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1953

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CYTOCHROMES AND THE SUCCINIC ACID OXIDASE SYSTEM OF POKY STRAINS OF NEUROSPORA*

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(Received for publication, September 11, 1952)

Recent investigations in this laboratory (1) have demonstrated that in *Neurospora crassa* growth characteristics representing at least two stable states are possible with the same constitution of nuclear genes. If a slow growing strain, designated *poky*, functions in a cross to wild type as the "maternal" or protoperithecial parent, then all progeny from the cross exhibit the *poky* character. If the cross is made in the reverse, or reciprocal manner, that is, with the wild type strain functioning as a protoperithecial parent, then all progeny are normal in growth rate.

During the course of the work on inheritance it was observed that mycelial pads from *poky* were characterized by a red color reminiscent of that of the heme pigments. Examinations of suspensions and extracts of the mold with a spectroscope yielded the information that *poky* mycelium contains large quantities of a substance having the absorption spectrum characteristic of cytochrome *c*, whereas the bands corresponding to cytochromes *a* and *b* were not visible. All three of these components were detected easily in the wild type strain. The experimental results presented here provide more precise information on the similarities and differences between these two strains with respect to the cytochrome and the succinic acid oxidase systems. Preliminary observations suggested that *poky* in *Neurospora* is analogous to *petite* in yeast (2) in which abnormalities in the succinic acid oxidase system have been reported, but this analogy is shown here to be only a partial one.

EXPERIMENTAL

Strains and Culture Methods—The origin of the *poky* strains has been described previously (1). Most of the enzyme preparations were made from *po*-1803-4A (1) or from a standard wild type P1347-2a.

Both strains were cultured at 25° in minimal medium (3) contained in

* This work was supported by funds from the Williams-Waterman Fund for the Combat of Dietary Diseases, the Rockefeller Foundation, and by funds from the Atomic Energy Commission administered through contract with the Office of Naval Research, United States Navy, contract No. N-6-onr-244, Task Order 5.

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Published in JOURNAL OF BIOLOGICAL CHEMISTRY 200:2 (1953), pp. 819-826.

2.5 or 5 gallon Pyrex bottles. In addition to inlet and outlet tubes for aeration, each bottle was equipped with a siphon tube to permit sterile sampling at various time intervals. A portion of mold from the last sample taken from each bottle of *poky* was subcultured on an agar slant and tested to make certain that reversion or contamination had not occurred. Yields of mold produced in given intervals of time are shown in Table II.

Cytochromes a, b, and c—With a Zeiss low dispersion spectroscope examinations were made of a high intensity light transmitted through standard quantities of thick suspensions of mycelium and cell-free preparations (described below). Sodium hydrosulfite or succinate was used as a reducing agent. Under these conditions wild type mycelium showed typical absorption bands corresponding to cytochromes *a*, *b*, and *c*, as seen, for example, in a preparation from heart muscle (4). Mycelium from *poky* at 3 or 4 days showed a very strong band at 550 mμ (cytochrome *c*), but at room temperature the α-bands of cytochromes *a* and *b* were not visible. However, when mycelium or cell-free preparations from a 3 day-old *poky* culture were suspended in 50 per cent glycerol and observed after freezing in liquid nitrogen (5), the cytochrome *a* and *b* bands were discernible. It was estimated that these components were present in less than 2 per cent of the normal concentrations.

More detailed observations were made on the cytochrome *c* content of wild type and *poky* mycelium. For this purpose samples of mold of different ages were dried *in vacuo* over P_2O_5 at 40°. Samples (5 to 30 mg.) of the finely powdered product were suspended in 0.2 ml. of dilute buffer contained in 8 $\times$ 15 mm. vials. All samples were reduced by addition of sodium hydrosulfite. A series of standard solutions containing cytochrome *c* (Sigma) in the range of 30 to 200 γ was prepared. By means of direct spectroscopic comparisons of the solutions and suspensions, and of mixtures of these, relative intensities of absorption bands were estimated visually. Calculated cytochrome *c* concentrations are given in Table I. Comparative values are obviously more significant than the absolute concentrations. The above method was utilized in preference to the more common acidic extraction procedure, since the latter gave very low yields of cytochrome *c* with this material. Subsequently, it was found that good extractions (approximately 70 per cent based on the direct spectroscopic method described above) could be obtained by treating the ground mold with half saturated $(NH_4)_2SO_4$ at pH 7.5.

