ENDOGENOUS CYCLIC ELECTRON TRANSPORT IN BROKEN CHLOROPLASTS

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Received 19 March 1980

1. Introduction

Cyclic electron flow around photosystem I plays an important role in providing sufficient ATP for the photosynthetic assimilation of CO_2 [1,2]. Cyclic activity may be observed in isolated intact chloroplasts [2,3] but is lost, along with $NADP^+$ reduction, during the isolation of naked thylakoids; however, addition of ferredoxin has been reported [4,5] to restore $NADP^+$ reduction and cyclic photophosphorylation (i.e. phosphorylation resulting from cyclic electron flow around photosystem I). This reconstituted cyclic phosphorylation was found to resemble cyclic phosphorylation in intact chloroplasts in being inhibited by antimycin (a specific inhibitor of cyclic electron flow [1,6,7]), by the quinone-analogue DBMIB [6,8] or by poor redox poising [1,5,6,9,10].

The reconstituted cyclic system has special value in studies of the cyclic pathway because there is no permeability barrier isolating substrates, inhibitors and membrane probes from the outer thylakoid surface. It has, however, the disadvantage of requiring large amounts of exogenous ferredoxin while still not giving restoration of cyclic electron flow at the rates seen in intact chloroplasts. An improved procedure is reported here that gives envelope-free thylakoids retaining almost full activity of cyclic electron flow.

A spectroscopic characteristic of intact chloroplasts is the slow (milliseconds rise time) electrochromic shift which, following flash activation, is superimposed on the fast (nanoseconds rise time) shift which has been extensively studied in broken chloroplasts [11]. The slow rise has been seen in chromatophores [12] and whole algae [13] but not in broken chloroplasts. In intact chloroplasts it was found to correlate with cyclic electron flow parameters (cytochrome turnover, ΔpH and phosphorylation) and was thus suggested to reflect an electrogenic step specific to cyclic electron flow [6,9,10]. Although the nature of this electrogenic step is unknown, the resulting absorbance transient can be used as a specific indicator of cyclic electron flow. In this work, the slow electrochromic shift and other bioenergetic parameters associated with native cyclic electron flow were studied in chloroplasts that had been prepared intact and then osmotically shocked prior to assay. The presence or absence of Mg^{2+} during osmotic rupture strongly affected the retention of cyclic electron transport activity.

Abbreviations: 9-AA, 9-aminoacridine; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; ΔpH, pH gradient across the thylakoid membrane (inside acidic); MES, 2-(*N*-morpholino)-ethane sulphonic acid

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2. Materials and methods

Intact chloroplasts were isolated from spinach leaves as previously described [1], except for the substitution of MES as buffer in the isolation medium. Intactness, assayed from the ratio of ferricyanide reduction rates before and after osmotic shock, was between 70 and 85%. Flash-induced absorbance changes were measured in the single beam spectrophotometer previously described [9,10] with a computer-based signal averager (Datalab DL922 transient recorder and Digital Equipment Corp. PDP 11/34A computer). Forty preilluminating flashes were given before averaging 64 traces (518 nm) or 256 traces (554 and 564 nm) to obtain the change shown here. The flash frequency was 2.0 Hz.

Photophosphorylation was measured by following the light induced pH increase with a glass electrode [14], while 9-aminoacridine fluorescence changes were measured as previously described [15]; actinic light (Corning 2-58) intensity was 230 W/m^2 at the surface of the cuvette in both cases.

Temperature was maintained at 20°C for all experiments, and continuous stirring employed for phosphorylation and fluorescence measurements. Chlorophyll concentrations were determined by the method in [16].

Nigericin was a kind gift from Dr R. L. Hamill (Eli Lilly Laboratories). Ferredoxin was obtained from Sigma (spinach) or Miles-Yeda (Swiss chard); similar results were obtained with both.

3. Results

Fig.1 shows the flash induced absorbance changes at 518 nm from intact chloroplasts (fig.1a), from chloroplasts shocked gently (i.e. with minimal agitation) in the presence of 5 mM $MgCl_2$ (fig.1b) and from chloroplasts shocked in the absence of Mg^{2+} ion (fig.1c). Those shocked in the presence of Mg^{2+} retained the ability to generate the millisecond antimycin (or DBMIB) sensitive rise in the flash induced P518 response, while those shocked without added Mg^{2+} substantially lost this feature. Both shock treatments caused complete rupture of the chloroplast envelope, as judged by the rates of light induced ferricyanide reduction (results not shown).

