Synthesis of Pantoic Acid and Its Congeners by *Neurospora.* (22936)

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Wagner and Haddox(1) reported that the *pantothenicless* mutant (5531) of *Neurospora crassa,* when grown in aerated cultures for periods of longer than four days, synthesized in appreciable quantity what appeared to be pantothenic acid. This together with the fact that acetone-dried mycelia of the mutant could effect the synthesis of pantothenic acid from its precursors(2) was significant in biochemical genetics theory in casting doubt on the hypothesis that the mutant lacked the enzyme system for synthesizing pantothenic acid. These observations have prompted us to study the growth factors of the pantothenic acid group which are elaborated into the culture medium by a wild type and a *pantothenicless* strain of *Neurospora crassa.*

Materials and methods. Neurospora crassa 5256A (Emerson wild type) was grown on the medium described by Horowitz and Beadle(3). For growth of the *pantothenicless* mutant 5531A the medium was supplemented with pantothenic acid. Both wild type and mutant were obtained from Dr. R. P. Wagner, Department of Zoology, University of Texas, Austin. Further details on the conditions of growth are given in Table II. Pantothenic acid and related compounds were determined microbiologically with *Saccharomyces carlsbergensis*(4), *Lactobacillus arabinosus*(5), *Acetobacter suboxydans*(6) at pH 5.5, *pantothenicless N. crassa* and *Lactobacillus helveticus*(7). Specificity of the response of these organisms to pantothenic acid and related compounds is summarized in Table I. The nutritional response of *N. crassa* to these compounds resembles closely that of the yeast, *Saccharomyces carlsbergensis.* Coenzyme A was determined by the arsenolysis method of Stadtman, *et al.* (15). An extract from sonically disrupted *Escherichia coli* cells served as the source of transacetylase. The bioautograph technic of Winsten and Eigen(16) was employed to differentiate related growth factors. The plates were poured with the pantothenate-free assay medium for *A. suboxydans* to which was added 2% agar. When heavily seeded with

TABLE I. Activity of Various Pantothenic Acid-Related Compounds in the Growth of *N. crassa* 5531A and Other Microorganisms.

Compound	Activity (equivalent basis)				
	*N. crassa**	*A. suboxydans*§	*S. carlsbergensis*	*L. arabinosus*	*L. helveticus*
D-Pantothenic acid	1	1	1	1	1
D-Pantoic acid	0	1 (6)	0 (4)		
D-Pantolactone	0	0 (6)	0 (10)	0 (12)	
D-Pantothenic acid 4'-phosphate	0	0 (8,9)	0 (8)‖	0 (8)	
D-Pantothenyl-L-Cystine	<.001†	.1 (6)	<.02 (11)	<.02 (11)	<2 (11)
D-Pantethine	.002‡	2.8 (6)	0 (7)	.41 (7)	100 (7)
DL-Pantetheine 4'-phosphate	0	5.9 (6)		.005 (13)	17 (14)
D-Pantethine 4'-phosphate	0	2.7 (6)			
Coenzyme A	0	10.8 (6)	0 (7)	0 (7)	0 (7)

Numbers in parentheses refer to literature citation.

* Based on dry wt of washed mycelium after 48 hr growth in 25 ml standing cultures.

† By assay with *S. carlsbergensis* this sample of pantothenycystine had a relative activity (pantothenic acid = 1) of 0.0006 on an equivalent basis.

‡ By assay with *S. carlsbergensis* this sample of pantethine had a relative activity (pantothenic acid = 1) of 0.0001 on an equivalent basis.

§ Comparative activities at pH 6.

‖ Activity for *Saccharomyces cerevisiae.*

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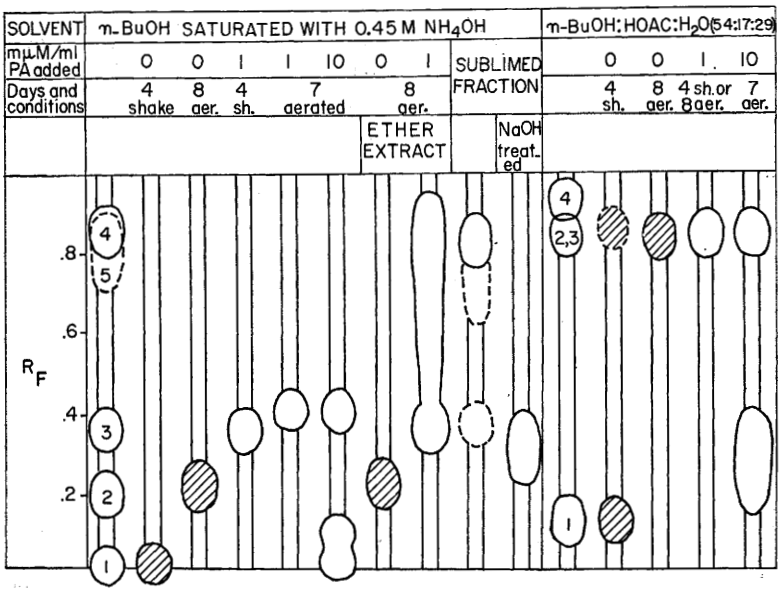

