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EFFECTS OF GIBBERELLIC ACID AND L-METHIONINE ON C_1 METABOLISM IN AERATED CARROT DISKS*

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Key Word Index—*Daucus carota*; Umbelliferae; carrot; storage tissue disks; GA_3; L-methionine; folate derivatives; enzyme activity; effect of aeration; regulation.

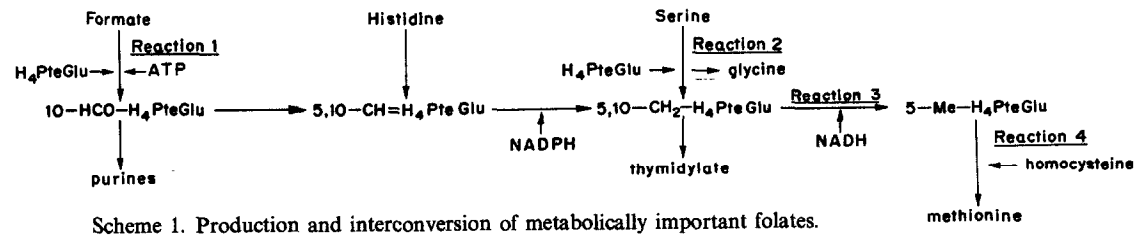
Abstract—Aeration of carrot storage tissue disks in water was accompanied by net folate synthesis and by changes in the specific activities of key folate-dependent enzymes. Disks aerated in 0.1 mM gibberellic acid (GA_3) for 48 hr contained higher concentrations of methyltetrahydrofolates but aeration in 5 mM L-methionine reduced net folate synthesis. Gibberellic acid also increased the specific activities of 5,10-methylenetetrahydrofolate reductase (E.C. 1.1.1.68), serine hydroxymethyltransferase (E.C. 2.1.2.1) and 5-methyltetrahydrofolate: homocysteine transmethylase. The levels of these enzymes in disks aerated in L-methionine (5 mM) were comparable or slightly higher than those of disks aerated in water. Activity of the reductase and 10-formyltetrahydrofolate synthetase (E.C. 6.3.4.3) was inhibited by L-methionine *in vitro*. Aeration increased ability to incorporate formate [^{14}C] into serine, glycine and methionine. Disks aerated for 36 hr in 0.1 mM GA_3 incorporated greater amounts of ^{14}C into free methionine but those aerated in L-methionine (5 mM) had less ability to metabolize formate and the specific radioactivities of free glycine, serine and methionine were low.

INTRODUCTION

Aeration of storage tissue disks is accompanied by rapid changes in several physiological and biochemical processes [1–7]. Recently we have reported [8] that these include synthesis of metabolically important folate derivatives. In carrot disks, aeration increased synthesis of methyl- and formyltetrahydrofolates as well as the specific activities of key enzymes for production and interconversion of C_1 units [8]. It was suggested that synthesis and turnover of methylfolates in this system was related to an observed net synthesis of methionine. In this connection, C_1 units appeared to arise principally from serine (Scheme 1, reaction 2). However, the presence of relatively high levels of 10-formyltetrahydrofolate synthetase suggests that C_1 units may be synthesized at the formyl level of oxidation (Scheme 1, reaction 1). We have now assessed the importance of this reaction by pulse feeding formate [^{14}C] to carrot disks undergoing aeration in sterile water.

In animal tissues and several microbial species, L-methionine controls the flow of C_1 units through methylfolate [9–11]. In contrast, there is little information on the regulation of C_1 metabolism in higher plants apart from observations [12,13] that L-methionine inhibits 5-methyltetrahydrofolate:homocysteine transmethylase activity (Scheme 1, reaction 4). To determine whether folate metabolism in carrot disks is regulated by methionine we have now examined the effects of this amino acid on (a) net folate synthesis, (b) the flow of formate carbon to methionine and (c) the activities of four folate-dependent enzymes.

