of illumination. Yeast cells treated with solid CO_2, on the other hand, are smaller and seem to have undergone internal disorganization (Fig. 2). Only in very rare instances can vacuoles be seen, and the granules are irregularly dispersed or clumped together. This alteration in appearance was not caused by the incubation treatment which preceded freezing.

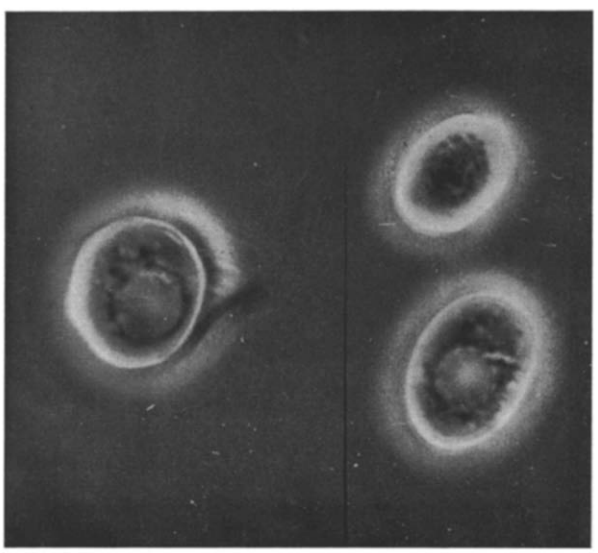

Fig. 1. Fresh cells of bakers' yeast before freezing.

It was thought that electron micrographs would reveal more of the structure of the cell. We therefore attempted to make yeast preparations suitable for electron microscopy after ultra-thin sectioning[10]. It was difficult to obtain adequate fixation without complete loss of detail, especially as considerable shrinkage occurred during dehydration. After some experimentation, the method selected was: fixation with 1% OsO_4 in 0.57 *M* Michaelis veronal-acetate buffer pH 7.4 for 30 min, dehydration by passage through graded ethanol, and embedding in methacrylate polymer (2 vols.

References p. 512.

methyl methacrylate: 7 vols. butyl methacrylate[11]). The micrographs obtained were no more informative than those obtained by phase-contrast microscopy. Alteration of pH, use of a lower concentration of OsO_4, or use of other fixatives gave even less definition.

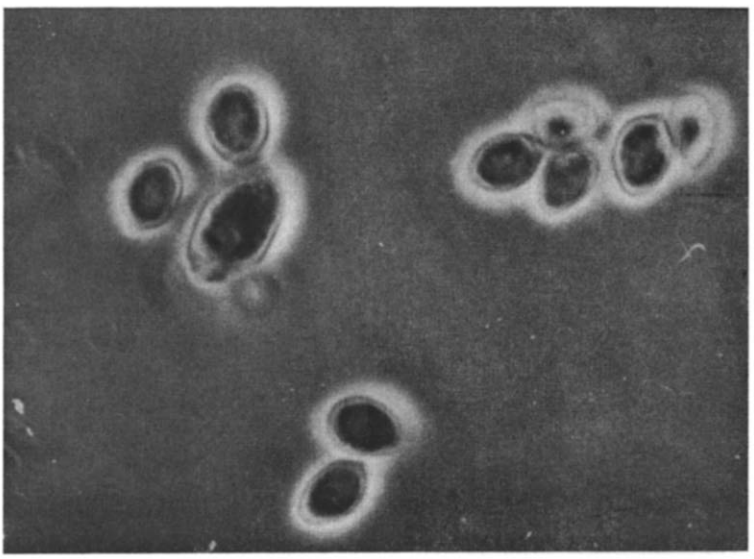

Fig. 2. Yeast cells after freezing with solid CO_2. Magnification 1800 ×. Leitz Ortholux phase-contrast equipment.

Oxidation of ethanol and succinate

It has been claimed[5] that suitable heat pretreatment before CO_2-freezing of cells destroys their ability to respire acetate or ethanol while preserving the ability to oxidize succinate.

We have confirmed the one-step nature of the succinate oxidation to fumarate + malate only.

