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STUDIES ON THE RESPIRATORY SYSTEM OF THE POKY STRAIN OF NEUROSPORA*

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It was shown recently that the *poky* strain of *Neurospora* accumulates large amounts of cytochrome *c*, but is deficient in cytochromes *a* and *b* and thus in cytochrome oxidase and succinic acid oxidase activities (1). These findings, which suggest that respiration in *poky* does not utilize the cytochrome system, led to a study of the respiratory system of *poky* compared with that of wild type. The experiments reported here are concerned with the effects of the inhibitors cyanide and azide on the respiration of intact mycelium of *poky* and wild type, the oxygen uptake of cell-free extracts in the oxidation of their endogenous substrates, the cofactor requirement for this oxidation, and the effects of azide and of oxygen tension. Evidence presented confirms that, in *poky*, respiration does not depend upon the cytochromes, but upon another terminal oxidase system in which flavin-adenine dinucleotide (FAD) enzymes appear to be concerned.

Materials and Methods

Strains and Culture Methods—The *poky* and wild type strains used in the course of this work, as well as the methods of culture, were described in previous papers (1, 2).

Respiration of Intact Mycelium—Manometric experiments were carried out with Warburg manometers, at 35°, in air, unless otherwise specified, with KOH in the center well.

Samples of mold were washed three times with 20 volumes of 0.05 M phosphate buffer, pH 7.0, and were resuspended in fresh buffer. The *poky* mycelium grown under forced aeration contained very short hyphae and gave a homogenous suspension which could easily be pipetted, even when the mold was 7 days old. With wild type, however, the respiration of only very young mycelium (8 to 26 hours) could be measured satisfactorily,

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as older mold, consisting of longer hyphae, did not form a homogenous suspension. The respiration of the mycelium was measured in 0.05 M phosphate buffer (pH 7.0) in the presence of glucose (0.05 M). It should be noted that the endogenous respiration of the washed mycelium grown with 15 gm. of sucrose per liter was not modified by addition of sucrose or glucose. In each experiment an aliquot of washed mold was dried at 110° for determination of the dry weight.

Cell-Free Extracts—The mold was washed three times with 20 volumes of distilled water, filtered with suction, and ground by hand in a mortar in the cold room (2°) with 0.5 part of sand and 1 to 3 parts of cold phosphate-mannitol buffer (0.05 M phosphate, pH 7.0, containing 75 mg. of mannitol per ml.). The mixture was centrifuged for 5 minutes at 1000 × *g*, and the supernatant solution was used as such (crude extract), or dialyzed at 2° for 4 hours against 0.05 M phosphate, pH 7.0 (dialyzed extract), or fractionated by centrifugation at 60,000 × *g* for 30 minutes in a model L Spinco refrigerated centrifuge to yield a clear supernatant solution and a pellet of sedimented particles. The particles were washed once in phosphate-mannitol buffer, centrifuged as above, and finally resuspended in one-fourth of the original volume of cold phosphate-mannitol buffer.

The dialysate was prepared by dialyzing 20 ml. of 5 day-old *poky* crude extract against 100 ml. of distilled water for three hours at room temperature. The dialysate was then concentrated to 5 ml. *in vacuo* at 50°.

Analytical Methods—Phosphogluconic acid dehydrogenase activity was measured in acetone powder of the mold according to the method of Horecker and Smyrniotis (3).

Catalase was estimated by a procedure similar to that of Bonnichsen *et al.* (4). Hydrogen peroxide solution (0.4 ml. of solution containing 1.5 ml. of 30 per cent H_2O_2 in 50 ml. of water) was added to 0.2 ml. of 0.5 M phosphate buffer (pH 7.0) and 8.4 ml. of water in a 50 ml. Erlenmeyer flask. 1 ml. of enzyme solution (1 ml. of buffer for the control) was added rapidly at zero time, and, after 20, 40, and 90 seconds, 2 ml. samples were withdrawn, mixed with 10 ml. of 2 per cent sulfuric acid solution, and titrated with 0.01 M potassium permanganate. The experiments were performed at 22°.