Gibberellic acid (GA_3) is known to enhance the incorporation of nucleic acids into DNA in artichoke disks [6] and increases methylation of RNA in the barley aleurone system [14]. In this latter system, GA_3 clearly



Scheme 1. Production and interconversion of metabolically important folates.

increases the activity of *t*RNA methylase [15]. As aeration of storage tissue disks is accompanied by synthesis of gibberellins [16] and by methylation of *t*RNA [17,18] it follows that addition of GA_3 to the aeration medium may have pronounced effects on the biosynthesis of methylfolates.

* The abbreviations used for derivatives of pteroylglutamic acid are those suggested by the IUPAC–IUB Commission as listed in (1967) *Biochem. J.* **102**, 15, e.g. 5-Me-H_4PteGlu = N^5-methyltetrahydropteroylmonoglutamate.

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The present paper shows that aeration of carrot disks increased their abillity to incorporate formate [^{14}C] into serine and methionine. Aeration in the presence of L-methionine and GA_3 respectively, affected C_1 metabolism by changing folate pool size, the activities of certain folate-dependent enzymes, and the ability to incorporate formate [^{14}C] into free and protein methionine.

RESULTS

Total folate contents were determined during a 48 hr aeration period in water, 0.1 mM GA_3 and 5 mM L-methionine, respectively, as summarized in Fig. 1. Greatest net folate synthesis occurred in GA_3-treated disks which had been aerated for 48 hr. For example, such disks contained 550 ng PteGlu equivalents/g fr. wt compared to the 405 ng/g fr. wt of the water controls. In contrast, disks aerated in L-methionine solution had maximal levels of 365 ng/g fr. wt. The nature of these folates was examined by γ-glutamylcarboxypeptidase treatment and differential microbiological assay. This revealed (Fig. 1) that treatment with GA_3 and L-methionine affected the levels of polyglutamyl folates and changed the proportions of methyl and formyl derivatives. In this respect, GA_3 increased the levels of Me-H_4PteGlu$_n$ in disks aerated for 48 hr and raised the levels of Me-H_4PteGlu throughout the aeration period. As a result the total levels of methylfolates in GA_3-treated disks were some 45% higher than those of the controls. Greater levels of conjugated formylfolates were also present in GA_3-treated disks especially during the early stages of aeration.

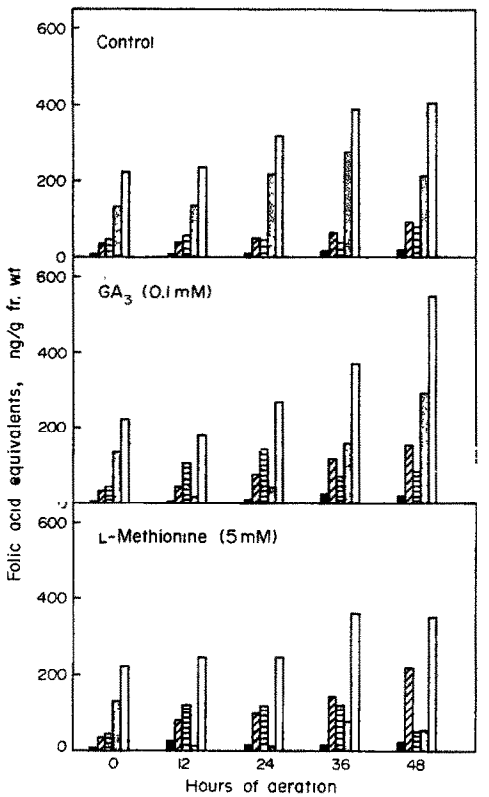

Fig. 1. Effect of treatment and aeration on folate levels in carrot disks. Disks (1 × 9 mm) were aerated in water (control), GA_3 and L-methionine as indicated. Extracts were prepared and assayed using *L. casei* and *S. faecalis*. Polyglutamyl folates were assayed after γ-glutamylcarboxypeptidase treatment. □, Total folates after γ-glutamylcarboxypeptidase treatment; ■, formyl mono- and diglutamyl folates; ▨, methyl mono- and diglutamyl folates; ▤, formyl polyglutamates; ▩, methyl polyglutamates. Data represents the mean of two separate determinations.

