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# THE CARBON MONOXIDE-REACTIVE HAEMOPROTEINS OF YEAST

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SUMMARY

The yeasts *Saccharomyces cerevisiae*, *Candida utilis* and *Saccharomyces carlsbergensis* were shown to have two CO-binding haemoproteins in addition to cytochrome $a_3$. These haemoproteins were separated by a method described. One, contained in the particulate fraction of the cells, was extracted by sodium deoxycholate and precipitated at 30% $(NH_4)_2SO_4$ saturation. The visible spectrum was that of a B-type cytochrome, but it reacted with carbon monoxide to produce CO-difference transmission minima at 408 mμ, 540 mμ and 572 mμ. It did not form a complex with either $CN^-$ or $O_2$. The other haemoprotein was separated from the non-particulate cellular fraction and precipitated at 65% $(NH_4)_2SO_4$ saturation. Like the haemoglobin of blood, this haemoprotein can be oxygenated, reduced and oxidized and can react with $CN^-$ and CO. The CO-difference transmission minima are situated at 419 mμ, 532 mμ and 568 mμ.

CO-difference reflectance spectrophotometry of *C. utilis* cells was employed for semi-quantitative estimation of the carbon monoxide complex of cytochrome $a_3$ (CO–$a_3$) and the CO complex of those haemoproteins with a 570 mμ CO-difference reflectance minimum (CO–$Hp_{570}$). The concentration of CO–$a_3$ and CO–$Hp_{570}$ varied with glucose concentration and culture age. Evidence is presented for contribution to the CO–$Hp_{570}$ of *C. utilis* by both cytochrome *o* and haemoglobin in high glucose cultures and by cytochrome *o* alone in low glucose cultures.

## INTRODUCTION

Two haemoproteins, besides A-type cytochromes, which are capable of complexing with CO, have been reported in microorganisms. These pigments have not, however, been reported as occurring together in the one organism. Various workers have observed one of them, an oxygenated ferrous haemoprotein, similar to the oxyhaemoglobin of blood, in yeast[1–5] and moulds[6,7]. The name endogenous haemoglobin[2] was given to this haemoprotein of baker's yeast. Like the haemoglobin of blood, it combined reversibly with molecular oxygen[2]; the oxygenated complex had an α-band at 583 mμ which disappeared on reaction with CO (refs. 1–3), reduction with dithionite[3] and oxidation with $K_3[Fe(CN)_6]$ (ref. 6).

Abbreviations: CO–$Hp_{570}$, CO complex of any haemoprotein with a 570 mμ minimum in its CO-difference reflectance or CO-difference transmission spectrum; CO–$a_3$, CO complex of cytochrome $a_3$.

The other CO-binding haemoprotein was first observed by CHANCE[8] as being responsible for three maxima at about 416 mμ, 540 mμ and 568 mμ in the CO-difference absorption spectrum of *Staphylococcus albus*. It was demonstrated to be a terminal oxidase[9] and was designated cytochrome *o* (ref. 10). It has been reported in a number of bacteria[8–19] and colourless algae (Cyanophyta)[20,21]. It has been demonstrated in *Staphylococcus aureus*[14] and Vitreoscilla[21] to have a reduced spectrum with bands in the region characteristic of B-type cytochromes. The nature of the prosthetic group has not, however, been elucidated; there is evidence both for[8,14,21] and against[22] protohaem being the prosthetic group. It has some properties in common with Rhodospirillum haem protein (see ref. 23).

Evidence is now presented for the occurrence of both cytochrome *o* and haemoglobin in yeast. The relative concentrations of the CO-reactive haemoproteins of *C. utilis* varied with the glucose concentration of the culture medium and age of the culture.

## MATERIALS AND METHODS

### *Organisms*

The yeast *Saccharomyces cerevisiae* was a "yeast foam" kindly supplied by Dr. P. Slonimsky. A laboratory strain of the yeast *Candida utilis* was originally obtained from the British Type Culture Collection.

