PROMOTION BY ZINC OF THE FORMATION OF CYTOCHROMES IN USTILAGO SPHAEROGENA [1]

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Ustilago sphaerogena is a smut fungus which is parasitic on the grass *Echinochloa crus-galli* but may be cultured saprophytically in the sporidial stage of its life cycle. The form, rate of growth, and color of the sporidia were found (29) to be dependent upon the medium in which the fungus is grown. In a medium containing 2 % yeast extract and 2 % sucrose, large, rapidly growing pink sporidia which bud in the manner of yeasts are produced in shake-culture. However, in other organic and inorganic media, filamentous, slower growing, white sporidia are produced. The difference in color is striking and was found (29) to be due to the pigment, cytochrome c. The effect of the nutrient medium on the production of other cytochromes and cytochrome oxidase as well as cytochrome c is presented here. Evidence is given that the substance in yeast extract responsible for the high level of cytochrome in this organism is zinc.

MATERIALS AND METHODS

CULTURE OF THE SMUT: All cultures used were derived from one single-spore isolate of *Ustilago sphaerogena* except in the analyses of cultures of different ages, for which another isolate was used. A 2 % Difco yeast extract medium containing 2 % sucrose was used whenever yeast extract (Y.E.) is designated, unless otherwise described. Modified Czapek's medium and double-strength Henderson-Snell medium (Medium B) (14) without the purines and pyrimidines were used particularly in some of the early experiments. Medium B was later revised to the composition given below,[3] and this revised, synthetic medium is designated as Medium A. All media were sterilized by autoclaving for 15 minutes at 15 pounds pressure.

Cultures were grown in 150 ml of liquid media in 500-ml Erlenmeyer flasks kept on a horizontal shaker in a controlled temperature room at 23 to 25° C. The rate of shaking was 94 cycles per minute, each cycle consisting of two 10.2-cm strokes.

Sporidia were harvested by centrifugation at 4° C for 5 minues at 950 × g after 2 days' growth in a yeast extract medium or 3 days in synthetic media. The cells were washed 3 times with distilled water by re-suspension and re-centrifugation.

MANOMETRIC PROCEDURES: Sporidial respiration was measured at 20° C using conventional Warburg manometric techniques. Suspensions of washed cells containing 3 to 10 mg dry weight of cells and buffered at pH 6.0 with M/30 phosphate were used in each vessel. The concentration of cells was adjusted to fall within this range for each determination, as the Q_{O_2} is affected by cell concentration but is constant within this particular range. A 0.073 M concentration of sucrose was employed as the substrate, although glucose gives similar results. Three-tenths ml of 10 % KOH was used in the center well, and the total liquid volume in the main chamber was 3.0 ml. The vessels were shaken at a rate of 84 strokes per minute, each stroke consisting of two, 7.6-cm arcs. Dry weights were obtained by filtering suspensions through asbestos-lined Gooch crucibles and drying the cells at 85° C for 24 hours.

Sporidia were homogenized by grinding in a mortar with alumina (ALCOA No. A-301). The quantity of alumina used was 1 to 2 times the volume of the packed cells. The mixture was ground until it became fluid, alumina added again to form a thick paste, and the paste re-ground until it again became liquid. The slurry was transferred with 15 ml of 0.22 M sucrose and centrifuged at 978 × g for ten minutes at 4° C. The cell-free supernatant was then used as the enzyme preparation, either directly or after diluting by 1/3 with 0.22 M sucrose. Other methods of homogenization were tried but did not effectively break the cells of this organism.

The cytochrome oxidase activity in homogenates was determined essentially by the manometric method of Schneider and Potter (22). All determinations were made at 20° C in Warburg vessels as described for the measurement of respiration. Cytochrome c solutions were prepared in distilled water from the product of the Sigma Chemical Company, assuming a molecular weight of 16,500. A final concentration of 4×10^{-5} M cytochrome c was used throughout. Sixtieth molar ascorbic acid neutralized with KOH was used as the reductant, and 0.4×10^{-3} M $AlCl_3 \cdot 6H_2O$ and M/15 phosphate buffer at pH 7.0, all final concentrations, completed the system. Four concentrations of homogenate (0.25, 0.50, 0.75, and 1.0 ml) were used and sucrose solution added to give a total volume of 3.0 ml and a final sucrose concentration of 0.073 M.

All data reported here are based on the O_2 uptake during a half-hour period beginning 10 minutes after the ascorbate was tipped in. No appreciable O_2 consumption was observed before ascorbate was added, and a 5 or 10 minute lag followed the addition of ascorbate. A constant rate was then maintained for a half hour after which a decrease in O_2 uptake was observed.

A correction for the autoxidation of ascorbate was made by subtracting a value based on the pH of the vessel contents measured at the end of the run.

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[3] Composition of Medium A in quantities per l: 20 gm sucrose, 3.0 gm $NH_4C_2H_3O_2$, 3.0 gm K_2HPO_4, 0.80 gm $MgSO_4 \cdot 7H_2O$, 0.63 gm citric acid, 80 mg $FeSO_4 \cdot 7H_2O$, 0.14 mg $MnSO_4 \cdot 4H_2O$, 0.02 mg $CuSO_4 \cdot 5H_2O$.

