Phytochemistry, Vol. 21, No. 7, pp. 1485–1490, 1982.
Printed in Great Britain.

0031-9422/82/071485-06$03.00/0

RELATIONSHIPS BETWEEN GLYCOLLATE AND FORMATE METABOLISM IN GREENING BARLEY LEAVES

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(*Received* 23 *April* 1981)

Key Word Index—*Hordeum vulgare*; Gramineae; barley leaves; greening; enzymes of glycollate and formate metabolism.

Abstract—The activities of enzymes catalysing glycollate oxidation, formate production and folate-dependent formate utilization were examined in the primary leaves of *Hordeum vulgare* cv Galt. Seedlings were grown for 6 days in darkness and then transferred to continuous light (500 μeinsteins/m^2 per sec) for up to 5 days. Cell-free extracts of the primary leaves contained glycollate oxidase (EC 1.1.3.1), 10-formyltetrahydrofolate synthetase (EC 6.3.4.3), 5, 10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) and ability to enzymically decarboxylate glyoxylate. These activities increased during greening and at the end of the light treatment were 70–450% higher than etiolated controls. Greened primary leaves also incorporated [^{14}C]formate at rates that were three- to four-fold higher than shown by etiolated leaves. The specific activity of 10-formyltetrahydrofolate synthetase was decreased by 20–35% when the leaves were greened in the presence of 10 mM hydroxysulphonate. This inhibitor also reduced the incorporation of [^{14}C]formate by up to 45%. A potential flow of carbon from glycollate to 10-formyltetrahydrofolate via glyoxylate and formate was suggested by the data.

INTRODUCTION

Studies *in vivo* [1–4] suggest that higher plants generate formyltetrahydrofolate by activation of formate. This activation centres on 10-formyltetrahydrofolate synthetase, an enzyme of widespread occurrence in plants [5–8] as well as a variety of other organisms [9]. In these species, formate appears to be the principal source of this important folate derivative and recent studies of the metabolic origins of formate suggest that glycollate is an important precursor. For example, it is clear that tobacco leaf extracts [10] and spinach beet peroxisomes [11] catalyse the formation of this acid from C-2 of glycollate. The reaction involves oxygen uptake and the intermediary formation of glyoxylate. This latter compound is non-enzymically decarboxylated by hydrogen peroxide in peroxisomes despite the presence of catalase within this organelle [11]. Chloroplasts and plant mitochondria are also capable of generating formate from C-2 of glyoxylate. In tobacco leaf mitochondria formate is formed in a reaction with requirements for oxygen, thiamine pyrophosphate and divalent ions [12]. Similar reactions have been reported for illuminated envelope-free spinach chloroplasts [13] and for *Euglena* [14].

In the present studies we have examined the possible metabolic relationships between glycollate and formate in greening barley leaves. Exposure of etiolated seedlings to light provides a dynamic system undergoing rapid metabolic change [16–21] and in which acquisition of photosynthetic ability [16–18, 22–24] is accompanied by carbon flow through the glycollate pathway [19, 25–27]. The diverse biosynthetic activity accompanying greening would conceivably have requirement for C-1 units [28, 29]. The present work supports this view and suggests a precursor role for glycollate in 10-formyltetrahydrofolate synthesis.

RESULTS

Changes in enzyme activities during greening

In preliminary studies, dark-grown 6-day-old barley seedlings were transferred to different light intensities for 48 hr. The primary leaves were then analysed for chlorophyll, soluble protein and glycollate oxidase activity (Table 1). Maximum enzyme and protein levels were found in leaves receiving 400–500 μeinsteins/m^2 per sec. When the latter intensity was provided for periods up to 5 days (Fig. 1), it was clear that greatest increases in these components and chlorophyll occurred within 48 hr of providing light. In contrast to these increases, the free amino acid pool of illuminated leaves declined during greening

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Table 1. Effect of light intensity on chlorophyll, soluble protein, and glycollate oxidase during greening

Light intensity (μeinsteins/m^2 per sec)	Total chlorophyll (mg/g fr.wt)	Soluble protein (mg/g fr.wt)	Glycollate oxidase (μmol/min/g fr.wt)
0	0	10.2 ± 0.3	0.4 ± 0.01
100	1.04 ± 0.03	13.8 ± 0.3	1.4 ± 0.03
200	1.38 ± 0.09	18.2 ± 0.6	1.6 ± 0.08
400	1.19 ± 0.08	20.3 ± 0.3	1.9 ± 0.06
500	1.24 ± 0.07	18.7 ± 0.6	1.9 ± 0.12
600	1.16 ± 0.01	19.5 ± 0.2	1.7 ± 0.10

6-Day-old etiolated leaves were greened for 2 days at the light intensities indicated. Data are mean values (±s.e.m.) from three separate experiments assayed in triplicate.

