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SYNTHESIS OF FOLATE DERIVATIVES IN AERATED STORAGE TISSUE DISKS*

PAUL FEDEC† and EDWIN ALBERT COSSINS
Department of Botany, University of Alberta, Edmonton, Canada T6G 2E9

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Key Word Index—*Beta vulgaris*; Chenopodiaceae; *Brassica campestris*; Cruciferae; *Daucus carota*; Umbelliferae; *Solanum tuberosum*; Solanaceae; storage tissue disks; folate derivatives; effect of aeration.

Abstract—Microbiological assay of extracts prepared from carrot, potato, turnip and beet storage tissue disks revealed that folate derivatives were synthesized during a 48 hr aeration period in sterile distilled water. The composition of the folate pool in carrot was examined by DEAE-cellulose column chromatography, γ-glutamylcarboxypeptidase treatment and differential assay of individual derivatives using *Lactobacillus casei* and *Streptococcus faecalis*. The principal folates were polyglutamates of formyl and methyl tetrahydrofolate. Smaller quantities of the corresponding mono- and di-glutamates were also detected. The latter derivatives occurred in pools having a high degree of metabolic turnover. The specific activities of three enzymes catalyzing production of these derivatives from tetrahydrofolate increased during the first 12 hr of aeration. Amino acid analyses revealed that folate synthesis in carrot disks was accompanied by depletion of free serine and by net synthesis of free and protein methionine.

INTRODUCTION

During the past twenty years the physiological and biochemical changes which accompany aeration of higher plant storage tissue disks have received detailed study. These have included increased rates of respiration [1–4], enzyme activation [5–7], synthesis of proteins [8], RNA and DNA [9, 10], increased ability to metabolize exogenous substrates [11, 12] and response to growth regulating substances [13, 14].

From a consideration of basic metabolism it follows that many of these changes will depend on a pool of C-1 units presumably generated during the aeration period. In this regard, reduced derivatives of folic acid with formyl and methyl substituent groups would have greatest importance [15]. However, to our knowledge, there have been no studies of folate synthesis or its control in this interesting higher plant system.

The present paper presents evidence that both formyl and methyl folates are synthesized when storage tissue disks are aerated. Experiments with antagonists of C-1 metabolism suggested that the tissue pools of these compounds were in high state of metabolic turnover. The specific activities of key enzymes for synthesis of these derivatives from tetrahydrofolate were also found to increase during aeration. A preliminary report of this work has already appeared [16].

RESULTS

When freshly prepared tissue disks of four different species were analyzed for folates (Fig. 1) it was clear that polyglutamyl derivatives accounted for at least 75% of the total folate assayed by *Lactobacillus casei*. When the disks were aerated, changes occurred in the proportions of polyglutamyl and unconjugated derivatives. In

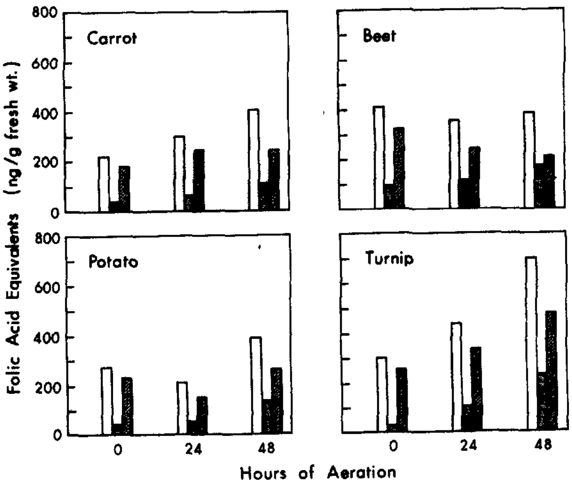

Fig. 1. Effect of aeration on pteroylglutamates in storage tissue slices. □, Total levels after pea cotyledon γ-glutamylcarboxypeptidase treatment; ▨, levels before carboxypeptidase treatment; ■, levels of polyglutamyl derivatives. All assays were performed using *L. casei* and represent the mean of three separate determinations.