### *Culture medium*

Each l of culture medium contained: 9 or 54 g glucose (referred to, respectively, as low and high glucose cultures), 5 g Difco yeast extract, 5 g Difco bactopeptone, 1.8 g citric acid, 6.0 g $KH_2PO_4$, 1.8 g $Na_2HPO_4 \cdot 12H_2O$, 6.5 g $(NH_4)_2SO_4$, 104 mg HCl, 50.5 mg MgO, 27.0 mg $FeCl_3 \cdot 6H_2O$, 10.0 mg $CaCO_3$, 5.0 mg $MnCl_2 \cdot 4H_2O$, 2.0 mg ZnO, 1.2 mg $CoCl_2 \cdot 6H_2O$, 0.85 mg $CuCl_2 \cdot 2H_2O$ and 0.3 mg $H_3BO_3$.

### *Cultural conditions*

The cultures were incubated aerobically at 30° on a reciprocal shaker (stroke 8.6 cm) at a rate of 44 complete oscillations/min. The yeast was harvested at times indicated by centrifuging at 4000 × *g* for 10 min and washed twice with cold 0.1 M phosphate buffer, pH 7.5.

### *Preparation of cell-free fractions*

The scheme for separation of the haemoproteins into soluble fractions appears in Fig. 1. All operations were performed at 4°, except where indicated otherwise. Cell walls were disrupted by shaking in 0.1 M phosphate buffer, pH 7.5, with 3-mm diameter glass beads on a wrist shaker (Griffin Flask Shaker). The preparation was centrifuged at 6000 × *g* for 20 min and the supernatant then centrifuged at 30000 × *g* for 50 min to give Residue $R_1$ and a red-coloured Supernatant S1.

Residue $R_1$ was incubated with 0.1 % sodium deoxycholate in 0.05 M phosphate buffer, pH 7.5, at 37° for 30 min. Treatment was continued for a further 15 h at 4° before centrifuging at 30000 × *g* for 50 min. The Supernatant $SR_1$ was brought to 30 % $(NH_4)_2SO_4$ saturation with solid $(NH_4)_2SO_4$ (16 g to each 100 ml) and the suspension allowed to stand for 15 h before centrifuging at 25000 × *g* for 30 min. The

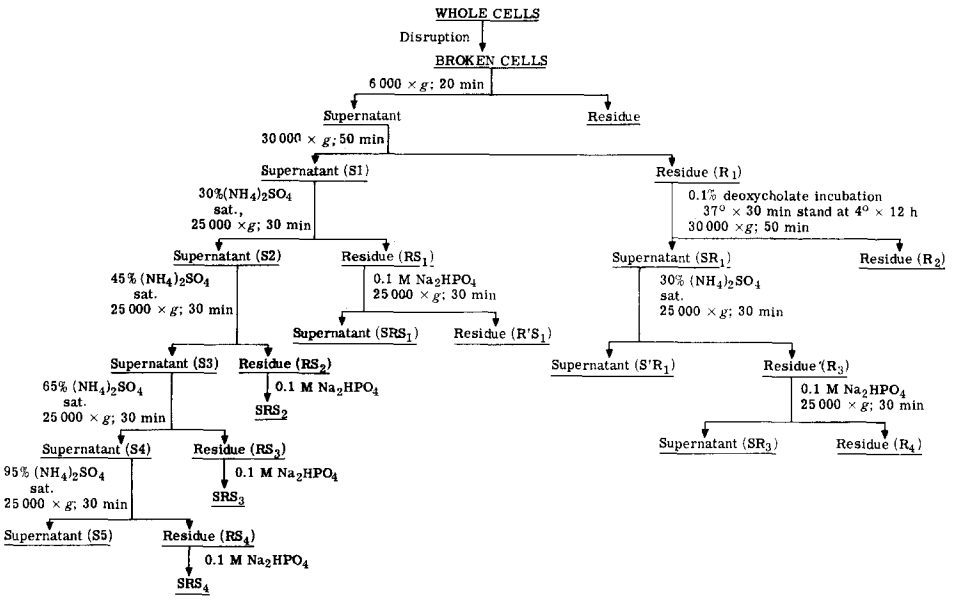


Fig. 1. Scheme for the separation of yeast haemoproteins.

Supernatant $S'R_1$ contained very little pigment and was therefore discarded. The corresponding Residue $R_3$ was extracted for 15 h with a vol. of 0.1 M $Na_2HPO_4$ equal to the vol. of the original cell suspension and then centrifuged at 25 000 × *g* for 30 min to give Residue $R_4$ and a greenish-brown Supernatant $SR_3$.

