Changes in the Structure and Enzyme Activity of *Saccharomyces cerevisiae* in Response to Changes in the Environment

By E. S. POLAKIS,* W. BARTLEY* and G. A. MEEK*
Department of Biochemistry and Department of Human Anatomy, University of Oxford, South Parks Road, Oxford

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The first report, as a result of work with the electron microscope, of cytoplasmic inclusions in cells of *Saccharomyces cerevisiae* having the characteristic ultrastructural features of plant and animal mitochondria (Palade, 1953) was published by Agar & Douglas (1957), although the characteristic cristae are barely discernible in their published micrographs. Later electron micrographs of cells fixed in potassium permanganate published by Vitols, North & Linnane (1961) show characteristic mitochondria about 0·5 μ in length in aerobically grown *S. cerevisiae* cells. Linnane, Vitols & Nowland (1962) have shown that, in the yeast *Torulopsis utilis*, anaerobically grown cells do not contain characteristic mitochondria or cytochromes; the cytoplasm of these cells, however, contains a reticular system of membranes, vacuoles containing electron-dense granules, and concentric membrane systems resembling myelin forms. The aerobically grown cells, however, show characteristic mitochondria, no reticular membrane system, and vacuoles with far fewer and less-electron-dense granules. Heyman-Blanchet, Zajdela & Chaix (1959) claim to have isolated mitochondria-like structures from anaerobically grown yeast cells, but we have failed so far to detect any mitochondria in anaerobically grown *S. cerevisiae* cells by electron microscopy.

Slonimski (1953, 1955) and Tustanoff & Bartley (1962) have shown that *S. cerevisiae* cells, when grown anaerobically with glucose as substrate, lose their capacity for oxidation. When this anaerobic yeast is incubated in the presence of oxygen and a low concentration of glucose, respiration develops. Under the conditions of Slonimski (1953) respiration develops gradually over several hours; under the conditions of Tustanoff & Bartley (1962) respiration becomes appreciable and is maximal at about 2½ hr.

In our experience such anaerobically grown yeast does not develop the ability to oxidize acetate, but yeast grown aerobically on low glucose concentrations eventually becomes able to oxidize acetate. The present paper shows that the growth conditions of the yeast determine what substrates can be oxidized, and that there is a correlation between the enzyme content of the yeast, the magnitude of respiration and the type of intracellular organelle developed within the cell.

METHODS

Special chemicals. Chemicals were obtained from the following manufacturers: casein hydrolysate (acid) L 41 from Oxo Ltd., London; yeast extract from Difco Laboratories, Detroit, Mich., U.S.A.; wheat-germ oil from Vitamins Ltd., London; Tween 80 and ergosterol from L. Light and Co. Ltd., Colnbrook, Bucks.; cytochrome *c* (type III) from Sigma Chemical Co., St Louis, Mo., U.S.A.; bovine plasma albumin from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex; sodium succinate, NAD, NADP, $NADH_2$ and alcohol dehydrogenase from C.F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride from British Drug Houses Ltd., Poole, Dorset. The monopotassium isocitrate from *Bryophyllum crenatum* was a gift from Professor H. B. Vickery.

Maintenance and growth of yeast. Saccharomyces cerevisiae strain no. 77 of the National Collection of Yeast Cultures (Brewing Industry Research Foundation, Nutfield, Surrey) was used in the present work. The organism was maintained aerobically on agar slopes containing inorganic salts, 2·25% (w/v) of Difco malt extract, 0·05% (w/v) of Difco yeast extract and 0·5% (w/v) of sucrose. The yeast was grown for 48 hr. at 30° and subcultured monthly.

Growth of anaerobic cells. The medium for the anaerobic bulk growth of the yeast contained (per litre): 20 g. of glucose, 10 g. of Difco yeast extract, 5 g. of Oxoid casein hydrolysate (acid), 9 g. of KH_2PO_4, 0·3 g. of $CaCl_2$, 0·5 g. of $MgSO_4,7H_2O$, 6 g. of $(NH_4)_2SO_4$, 5 ml. of sodium lactate (70–72%, w/w), 0·15 ml. of wheat-germ oil, 5 ml. of Tween 80 and 20 mg. of ergosterol dissolved in 5 ml. of ethanol. The inoculum of yeast was 0·93 mg. dry wt. of cells/g. of glucose in the medium. Growth was in a wide-based conical stoppered flask (Fernbach flask), with a gas inlet and outlet, which was continuously flushed with a slow stream of oxygen-free nitrogen and shaken at 30° for 10 hr. The yeast was harvested just before the onset of the stationary phase, since Tustanoff & Bartley (1962) showed that cells at this stage adapted most readily under these conditions (cf. Slonimski, 1953). The harvested cells were washed three times with ice-cold water and finally suspended in water at a concentration of 100 mg. wet wt./ml.

