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Co-accumulation of Prephenate, L-Arogenate, and Spiro-arogenate in a Mutant of *Neurospora**

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A mutant strain of *Neurospora crassa* blocked in each of the initial steps of tryptophan, tyrosine, and phenylalanine biosynthesis was previously shown to accumulate and secrete prephenate and L-arogenate (Jensen, R. A., Zamir, L. O., St. Pierre, M., Patel, N., and Pierson, D. L. (1977) *J. Bacteriol.* 132, 896–903). We now report the co-accumulation of yet another compound which was identified (Zamir, L. O., Tiberio, R., Jung, E., and Jensen, R. A. (1982) *J. Biol. Chem.* (1983) 258, 6486–6491) as the lactam derivative of L-arogenate. This structure, spiro-arogenate, undergoes a facile acid-catalyzed conversion to L-phenylalanine (as does L-arogenate). Since L-arogenate is conveniently quantitated as 5-dimethylaminonapthalene-1-sulfonyl (dansyl)-phenylalanine following acidification and dansylation, the unknown presence of spiro-arogenate may easily lead to overestimation of L-arogenate present in mixtures. Reliable quantitative assays for both L-arogenate and spiro-arogenate in mixtures were designed utilizing [^{3}H]dansyl-chloride and exploiting the inability of the spiro-arogenate molecule to be dansylated in contrast to L-arogenate. The initial appearance of spiro-arogenate during accumulation lagged behind prephenate and L-arogenate, and spiro-arogenate accumulation leveled off after 5 days while prephenate and L-arogenate accumulations continued. It seems likely that spiro-arogenate is derived directly from L-arogenate. Prephenate, L-arogenate, and spiro-arogenate comprised about 70, 15, and 15% fractions of the total accumulation in a representative accumulation experiment designed to maximize spiro-arogenate yields. Modest variations in co-metabolite ratios were obtained under nutritional conditions where carbon source, growth temperature, duration of incubation time, and amino acid additions were experimental variables.

L-(8S)-Arogenate (β-(1-carboxy-4-hydroxy-2,5-cyclohexadien-1-yl)alanine) is an intermediate of aromatic amino acid biosynthesis in many, but not all, microbes and plants. The arogenate route to L-tyrosine now seems to be distinctly more common in nature than the earlier known routing through 4-hydroxyphenylpyruvate. Phenylalanine biosynthesis also may proceed via arogenate (rather than phenylpyruvate) in a growing list of organisms so far studied. The chemistry of the L-arogenate structure has been fully documented (1) and the most recent update of the diversity of pathway arrangements in which L-arogenate participates was reviewed in Ref. 2.

A limiting aspect of research requiring L-arogenate for use as enzyme substrate, nutrient compound, or standard is likely to be the amount and quality of L-arogenate available. One means of preparation has been enzymological, either via partially purified prephenate aminotransferase (3) or with enzyme immobilized on a matrix of phenoxyacetylcellulose (4). To date L-arogenate has usually been isolated from the culture fluid of a multiply blocked mutant of *Neurospora crassa* (5), a strain initially developed for accumulation of prephenate (6).

The mutations in this strain of *N. crassa* (available as ATCC 36373) produce an enormous imbalance of aromatic pathway keto acids under conditions of starvation for phenylalanine and tyrosine. Prephenate accumulates while phenylpyruvate and 4-hydroxyphenylpyruvate biosynthesis are totally prevented. An aromatic aminotransferase partially purified from *N. crassa* possesses overlapping substrate specificities for all three keto acids.[1] Under physiological conditions which maximize keto acid imbalance in the mutant, the aminotransferase is restricted to prephenate as substrate since phenylpyruvate and 4-hydroxyphenylpyruvate are absent. The elevated concentration of prephenate offsets the relatively poor affinity of the aromatic aminotransferase for this substrate. The formation of significant amounts of L-arogenate by transamination presumably promotes the further biosynthesis of the lactam derivative denoted spiro-arogenate. The biochemical arrangement to be found in the *N. crassa* mutant is shown in Fig. 1. The structure shown for spiro-arogenate has been proven recently (7). It appears that in the mutant a route yielding spiro-arogenate has either been initiated or has been highly amplified.

