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Control of Pantothenate Accumulation in *Agrobacterium tumefaciens*

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Two pantothenate-requiring mutants of *Agrobacterium tumefaciens* have been isolated. One of them (strain WMP-1) is unusual in that growth levels equivalent to the parent strain are achieved only when the medium is additionally supplemented with aspartate or another compound related to the tricarboxylic acid cycle. Extracts of cells grown on limiting aspartate were found to contain four times more ^{14}C-pantothenate than those grown at optimal aspartate concentrations. This difference was found in both the perchloric acid-soluble and -insoluble fractions, presumably the coenzyme A pool and acyl carrier protein, respectively. These findings are discussed in terms of membrane integrity and the control of fatty acid biosynthesis.

Most of the pantothenate taken up by microorganisms is converted to coenzyme A (24, 25). Pantothenate and coenzyme A deficiencies manifest themselves in a wide variety of ways, but Guirard and Snell (8) concluded that these are all compatible with the sole role of coenzyme A as the carrier and activator of acetyl and other acyl groups. As an example, Holden et al. (11, 12) observed pantothenate deficiencies to be accompanied by a decreased ability to accumulate glutamate and other amino acids. Significantly, this was not due to defective uptake mechanisms but to a leakiness in the membrane that prevented the maintenance of the amino acid pools once formed. This defect could be corrected by the addition of exogenous fatty acids (10). Their interpretation is reasonable in view of the findings of Vagelos and co-workers (1, 6, 26) that coenzyme A is the immediate precursor of the 4'-phosphopantetheine moiety of the fatty acid synthetase acyl carrier protein.

Nutritional interdependency is well known (8). Vitamin requirements may be met by the addition of either the vitamin itself or the various end products of the metabolic reactions for which the vitamin was needed. The replacement of biotin by exogenous aspartate and fatty acids has been well studied (3). For those vitamins involved in fatty acid, and thus membrane, biosynthesis (i.e., biotin and pantothenate), apparent vitamin and amino acid nutritional requirements may be related only secondarily. Membrane defects may change permeability and pool size, as well as the functioning of membrane-bound proteins such as the permeases.

The addition of free fatty acids can eliminate 80% of the pantothenate requirement in *Streptotoccus faecalis* (29) and correct the glutamate pool deficiency in *Lactobacillus plantarum* (10). We want to know whether these interdependent effects of pantothenate deficiency are true of other bacteria, particularly those whose membrane fatty acid and phospholipid compositions are atypical. *Agrobacterium tumefaciens* was the organism of choice because it is known (5, 15) to contain methylated phospholipids such as phosphatidyl choline that are not present in the vast majority of bacteria (19). We have previously used this microorganism to study the unique membrane lipid methylations yielding phosphatidyl choline (14) and the cyclopropane fatty acids (13).

Preliminary experiments indicated that pantothenate metabolism in *A. tumefaciens* strain WMP-1 was influenced by the pool size of compounds related to the tricarboxylic acid cycle in a manner exactly opposite to what one would expect if aspartate were acting solely as the precursor of the β-alanine moiety of pantothenate. An understanding of this relation could lead to a better understanding of the

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control of fatty acid synthesis and the rate of pantothenate utilization.

Carbohydrate metabolism in *A. tumefaciens* has been characterized by radiorespirometry (L. Arthur, G. St. Julian, and L. Bulla, *unpublished data*). Functional pentose phosphate, Entner-Doudoroff, and tricarboxylic acid cycles are present to the exclusion of the Embden-Meyerhof-Parnas path. The absence of glycolysis is another variable that conceivably could alter the pantothenate metabolism.

MATERIALS AND METHODS

Strains used. *A. tumefaciens* WP-3 (NRRL B-3738) is a pantothenate-requiring mutant derived from the wild-type strain W (NRRL B-36); strain WMP-1 (NRRL B-3737B) is a mutant requiring both methionine and pantothenate that is derived from the methionine-requiring strain WM-11 (NRRL B-3557), which itself had been previously (13) derived from strain W also. The pantothenate-requiring mutants were isolated by a procedure in which *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (300 μg/ml) was used as the mutagen, followed by enrichment in media (13) supplemented with 2 μg of calcium pantothenate/ml. Mutant growth in response to exogenous pantothenate and amino acids was determined turbidimetrically (Klett no. 66 filter), after aerobic incubation at 25 C. When strain WMP-1 is grown in basal medium (BM; 13) supplemented with methionine, pantothenate, and aspartate, 250 Klett units is found to equal 1.0 mg (dry weight) of cells per ml.