Growth of aerobic cells. The conditions of growth were as given for anaerobic growth except for the omission from

* Present address: University of Sheffield, Sheffield 10.

the medium of sodium lactate, Tween 80, wheat-germ oil and the ethanolic solution of ergosterol. A stream of oxygen was blown through the culture and samples were taken periodically to test the ability to oxidize glucose, ethanol or acetate, and for electron microscopy.

Development of respiration with various substrates. Yeast (4 mg. dry wt.) was incubated in Warburg manometers with air as the gas phase at 25° or 35°. The medium contained KH_2PO_4 (66 mM) and the substrate was glucose (33 mM), ethanol (100 mM) or acetate (100 mM). Casein hydrolysate (6 mg.) was added to each vessel when the cells had been grown anaerobically. The final volume was 3·0 ml. and the centre well contained 0·2 ml. of 10N-KOH and filter paper. The yeast was added to the side bulb and tipped into the reaction mixture after temperature equilibration, and oxygen consumption was measured. The dry weight of the yeast was measured in a twice-washed suspension by drying overnight at 105°. Samples for electron microscopy were taken at suitable times.

Preparation of cells for electron microscopy. The cells were harvested by centrifuging and prepared for electron microscopy essentially as described by Thyagarajan, Conti & Naylor (1961). After being stained with permanganate and fixed in 2% (w/v) osmium tetroxide the cells were dehydrated in an alcohol series, treated with epoxypropane and embedded in British Ciba Araldite. The blocks were polymerized at 60° for 2 days, and were then sectioned at 50–80 mμ on a Porter–Blum or LKB ultramicrotome. Sections were picked up on bare 200-mesh grids, stained by floating them on Millonig's (1961) lead solution and examined in a modified Siemens Elmiskop I electron microscope (Meek, 1958).

Cell disruption. Yeast cells (20%, w/v) in water were disrupted in a French pressure cell at 7000–8000 lb./in.^{2} with a Wabash hydraulic press. The suspension of disrupted cells (10 ml.) was layered on 25% (w/v) sucrose solution (20 ml.) and centrifuged for 10 min. at 500*g* to remove cell walls. The upper layer was removed and used for measurement of enzyme activities. All manipulations were carried out at 0°.

Analytical methods. Glucose was determined by the glucose-oxidase method of Huggett & Nixon (1957), ethanol by the method of Bonnichsen & Theorell (1951) and acetate by a method essentially as described by Serlin & Cotzias (1955). Protein was determined by a modification of the biuret method made by Professor Utter.

Measurement of enzyme activities. Succinate–tetrazolium-oxidoreductase activity was measured in the disrupted-cell preparation before centrifuging. All other enzyme activities were determined in the upper layer remaining after centrifuging. Cytochrome *c*-oxidase activity was measured at 25° with a modification of the method of Minnaert (1961). Cytochrome *c* was reduced by an equivalent amount of ascorbic acid. The reaction mixture contained 2·4 ml. of 0·1M-potassium phosphate buffer, pH 6·9, containing EDTA (1 mM), 0·1 ml. of reduced cytochrome *c* (6 mg./ml.), 0·15 ml. of bovine plasma albumin (10%, w/v), the cell suspension and water to 3 ml. The decrease in extinction at 550 mμ was measured every 15 sec. for 3 min. The extinction of the completely oxidized cytochrome *c* was measured after the addition of $K_3Fe(CN)_6$ solution. $NADH_2$–cytochrome *c*-oxidoreductase and $NADH_2$-oxidase activities were measured at 25° by the methods of Green & Ziegler (1963). Succinate–tetrazolium-oxidoreductase activity was measured by the method of Pennington (1961). The incubation was for 20 min. at 35° and sucrose was omitted from the incubation medium. Isocitrate–tetrazolium-oxidoreductase activity was measured by a similar method. The incubation mixture contained 0·1 ml. of 0·5M-potassium phosphate buffer, pH 7·4, containing semicarbazide hydrochloride (0·2M), 0·1 ml. of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (1%), 0·1 ml. of 0·05M-isocitrate, 7 μl. of 0·05M-NAD, 7 μl. of 0·025M-NADP, the cell suspension and water to 3 ml. The incubation was for 20 min. at 35° and the reaction was stopped by the addition of 1 ml. of 2N-$HClO_4$. The formazan was extracted into 4 ml. of chloroform and the extinction was measured at 497 mμ.

RESULTS

Growth of yeast on different glucose concentrations. After 12 hr. glucose was absent from the medium (see Table 2) and growth was taking place on ethanol and acetate. Between 24 and 36 hr. of growth only the yeast grown on 1·8% (w/v) glucose increased in weight. Thus the yeast grown on 5·4% (w/v) glucose virtually ceased to grow after 12 hr. (Table 1) in spite of one third of the carbon for growth still

Table 1. *Respiration of yeast grown on different glucose concentrations*

Tests of respiration on the substrate given were made in Warburg vessels as described in the Methods section. Endogenous respiration was subtracted. The numbers of generations were calculated on the basis of the increases in dry weight.