Spiro-arogenate is a ninhydrin-negative compound which can account for spurious overestimates of L-arogenate concentrations in mixtures since both compounds readily undergo an acid-mediated conversion to phenylalanine, a key step in the most convenient procedures for assay of L-arogenate. In this report we define the relationship of culture conditions to the differential formation of the three cyclohexadienyl compounds in *Neurospora*. An improved technique is described that employs tritiated dansyl[2]-chloride to monitor levels of L-arogenate and spiro-arogenate in accumulation protocols

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[1] N. Patel and R. Jensen, unpublished data.

[2] The abbreviations used are: dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; AGN, arogenate; SPN, spiro-arogenate; PHE, phenylalanine; PPA, prephenate.

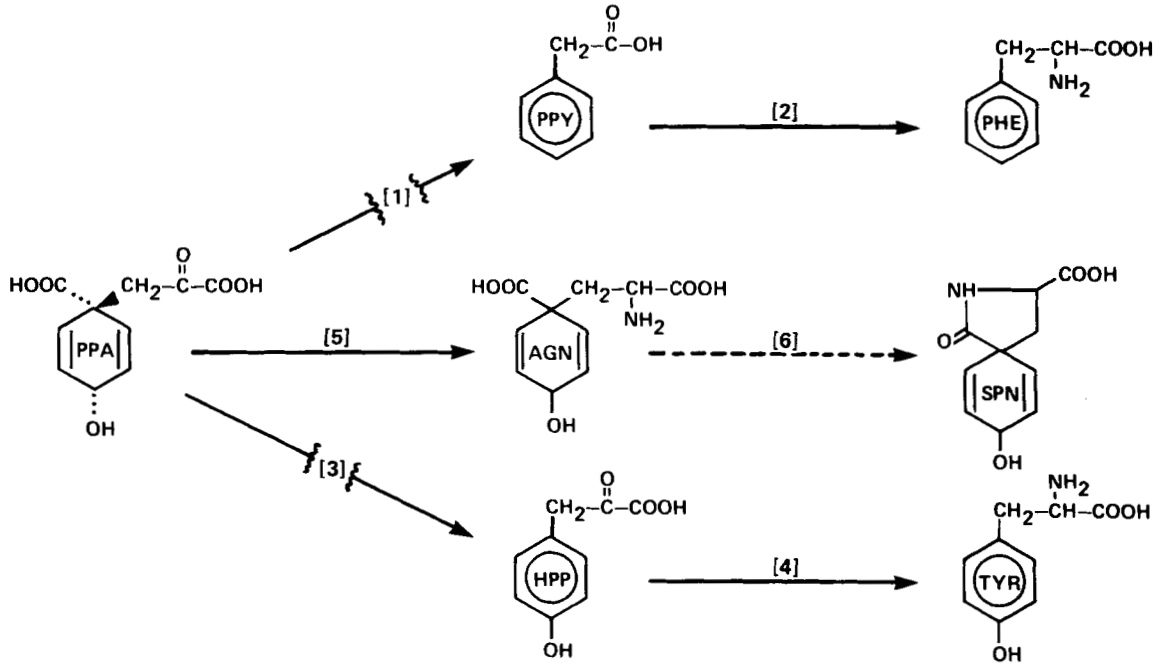

FIG. 1. **Formation of arogenate and spiro-arogenate in a triple mutant of *N. crassa* deficient in enzymes [*1*], [*3*], and anthranilate synthase (not shown).** As a consequence of these blocks the mutant accumulates PPA, AGN, and SPN under conditions of starvation for phenylalanine PHE and tyrosine (*TYR*). Other abbreviations: phenylpyruvate (*PPY*), and 4-hydroxyphenylpyruvate (*HPP*). Enzymes shown are [*1*], prephenate dehydratase; [*2*], phenylpyruvate aminotransferase; [*3*], prephenate dehydrogenase; [*4*], 4-hydroxyphenylpyruvate aminotransferase; [*5*], prephenate aminotransferase; and [*6*], a postulated "arogenate spirase."

where carbon source, temperature, or nutritional supplements were experimental variables.