The orcinol pentose reaction was carried out according to the procedure of Mejbaum (5).

Total reducing sugar was determined colorimetrically by use of the reagents described by Dubois *et al.* (6). Samples (1 ml.) containing 10 to 100 γ of glucose were mixed with 1 ml. of 1 per cent phenol and 5 ml. of concentrated H_2SO_4 and allowed to stand 30 minutes. They were then compared with known standards by means of a Klett colorimeter. Particle preparations for sugar determinations were made in 0.2 M phosphate buffer (pH 7) without mannitol.

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For the purpose of following sugar disappearance from culture media, samples were chromatographed on paper cylinders (7) of Whatman No. 1 paper in a solvent consisting of 1 part of 1 per cent NH_4OH and 5 parts of *n*-propanol. Samples containing 2 to 20 γ of glucose (in 0.005 ml.) were placed on the paper near the unknowns. Chromatograms were developed by spraying dried sheets with 4 per cent benzidine in glacial acetic acid. After the sprayed sheets were heated at 100° for 10 minutes, sugar concentrations in the sample were estimated by a comparison of the intensities of the spots from the unknowns with those from the knowns.

For qualitative determination of flavins, the acetic acid-butanol solvent described by Crammer (8) was used for ascending chromatograms with Whatman No. 1 paper. 1 part of mold (wet weight) and 4 parts of 80 per cent methanol were ground with sand in the cold room (+2°), left standing for 10 minutes, and then centrifuged for 10 minutes at 1000 × *g*. The supernatant solution was used directly for paper chromatography.

Samples for quantitative determination of flavin and niacin were hydrolyzed by autoclaving at 15 pounds per sq. in. for 15 minutes in the presence of 10 parts of 0.1 M HCl. The resulting solutions were adjusted to pH 5 by addition of sodium acetate (2.5 M), and the concentrations of the vitamins were determined microbiologically with *Lactobacillus casei* and *Lactobacillus arabinosus* for riboflavin and niacin respectively (9).

Protein nitrogen was determined by nesslerization after digestion of trichloroacetic acid-precipitated material with sulfuric acid and H_2O_2.

Materials—The diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) used were cozymase 65 and TPN 80 from the Sigma Chemical Company. Glucose-1-phosphate and glucose-6-phosphate were obtained from the Schwarz Laboratories, Inc. The sample of 6-phosphogluconic acid was kindly supplied by Dr. R. S. Bandurski.

Results

Respiration of Intact Mycelium—The results presented in Table I show that (1) the Q_{O_2} of the slow growing strain, *poky*, is roughly one-third that of the wild type mold; (2) the respiration of *poky* is not markedly affected by azide or cyanide, while that of wild type is strongly inhibited; (3) even with wild type there is an appreciable amount of azide- or cyanide-insensitive respiration. Other results, not shown in Table I, demonstrated that the wild type respiration is not increased by using pure oxygen in the Warburg vessel, while the respiration of *poky* doubles under these conditions.

Respiration in Cell-Free Extracts—As is illustrated in Table II, the Q_{O_2} (N) of extracts of 3 day-old wild type was increased from 30 to 78.5 by addition of DPN and to 110 by addition of TPN. With 5 day-old wild type, DPN was relatively ineffective. The oxygen uptake by preparations

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from mold of either age was reduced about 50 per cent by 0.002 M azide in the presence of TPN, while with young *poky* (4 days old) azide had either no effect or it produced a slight stimulation. With 6 day-old *poky*, azide caused some inhibition. In contrast to those from wild type, preparations from *poky* did not show a dependence on DPN or TPN at any of the ages investigated. The results from experiments on the respiration of cell-free

TABLE I

Respiration of Intact Mycelium and Effects of Cyanide and Azide

	Age of culture	Q_{O_2}, μl. O_2 uptake per hr. per mg. dry weight	Per cent inhibition			
			0.003 M NaN_3	0.001 M NaN_3	0.003 M KCN	0.001 M KCN
	hrs.					
Wild type	8	70–75	80	67	88	55
	26	68–76	71	50	82	52
Poky	40	26	19	0	8	0
	48	22	14	0	4	0
	96	30	7	0	0	3
	168	33	0	0	0	18