Nutritional characterization. Inocula for all subsequent experiments were grown for 24 to 48 hr on yeast extract-nutrient agar slants. A loopful of cells was suspended in saline for 0.5 hr, and 0.2 ml was inoculated into 25 ml of BM with the appropriate supplement. Growth response to added nutrients was determined from the turbidity ratio of supplemented and unsupplemented cultures after incubation times varying from 20 to 35 hr. When 0.2 ml of the WMP-1 saline suspension was plated on BM with methionine, no pantothenate-independent colonies were detected after 3 days.

Most of the following pool size and pantothenate uptake experiments were done with strain WMP-1; 500-ml cultures were grown aerobically in Fernbach flasks. After their turbidities were determined, all of the cultures were harvested at the same time. Since the doubling time is known to increase as the supply of methionine or aspartate decreases, this means our cultures are not at precisely the same stage of growth. All cells were centrifuged before the fastest growing culture (BM with 75 μg of methionine, 150 μg of L-aspartate, and 4 μg of calcium pantothenate/ml) entered the stationary phase. Our subsequent experiments on pantothenate uptake and accumulation compare cells from amino acid-limited cultures in early exponential phase with cells from cultures with optimal amounts of the same amino acid and in late exponential phase. Since both cultures are still exponential, not many cyclopropane fatty acids would be expected to have been formed yet (18).

Accumulation of pantothenate. Cells were harvested after 20 to 35 hr of growth and suspended in 0.01 M potassium phosphate buffer at *p*H 7.0 containing 0.01 M 2-mercaptoethanol (1). Cytoplasmic extracts were obtained by breaking the cells in a French press, whereupon the cellular residue was centrifuged at 22,000 × *g* for 30 min. Estimation of viable count indicated that about 80% of the WMP-1 cells were ruptured, independent of whether they had been grown on optimal or limiting aspartate. This finding eliminates the possibility that our observed differences in pantothenate accumulation are really due to a differential degree of cell rupture.

The pantothenate concentrations in the cytoplasmic extracts, expressed as nanomoles per milligram of protein, were determined in two ways. (i) Coenzyme A was determined from its concentration effect on phosphotransacetylase catalysis in the presence of excess acetyl phosphate (1). This indirect method is valid because most of the added pantothenate is converted to coenzyme A. (ii) Pantothenate accumulation was measured directly by growing the cells in media to which 1.60 μmoles of ^{14}C-pantothenate (0.84 Ci/mole) had been added. These cytoplasmic extracts were adjusted to contain identical amounts of protein, whereupon they were treated with 1 M $HClO_4$ (1). The amounts of ^{14}C-pantothenate in the cytoplasmic extracts and aqueous suspensions of the acid-insoluble fractions were determined. The perchloric acid-soluble fraction is presumed to represent the joint pool of pantothenate and coenzyme A, whereas all protein-bound pantothenate, particularly the acyl carrier protein of fatty acid synthetase, would be perchloric acid insoluble.

Protein concentrations were determined by the method of Lowry et al. (20).

Uptake of pantothenate. WMP-1 cells grown under aspartate-limiting conditions were harvested, washed, adjusted to a final turbidity of 200 Klett units, and equilibrated for 15 min in media (13) either containing or not containing 4 μg of aspartate/ml at 25 C. The procedure of Britten and McClure (2) was modified to measure ^{14}C-pantothenate uptake and pool formation. ^{14}C-pantothenate (final concentration, 5.7×10^{-7} M) was added to 20 ml of the cell suspensions, and samples were taken at intervals to determine the amount of ^{14}C-pantothenate that was taken up into either whole cells or trichloroacetic acid-insoluble material. Membrane filters (0.45 μm pore size) were used to collect the samples, and the pool size was determined by subtracting the trichloroacetic-insoluble ^{14}C-pantothenate from that of whole cells. Since these experiments were conducted at 25 C rather than at 0 C, we are not measuring the size of a preexisting pool by exchange, but, instead, are comparing the ability to form a pantothenate pool and the maximum such a pool can achieve at a given exogenous pantothenate concentration.