		$Q_{O_2}^{air}$											
Time of growth (hr.)		12				24				36			
Initial concn. of glucose medium (%)		0·3	0·9	1·8	5·4	0·3	0·9	1·8	5·4	0·3	0·9	1·8	5·4
Yield of cells (g. wet wt. of yeast/100 ml. of medium)		0·29	0·80	1·42	3·84	0·77	2·30	2·57	4·15	0·69	2·36	3·42	3·88
No. of generations		7·7	7·6	7·6	7·4	9·4	9·0	8·5	7·4	9·1	9·5	9·1	7·2
Substrate	Final concn. (mM)												
Glucose	33	52	41	23	12	171	168	112	18	83	94	89	7
Ethanol	100	83	63	25	17	208	201	91	25	52	117	104	6
Acetate	100	0	0	0	0	132	150	50	10	4	0	1	5

being available. This was largely consumed by 24 hr. (Table 2) without further growth. On the other hand the cells grown with 1·8 % of glucose present in the medium initially were able to utilize ethanol for further growth. After 24 hr. of growth the concentration of substrate was low in all the media and probably limited growth. It is possible that nitrogen lack was preventing the growth of the yeast initially in 5·4 % glucose after 12 hr. The cultures at the same times were of different physiological ages and had passed through different numbers of cell divisions.

Changes in the structure of yeast cells on developing respiration. Plate 1 shows the structure of the anaerobically grown yeast cells and Plate 2 the structure of these cells when respiration had developed after 2·5 hr. of aerobic incubation at 25° in the glucose adaptation medium. Initially the $Q_{O_2}^{air}$ was zero. The $Q_{O_2}^{air}$ reached a maximum of 40 at 2¼ hr., just before sampling for electron microscopy. The most obvious change in the cell structure on the development of respiration was the appearance of vacuolar-like areas containing sparse granular material. Only 2 % of the anaerobic cells had the vacuolar structure initially but about 50–70 % of the respiring cells had these structures. The anaerobic yeast cells contained few membranous structures. By contrast many extranuclear membranous structures can be seen in the respiring yeast; none of these, however, appear to be mitochondria. The vacuolar area contained one or more densely staining bodies having the same appearance as the dense granules in mitochondria described by Novikoff (1961).

The relationship between the development of the changes in cell structure and the development of respiration was investigated at 35°. After successive intervals (0, 15, 30, 75 and 90 min.) samples of cells were fixed and electron micrographs were made. Respiration first became measurable manometrically at 20 min. but did not increase appreciably until 60 min. After 90 min. of incubation the $Q_{O_2}^{air}$ was 76. The yeast initially had slightly more vacuolar structures (about 8 %) than in those incubated at 25°. At the time of the first sample (15 min.), about 30 % of the cells had developed the characteristic vacuolar structure although at this time no respiration was apparent. E. R. Tustanoff & W. Bartley (unpublished work) showed that at this time cytochrome c oxidase was already being synthesized by these cells. The percentage of cells having the vacuolar structure at successive times were: 30 min., 50 %; 60 min., 54 %; 75 min., 43 %; 90 min., 56 %. From the thickness of the sections and the geometry of the system it may be assumed that if 50 % of the cells bear vacuolar structures as seen in the electron microscope then almost all the cells must contain one vacuole.

Table 2. *Enzyme activities of aerobically grown yeast cells*

Cytochrome c-oxidase, $NADH_2$–cytochrome c-reductase and $NADH_2$-oxidase activities are given as $10^2 \times \mu$moles utilized/min./mg. of protein. Succinate-dehydrogenase activity is given as the absorption of the formazan in 4 ml. of ethyl acetate at 490 mμ/min./mg. of protein. Isocitrate-dehydrogenase activity is given as the absorption of the formazan in 4 ml. of chloroform at 497 mμ/min./mg. of protein.

Time of growth (hr.)	12				24				36			
Initial concn. of glucose in medium (%)	0·3	0·9	1·8	5·4	0·3	0·9	1·8	5·4	0·3	0·9	1·8	5·4
Enzyme activity												
Cytochrome c oxidase	6·5	6·2	5·0	2·4	9·7	8·9	4·6	0·7	4·3	3·9	9·6	0·3
$NADH_2$–cytochrome c reductase	4·6	4·1	3·3	2·2	35·8	60·5	11·4	2·8	10·2	11·2	16·8	1·7
$NADH_2$ oxidase	2·1	2·3	1·8	1·2	13·4	19·2	4·9	1·3	3·3	3·2	7·9	1·1
Succinate dehydrogenase	< 0·010	< 0·010	< 0·010	< 0·010	0·050	0·090	0·031	0·018	< 0·010	0·028	0·026	0
Isocitrate dehydrogenase	0·014	0·016	< 0·010	< 0·010	0·051	0·068	0·067	0·049	0·011	0·021	< 0·010	0
Substrate in the growth medium (μmoles/ml.)												
Glucose	0	0	0	0	0	0	0	0	0	0	0	0
Ethanol	17·3	82·4	153·7	337·0	0	2·8	0	25·8	0	0	0	24·7
Acetate	3·0	4·5	6·2	22·1	2·3	2·0	4·3	15·2	0	0	0	12·5