TABLE II

*Oxygen Uptake by Crude Extracts and Effects of DPN, TPN, and Azide**

	Age of culture		+ Azide	+ DPN	+ TPN	+ DPN + azide	+ TPN + azide	+ TPN + DPN
	days							
Wild type	3	30.0		78.5	110.0	37.5	52.0	
	5	0	0	13.0	87.0	12.6	54.0	99
Poky	4	15.9		17.0	19.3	21.2	26.4	
	6	75.0	55.0	82.5	75.0	73.5	51.0	78

* The results are given as Q_{O_2} (N) (microliters of O_2 uptake per mg. of protein nitrogen per hour). The vessels contained added DPN or TPN in the amount of 0.1 mg. per 2.5 ml. of reaction mixture. Azide, 0.002 M.

extracts in the presence of pure oxygen were qualitative, like those obtained with intact mold. The use of oxygen in the place of air caused an increase in the Q_{O_2} (N) of *poky* (3 to 5 day-old cultures) of 100 per cent and an increase in the case of wild type (3 to 4 day-old cultures) of 20 to 60 per cent.

Oxygen Uptake by Dialyzed Extracts—Cell-free preparations from 3 or 4 day-old *poky* cultures lost nearly all of their activity after a 3 hour dialysis, but the addition of TPN was sufficient to restore the oxygen uptake to the level of the undialyzed preparation. The results of a typical ex-

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periment with a dialyzed extract of 5 day-old *poky*, with and without the addition of TPN and dialysate, are given in Fig. 1. When TPN alone was added to the dialyzed extract, the oxygen uptake became progressively slower than that of the crude extract plus TPN. However, in the presence of the dialysate, the oxygen uptake remained constant over a period of at least 1 hour. It was higher than that of the crude extract plus TPN, and it did not show any lag period in the beginning of the experiment. With a

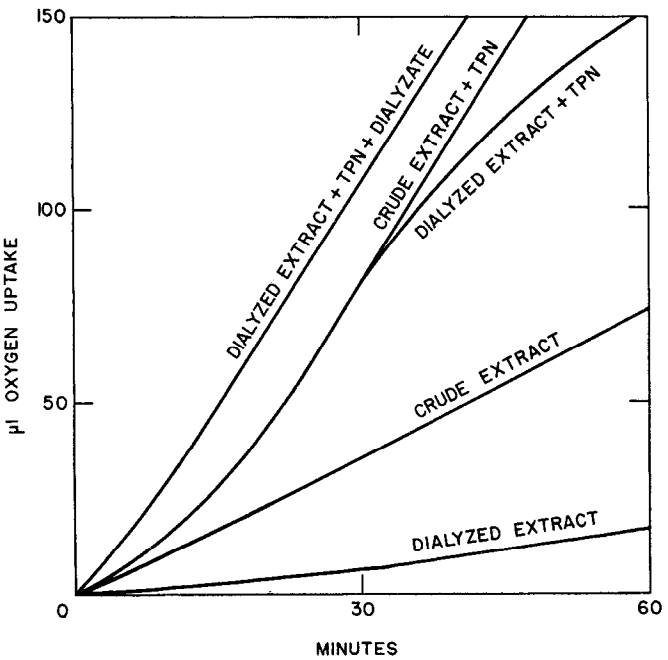

FIG. 1. Oxygen uptake by extracts from 5 day-old *poky*. The manometric flasks contained 0.6 ml. of crude extract or the corresponding amount of dialyzed extract and enough phosphate-mannitol to bring the final volume to 2.5 ml. TPN (0.1 mg.) was added to each flask.

poky extract that had been submitted to a 6 hour dialysis, both TPN and the dialysate were necessary to restore the oxygen uptake by the system.