Radioactivity measurements. D-Pantothenic-*1,3-*^{14}*C* acid was obtained from International Chemical & Nuclear Corp. Radioactivity was determined in a Beckman LS-100 scintillation counter

with corrections for dilution, quenching, and background. Samples containing water were counted in a dioxane solution (18); all others were made in toluene containing 4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] benzene per liter.

RESULTS

Mutant growth requirements. Exogenous pantothenate was found to be required for the growth of mutant strains WP-3 and WMP-1 in BM containing optimal methionine (75 μg/ml). The parent strains W and WM-11 do not require pantothenate. Either 0.4 μg of calcium pantothenate or 5 μg of DL-pantoyl lactone per ml satisfied the pantothenate requirement. The fact that pantoyl lactone could replace pantothenate immediately classifies our mutants as defective in the synthesis of pantoic acid rather than the other component of pantothenate, namely, β-alanine. The greater requirement for DL-pantoyl lactone is consistent with Novelli's (23) observation that pantothenate synthetase is specific for L-pantoic acid, and thus preliminary hydrolysis of the lactone is required.

Strain WP-3 appears to be a normal vitamin auxotroph in that added pantothenate restored growth to parental levels. The situation with WMP-1 is more complex, however. Parental growth levels could be achieved only when the pantothenate or pantoyl lactone was supplemented by the addition of aspartate (Fig. 1) or another compound related to the tricarboxylic acid cycle (Table 1). Figure 1 shows that maximal growth required the addition of methionine, pantothenate, and aspartate to the basal medium. Omission of any one prevented growth. Those cultures that did grow in the absence of either aspartate or pantothenate did so after long lag periods and were found to be revertants. The revertants were uniformly independent of exogenous pantothenate. Note that the absence of aspartate has selected for strains that no longer require pantothenate, even though pantothenate was present in the medium which contained no aspartate. This result indicates that the double requirement results from a pleiotropic mutation rather than a fortuitous double mutation.

Strain WMP-1 is indeed a pantothenate mutant because: (i) when pantothenate was omitted no growth was observed; (ii) no other compound has yet been found to substitute for pantothenate; (iii) in the absence of exogenous aspartate the addition of pantothenate beyond those amounts required by a typical pantothenate auxotroph such as strain WP-3 was with-

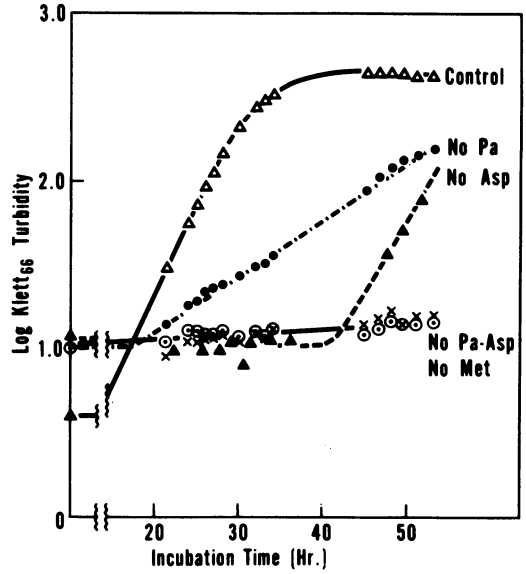

FIG. 1. *Growth of strain WMP-1 aerobically at 25 C in response to external nutrients. The control medium (Δ) contained a complete supplement of methionine (75 μg of Met/ml), calcium pantothenate (4 μg of Pa/ml), and aspartic acid (150 μg of Asp/ml) in a basal medium. (×) Pa and Asp are missing; (⊙) Met is missing; (●) Pa is missing; (▲) Asp is missing.*

TABLE 1. *Growth stimulation in Agrobacterium tumefaciens by tricarboxylic acid cycle-related compounds*

Supplement (300 μg/ml)[a]	Klett$_{66}$ turbidity ratio[b] of strains		
	WM-11	WP-3	WMP-1
None (control)[b]	1.0	1.0	1.0
L-Aspartic acid	2.3	1.7	5.5
Succinic acid	2.4	1.0	5.0
Fumaric acid	2.1	1.3	4.1
Malic acid	2.2	0.3	2.4
α-Ketoglutaric acid	2.3	0.7	6.6
L-Glutamic acid[c]			10.0
β-Alanine[d]			1.1
Biotin[e]			3.0

[a] All media neutralized with KOH to pH 6.8.