Table 1. Effect of aeration on key enzymes of folate metabolism of treated carrot disks

Enzyme and treatment	Time of aeration (hr) 0	12	24	36	48
10-Formyltetrahydrofolate synthetase					
Water control	918	1300	1460	1550	1850
0.1 mM GA_3		1240	1310	1630	1900
5.0 mM L-methionine		1210	1300	1150	970
Serine hydroxymethyltransferase					
Water control	0 98	1 99	1 63	1.66	1.93
0 1 mM GA_3		2 19	2 85	2 30	3.60
5.0 mM L-methionine		2.00	1.97	1.65	1.65
Methylenetetrahydrofolate reductase					
Water control	1.38	3 19	2.69	1.37	0.74
0 1 mM GA_3		3.76	2.78	2.41	1.22
5.0 mM L-methionine		2.71	2.44	2.50	2.12
Methyltetrahydrofolate homocysteine transmethylase*					
Water control	152	149	202	132	65
0.1 mM GA_3		256	281	428	215
5.0 mM L-methionine		160	197	228	247

* Expressed as pmol product formed/hr/mg protein at 30°. All other enzyme activities are expressed as nmol product formed/hr/mg protein at 30°. All assays were performed in duplicate and results are expressed as the mean.

L-methionine also affected the proportion of formyl and methyl derivatives in the folate pool (Fig. 1). Compared to the controls, such disks contained less conjugated methylfolates, particularly after 12 and 24 hr, but tended to accumulate greater amounts of Me-H_4PteGlu. As a result the total methylfolate concentration after 48 hr was decreased by *ca* 8% compared to the controls. Methionine-treated disks also contained greater concentrations of formyl polyglutamates during the first 36 hr of aeration.

Aeration in GA_3 and L-methionine also affected the levels of key folate-dependent enzymes (Table 1). In the control disks, the specific activity of 10-formyltetrahydrofolate synthetase rose with aeration. Treatment with GA_3 did not alter this trend but aeration in L-methionine gave lower specific activities and these declined as the aeration period was extended. Serine hydroxymethyltransferase, which also mediates synthesis of C_1 units, was increased by aeration and highest levels were achieved by GA_3-treated disks. However, methionine did not alter the levels of this transferase. The levels of methylenetetrahydrofolate reductase, a key enzyme in methylfolate synthesis (Scheme 1, reaction 3) rose in all three treatments during the initial stages of aeration and then declined. Highest reductase levels were found in GA_3-treated disks and the subsequent decline was less. Methionine-treated disks contained relatively high levels of this reductase throughout the aeration period. In the controls, the transmethylase, catalyzing methionine synthesis, was most active after 24 hr of aeration but GA_3-treated disks had greatest enzyme activity and this was maximal after aeration for 36 hr. In the presence of L-methionine, transmethylase activities rose throughout the aeration period and were generally higher than those of the controls.

Methionine did not drastically reduce production of the reductase or transmethylase (Table 1) although such

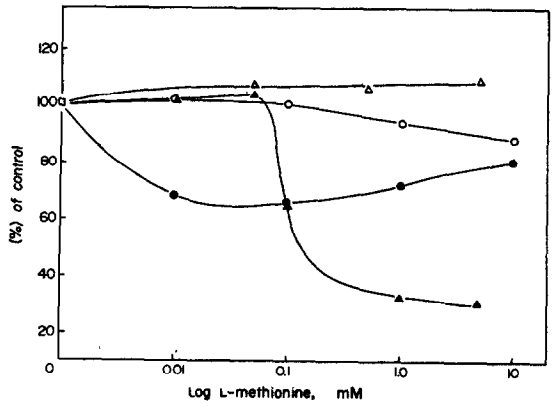

Fig. 2. Effect of L-methionine on the activity of folate-dependent enzymes. Disks were aerated in water for periods sufficient to reach maximal specific enzyme activities; 12 hr for 5,10-methylenetetrahydrofolate reductase (●) and 10-formyltetrahydrofolate synthetase (▲); 24 hr for 5-methyltetrahydrofolate: homocysteine transmethylase (○) and serine hydroxymethyltransferase (△). L-methionine was added to the standard reaction system prior to addition of L-serine-[3-^{14}C]. Data represent the mean of two separate determinations.