MATERIALS AND METHODS

Microbial Strain

A strain of *N. crassa* having multiple auxotrophic blocks was originally described by Metzenberg and Mitchell (6) as 75001/5212/C-167, and is now available from the American Type Culture Collection as ATCC 36373. Because of mutant deficiencies in genes specifying prephenate dehydrogenase, prephenate dehydratase, and anthranilate synthase, auxotrophic requirements for growth are satisfied by L-tyrosine, L-phenylalanine, and L-tryptophan. Our strain was obtained from R. L. Metzenberg (University of Wisconsin), and is maintained on agar slants of Westergaard synthetic crossing medium (8) supplemented with L-phenylalanine, L-tyrosine, and L-tryptophan (1). Once conidia have formed, the slants are stored at 4 °C. Periodically the conidial population is checked to guard against reversion to prototrophy by testing the nutritional requirements of individual conidial colonies; the phenylalanine marker is especially revertible.

Chemicals and Supplies

Amino acids, shikimate, and other biochemicals were from Sigma, and tritiated dansyl-chloride in acetone was from Schwarz/Mann Radiochemicals.

Growth and Accumulation Procedures

The growth and accumulation medium, a modified version of Westergaard medium, contained (per liter): KNO_3, 1.0 g; K_2HPO_4, 1.4 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; NaCl, 0.1 g; biotin, 5 μg; $CaCl_2 \cdot 2H_2O$, 0.1 g; the indicated amount of sucrose (usually present at 1%); L-tyrosine, 25 mg; L-tryptophan, 25 mg; L-phenylalanine, 5 mg; and 0.1 ml of trace element solution consisting of citric acid monohydrate, 5.0 g; $ZnSO_4 \cdot 7H_2O$, 5.0 g; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 1.0 g; $CuSO_4 \cdot 5H_2O$, 0.25 g; $MnSO_4 \cdot H_2O$, 0.05 g; H_3BO_3, 0.05 g; and $Na_2MoO_4 \cdot 2H_2O$, 0.05 g. All of the above were dissolved in enough water to make 100 ml of solution. The concentrations of phosphate salts were doubled from the original Westergaard medium recipe in order to help maintain alkaline pH during the accumulation. The pH was adjusted to 7.6 with 10 N KOH before autoclaving. Glucose, fructose, and ribose were autoclaved separately and added to medium aseptically. Sucrose and other nutrient supplements were autoclaved with the media.

Cultures were started with 1-ml inocula of a conidial suspension containing 3×10^6 spores per ml into 180 or 200 ml of medium in 250-ml flasks. Mixing and aeration were accomplished by sparging with humidified air. Freshly inoculated cultures were incubated for 3–4 h at 37 °C to enhance spore germination. Thereafter, the cultures were maintained in a 23 °C water bath unless noted otherwise. After the second day of growth, the pH was monitored. At approximately 24-h intervals, 10 N NaOH was added dropwise to maintain the pH at 7.5 ± 0.2 unit.

Determination of L-Arogenate and Spiro-arogenate Concentrations

Both arogenate and spiro-arogenate convert to phenylalanine readily at acidic pH and can then be quantitated as dansyl derivatives of phenylalanine, using [^{3}H]dansyl-chloride. Dansylated samples were spotted on polyamide thin layers and developed bidimensionally prior to recovery of [^{3}H]dansyl-phenylalanine for scintillation counting. The presence of spiro-arogenate does not interfere with the radio-dansyl assay for arogenate since spiro-arogenate lacks a free amino group for dansylation. Subsequent acidification produces a mixture of tritiated dansyl-phenylalanine (previously was L-arogenate) and nonradioactive phenylalanine (previously was spiro-arogenate). If the mixture is first acidified prior to dansylation, then the tritiated phenylalanine represents the combined total of both compounds. Hence, if steps of dansylation and acidification are carried out in different order in separate assays, the fractional portion of spiro-arogenate in a mixture can be calculated as illustrated under Table I. The data in Table I illustrate the reliability of the radio-dansyl assay technique, as well as the degree of variation encountered from different accumulation experiments.