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

† Present address: Department of Biology, University of Saskatchewan, Saskatoon, Canada.

carrot and turnip, both classes of folates increased. In potato, increases were evident in unconjugated derivatives but the polyglutamate content fluctuated. In beet disks, total folate content remained relatively constant but unconjugated derivatives appeared to be formed at the expense of polyglutamyl folates.

As bacteria are known to contain relatively high folate concentrations [15], it was important to determine whether these changes were in part due to progressive bacterial contamination during aeration. In considering this possibility, however, it was concluded that bacteriostatic compounds would interfere in the microbiological assay procedure and might also affect metabolic processes within the disks [17]. Attempts to minimize bacterial contamination, therefore, included aseptic handling of the tissues, thorough sterilization of equipment and frequent changes of the aeration medium. Despite these precautions some contamination developed and serial dilution analysis showed that 58 and 143 bacteria/ml were present in the aerating solution after 24 and 48 hr respectively. These relatively low levels of bacteria have been regarded as tolerable in earlier work [18] and comparisons with more heavily contaminated disks showed that folate levels still increased by approximately the same amount. We concluded, therefore, that folates are synthesized by the plant tissue during aeration.

To study the nature of individual folates and their synthesis during aeration, carrot tissue extracts were assayed after DEAE-cellulose chromatography. Data for freshly cut and aerated disks are given in Fig. 2. Two peaks (*a* and *b*), occurring early in the elution sequence, gave growth responses to both assay bacteria typical of formyl derivatives. These compounds also occupied positions in the elution sequence corresponding to authentic 10-HCO-H_4PteGlu and 10-HCO-H_4PteGlu$_2$ and therefore appear identical to the compounds found in other plants by Roos and Cossins [19]. The large peak *c* co-chromatographed with authentic 5-Me-H_4PteGlu and did not support growth of *Streptococcus faecalis*. Using similar criteria, peaks *d* and *e* were identified as H_4PteGlu and 5-Me-H_4PteGlu$_2$ respectively. Peak *f*, which was not detected in freshly cut disks, occupied a position in the elution corresponding to authentic 5-HCO-H_4PteGlu$_3$ and 5-Me-H_4PteGlu$_3$ and supported the growth of both assay organisms.

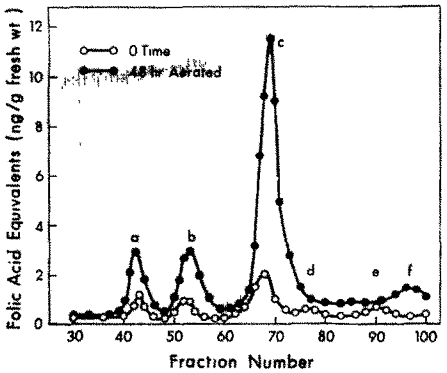

Fig. 2. Chromatography of pteroylglutamate derivatives before and after aeration of carrot slices. After DEAE-cellulose chromatography, the fractions were assayed for folate content using *L. casei*. The derivatives shown are: a, 10-HCO-H_4PteGlu; b, 10-HCO-H_4PteGlu$_2$; c, 5-Me-H_4PteGlu; d, H_4PteGlu; e, 5-Me-H_4PteGlu$_2$; f, unidentified. *S. faecalis* gave positive responses to compounds in peaks a, b and d but not in c. Each point is the mean of values from duplicate assays from two separate experiments.

Table 1. Effect of aeration and L-ethionine on folate derivatives of carrot tissue disks

Derivatives	Hours of aeration 0	12	24	36	48
Control treatment					
Formyl mono & diglu	6	7	9	14	19
Methyl mono & diglu	36	39	50	61	92
Total mono & diglu	42	46	59	75	111
Formyl polyglutamates	46	54	45	37	79
Methyl polyglutamates	134	134	215	276	214
Total polyglutamates	180	188	260	313	293
Total folates	222	234	319	388	404
L-Ethionine treatment					
Formyl mono & diglu		18	13	16	15
Methyl mono & diglu		48	52	103	122
Total mono & diglu		66	65	119	137
Formyl polyglutamates		47	46	57	43
Methyl polyglutamates		67	159	193	370
Total polyglutamates		114	205	250	413
Total folates		180	270	369	550

Carrot slices (1 × 9 mm) were aerated in H_2O or 10 mM L-ethionine for the periods shown. Extracts were prepared, assayed for mono- and di-glutamyl folates using *L. casei* and *S. faecalis*, treated with γ-glutamylpeptidase and reassayed for total folate content. Data are expressed as ng PteGlu equivalents/g fr. wt disks and are averages of duplicate assays.