repression is common in microorganisms and animal tissues [10,11,19–21]. To determine whether this amino acid might inhibit enzyme activity *in vitro*, extracts were prepared from disks aerated in water. After gel filtration, enzyme activities were determined as shown in Fig. 2. Additions of methionine did not inhibit serine hydroxymethyltransferase activity. However, inhibition of the other folate-dependent enzymes was apparent. Methylenetetrahydrofolate reductase was most sensitive in this respect; at 10 μM L-methionine more than 30% inhibition occurred. At higher concentrations (1 mM) the synthetase was inhibited by *ca* 70%. Unlike the homocysteine transmethylase of pea seedlings [12], the enzyme of carrot disks was not appreciably inhibited at physiological concentrations of methionine.

In some species methyl group biogenesis within the folate pool is regulated by *S*-adenosylmethionine [9] which strongly inhibits methylenetetrahydrofolate reductase activity. The reductase of carrot disks was not, however, inhibited when increasing concentrations (up to 5 mM) of *S*-adenosylmethionine were added to the reaction system.

As GA_3 and methionine altered the concentration of folates and related enzymes, it follows that these treatments should affect the flow of C_1 units within the folate pool. To examine this, treated disks were examined for ability to incorporate formate [^{14}C] into amino acids related to C_1 metabolism. This was found in both freshly cut and aerated disks but maximal incorporations occurred after aeration for 36 hr (Table 2). Aeration also enhanced, by *ca* 3-fold, the labelling of other products. CO_2 was the chief product but methionine-treated disks metabolized less formate than those aerated in water or GA_3.

Analysis of the free protein amino acid fractions (Table 3) showed that the methionine and GA_3 treatments altered the specific radioactivities of acids related to C_1 metabolism. Aeration increased the labelling of serine,

Table 2. Incorporation of formate [^{14}C] by freshly cut and aerated carrot tissue disks

	Freshly cut disks		Disks aerated for 36 hr in Water (control)		0.1 mM GA_3		5 mM L-methionine	
Fraction	cpm	% of total	cpm	% of total	cpm	% of total	cpm	% of total
CO_2	184000	61.9	1010000	73.3	742000	73.3	536000	65.3
Lipids	390	0.1	1900	0.1	11000	1.1	180	0.02
Sugars	5000	1.7	5700	0.4	9100	0.9	11000	1.3
Organic acids	40200	13.5	131000	10.0	75400	7.4	54800	6.7
Amino acids								
Free	49800	16.8	132000	9.7	95400	9.4	211000	25.7
Protein	17800	5.9	89000	6.6	79400	7.8	7200	0.9
Total	298000		1360000		1010000		820000	

25 Disks from each treatment were incubated in duplicate in closed 25 ml Warburg flasks containing 1 μmol formate [^{14}C] (2.5 μCi/μmol; pH 5.9) in 4 ml H_2O for 1 hr at 30°. Results are expressed as cpm/25 disks extracted and are the mean of two separate determinations.