Changes in yeast structure when ability to oxidize acetate is developed. Yeast grown aerobically for 12 hr. in media containing 0·3, 0·9, 1·8 and 5·4 % of glucose was unable to oxidize acetate although it was able to oxidize glucose and ethanol. At this stage the yeast had no mitochondria but had many vacuolar structures similar to those developed in anaerobically grown cells on exposure to oxygen (see Plate 2). Plate 3 shows the yeast after 24 hr. of growth when it had developed the ability to oxidize acetate and had maximum rates of respiration with glucose and ethanol (Table 1). The activities of respiratory and tricarboxylic acid-cycle enzymes were high at this time (Table 2). A large number of mitochondria can be seen but the vacuolar structures have almost disappeared. The yeast cells lost the ability to oxidize acetate after 36 hr. of growth and, although mitochondria were still present, the average number seen in section fell from 7 to 4.

Changes with age in respiration and enzymic activities of aerobically grown yeast: (*a*) Respiratory changes. Table 1 gives the $Q_{O_2}^{air}$ values with glucose, ethanol and acetate as substrates of yeast cells grown aerobically on four different glucose concentrations for various periods. After 12 hr. of growth glucose had disappeared from the growth medium but ethanol and acetate were still present. All the yeast samples were able to respire with glucose or ethanol as substrates but were unable to oxidize acetate. The $Q_{O_2}^{air}$ values were low and were inversely proportional to the concentrations of glucose originally present in the growth media. After 24 hr. of growth ethanol had almost disappeared from all but the medium originally having 5·4 % of glucose (Table 2), but acetate was present in all the media. The cells grown on 5·4 % glucose showed little growth in the period 12–24 hr. and the $Q_{O_2}^{air}$ values increased only slightly. By contrast the $Q_{O_2}^{air}$ values with glucose or ethanol as substrate increased four- to five-fold at 24 hr. compared with the values at 12 hr. and the cells became able to oxidize acetate.

After 36 hr. of growth ethanol and acetate were consumed in all but the medium originally containing 5·4 % glucose. There was a decline in all the $Q_{O_2}^{air}$ values (except for the $Q_{O_2}^{air}$ value with ethanol of cells grown on 1·8 % glucose) and respiration when tested with acetate as substrate ceased.

(*b*) Enzyme changes. Table 2 gives the activities of cytochrome *c* oxidase, $NADH_2$–cytochrome *c*-oxidoreductase, $NADH_2$ oxidase, succinate–tetrazolium oxidoreductase and isocitrate–tetrazolium oxidoreductase in the aerobically grown cells.

After 12 hr., when all glucose had been consumed, at the end of the period of growth on glucose the respiratory enzymes had low activities in spite of the vigorous oxygenation of the cultures during growth. The initial glucose concentrations present in the growth media affected the activities of the enzymes. Thus cells grown on 5·4 % glucose had about one-half the activity of cells grown on 0·3 or 0·9 % glucose. The activities of succinate–tetrazolium oxidoreductase and isocitrate–tetrazolium oxidoreductase were low, especially in the cells grown on the two higher glucose concentrations.

After 24 hr. of growth (12 hr. after the consumption of glucose) when acetate could be oxidized and mitochondria had appeared the enzyme activities reached high values. Cytochrome *c*-oxidase activity increased by about 50 % in the two media which originally had the lowest glucose concentration. In the other two media the cytochrome *c*-oxidase activity fell. With all the other enzyme activities the cells grown in the three lower glucose concentrations showed increases of two- to eight-fold in the activities of their oxidative enzymes. The cells grown in 5·4 % glucose showed only small increases in enzyme activity, the greatest being a fivefold increase of isocitrate–tetrazolium-oxidoreductase activity. Activities were higher with lower glucose concentrations.

After 36 hr. of growth all the activities (with the exception of those of cells grown originally in 1·8 % glucose) declined markedly, the greatest decline being with the cells grown on the highest glucose concentration.

DISCUSSION

S. cerevisiae is an organism able to utilize many substrates for its growth. Thus it requires great plasticity of its enzyme make-up. From the experiments described above and those of E. R. Tustanoff & W. Bartley (unpublished work) and Strittmatter (1957) it appears as if it is the nature of the carbon source utilized rather than the presence of oxygen that determines the type of metabolism. The work described above shows that complex structures such as mitochondria are produced only when the substrate presented makes this necessary. Ephrussi, Slonimski, Yotsuyanagi & Tavlitzki (1956) have described changes in the structure of the yeast cell during the growth cycle, and Yotsuyanagi (1962*a*, *b*) has described changes of the mitochondrial structure during the aerobic growth of yeast and comparative studies of mitochondria of normal and of respiration-deficient mutants of yeast. Linnane *et al.* (1962) have described premitochondrial structures in anaerobically grown yeast. Vitols *et al.* (1961) have presented electron-microscope pictures of mitochondria in aerobically grown yeast. Although we find little in the way of structures in our anaerobically grown yeast (cf. Heyman-Blanchet *et al.* 1959) we have shown the sequence of the development of structures which culminated in the