Three types of commercial bakers' yeast were subjected to CO_2-treatment involving temperature changes of various magnitudes. This was achieved by bringing the yeast to a thermostatically controlled temperature in the following ways before CO_2-freezing: (1) at least 4 h at −12°; (2) 4 h at 1°; (3) 3 h at 20°; (4) 1 h at 48°; (5) 1 h at 53°. After freezing, the cells were washed once with 19 vols. 0.1 *M* phosphate buffer pH 6.5 and resuspended in a further 19 vols. Table I shows that in the case of Nycander's and Barrett's yeasts, oxidation of ethanol decreased progressively as the temperature change increased, while that of succinate was almost unaffected. With Effront yeast, however, little decrease in ethanol oxidation was observable until the yeast was heated to above 50° before freezing. Cells heated at 53° for 1 h showed negligible ethanol oxidation even without CO_2-treatment.

The wide divergence between various commercial strains of bakers' yeast in their ability to withstand freezing may be due to differences in cell wall structures. These results indicate the difficulty of using such data obtained with one strain to formulate a general mechanism of ethanol oxidation. Even using one commercial type over a period of months, we have observed considerable variation in enzymic activity and resistance to freezing.

References p. 512.

TABLE I

OXIDATIONS IN THREE STRAINS OF BAKERS' YEAST AFTER CO_2-FREEZING

Pretreatment of yeast as described in the text. The conventional Warburg apparatus contained *ca.* 30 mg dry wt yeast and 0.01 *M* substrate. $Q_{O_2}^{30°}$ values are means of several experiments.

	$Q_{O_2}^{30°}$								
	Effront			*Nycander's*			*Barrett's*		
	Blank	*Ethanol*	*Succinate*	*Blank*	*Ethanol*	*Succinate*	*Blank*	*Ethanol*	*Succinat*
Fresh cells	4	34	5	7	32	8	10	28	10
Treatment 1	7	31	14	5	30	12	3	22	8
Treatment 2	6	32	12	5	13	13	2	9	8
Treatment 3	4	15	11	4	10	11	1	6	7
Treatment 4	5	25	14	0	0	7	0	1	7
Treatment 5	0	0	6	—	—	—	—	—	—

Dehydrogenase activities of frozen cells

It is well known that intact yeast cells are impermeable to di- and tricarboxylic acids, whereas cells which have undergone treatment with liquid air[2] or solid CO_2[5] are permeable. CO_2-frozen cells showed dehydrogenase activities (Table II) of the same order of magnitude, except in the case of ethanol dehydrogenase, as those found in cell-free extracts of untreated yeast[12]. The activities were considerably higher than those reported by LYNEN AND NECIULLAH[2]. With unwashed frozen cells it was found that several of the dehydrogenases, usually coenzyme-dependent, showed appreciable activity without added coenzyme. After washing the cells with water or with 0.1 *M* phosphate buffer pH 6.5, these dehydrogenases showed the expected coenzyme-dependence (Table III). The washings had virtually no enzymic activity towards the substrates tested, but restored the activities of those dehydrogenases which in washed cells required addition of coenzymes. Similar results were obtained with washings which

TABLE II

DEHYDROGENASE ACTIVITIES OF UNWASHED CO_2-FROZEN YEAST

Immediately after thawing, yeast was suspended in 19 vols. water. Each Thunberg tube contained 5 mg dry wt yeast. Q_{MB} values are means of six experiments.

Substrate	*Additions*	Q_{MB}
None	None	< 1
None	0.1 mg DPN	1
None	0.1 mg DPN 0.1 mg TPN	5
Succinate	None	7
Lactate	None	19
Citrate	0.1 mg DPN 0.1 mg TPN 0.001 *M* $MnCl_2$ 0.001 *M* A-5-P	8
*iso*Citrate	as for citrate	48
Ethanol	0.1 mg DPN	21
L-Malate	0.1 mg DPN	23
Fumarate	0.1 mg DPN	1.5
L-Glutamate	0.1 mg TPN	19

References p. 512.

had been boiled for 2 min. Addition of the same amount of nucleotide coenzymes as contained in the washings resulted in the same increases in dehydrogenase activity of washed cells; the effect of the washings could therefore be ascribed to their coenzyme content.

TABLE III

COENZYME DEPENDENCE OF DEHYDROGENASES IN WASHED AND UNWASHED FROZEN CELLS

Unwashed cells = thawed cells suspended in 19 vols. water or 0.1 *M* Na, K-phosphate buffer pH 7.0.
Washed cells = cells suspended as above, centrifuged, and resuspended in another 19 vols. water or buffer, this process being repeated once more.
Washings = supernatant obtained from centrifugations. Blank values for unwashed cells are given in Table II; those for washed cells were even lower.