Dansylation Procedures

Dansylation of Phenylalanine Standards—To 20 μl of a standard phenylalanine solution, 2 μl of H_2O, 2 μl of 3.4 M potassium carbonate buffer (pH 10), and 10 μl of 9:1 solution of [^{3}H]dansyl-chloride (1 mCi/ml, 24.9 Ci/mmol): unlabeled dansyl-chloride (5% w/w in acetone) were combined in a culture tube (6 × 50 mm) and sealed with parafilm. The samples were incubated for 30 min at 37 °C, dried in a desiccator, and then dansyl derivatives were dissolved from the residue by adding 50 μl of chilled methanol. The samples (sealed) were

TABLE I

Analytical determination of AGN and SPN in mixtures from accumulation media

Replicate cultures[a]	AGN[b]	SPN[b]
1	0.28 ± 0.02	0.19 ± 0.04
2	0.27 ± 0.02	0.13 ± 0.04
3	0.25 ± 0.02	0.18 ± 0.04
4	0.30 ± 0.02	0.42 ± 0.04

[a] Each culture was grown independently at 23 °C for 6 days in the standard 1% sucrose medium described under "Materials and Methods" prior to assay of culture medium.

[b] AGN was measured as [^{3}H]dansyl-PHE through use of steps (also see under "Materials and Methods") represented as follows:

$$\text{SPN} + \text{AGN} \xrightarrow{[^3\text{H}]\text{dansyl-Cl}} \text{SPN} + [^3\text{H}]\text{dansyl-AGN} \xrightarrow{\text{H}^+} \text{PHE} + [^3\text{H}]\text{dansyl-PHE}.$$

SPN concentrations were calculated by first taking the total of (AGN plus SPN) measured as follows:

$$\text{SPN} + \text{AGN} \xrightarrow{\text{H}^+} \text{PHE} \xrightarrow{[^3\text{H}]\text{dansyl-Cl}} [^3\text{H}]\text{dansyl-PHE}.$$

Subtraction of the value obtained for AGN alone from that obtained for the sum (SPN + AGN) equals the calculated values for SPN. Background radioactivity (mostly trace amounts of phenylalanine in the culture sample) was measured in control samples in which acidification was omitted. In each case above, this value was 0.01 mM ± 0.002. Concentrations of dansyl-phenylalanine formed in reaction mixtures were read from dansyl-phenylalanine standard curves prepared from authentic phenylalanine. The concentration of each compound was determined by averaging the values obtained with 2–3 separate measurements. The indicated standard deviation was estimated from the range of values according to the method of Dean and Dixon (9).

allowed to stand on ice for 30 min, with occasional vortexing. If the residue adhered to the tube, the needle of the syringe used for spotting was used to loosen the residue. Five μl of the methanol extract was spotted on a polyamide plate (5 × 5 cm).

Dansylation of L-Arogenate and Spiro-arogenate—Ten μl of samples, 12 μl of H_2O, and 1 μl of 1 N HCl were combined in the culture tube and incubated for 20 min at 37 °C. After acid conversion was complete, 1 μl of 1 N NaOH, 2 μl of potassium carbonate buffer, and 10 μl of [^{3}H]dansyl-chloride:5% dansyl-chloride in acetone (9:1) were added. Samples were incubated for 30 min at 37 °C, chilled on ice, and evaporated to dryness, as above. Five μl of the methanol extract were spotted on polyamide.