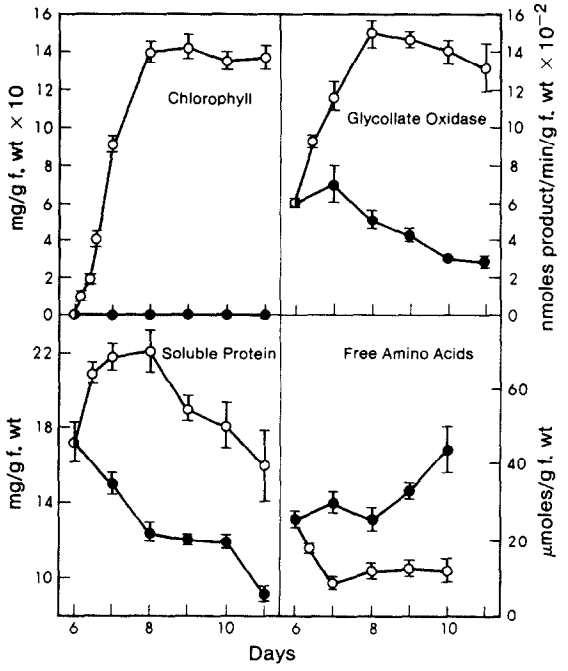

Fig. 1. Changes in chlorophyll, glycollate oxidase, soluble protein and free amino acids during greening. 6-Day-old etiolated leaves were either greened (○) at 500 μeinsteins/m^2 per sec, or held in the dark as controls (●). Samples were taken during a subsequent 5-day period. Each data point represents a mean value ±s.e.m., obtained from triplicate assays performed in each of three separate experiments.

and by 4 days of light was only 25% of that found in etiolated controls (Fig. 1).

The sharp increase in glycollate oxidase activity during greening may represent a potential source of formate synthesis [11]. To examine this, barley leaf extracts were assayed for glyoxylate decarboxylation by the method of Halliwell and Butt [11]. The data in Fig. 2(a) show that ability to generate formate from glyoxylate increased with leaf age and that illuminated leaves had consistently higher activities. Boiled extracts failed to produce formate in these assays. Extracts of greening leaves also contained greater levels of 10-formyltetrahydrofolate synthetase (Fig. 2b) and 5, 10-methylenetetrahydrofolate dehydrogenase (Fig. 2c). In this regard, light appeared to prevent the sharp decline in synthetase activity shown by the etiolated controls. Dehydrogenase activity also declined in 6–8-day-old etiolated leaves but rose by more than 40% when light was supplied (Fig. 2c).