[b] Growth stimulation was recorded by Klett$_{66}$ turbidity ratios (no. 66 filter) of supplemented cultures to an unsupplemented control. WM-11 control = 38 Klett$_{66}$ units after 24 hr; WP-3 = 30 after 30 hr; WMP-1 = 9 after 30 hr. All controls contained pantoyl lactone (20 μg/ml) and methionine (100 μg/ml) in basal medium at 25 C.

[c] L-Glutamic acid (150 μg/ml of medium) and 4 μg of calcium pantothenate in place of pantoyl lactone. The cultures were incubated for approximately 35 hr. Growth stimulation with aspartate in place of glutamate was 13.7 in this experiment.

[d] β-Alanine (120 μg/ml), incubated for 44 hr.

[e] Biotin (0.8 μg/ml), incubated for 24 hr.

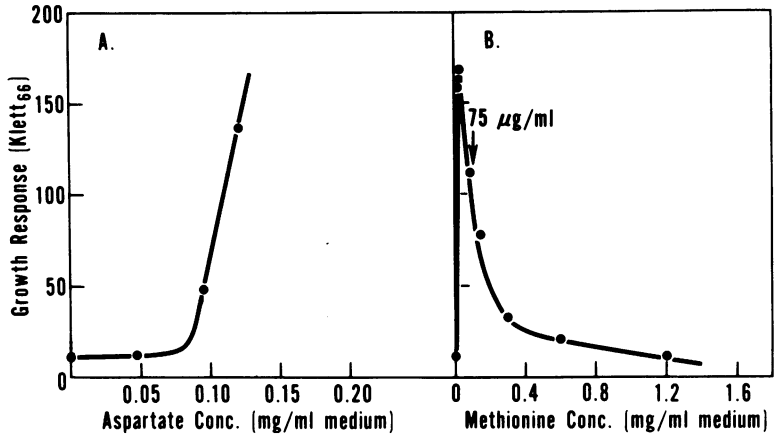

FIG. 2. *Growth response of strain WMP-1 (Agrobacterium tumefaciens mutant) to exogenous* L-*aspartate (A) and* L-*methionine (B) in a basal medium (BM) containing* DL-*pantoyl lactone (20 µg/ml used in place of pantothenate). The culture of curve A contained excess methionine (75 µg/ml) and that of curve B contained excess aspartate (120 µg/ml) as supplements also. All cultures were grown aerobically at 30 C for either 48 hr (A) or 20 hr (B).*

out effect; (iv) in the absence of exogenous pantothenate intracellular coenzyme A levels were observed to decrease dramatically in both of our presumed pantothenate auxotrophs (see Table 3).

In an effort to understand why strain WMP-1 required aspartate, a variety of compounds related to aspartate metabolism were tested for their ability to substitute for aspartate. These are listed in Table 1. The relative stimulation by tricarboxylic acid cycle-related compounds was much greater for strain WMP-1 than for WM-11 or WP-3, but this was only because the unsupplemented WMP-1 control did not grow at all whereas the other two did. The addition of tricarboxylic acid cycle-related compounds served to raise the growth of WMP-1 to the approximate level of the other strains.

Because aspartate is the metabolic precursor of β-alanine, it is important to note that β-alanine was without effect. This reinforces our conclusion that the effects observed in Table 1 represent a general requirement for tricarboxylic acid cycle intermediates rather than for the pantothenate precursor, β-alanine.

