STIMULATION BY PHENETHYL ALCOHOL OF AEROBIC FERMENTATION IN MUCOR ROUXII

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In the absence of oxygen, some Mucor species exhibit a yeast-like morphology and reproduce by budding. Bartnicki-Garcia and Nickerson (1962 a,b) showed that the yeast-like phase of Mucor rouxii took place only when hexoses were fermented and that it could be converted to the hyphal phase after a few hours of exposure to air. Haidle and Storck (1966 a,b) found that this conversion which required the synthesis of RNA and proteins was accompanied by the appearance of cytochrome oxidase activity.

Recently Terenzi and Storck (1967) reported that a yeast-like phase was obtained in aerobiosis provided 0.2% (v/v) of phenethyl alcohol (PEA) and a hexose (2-5%) were included in the growth medium. In the present paper it is shown that aerobic yeast-like growth involves an increase of alcoholic fermentation and a concomitant decrease of respiration. The data further suggest that PEA acts like an uncoupling agent and stimulates a Crabtree effect.

Materials and Methods: M. rouxii (NRRL 1894) was kindly supplied by Dr. C. W. Hesseltine. The synthetic medium (BG) of Bartnicki-Garcia and Nickerson (1962a) was used in all the experiments described. Ethanol production was determined with the spectrophotometric procedure of Bonnichsen and Lundgren (1957). Manometric experiments were performed at 27° with a Warburg respirometer according to Umbreit et al. (1957). Cytochrome oxidase activity was measured under condi-

tions described by Haidle and Storck (1966b). PEA was obtained from Eastman Kodak Co., Rochester, N. Y., and from K & K Laboratories, Plainview, N. Y. All the other chemicals used including sugars were of reagent grade.

Results: The morphological similarity between the anaerobically and PEA induced yeast phases suggested that they might have the same metabolic basis. A comparison was therefore made of the cytochrome oxidase activities of extracts from anaerobic and aerobic cultures done with and without PEA.

Table 1. Inhibitory action of PEA on the expression of cytochrome oxidase activity.

Treatment[1]		Morphology	Cytochrome Oxidase[2]
(a) Anaerobiosis:	18 hrs	Y	0
(b) Anaerobiosis: by Aerobiosis:	12 hrs followed 6 hrs	75%F - 25%Y	0.035
(c) Aerobiosis:	18 hrs	F	0.071
(d) Aerobiosis:	20 hrs; 0.20% PEA	Y	0.030
(e) Aerobiosis:	20 hrs; 0.10% PEA	F	0.044

[1]Spores were incubated in BG medium supplemented with 1% glucose in anaerobiosis and in aerobiosis with or without PEA. In treatment (b), yeast-like cells were exposed to air for a duration of 6 hrs following a 12-hr period of anaerobiosis (Y = yeast, F = filament).

[2]Specific activity expressed in rate constant per mg protein of crude cell extract (see Materials and Methods).

As shown in Table 1, anaerobic yeast-like cells had no activity, but after exposure to air for a period of 6 hrs the activity was equal to that of cells incubated with PEA and air. (Addition of PEA to cell extracts had no effect.)

Since a high glucose concentration is a prerequisite for yeast-like phase, the respiration of cells grown in the presence of 0.5 and 5% glucose was measured. As shown in Table 2, the specific activity of both cytochrome oxidase and of O_2 uptake was slightly lower after growth with 5%, but CO_2 production was substantial. Assuming a respiratory quotient (Q_{CO_2}/Q_{O_2}) of 1.0, one finds that 3.4 μmoles of glucose were fermented per μmole of glucose respired, indicating according to De Deken (1966) a Crabtree effect.

Table 2. Effect of glucose concentration on the respiratory activity of PEA treated cells.

	Specific Activities	
Glucose	0.5% (F)	5% (Y)
Cytochrome Oxidase (rate constant/mg protein)	0.0125	0.0113
$\mu l\ O_2$/hr/mg DW	37.5	35.0
$\mu l\ CO_2$/hr/mg DW	0	39.8
$\frac{\mu l\ CO_2}{\mu l\ O_2}$	0	1.14
$\frac{\mu moles\ glucose\ (CO_2)}{\mu moles\ glucose\ (O_2)}$	0	3.41

Cells were grown in the presence of 0.23% PEA either with 0.5% or 5% glucose. PEA was added 7 hrs after the beginning of the incubation. After 48 hrs cells were transferred directly into manometric vessels and assayed for a period of one hour. With 0.5% glucose the growth was exclusively filamentous.

As shown in Table 3, ethanol accumulated in all cultures, but in greater amount per mg dry weight after growth with PEA and 5% glucose. A value of 44.9, close to 39.8 (Table 2), was obtained after conversion of 96.3 μmoles ethanol/mg dry weight/48 hrs into $\mu l\ CO_2$/hr/mg dry weight. Additional experiments revealed that ethanol could not be detected after 48 hrs of growth in the presence or absence of PEA but without sugar. Ethanol and CO_2 productions appeared therefore

Table 3. Effect of glucose concentration and PEA on ethanol production and growth yield.