In order to confirm the conclusion that an alteration of the cytochrome system is associated with the cytoplasmically inherited *poky* character, strains derived from each spore pair of *poky* and not *poky* asci were examined. The asci used were *po*-1720, *po*-1803, and *po*-1852 (all *poky* strains), from crosses in which the "maternal" parent was *poky* and the fertilizing

parent wild type, and 1723 and 1849 (all not *poky* strains) from the reciprocal crosses (1). All strains from the *poky* asci were deficient in cytochromes *a* and *b* and all had a great excess of cytochrome *c*. All strains from the not *poky* asci were, on the other hand, normal with respect to cytochromes.

Enzyme Preparations—Samples of mold were washed four times in 20 parts of distilled water. After filtration with suction, the moist pads were chilled at 0° and then mixed with 0.5 part of sand contained in a cold mortar. After addition of 2.5 parts of cold buffer (0.05 M phosphate, pH 7.0, supplemented with 75 gm. of mannitol per liter) the mixture was ground vigorously for 5 minutes. The resulting slurry was then diluted with cold buffer to give a total of 5 parts of buffer to 1 part of moist myce-

TABLE I

Cytochrome c in Wild Type and Poky Strains of Neurospora

Age of culture	Cytochrome *c*, estimated per cent of dry weight of mold	
	Poky	Wild type
days		
1		0.15
2	3.0	0.20
3	1.6	0.18
4	1.2	0.18
6	0.7	0.22
8	0.5	

lium. After a thorough mixing, cell debris and sand were removed by centrifugation at 40 × *g* (bottom of tube) for 10 minutes in a model L Spinco refrigerated centrifuge. Residues were discarded, and the cell-free supernatant suspensions were again centrifuged. In most experiments a force of 60,000 × *g* was applied for 40 minutes at this stage of preparation, although it was observed that most of the particles that carry the succinic acid oxidase system were sedimented at forces of less than 6000 × *g*. Particle fractions obtained in this fashion were suspended in buffer, centrifuged, and resuspended in the original volume for use in the determination of enzyme activities. Aliquots were removed and treated with trichloroacetic acid to precipitate the proteins and nitrogen was determined by nesslerization after digestion of the precipitates with H_2SO_4 and H_2O_2. The results are given in Table II. Satisfactory preparations were also obtained by use of phosphate buffer without mannitol, but it was found to be essential that buffer be added to the moist mold before grinding. When this was not done, a part of the succinic acid dehydrogenase activ-

ity was found in the non-sedimented fraction. All of the supernatant solutions from the particle preparations that were used in the experiments described here were tested and found to be practically devoid of succinic acid oxidase, cytochrome oxidase, and succinic acid dehydrogenase activi-

TABLE II

Comparisons of Succinic Acid Oxidase, Cytochrome Oxidase, and Succinic Acid Dehydrogenase Activities of Particle Preparations from Wild Type and Poky Strains of Neurospora

The data on growth rates and protein nitrogen of the preparations are included.

Age of culture	Weight of washed mold (dry)	Protein N of particle suspension	Q_{O_2} (N) values (μl. O_2 per mg. N per hr.)		
			Succinic acid oxidase	Cytochrome oxidase	Succinic acid dehydrogenase*
			Wild type P1347-2a		
days	*gm. per l.*	*mg. per ml.*			
1	0.27	0.096	350	1580	16
1†	1.4	0.28	223	445	17
3	2.8	0.38	131	390	22
3†	3.6	0.32	176	389	35
6	3.3	0.35	251	362	33
7†	4.0	0.33	232	451	34
			poky 1803-4A		
3	0.14	0.53	0‡	0‡	39
3†	0.34	0.32	23	40	39
4	0.66	0.35	0‡	9	30
5	1.3	0.42	160	116	27
6	1.4	0.30	276	168	48
7	1.7	0.36	218	195	35
9	1.7	0.35	227	165	33

* Methylene blue calculated as microliters of O_2.

† Mold grown from a very heavy inoculum.

‡ No activity detected <5 μl.

ties. It should be noted, however, that while all the particle preparations from *poky* were much more highly colored than those from wild type, the supernatant solutions from *poky* were also pink due to dissolved cytochrome *c*. It is not known whether this is an artifact due to the preparative method or a characteristic of *poky*.

Determinations of Enzyme Activities—Relative rates of reactions were determined for succinic acid oxidase, cytochrome oxidase, and succinic acid dehydrogenase with the particle preparations described above. The reac-

tions were carried out at 35° with all reagents dissolved in phosphate-mannitol buffer. Appropriate controls were set up in each experiment, but only the corrected data are shown here.