These values were obtained by determining the rate of O_2 uptake in the absence of homogenate. Autoxidation is slight at pH 7.0 or below, but above 7.0 begins to rise, becoming very rapid at pH's above 8.0 O_2 uptake, when corrected by this method and plotted against low enzyme concentrations, usually extrapolates to zero activity at zero enzyme concentration. Assays for ascorbic acid oxidase were negative, and no appreciable O_2 uptake is observed in the absence of added cytochrome c.

Activity is expressed as μl of O_2 per hour per mg dry weight of homogenate (Q_{O_2} homogenate). The method of homogenizing does not permit the calculation of activity on the basis of dry weight of original cells, as only a portion of the cells are broken. The homogenate dry weight was obtained by centrifuging an aliquot for 40 minutes at 15,000 × g. The pellet formed was re-suspended in distilled water, recentrifuged, and the washed pellet then dried for 24 hours at 85° C. One to 4 mg/ml homogenate were obtained.

The increase in rate of O_2 uptake is not proportional to the increase in concentration of the homogenate at higher homogenate concentrations. A decrease in O_2 uptake is sometimes found when 1.0 ml homogenate is used (table II). No attempt was made to eliminate this decrease by means of purification of the enzyme preparation. The Q_{O_2} of homogenate was obtained from the plot of homogenate concentration against the rate of O_2 uptake corrected for autoxidation of ascorbate.

The inhibition of oxygen uptake by 0.001 and 0.0001 M sodium azide and potassium cyanide was calculated from simultaneous determinations at 4 homogenate concentrations, with and without the inhibitor. CO inhibition was determined in the dark at 4 homogenate concentrations with an atmosphere of 95 % CO and 5 % O_2 and compared to a system with 95 % N_2 and 5 % O_2. A light intensity of 100 fc at the manometric vessel level was used to test for light reversal of the inhibition.

SPECTROPHOTOMETRIC PROCEDURES: The conventional methods of cytochrome c extraction employing hot or cold trichloracetic acid, 0.1 M phosphate, or 0.1 *N* sulfuric acid fail to extract the pigment from this organism. Plasmolysis, homogenization, and subjection of the sporidia to alternate periods of freezing and thawing also fail to release the cytochrome c. A method was therefore worked out for the quantitative determination of cytochrome c in intact cells. Such a method has a definite advantage over methods involving extraction of the pigment, since it avoids unassessed errors arising from incomplete extraction. Recently, however, Neilands (19) has found that cytochrome c may be extracted from *Ustilago sphaerogena* by raising the pH of the suspending medium to 10. Neilands found that the absorption spectrum of the extracted smut-cytochrome c is very similar to that of animal cytochrome c, but that the fungus pigment has a higher molecular weight and lower isoelectric point than the beef heart preparation.

For the investigations described here, a spectrophotometric method was used. It is based on the difference in absorption between a suspension of cells in which the cytochrome c is reduced and one in which the pigment is oxidized. Reduction is readily achieved by the addition of 20 mg ascorbic acid and 0.05 ml of 0.02 M KCN to 3.0 ml cell suspension containing 15 to 45 mg dry weight of cells. Potassium ferricyanide, 0.05 ml of a 0.02 M solution/3 ml suspension, can be used to oxidize the endogenous cytochrome but only after the suspension has been heated for 1.5 hours at 60° C. Because heating changes the background absorption, a suspension was heated and then separated into aliquots to be oxidized and reduced.

All measurements were made on a Beckman photoelectric quartz spectrophotometer, Model DU, using 1-cm, matched, Corex cells. The measurements were made by determining the percentage transmission of the reduced suspension when an oxidized suspension was used as a blank adjusted to 100 % transmission. The absorption spectrum obtained in this manner with solutions of oxidized and reduced cytochrome c is similar to that calculated by Lundegårdh (16) as the ox/red spectrum. He showed that the height of the absorption bands of cytochrome c, when plotted as the difference between the extinction of the reduced and oxidized forms, varied approximately linearly with the percentage of reduced cytochrome c. Chance (3), using suspensions of microorganisms, demonstrated that the difference spectrum is a valid measure of the pigment content.

In the assay for smut cytochrome c, measurements were taken in the above manner at two wave lengths, 540 and 550 mμ. At the former wave length, absorption of the oxidized and reduced forms is almost equal, and at the latter, absorption of the reduced form is maximal for the visible portion of the spectrum. The increase in absorption at 550 mμ over that at 540 mμ is used as the basis for calculating the concentration of cytochrome c. This increase is referred to here as the relative absorption. The optical density is then calculated as log I_0/I, where I_0 is the intensity of the incident light, in this case the transmission of the oxidized form, or 100 %. I is the intensity of the transmitted light, in this case the lower transmission whose reduction is due solely to the absorption by reduced cytochrome c. I is calculated by substracting from 100 the difference in the percentage transmission at the two wave lengths specified. The relative optical density then becomes: $2 - \log\ [100 - (\%\ \text{transmission}_{540\ m\mu} - \%\ \text{transmission}_{550\ m\mu})]$.