Guirard and Snell (8) concluded that a typical aspartate auxotroph required only 10 µg of aspartate/ml to achieve maximal growth. Figure 2B shows that the methionine requirement in strain WMP-1 appears normal in that less than 50 µg/ml was required for maximal growth. The aspartate requirement (Fig. 2A) was more extensive; 100 to 150 µg of aspartate/ml was required, depending on the temperature chosen. We consider a dependency of this magnitude to be consistent with the need to maintain a pool of tricarboxylic acid cycle intermediates in the presence of a leaky membrane (10-12). The inhibition evident (Fig. 2B) at high concentrations of methionine may be due to false feedback inhibition by methionine on earlier metabolic reactions common to other end products derived from aspartate (28). An identical methionine inhibition of growth was shown by the methionine-independent strain W. For reasons that are not entirely clear, this methionine inhibition was not present in the methionine-dependent strain WM-11, the intermediary derived from strain W and from which WMP-1 was isolated (13).

Pantothenate accumulation. ^{14}C-pantothenate accumulation was evaluated by comparison of extracts of cells from 1-day-old cultures grown under nutritionally complete conditions with those of cells whose growth had been limited by suboptimal concentrations of aspartate. Total radioactive counts were measured both in the whole extract and in the perchloric acid-insoluble fraction. This latter fraction consists primarily of the fatty acid synthetase acyl carrier protein (1), whereas the pantothenate and coenzyme A pools are perchloric acid-soluble. We thought that information on total pantothenate accumulation and its intracellular distribution at both limiting and optimal aspartate concentrations might reveal why the WMP-1 strain required them both.

Table 2 shows that, when aspartate was limiting, strain WMP-1 accumulated approximately four times as much ^{14}C-pantothenate as

TABLE 2. *^{14}C-pantothenate accumulation by strain WMP-1*

Limiting supplement[a]	Strain	Cultural turbidity ($Klett_{66}$)	^{14}C-pantothenate (nmoles per mg of protein)[b]		
			Cytoplasmic extract	Perchloric acid precipitate	Perchloric acid soluble
Excess (control)	WM-11	284	1.36	0.65	0.71
Excess (control)	WP-3	135	2.80	1.89	0.91
Excess (control)	WMP-1[c]	236	1.84	1.12	0.72
Asp-lim	WMP-1[c]	28	8.28	4.35	3.93

[a] Medium supplements are as described in Table 3. All cultures contained an excess of ^{14}C-pantothenate (1.60 μmoles or 2.98×10^6 disintegrations per min per 500 ml of medium).

[b] ^{14}C-pantothenate (nanomoles) per milliliter was determined by dividing the total radioactivity in the $HClO_4$-precipitate from 1 ml of cytoplasmic extract by the specific activity (disintegrations per minute divided by nanomoles of ^{14}C-pantothenic acid). Protein concentrations are the total protein in 1 ml of cytoplasmic extract.

[c] The turbidity of WMP-1 cultures was measured after 30 hr of incubation, when the cells were collected by centrifugation.

when it was present in optimal amounts. This difference was observed in both the perchloric acid-soluble and -insoluble fractions.

These observations are reinforced by a similar set of experiments (Table 3) which differed from those in Table 2 only in that nonradioactive pantothenate was added initially and the cell extracts were assayed for coenzyme A enzymatically. Here too, we see that the coenzyme A pool, and thus the amount of pantothenate accumulated, was markedly higher in strain WMP-1 when its rate of growth had been limited by deficient levels of aspartic acid or methionine. It is comforting to note that the internal control of pantothenate-limited growth does indeed give negligible coenzyme A concentrations.

This interdependence of coenzyme A level with growth condition seems to apply only to WMP-1. Coenzyme A levels in both the methionine auxotroph WM-11 and the pantothenate auxotroph WP-3 remained unchanged in the various media, even though they consistently differed from each other by a factor of five. Two separate methods of assay agreed in indicating that strain WMP-1 accumulates exogenous pantothenate to a greater extent if its growth is

TABLE 3. *Coenzyme A pool in cytoplasmic extracts under growth-limiting conditions*

Exogenous requirement	Strain	Limiting supplement[a]			
		Control	Pa-lim	Met-lim	Asp-lim
Met	WM-11	1.6[b]		1.9	1.9
Pa	WP-3	0.3	0.0[c]	0.2	0.4
Pa-Met-Asp	WMP-1[d]	0.9	0.2	1.6	1.8

[a] Control cultures contained an excess of all three supplements: pantothenate (4 μg/ml), methionine (75 μg/ml), and aspartic acid (150 μg/ml). Limiting supplements of growth medium (1 ml) were as follows: 0.12 μg of calcium pantothenate (Pa-lim), 10 μg of L-methionine (Met-lim), and 9 μg of L-aspartic acid (Asp-lim). The cytoplasmic extracts were obtained by French pressure disruption of cells grown aerobically for approximately 24 hr.

