Porphyrin Metabolism in Yeast

By J. E. KENCH (Sir Halley Stewart Research Fellow) AND J. F. WILKINSON

Department of Clinical Investigation and Research, Manchester Royal Infirmary

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As an approach to the problem of porphyrin and haemoglobin formation in the red blood corpuscle, we have investigated the production of coproporphyrin by yeast. Rimington (1943*a*) has outlined a scheme for the separation of intermediates in coproporphyrin formation and in a preliminary communication (Kench & Wilkinson, 1945), we have described conditions under which the formation of pigment may be markedly increased. Further studies have since been carried out on the fate of the chromoproteins, cytochrome *c*, catalase and peroxidase in yeast, and also on the stability of coproporphyrin; these we describe in this paper.

EXPERIMENTAL

Materials and methods

Throughout the investigation, we have worked with top-fermentation brewers' yeast, filtered and pressed to a firm consistency. To 250 g. yeast at 18–20° were added at 0 hr. 4 g. (NH₄)₂CO₃ and 10 g. glucose with thorough stirring. Many cells were evidently broken down by this harsh treatment and the preparation quickly became fluid. Samples were withdrawn every 24 hr. and the coproporphyrin content determined fluorometrically in slightly alkaline solution (Rimington, 1943*b*) after ether extraction and removal into HCl. For the extraction, 10–20 g. yeast preparation were diluted to 200 ml. with water, and the suspension acidified with 10 ml. glacial acetic acid/100 ml., and shaken repeatedly with ether (5 times with 200 ml.) until no porphyrin fluorescence could be detected by U.V. light in the separated ether phase, after washing to remove interfering blue-fluorescent substances. A few ml. of

ethanol were added after each shaking to break up the emulsion, and to facilitate removal of the pigment. The possible effects of breakdown of cytochrome *c*, catalase and peroxidase on the pigment yield were tested in turn, and are described separately.

Cytochrome *c*. Cytochrome *c* was prepared from ox-heart muscle (7 kg.) by the method of Keilin & Hartree (1937) as a concentrated aqueous solution, the purity of which was checked by determination of its iron content with $\alpha\alpha'$ -dipyridyl, and porphyrin content photometrically, by the pyridine haemochromogen method.

Peroxidase. Peroxidase was prepared from horse radish (3 kg.) by the method of Keilin & Mann (1937), but the enzyme was obtained only in small yield.

Catalase. Catalase was obtained from ox-liver (5 kg.) by the method of Sumner & Dounce (1937) by repeated precipitation with dioxan and (NH₄)₂SO₄. After final dialysis, the enzyme was precipitated with dioxan, filtered by suction, and dried *in vacuo*.

RESULTS

*Cytochrome *c**

The cytochrome *c* solution was added to the yeast preparation, with or without carbonate and glucose, at the beginning of the experiment, and the coproporphyrin formation followed (Table 1). A second experiment was made with yeast cells previously ruptured by alternate freezing and thawing. Cytochrome *c* was determined in the mixtures by centrifuging the yeast and assaying ferrocytochrome *c* in the supernatant after treating the latter with 0.1N-NaOH and Na₂S₂O₄ (Table 2). It is

Table 1. *Cytochrome *c* breakdown and coproporphyrin formation in yeast*
(250 g. fresh top-fermentation brewers' yeast. Pure cytochrome* added in aqueous solution.)

Duration of exp. (hr.)	Coproporphyrin ($\mu\text{g.}/\text{g.}$ fresh yeast)			
	Yeast + 100 ml. H ₂ O	Yeast + 100 ml. cytochrome solution	Yeast + 4 g. (NH ₄) ₂ CO ₃ + 10 g. glucose + 100 ml. H ₂ O	Yeast + 4 g. (NH ₄) ₂ CO ₃ + 10 g. glucose + 100 ml. cytochrome solution
0	0.5	0.5	0.5	0.5
16.5	0.0	1.6	47.1	11.6
65.0	0.0	1.4	25.9	7.2
120.0	0.0	4.9	9.5	20.8
256.0	3.3	2.8	10.7	11.6
305.0	2.4	0.0	7.4	13.9
355.0	3.1	0.0	2.3	5.3

* Cytochrome, dialyzed free from electrolytes, containing 9.5 mg./100 g. porphyrin, checked by Fe content with $\alpha\alpha'$ -dipyridyl. 100 ml. of solution added to yeast preparation to provide 38 $\mu\text{g.}$ porphyrin/g. yeast.