Supernatant $S_1$ was fractionated with $(NH_4)_2SO_4$. The serial additions of the salt were equal to 16 g to each 100 ml (30% saturation), 8.8 g to each 100 ml (45% saturation), 12.3 g to each 100 ml (65% saturation) and 15.6 g to each 100 ml (95% saturation). After each addition the preparation was stood for 15 h before centrifuging at 25 000 × *g* for 30 min. Supernatants S2, S3, S4, and S5 and Residues $RS_1$, $RS_2$, $RS_3$ and $RS_4$ were obtained at 30%, 45%, 65% and 95% saturations, respectively. The four residues were treated with 3 ml of 0.1 M $Na_2HPO_4$ per 10 ml of original cell suspension. $RS_1$ did not dissolve completely and was centrifuged at 25 000 × *g* for 30 min to give $SRS_1$ and $R'S_1$. $RS_2$, $RS_3$ and $RS_4$ dissolved completely to yield $SRS_2$, $SRS_3$ and $SRS_4$, respectively.

*Reflectance spectrophotometry*

CO-difference reflectance spectra were obtained as follows: after harvesting, the yeast cells were washed once with 0.15 M phosphate buffer, pH 7.0, and then resuspended in the same buffer (14 g wet cells: 240 ml buffer). Sodium dithionite (200 mg/l) was added to the suspension, mixed and allowed to stand for 10 min. Half the dithionite-reduced suspension was gassed for 20 min with coal gas while the other half remained untreated, to provide the CO-reduced and reduced samples, respectively. The cells were collected by centrifugation and CO-difference reflectance spectra scanned as described by RICKARD, MOSS AND ROPER[24], with the CO-reduced cells in the sample port and dithionite-reduced cells in the reference port. Scans were recorded on two reflectance scales, Mode 1T and its 5-fold expansion, 5T.

*Transmission spectrophotometry*

Transmission scans of cell fractions were recorded at 1T and 5T on a Perkin-Elmer Model 350 double-beam recording spectrophotometer. Glass cuvettes of 3-ml capacity and 1-cm light path were employed. For absolute spectra, distilled water was used as reference. Crystalline sodium dithionite was used for reduction and crystalline $K_3[Fe(CN)_6]$ for oxidation of the yeast fractions. For transmission scanning of CO-difference spectra, the dithionite-reduced sample was gassed for 30 sec with coal gas and scanned against a dithionite-reduced reference sample. Crystalline KCN was added to the dithionite-reduced and ferricyanide-oxidized yeast fractions for the formation of ferrous and ferric cyanide complexes, respectively. All spectra were recorded at 20°. For the pyridine haemochrome spectra, a slightly modified method to that described by HORIE AND MORRISON[25] was employed. Instead of using 0.5 ml of the sample, a solid portion of the sample was dissolved in 1 ml of 0.1 M NaOH and then 1.0 ml of pyridine and 0.5 ml of distilled water added.

*Semi-quantitative estimation of CO–haemoprotein in the intact cells*

The concentration of each CO–haemoprotein was estimated in arbitrary units by measuring the difference between its α-band minimum and the corresponding maximum in the CO-difference reflectance spectrum. This method of determination is based on the assumption that the same pigment, in the CO-reduced form in the sample port and the reduced form in the reference port, was responsible for the minimum and maximum respectively. The level of the total CO–haemoproteins with a minimum at 570 mμ ($CO–Hp_{570}$) was estimated as the difference between the reflectance at this minimum and at the corresponding maximum at 555 mμ. Similarly, the difference between the reflectance at the 590 mμ minimum and at its corresponding maximum at 600 mμ was regarded as the level of the carbon monoxide–cytochrome $a_3$ complex ($CO–a_3$). The differences are recorded in reflectance units, each unit being equal to 1 % on the reflectance scale. Duplicate cultures agreed within 5%.

*Protein determination*

The protein in the soluble extract was determined by the method of LOWRY *et al.*[26].

## RESULTS

*Identification of CO-reactive haemoproteins*

The CO-difference reflectance spectra of intact cells of *S. cerevisiae* and *C. utilis* grown for 24 h in low glucose had minima at 420 mμ, 535 mμ, 570 mμ and 590 mμ, and maxima at 443 mμ, 518 mμ, 555 mμ and 600 mμ. The 590 mμ, and to some extent the 420 mμ, minima are evidently due to the cytochrome $a_3$–CO complex. The spectral properties of the pigments responsible for the 535 mμ and 570 mμ minima were examined more fully in cell fractions.