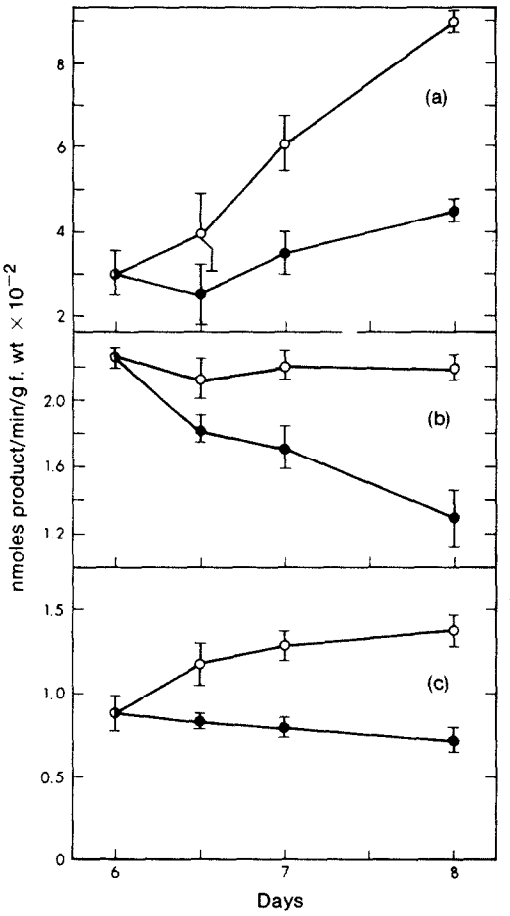

Fig. 2. Changes in enzyme activities during the first 2 days of greening. 6-Day-old etiolated leaves were either greened (○) at 500 μeinsteins/m^2 per sec, or held in the dark as controls (●). Cell-free extracts were prepared from leaves harvested during a subsequent 2-day period and assayed for: (a) enzymic glyoxylate decarboxylation; (b) 10-formyltetrahydrofolate synthetase; and (c) 5, 10-methylenetetrahydrofolate dehydrogenase.

Incorporation of [^{14}C]formate by greened and etiolated leaves

The enzyme data suggest that greened leaves have a greater potential for production and activation of formate. To examine this further we compared the fates of [^{14}C]formate in leaves greened for 2 days and in etiolated leaves of identical age. The results of these experiments (Table 2) showed that greened leaves incorporated significantly more formate carbon into all of the fractions examined. The free amino acids were heavily labelled in both cases, with serine containing more ^{14}C than any other individual compound. The radioactivity of this product was six-fold higher in the leaves that had been illuminated whereas a seven-fold increase was noted for glycine (Table 2). These differences were not due to formate oxidation and refixation of the resulting $^{14}CO_2$ in the light as parallel [^{3}H]formate feeding experiments of 8-day-old tissues [30] also showed heavier labelling of these products in greened leaves.

Table 2. Incorporation of [^{14}C]formate by 8-day-old barley leaf sections

Fraction	Radioactivity recovered (dpm/3 leaves) ± s.e.m. × 10^{-3}	
	8-day-old greened leaves	8-day-old etiolated leaves
Ether solubles	48.0 ± 2.5	10.7 ± 2.0
Sugars	437.3 ± 14.8	16.6 ± 1.6
Organic acids	626.8 ± 21.8	216.4 ± 18.6
Free amino acids	848.2 ± 18.3	267.0 ± 18.1
aspartate	146.5 ± 22.3	51.7 ± 2.2
serine	297.0 ± 6.6	59.7 ± 6.6
glutamate	31.3 ± 3.3	29.6 ± 3.5
glycine	146.1 ± 25.7	19.7 ± 0.3
alanine	142.8 ± 3.8	12.8 ± 1.4
methionine	n.d.	5.0 ± 1.5
Total ^{14}C incorporated	1960.3 ± 57.4	510.7 ± 40.3

After a 15-min pre-incubation period, 12.5 μCi of sodium [^{14}C]formate sp. act. 60.7 μCi/μmol were fed to 8-day-old barley leaf sections (derived from three primary leaves, *ca* 0.31 g fr. wt) for 20 min. Feeding to greened material was in the light (500 μeinstein/m^2 per sec). Etiolated sections were incubated in the dark. The data are mean values (±s.e.m.) obtained from three separate experiments run in duplicate. n.d.—not detected.

If a substantial part of the glycollate oxidized by greening barley leaves was converted to formate it follows that carrier amounts of formate may dilute the flow of glycollate carbon to other products. This possibility was examined in the experiments summarized in Table 3. Although the total incorporation of glycollate carbon was not affected by pre-incubation in 0.6 and 6 mM formate, labelling of the ether-soluble fraction was significantly reduced. The major amino acids formed from glycollate were glycine, serine and aspartate. Formate decreased the specific radioactivities of these products but, for glycine and serine, this was mainly due to an increase in pool size rather than a substantial reduction in carbon flow.