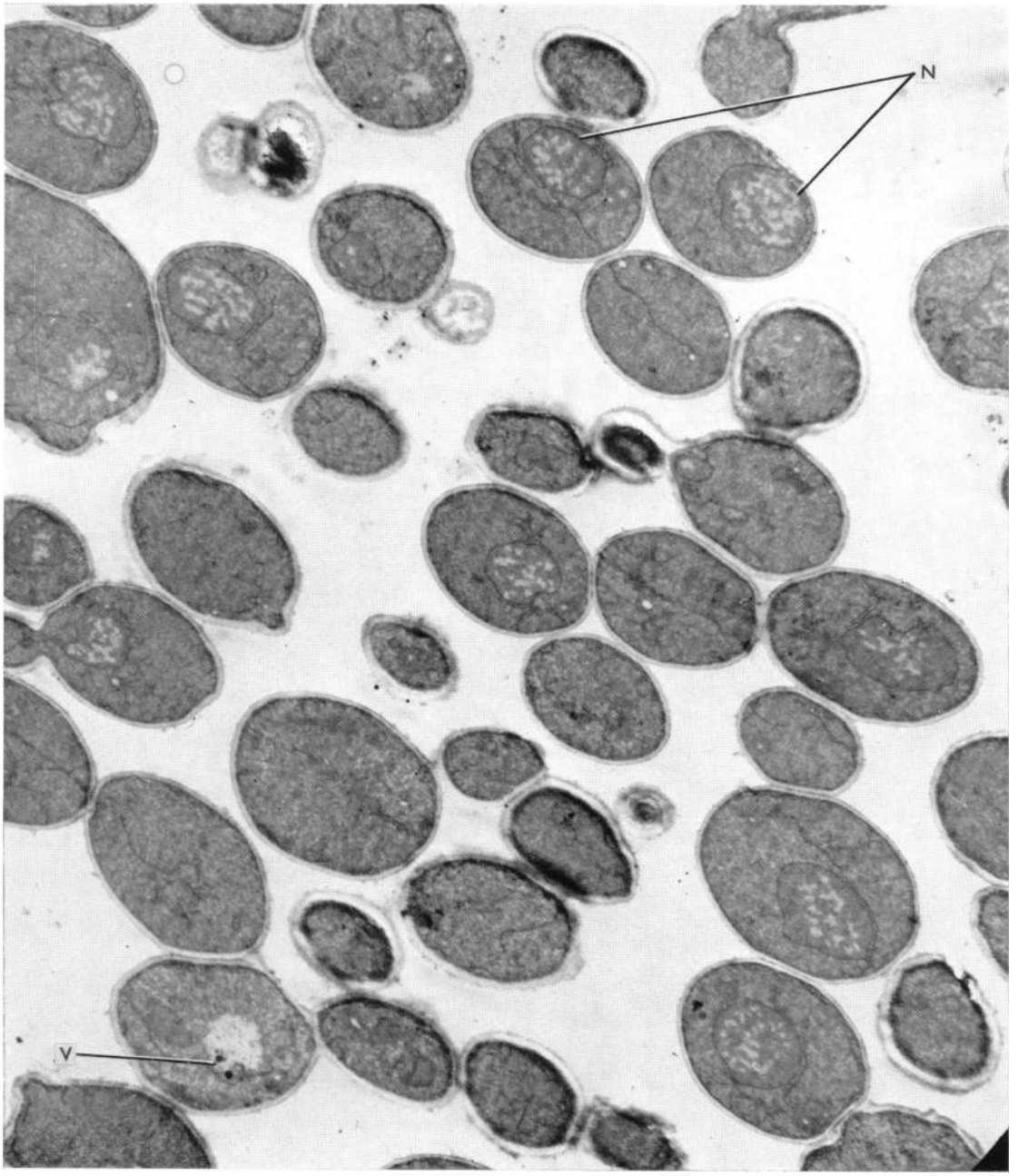

Plate 1. Electron-microscope picture of sections through yeast cells grown anaerobically on 5·4% glucose. Only 2% of the yeast cells have the vacuolar structures shown in Plate 2. N, Nucleus; V, vacuolar structure. Magnification: ×8300.