Dansylation of Arogenate—Ten μl of sample, 2 μl of potassium carbonate buffer, 12 μl of H_2O, and 10 μl of 9:1 [^{3}H]dansyl-chloride:5% dansyl-chloride in acetone were combined and incubated in sealed tubes for 30 min at 37 °C. After incubation the dansyl-arogenate was converted to dansyl-phenylalanine by addition of 2 μl of 5.1 M $HClO_4$. The tubes were sealed and placed in a 37 °C water bath for 30 min. One μl of potassium carbonate buffer was added to neutralize the reaction mixture. The samples were then evaporated to dryness, and the dansyl-phenylalanine (from dansyl-arogenate) was extracted into 50 μl of methanol, as above. Five μl of the methanol extracts was spotted on the polyamide plate.

In experiments where phenylalanine was present as a nutritional supplement, arogenate was separated from phenylalanine and spiro-arogenate in samples taken for analytical assay prior to dansylation. Ten-μl samples of each supernatant (and standard L-phenylalanine solutions) were spotted on a silica plate (7 × 5 cm) and developed in EtOH:$CHCl_3$:NH_4OH (4:1:1). The broad arogenate band (R_F = 0.25–0.35) was then eluted and dansylated with [^{3}H]dansyl-chloride. Spiro-arogenate was not measured, as it was not well separated from the phenylalanine.

Development of Thin Layer Chromatography Plates

Plates were usually developed in two dimensions. The solvent for the first development was NH_4OH:H_2O (1:4). Plates were thoroughly dried and developed in the second dimension in benzene:acetic acid:pyridine (50:5:1). The separation and quantitation of dansyl-arogenate and dansyl-phenylalanine is illustrated in Fig. 2. Dansyl-arogenate is more mobile than dansyl-phenylalanine in solvent 1. Prior to development in solvent 2, the dry plates are exposed to formic acid vapors for 15 min. This converts the separated dansyl-arogenate to dansyl-phenylalanine. Chromatographic development in the second direction is then carried out as shown in Fig. 2. Although conversion of dansyl-arogenate to dansyl-phenylalanine after dansylation is not essential, the radioactivity of the dansyl-phenylalanine spot is measured more accurately than the dansyl-arogenate spot. This is especially true when the reaction mixtures contain high salt concentrations, which enhances tailing of the dansyl-arogenate spot. The positions of dansylated compounds were visualized under short-wave (254 nm) ultraviolet light so that appropriate spots could be excised from the developed TLC plates and then placed in a scintillation vial containing 15 ml of Aquasol for tritium counting.

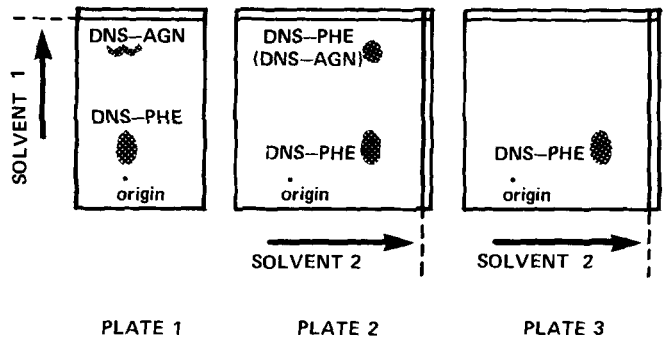

FIG. 2. **Resolution and quantitation of L-arogenate and L-phenylalanine in mixtures via two-dimensional thin layer chromatography.** The drawings are diagrams made from photographs taken under ultraviolet illumination. A number of spots corresponding to dansylic acid, dansyl-amide, and a few unknown dansyl derivatives are not shown for clearer presentation. *Plate 1*, one-dimensional separation of dansyl-phenylalanine (*DNS-PHE*) and dansyl-arogenate (*DNS-AGN*). Dansyl-arogenate migrates almost to the solvent front. *Plate 2*, a plate developed in solvent 1 as shown on the *left* with Plate 1 was exposed to formic acid vapors (converts dansyl-AGN to dansyl-PHE *in situ*) and then developed in solvent 2. This yields two dansyl-phenylalanine spots, the upper spot representing the amount of dansyl-arogenate originally applied at the origin. *Plate 3*, the mixture of dansyl-phenylalanine and dansyl-arogenate was acidified prior to application of the sample of the origin. Bi-dimensional thin layer chromatography was then carried out exactly as with Plate 2. This yielded a single spot of dansyl-phenylalanine representing the total amount of dansyl-arogenate plus dansyl-phenylalanine present in the sample.