The relative optical density of solutions of pure cytochrome c determined in this manner is proportional to the concentration of cytochrome c from 4×10^{-5} M to 3×10^{-7} M. Cell suspensions also follow the Lambert-Beer law, except in very dilute solutions (fig 1). Successive additions of aliquots of a solution of pure cytochrome c to a cell suspension also give a linear relationship between relative opti-

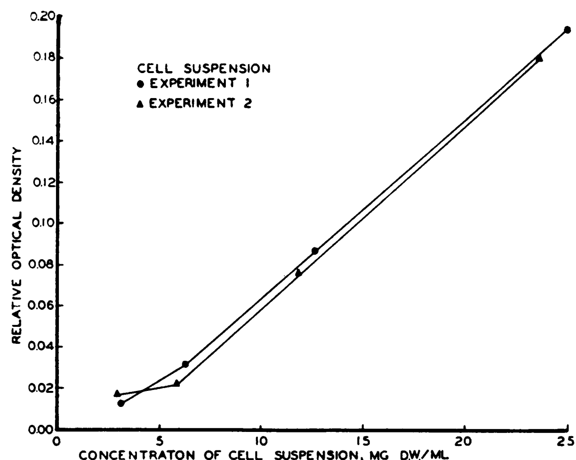

FIG. 1. The relationship between the concentration of endogenous cytochrome c in cell suspensions and the relative optical density.

cal density and the concentration of added cytochrome c.

Using the relative optical density as calculated above, the relative extinction coefficient (a) of cytochrome c was determined from solutions of cytochrome c of known concentration (c) and the diameter of the vessel (l = 1.0 cm). From the Lambert-Beer law, $c = \frac{\log I_o/I}{a\,l}$, concentrations of cytochrome c in unknown solutions and in cell suspensions were then calculated using this coefficient. Concentrations of cytochrome c are expressed here as a percentage of the dry weight of the cells. Dry weights were determined as described for the measurement of Q_{O_2}.

SPECTROSCOPIC PROCEDURES: Cytochromes a and b were detected and studied by visual observation of their absorption bands at 605 and 565 mμ, respectively. A diffraction grating spectroscope and a tungsten filament lamp were used for this purpose.

EXPERIMENTAL RESULTS

CYTOCHROME SYSTEM OF CELLS GROWN IN YEAST EXTRACT: The maximal cytochrome c concentration in cells cultured in Y.E. is found after 2 days' growth and in modified Czapek's medium after 3 days (table I). Cells produced in other synthetic media, like those from Czapek's medium, contain maximal quantities of cytochrome c after three days' growth. The isolate used in this particular analysis has a lower and more variable cytochrome c content than the isolate used in the other investigations reported here. Slightly more than 1 % Y.E. is required for the production of maximal concentrations of cytochrome c (fig 2).

TABLE I

CYTOCHROME C CONTENT OF CELLS FROM CULTURES OF DIFFERENT AGES

AGE IN DAYS	Y.E. MEDIUM	CZAPEK'S MEDIUM
	% OF DRY WEIGHT	
1	0.08	...
2	0.48	0.03
3	0.43	0.07
4	0.21	0.07
5	0.30	0.05
6	0.22	0.03
7	0.14	0.03

Differences in the levels of other cytochromes are also observed in cells from different media. Spectroscopic examination of cell suspensions from Y.E. reveals, along with the striking absorption bands of reduced cytochrome c at 550 and 520 mμ, faint bands of cytochrome a and b at 605 and 565 mμ, respectively. Suspensions of white cells obtained from synthetic media show no regions of absorption, or, at best, only a faint band of cytochrome c at 550 mμ.

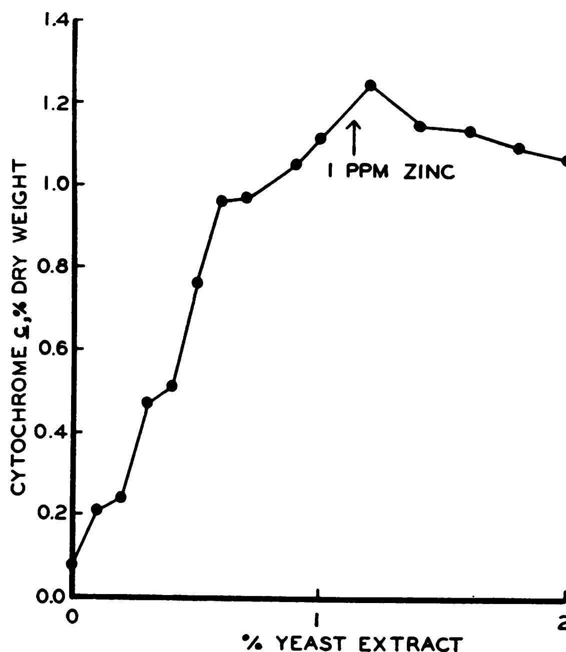

FIG. 2. The influence of yeast extract concentration on cytochrome c production. The concentration of yeast extract which contains 1.0 ppm Zn is indicated by the arrow.