Table 2. *Cytochrome c experiment repeated with ruptured yeast cells*

(100 g. brewers' yeast + 2 g. $(\text{NH}_4)_2\text{CO}_3$ + 4 g. glucose. The cells were ruptured by alternate freezing and thawing and a second cytochrome *c** preparation added for test.)

Duration of exp. (hr.)	Coproporphyrin found ($\mu\text{g./g.}$ fresh yeast)		
	Cytochrome <i>c</i> recovered (as porphyrin $\mu\text{g./g.}$ yeast)	Yeast preparation + 13 ml. H_2O	Yeast preparation + 13 ml. H_2O
0	118.0	—	—
19	—	4.1	4.5
41	—	2.1	1.9
96	65.0	—	—
116	—	—	2.4
136	56.2	—	—

* Cytochrome *c* solution, 13 ml. containing 11.8 mg. porphyrin, added to 100 g. yeast to give a concentration of cytochrome *c* in terms of coproporphyrin of 118 $\mu\text{g./g.}$ yeast.

apparent from this evidence that nearly half of the added cytochrome *c* had been destroyed during the first 96 hr. of the experiment with the possibility of liberation of 53 $\mu\text{g.}$ coproporphyrin/g. yeast. There was, however, no increase in the porphyrin content. It seems probable, therefore, that coproporphyrin arising in these yeast preparations is not derived by simple fission of the prosthetic group from the protein moiety, but we are not justified in applying this argument to the still larger quantities of coproporphyrin found transitorily in wort towards the end of fermentation (Kench & Wilkinson, 1945), because the conditions of the experiments are quite different.

Peroxidase

The peroxidase preparation containing 30 $\mu\text{g.}$ of combined protoporphyrin was assayed in an appropriately small yeast preparation. After 65 hr., no coproporphyrin could be detected in the peroxidase specimen; size of the samples precluded determination of enzyme activity (Table 3). We are at a loss to account for the absence of coproporphyrin following peroxidase treatment, but the change may be brought about by toxic impurities contained in our preparation.

Table 3. *Coproporphyrin formation in yeast following addition of peroxidase*

(5 g. yeast + 0.08 g. $(\text{NH}_4)_2\text{CO}_3$ + 0.2 g. glucose. Peroxidase F2 added, Purpurogallin Number 35.7, containing 30 $\mu\text{g.}$ porphyrin.)

Duration of exp. (hr.)	Coproporphyrin found ($\mu\text{g./g.}$ yeast)	
	Yeast preparation	Yeast preparation + peroxidase
65	13.5	0.0

Catalase

The pure product, which was almost black in colour, was added to standard yeast preparations and the subsequent changes in coproporphyrin and catalase studied. The observations are presented in Table 4. Fresh yeast was found to have a low

Table 4. *Catalase breakdown and coproporphyrin formation in yeast*

(250 g. brewers' yeast + 4 g. $(\text{NH}_4)_2\text{CO}_3$ + 10 g. glucose; ox-liver catalase* added.)

Experiment	Duration of exp. (hr.)	Catalase activity (Kat. f.)	Coproporphyrin found ($\mu\text{g./g.}$ fresh yeast)
250 g. yeast alone	0	0.0887	—
	16.5	—	2.3
	65	—	0.0
	120	—	0.5
	305	—	1.4
	864	0.0060	2.3
250 g. yeast + catalase ($\equiv 215 \mu\text{g.}$ porphyrin/g. yeast)	0	1460	0.0
	22	—	—
	72	—	0.0
	96	237	—
	864	104.1	0.0
250 g. yeast + $(\text{NH}_4)_2\text{CO}_3$ + glucose	16.5	—	6.9
	43	—	21.1
	68	—	14.4
	256	—	18.5
	864	0.0029	14.5
250 g. yeast + $(\text{NH}_4)_2\text{CO}_3$ + glucose + catalase ($\equiv 153.5 \mu\text{g.}$ porphyrin/g. yeast)	0	1016	—
	22	—	2.0
	48	487	—
	72	347	6.9
	96	426	—
	864	318	1.4