Assay for Prephenate

Prephenate was quantitatively converted to phenylpyruvate, and absorption was measured at 320 nm, using a molar extinction coefficient of 17,500 (6) for calculations of phenylpyruvate concentrations. An appropriate control was included to determine the background absorption in samples not subjected to acidification.

RESULTS

Time Course of Metabolite Accumulation—The *N. crassa* mutant was grown for 6.5 days as described under "Materials and Methods" in order to monitor the accumulated concentrations of spiro-arogenate, arogenate, and prephenate. Arogenate increased at a near exponential rate, reaching a maximum concentration that usually did not exceed 0.40 mM (Fig. 3). The kinetics of arogenate accumulation closely resembled that previously reported for *N. crassa* grown in a New Brunswick fermentor (5). In that case, as in the current growth procedure, large mycelial clumps formed. This may have limited the viability per mass unit of cells, resulting in lower arogenate levels than those found in another procedure (5) where mycelia were largely dispersed.

Formation of spiro-arogenate paralleled that of arogenate until the fifth day, when the spiro-arogenate accumulation abruptly ceased. The appearance of arogenate before spiro-

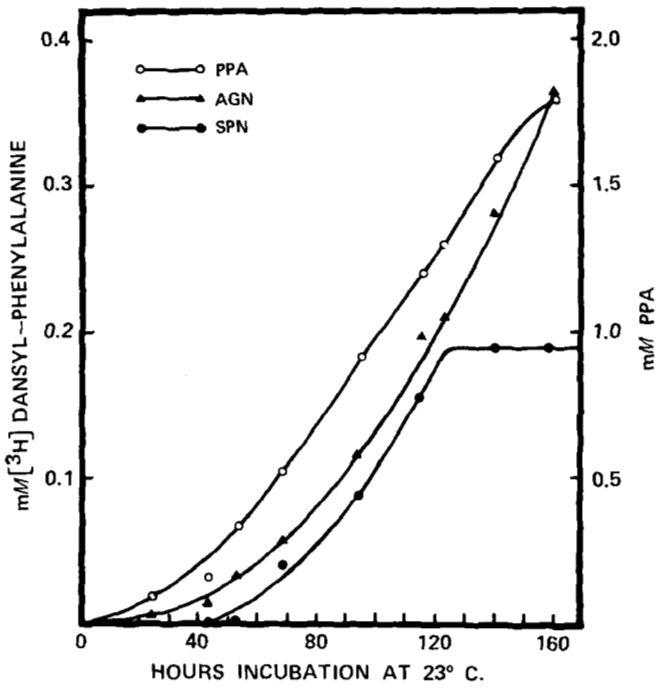

FIG. 3. **Kinetics of co-accumulation of PPA, AGN, and SPN molecules during growth in 1% sucrose medium.** AGN and spiro-AGN were measured as described under "Materials and Methods" and are expressed as [^{3}H]dansyl-phenylalanine concentration (*left ordinate*). The ratio of PPA:AGN continuously decreased from a high value of 16 at 24 h to 5 at 159 h.

TABLE II
Effect of growth temperature upon accumulation

Each culture was grown for 6 days in the standard 1% sucrose medium described under "Materials and Methods."

Growth temperature	PPA	AGN	SPN
°C ± 1 °C		mM	
23	1.1	0.30	0.42
30	1.8	0.42	0.46
37	2.0	0.26	0.33

arogenate is consistent with the probability on chemical grounds that arogenate is an immediate precursor of spiro-arogenate.