Glucose	Morphology	PEA[2] (0.23%)	Ethanol[1] μmoles	Dry Weight[1] mg	μmoles Ethanol/mg Dry Weight
1.0	Filaments	(-)	2,786	346.1	8.0
1.0	Filaments	(+)	320	80.0	4.0
5.0	Filaments	(-)	6,368	359.0	17.7
5.0	Yeast	(+)	2,630	27.3	96.3

[1]Ethanol and dry weight were determined after 48 hrs incubation.

[2](+) PEA added 7 hrs after incubation was started
(-) no PEA added control.

to be greater under conditions required for the exhibition of yeast-like morphology. In order to study metabolic responses to PEA, growth was allowed to proceed in its absence. The 16 to 17 hr cultures were transferred to Warburg flasks either directly or after centrifugation and suspension in fresh medium. In the first experiment cells grown with 5% glucose were washed and suspended in medium with 0.5% glucose and PEA was added from the sidearm. After 6 hrs, 1,255 and 1,197 µl of CO_2 and 56.55 and 58.50 µM of ethanol had been produced in duplicate flasks. The last two values correspond theoretically and respectively to 1,266 and 1,310 µl of CO_2 which are similar to those found manometrically. The combined effects of PEA and of glucose concentration are shown in Table 4. Prior to PEA addition the rate of O_2 uptake was independent of the glucose concentration.

Table 4. Influence of glucose concentration and PEA on the rate of fermentation and respiration.*

	Glucose Concentration (gr/100 ml)							
	0.05%		0.10%		0.20%		5.0%	
Time (min)	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2
30	0	90	0	87	0	75	5	69
PEA to 0.22% added								
30	0	80	0	75	0	66	29	66
60	0	174	1	155	10	136	98	118
90	0	292	2	245	43	218	208	183
120	0	423	49	350	89	312	355	255
150	0	576	78	475	145	426	530	353
180	0	723	110	611	218	552	729	446
210	0	828	136	763	299	700	952	552
240	0	903	142	871	355	807	1120	636
270	0	1020	142	1045	439	985	1383	750
360	-	-	142	1378	578	1535	-	-

*Cells were grown for 17 hrs in BG medium supplemented with 5% glucose, washed and resuspended in BG medium with various glucose concentrations.

With 0.05% glucose or 8.25 μmoles/flask, no CO_2 production was observed. With 0.10% and 0.20% glucose, while O_2 uptake continued, CO_2 evolution stopped respectively after 240 min. and 360 min. suggesting that at these times the amounts of glucose remaining in the Warburg flasks should be equal to or lower than 8.25 μmoles. Assuming ratios of 6 μmoles O_2/μmole glucose and 2 μmoles CO_2/μmole glucose respectively for respiration and fermentation, it was calculated that the amount of remaining glucose was equal to 6.3 μmoles in the 0.10% system (after 240 min.) and to 8.2 μmoles in the 0.20% system (after 360 min.). In the 5% system, the ratio μmoles glucose fermented over μmoles glucose respired (using the same assumptions as above) was equal to 1.27 and 5.5 respectively 30 min. and 270 min. after PEA addition. Experiments to be reported elsewhere and performed with glucose uniformly labeled with radioactive carbon have shown that all the CO_2 evolved originated from glucose. Additional experiments have further revealed: 1) In the absence of glucose but in the presence of PEA no CO_2 was evolved and the O_2 uptake was negligible; 2) With 5% galactose, mannose or fructose, CO_2 production was enhanced by PEA, but not with 5% maltose, sucrose or xylose; 3) We were able to establish that growth in the presence of 5% glucose (or another hexose) was a prerequisite for the production of a Crabtree effect by PEA.

Discussion: Our results demonstrate that the inhibition of growth yield and the induction of yeast-like morphology by PEA coincides with an enhancement of alcoholic fermentation provided the glucose concentration is equal to or higher than 2%. This enhancement results in the establishment of ratios (μmoles glucose fermented/μmoles glucose respired) greater than one indicating by analogy with the observations of De Deken (1966) on yeast, the existence of a Crabtree effect. Two working hypotheses, which are amenable to an experimental test, can be made: 1) In M. rouxii and related organisms, yeast-like morphology appears only when the inhibition of

respiration by fermentation has reached a certain level. 2) Since Silver and Wendt (1967) showed that PEA altered the permeability in Escherichia coli, it is suggested that in M. rouxii, PEA modifies the permeability of mitochondria in such a way as to permit leakage of ATP, metal ions or any other type of molecule essential for the regulation of the balance of respiration

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