Succinic acid oxidase and cytochrome oxidase activities were determined manometrically. In the former case the Warburg vessels with the complete system contained 2.4 ml. of enzyme preparation and 0.4 ml. of 4×10^{-4} M cytochrome *c* (Sigma). Side arms contained 0.2 ml. of sodium succinate (15 mg. per ml.) and the center wells 0.1 ml. of 4 N KOH on a 1 inch square of filter paper. For determinations of cytochrome oxidase activities the vessels with the complete system contained 2.0 ml. of enzyme, 0.4 ml. of buffer, and 0.3 ml. of 4×10^{-4} M cytochrome *c* (Sigma). Side arms contained 0.3 ml. of 0.25 M ascorbate and the center wells KOH as above.

Succinic acid dehydrogenase activity was determined by the Thunberg method. Rates of decolorization of methylene blue were followed by use of a Klett colorimeter. Tubes with the complete system contained 7.5 mg. of sodium succinate and 0.05 mg. of methylene blue in 5.5 ml. of buffer and the side arms contained 0.5 ml. of enzyme preparation. Initial rates of reaction were determined directly from colorimeter readings taken at 5 minute intervals.

Data obtained from these experiments, corrected for blanks, are summarized in Table II. Several significant points can be observed from the following results: (1) The growth characteristics of *poky*, relative to wild type, in submerged, aerated cultures are comparable to those shown previously (1) with standing cultures. The availability of oxygen does not appear to be an important factor in the determination of the growth characteristics of *poky*. (2) Succinic acid oxidase and cytochrome oxidase activities are very low during early stages of growth of *poky*. The former activity reaches a normal level in 6 day-old cultures, but the latter does not reach much more than half the normal value. These results are in accord with the spectroscopic observations on the cytochromes as reported above. (3) The level of succinic acid dehydrogenase activity in *poky* is normal or above normal in all preparations tested. This observation is somewhat surprising if it is assumed that succinic acid oxidase is identical with cytochrome *b*, since spectroscopic observations showed that young *poky* cultures contain only very small amounts of *b*.

The results reported in Table II for succinic acid oxidase activity were made in the presence of added cytochrome *c* (6). In all cases but one, activities found without this addition were at least two-thirds as high as the values given. The one exception was the preparation from the first wild type culture (Table II). Here the succinic acid oxidase activity was only one-tenth of that given in Table II when cytochrome *c* was omitted

from the reaction mixture. Wild type mycelium from this 1 day-old culture did not show spectroscopic abnormalities and it seems likely that the difference in this material is due to difficulties in "extraction" of particles, since the mold at this age is slimy and very difficult to grind properly.

"Cytochromase"—During the investigations on the succinic acid oxidase systems described above, it was noted that the reaction mixture, in vessels containing cytochrome *c*, succinate, and particle preparations from 3 or 4 day-old *poky* cultures, changed in color from red to green. The rate of this change was found to decrease with an increase in age of the mold. No change was observed with 6 day-old material. Examination of the absorption spectrum of the reaction mixture yielded the information that the cytochrome *c* originally present had been converted to a substance with a spectrum resembling that of biliverdin. The reaction requires oxygen, but oxidized cytochrome *c* is not acted upon. Succinate was found to be by far the most satisfactory substrate for providing reduced cytochrome in the system, but ascorbate and malate had significant effects. Glutathione, isocitrate, and 6-phosphogluconate were not effective. When coupled to succinate the reaction was inhibited completely by malonate (0.01 M), partially by cyanide (0.001 M), but not appreciably by azide (0.001 M) or arsenate (0.001 M). The supernatant solution from the high speed centrifugation showed a strong inhibitory action on the system, a fact that perhaps accounts for the simultaneous presence in the mold of a high concentration of cytochrome *c* and an enzyme system that destroys this substance. Efforts to demonstrate the existence of the cytochrome-destroying system in preparations from wild type were unsuccessful. In the presence of succinate and azide (to inhibit cytochrome oxidase) added cytochrome *c* was reduced but it was not converted to the green pigment.

Particle preparations from *poky* and wild type were mixed in the presence of succinate and azide (0.001 M) (35°) and samples were removed at intervals and reduced with hydrosulfite. Examination of these samples with the spectroscope showed that the absorption bands of cytochromes *a*, *b*, and *c* all disappeared within 30 minutes. On the other hand, the *poky* preparation did not act on hemoglobin, heme, or catalase.