[b] Nanomoles of coenzyme A per milligram of cytoplasmic protein.

[c] No coenzyme A was detected in WP-3 cells (3.2 mg of cytoplasmic protein per ml) if exogenous pantothenate limited growth.

[d] The values shown are the mean of two separate experiments with strain WMP-1.

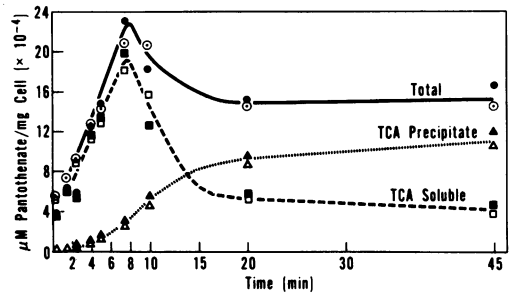

FIG. 3. *Effect of exogenous aspartate on ^{14}C-pantothenate (5.7×10^{-7} M) uptake by whole cells (●, ⊙), trichloroacetic acid-soluble pool (■, □), and trichloroacetic acid-precipitated materials (▲, △). Strain WMP-1 was grown under an aspartate-limiting condition (see Table 3, Asp-lim). The washed cells (0.8 mg of dry cells/ml) were preincubated for 15 min either with 4 μg of aspartate/ml (closed symbols) or without aspartate (open symbols) in the basal medium containing 75 μg of methionine/ml. The soluble pool size (■, □) was determined by subtracting the uptakes of trichloroacetic acid-insoluble material from whole cells.*

aspartate limited. We have not conducted similar experiments under a sufficient number of nutritional conditions to state whether these differences are specific for tricarboxylic acid cycle components or whether they derive from general considerations regarding differences in growth rate and stage of growth. In any case, we believe the differences are a manifestation

of fatty acid synthesis control mechanisms.

Pantothenate uptake. The fact that aspartate concentrations can influence overall pantothenate accumulation in strain WMP-1 could mean that it was influencing the cells' ability either for uptake or for retention once taken up (10–12). Figure 3 shows a comparison of strain WMP-1 short-term uptake ability for pantothenate in the presence and absence of exogenous aspartate. The cells had been grown on limiting aspartate previous to harvesting for the uptake experiment. In either case, ^{14}C-pantothenate was taken up rapidly and achieved maximal concentration in the soluble pool within 7 to 8 min. Thereafter, the ^{14}C-pantothenate pool decreased as it was incorporated in trichloroacetic acid-insoluble material. This too occurred at the same rate, regardless of aspartate concentration.

DISCUSSION

We have shown that *A. tumefaciens* strain WMP-1 requires both pantothenate and a tricarboxylic acid cycle-related compound for growth. The biochemical nature of this interdependency is of immediate interest. This difference between strain WMP-1 and other pantothenate auxotrophs may be caused by the genetic location of the pantothenate lesion or by the simultaneous presence of the methionine auxotrophy. A similar dependence of pantothenate function on sufficient tricarboxylic acid cycle intermediates has been found for naturally occurring strains of *Erwinia* (7) and *Bacillus thuringiensis* (K. W. Nickerson, *unpublished data*).

The primary metabolic role of pantothenate is to act as a precursor for coenzyme A synthesis. Since exogenous free fatty acids can replace 80% of an organism's nutritional requirement for pantothenate (29), we thought it reasonable to examine the unique features of strain WMP-1 with a view toward possible fatty acid biosynthetic controls. That such controls exist is evident from the work of Henderson and McNeill (9), in which *Lactobacillus plantarum* fatty acid biosynthesis is subject to feedback regulation by exogenous long-chain fatty acids. It is also implicit in work (16, 22) showing pronounced changes in fatty acid and phospholipid composition in response to changing growth conditions; i.e., changes in temperature, oxygen tension, stage of growth cycle, growth rate, or nutritional limitations of the carbon or nitrogen supply.