Table 3. Incorporation of formate [^{14}C] into free and protein amino acids by freshly cut and 36 hr aerated disks

	Freshly cut disks		Water control				Aerated for 36 hr 0.1 mM GA_3				5 mM L-methionine			
	Free	Protein	Free		Protein		Free		Protein		Free		Protein	
Amino acid	cpm/ nmol	cpm/ nmol	cpm/ 25 disks	cpm/ nmol	cpm/ 25 disks	cpm/ nmol	cpm/ 25 disks	cpm/ nmol	cpm/ 25 disks	cpm/ nmol	cpm/ 25 disks	cpm/ nmol	cpm/ 25 disks	cpm/ nmol
Methionine sulfoxide	n.d.	n.d.	4480	11.8	n.d.	n.d.	2580	5.80	n.d.	n.d.	530	1.89	n.d.	n.d.
Aspartate	4.31	3.09	11900	10.2	7730	6.55	8210	9.72	6660	4.35	12300	8.55	1150	0.83
Serine	11.5	26.8	88900	44.7	44200	54.6	52300	19.7	35600	43.4	58600	17.3	960	1.04
Glutamate	2 83	0.57	16700	8.66	1530	1 33	6990	5.46	1070	0.89	4030	3.99	400	0.30
Glycine	19.4	1.69	2660	44.3	3830	3.65	300	7.50	1660	1.54	2050	26.0	1410	1.20
Alanine	0 93	0.36	4330	8.49	750	0.69	300	0.81	840	0.81	840	1.02	140	0.05
Methionine	13.3	38.2	840	84.0	5470	124	1750	175	2670	103	109000	32.9	240	13.3

Carrot disks were either freshly prepared or pre-aerated in the three treatment solutions. 25 Disks from each treatment were incubated in duplicate in closed 25 ml Warburg flasks containing 1 μmol formate [^{14}C] (2.5 μCi/μmol; pH 5.9) in 4 ml H_2O for 1 hr at 30°. Considerably lower amounts of ^{14}C were also detected in threonine, proline, cysteine, valine and isoleucine. n.d.—not detected.

glycine and methionine, the latter displaying the highest specific radioactivity. GA_3 increased the labelling of free methionine by more than 100% but reduced the specific activities of free serine and glycine. Methionine reduced the concentration of ^{14}C in all three amino acids. Radioactivity also accumulated in free methionine, the pool of which was much enlarged by uptake from the aeration medium. As a result, protein labelling in these disks was greatly reduced.

DISCUSSION

Changes initiated by aeration of storage tissue disks are largely developmental in nature [7]. Consistent with this is the synthesis and methylation of *t*RNA [17,18], an event which commonly accompanies development in other systems [22–27]. This methylation has been implicated in amino acylation [28,29] and is generally dependent on methyl groups derived from methionine. In carrot disks, where net methionine synthesis accompanies aeration [8], these groups would be largely generated within the folate pool. Data in Fig. 1 and Table 1 show that aeration increased methylfolate content and the levels of enzymes which mediate methyl group turnover. Formate may be an important precursor of these C_1 units as aerated disks contained formyltetrahydrofolate synthetase (Table 1) and readily utilized this compound for synthesis of amino acids related to folate metabolism (Table 3).

GA_3, added to the aerating medium, tended to accentuate these changes. Thus this growth substance stimulated methylfolate synthesis (Fig. 1, Table 1) and the incorporation of formate into free methionine (Table 3). Although gibberellins have diverse physiological roles in plants [30], these effects on C_1 metabolism could be related to their effects on RNA and its methylation [14,15,30].

Disks aerated for 48 hr in 5 mM methionine contained less total methylpolyglutamates than the controls (Fig. 1) and less formate [^{14}C] was incorporated into amino acids deriving C_1 units from the folate pool (Table 3). These data and the inhibition of key folate-dependent enzymes by L-methionine (Fig. 2) suggest a regulation of C_1 metabolism by this amino acid or its metabolite. Such control is well documented for other species and centres on methyltetrahydrofolate:homocysteine transmethylase [11–13], methylenetetrahydrofolate reductase [11,31,32] and serine hydroxymethyltransferase [19,33–36]. In carrot disks, methionine pool size may regulate the reduction of CH_2-H_4PteGlu and generation of 10-HCO-H_4PteGlu by affecting enzyme activities (Fig. 2, Table 1). It remains to be determined whether these enzymes possess L-methionine binding sites for control of activity.