Spectroscopic observation of cells grown in Y.E. also reveals that cytochrome oxidase is present and functional in the intact cell. The band of reduced cytochrome c disappears upon aeration of a suspension and re-appears when aeration is stopped. The activity of the oxidase in homogenates was also investigated. The optimum pH is about 8.0 in phosphate buffer. The effect of pH and buffer composition is shown in figure 3. Because of the high autoxidation above neutrality, activities were determined at pH 7.0 rather than at the optimum pH.

The activity of cytochrome oxidase in homo-

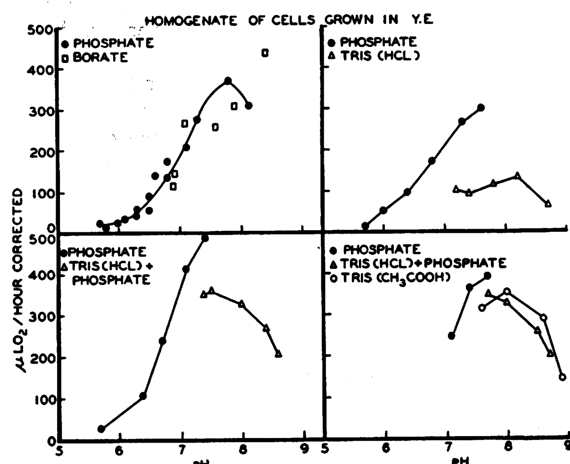

FIG. 3. The influence of pH and buffer composition on the cytochrome oxidase activity of homogenates. All buffers were used at a concentration of M/15, and 1.0 ml homogenate was used in each vessel. Phosphate and boric acid–KCl do not maintain the pH of the system well at pH 8 and above. Tris-(hydroxymethyl)-aminomethane, adjusted to pH 7.0 to 9.0 with HCl, buffers the reaction mixture very well but inhibits O_2 uptake, although this buffer has been recommended for use in some biological systems (13). The addition of phosphate or the use of acetic acid in place of HCl partially abolishes this inhibition.

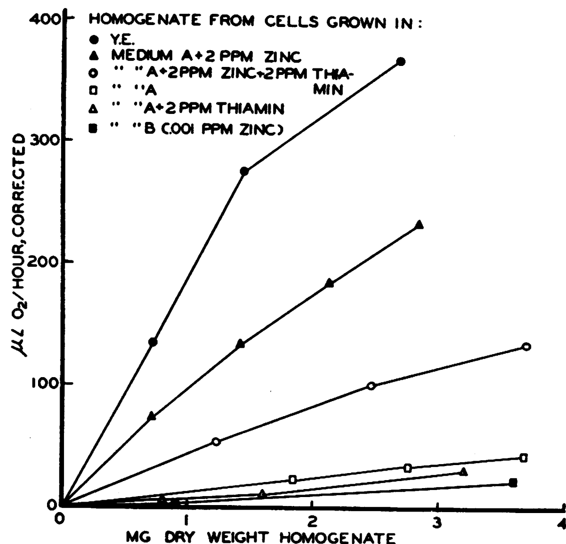

FIG. 4. Cytochrome oxidase activities at varied concentrations of homogenates of cells grown in different media.

genates of cells from Y.E. is higher than that in homogenates of cells from synthetic media (fig 4). The identity of the active enzyme as cytochrome oxidase was further substantiated by the effect of cyanide, azide, and CO and the light reversibility of the carbon monoxide inhibition (tables II, III).

In the experiments reported here, the activity of cytochrome oxidase was studied using animal cytochrome c prepared by the Sigma Chemical Company. The cytochrome c from *U. sphaerogena* is also catalytically active with the cytochrome oxidase of the smut, and the activity is of the same order of magnitude as found with animal cytochrome c.

Despite striking differences in cytochrome oxidase activity of homogenates from cells grown in Y.E. and synthetic media, there are no significant differences in the respiratory rates of intact cells as long as the pH of the nutrient medium is maintained above 4.5 during growth (table V). The Q_{O_2} of the intact cells with sucrose is about 30. Assays for ascorbic acid oxidase, lipoxidase, tyrosinase, and catecholase were negative. The absence of these other oxidases and presence of cytochrome oxidase in homogenates and the observed oxidation-reduction in cells point to cytochrome oxidase as a participant in the respiration of the cells. Despite these facts, this smut, like the fungus *Myrothecium verrucaria* (6), is not inhibited by azide, cyanide, or CO although shown to have a cytochrome oxidase system.