	Dry weights (mg/ml)	*Additions*	Q_{MI}		
			Ethanol	L-*Malate*	L-*Glutamate*
Unwashed cells	12.0	—	5	16	18
Unwashed cells		0.1 mg DPN	20	34	21
Washed cells	9.3	—	< 1	2	< 1
Washed cells		0.1 mg DPN	19	20	21
Washed cells		DPN equal to content of washings	12	14	15
Washings	2.6	0.1 mg DPN	< 1	< 1	< 1
Washed cells + washings	—	—	6	14	10
Washed cells + washings		0.1 mg DPN	24	23	24
Washed cells + boiled washings	—	—	8	15	7

The amount of material which appeared in the washings was quite large, as much as 20% of the original dry weight. This would account for most of the material lost on dialysis of extracts (see Table V). Some of the constituents of the washings are shown in Table IV.

TABLE IV

COMPOSITION OF WASHINGS OF FROZEN CELLS

Substance	*Method of estimation*	*mg dry wt per 100 mg dry wt washings*
Organic acids	Ether extraction	1.0
(Succinic acid	Succinoxidase	0.85)
Amino-acids	Ninhydrin colour	32.0
Protein	Biuret colour	14.8
Total Carbohydrate*	Anthrone colour	36.2
Inorganic salts	Ashing	19.3
DPN + TPN	Cyanide complex	0.9

* The carbohydrate present did not reduce Benedict's solution.

Intracellular enzyme distribution

10-sec extracts of both fresh cells and CO_2-frozen cells were prepared in our shaker and fractionated, as described under METHODS. Table V shows that the granules accounted for less of the dry weight in the case of CO_2-frozen cells than in the case of the fresh cells. Dialysis of the supernatant and of the whole extract against 0.9% KCl

References p. 512.

caused a large decrease in dry weight. In comparative experiments (see METHODS) the proportion of the dry weight lost in each case was greater with frozen than with fresh cells. The fact that the absolute increase in dialysable material was the same for the whole extract as for the supernatant suggests that the latter contained the additional material which had been rendered dialysable.

TABLE V

DRY-WEIGHT DISTRIBUTION IN EXTRACTS OF FRESH AND FROZEN CELLS

Values are means of seven determinations.

Fraction	*CO_2-frozen cells*			*Fresh cells*			*Difference in dialysis loss (mg)*
	dry wt (mg) (1)	*percentage of W* (2)	*wt loss on dialysis (mg)* (3)	*dry wt (mg)* (4)	*percentage of W* (5)	*wt loss on dialysis (mg)* (6)	(3)−(6)
W	17.9	100	9.3	17.1	100	7.9	1.4
R	3.0	16.8	—	3.4	19.9	—	—
S	14.0	78	8.3	13.6	79.5	6.9	1.4
RW	1.3	7.3	—	1.2	7.0	—	—

The activities of a number of dehydrogenases in extracts of fresh yeast cells have already been reported by SLONIMSKI AND HIRSCH[13] and NOSSAL[12]. The activities in extracts of CO_2-frozen cells (Table VI) did not differ greatly from those in fresh cells, except that the ethanol and malic dehydrogenases were slightly less active. Some differences are demonstrated more clearly in Table VII, which compares the distribution of several enzymes between the granules and supernatants of extracts from fresh and CO_2-frozen cells. The distribution of *iso*citric dehydrogenase was similar in both types of extracts. Using citrate as substrate, higher activity was found in the supernatant of frozen-cell extracts than in the granules. Aconitase, which converts citrate to *iso*citrate, was also shown to have higher activity in the supernatant of these extracts than in the granules. This suggests that aconitase is more readily detached from the granules than *iso*citric dehydrogenase. A larger proportion of the total ethanol and malic dehydrogenase activities was located in the supernatant in the case of frozen-cell extracts.

TABLE VI

DEHYDROGENASES IN DIALYSED WHOLE EXTRACTS OF FROZEN CELLS

Q_{MB} values are means of at least four experiments.

Substrate	*Additions*	Q_{MB}
None	0.1 mg TPN 0.1 mg DPN	< 1
Succinate	—	5
Lactate	—	21
*iso*Citrate	0.1 mg TPN 0.1 mg DPN 0.001 *M* $MnCl_2$ 0.001 *M* A-5-P	23
L-Glutamate	0.1 mg TPN	21
Ethanol	0.1 mg DPN	42
L-Malate	0.1 mg DPN	20
Fumarate	0.1 mg DPN	5

References p. 512.