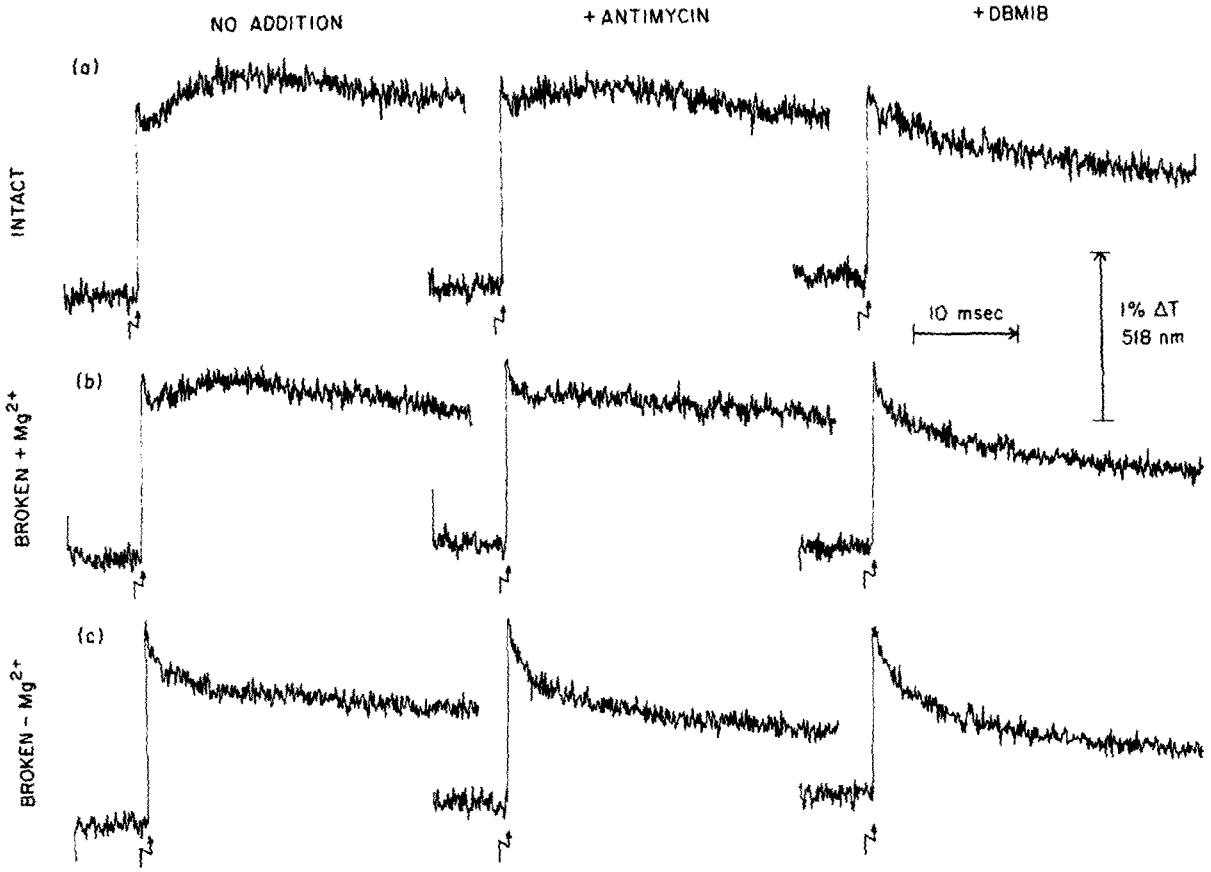

Fig.1. Flash-induced 518 nm absorbance changes in intact and osmotically shocked chloroplasts. Reaction mixture contained in 1.5 ml: sorbitol 0.35 M, Tricine-KOH 50 mM (pH 8.1), phosphate 0.25 mM, $MgCl_2$ 5 mM, KCl 50 mM, nigericin 1.8 μM, catalase 750 units and chloroplasts (50 μg chlorophyll/ml). Where indicated, 13 μM antimycin or 1.3 μM DBMIB were added. Chloroplasts were shocked by gently mixing with 0.75 ml of 1 mM Tricine (pH 8.2) and 10 mM $MgCl_2$ for 30 sec (b) or shocked by shaking with 1 mM Tricine (lacking $MgCl_2$) for 1 min (c), then adding double strength reaction mixture to give the above final concentrations. Other details are given in Section 2.