The effect of hydroxysulphonate on formate metabolism

In leaf peroxisomes [11] the enzymic production of formate from glyoxylate is closely coupled to glycollate oxidase activity. In *Euglena* [14], conditions that repress or inhibit glycollate oxidation also affect ability to metabolize formate. The data in Table 4 show that α-hydroxy-2-pyridinemethane sulphonate (α-HPMS), a competitive inhibitor of glycollate oxidase [31–33], also inhibited formate metabolism in greening barley leaves. In these experiments, the primary leaves were excised from 7-day-old seedlings that had been grown in the dark. After illumination for 24 hr in the presence of 0.1–10 mM α-HPMS the leaves were incubated with [^{14}C]formate for 20 min. The inhibitory effect of α-HPMS on formate metabolism applied to all of the fractions containing ^{14}C and the amount of inhibition was related to α-HPMS concentration. The data imply that the ability to utilize formate during greening was decreased when glycollate oxidation was curtailed. It is also possible that the level of this enzyme *in vivo* is modulated by the endogenous level of formate [34]. Some support for this latter view was obtained (Table 5) when the α-HPMS-treated leaves were examined for 10-formyltetrahydrofolate synthetase. Leaves greened in the presence of 10 mM α-HPMS contained less enzyme activity than the controls. Desalting these extracts increased synthetase activities but those of the 10 mM α-HPMS-treated tissues remained about 20% lower than the controls.

DISCUSSION

There is considerable debate in the literature regarding the possibility that glyoxylate represents a branch point in the glycollate pathway. Consistent with this view are recent studies [35, 36] indicating that a significant release of photorespiratory CO_2 can occur from this pathway intermediate. Halliwell [7] has suggested that the accompanying formate production may be utilized via the 10-formyltetrahydrofolate synthetase reaction. Subsequent reduction of the C-1 unit to 5, 10-methylenetetrahydrofolate would conceivably provide substrate for serine hydroxymethyltransferase. Furthermore, operation of this folate-dependent sequence would tend to retain C-2 of glycollate within the conventional glycollate pathway intermediates. The results of the present work point to a potential for these reactions in young barley leaves. For example, relationships between glycollate and 10-formyltetrahydrofolate are implied by the enzyme data (Figs. 1 and 2), by the [^{14}C]formate feeding experiments (Table 2) and by the inhibitory effect of α-HPMS on formate metabolism (Tables 4 and 5).

The levels of glycollate oxidase, the glyoxylate → formate reaction and 10-formyltetrahydrofolate synthetase during greening (Figs. 1 and 2) imply that formate activation would be a rate-limiting step in the conversion of glycollate to 10-formyltetrahydrofolate. However, this assumes that the precursor of gly-

Table 3. Effect of formate on the [2-^{14}C]glycollate metabolism of partially greened barley leaves

	Sodium formate concentration (mM)		
	0	0.6	6.0
Ether solubles	121.4 ± 20.7	52.7 ± 18.6	28.5 ± 6.0
Sugars	162.3 ± 19.5	153.5 ± 26.5	176.1 ± 73.8
Protein amino acids	7.2 ± 0.6	5.6 ± 0.5	5.2 ± 0.1
Soluble amino acids	938.5 ± 107.3	1178.3 ± 62.4	976.9 ± 158.1
aspartate	25.5 ± 5.4	14.5 ± 4.1	9.7 ± 2.1
sp. act	109.6 ± 24.8	46.8 ± 4.0	28.6 ± 4.6
μmol*	0.25 ± 0.05	0.31 ± 0.06	0.36 ± 0.10
serine	37.4 ± 3.1	50.9 ± 7.9	50.3 ± 11.0
sp. act.	147.3 ± 29.5	113.7 ± 18.1	75.3 ± 14.9
μmol*	0.27 ± 0.03	0.47 ± 0.04	0.73 ± 0.19
glycine	870.0 ± 99.5	1135.7 ± 98.1	838.6 ± 159.7
sp. act.	4218.2 ± 112.5	2880.0 ± 118.3	2473.8 ± 210.1
μmol*	0.22 ± 0.03	0.40 ± 0.11	0.34 ± 0.05
Total [^{14}C] incorporated	1229.4 ± 148.1	1390.1 ± 108.0	1186.7 ± 238.0

*Pool size/3 leaves ± s.e.m.

Radioactivities are expressed as dpm incorporated/3 leaves × 10^{-3} ± s.e.m. Sp. act.—specific activities expressed as dpm/μmol × 10^{-3} ± s.e.m. Sections (derived from three primary leaves, *ca* 0.22 g fr. wt) of 7-day-old leaves, greened for 24 hr, were pre-incubated with Na formate for 20 min. [2-^{14}C]Glycollate, 2μCi, sp. act. 55 μCi/μmol was then fed for 20 min. Light was supplied (500 μeinsteins/m^2 per sec) during pre-incubation and feeding. Data are mean values ± s.e.m. obtained from three separate experiments run in duplicate. The pool sizes of other amino acids were not affected by the formate treatment.