Substrate for Respiration—Cell-free extracts, as well as intact mold, have a high endogenous respiration, and it was not possible to suppress it by short dialysis (6 to 8 hours). As shown below, the intact mold and the extracts contain a considerable amount of polysaccharide which presumably acts as substrate. Among the substances tested by being added to crude or dialyzed extracts, the following increased the oxygen uptake, but only in the presence of added TPN: glucose-1-phosphate, glucose-6-phosphate, and 6-phosphogluconic acid. The last was by far the most effective.

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There was, however, a marked variation from one cell-free preparation to another with respect to capacity for oxidation of these added substrates. The orcinol pentose reaction (5) was found to be positive at the end of a manometric experiment without added substrate, and in the presence of 6-phosphogluconic acid this reaction showed that a larger amount of pentose had been formed. The activity of phosphogluconic acid dehydrogenase was estimated and it was found that 1 gm. of acetone powder from wild type had an activity of 7.8 units. There was no significant difference between wild type and *poky* in this respect, although the activity found in acetone powder does not necessarily correspond with the activity of the enzyme in the mold itself.

TABLE III

Utilization of Sucrose by Wild Type and Poky Strains of Neurospora

	Age of culture	Dry weight per 20 ml.	Sugar remaining in culture medium (per 20 ml.)*			Glucose after hydrolysis of whole mold	
			Sucrose	Glucose	Fructose		
	days	*mg.*	*mg.*	*mg.*	*mg.*	*mg. per 20 ml.*	*per cent dry weight*
Wild type	1.5	13	20	120	120	4.5	35
	2.0	41	0	90	110	11	27
	2.5	55	0	40	80	20	36
	3.0	81	0	10	60	20	25
Poky	3.8	3.5	20	120	120	1.2	35
	5.5	18	0	100	120	6	33
	9.5	36	0	0	0	14	39

* The flasks contained 300 mg. of sucrose per 20 ml. at the beginning of the experiment.

Since the above experiments indicated that both wild type and *poky* contain a sizable reservoir of substrate, probably polysaccharide, experiments were carried out to provide more detailed information on this question. Results showing the course of utilization of sucrose by the two strains are presented in Table III. Glucose was estimated by the chromatographic procedure described above. For estimation of the total glucose in the mold the pads were dried *in vacuo* and autoclaved at 15 pounds per sq. in. for 2.5 hours in the presence of 20 parts of 0.5 M HCl. Free glucose was found to be present to the extent of about 0.5 to 2 per cent of the dry weight of washed mold. The chromatographic experiments also demonstrated that intact mycelium from either wild type or *poky* yielded, on hydrolysis, no significant amount of any sugar besides glucose, even though the cultures were grown in the presence of sucrose, fructose, glucose, mannose, or acetate. The mold does contain a variety of sugar phosphates

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and some free fructose, but these substances are not present in sufficient quantities to affect the results described above. No glucosamine was observed on any of the chromatograms.

In further experiments wild type and *poky* mycelia were ground with sand and separated by centrifugation into large particles and soluble fractions, as described under the section on methods. These fractions as well as samples of the whole mold were hydrolyzed with acid, and sugars were determined colorimetrically (6). The results obtained, calculated as glucose in mg. per gm. of dry mold, were as follows: wild type (2.5 days), whole mold 440, particles 44, soluble 72; *poky* (3.5 days), whole mold 370, particles 12, soluble 95. These data are in reasonable agreement with those from the chromatographic procedure, but they show in addition that (1) about 75 per cent of the glucose produced by hydrolysis of the whole mold is not obtained in the cell-free extracts, and (2) the distribution of bound sugar between the particles and the soluble fraction is quite different in *poky* and wild type. *Poky* particles are quite deficient in glucose. Polysaccharides have been isolated from the soluble fractions of *poky* and wild type by alcohol precipitation after removal of protein with trichloroacetic acid. These products are very soluble in water and they give no color with iodine. They yield only glucose on hydrolysis.