TABLE VII

DISTRIBUTION OF DEHYDROGENASES IN EXTRACTS OF FRESH AND FROZEN CELLS

	CO_2-frozen cells			Fresh cells		
	Q_{MB}		% activity* due to granules	Q_{MB}		% activity* due to granules
	Granules	Dialysed supernatant		Granules	Dialysed supernatant	
*iso*Citrate + 0.1 mg TPN	25	17	43	26	24	36
Citrate + 0.1 mg TPN	4.5	5.5	29	6.3	2.2	61
L-Malate	2.0	4.9	15	5	14	16
L-Malate + 0.1 mg DPN	41	83**	25	40	34	39
Fumarate + 0.1 mg DPN	2.6	7.3	17	5.0	4.8	36
Ethanol	< 1	< 1	—	44	< 1	> 95
Ethanol + 0.1 mg DPN	36	83	18	64	37	48
Dry wt (mg)	2.4	4.8		3.2	6.0	

* The total activity of the granules, and similarly of the supernatant, is calculated as specific activity (Q_{MB}) X dry wt.

The percentage activity is thus $\frac{\text{granular activity} \times 100}{\text{granular activity} + \text{supernatant activity}}$

** The apparent increase in total activity is due to loss of reoxidizing systems.

It has already been shown that nucleotide coenzymes are removed from frozen cells by washing. This fact, together with the values recorded in Table VII for ethanol and malic dehydrogenase activity without added coenzyme, suggests that the coenzyme originally attached to these enzymes was lost on freezing.

HUENNEKENS AND GREEN[14] described this phenomenon for DPN attached to the malic dehydrogenase of animal mitochondria, but gave no details of their method of freezing.

Both ethanol and malic dehydrogenase have been shown to require DPN in extracts of fresh yeast[12]. In the dialysed supernatant from these extracts, malic dehydrogenase was partially saturated with DPN. However, DPN did not seem to be associated with the enzyme in the granules of fresh cells, nor in the granules or dialysed supernatant of frozen cells. In the latter three cases, addition of DPN fully reactivated the enzyme. On the other hand, it was in the granules of fresh cells, and not in the dialysed supernatant, that ethanol dehydrogenase was found to be partially saturated with DPN. Once again, DPN did not seem to be associated with the enzyme in the granules or dialysed supernatant of frozen cells. Addition of DPN also caused reactivation of the ethanol enzyme. It is suggested that these facts can be explained by assuming a fairly stable enzyme-coenzyme complex which is disrupted by freezing or prolonged disintegration. DPN binding with crystalline yeast alcohol dehydrogenase[15] and with 3-phosphoglyceraldehyde dehydrogenase[16] has been demonstrated.

The systems which reoxidize methylene blue, causing a return of colour, when fumarate and malate are used as substrates for fresh-cell extracts[12], did not seem to operate in frozen-cell extracts, as no colour-return was ever observed. Therefore the overall rates of decolorization with these substrates were usually greater in frozen than in fresh cells. It is concluded that the reoxidizing systems were disrupted by freezing.

References p. 512.

TABLE VIII

FUMARASE AND ACONITASE DISTRIBUTION IN FRESH AND FROZEN-CELL EXTRACTS

Each value is the mean of three complete series of experiments.

Fraction	CO_2-frozen cells					Fresh cells				
	Dry wt (mg)	Fumarase		Aconitase		Dry wt (mg)	Fumarase		Aconitase	
		Specific activity	Contribution per ml W	Specific activity	Contribution per ml W		Specific activity	Contribution per ml W	Specific activity	Contribution per ml W
W	15.1	144	2190	43	650	16.3	156	2530	45	740
S	12.8	162	2050	50	630	13.1	176	2310	56	730
R	2.2	110	250	20	47	2.3	149	340	35	82
RW	0.15	> 200	44	> 25	4	0.0	high*	46	high	7

* 1 ml caused a change in optical density of 0.046 per min. Thus the activity per ml can be calculated, but not the specific activity.

