THE IN VITRO SYNTHESIS OF PANTOTHENIC ACID BY PANTOTHENICLESS AND WILD TYPE NEUROSPORA

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It has been demonstrated by Wagner and Guirard[1] that the resting mycelium of wild type *Neurospora* is able to synthesize pantothenic acid in the presence of β-alanine and pantoyl lactone (DL-α-hydroxy-β,β-dimethyl-γ-butyrolactone). The *in vivo* synthesis of pantothenate from these precursors cannot be performed, however, by the pantothenicless mutant 5531. If the wild type mycelium is treated with acetone to kill the cells and then dried with acetone, the resultant preparation can also be shown to possess an active enzyme system capable of carrying out the above synthesis *in vitro*.[1]

This present communication is concerned with an extension of the investigation of *in vitro* synthesis of pantothenate by *Neurospora*. Three strains, Emerson wild type 5256A, and two pantothenicless mutants, 5531A and 34556A, were used. Both mutants appear to be due to a change at the same gene locus. The two differ in that 5531 was induced by x-rays and 34556 by ultra-violet light.[2]

Experimental.—Activity of Intact and Water Extracted, Dried Mycelium: Fresh Mycelium of 5256, 34556, and 5531 was treated with acetone according to the method previously described.[1] The resultant preparations are described in what follows as intact, acetone-dried mycelium. The activity of these preparations with respect to the production of pantothenic acid from the precursors, β-alanine and pantoyl lactone was tested at three different temperatures. In each case the reaction mixture consisted of 50 mg. of acetone-dried material/flask suspended in 25 ml. of 0.04 *M* potassium phosphate buffer at pH 6.5. In the case of those flasks to which precursors were added, an initial concentration of 0.004 *M* of each precursor was used. The flasks and contents were sterilized by autoclaving and cooled before the addition of the acetone preparations. Incubation was carried on for twenty-four hours with constant shaking at 22°, 27° and 35°C. The flasks were then steamed, the solids filtered out and the filtrate assayed for pantothenate with *Lactobacillus arabinosus* by the method of Skeggs and Wright.[3] The results are presented in table 1, as μg. pantothenate produced/50 mg. mycelium/twenty-four hours, both in the presence and absence of the two precursors.

Ten grams of each of the intact, acetone-dried preparations were washed in 1000 ml. of distilled water for several hours with constant shaking and then filtered. The residues (R) were dried with acetone and weighed.

In the case of each strain the residue was found to have lost ca. 50% of the original weight of the unextracted preparation. The filtrates were each reduced *in vacuo* to 200 ml. The water-insoluble residues (R) were tested for activity in the same way that the intact preparations were tested using 50 mg. of R per flask. The concentrated filtrates (S) were sterilized by Seitz filtration and tested for activity at 35° with and without the corresponding residues. An amount of S (1 ml.) equivalent to 50 mg. of intact, acetone mycelium was added to the flasks. Table 2 presents the results of these tests.

Activity of the Concentrated Water Extract: The concentrated filtrate (S) from each strain was added in several different ml. concentrations to a set of flasks containing the precursors and 50 mg. of the respective residues.

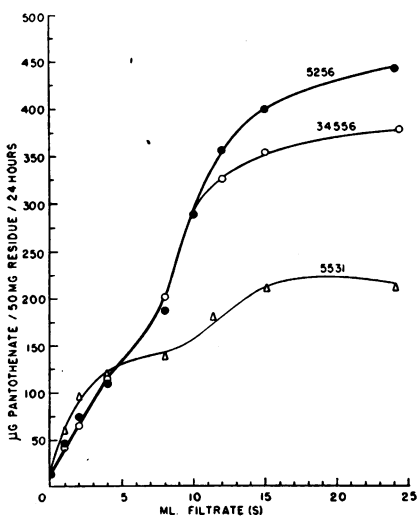

FIGURE 1

The effect of added excess filtrate (S) on the production of pantothenate.

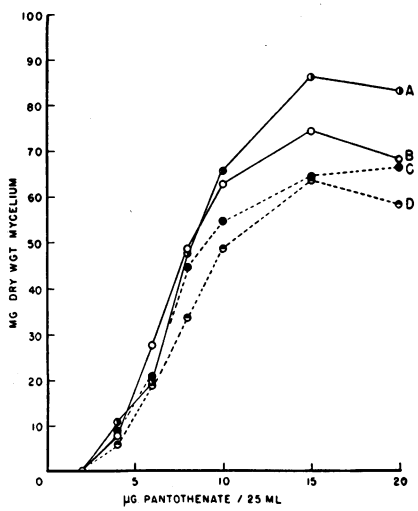

FIGURE 2

The effect of the precursors on the growth of the pantothenicless mutants. A, 5531 plus precursors; B, 5531 without precursors; C, 34556 without precursors, D, 34556 plus precursors.