Fraction $SRS_1$ of *S. cerevisiae* grown for 24 h in low glucose culture contained a small amount of A- and B-type cytochrome. Fraction $SRS_2$ was free from these cytochromes but had very weak bands similar in position to those of $SRS_3$. Fig. 2 shows the untreated, reduced and oxidized spectra of Supernatant $SRS_3$. In the untreated state its spectrum had bands centred at 412 mμ, 535 mμ, 575 mμ and, in some

samples, a weak one in the vicinity of 628 mμ. In the reduced spectrum the bands were centred at 420 mμ, 520 mμ, 551 mμ and, in some samples, there was an additional weak band centred at 600 mμ. The bands of oxidized $SRS_3$ were centred at 630 mμ and at about 535 mμ; compared with the bands of the untreated state, the 575 mμ band had disappeared, the 535 mμ band was very much weaker and the 630 mμ band was considerably stronger than the 628 mμ untreated band. The marked differences between the untreated, reduced and oxidized spectra indicate that the untreated

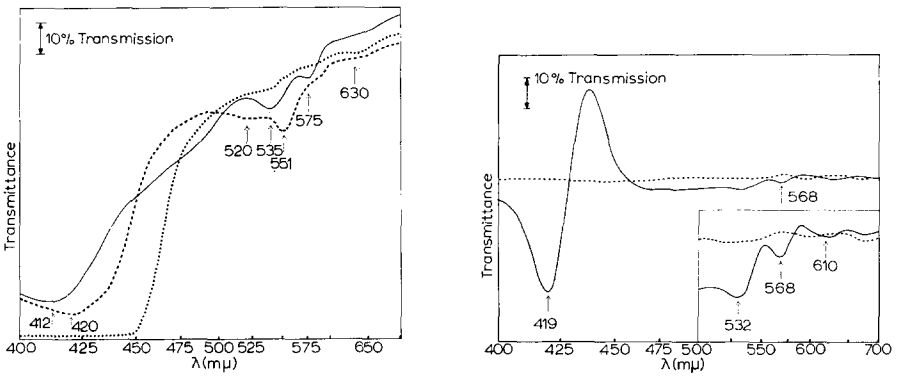


Fig. 2. Transmission spectra of $SRS_3$ of *S. cerevisiae* grown for 24 h in low glucose culture (27 mg protein/ml): ———, untreated sample; --------, reduced sample; ·····, oxidized sample.

Fig. 3. CO-difference transmission spectrum of Supernatant $SRS_3$ of *S. cerevisiae* grown for 24 h in low glucose culture (27 mg protein/ml): ———, CO-reduced *minus* reduced sample; ·····, reduced *minus* reduced base line. Full spectrum scanned on 1T; inset scanned on 5T expanded scale.

spectrum is that of an oxygenated complex of the $SRS_3$ pigment. The spectra of the three states closely resemble those of oxyhaemoglobin, haemoglobin and methaemoglobin, respectively (see Table I). Furthermore, the spectral changes after treatment of the pigment with $CN^-$ and CO were similar to the changes evoked when animal[27] haemoglobin complexed with these ligands: the bands of the cyanide ferrous complex were centred at 420 mμ, 534 mμ and 545 mμ and the bands of the cyanide ferric complex at 420 mμ and 535 mμ; the minima of the CO-difference transmission spectrum were at 419 mμ, 532 mμ, 568 mμ and 610 mμ, and the maxima at 437 mμ, 554 mμ and 592 mμ (see Fig. 3). The pigment contained in fraction $SRS_3$ is therefore considered to be a yeast haemoglobin.

The spectral characteristics of Supernatant $SRS_2$ of *C. utilis* cells from 36 h high glucose culture (see Table II) were similar to those of Supernatant $SRS_3$ of *S. cerevisiae* described above. Both yeasts therefore contained haemoglobin. No haemoglobin-like pigment was however detected in any fraction from 24 h low glucose cultures of *C. utilis*. The $SRS_3$ Supernatant of *C. utilis* contained the major portion of C-type cytochrome.