Data on the distribution in yeast extracts of the dehydrases fumarase and aconitase have been reported by HIRSCH[17] and NOSSAL[8,12]. Table VIII compares the distribution in extracts of fresh and frozen cells. The values designated "contribution" represent the change in optical density ($\times 10^3$)/min/ml of the original whole extract. The activities of both enzymes were slightly lower in extracts of frozen cells than in those of fresh cells. The ratio of the contribution of granules to that of supernatant was lower in frozen than in fresh cells in each case, but this altered ratio was more pronounced with aconitase than with fumarase.

Extracts of fresh cells were fractionated and the granules divided into two parts. One part was washed with 0.9% KCl as usual and tested for enzyme activity. The other part was suspended in 0.9% KCl, frozen in dry-ice for 30 min, thawed for 1 h at 2°, and the granules resedimented. The activities of the supernatant KCl, and the granules after suspension, are recorded in Table IX. The high activities of fumarase and aconitase in the KCl supernatant after freezing indicate solubilization of these enzymes by the treatment. Further, the total fumarase activity (per ml of original whole extract) of granules plus washings increased on freezing, possibly due to disruption of interfering systems[12], whereas aconitase appeared to be inactivated.

TABLE IX

FUMARASE AND ACONITASE ACTIVITIES OF GRANULES BEFORE AND AFTER FREEZING

Fraction	Dry wt. (mg)	Fumarase		Aconitase	
		Specific activity	Contribution per ml W	Specific activity	Contribution per ml W
W	15.3	166	2530	46	705
S	12.5	186	2330	60	740
R	2.6	128	343	21	52
RW	< 0.1	> 400	48	> 90	8
Frozen R	2.5	65	152	6	14
KCl supernatant after freezing	0.53	1365	570	28	15

References p. 512.

DISCUSSION

Sudden freezing and subsequent thawing markedly affect the appearance of yeast cells. Two striking features are usually observable — distortion or shrinkage of the cell outline and disappearance of the vacuole. No rupture of the external membrane could be observed. Many substances have been shown to diffuse out of frozen cells during washing, including pyridine nucleotides — even those which may have been attached to the cell constituents, such as cytoplasmic granules, before freezing.

Freezing appears to have a disruptive effect on these granules, judging from the distributions of dry weight, enzyme activity, and coenzymes. It would therefore be expected to interfere with the integration of the granular enzyme complex. Thus it has been found that oxidative phosphorylation, which occurs when succinate is oxidized by granules from fresh cell extracts, is abolished by freezing[18]. Disintegration of yeast cells by means of our high-speed shaker solubilizes a number of enzymes[12], but freezing enhances this effect, as shown by comparison of fresh and frozen cells disintegrated for the same period. The distribution of enzymes in 10-sec extracts of frozen yeast tended to resemble that in extracts of fresh yeast prepared by disintegrating for longer periods. A study of aconitase has indicated that it is very easily removed from its site on the granules, as has recently been found for animal mitochondria[19]. This may be one reason why citrate is not an effective substrate for the granular *iso*citric dehydrogenase in our preparations.

KREBS *et al.*[5] have dismissed the idea that the tricarboxylic acid cycle is the major mechanism of acetate oxidation in yeast on the following grounds: (1) Thermal shock affects acetate and succinate oxidation differentially. However, the acetate oxidizing system is presumably an enzyme complex and is therefore, like cyclophorase, susceptible to freezing. The system catalysing the one-step oxidation of succinate, being very much simpler, would be expected to be much more resistant to various treatments, as we have indeed found. (2) In frozen cells, malonate inhibits the oxidation of succinate but not of acetate. It has been suggested[20] that there may be different sites of acetate and succinate oxidation in frozen cells. Malonate may inhibit oxidation of added succinate by small granules (succinoxidase fragments split off respiratory granules by freezing) which are incapable of oxidizing acetate. In intact granules which will oxidize acetate, oxidation of the bound or "active" succinate produced is assumed not to be inhibited by malonate. The independence of the systems oxidizing acetate and succinate added at the same time seems likely from the strictly additive oxygen consumption (Tables 9 and 10 of reference)[5]. (3) Since added unlabelled succinate and α-ketoglutarate did not become radioactive during simultaneous oxidation of $CH_3{}^{14}COOH$, they were ruled out as intermediaries of acetate oxidation. Recent experiments[21] have shown that this interpretation may not be valid when pooling between intermediaries and added carriers is restricted. Further, we have found that cell-free yeast extracts, when incubated with $CH_3{}^{14}COOH$ and oxalacetate, form citric, α-ketoglutaric, succinic, fumaric, and malic acids with high radioactivity[21]. These results are similar to those obtained with *Micrococcus lysodeikticus* and *Escherichia coli* in noncarrier experiments[22, 23, 24]. (4) It is claimed that, apart from fumarase and aconitase, the enzymes of the cycle show activities too low to account for the rate of acetate oxidation. The following observations indicate that the enzymes have much higher activities than hitherto suspected. The fumarase activity of crushed frozen cells is 4–5 times higher than that of intact frozen cells[20],