Incubation was at 35° for twenty-four hours. The results are described by figure 1.

The Effect of the Precursors on the Growth of the Mutants: The mutants 34556 and 5531 were grown for four days at 35° and pH 6.5 in the presence of 0.004 *M* β-alanine and pantoyl lactone, and various concentrations of Ca pantothenate. A control series without the precursors, but with Ca pantothenate, was tested simultaneously. The results of this experiment are given in figure 2.

Growth of the Mutants on Pantothenate Synthesized in Vitro: The panto-

thenicless mutants were inoculated into media containing various concentrations of pantothenate synthesized by the acetone preparations of these same strains which had been previously assayed with *L. arabinosus*. The pH of the medium was at 6.5, the temperature, 35°, and the incubation period, four days. Controls were run simultaneously with Ca pantothenate. Figure 3 shows the growth response of 5531 to pantothenate synthesized by 5531 *in vitro*, and figure 4 the response of 34556 to its corresponding source of pantothenate. The data from the corresponding controls are included in each case for comparison.

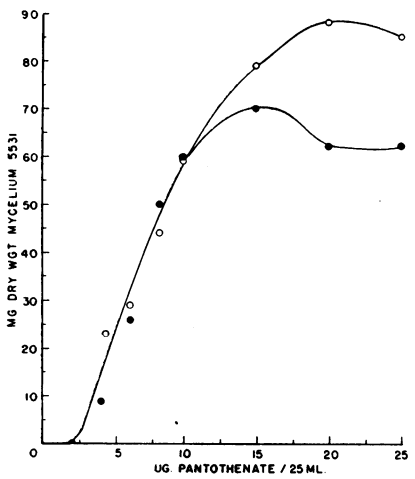

FIGURE 3

Growth of 5531 on Pantothenate synthesized *in vitro* by 5531.

O pantothenate synthesized by 5513

● Ca pantothenate control

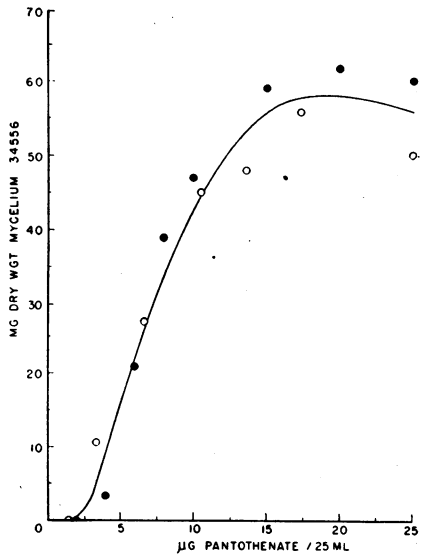

FIGURE 4

Growth of 34556 on pantothenate synthesized *in vitro* by 34556.

O pantothenate synthesized by 34556

● Ca pantothenate control

Discussion.—The data presented in tables 1 and 2 demonstrate that the pantothenicless mutants, 5531 and 34556, as well as the wild type *Neurospora*, possess an enzyme system capable of carrying out the *in vitro* synthesis of pantothenic acid from β-alanine and pantoyl lactone. There is a difference indicated in the system possessed by the mutants when compared to the wild type with regard to the effect of temperature, but the enzyme is active to the same degree in all three strains at 35°. The enzyme in each strain consists of at least two parts, a water-insoluble residue (R) and a water-soluble portion (S) which is inactive alone, but which increases the activity of R when combined with it.

The data in figure 1 when combined with the data in tables 1 and 2 show

that the concentration of S is limiting, with the concentration of precursor used, in the synthesis of pantothenate by the intact and washed acetone preparations. The amount of pantothenate synthesized in the presence of excess S, as shown in figure 1, leaves no doubt that there is present in all three strains a mechanism active in this synthesis. This considerable increase in pantothenate production cannot be accounted for by the pantothenate present in S, which is 0.31, 0.48 and 0.65 μg./ml. for 5256 (S), 5531 (S) and 34556 (S), respectively.