Fig. 4 shows the transmission spectra of the untreated, reduced and oxidized states of Supernatant $SR_3$ obtained from the particulate fraction of *S. cerevisiae* grown in low glucose culture for 24 h. The spectrum of the untreated sample had a Soret band centred at 413 mμ and a weak band centred at 598 mμ. On reduction with sodium dithionite the 413 mμ band disappeared, and bands centred at 428 mμ, 526 mμ and

TABLE I

SUMMARY OF TRANSMISSION MINIMA AND PROSTHETIC GROUPS OF THE CO-REACTIVE HAEMOPROTEINS OF *S. cerevisiae* GROWN FOR 24 h IN LOW GLUCOSE CULTURE

Data reported for other organisms are included. Values in parentheses indicate weak bands.

| *Source* | *Transmission minima (mμ)* | | | | | *Prosthetic group* | *Haemoprotein* |
|---|---|---|---|---|---|---|---|
| | *Untreated (oxygenated) spectrum* | *Reduced spectrum* | *Oxidized spectrum* | *CO-difference spectrum* | *Cyanide spectrum* | | |
| *S. cerevisiae* Non-particulate fraction $SRS_3$ | 412, 535, 575 (628) * | 420 (520) 551 (600) * | (535) 630 | 419, 532, 568 (610) | 420, 533–535, 545 ($Fe^{2+}$) 420, 535 ($Fe^{3+}$) | Protohaem | Haemoglobin |
| *S. cerevisiae*, Particulate fraction $SR_3$ | 413 | 428, 526, 557 | | 408, 540, 572 | 428, 526, 557 ($Fe^{2+}$) | Protohaem (?) | Cytochrome *o* |
| | (598) | 440, 598 | (590) | 590 (610) | 437, 596 ($Fe^{2+}$) | Haem *a* | Cytochrome $a_3$ |
| Animals[27] | 412–415, 540–542, 576–578 | 430, 555 | 405–407, 500, 630 | 418, 538–540, 568–572 ** | 412–416, 540 ($Fe^{3+}$) | Protohaem | Haemoglobin |
| *S. aureus*[14] | | 427, 557 | | 415, 538, 568 | 427, 557 | Protohaem | Cytochrome *o* |
| Vitreoscilla[20,21] | | 423–428, 553–559 | 398–399 | 418–419, 532–534 566–570 | 416, 540 | Protohaem | Cytochrome *o* |

* Present only in some samples.
** CO-absorption spectrum.

TABLE II

TRANSMISSION MINIMA OF CO-REACTIVE HAEMOPROTEINS OF *C. utilis*

Values in parentheses indicate weak bands.

| *Initial glucose concn. in growth medium* (%) | *Incubation time* (*h*) | *Fraction* | *Transmission minima* (*mμ*) | | | | *Haemoprotein* |
|---|---|---|---|---|---|---|---|
| | | | *Untreated spectrum* | *Reduced spectrum* | *Oxidized spectrum* | *CO-difference spectrum* | |
| 0.9 | 24 | $R_2$ from particulate cellular components* | 412 (526) | 442, 600<br>426, 526, 557 | —<br>— | 588<br>407, 537, 571 (606) | Cytochrome $a_3$<br>Cytochrome *o* |
| 0.9 | 24 | $SR_3$ from particulate cellular components | 412 (526) | 426, 526, 557 | — | 407, 537, 571 (606) | Cytochrome *o* |
| 5.4 | 36 | $SR_3$ from particulate cellular components | 416 | 442, 598,<br>426, 526, 557 | —<br>— | 590<br>407, 537, 571 | Cytochrome $a_3$<br>Cytochrome *o* |
| 5.4 | 36 | $SRS_2$ from non-particulate cellular components | 414, 540, 575 | 432, 551 | (528) 624 | 408, 532, 568 (610) | Haemoglobin |

* Reflectance minima.

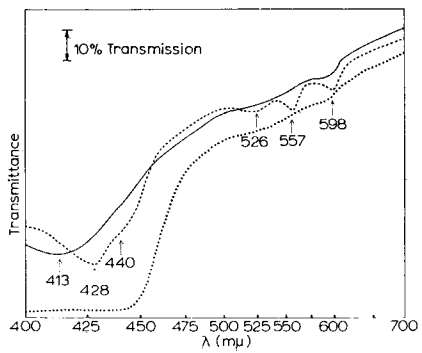


Fig. 4. Transmission spectra of Supernatant $SR_3$ of *S. cerevisiae* grown for 24 h in low glucose culture (6.1 mg protein/ml): ———, untreated sample; - - - - - -, reduced sample; ·····, oxidized sample.