FACTORS RESPONSIBLE FOR CYTOCHROME PRODUCTION: Because of the high levels of cytochrome c produced under favorable conditions, its presence can be detected and the amount estimated roughly by visual observation of the color of packed cells. Using color as a criterion of cytochrome content, the factor in yeast extract responsible for cytochrome c

TABLE II

CO INHIBITION OF CYTOCHROME OXIDASE ACTIVITY OF HOMOGENATES OF CELLS GROWN IN Y.E. PERCENTAGE INHIBITION GIVEN IN PARENTHESES

TIME PERIOD		95 % N_2, 5 % O_2				95 % CO, 5 % O_2		
		ML HOMOGENATE				ML HOMOGENATE		
		0.25	0.50	0.75	1.0	0.25	0.50	1.00
		μL O_2/HOUR, CORRECTED FOR AUTOXIDATION						
First 30 min.	Dark	93	136	145	116	3 (95%)	20 (85%)	35 (70%)
Second 30 min.	Light	84	112	118	94	19 (78%)	43 (62%)	83 (12%)
Third 30 min.	Dark	79	101	106	83	1 (99%)	10 (91%)	32 (61%)
Fourth 30 min.	Light	76	95	94	72	19 (75%)	27 (71%)	68 (5%)

TABLE III

CO, CYANIDE, AND AZIDE INHIBITION OF CYTOCHROME OXIDASE ACTIVITY OF HOMOGENATES OF CELLS GROWN IN Y.E.

INHIBITOR	CONCENTRATION OF INHIBITOR	% INHIBITION AT 1.0 MG DRY WEIGHT OF HOMOGENATE
CO–dark	95 %	76
CO–light	95 %	30
KCN	0.001 M	99
KCN	0.0001 M	86
NaN_3	0.001 M	88
NaN_3	0.0001 M	53

production was determined. Initial experiments showed the active substance to be a dialysable, heat stable, ether insoluble component of Difco yeast extract. Neilands (unpublished) observed some formation of cytochrome c in a modified, double-strength Henderson-Snell medium, more than in other synthetic media but less than in yeast extract. This medium contains inorganic salts, sucrose, vitamins, and high concentrations of the 10 essential amino acids. Experiments with this basal medium indicated that the complete amino acid fraction was usually required for cytochrome c production, but sporadic appearance of pink cells was also observed with arginine, isoleucine, and especially methionine. Spectroscopic checks confirmed the presence of cytochrome in these cells. It could not, however, be obtained at will, suggesting the possibility that a contaminating substance was responsible for cytochrome c formation in these cultures. The recognized avidity of amino acids for heavy metals (1) and the release of heavy metals from new glassware (26) further suggested that the contaminant required might be a metallic ion. Tests with dithizone indicated the presence of relatively high concentrations of heavy metals in the amino acid solutions employed. Further credence for this suggestion was found in the observation of Allen (2) that heavy metals other than Fe were involved in cytochrome production by this smut.

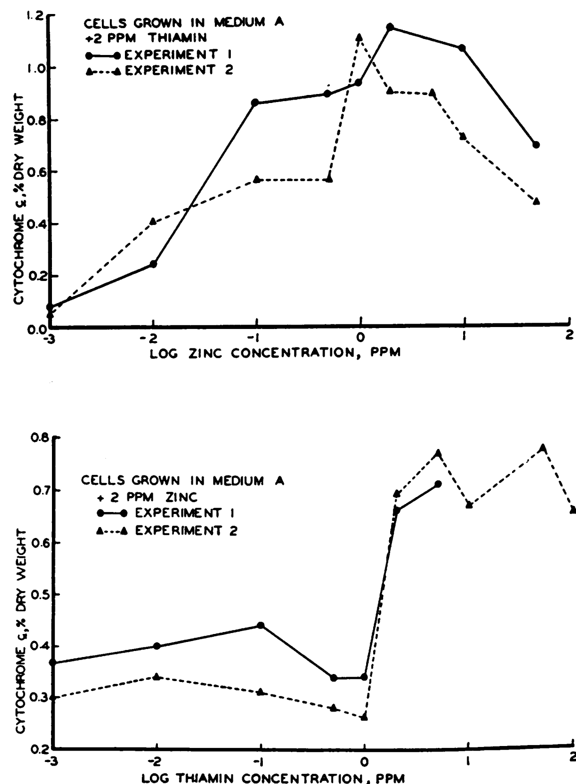

FIG. 5. The influence of Zn and thiamin concentration on cytochrome c production.

The pH of the various synthetic media employed in the preceding experiments dropped rapidly to 2.0 or 3.0 during the growth of the smut. In an attempt to find a synthetic medium in which the pH was maintained during growth at a level comparable

TABLE IV

CYTOCHROME c CONTENT OF CELLS GROWN IN DIFFERENT MEDIA *

EXPT. NO.	Y.E.	MEDIUM A: NO SUPPLEMENTS	+2 PPM THIAMIN	+2 PPM ZN	+2 PPM THIAMIN +2 PPM ZN
	% OF DRY WEIGHT				
1	0.78	0.14	0.07	0.45	0.59
2	0.76	0.10	0.06	0.52	1.14
3	0.90	0.03	0.09	0.52	0.90
4	1.06	0.05	0.01	0.44	0.52
5	0.77	0.01	0.01	0.27	0.69
Av.	0.85	0.07	0.05	0.44	0.77

* Y.E. cultures two days old. Other cultures three days old.

to that of yeast extract (pH 5.0 to 6.0), the medium given in footnote 3 was devised. Ammonium acetate was used instead of the nitrate or ammonium compounds of other synthetic media and was found to maintain the pH above 4.5 during growth. Increases in Mn, Cu, and Fe concentrations over those used in previous media did not effect cytochrome c production. However, the addition of higher concentrations of Zn did promote the production of pink cells. Because an effect of the complete vitamin solution had also been noted with the modified Henderson-Snell medium previously employed, vitamins were added to the revised medium. From experiments with vitamins added singly to this revised medium, it was found that thiamin, although ineffective when added without Zn, increased the amount of cytochrome produced in the presence of Zn.