DISCUSSION

Fig. 1 gives a generalized summary of the changes that occur with time in the succinic acid oxidase system of *poky*. Values are plotted relative to those of wild type. The wild type system is not completely unchanging with time in the normal strain, as can be noted in Tables I and II, but the variations are relatively minor as compared to those of *poky*. Values for "cytochromase" activity are not included in Fig. 1, since this system has not been detected in the wild type strain. As noted previously, the sys-

tem is very active in *poky* preparations from 3 or 4 day-old cultures but no activity was observed in 6 day-old material. This result is in keeping with the data summarized in Fig. 1 which show that with increasing age *poky* becomes more like wild type. It should be noted, however, that *poky* never becomes entirely normal and that the observed changes in enzyme patterns are repeated through successive transfers.

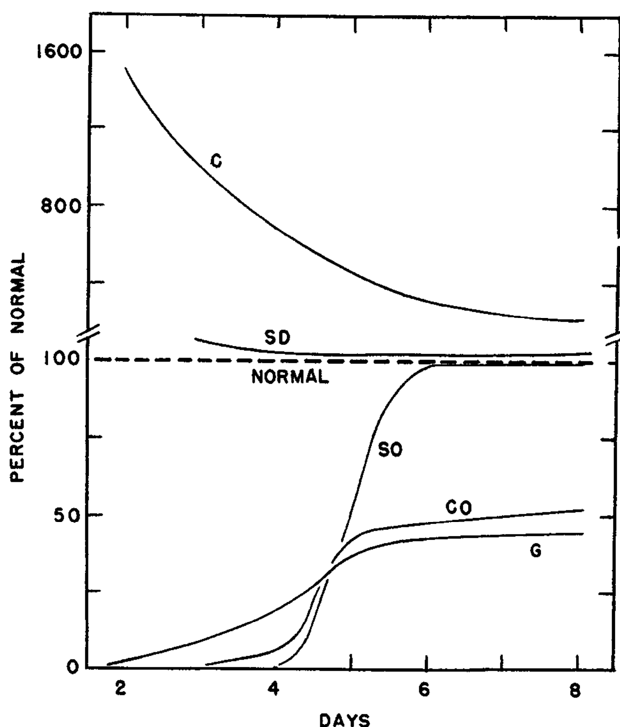

FIG. 1. Diagram showing relative growth (*G*), cytochrome *c* content (*C*), succinic dehydrogenase activity (*SD*), succinic acid oxidase activity (*SO*), and cytochrome oxidase activity (*CO*) of *poky* as compared to wild type. The latter is represented by the horizontal dotted line at the level of 100 per cent.

Young *poky* cultures are similar to the *petite* strains of yeast described by Slonimski and Ephrussi (7) in that both are deficient in succinic acid oxidase and cytochrome oxidase activities. Both possess abnormally high concentrations of cytochrome *c*, but this accumulation appears to be much more extreme in *poky*. A striking difference between the yeast and the mold strains is that *poky* has a normal level of succinic acid dehydrogenase activity, whereas *petite* is reported to be deficient in this enzyme.

The experiments on *poky* described here have raised one other question of considerable interest. The data presented show that as the concentra-

tion of cytochrome *b* changes some 50-fold or more, starting at a very low level, there is no significant change in the activity of succinic acid dehydrogenase. These results seem to be in agreement with those of Tsou (8) and Chance (9) which indicate that cytochrome *b* and succinic acid dehydrogenase are not identical. Such a conclusion is not entirely justified on the basis of the present data, since even the youngest *poky* cultures observed contained traces of cytochrome *b*. However, by use of the "cytochromase" system which destroys *b* it should be possible to obtain further supplementary information. Preliminary results are in accord with the tentative conclusion stated above.

SUMMARY

The succinic acid oxidase system of a strain of *Neurospora crassa* which carries the cytoplasmically inherited slow growth character, *poky*, has been shown to differ markedly from that of the wild type mold.

1. The *poky* strain accumulates large amounts of cytochrome *c*.
2. Young *poky* is deficient in cytochromes *a* and *b*, and thus in succinic acid oxidase and cytochrome oxidase activities.
3. With increasing age of *poky* cultures an extreme change in the composition of the cytochrome system occurs.
4. Young *poky* cultures contain a system which destroys the cytochromes. This system has not been detected in wild type strains.
5. Succinic acid dehydrogenase activity in particle preparation from *poky* appears to be independent of the concentration of cytochrome *b*.

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