557 mμ (attributable to B-type cytochrome) and at 440 mμ and 598 mμ (attributable to A-type cytochrome) appeared. No bands characteristic of C-type cytochrome were present, cell fractionation having succeeded in relegating the C-type cytochrome to fraction $SRS_4$. On oxidation with $K_3[Fe(CN)_6]$, the bands of the untreated sample almost disappeared, leaving only an extremely weak band centred at approx. 590 mμ.

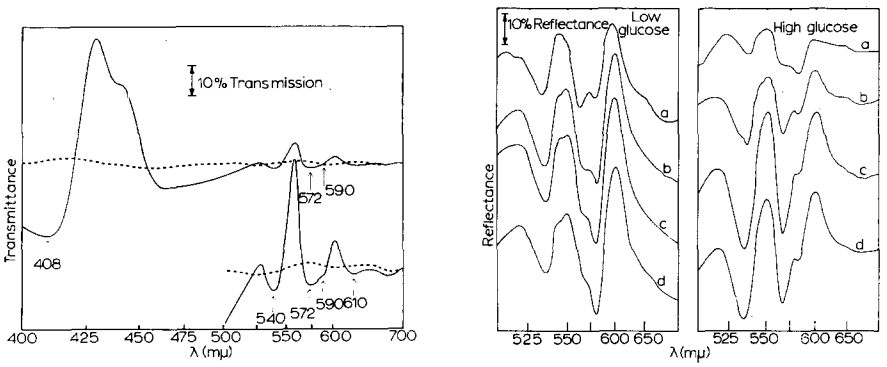


Fig. 5. CO-difference transmission spectrum of Supernatant $SR_3$ of *S. cerevisiae* grown for 24 h in low glucose culture (6.1 mg protein/ml): ———, CO-reduced *minus* reduced sample; – – – –, reduced *minus* reduced base line. Full spectrum scanned on 1T; inset scanned on 5T expanded scale.

Fig. 6. CO-difference reflectance spectra (5T expanded scale) of intact *C. utilis* cells, grown in low and high glucose culture, harvested at 12 h (a); 24 h (b); 36 h (c); and 48 h (d).

$CN^-$ shifted the 440 mμ and 598 mμ bands of the reduced state to 437 mμ and 596 mμ, respectively, apparently by forming a cytochrome $a_3$ complex; there was no evidence for complex formation between cyanide and B-type cytochrome, since the 428 mμ 526 mμ and 557 mμ bands remained unaffected. Fig. 5 shows the CO-difference transmission spectrum of Supernatant $SR_3$. The minima were situated at 408 mμ, 540 mμ, 572 mμ, 590 mμ and 610 mμ, and the maxima at 430 mμ, 440 mμ, 528 mμ, 558 mμ, and 601 mμ. The 590 mμ band is assignable to cytochrome $a_3$–CO, but the $a_3$–CO Soret band, reported at 432 mμ in the action spectrum of CASTOR AND CHANCE[9], was apparently masked by the CO-difference transmission maxima at 430 mμ and 440 mμ. It is considered that the CO-difference bands at 408 mμ, 540 mμ and 572 mμ were sufficiently similar to the CO-difference bands of cytochrome *o* in other microorganisms to justify attributing them to the presence of this cytochrome in *S. cerevisiae* (see DISCUSSION). Supernatant $SR_3$, the soluble extract of the intracellular particles, therefore contained cytochromes *a*, $a_3$, *o* and B-type. We can attempt no explanation for the 610 mμ CO-difference transmission minimum in this fraction.

Similar spectral characteristics possessed by Supernatant $SR_3$ of *C. utilis* cells harvested at 36 h from high glucose culture (see Table II) indicate the occurrence of cytochrome *o* and cytochrome $a_3$ in this yeast also. The cytochrome *o* of low glucose cultures of *C. utilis* harvested at 24 h was successfully separated in Supernatant $SR_3$ from cytochrome $a_3$ which was relegated to Residue $R_2$.