References p. 512.

indicating incomplete permeability in the latter. In cell-free extracts, malic and *iso*citric dehydrogenase activities are of the same order of magnitude as that of ethanol dehydrogenase[12]. The condensation of acetyl-coenzyme A with oxaloacetate is sufficiently rapid to account for acetate oxidation in fresh cells (LYNEN[25]). Finally, fresh cells at pH 2.5 oxidize approx. 0.1 *M* fumaric acid rapidly[20] (Q_{O_2} values of up to 25 did not represent a maximum) and extensively.

It is thus possible to reinterpret the results of earlier work in such a way that the tricarboxylic acid cycle is no longer excluded from being the major pathway of acetate metabolism in yeast.

ACKNOWLEDGEMENTS

We are grateful to Prof. Sir C. STANTON HICKS of the Physiology Department for the loan of Leica photomicrography equipment, DR. S. G. TOMLIN of the Physics Department for assistance with the electron microscopy, and the Effront Yeast Co., South Yarra, Victoria, for a regular supply of yeast.

SUMMARY

1. Some changes in the structure and metabolism of yeast cells which occur on freezing with solid CO_2 are described.

2. CO_2-frozen cells, besides being permeable to added organic acids, lose organic acids, amino acids, protein, carbohydrate, coenzymes and inorganic salts when washed with water or buffer.

3. The ability of such cells to respire ethanol and succinate may not be a reliable guide to the normal metabolism of these substrates, since the response to varying degrees of temperature-shock differs according to the strain of bakers' yeast used.

4. Freezing reduces the amount of sedimentable material in cell-free extracts prepared by ultra-rapid shaking. In this respect, the effects of freezing resemble those obtained by disintegrating fresh cells for longer periods. Partial solubilization of granular fumarase, aconitase, and ethanol and malic dehydrogenases occurs, and aconitase is partially inactivated.

5. The coenzyme-independent ethanol and malic dehydrogenase activities found in extracts of fresh yeast are lacking in extracts of frozen yeast. The possibility of coenzyme-binding, the disruptive action of thermal shock and the evidence for the participation of the tricarboxylic acid cycle in acetate oxidation by bakers' yeast, are discussed.

RÉSUMÉ

1. La congélation par CO_2 solide de cellules de levure s'accompagne de modifications dans leur structure et leur métabolisme.

2. Ces cellules, outre qu'elles sont perméables aux acides organiques ajoutés au milieu, perdent des acides organiques, des aminoacides, des protéines, des glucides, des coenzymes et des sels minéraux quand elles sont lavées avec de l'eau ou avec un tampon.

3. La capacité de ces cellules d'oxyder l'éthanol et le succinate ne constitue pas forcément un indice sûr du métabolisme normal de ces substrats, puisque la réponse à des degrés différents de choc de température varie selon la souche de levure de boulangerie utilisée.

4. La congélation diminue la quantité de matériel sédimentable dans les extraits acellulaires préparés par agitation ultra-rapide. A cet égard, les effets de la congélation ressemblent à ceux qu'on obtient en désintegrant des cellules fraîches pendant des temps plus longs. On observe une solubilisation partielle des fumarase, aconitase, éthanol et malique déshydrogénases granulaires et une inactivation partielle de l'aconitase.

5. Les activités éthanol et malique-déshydrogénasiques, indépendantes du coenzyme, que l'on trouve dans les extraits de levures fraîches, disparaissent dans les extraits de levures congelées. La possibilité d'une fixation du coenzyme, l'action disruptrice du choc thermique et la preuve d'une participation du cycle de l'acide tricarboxylique au cours de l'oxydation de l'acétate par la levure de boulangerie sont discutées.

References p. 512.

ZUSAMMENFASSUNG

1. Einige Veränderungen in der Struktur und im Stoffwechsel von Hefezellen werden beschrieben, die auftreten nach Gefrieren mit fester CO_2.