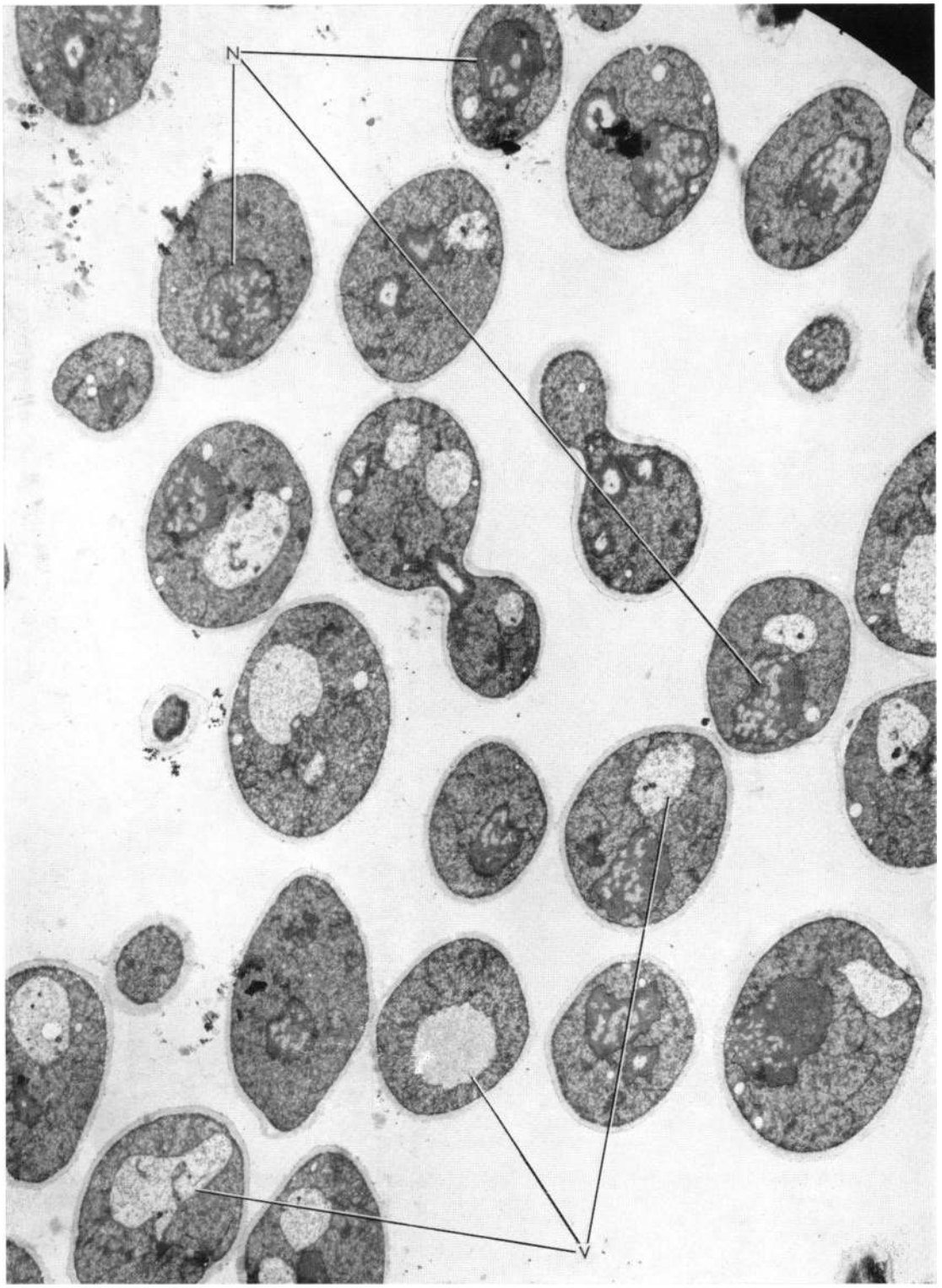

Plate 2. Electron-microscope picture of sections through yeast cells grown anaerobically in 5·4% glucose and adapted to respire on glucose. 70% of the cells have vacuolar structures. N, Nucleus; V, vacuolar structure. Magnification: ×8300.