TABLE 1

THE ACTIVITY OF INTACT, ACETONE-DRIED MYCELIUM

ACETONE PREPARATION	22°		27°		35°	
	O	P	O	P	O	P
5256	4.0	12.5	2.8	55.0	2.3	48.0
5531	0.51	0.55	1.8	13.0	1.5	47.0
34556	4.2	8.3	1.5	9.5	1.5	45.0

μg. pantothenate produced/50 mg. intact, acetone-dried mycelium/twenty-four hours by 5256 (wild type) and two pantothenicless strains, 5531 and 34556, at three different temperatures. O designates absence and P presence of both pantoyl lactone and β-alanine.

TABLE 2

THE ACTIVITY OF WATER-SOLUBLE AND WATER-INSOLUBLE FRACTIONS

REACTION MIXTURE	5256	5531	34556
Residue alone	0.43	0.55	0.90
Residue + precursors	12.0	13.0	12.0
Filtrate + precursors	0.42	0.65	0.65
Residue + filtrate + precursors	48.0	78.0	68.0

μg. pantothenate produced/50 mg. residue/twenty-four hours. The amount of filtrate added was equivalent to 50 mg. of intact acetone dried mycelium. Temperature at 35°C.

The water-soluble factors appear to be identical in activity for the three strains, since they can be substituted for one another with results comparable to those given in figure 1. In addition the factor or factors are dialyzable and heat stable while the water-insoluble fraction is not. Further work has been done on the coenzyme indicated, and these results will be recorded elsewhere. It is evident that 34556, 5531 and 5256 each have the same or a similar coenzyme involved in the production of pantothenic acid from β-alanine and pantoyl lactone.

In all of the experiments discussed above the production of pantothenate was determined by assaying with *L. arabinosus*. It was necessary, therefore, to establish the activity of the pantothenate for the mutants. Since the reaction mixtures containing pantothenate also had uncombined pantoyl lactone and β-alanine present, the effect of these compounds on the growth of the mutants was determined as shown in figure 2. It can be seen from these data that no growth occurs in the absence of panto-

thenate with the precursors present, and there is no increase in growth over the controls in the presence of the precursors and limiting amounts of pantothenate. There does seem to be a slight but consistent beneficial effect of the precursors on the growth of 5531 at optimal concentrations of pantothenate.

In view of the inactivity of the precursors, the data in Figures 3 and 4 definitely establish the activity of the pantothenate synthesized by 5531 and 34556 *in vitro* for these mutants. The results reported here were obtained by using pantothenate synthesized in the presence of excess coenzyme. Similar results were obtained by using the pantothenate synthesized by intact, acetone-dried mycelium, and also with pantothenate synthesized by wild type.

These findings are of interest in connection with the current hypotheses dealing with the postulated gene enzyme relationship. According to one of these hypotheses[4] 5531 and 34556 are mutant because they are incapable of synthesizing their own pantothenic acid due to a blocking of the reaction coupling β-alanine and pantoyl lactone. The block may be caused by the absence of the necessary enzyme or coenzyme or by inhibition. This interpretation is supported by the fact that (1) wild type is stimulated to produce pantothenic acid by the presence of these two compounds,[1] (2) by the demonstration that these compounds together do not support growth of the mutants, and (3) by the report that these precursors accumulate in the medium when the mutant 5531 is grown in the presence of pantothenate.[5] (Strain 34556 has not as yet been tested for accumulation of precursors.) The data presented in this report rule out the enzyme absence hypothesis for these mutants, assuming that we are correct in concluding that it is this particular step in the synthesis of pantothenate which is involved. It is then necessary to conclude that the mutants possess a mechanism which is active *in vivo* in preventing this enzyme system from operating in pantothenate synthesis.

Summary.—The pantothenicless mutants 5531 and 34556 of *Neurospora* possess, like wild type, an enzyme system for the synthesis of pantothenic acid from β-alanine and pantoyl lactone *in vitro*.

* A portion of this work was done while the author was a research fellow at the California Institute of Technology during the summer of 1948.

[1] Wagner, R. P., and Guirard, B. M., these PROCEEDINGS, **34**, 398–402 (1948).

[2] Houlahan, M., Private Communication.

[3] Skeggs, H. R., and Wright, L. D., *J. Biol. Chem.*, **156**, 21 (1944).

[4] Beadle, G. W., *Chem. Rev.*, **37**, 15–96 (1945).

[5] Tatum, E. L., and Beadle, G. W., *Ann. Missouri Botan. Garden*, **32**, 125–29 (1945).