The concentration of these individual folates increased dramatically as the disks were aerated. For example, the principal derivative, 5-Me-H_4PteGlu increased nearly 6-fold during the 48 hr aeration. Chromatography of extracts prepared after 12, 24 and 36 hr of aeration gave folate levels intermediate between the extremes shown in Fig. 2. Further evidence that folate synthesis occurred during aeration of carrot disks was obtained in experiments with aminopterin and L-ethionine.

When freshly cut disks were aerated in 0·02 M aminopterin the pools of formyl and methyl folates were progressively depleted. In such experiments, however, the rate of depletion varied for each derivative. The pools of formyl mono- and diglutamyl derivatives were not decreased after 24 hr of treatment but the methyl folate pool was only 3% of that present in control disks. With longer periods of aminopterin treatment marked depletion of the 10-HCO-H_4PteGlu$_2$ pool occurred until it was only 10% of the control at 48 hr. In contrast, aminopterin reduced the 10-HCO-H_4PteGlu pool size by only 25% after 48 hr of aeration.

L-ethionine, which blocks transmethylation reactions mediated by *S*-adenosylmethionine, also affected the concentration of folate derivatives in carrot disks (Table 1). In this case, however, the net synthesis of folates accompanying aeration was not curtailed but slightly enhanced. Ethionine had greatest effect initially on the methyl pools of mono- and diglutamyl folates. With prolonged aeration methyl polyglutamyl folates accumulated in the ethionine treated disks.

Aeration of carrot storage tissue disks also affected the specific activities of enzymes which catalyze synthesis of formyl and methyl tetrahydrofolates (Table 2). After 12 hr aeration, the greatest increase was shown by the reductase which produces 5-Me-H_4PteGlu from methylene tetrahydrofolate. The activity of serine hydroxymethyltransferase was doubled whereas the formyl tetra-

Table 2. Effect of aeration on key enzymes of folate metabolism

Enzyme	Activity* after hours of aeration 0	12
10-formyltetrahydrofolate synthetase	918	1300
Serine hydroxymethyltransferase	0·98	2·0
Methylenetetrahydrofolate reductase	1·4	3·2
Methyltetrahydrofolate:homocysteine transmethylase	152†	149†

* Nmol product formed/hr/mg protein at 30° except for last enzyme† expressed as pmol product formed/hr/mg protein at 30°. All assays were performed in duplicate and the results are expressed as the mean.

Table 3. Changes in amino acids during aeration of carrot disks†

	Amount present after hours of aeration								
	0			24			48		
Amino acid	Free	Protein	Total	Free	Protein	Total	Free	Protein	Total
Lys	0·19	2·19	2·38	0·05	1·78	1·83	0·02	2·82	2·84
His	n.d.	0·72	0·72	n.d.	0·59	0·59	n.d.	0·83	0·83
Arg	n.d.	0·56	0·56	n.d.	0·55	0·55	n.d.	0·83	0·83
Asp	2·85	3·89	6·74	2·58	4·23	6·81	1·99	5 68	7·67
Thr	t	2·51	2·51	t	2·50	2·50	n.d.	3·35	3·35
Ser	8·70	3·06	11·76	3·81	3·05	6·86	4·84	3·99	8·83
Glu	11·00	4·93	15·93	8·67	4·86	13·53	3·16	6·49	9·65
Pro	n.d.	2·29	2·29	n d.	2·25	2·25	0·07	3·11	3·18
Gly	0·23	3·92	4·15	0·04	4 13	4 17	0·05	6·66	6·71
Ala	13·20	4·02	17·22	2·69	4·02	6·71	1·24	5·35	6·59
Cys	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	t	n.d.	t
Val	1·10	3·28	4·38	0·47	3·05	3·52	0·31	4 39	4·70
Met	n.d.	0·09	0·09	t	0·22	0·22	0·17	0·36	0·53
Ile	0·58	1·96	2 54	0·14	1·70	1·84	0·08	3·77	3·85
Leu	0·31	3·06	3·37	0·07	2·60	2·67	0.03	4·32	4·35
Tyr	n.d.	0·64	0·64	n.d.	0·51	0·51	n.d.	0·85	0·85
Phe	n.d.	0·61	0·61	n.d.	0·55	0·55	n.d.	0·89	0·89
Total	38·16	37·73	75·89	18·52	36.59	55·11	11·96	53·69	65·65