Table 4. The effect of α-HPMS on [^{14}C]formate metabolism

	α-HPMS concentration (mM)			
	0	0.1	1	10
Ether solubles	29.4 ± 3.5	23.5 ± 1.0	17.1 ± 5.3	4.8 ± 0.5
% inhibition		20.0	41.8	83.7
Sugars	178.0 ± 14.1	151.1 ± 10.2	122.2 ± 10.5	44.5 ± 7.4
% inhibition		15.1	31.4	75.0
Organic acids	314.3 ± 4.8	277.5 ± 9.9	250.9 ± 24.7	156.6 ± 16.6
% inhibition		11.7	20.7	50.2
Soluble amino acids	343.5 ± 9.9	272.1 ± 11.0	234.4 ± 8.7	103.9 ± 16.5
% inhibition		20.8	31.8	69.7
Total ^{14}C incorporated	865.2 ± 32.4	724.2 ± 32.1	624.4 ± 49.2	309.8 ± 41.0

Radioactivities are expressed as dpm incorporated/three leaves × 10^{-3} ± s.e.m.

7-Day-old etiolated leaves were greened for 24 hr in the presence of α-HPMS. After a 15-min pre-incubation, 12.5 μCi of [^{14}C]formate (sp. act. 60.7 μCi/μmol) were fed to sections of three greened leaves (*ca* 0.22 g fr. wt) for 20 min. Pre-incubation and feeding were in the light (500 μeinsteins/m^2 per sec). Data are mean values ± s.e.m., obtained from two separate experiments run in duplicate.

collate and cofactors for 10-formyltetrahydrofolate synthesis such as tetrahydrofolate and ATP are not rate-limiting during the greening period. These assumptions have not been verified in the present work.

The magnitude of carbon flow through the suggested glycollate → formate → 10-formyltetrahydrofolate sequence is difficult to assess. The lack of appreciable dilution of the serine pool in the formate competition experiments (Table 3) raises doubt about the quantitative importance, in serine synthesis, of the methylenefolate arising from formate. Similarly, the mitochondrial site of the glycine → serine reaction in leaves [29] and the predominantly cytosolic locale of 10-formyltetrahydrofolate synthetase [7, 8, 29] may prevent equilibrium between methylenefolate

Table 5. 10-Formyltetrahydrofolate synthetase activity of barley leaves, greened in the presence of α-HPMS

Leaf extract	α-HPMS concentration (mM)		
	0	1	10
Crude			
nmol/min per g fr. wt	101.1 ± 6.3	97.1 ± 2.0	70.2 ± 2.3
% inhibition		4.0	30.6
nmol/min per mg protein	6.9 ± 0.5	6.2 ± 0.2	4.3 ± 0.1
% inhibition		10.1	37.7
Desalted			
nmol/min per g fr. wt	228.2 ± 6.5	226.6 ± 8.2	172.9 ± 6.6
% inhibition		0.7	24.2
nmol/min per mg protein	28.9 ± 0.7	27.2 ± 0.7	22.1 ± 0.6
% inhibition		5.9	23.5

The inhibitor was supplied during greening as outlined in Table 4. Data are mean values (±s.e.m.) from two separate experiments assayed in triplicate.

produced from exogenous formate and that arising during glycine cleavage. On the other hand, formate did effectively dilute the contribution of glycollate [2-^{14}C] to the ether-soluble fraction (Table 3). This fraction was also labelled when formate-[^{14}C] was supplied (Table 2) and 10 mM α-HPMS reduced this incorporation by 84% (Table 4). The nature of these products was not determined but the presence of formate and glycollate carbon in this fraction strengthens the view that both metabolites were, to some extent, utilized by common pathways during greening.

EXPERIMENTAL

Materials. Na [^{14}C]formate, and [^{14}C]toluene standard were purchased from Amersham-Searle, Des Plaines, IL. [2-^{14}C]Glycollic acid was obtained from ICN, Irvine, CA. α-HPMS was obtained from Terochem Laboratories Ltd, Edmonton, Alberta. Other chemicals, of the highest quality commercially available, were purchased from Sigma, St Louis, and Fisher Scientific, Edmonton, Alberta.