Distribution of Flavin and Niacin—Paper chromatography of extracts of both *poky* and wild type mycelium demonstrated that essentially all of the flavin present was in the form of FAD. That FAD-X was not present was demonstrated by Dr. Hugh S. Forrest using the chromatographic procedure of Dimant *et al.* (10).

Quantitative estimates of riboflavin and niacin in whole mold, in particles, and in soluble fractions were made by use of standard microbiological procedures with *L. casei* and *L. arabinosus* (9). Results of these determinations are summarized in Table IV. These data show that *poky* contains nearly twice as much total riboflavin and niacin as wild type. The relative increase of flavin in the particles of young *poky* is perhaps significant in relation to the cytochrome independence of the respiration of *poky* at this age. On the other hand *poky* particles contain only a little more niacin than normal and the principal increase is found in the soluble fraction.

Catalase—Values for catalase activity (*Kat. f.* (4)) were found to be 2.1 and 1.9 for 2 and 4 day-old wild type, whereas the corresponding values for *poky* were 2.5, 2.2, and 2.5 for 2, 4, and 5 day-old cultures respectively.

Interactions between Particles and Soluble Fractions—The experiments described above have demonstrated that both the particles and the soluble fractions of *poky* and wild type strains of *Neurospora* differ in several respects. It was also found that neither fraction alone from either strain

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is capable of carrying on a significant amount of respiration. Therefore, as a basis for further examinations of the respiratory similarities and dif-

TABLE IV

Riboflavin and Niacin Content of Wild Type and Poky Strains of Neurospora

	Strain	Age of culture	Dry mold	Protein N		Ratio, particles / supernatant
				Particles	Supernatant solution	
		days	*γ per gm.*	*γ per mg.*	*γ per mg.*	
Riboflavin	Wild	2.5	46	1.0	1.5	0.67
	"	4.5	57	0.95	1.1	0.76
	Poky	3.5	100	1.5	1.3	1.15
	"	5.5	94	1.1	1.6	0.69
Niacin	Wild	2.5	230	3.2	19	0.17
	"	4.5	300	2.7	11	0.24
	Poky	3.5	470	3.8	34	0.11
	"	5.5	400	4.2	35	0.12

TABLE V

Interaction between Particles and Soluble Portions

Oxidizing systems produced by mixing wild type particles (WP), wild type supernatant solution (WS), *poky* particles (PP), and *poky* supernatant solution (PS) in all combinations of particles and supernatant solutions. Each flask contained TPN (0.1 mg.) and enzyme preparation in a total of 2.5 ml. of phosphate-mannitol buffer. The results are given in microliters per hour. Each fraction alone did not take up an appreciable amount of oxygen.

Mixtures	From 3 day wild type and 5 day *poky* cultures			From 3 day wild type and 3.5 day *poky* cultures		
	Oxygen uptake		Per cent inhibition	Oxygen uptake		Per cent inhibition
		+ 0.001 M azide			+ 0.001 M azide	
WP + WS	102	60	41	150	95	36
PP + PS	225	180	20	90	85	5
WP + PS	230	84	64	221	81	63
PP + WS	154	95	38	35	20	43

ferences between the particles and the soluble portions from the two strains, preparations were made and oxygen consumption was measured in mixtures of various combinations of fractions in the presence and absence of azide. Some results are summarized in Table V. Fractions from each strain were mixed in proportion to the amount of mold from which they were derived and TPN was added to the mixtures in all cases. It is clear from these

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data that the soluble fractions from either wild type or *poky* will interact with particles from either source to give systems capable of oxygen uptake, but it should be noted that the *poky* supernatant solution added to either *poky* particles or wild type particles is far more effective than wild type supernatant solution. The inhibition by azide in non-homologous mixtures (WP + PS and PP + WS) is much greater than would be expected from a consideration of the results with the homologous mixtures. For example, in the second experiment (Table V), the oxygen uptake for PP + PS was 90 with only 5 per cent inhibition by azide, while the same quantity of particles (PP) when mixed with the wild type soluble fraction (WS) gave an oxygen uptake of only 35, and yet in the latter case azide produced an inhibition of 43 per cent. The azide may, of course, be inhibitory to more than one reaction.