Our finding that growth on limiting aspartate forces the cells to produce four times as much coenzyme A and acyl carrier protein as normal is a further indication of fatty acid biosynthetic control. The interdependence of pantothenate and tricarboxylic acid-cycle compounds is reasonable because the rate of fatty acid synthesis is determined by the enzyme acetyl coenzyme A carboxylase which converts acetyl coenzyme A to malonyl coenzyme A (27). This important enzyme is activated by the di- and tricarboxylic acids of the tricarboxylic acid cycle, and in their absence very little fatty acid is formed (27). Added aspartate would then allow the fatty acid biosynthetic machinery already present to function more efficiently and so eliminate the need for additional machinery. This is fundamentally different from the observation of Maas and Davis (21) that in *Escherichia coli* L-aspartate acts as an inhibitor of pantothenate synthesis by competing with β-alanine for the pantothenate synthetase enzyme.

Holden and his colleagues (10–12) have shown that an abnormally large amino acid requirement may result from a leaky membrane and the consequent inability of the organism to maintain a sufficiently large pool size. A selectivity must exist with regard to which pools are susceptible to loss through a leaky membrane. Obviously, the coenzyme A pool is larger (Tables 2 and 3) under the aspartate-limited conditions postulated to accentuate leakiness. The aspartate pool is especially important because of its role in activating fatty acid synthesis. If its concentration drops below a certain level, a vicious circle is created. Aspartate is lost through a leaky membrane because there is not enough aspartate to provide adequate synthesis of fatty acids. The pool of tricarboxylic acid cycle intermediates may be especially sensitive to loss through a leaky or defective membrane because the enzymes responsible for their formation are localized in that membrane.

The methionine auxotrophy already present in strain WM-11 may be responsible for differentiating WMP-1 from other pantothenate auxotrophs which do not require aspartate. Strain WM-11 is also stimulated by added tricarboxylic acid cycle intermediates (Table 1). However, this stimulation is enhanced in the pantothenate auxotroph WMP-1 to the point where it is an absolute requirement. We have already presented evidence (13) that strain WM-11 differs from its parent strain W in its mode of methylating cellular lipids. This is particularly true of the cyclopropane fatty

acids. A somewhat different specificity of membrane methylation patterns could explain the greater susceptibility to membrane leakage in both WM-11 and WMP-11. One possible cause of this difference in methylation would involve the known ability (17) of excess methionine to repress its own synthesis by repression of 5,10-methylenefolate-H_4 reductase. This enzyme converts 5,10-methylenefolate-H_4 to 5-methylfolate-H_4, the immediate precursor of the methionine methyl group. The amounts of methionine added to our methionine auxotrophs should be sufficient to repress the reductase, thus totally eliminating 5-methylfolate-H_4 production. If the 5-methylfolate-H_4 had an independent role in lipid methylation, that role would be lost, and the methionine auxotrophs would have to rely exclusively on the *S*-adenosyl methionine route to achieve methylation. Postulating an alternative route for methylation does not conflict with previous work (14) proving the existence of the *S*-adenosyl methionine route.

We should at least mention a few other possible reasons why strain WMP-1 requires aspartate. The pantothenate lesion may cause the metabolite immediately preceding the block to accumulate. At an elevated concentration, this metabolite might become inhibitory and require tricarboxylic acid cycle intermediates in excess to overcome that inhibition. The only two candidates would be 2-keto isovalerate and 2-keto pantoate. If true, the tricarboxylic acid requirement should have been evident in most other pantothenate auxotrophs with defective pantoic acid synthesis.

Alternatively, the aspartate may have been relieving a shortage in C_4 intermediates caused by a defective phosphoenolpyruvate carboxylase. This anaplerotic mechanism could be defective either because of a biotin deficiency (note the biotin stimulation in Table 1) or because the phosphoenolpyruvate carboxylase activator (4) acetyl coenzyme A is in short supply. This is certainly a reasonable supposition in pantothenate-deficient organisms, as Grula et al. (7) have pointed out, but this does not explain our observation that coenzyme A and acyl carrier protein levels decrease when sufficient aspartate is added.

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