One ppm Zn in the presence of thiamin promotes the formation of maximal quantities of cytochrome c (fig 5). Fifty ppm slightly inhibits cytochrome c production, and white cells are again formed when

TABLE V

CYTOCHROME OXIDASE ACTIVITY OF HOMOGENATES OF CELLS GROWN IN DIFFERENT MEDIA

	Medium A			
Yeast extract	No supplements	+2 ppm thiamin	+2 ppm Zn	+2 ppm Zn +2 ppm thiamin
	Q_{O_2} of homogenate			
248	25.5	5.0	98	61
220	18.0	2.0	98	56
190	7.0	..	..	54
187	6.5	..	..	52
170	6.0	..	..	44
169	3.5	..	..	..
156	...	..	..	..
Av. 191	11.1	3.5	98	53
Respiration of cells grown in different media. Values as Q_{O_2}.				
Av. 32	29	29	28	30

the concentration is increased to 100 ppm Zn. The optimal concentration of thiamin for cytochrome c production is 2.0 ppm in the presence of 1.0 ppm Zn (fig 5). Thiamin maintains the pH of the nutrient medium at 6.0 during growth, instead of the usual pH of 4.5, but this effect is observed at a concentration of 0.01 ppm, far lower than the optimal concentration for cytochrome c production. Only white cells are produced if thiamin is added to the basal medium in the absence of Zn, and there is no increase in cytochrome c (table IV). In the revised synthetic medium containing 1 ppm Zn and 2 ppm thiamin, cytochrome c production is comparable to that in a yeast extract medium (table IV). The effect of yeast extract is closely correlated with the concentration of Zn in a yeast extract solution (fig 2).

Spectroscopic observation revealed that cytochromes a and b as well as c are present in cells grown in the nutrient medium with Zn but are not detectable in cultures from low Zn media. The cytochrome oxidase activity of homogenates is likewise higher for cells grown in Zn sufficient media but falls short of that obtained from cells grown in yeast extract (fig 4). Thiamin does not bring about an additional increase, as it does with cytochrome c production, but reduces the activity below that found when Zn alone is added (table V). The inhibition of cytochrome oxidase by cyanide and azide is of the same magnitude in homogenates of cells from synthetic media as from yeast extract media (table VI).

The similarity in the effects of Zn and of yeast extract is not confined to the production of cytochromes but extends to the morphology of the sporidia. Cells grown in Y.E. are ovoid with dense homogeneous contents, whereas those grown in media containing little Zn are filamentous and vacuolate. When 1.0 ppm Zn is added to the basal medium, the cells formed are shorter, wider, less vacuolate, and branch frequently. Although not completely identical, they resemble the cells from Y.E. much more closely than those from media low in Zn. Thiamin, on the other hand, has no effect on cell morphology in either the presence or absence of Zn.

Zn is required for the growth of this smut, but the optimal concentration is 0.001 ppm, far less than the optimal concentration, 1.0 ppm, for cytochrome production. No inhibition of growth is observed at Zn concentrations as high as 100 ppm. A slight increase in growth is observed when thiamin alone is added to the basal medium. Only half of the total growth from Y.E. is obtained from the synthetic medium supplemented with both Zn and thiamin. Maximum total growth is obtained in two days in Y.E. and in three days in synthetic media. Neither thiamin nor Zn in high concentrations affects the rate of growth.

Re-examination of the contribution of other components of the synthetic medium showed that neither Mn nor Cu could replace Zn for cytochrome production. If either is required for cytochrome production or growth, the amounts necessary are small and are supplied as contaminants in the unpurified medium.

TABLE VI

CYANIDE AND AZIDE INHIBITION OF CYTOCHROME OXIDASE ACTIVITY OF CELLS GROWN IN DIFFERENT MEDIA

		ML homogenate							
		0.25		0.50		0.75		1.00	
Medium	Concentration of inhibitor	KCN	NaN_3	KCN	NaN_3	KCN	NaN_3	KCN	NaN_3
		% inhibition							
Y.E.	0.001 M	100	92	100	90	98	87	99	82
Y.E.	0.0001 M	87	57	87	49	85	37	79	34
Medium B	0.0001 M	...	..	91	77	..	..	76	46
Medium A	0.0001 M	...	..	96	..	..	..	92	43
Medium A +2 ppm Zn +2 ppm thiamin	0.0001 M	...	..	97	76	..	..	93	33

Cu inhibits growth at concentrations greater than 0.25 ppm in the absence of Zn, but in the presence of added Zn, higher concentrations of Cu are required to inhibit. Different samples of both zinc chloride and zinc sulfate were equally effective in promoting maximal cytochrome production.