The reduced pyridine haemochrome spectrum of the haemoglobin-containing fractions of both yeasts had bands centred at 419 mμ, 523 mμ and 554 mμ. This corresponded precisely with the pyridine haemochrome spectrum of haem isolated

from ox blood. Supernatant $SR_3$ of both yeasts also produced reduced pyridine haemochrome bands at 419 mμ, 523 mμ and 554 mμ, in addition to bands at 430 mμ and 586 mμ which corresponded with haem *a*. Protohaem is therefore undoubtedly the prosthetic group of yeast haemoglobin and most probably also that of the cytochrome *o* in fraction $SR_3$.

*Concentrations of CO–$Hp_{570}$ and CO–$a_3$*

Fig. 6 shows the spectra of *C. utilis* at various stages of culture in low and high glucose media. Distinctive changes occurred with incubation time. Semi-quantitative evaluation of the changes, expressed in reflectance units (see MATERIALS AND METHODS), appears in Table III.

During 12 and 48 h in low glucose culture, CO–$Hp_{570}$ first increased slightly and then decreased. At 12 h this pigment was lower in high glucose culture than low glucose culture but increased by 36 h to a maximum concentration. In low glucose culture CO–$a_3$ increased extensively between 12 and 24 h and rose to a maximum by 48 h. Its concentration also increased with time in high glucose culture but was always lower than in low glucose. The relative changes in CO–$Hp_{570}$ and CO–$a_3$ with incubation time are seen more clearly when expressed as a CO–$Hp_{570}$/CO–$a_3$ ratio (Table III): in low glucose culture the ratio decreased with time whilst in high glucose it increased up to 36 h and then declined.

The CO–$Hp_{570}$/CO–$a_3$ ratios of *S. cerevisiae* cells were higher than comparable *C. utilis* ratios, particularly when high glucose grown cells were compared.

Glucose was depleted by 12 h in all cultures examined.

TABLE III

CONCENTRATION OF CO-REACTIVE HAEMOPROTEINS IN *C. utilis* AND *S. cerevisiae*

| *Organism* | *Initial glucose concn. in growth medium* (%) | *Incubation time* (*h*) | *Concentration of CO–$Hp_{570}$* (*reflectance units*) | *Concentration of CO–$a_3$* (*reflectance units*) | *CO–$Hp_{570}$/CO–$a_3$* |
|---|---|---|---|---|---|
| *C. utilis* | 0.9 | 12 | 23.5 | 23.9 | 0.98/1 |
| | | 24 | 25.2 | 40.4 | 0.62/1 |
| | | 36 | 23.2 | 43.4 | 0.54/1 |
| | | 48 | 18.5 | 45.0 | 0.41/1 |
| | 5.4 | 12 | 11.0 | 10.8 | 1.02/1 |
| | | 24 | 18.0 | 13.0 | 1.38/1 |
| | | 36 | 34.5 | 18.8 | 1.83/1 |
| | | 48 | 32.0 | 21.0 | 1.53/1 |
| *S. cerevisiae* | 0.9 | 24 | 17.0 | 21.4 | 0.79/1 |
| | 5.4 | 24 | 14.5 | 5.2 | 2.80/1 |

DISCUSSION

The spectral properties of fractions extracted from *S. cerevisiae* and *C. utilis* provide evidence for the existence in both these yeasts of two CO-binding haemoproteins besides cytochrome $a_3$. Preliminary studies indicate that they are present also in the yeast *Saccharomyces carlsbergensis*. Since these two distinct CO-binding

haemoproteins both have CO-difference transmission minima in the regions 532–540 mμ and 568–572 mμ, the presence of one makes the detection of the other more difficult. It is in fact impossible to study either of them in unfractionated cells unless previous fractionation studies have established the absence of the other.

One pigment reacted similarly to the haemoglobin of blood in that it could be oxygenated as well as reduced and oxidized. Haemoglobin was apparently not present in the aerobically-grown *S. cerevisiae* cells employed by CHAIX[5] in her fractionation studies. Its presence was, however, indicated by a 575 mμ band in her unfractionated anaerobically-grown *S. cerevisiae* cells which, unlike aerobic cells, do not have high cytochrome concentrations to mask their haemoglobin spectrum. If CHAIX's observation[5] that the intensity of the 575 mμ band (and presumably the haemoglobin concentration) of anaerobic cells increases with the age of the culture is also true for aerobic cells, then her fractionation studies with late exponential phase aerobic cells may not have been timed as fortuitously as our studies, where stationary phase cells were employed. Supporting this explanation for the differences between CHAIX's[5] and our fractionation studies is an observation made on mould by KEILIN AND TISSIÈRES[6]. They reported that in *Neurospora crassa* haemoglobin appeared in the culture only after the third day and reached a maximum concentration on the fifth day, after which it gradually disappeared.