FIG. 1. Bioautographs of culture filtrates of *Neurospora* using *A. suboxydans* as the indicating organism. Shaded spots are from wild type extracts, unshaded spots from mutant 5531A extracts. Reference compounds: (1) coenzyme A, (2) pantothenic acid (PA), (3) pantoic acid, (4) pantethine, (5) pantolactone.

a washed suspension of *A. suboxydans* in a top layer of agar, growth zones in response to topical application of active compounds were readily detected after 24 hours at 30°C.

Results. The predominant active compound for *A. suboxydans* produced by 4-day cultures of wild type *Neurospora* is coenzyme A (Fig. 1). Assuming coenzyme A to be 5.5-10 times as active as pantothenic acid for *A. suboxydans*(6,14) at pH 5.5 on the molar basis, coenzyme A could account for 44-80% of the activity of the culture filtrate for *A. suboxydans* (Table II). By microbiological assays small amounts of pantothenic acid and pantetheine could be detected (Table II). With longer growth and aeration of the culture, coenzyme A disappeared while pantothenic acid and pantetheine increased in concentration. Bioautographs of these culture filtrates (Fig. 1) confirmed the presence

TABLE II. Assay of *Neurospora* Culture Filtrates for Various Pantothenic Acid-Related Compounds.

Growth conditions	Days	Pantothenic acid added (mμM/ml)	Pantothenic acid equiv. (mμM/ml) *A. suboxydans*	*S. carlsbergensis*	*L. arabinosus*	LBF as pantetheine* (mμM/ml)	Co A (mμM/ml) by arsenolysis
Wild type	4, standing	0	24	.8	.7	<.3	
	4, shaking	0	25	1.8	1.6	.5	
	4, "	0	88	3			7
	8, aerated	0	32	6.5†		1.2	0
	8, "	0	11	3	2	1.4	
5531A	4, standing	1	20	.6	.7	<.3	
	4, shaking	1	28	.6	.5	<.3	
	8, aerated	1	35	1‡		.4	0
	7, "	1	53	.5	.2	<.1	
	7, "	10	73	10	9	.4	

* One LBF unit = 0.17 mμmoles pantetheine.
† 7 mμM/ml pantothenic acid by *N. crassa* 5531A assay.
‡ <2 " *Idem*

of pantothenic acid. Taking pantetheine (LBF) to be approximately 4 times as active as equimolar amounts of pantothenic acid at pH 5.5(14), 32-72% of the activity for *A. suboxydans* in 7-8-day aerated cultures of *Neurospora* would appear due to pantetheine and pantothenic acid. The form in which LBF occurs has not been determined; it does not appear to be pantetheine itself. Although pantothenic acid 4'-phosphate has been detected in spent *N. crassa* culture filtrates(14, 17), this compound is reportedly inactive for *A. suboxydans*(8,9) and would not add to the activity for this organism.

Culture filtrates of the *pantothenicless* mutant of *Neurospora*, grown with minimal but sufficient added pantothenic acid to give good growth (1 mμmole per ml), showed high activity for *A. suboxydans*, but did not exhibit any coenzyme A activity (Fig. 1). The pantothenic acid content of such filtrates was equal to or slightly less than that originally added to the medium and only traces of LBF activity could be detected (Table II). Bioautographs of 4- and 7-day cultures showed only a single growth zone which corresponded to pantoic acid (Fig. 1). Tatum and Beadle (18) have reported the accumulation of pantoic acid by the *pantothenicless* mutant of *Neurospora* but without experimental details.