Although nitrate-, ammonia-, and amino-N are adequate N sources for the growth of this fungus, the ammonium ion is specifically required for cytochrome production. When amino acids or nitrates are employed, only white cells are obtained even though all other conditions for cytochrome production, the presence of Zn and the maintenance of pH above 4.5 during growth, are fulfilled. The presence of acetate serves only to maintain the pH at this level and is not specifically required for pigment production. Pink cells are produced when the pH is maintained above 4.5 by means other than the use of acetate. The maintenance of this pH, however, is necessary for cytochrome production. If a salt such as NH_4Cl is used, the pH falls to 2.0 during growth and little cytochrome is formed.

SYNTHESIS OF CYTOCHROMES BY SUSPENSIONS OF WASHED CELLS: When *Ustilago sphaerogena* is grown in 1 % Y.E. without added carbohydrate, cell masses of a tan color are obtained. No bands of cytochromes a, b, and c appear in suspensions of these cells, but a broad, ill-defined absorption band is found at 555 mμ. When washed cells of this nature are suspended in a medium containing sucrose and phosphate, the band at 555 mμ disappears and the bands of the normal cytochromes appear within 8 hours. Sucrose and phosphate are the only supplements required for this transformation. When white cells from a Zn-deficient synthetic medium are washed and suspended in a new medium for this period, Zn, ammonium ion, and Fe are required to bring about the formation of the cytochromes. Sucrose and thiamin increase further the amount of cytochrome c formed from such cells. No band at 555 mμ is observed in the Zn-deficient cells.

DISCUSSION

The high levels of cytochromes produced and the existence of well defined nutrient substances affecting their formation make this organism exceptionally favorable material for studying the synthesis and interrelationships of components of the cytochrome system. Beef heart muscle, generally used for the preparation of this pigment, contains only 191 μg cytochrome c per gm fresh weight of tissue (15). The pink mutant yeast obtained by Ephrussi and Slonimski (25) produces 0.41 % dry weight of cytochrome c, and rat tissues as much as 0.1 to 0.3 % dry weight (7, 21, 27). These values are all considerably lower than the average cytochrome c content of *U. sphaerogena* sporidia grown in Y.E., 0.85 %, or in medium A supplemented with 1 ppm Zn and 2 ppm thiamin, 0.77 %.

Although neither Zn nor thiamin have previously been found to influence the formation of cytochrome or cytochrome oxidase, several other nutrients have been reported to influence their production. Fe is, of course, necessary, and Fe-deficiency has been shown to reduce cytochrome synthesis in yeast (9, 31). Cu increases the level of the cytochrome pigments (5) and of cytochrome oxidase (23) in rat tissues and in yeast (9, 31), but we have found no evidence for a comparable role of Cu in *U. sphaerogena*. Rats fed with glycine-2-C^{14} were shown by Drabkin (8) to incorporate the C^{14} into cytochrome c in the liver. The utilization of glycine in hemoglobin synthesis in duck blood has been shown by Shemin and Wittenberg (24). They found that the entire porphyrin was synthesized from acetate and glycine through a tricarboxylic acid intermediate. In *U. sphaerogena,* however, glycine cannot replace the ammonium ion required for cytochrome formation. Neither glycine nor acetate enhance cytochrome production in the smut, although they support good growth of white sporidia in the absence of other N and C sources. Tipton (28) found no decrease in cytochrome oxidase level in thiamin deficient rat tissues.

The production of cytochrome is not controlled merely by nutrient conditions, however. Ephrussi and Slonimski (10) showed that O_2 was necessary for cytochrome formation in yeast and that the production of cytochrome oxidase depended upon the presence of the appropriate cytoplasmic particles (25). Chen, Ephrussi, and Hottinguer (4) described the genetic mechanism for the inheritance of the ability to produce components of the cytochrome system. In insects, Williams (30) has shown a correlation between hormone concentration and cytochrome oxidase level during metamorphosis, and Tipton (28) and Drabkin (8) have found that thyroxine administration increased the level of cytochrome oxidase in rat tissues.

In *U. sphaerogena,* however, Zn is the limiting factor for cytochrome production. The function of the high cytochrome oxidase and cytochrome pigment level resulting from the addition of Zn is not clear. These high levels do not provide for a higher rate of respiration, which is of the same magnitude in both pink and white cells. If the greater concentrations of cytochrome and cytochrome oxidase are of value to the organism, it must therefore be in some function other than respiration, possibly connected with the parasitism of the fungus.

There have been several reports of Zn affecting the synthesis of other enzymes. Nason (17) found that Zn deficiency in Neurospora inhibited the condensation of serine and indole to form tryptophane, but tryptophane cannot replace Zn in *U. sphaerogena.* Aldolase (20), alcohol dehydrogenase (18), and pyruvic oxidase (12) have all been reported to decrease or disappear entirely from Zn deficient organisms.

In none of the above examples is the specific site of action of Zn known. In *U. sphaerogena,* Zn is most probably concerned in the formation of the porphyrin nucleus, because the level of several of the

cytochromes is increased. The importance of Zn in carbohydrate metabolism in fungi (11) and the role of an intermediate of the tricarboxylic acid cycle in porphyrin formation are also consistant with this hypothesis. However, no direct evidence is available which would exclude an action in the formation of the protein moiety of the cytochromes or in the incorporation of iron into the porphyrin nucleus.