* Ox-liver catalase, original solution Kat. f. 888 containing 11.8 mg. porphyrin/100 g. The enzyme activity Kat. f. is calculated from the expression

$$\frac{K}{\text{wt. preparation tested (g.)}},$$

where K (the velocity coefficient) = $\frac{2.303}{t} \log_{10} \left(\frac{a}{a-x} \right)$ as described by Stern (1932).

catalase activity, which had decreased to 7 % of its original value after 864 hr. Breakdown of catalase proceeded most rapidly in yeast alone where no coproporphyrin could be detected, whilst decomposition was retarded in porphyrinogenic preparations containing added $(\text{NH}_4)_2\text{CO}_3$ and glucose. In the former preparation, a breakdown of catalase could have provided 182 $\mu\text{g.}$ porphyrin/g. yeast, in the latter 89 $\mu\text{g./g.}$ over the 96 hr. period. The yields, however, were well below these values, supporting the conclusion that although the chromoproteins, cytochrome *c* and catalase are broken down, coproporphyrin is not one of the products of

disruption. Such a conclusion presupposes that coproporphyrin itself is a stable constituent of the system, which, once formed by synthesis or catabolism, persists unchanged for the remainder of the experimental time.

Further studies were made with a view to determining if coproporphyrin was indeed an end product or an intermediate of metabolism.

The pigment was extracted from yeast, purified by the usual methods, and evaporated to dryness from ethereal solution. Known amounts of this porphyrin were dissolved in yeast preparations after varying intervals of time, and the response subsequently examined (Table 5). The results show

during the initial 48 hr., it is possible for 87 μ g. of free coproporphyrin to be liberated/g. yeast. If such an amount of extra coproporphyrin were actually added during this period, we should expect that rather less than one-half would be metabolized, leaving 45 μ g. determinable porphyrin. Actually, only 2 μ g./g. coproporphyrin could be accounted for after 22 hr. On the other hand, catalase equivalent to 180 μ g. coproporphyrin/g. yeast was disrupted during 96 hr., during which time no trace of pigment could be detected. In each case the yeast preparations without added catalase, and those in which the catalase content was low, gave higher yields of coproporphyrin.

Table 5. *Metabolism of coproporphyrin by yeast*

Experiment	Duration of exp. (hr.)	Coproporphyrin added (μ g./g. yeast)	Coproporphyrin recovered (μ g./g. yeast)
50 g. yeast A + 1 g. $(\text{NH}_4)_2\text{CO}_3$	20	50	6.3*
+ 2 g. glucose	97	—	29.1
100 g. yeast A + 2 g. $(\text{NH}_4)_2\text{CO}_3$	0	50	—
+ 4 g. glucose	20	—	36.7 (1.9)*
	41	—	55.5
100 g. yeast B + 2 g. $(\text{NH}_4)_2\text{CO}_3$	0	94.3	—
+ 4 g. glucose	20	—	54.0 (97.5)†
50 g. yeast C + 1 g. $(\text{NH}_4)_2\text{CO}_3$	0	50	—
+ 2 g. glucose	20	—	54.9
100 g. yeast C + 2 g. $(\text{NH}_4)_2\text{CO}_3$	0	47.1	—
+ 4 g. glucose	44	—	53.1
50 g. yeast D + 1 g. $(\text{NH}_4)_2\text{CO}_3$	96	50	—
+ 2 g. glucose	115	—	39.3
100 g. yeast D + 2 g. $(\text{NH}_4)_2\text{CO}_3$	0	132	—
+ 4 g. glucose	20	—	67.0 (123.1)†
	0	350	1.4*
	20	—	193.0 (365, † 4.1*)

* Without added coproporphyrin.