The CO-difference transmission spectrum of the haemoglobin isolated from yeast could perhaps be mistaken for the cytochrome *o* of *Staphylococcus albus*[10] and *Staphylococcus aureus*[14]. Unlike yeast haemoglobin, however, complex formation between cytochrome *o* of *S. aureus* and $CN^-$ could not be demonstrated[14]. A CO-binding haemoprotein that even more closely resembles the haemoglobin of yeast is the $CN^-$-reactive cytochrome *o* of Vitreoscilla reported by WEBSTER AND HACKETT[21]. The important distinction here, however, lies in lack of evidence for an oxygenated complex of the Vitreoscilla pigment.

The failure of the two CO-binding pigments in Supernatant $SR_3$ of the yeasts to manifest oxygenated spectra is a criterion of their non-haemoglobin nature. One is obviously cytochrome $a_3$ and the other we have designated cytochrome *o* because of certain similarities shared with the cytochrome *o* of other organisms. The yeast cytochrome *o* did not react with $CN^-$ and its spectral characteristics closely resemble those of *S. aureus* cytochrome *o* reported by TABER AND MORRISON[14].

The differences in fractionation behaviour and spectral properties between yeast haemoglobin and cytochrome *o* leave little doubt that they are two distinct pigments and that they probably play different physiological roles. We have already assumed a terminal oxidase role for the latter in designating it cytochrome *o*; until its terminal oxidase activity and unequivocal identity with bacterial cytochrome *o* are demonstrated, such designation must remain a tentative, but convenient, one.

The semi-quantitative estimation of the CO-reactive haemoproteins of *C. utilis* indicate that cytochrome $a_3$ participates in the phenomenon whereby mitochondrial content, respiratory activity, respiratory enzymes and cytochromes of yeast are repressed by glucose (refs. 28–35, *etc.*). CO–$Hp_{570}$ of *C. utilis* is a notable exception in being in higher concentration in late high glucose culture than late low glucose culture. In low glucose culture the CO–$Hp_{570}$/CO–$a_3$ ratio fell with incubation time, whilst in high glucose culture it rose up to 36 h and then declined slightly. It therefore appears that cells in low glucose culture become more dependent with time on cytochrome $a_3$

and less dependent on the haemoprotein(s) responsible for CO–$Hp_{570}$. If it is assumed that the absence of haemoglobin in 24 h low glucose cultures indicates general lack of this pigment in all stages of low glucose culture, then the increasing dependence of low glucose cultures on cytochrome $a_3$ with time is counteracted by a lessening dependence on cytochrome *o*. This suggestion is in agreement with CASTOR AND CHANCE[10], who reported that, whereas in exponential phase *Escherichia coli*, *Proteus vulgaris* and *Aerobacter aerogenes* contained cytochrome *o* as their only terminal oxidase, in stationary phase cytochrome $a_1$ and/or $a_2$ contributes to the terminal oxidative activity.

The increase in CO–$Hp_{570}$ with time in late high glucose cultures of *C. utilis* could be related to ethanol oxidation after glucose depletion. Notable in this respect is that so far haemoglobin has only been detected in *S. cerevisiae*, aerobically grown *Saccharomyces carlsbergensis* and high glucose aerobically grown *C. utilis*. All these cells have lower respiratory activity and produce more ethanol than aerobic low glucose grown *C. utilis*, in which, after 24 h incubation, haemoglobin is lacking. The possibility, and its inherent assumptions, of haemoglobin serving as a link between free oxygen and terminal oxidases has been discussed, together with other possible roles for this pigment, by KEILIN[2]. The present finding that haemoglobin occurs after glucose depletion in cultures with high fermentation activity is compatible with the suggestion that it does serve a role in enhancing respiratory activity.

The possibility cannot be overlooked, however, that yeast haemoglobin is a precursor or degradation product of yeast cytochrome *o*.

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