Growth conditions. Barley seeds (*Hordeum vulgare*, cv Galt) were surface sterilized in 1% Na hypochlorite, sown in Vermiculite and grown in darkness at 22° and 30% r.h. for 6 days. Etiolated plants were either illuminated or maintained in the dark as controls. Illumination was by combined cool-white fluorescent lamps and incandescent bulbs giving an intensity of 500 μeinsteins/m^2 per sec at the level of the seedlings. Light intensity was measured using a LI-1905 quantum sensor (Lambda Instruments Corp., Lincoln, NB). Following the treatment period primary leaves were harvested by excision just above the coleoptile sheath. The entire excised leaf was used as experimental material.

Enzyme studies. Leaf samples (*ca* 1 g fr. wt) were homogenized at 2° using 6 ml of extraction buffer (see below) containing 1 g insoluble polyvinylpyrrolidone [37]. The homogenate was centrifuged at 18 000 *g* for 20 min. Low MW compounds were removed by passage through a 1 × 18 cm column of Sephadex G-15. With the exception of 5, 10-methylenetetrahydrofolate dehydrogenase, which was found to be labile, all enzyme assays were performed using the desalted extract. In each case, optimal activities with respect to pH, substrate and co-factor concentrations were determined using material harvested throughout the treatment period. Assays were carried out at 30°. 10-Formyltetrahydrofolate synthetase was assayed by the method of ref. [5], and 5, 10-methylenetetrahydrofolate dehydrogenase by that of ref. [38]. The extraction buffers for these enzymes were 0.05 M Tris (pH 8) containing 0.01 M 2-mercaptoethanol, and 0.01 M KPi (pH 7.5) containing 0.1 M 2-mercaptoethanol and 20% glycerol respectively. Glycollate oxidase was assayed according to ref. [19], using an extraction buffer of 0.05 M Tris (pH 8) containing 0.01 M 2-mercaptoethanol. A standard curve was prepared using Na glyoxylate. Enzymic glyoxylate decarboxylation was determined by the method of ref. [11] using 0.02 M glycylglycine (pH 7.5) as extraction buffer. Activities were corrected for non-enzymic decarboxylation and for possible inhibition of colour development by the desalted extract. Standard curves were prepared using Na formate.

Estimation of protein and chlorophyll. The method of ref. [39] was used, with crystalline egg albumin as reference standard for all protein determinations. Chlorophyll was measured by the method of ref. [40].

Na [^{14}C]*formate feeding experiments.* 6-Day-old etiolated seedlings were greened for 2 days or held in the dark as controls. Three representative primary leaves were cut at the coleoptile tip and sectioned transversely (2 mm). These tissues were then placed in a Warburg flask containing 2.8 ml of half-strength Hoagland–Epstein soln, pH 7.5 [41]. The flasks were attached to manometers in a closed system and were illuminated in a Warburg apparatus (Bronwill Scientific Inc., NY). Illumination was by incandescent bulbs, with a mean light intensity at the base of the flask of 500 μeinsteins/m^2 per sec. Radioisotope feeding was at 25° with constant shaking. Pre-incubation and feeding was in the light to greened material, and in the dark to etiolated. After a 15 min pre-incubation, 12.5 μCi Na [^{14}C]formate (60.7 μCi/μmol) was added as a 0.2-ml aliquot and incubation continued for 20 min. Leaf segments were then homogenized in EtOH (95%) at 2°. After centrifugation (1800 *g* for 20 min), the residues were washed 3× with 10 ml H_2O. The combined supernatants were dried *in vacuo* at 40°. Residual labelled formate was removed by sequential washing and re-drying with 20 ml 8 M HCOOH, and H_2O. After extraction of lipids and pigments with Et_2O, the residue was

redissolved in 15 ml H_2O and fractionated with ion exchange resins [42]. The amino acid fraction was further analysed using an amino acid analyser [43]. Radioactivities were measured by scintillation counting in Bray's soln [44], and were corrected for quenching using a [^{14}C]toluene standard.

Acknowledgements—This work was supported by a grant awarded to one of us (E.A.C.) by the Natural Sciences and Engineering Research Council of Canada (NSERC) and carried out while D.J.G. was the recipient of an NSERC postgraduate scholarship. The data presented form part of a Ph.D. thesis submitted by D.J.G. to the University of Alberta.

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