† Control experiment with autoclaved yeast.

that only occasionally is it possible to recover all the added pigment, not only during active fermentation, but equally so at the end of the observation period of 96 hr., when active fermentation had ceased. Neither haem nor haematin was formed as the result of the addition of extra porphyrin, and the route by which the porphyrin was removed has not yet been determined.

It is evident, therefore, that coproporphyrin plays the part of an intermediate in metabolism, and its accumulation represents the net result of imbalance between the rates of its removal and formation. Of the amount of pigment formed, a high proportion can be rapidly metabolized even when the concentration is approaching the peak levels observed at 96 hr. and onwards.

In the light of these observations, it is necessary to study more closely the question of breakdown of cytochrome *c* and catalase. Measurements of catalase capability show that by decomposition

Similarly with cytochrome *c*, we cannot reconcile the rate of disappearance of the chromoprotein with the low yields of porphyrin, even allowing for lability of the latter, on the basis of simple fission of the protein and the prosthetic groups.

DISCUSSION

The results presented in the foregoing section show that both cytochrome *c* and catalase are unstable in the yeast preparations, but we were unable to detect augmentation of porphyrin production as a result of breakdown. Actually the reverse is true, an effect which calls for further investigation.

Coproporphyrin, itself, is undergoing change and accumulates apparently owing to the relative rates of formation and removal being out of phase. However, in considering the fate of cytochrome *c* and catalase in relation to the observed changes of coproporphyrin, we feel justified in concluding that

the chromoproteins are not the source of the porphyrin.

Consequently, we must assume that the reactions by which porphyrin is formed, to be subsequently metabolized, are now out of harmony, and if we may interpret our data in terms of the interesting experiments of Conway & Breen (1945) on 'ammonia' yeast, it would appear probable that the phenomenon is due to excessively rapid cell division.

Regarding the origin of the coproporphyrin, certain facts seem worthy of mention. The yeast preparations we have used resemble in composition those described by Neuberg & Ursum (1920) as leading to the Third Form of Fermentation, in which, by use of alkali carbonates, borates, or triphosphates at a concentration of 0.1–0.2M, increased amounts of the by-products, acetaldehyde, acetic acid and glycerol were produced. Under the most favourable conditions (with 4% $(\text{NH}_4)_2\text{CO}_3$) as much as 41.3% of sugar was fermented in this way. We have tested other substances of this fixation group, e.g. BaCO_3 , MgO and CaCO_3 , but these were ineffective in porphyrin production as also was Na_2SO_3 in Neuberg's Second Form of Fermentation (Neuberg & Hirsch, 1919). Other ammonium salts, viz. the chloride, sulphate and oxalate, appeared to be toxic, and we conclude that the primary effect of $(\text{NH}_4)_2\text{CO}_3$ is to supply a ready source of nitrogen for rapid proliferation of the yeast cells and that the pigment does not arise from by-products of fermentation. This is also supported by the occurrence of the highest recorded yield in wort, wherein by-products are necessarily reduced to a minimum.

Yeast preparations in an atmosphere of oxygen

behaved little differently from controls in air, but in nitrogen the level of porphyrin was maintained after 312 hr. at a value twice that of oxygenated and control preparations. This suggests that the removal of porphyrin from the system is expedited by the presence of oxygen, suggesting that the accumulation of porphyrin may be partly associated with oxygen lack in the cells. Experiments are being continued on the pigment yield in relation to the rate of cell division.

SUMMARY

1. An account has been given of studies on the formation of coproporphyrin in yeast preparations.
2. Catalase and cytochrome *c*, chromoproteins each of which contains protoporphyrin as a constituent of its prosthetic group, are both disrupted in yeast, whether present intracellularly or added extracellularly.
3. No porphyrin could be detected as the result of breakdown of the chromoproteins.
4. Coproporphyrin itself is not a stable constituent of the system, and appears to be an intermediate of metabolism. It does not appear to be formed by interaction of abnormal by-products of fermentation, but may be associated with high rates of cellular division.

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