† Disks were prepared from greenhouse-grown carrot tissue after storage for 2 months at 4°. Mean values expressed as μmol/g fr. wt tissue after duplicate analysis. n.d.—not detected; t—trace amounts.

hydrofolate synthetase increased by approximately 40%. Although the specific activity of the transmethylase was not altered by aeration of the disks for 12 hr, after 24 hr aeration specific enzyme activities slightly in excess of 200 were found.

In other experiments with carrot disks, the levels of free and protein amino acids were examined during a 48 hr aeration period to determine whether changes occurred in pools related to C-1 metabolism. As shown in Table 3, total free amino acids declined during aeration whereas total protein amino acid levels rose. Of particular interest to the present study was the substantial net synthesis of methionine and the initial decrease in the level of free serine.

DISCUSSION

In common with other plant tissues [19–22], the folates of storage tissues were mainly highly conjugated (Fig. 1). In carrot, these folates were methyl derivatives of $H_4PteGlu_n$ and their concentration increased as the disks were aerated (Table 1). In addition, folates with 1-3 glutamyl residues were also present in these tissues as judged by growth of the assay organism prior to γ-glutamylcarboxypeptidase treatments. These folates also increased in concentration when disks of the four species were aerated (Fig. 1). In carrot, potato and turnip, this increase was accompanied by net folate synthesis but in beet it appeared to occur at the expense of polyglutamyl derivatives. Conceivably this decline in polyglutamyl content could be catalyzed by a γ-glutamylcarboxypeptidase as such enzymes now appear to be ubiquitous to living tissues [15].

Evidence for folate synthesis in carrot was also obtained when disks were aerated in the presence of aminopterin. This folate analogue, which blocks generation of $H_4PteGlu$ by inhibition of dihydrofolate reductase, caused rapid depletion of the 5-Me-$H_4PteGlu$ pool. In contrast, the pools of formyl folates were less drastically affected, a situation which we have noted in studies of other plant tissues [19, 20]. Clearly, in carrot disks, the formyl and methyl tetrahydrofolates must have different rates of turnover and be derived from pools of $H_4PteGlu$ which, in turn, are generated from $H_2PteGlu$ at different rates. In this respect, the 5-Me-$H_4PteGlu$ pool may be derived from a metabolic pool of 5,10-CH_2-$H_4PteGlu$ which is also utilized in the thymidylate synthetase reaction.

The methionine analogue, ethionine, is known to inhibit several processes in higher plants including the auxin stimulation of cell elongation [23], the synthesis of α-amylase [24], phenylalanine ammonia lyase [25], and invertase [26] as well as the methylation of RNA [26]. As methionine reverses these ethionine effects, it follows that the analogue in plants, as in other systems, blocks key transmethylation reactions in which *S*-adenosylmethionine acts as methyl donor. Tissues treated with ethionine should, therefore, tend to accumulate methionine and its immediate precursors. The data in Table 1 show that mono- and diglutamyl forms of methyltetrahydrofolate accumulated in ethionine-treated disks particularly after aeration for 36–48 hr. At 48 hr, this accumulation of methyl folates also included polyglutamyl derivatives. Accordingly, it may be argued that 5-Me-$H_4PteGlu$ and 5-Me-$H_4PteGlu_2$ are major precursors of methionine in carrot disks and that their methyl groups are actively generated, presumably from 5,10-CH_2-$H_4PteGlu$, during the aeration period.