The ratio of prephenate/arogenate decreased from 16 to 5 over a typical 6-day period. This may be expected as the equilibrium in the transamination reaction is approached.

Effects of Temperature—Cultures were compared during growth at three incubation temperatures (Table II). The highest levels of arogenate and spiro-arogenate were accumulated at 30 °C, although the highest level of spiro-arogenate relative to its co-metabolites was achieved at 23 °C. Large decreases in both metabolites were noted when cultures were grown at 37 °C. A similar decrease in arogenate at 37 °C was previously noted (5).

Effects of Nutritional Supplements—The formation of arogenate *in vivo* arises from transamination of prephenate. Therefore, the nutritional supplements chosen as experimental variables in accumulation experiments were compounds expected to increase arogenate and/or spiro-arogenate concentrations by either: (i) increasing the intracellular level of prephenate and/or (ii) providing high levels of amino-donor substrates for prephenate aminotransferase.

Most supplements produced only minor effects on L-arogenate accumulation (Table III). Leucine, known to be a good amino donor for prephenate aminotransferases, did increase the concentration of L-arogenate by 30%. L-Phenylalanine, although a good amino donor for the prephenate aminotransferase reaction in *N. crassa in vitro*,[1] seemed to decrease the L-arogenate level. The combination of L-phenylalanine and shikimate did not lead to increased production of L-arogenate in these experiments.

Most amino acid supplements had little effect on L-arogenate accumulation but did tend to reduce the level of prephenate accumulated in the supernatant. (In the amino acid mixture-supplemented culture, where the additions are much lower than in the other cultures, this effect was less marked.) As expected, the presence of shikimate increased the accumulation of prephenate. However, when shikimate and an amino acid were both present in the medium, the amino acid effect (reduced prephenate levels) appeared to dominate over the enhancing effect of shikimate. All of the supplemented cultures accumulated less spiro-arogenate than did the unsupplemented culture. The spiro-arogenate level was not measured in the supernatants containing additional phenylalanine, due to technical difficulty in separating spiro-arogenate from phenylalanine.

Effects of Carbon Source upon Accumulation—The effects of six different carbon sources upon arogenate and spiro-arogenate accumulation were tested (Table IV). Cultures containing sucrose, glucose, or fructose produced comparable levels of arogenate, fructose possibly being the best substrate. In these experiments a slightly higher concentration of spiro-arogenate was achieved using glucose rather than sucrose as the carbon source. Both fructose and glucose media yielded a prephenate/arogenate ratio approximately twice that of sucrose, consistent with previous findings (5).

TABLE III
PPA, AGN, and SPN accumulation in nutritionally supplemented media

Cultures were started by inoculating 50-ml volumes of medium described under "Materials and Methods." After 2 days of growth at 23 °C, 150 ml of medium supplemented as indicated was added to the cultures. Each culture contained 1% sucrose as the carbon source.

Supplementation[a]	PPA	SPN	PHE	AGN
		mM		
None	1.50	0.18	0.01	0.25
L-Leucine	0.91	0.04	0.12	0.36
L-Leucine + shikimate	0.62	0.13	0.08	0.27
Shikimate	1.80	0.09	0.01	0.20
L-Glutamate	0.48	0.08	0.09	0.27
L-Glutamate + shikimate	0.80	0.03	0.01	0.30
Amino acid mixture[b]	1.20	0.07	0.01	0.23
L-Tyrosine	1.45	0.14	0.03	0.23
L-Phenylalanine[c]	0.49			0.18
L-Phenylalanine + shikimate[c]	0.64			0.15

[a] Additions to the media were made to give a final concentration of 1 mg/ml.

[b] L-Methionine, L-glutamate, and L-leucine were added to yield final concentrations of 200 μg/ml each.

[c] When L-PHE was present in the culture medium, only PPA and AGN concentrations were measured. AGN was separated from PHE by TLC prior to measurement of AGN (see Fig. 2). The migration positions of SPN and PHE were too close to achieve satisfactory separations.