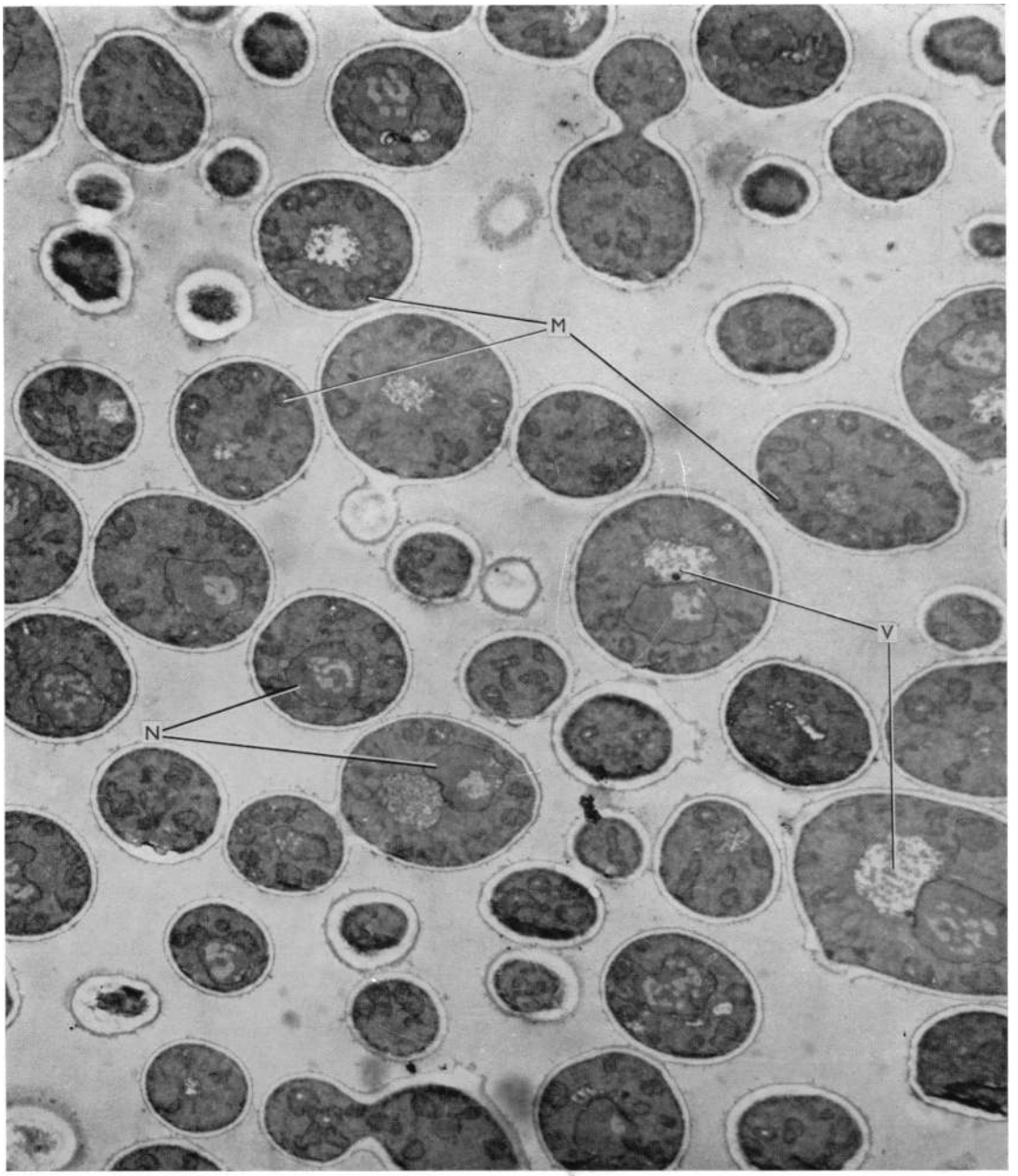

Plate 3. Aerobically grown yeast after 24 hr. of growth when oxidation of acetate appears. Initially the appearance was as in Plate 2. Mitochondria are present in every cell. N, Nucleus; V, vacuolar structures; M, mitochondria. Magnification: × 8300.

formation of mitochondria as the metabolism of the yeast changed with the change of the substrate presented to it. Correlated with the changes in structures are the changes in the enzyme make-up of the yeast and the ability to oxidize different substrates. Thus no respiration occurs unless the yeast has the vacuolar structures shown in Plate 2. The results are consistent with the vacuolar structures being the physical expression of $NADH_2$ oxidase. Later, when acetate becomes the major substrate requiring tricarboxylic acid-cycle enzymes for its oxidation, the vacuolar structures are replaced by mitochondria and the enzymes found in the yeast change in accordance with what would be expected from this structural change.

Effect of glucose on enzyme synthesis. Glucose is known to be a repressor of the synthesis of oxidative enzymes in many micro-organisms (see, for example, Magasanik, 1961). It may be that the enzymes associated with lipoprotein structures were particularly susceptible to the repressive action of glucose. The analysis of the enzyme activities presented above is in accordance with this idea. Thus the structurally bound enzymes such as succinate-tetrazolium oxidoreductase are influenced by the glucose concentration much more than is the soluble isocitrate–tetrazolium oxidoreductase. Owing to this repressive effect of glucose yeast cells are unable to respire on acetate as long as glucose is present in the growth medium. Our results on the oxidation of acetate by yeast cells at different stages of growth are in agreement with those of Eaton & Klein (1954), and our 12 hr. growing cells can be said to correspond to the 'young' cells described by these workers. One puzzling feature is the effect that high concentrations of glucose have in conditioning the future behaviour of the yeast. After growing on 5·4% glucose the yeast never gains the ability to oxidize acetate in spite of glucose being absent from the medium for several hours. It is possible that during the growth on high glucose concentration carbohydrate stores are laid down which later release glucose after this has vanished from the medium. In fact Chester (1963) has shown that anaerobically grown yeast has a higher carbohydrate content which is utilized after all the glucose in the medium has been consumed and that the loss of reserve carbohydrate is stimulated under aerobic conditions. As yeast cells grown aerobically on 5·4% glucose behave similarly to anaerobic cells this explanation seems possible. An alternative possibility is that the growth medium may have been depleted of an essential constituent during the yeast growth. Since the total weight of yeast produced on 5·4% glucose is higher than in the other media some factor could be limiting in this case but not with the lower concentrations of glucose. The fact that yeast cells grown for 12 hr. on 5·4% glucose grow only slightly in the subsequent 12 hr. in spite of the presence of ethanol and acetate in the grown medium is agreement with this explanation.