The low specific radioactivities of protein amino acids in the L-methionine-treated disks suggest that this treatment also reduced the rate of protein synthesis (Table 3). Such an effect could result from a depletion of ATP. In this respect, Davies [37] has shown that turnip disks convert exogenous methionine into *S*-adenosylmethionine and as a result significant amounts of adenosine are removed from participation in oxidative phosphorylation. An inhibition of protein synthesis by methionine in carrot disks may also explain why the specific activities of CH_2-H_4PteGlu reductase and Me-H_4PteGlu transmethylase were apparently higher in the methionine-treated disks (Table 1). This assumes, however, that both enzymes have low rates of turnover. Similarly, the pronounced decrease in HCO-H_4PteGlu synthetase levels in these disks (Table 1) could arise if this enzyme had a relatively high rate of turnover during the aeration period.

In common with other species [9] the folates of carrot disks are principally polyglutamyl derivatives (Fig. 1). Although precise roles for these compounds in C_1 metabolism require elucidation there is growing evidence for their metabolic importance [38,39]. In this connection, more than half the folates of carrot disks aerated in water were methylpolyglutamates and methionine greatly reduced their concentration (Fig. 1). As methylpolyglutamate concentration was also affected by L-ethionine [8] it follows that these complex folates may be an important source of methyl groups in this dynamic system.

EXPERIMENTAL

Materials. Source, disk preparation and aeration of carrot tissue (*Daucus carota* L. var. Nantes Coreless) were as previously described [8]. L-Serine-[3-^{14}C], sodium formate-[^{14}C] and tetrahydrofolate-N^5[*methyl*-^{14}C] were purchased from the Amersham/Searle, U.S.A. Tetrahydrofolic acid was obtained from Sigma Chem. Co.

Folate and enzyme assays. Samples (50 disks) were extracted [8], fractionated and assayed with *Lactobacillus casei* (ATCC 7469), *Streptococcus faecalis* (ATCC 8043) or *Pediococcus cerevisiae* (ATCC 8081) as previously described [40]. Polyglutamyl derivatives were hydrolyzed using a glutamic carboxypeptidase prepared from pea cotyledons [40]. N^5,N^{10}-methylenetetrahydrofolate reductase, N^5-methyltetrahydrofolate:homocysteine transmethylase, serine hydroxymethyltransferase, and N^{10}-formyltetrahydrofolate synthetase were assayed as described previously [8].

Formate-[^{14}C] feeding. Samples of 25 freshly-cut or aerated disks were transferred to 25 ml Warburg flasks containing 1 μmol formate[^{14}C] (2.5 μCi/μmol; pH 5.9) in 4 ml H_2O. The centre well contained a filter paper wick saturated with 20% KOH. Disks were incubated with shaking for 1 hr at 30°. A control flask containing formate[^{14}C] and KOH-impregnated filter paper was included to monitor possible release of ^{14}C from the incubating soln. $^{14}CO_2$ values were corrected using this figure. After incubation, the disks were removed, washed in H_2O and boiled in 10 ml EtOH for 5 min. After homogenizing (Ten Broeck homogenizer), homogenate was centrifuged for 15 min at 18000 *g*. Supernatant was decanted and the pellet resuspended in 10 ml 50% EtOH followed by centrifugation. The pellet was then washed in 10 ml H_2O. Combined supernatants were evaporated to dryness *in vacuo* at 35° and then the residue was redissolved in 10 ml H_2O. Lipids were extracted with 3 ml toluene and 2 ml petrol and then assayed for ^{14}C by lipid scintillation spectrometry [41]. Fractionation of H_2O-soluble compounds was accomplished by use of ion exchange resins [42]. Free amino acids were separated [43] by an amino acid analyzer as described in ref [41]. Protein was hydrolyzed [44] for subsequent analysis of protein amino acids.

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