Table 1
Photophosphorylation in freshly broken chloroplasts

Additions	Initial phosphorylation rate	
	μmol ATP/mg chlorophyll/h	%
None	40.1	100
DCMU (0.33 μM)	45.0	112
DCMU + antimycin (3.3 μM)	23.7	59
Dio-9 (4 μg/ml)	4.6	11
None (shocked in absence of Mg^{2+})	14.9	–

Intact chloroplasts were shocked in 0.6 ml of 10 mM $MgCl_2$ for 30 s, then 0.6 ml of double strength reaction mixture was added to give: sorbitol 0.33 M, orthophosphate 1 mM, KCl 20 mM, Tris 1 mM (pH 8.0), $MgCl_2$ 5 mM, ADP 0.8 mM and chloroplasts (40 μg chlorophyll/ml). Final pH was 7.9 and temperature 18°C. Red light intensity was 1000 W/m². Under similar conditions, 0.33 μM DCMU inhibited electron transport to ferricyanide by 61%

Chloroplasts shocked in the presence of Mg^{2+} (as in fig.1b) supported high rates of photophosphorylation (50 to 120 μmol ATP/mg chlorophyll/h). Data in table 1 show that in red light, phosphorylation was slightly stimulated by DCMU, even at concentrations which caused greater than 60% inhibition of linear electron flow; this phosphorylation was sensitive to antimycin (showing it to be driven by endogenous cyclic electron flow) and to the energy transfer inhibitor Dio-9. Under far-red illumination DCMU, as well as antimycin, *inhibited* phosphorylation (results not shown) in agreement with a report [3] showing the need for adequate electron supply from photosystem II to optimize the redox poise of carriers in the cyclic pathway.