SUMMARY

1. Yeast cells were grown anaerobically and aerobically on glucose at different concentrations and their respiration and enzyme contents were measured. The structure of the cells was examined in the electron microscope.

2. Yeast cells converted glucose aerobically into cell mass more efficiently at lower glucose concentrations.

3. Respiration and cytochrome *c*-oxidase, $NADH_2$-oxidase, $NADH_2$–cytochrome *c*-oxidoreductase, succinate-dehydrogenase and isocitrate-dehydrogenase activities varied inversely with the concentration of glucose in which the yeast grew for the first 12 hr.

4. After 12 hr. of growth the yeast could oxidize glucose and ethanol but not acetate. After 24 hr. of growth acetate was oxidized by the cells at rates that varied inversely with the glucose concentration originally present in the growth medium. After 36 hr. of growth the ability to oxidize acetate was lost but respiration with glucose or ethanol remained.

5. No mitochondrial structures were seen in yeast grown anaerobically. In yeast grown aerobically on glucose or in anaerobically grown yeast that had developed respiration vacuolar structures appeared as respiration developed. Mitochondria appeared in yeast at a time when acetate could be oxidized. Mitochondrial numbers diminished as ability to oxidize acetate was lost.

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Quantitative Analysis of Phospholipids by Thin-Layer Chromatography

BY V. P. SKIPSKI, R. F. PETERSON AND MARION BARCLAY
Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, Rye, N.Y., and the Sloan-Kettering Division, Graduate School of Medical Sciences, Cornell University Medical College, New York 21, *N.Y., U.S.A.*

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The quantitative analysis of phospholipid mixtures extracted from different tissues still poses a problem. Several techniques are available (McKibbin, 1957; Lea, Rhodes & Stoll, 1955; Hanahan, Dittmer & Warashina, 1957; Marinetti, Erbland & Kochen, 1957; Schmidt, Fingerman & Thannhauser, 1962; Dawson, 1960; Collins, 1959; Rauser, Bauman, Kritchevsky, Heller & O'Brien, 1961) which have been used by investigators according to their preferences or the character of the problem. Each method has some inherent disadvantages. Several investigators have made use of thin-layer chromatography for quantitative analysis of phospholipids (Wagner, 1961; Jatzkewitz, 1961; Habermann, Bandtlow & Krusche, 1961; Honegger, 1962; Doizaki & Zieve, 1963; Robinson & Phillips, 1963). Recent progress in the qualitative separation of phospholipids by thin-layer chromatography (Skidmore & Entenman, 1962; Skipski, Peterson & Barclay, 1962; Skipski, Peterson, Sanders & Barclay, 1963) has facilitated adaptation of this technique to an improved, efficient and quantitative procedure. The present paper describes the application of thin-layer chromatography to the quantitative analysis of phospholipids in animal tissues.

MATERIALS AND METHODS

Standards and tissue phospholipids. The origins of most of the reference compounds have been described by Skipski *et al.* (1962); additional phospholipids used were phosphatidic acids (generously given by Dr E. Baer, University of Toronto, and by Dr L. E. Hokin, University of Wisconsin), sodium salt of monophosphatidylinositol isolated from wheat sprouts (generously given by Dr M. Faure, Pasteur Institute, Paris) and lysophosphatidylcholine (General Biochemicals, Chagrin Falls, Ohio, U.S.A.).

Lipids were extracted from the pooled livers of five male adult albino rats with methanol–chloroform (1:2, v/v) and purified by the method of Folch, Ascoli, Lees, Meath & LeBaron (1951).

Preparation of plates. It is advisable to use a thicker layer of silica gel on plates for quantitative analysis of phospholipids than is generally used for qualitative analysis (0·5 mm. on adjustable Desaga applicator). Forty g. of Camag (Muttenz, Switzerland) silica gel, without calcium sulphate binder, was slurried with 90 ml. of 1 mM-Na_2CO_3 solution and transferred to the applicator. The plates (200 mm. × 200 mm.) were prepared in the usual manner (Stahl, 1958, 1962). This amount of slurry was sufficient to prepare three or four plates. The plates were allowed to dry at room temperature for 1–2 hr. and could then be stored for several days. Before the experiment, the plates were activated at 110° for 1 hr.

Application of samples. Samples (50 μl. or less) were applied to the thin-layer chromatography plate with standardized micropipettes. Where the concentration of the applied material was high (approx. 30–40 mg./ml.) a correction factor was used for the lipid phosphorus remaining on the tip of the pipette after delivery. It was necessary to apply to a plate such a quantity of total lipid that each phospholipid species separated would contain at least 0·4–0·5 μg. of phosphorus. Reference compounds, ranging in amounts from 15 to 50 μg., were applied together with tissue lipid extracts. For recovery experiments, approx. 100 μg. (3–4 μg. of phosphorus) was applied.