The serine hydroxymethyltransferase reaction is now generally regarded as the major source of 5,10-CH_2-$H_4PteGlu$ in living cells [15]. The specific activity of this enzyme and that of methylenetetrahydrofolate reductase, which forms 5-Me-$H_4PteGlu$, both increased substantially (Table 2) prior to increases in folate and methionine concentration (Tables 1 and 3). Furthermore, the levels of free serine in carrot disks declined by more than 50% during the first 24 hr of aeration (Table 3). These changes suggest that C-1 units required to support the *de novo* synthesis of methionine are largely derived from serine during the aeration period. If these conclusions are correct, the metabolism of serine via the folate pool would have major importance in the macromolecular syntheses which are characteristically initiated when storage tissue disks are aerated.

EXPERIMENTAL

Plant material. Roots of *Beta vulgaris*, *Brassica rapa* and *Solanum tuberosum* were purchased locally and stored in plastic bags at 4° in darkness until required. *Daucus carota* L. var. Nantes Coreless plants were raised in growth chambers, receiving 32 000 lx during 14 hr days in a day–night temperature regime of 24° and 20°. After 5 months, the roots were harvested, washed and stored in plastic bags at 4° in darkness. Carrots used as experimental material had been stored under these conditions for a minimum of 1 month but no longer than 3 months.

Preparation and aeration of tissue disks. Equipment used for preparation of disks was sterilized by prior autoclaving or washing with EtOH. Sterile H_2O was used throughout for rinsing and aerating the disks. Solns of aminopterin (0·02 M) and L-ethionine (0·01 M) were Millipore filtered before use. Roots from storage were washed in soapy water, rinsed 4 × in sterile H_2O and cylinders of tissue were removed using a No. 2 cork borer. Disks (1 × 9 mm) were then cut from the cylinders using a mechanical cutter. The disks were thoroughly rinsed in sterile H_2O and suspended in the same (4 disks/ml) contained in 500 ml Erlenmeyer flasks. Moist sterile air was drawn through the flasks at room temp. The disks were resuspended in fr. sterile H_2O every 12 hr.

Assessment of bacterial contamination. Serial dilutions of the medium were plated on agar plates containing tryptone–yeast extract and incubated at 37° for 24 hr. Colony counts were then made and used as a measure of bacterial contamination.

Folate analyses. Samples of 50 disks (1·2–1·4 g fr. wt) were placed in 10 ml of 2% (w/v) ascorbic acid (pH 6·0) and maintained at 95° for 10 min. Folates were then extracted, fractionated and assayed with *Lactobacillus casei* (ATCC 7469) and *Streptococcus faecalis* (ATCC 8043) as previously described [19]. Conjugated derivatives were hydrolyzed using a γ-glutamylcarboxypeptidase from pea cotyledons [19].

Enzyme assays. Carrot disks were homogenized in 50 mM K Pi buffer (pH 6·9) containing 2 mM 2-mercaptoethanol at 4°. After centrifugation (18000 *g* for 20 min) the cell-free extract was desalted by passage through a 1·5 × 7 cm column of Sephadex G-15. Preliminary enzyme assays were carried out to determine optimal substrate concentrations and pH optima. $N^5 \cdot N^{10}$-methylenetetrahydrofolate reductase (E.C. 1.1.1.68) was assayed according to Donaldson and Keresztesy [27] using [Me-^{14}C]-5-Me-H_4PteGlu as substrate. N^5-Methyltetrahydrofolate : transmethylase (E.C. 2.1.99) was assayed as previously described [28]. Serine hydroxymethyltransferase (E.C. 2.1.2.1) was measured by the method of Taylor and Weissbach [29]. N^{10}-Formyltetrahydrofolate synthetase activity was assayed [30] using cell-free extracts which contained 0·1 mM reduced glutathione in addition to the above extraction buffer. Radioactive products were assayed for ^{14}C by liquid scintillation spectrometry [31]. Protein was measured colorimetrically utilizing crystalline egg albumin as a reference standard [32].

Analysis of free and protein amino acids. Free amino acids were extracted from carrot disks by the method of Splittstoesser [33]. Protein was hydrolyzed [34] and amino acids were recovered from the hydrolyzate using Dowex 50W × 8 (H^+ form) resin. Individual amino acids were separated [35] by a Beckman automatic amino acid analyzer employing the buffer sequence and column resins described previously [31].

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