Fractionation of N. Crassa 5531A Culture Filtrates. To confirm the presence of pantoic acid a partial purification of the substance produced by the mutant was made. The mold was grown in 5 liter lots for 7-9 days with aeration. The culture medium (activity ≏ 1-2 mμmoles pantoic acid per mg dry wt) was filtered, the pH of the filtrate adjusted to 3, and 20 g Darco G-60 added per liter of filtrate. After stirring for 1 hour the carbon was filtered off, washed with a small amount of water, and eluted with 0.2 of the original volume of acetone containing 1% concentrated ammonia. The acetone was evaporated *in vacuo* and the residue concentrated to a point where considerable crystalline material (ammonium acid tartrate) was formed. This was filtered off. The recovery of active material at this point was 21-27%. When the adsorption and elution was done in small test batches recoveries of the order of 50% were realized. The filtrate was adjusted to pH 9 and extracted with 3 volumes of ether. The extract contained a negligible amount of active material. The aqueous phase was acidified to pH 2, extracted 4 times with 3 volumes of ether, and the ether extract concentrated *in vacuo* to dryness. The ether extract contained 43% of the active materials in the charcoal eluate. The active material in the ether extract was chromatographed on paper. It migrated as a mixture of pantoic acid and its lactone. To an aliquot of the ether extract (≏25,000 mμmoles pantoic acid) was added 0.5 ml 6N hydrochloric acid. The solution was taken to dryness *in vacuo* and the resulting residue sublimed at 100° and 0.02-0.03 mm for 2 hours. The sublimate showed activity equivalent to 500 mμmoles of pantoic acid before and 15,000 mμmoles of pantoic acid after sodium hydroxide saponification: the non-sublimed residue showed activity equivalent to 200 and 11,000 mμmoles of pantoic acid respectively, before and after sodium hydroxide treatment. The increase in activity results from conversion of pantoyl lactone to the salt of the free acid. The sublimate contained 170 mμmoles of pantoic acid per mg (2.5%). Bioautographs of the fractions clearly indicated the active material to be pantoic acid (Fig. 1). As a control, a sample of wild type culture filtrate from a 10-day aerated culture was adsorbed with charcoal in the same fashion, eluted, and the eluted material extracted 3 times at pH 2.0 with 7 volumes of ether. Only 12% of the active material in the eluate was extracted by the ether. When a solution of pantothenic acid containing 1 μmole per ml was subjected to the same ether extraction procedure, 13% appeared in the extract. The ether extract from wild type *Neurospora* chromatographed as pantothenic acid (Fig. 1).

When the *pantothenicless* mutant was grown in the presence of a large excess of pantothenic acid (10 mμmoles per ml) for 7 days with aeration, no change in the pantothenic acid content of the filtrate was observed and no significant increase in LBF activity (Table II). Bioautographs of the culture filtrate in-

dicated, however, the presence of coenzyme A and other active compounds in addition to pantoic acid.

Discussion. Wagner and Guirard(19) surmised that the *pantothenicless* mutant of *Neurospora* lacked the enzyme for coupling pantoic acid and β-alanine since wild type mycelium synthesized pantothenic acid from its precursors whereas mycelium from the mutant did not. In accord with this interpretation, we have been unable to demonstrate synthesis of pantothenic acid by growing cultures of this mutant, but have demonstrated accumulation of the precursor, pantoic acid. Wagner reported, however, that acetone-dried mycelium of this mutant could effect this synthesis from the precursors(2) and that on growing the mutant for 8 days under aeration in the presence or absence of precursors a considerable amount of pantothenic acid was synthesized as judged by assay with *L. arabinosus* and bioautographs(1). It was concluded, therefore, that the coupling enzyme was present, but ordinarily was inactive due perhaps to presence of an inhibitor which was destroyed by acetone drying or long aeration.

Although we have been unable to confirm synthesis of pantothenic acid in aerated cultures of the mutant strain, it is quite possible that unrecognized variables in the cultural conditions are responsible for this difference in result. Failure of growing cultures of the organism to synthesize pantothenate is, of course, consistent either with absence of the coupling enzyme or with inactivity of this enzyme during growth due to presence of an inhibitor.

Summary. 1. Synthesis of pantothenic acid and related compounds by wild type *Neurospora crassa* and a *pantothenicless* mutant of this organism, 5531A, was compared by growing these organisms for 4-8 days and determining the substances produced in the culture filtrates by microbiological and chromatographic methods. Culture filtrates of wild type *Neurospora* at 4 days contained principally coenzyme A; on longer incubation the coenzyme A decreased while the pantothenic acid and pantetheine (LBF) in the medium increased. Little or no pantoic acid was present. Culture filtrates of the *pantothenicless* mutant contained substantial amounts of pantoic acid which arose by synthesis; no synthesis of pantothenic acid or coenzyme A was observed when the culture was grown with minimal amounts of pantothenic acid. When larger amounts of pantothenic acid were added, small amounts of coenzyme A were detected in the culture filtrate. 2. Of the compounds so far implicated as intermediates in synthesis of coenzyme A, only pantothenic acid supports growth of this *pantothenicless* mutant.

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