2. CO_2-gefrorene Zellen werden nicht nur für hinzugefügte organische Säuren permeabel, sondern verlieren beim Waschen mit Wasser oder Puffer organische Säuren, Aminosäuren, Eiweiss, Kohlenhydrate, Coenzyme und organische Salze.

3. Die Fähigkeit solcher Zellen, Alkohol und Succinat zu veratmen, mag kein zuverlässiger Führer zu dem normalen Stoffwechsel dieser Substrate sein, da die Antwort auf verschiedene Grade des Temperaturschocks verschieden ist je nach der Art der benutzten Bäckerhefe.

4. Das Gefrieren reduziert die Menge des sedimentierbaren Materials in zellfreien Extrakten, die durch "ultra-rapid" Schütteln hergestellt wurden. In dieser Hinsicht gleicht die Gefrierwirkung den Wirkungen, die durch langes Disintegrieren frischer Zellen erhalten werden. Eine teilweise Auflösung der granulären Fumarase, Aconitase und Alkohol- wie Äpfelsäure-Dehydrogenase tritt ein, und die Aconitase ist teilweise inaktiviert.

5. Die Aktivitäten der Coenzym unabhängigen Alkohol- und Äpfelsäure-Dehydrogenase, die in frischen Hefe-Extrakten gefunden werden, fehlen in Extrakten der gefrorenen Hefe. Die Möglichkeit einer Coenzymbindung, die spaltende Wirkung der thermischen Schocks und die Beweise für eine Teilnahme des Tricarbonsäurezyklus bei der Acetatoxydation durch die Bäckerhefe werden diskutiert.

REFERENCES

1 E. J. CONWAY AND M. DOWNEY, *Biochem. J.*, 47 (1950) 282.
2 F. LYNEN AND N. NECIULLAH, *Ann.*, 541 (1939) 203.
3 H. WIELAND, K. RAUSCH AND A. F. THOMPSON, *Ann.*, 521 (1936) 214.
4 S. BLACK, *Arch. Biochem.*, 34 (1951) 86.
5 H. A. KREBS, S. GURIN AND L. V. EGGLESTON, *Biochem. J.*, 51 (1952) 614.
6 P. M. NOSSAL, *Austral. J. Exp. Biol. Med. Sci.*, 31 (1953) 583.
7 E. RACKER, *Biochim. Biophys. Acta*, 4 (1950) 211.
8 P. M. NOSSAL, *Biochim. Biophys. Acta*, 11 (1953) 596.
9 S. P. COLOWICK, N. O. KAPLAN AND M. M. CIOTTI, *J. Biol. Chem.*, 191 (1951) 447.
10 A. J. HODGE, H. E. HUXLEY AND D. SPIRO, *J. Histol. and Cytol.*, 2 (1954) 54.
11 G. E. PALADE, *J. Exp. Med.*, 95 (1952) 285.
12 P. M. NOSSAL, *Biochem. J.*, 57 (1954) 62.
13 P. P. SLONIMSKI AND H. M. HIRSCH, *Compt. rend.*, 235 (1952) 741.
14 F. M. HUENNEKENS AND D. E. GREEN, *Arch. Biochem.*, 27 (1950) 478.
15 J. E. HAYES Jr. AND S. F. VELICK, *J. Biol. Chem.*, 207 (1954) 225.
16 S. F. VELICK, J. E. HAYES Jr. AND J. HARTING, *J. Biol. Chem.*, 203 (1953) 527
17 H. M. HIRSCH, *Biochim. Biophys. Acta*, 9 (1952) 674.
18 M. F. UTTER AND D. B. KEECH, unpublished observations.
19 S. R. DICKMAN AND J. F. SPEYER, *J. Biol. Chem.*, 206 (1954) 67.
20 P. M. NOSSAL, *Ph. D. Thesis*, University of Sheffield.
21 P. M. NOSSAL, *Biochim. Biophys. Acta*, 15 (1954) 594.
22 H. J. SAZ AND L. O. KRAMPITZ, *J. Bact.*, 67 (1954) 409.
23 H. E. SWIM AND L. O. KRAMPITZ, *J. Bact.*, 67 (1954) 419.
24 H. E. SWIM AND L. O. KRAMPITZ, *J. Bact.*, 67 (1954) 426.
25 F. LYNEN, personal communication.

Received October 28th, 1954