Summary

1. A spectrophotometric method for the quantitative determination of cytochrome c in suspensions of intact cells is described.

2. The sporidia of *U. sphaerogena* grown in 1 % Y.E.–1 % sucrose medium are pink. The color is due largely to cytochrome c, which may constitute as much as 1.0 % of the dry weight of cells in this medium.

3. Sporidia grown in a simple synthetic medium, such as Czapek's or medium B, are white and contain a maximum of 0.08 % cytochrome c, although the yield of cell material is comparable with that obtained in a Y.E. medium and the Q_{O_2} of the cells is similar to that of the pink cells.

4. Y.E. also enhances the production of cytochromes a and b and of cytochrome oxidase. The oxidase has a pH optimum at about 8.0, and is catalytically active in oxidizing the cytochrome of smut sporidia in intact cells and in homogenates.

5. The substance in Y.E. responsible for high cytochrome production is Zn. It cannot be replaced by Fe, Cu, or Mn. Although only 0.001 ppm Zn is required for optimal growth, 1.0 ppm is optimal for pigment formation, which can be obtained in a completely synthetic medium.

6. The addition of 2.0 ppm thiamin results in a further increase in cytochrome formation in the presence of 1.0 ppm Zn, but thiamin is without effect in the absence of Zn and is not a required growth factor.

7. Cytochromes a and b and cytochrome oxidase are also increased by the addition of Zn to a synthetic medium, but not to the high levels produced in Y.E. Their production is not affected by thiamin.

8. The effect of Y.E. upon cell morphology is partially, but not completely, reproduced by Zn.

9. Ammonium ion is specifically required for the enhanced production of cytochrome c in the presence of Zn and thiamin. It cannot be replaced by nitrate or amino acids, although these compounds will serve as adequate sources of N for growth.

10. High cytochrome production requires that the pH be maintained above 4.5 during growth of the fungus and was achieved by the use of ammonium acetate.

11. Suspensions of washed cells from a medium containing Y.E. but no additional carbohydrate are low in cytochrome but will produce cytochrome if supplied with sucrose and phosphate. Similar cells from a Zn-deficient synthetic medium require Zn, ammonium ion, and Fe for cytochrome formation.

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AUXIN PHYSIOLOGY IN BEAN LEAF STALKS [1]

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Auxin production, auxin transport (8), and auxin inactivation (12) in tissues with relatively high auxin concentration have been extensively investigated and reviewed. Little attention, however, has been given to the auxin physiology of tissues with low auxin concentration. This paper reports an investigation of conditions associated with the low auxin concentration found in bean leaf stalks, a concentration of 0.4 μg equivalents of IAA per kg fresh weight (10).

In the authors' opinion, the extension of the term "auxin" to include all plant hormones, as well as all exogenous plant regulators (Zimmerman & Hitchcock, 19), ignores known differences between hormones and known differences between hormones and exogenous plant regulators, and is therefore confusing rather than helpful. In this paper the term "auxin" will be restricted to compounds manufactured by the plant (endogenous) which produce curvature in the standard Avena coleoptile assay. Synthetic compounds (exogenous) will be specifically named; indoleacetic acid will be designated by its abbreviation, IAA. Inactivation is considered a chemical alteration of a compound so that it no longer produces its characteristic chemical or biological reactions. An inactivator is a substance or biochemical system capable of inactivating a specific compound. Inhibition is a reduction or retardation of a response. An inhibitor is a substance which inhibits.

The material used in this investigation consisted of stalks bearing trifoliate leaves of greenhouse-grown Black Valentine beans. The leaves were in all cases fully expanded and showed no sign of senescence. Auxin was determined by the standard Avena coleoptile curvature assay (18), and IAA equivalents were calculated by the formula of van Overbeek (16). The blocks used in all experiments were 1.5 % agar, $2.7 \times 2.7 \times 1.0$ mm. Twelve blocks were used in each part of each experiment and each experiment was repeated at least once. The results given are based on the average curvature of 12 coleoptiles. Other details of method are included in the description of the experiments where they were used.

EXPERIMENTS

AUXIN PRODUCTION: No change in auxin concentration from the time the leaf is fully expanded until it is senescent (10) has been found in previous studies with bean leaf stalks on the plant. But a change in auxin concentration in excised bean leaf stalks was found in experiments here reported. In an experiment using three 35-gm lots of freshly cut bean leaf stalks, one lot was frozen, lyophilized, and its auxin extracted with ether. A second lot was submerged in water. The third lot was placed in air in a moist chamber. At the end of 24 hours the second and third lots were treated as the first had been, and their auxin extracted. The details of the method by which these extracts were obtained have already been described (10). The Avena assays of these three lots, reported as microgram equivalent of IAA per kilogram fresh weight, showed at the start of the experiment (first lot) an auxin concentration of 0.4 μg/kg; after 24 hours in air (second lot) a concentration of

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