Table 1 also shows that chloroplasts shocked in the

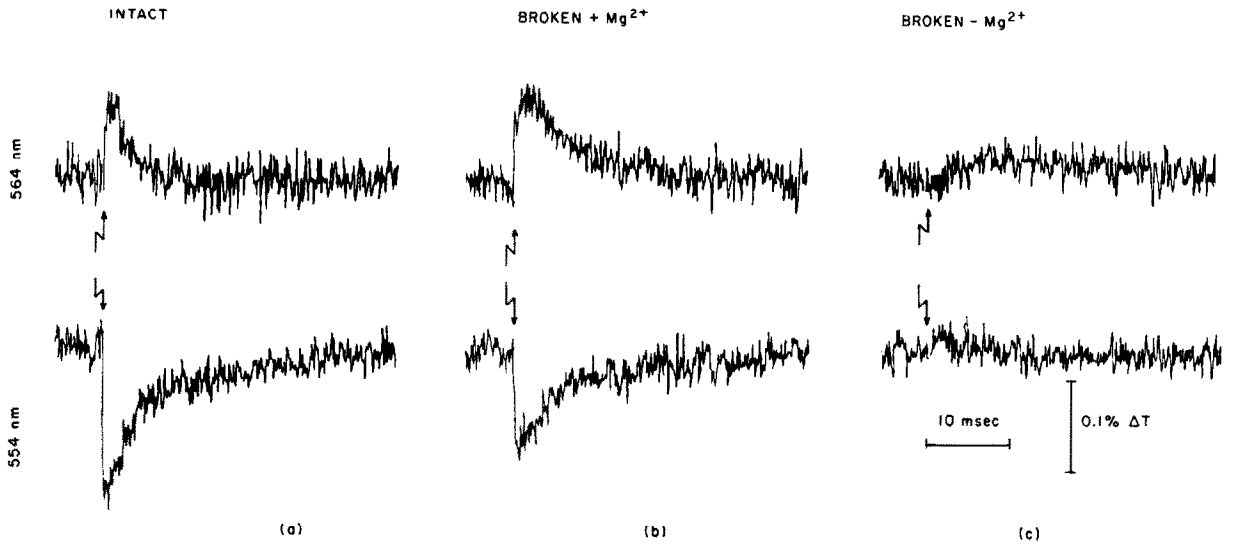

Fig.2. Flash-induced cytochrome *f* and b_{563} turnovers in intact chloroplasts and in chloroplasts broken in the presence or absence of $MgCl_2$. Final reaction mixtures contained in 1.5 ml: sorbitol 0.33 M, KCl 10 mM, phosphate 1 mM and Tris 10 mM at pH 8.2, nigericin 1.8 μM, $MgCl_2$ 5 mM where indicated, and chloroplasts (50 μg chlorophyll/ml). Chloroplasts were assayed intact (left traces) or after breakage as described for fig.1 (middle traces) or after shaking in Tris buffer without $MgCl_2$ and assaying in the absence of $MgCl_2$ (right traces). Traces were corrected for the contribution of the electrochromic shift by digital subtraction [23]; the correction factors were taken as the ratio of ΔT_{554} or ΔT_{564} to ΔT_{518} measured 35 ms after the flash, when the cytochrome changes were assumed to have relaxed fully.

absence of Mg^{2+} had a greatly diminished rate of phosphorylation compared to those shocked with Mg^{2+} present. Broken washed thylakoids prepared according to [17] also showed phosphorylation rates equal to about 30% of the rates obtained with chloroplasts shocked in the presence of Mg^{2+}. The data presented in fig.1 and table 1 strongly suggest that the endogenous coupled cyclic electron flow around photosystem I, seen in intact chloroplasts [2,3], may be retained if the chloroplasts are shocked gently in the presence of Mg^{2+}, but that this is lost when shocking is performed in the absence of Mg^{2+}. Fig.2 presents more direct evidence in support of this argument: here the flash induced turnovers of cytochromes f (λ_{max} = 554 nm) and b_{563} are compared for intact chloroplasts and for chloroplasts broken with or without added Mg^{2+}. Shocking in the presence of Mg^{2+} (fig.2b) only slightly slows the rates of turnover of both cytochromes (and decreases the extent of cytochrome f turnover), while shocking in the