TABLE IV
Effect of carbon source upon accumulation

Samples were taken after 5 days of growth and accumulation.

Carbon source (0.75%)[a]	AGN	SPN	PPA	Ratio, PPA/AGN
		mM		
Sucrose	0.28	0.10	1.1	4.1
Fructose	0.33	0.15	2.6	7.9
Glucose	0.28	0.18	2.4	8.6

[a] Ribose, acetate, and glycerol were also tested; cultures grew poorly and accumulations were low.

TABLE V

Effect of sucrose concentration upon metabolite accumulation

Samples from each culture grown at 23 °C were taken after 5 days of incubation.

% Sucrose	PPA	AGN	SPN
w/v		*mM*	
0.25	0.43	0.08	0.04
0.50	0.10	0.15	0.03
0.75	1.1	0.28	0.10
1.0	2.0	0.27	0.13
1.5	2.4	0.28	0.12

The concentration of sucrose that produced maximal yields of L-arogenate or spiro-arogenate was less than that required for maximal yields of prephenate. Table V shows that the amount of arogenate and spiro-arogenate accumulated remained constant above 0.75% sucrose. On the other hand, prephenate final accumulations were roughly proportional to the initial concentration of sucrose provided. The difference in accumulation response to high sucrose levels observed in comparing L-arogenate and prephenate suggests that once a saturating level of prephenate is reached (presumably between 0.07 and 1.1 mM prephenate), the aromatic aminotransferase catalyzes transamination of prephenate at the maximum rate possible under the conditions of growth.

DISCUSSION

Analytical Significance—The recognition of spiro-arogenate as a co-metabolite of accumulated L-arogenate and prephenate is important since spiro-arogenate, a lactam derivative which will not serve as a substrate for such enzymes as arogenate dehydratase or arogenate dehydrogenase, may be confused with L-arogenate (on the criterion of their common property of acid-catalyzed conversion to phenylalanine). Improved analytical techniques have been developed to discriminate between these compounds and to quantitate them.

Optimization of Protocol for Accumulation—Variation of nutritional supplements and growth temperatures resulted in relatively modest variations in ratios of the three co-metabolites. The composite of all data obtained indicates that if maximized yield of prephenate is sought, culture at 37 °C in 1.5% fructose for 6–7 days is optimal. If maximal arogenate yield is sought, culture at 30 °C in 0.75% fructose in the presence of L-leucine for 6–7 days is optimal. For spiro-arogenate, its relative yield will be enhanced with 5 days of accumulation in 0.75% glucose at 23 °C.

Spiro-arogenate as a Chemical Entity—Spiro-arogenate is an interesting structure from a chemical vantage point, and the procedures described here have accommodated the definitive characterization and proof-of-structure studies recently completed (7). The lactam structure of spiro-arogenate (Fig. 1) is consistent with its acid-catalyzed conversion to phenylalanine, its ninhydrin-negative character, and its lack of reactivity with dansyl-chloride. In the laboratory it is apparently difficult to convert L-arogenate to spiro-arogenate since the elimination of water from L-arogenate will preferentially yield L-phenylalanine. It seems likely that the crucial role of an enzyme in catalyzing conversion of L-arogenate to spiro-arogenate would be to make the secondary alcohol less accessible to dehydration, thus allowing elimination of water from the tertiary carboxyl group and the amino group.

Spiro-arogenate as a Biological Entity—The biological role of spiro-arogenate in *Neurospora* is uncertain. The abnormally high level of accumulated prephenate in the mutant may have effectively forced metabolite flow at an accelerated rate into a minor and hitherto undetected pathway. On the other hand, the apparent two-step pathway may be a dead-end consequence of the abnormal biochemical balance of the mutant. If by chance elevated intracellular prephenate tends to be toxic and, therefore, a selective disadvantage for the mutant strain, there may have been a long history in laboratory subculture of innocent selection for improved or newly recruited enzyme capabilities to channel metabolite flow away from prephenate (toward arogenate and spiro-arogenate).

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