DISCUSSION

The view that the respiratory system of young *poky* does not utilize the cytochromes, but is mediated by a cyanide- or azide-insensitive system, is supported by the following evidence. (1) *Poky* is deficient in cytochromes *a* and *b*, and thus in cytochrome oxidase and succinic acid oxidase activity (1). (2) Cytochrome *c*, the only component whose absorption band can be seen under the spectroscope in a preparation of washed *poky* particles, is reduced and is not readily oxidized by molecular oxygen; the same applies to added beef heart cytochrome *c*. (3) The respiration of intact mycelium of young *poky* is insensitive to cyanide and azide, two substances which are known to block respiration by combining with cytochrome oxidase. The respiration of wild type mycelium, which has a complete cytochrome system, is inhibited by these substances. (4) The oxygen uptake by cell-free extracts of young *poky* is insensitive to cyanide and azide, while that of wild type is inhibited. The oxygen uptake by crude extracts from 6 day-old *poky* becomes sensitive to cyanide and azide, although never as much as do extracts of wild type.

Poky mycelium has a Q_{O_2} of 25 to 35. Most of the cases of cyanide-resistant respiration reported in the literature concern organisms with a lower respiratory activity, as for instance *Bacillus delbrückii* (Q_{O_2} = 1 to 3) (11) in which the "yellow enzyme" was presumed to act as terminal oxidase (12). However, some organisms devoid of cytochromes have respiration rates very similar to that of *poky* (*i.e.*, one of the lactic acid bacteria, *Betabacterium breve* (Q_{O_2} = 37) (11), and in plants the spadix of *Arum* (Q_{O_2} = 32) (13)). While in these cases there is indirect evidence that flavoproteins act as oxidases, the terminal oxidase itself is still unknown. Two points seem to support the assumption that a flavin-adenine dinucleotide enzyme system acts as oxidase in *poky*. This strain contains about

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twice as much flavin as the wild type, nearly all of which is accounted for as FAD, and *poky* respiration is sensitive to oxygen tension, as flavin enzymes are known to be. However, it is not yet possible to arrive at a definite conclusion on this question.

In addition to the information given here on the differences that exist between the terminal oxidase systems of wild type and *poky* strains of *Neurospora*, data have also been presented that pertain to the more general problem of the nature of the over-all respiratory processes of the molds. It appears that in both of the strains under consideration exogenous carbohydrate is built into endogenous polysaccharides that provide the substrate reservoir which supports endogenous respiration. Polysaccharides are found in both particles and soluble fractions, but *poky* particles are relatively deficient in this respect. It is clear that the breakdown of hexose through pentose occurs in both of these *Neurospora* strains and the finding of an excess of TPN in *poky* (soluble fraction) suggests that the pentose pathway is especially prominent in this variant. However, the results with dialyzed extracts provide a basis for a detailed investigation of this question. The data on azide inhibition of oxygen uptake by homologous and non-homologous particle and supernatant fractions from *poky* and wild type indicate the existence of other differences between the fractions that have not yet been evaluated.

SUMMARY

1. Experiments on the respiration of intact mycelium and cell-free extracts of wild type and *poky* strains of *Neurospora* have confirmed the observation that *poky* is not dependent on the cytochrome system for terminal oxidation. Wild type is largely dependent on this system.

2. The oxidation of endogenous substrate in cell-free extracts of both *poky* and wild type has a marked requirement for TPN.

3. There is about twice as much flavin and niacin in *poky* as in wild type.

4. The respiration of intact *poky* mycelium is doubled in the presence of pure oxygen instead of air, while with wild type there is no significant difference under the two conditions.

5. *Neurospora* normally contains polysaccharides which appear to serve as a reservoir of substrate for endogenous respiration.

6. Results from experiments on respiration by mixed cellular fractions indicate that *poky* differs significantly from wild type *Neurospora* with respect to the supernatant solutions as well as the large cellular particles.

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