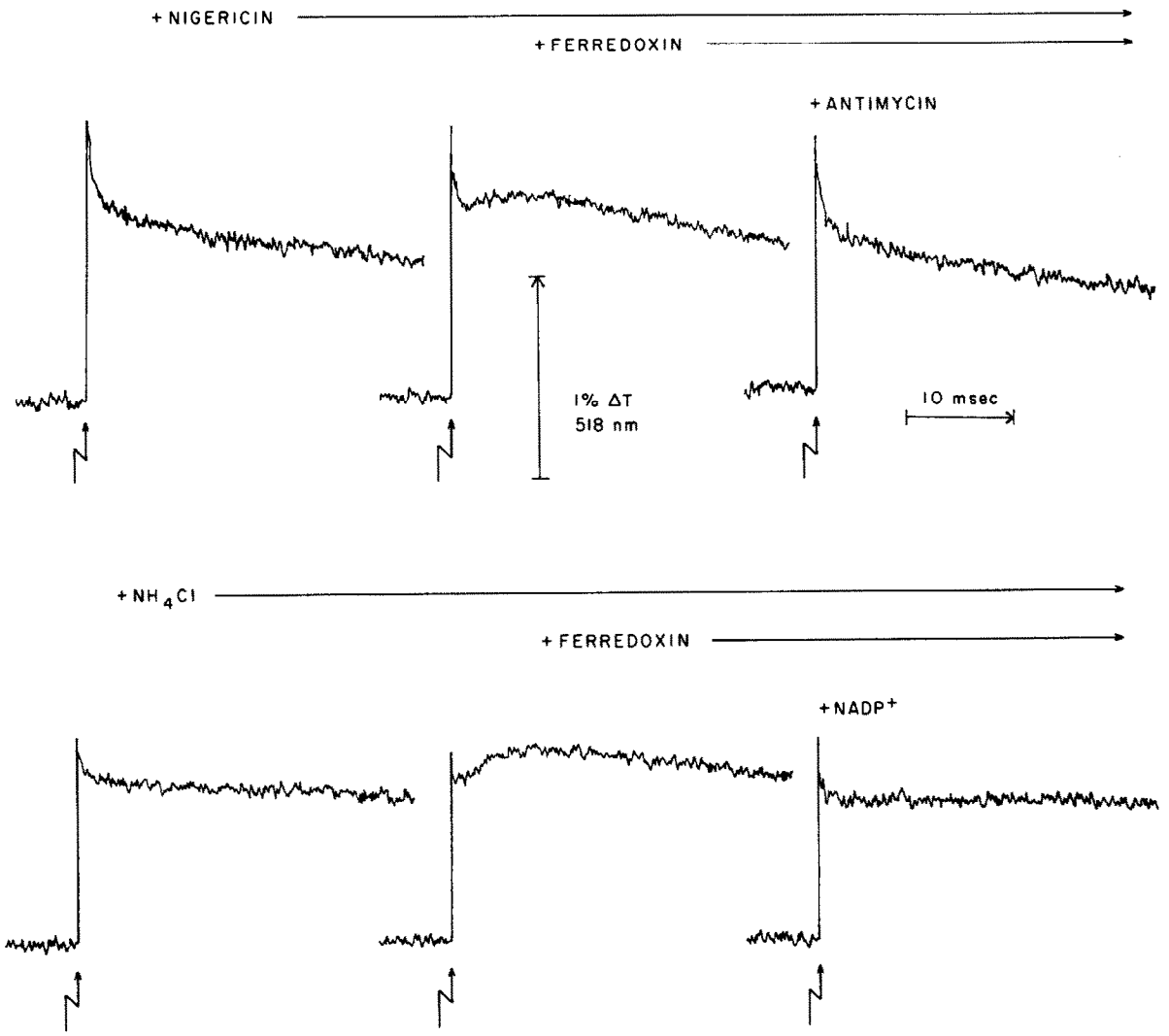

Fig.3. Restoration of the slow 518 nm change by ferredoxin in chloroplasts shocked without $MgCl_2$. Intact chloroplasts were broken in 0.75 ml of 1 mM Tricine (pH 8.2) and the mixture was shaken for 1 min; then 0.75 ml of double strength reaction mixture was added to give the final concentrations indicated in fig.1. Other concentrations where present were: nigericin 1.8 μM, NH_4Cl 3.3 mM, antimycin 6.7 μM, $NADP^+$ 130 μM, and ferredoxin 3.3 μM from Miles Yeda (upper traces) or Sigma (lower traces). Maximal restoration was achieved with 2 μM ferredoxin.

absence of Mg^{2+} (fig.2c) almost completely eliminates turnover of either cytochrome. Both these cytochromes have been implicated in cyclic electron flow [6,18] and thus the retention of turnover in fig.2b and its loss in fig.2c fully support the above hypothesis.

Arnon et al. [4,5] have shown that ferredoxin may induce an antimycin sensitive cyclic photophosphorylation in thylakoid preparations ('broken chloroplasts'). Fig.3 shows that addition of ferredoxin and Mg^{2+} to chloroplasts shocked in the absence of Mg^{2+} (as in fig.1c) does indeed restore the slow rise in the P518 response, indicative of cyclic electron flow, and that this restored rise is inhibited by antimycin. DBMIB caused similar inhibition (results not shown). Fig.3 (lower right trace) also shows that $NADP^+$ caused loss of the P518 slow rise, probably by acting as an electron acceptor from photosystem I and so preventing attainment of a suitable redox poise for cyclic electron flow [3]; methyl viologen gave a similar effect (result not shown).

The ability of ferredoxin to restore cyclic electron flow in chloroplasts shocked in the absence of Mg^{2+} is further demonstrated in table 2, where the light induced quenching of 9-AA fluorescence is reported for chloroplasts treated in the ways described. It is seen that chloroplasts shocked in the absence of added Mg^{2+} have a significantly lower capacity for cyclic phosphorylation (antimycin-sensitive quenching) than intact chloroplasts or those shocked with Mg^{2+}, but that this diminished capacity is increased by the addition of ferredoxin and Mg^{2+}. The first column also shows the restoration of photophosphorylation by ferredoxin and Mg^{2+}; the rates here may be compared with those presented in table 1.

4. Discussion

Cyclic electron flow and cyclic phosphorylation are known to occur in intact chloroplasts or in thylakoid preparations supplemented with exogenous ferredoxin [1,4,5,6,9,10]. The results presented here show that intact chloroplasts when gently shocked in the presence of Mg^{2+} retain this cyclic activity without the addition of any cofactor. The flash induced electrochromic shift, cytochrome b_{563} and f turnovers and formation of ΔpH in continuous light in these chloroplasts were comparable to intact chloroplasts in their sensitivity to antimycin and DBMIB (figs.1 and 2, and table 2).

Cyclic phosphorylation in intact as well as in ferredoxin-supplemented thylakoids is sensitive to the rate of electron input by photosystem II. In the absence of a terminal electron acceptor (HCO_3^- for intact and $NADP^+$ for broken chloroplasts), red illumination can over-reduce the system and thus inhibit the cycle. Under such conditions the addition of DCMU, which slows down electron transport from photosystem II,

Table 2
Light induced 9-Aminoacridine fluorescence quenching in intact and freshly broken chloroplasts

Conditions	9-Aminoacridine fluorescence quenching, %	
	Control	+Antimycin
Intact chloroplasts	74	50
Shocked[a]	73	46
Shocked[a] plus ferredoxin	73	44
Shocked[b]	62 (13.5)	34
Shocked[b] plus ferredoxin	73 (37.8)	40

Final reaction mixture contained in 1.5 ml: sorbitol 0.33 M, NaCl 10 mM, $MgCl_2$ 5 mM, Tricine 10 mM (pH 8.2), phosphate 1 mM, DCMU 0.13 μM (to enhance cyclic flow), 9-AA 10 μM and chloroplasts (19 μg chlorophyll/ml). Where indicated, 3.3 μM ferredoxin or 6.7 μM antimycin was added. Values in parentheses: phosphorylation rate (μmol ATP/mg chlorophyll/h) with 0.5 mM ADP added and Tricine 1 mM. Osmotic shock procedures: chloroplasts were [a]stirred for 1 min in 0.75 ml medium containing 10 mM $MgCl_2$ and 1 mM Tricine (pH 8.2), or [b]shaken for 1 min in 0.75 ml 1 mM Tricine; then 0.75 ml of double strength reaction mixture with $MgCl_2$ omitted[a], or present[b] was added. Illumination began 5 min after adding chloroplasts

was reported to stimulate cyclic phosphorylation [5,10,19]. Similar results were obtained for the chloroplasts broken with Mg^{2+} present: the relatively high rates of photophosphorylation measured in the presence of exogenous ADP and phosphate (but without artificial cofactors of electron flow) were further stimulated by DCMU at concentrations sufficient to inhibit most of the linear electron flow (table 1). Such stimulation of phosphorylation by DCMU and its inhibition by antimycin (table 1) suggest that it is mainly driven by cyclic electron flow.

Chloroplasts which were broken in the absence of Mg^{2+} lost most of this cyclic activity (figs. 2 and 3, and table 2). In accord with [4,5,19], the addition of Mg^{2+} and μM concentrations of ferredoxin restored cyclic activity (fig. 3 and table 2). The slow rise in the flash induced P518 response that was restored by ferredoxin was inhibited by antimycin and by $NADP^+$ (fig.3). The latter suggests that $NADP^+$ competes with the cycle for reduced ferredoxin and further supports the notion that the slow rise in P518 does not result from linear electron transport.

The above data clearly point to a role for Mg^{2+} in binding ferredoxin to the thylakoid. The addition of a few mM $MgCl_2$ to broken chloroplasts has been reported to increase specifically the quantum yield of ferredoxin-dependent $NADP^+$ reduction, which suggests an effect in the region of ferredoxin-$NADP^+$ reductase [20]. Mg^{2+} might be directly involved in binding of ferredoxin to the membrane at the reductase sites [21], or indirectly by affecting membrane surface potential or structure [22].

Acknowledgements

Thanks are owed to Catherine Chia for technical assistance. This research was carried out at Brookhaven National Laboratory under the auspices of the US Department of Energy.

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