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Uncouplers can shuttle between localized energy-coupling sites during photophosphorylation by chromatophores of *Rhodopseudomonas capsulata* N22

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1. Two models of the action of uncoupler molecules in inhibiting photophosphorylation in bacterial chromatophores are considered: either uncoupler molecules shuttle rapidly *between* energy-coupling sites, or uncoupler molecules that are bound to particular sites in the chromatophores for a time that is comparable with the turnover time of the photophosphorylation apparatus may uncouple by a co-operative 'substoichiometric' mechanism. 2. It is found that the titre of uncoupler necessary to cause complete uncoupling is lowered if the rate of photophosphorylation is initially decreased by partially restricting electron flow with an appropriate titre of antimycin A. 3. This result indicates that uncoupler molecules shuttle rapidly between energy-coupling sites, and is consistent both with models of energy *coupling* in which the energized intermediate between electron transport and phosphorylation is delocalized over the entire chromatophore membrane and those in which it is not. 4. If the rate of photophosphorylation is partially restricted with the covalent H^+-translocating ATP synthase inhibitor dicyclohexylcarbodi-imide, the titre of uncoupler necessary to effect complete inhibition of photophorphorylation is also decreased relative to that in which the covalent H^+-ATP synthase inhibitor is absent. 5. This important result appears to be inconsistent with models of electron-transport phosphorylation in which the 'energized state' of the chromatophore membrane that is set up by electron transport and utilized in photophosphorylation is delocalized over the entire chromatophore membrane.

Bacterial chromatophores have enjoyed widespread use as excellent experimental systems in studies directed towards the furthering of our understanding of the processes involved in electron-transport phosphorylation [for reviews see Gromet-Elhanan (1977), Crofts & Wood (1978), Clayton & Sistrom (1978), Baccarini-Melandri *et al.* (1981) and Barber (1982)]. As part of a general study of electron-transport phosphorylation in bacterial systems, we have recently presented and discussed experiments (Hitchens & Kell, 1982*a*) directed towards the achievement of a resolution of the current problem of whether the 'energized intermediate' between electron transport and ATP synthesis is delocalized over the entire chromatophore membrane, as in macroscopic versions of the chemiosmotic hypothesis of membrane energy-coupling processes (see, e.g., Mitchell, 1966, 1979*a*,*b*; Nicholls, 1982), or whether there exist more localized and direct free-energy-transferring interactions between electron transport and H^+-ATP synthase complexes in these and other energy-coupling membranes (see, e.g., Ernster, 1977; Del Valle-Tascon *et al.*, 1978; Rottenberg, 1978; Williams, 1978; Kell, 1979; Petty & Jackson, 1979; Fillingame, 1980; Melandri *et al.*, 1980, 1981; Baccarini-Melandri *et al.*, 1981; Conover & Azzone, 1981; Kell & Morris, 1981; Kell *et al.*, 1981; Malpress, 1981; Schuurmans *et al.*, 1981; Storey & Lee, 1981; Westerhoff *et al.*, 1981; Venturoli & Melandri, 1981).

In these experiments (Hitchens & Kell, 1982*a*) we carried out double-inhibitor titrations (see Baum *et al.*, 1971; Ernster, 1977; Kell *et al.*, 1978; Storey & Lee, 1981; Venturoli & Melandri, 1982), in which photophosphorylation by chromatophores of *Rhodopseudomonas capsulata* N22, incubated under

Abbreviations used: DCCD, dicyclohexylcarbodi-imide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; SF 6847, 3,5-di-t-butyl-4-hydroxybenzylidenemalononitrile; H^+-ATP synthase, H^+-translocating ATP synthase.

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conditions of saturating illumination, was partially decreased by the addition of the covalent H^+-ATP synthase inhibitor DCCD, and then titrated with the electron-transport inhibitor antimycin A. We were able to conclude (Hitchens & Kell, 1982*a*; cf. Melandri *et al.*, 1981; Venturoli & Melandri, 1982) that the localization of free-energy transfer during photophosphorylation appeared to be essentially complete in these chromatophores.

However, it was also found (Hitchens & Kell, 1982*a*) that full *uncoupling* of electron transport from phosphorylation in these chromatophores was achieved at concentrations of the uncoupler SF 6847 equivalent to approx. 0.3 molecules per electron-transport chain. After the earlier discovery of 'substoichiometric' uncoupling by SF 6847 (see Terada, 1981), two possible explanations of such behaviour were discussed by Terada & van Dam (1975). The first possibility, that all coupling sites in a given membrane-bound vesicle release their free energy into a common delocalized pool, and that the uncoupler works at its maximum turnover to lower the 'energization' of this pool, seems unlikely in view of the localized coupling demonstrated in these chromatophores (Hitchens & Kell, 1982*a*; cf. Venturoli & Melandri, 1982). The second mechanism of uncoupling discussed by Terada & van Dam (1975) was that SF 6847 simply shuttled rapidly from coupling site to coupling site during the uncoupling process. We also countenanced (Hitchens & Kell, 1982*a*) a novel mechanism for uncoupling by SF 6847, based on certain more recently discovered features of uncoupling (see, e.g., Hanstein, 1976; Decker & Lang, 1978), wherein uncoupler molecules bind to proteinaceous components of the energy-coupling membrane, causing co-operative (i.e. 'substoichiometric') and uncoupling conformational changes in energy-coupling membranes that do not occur in the absence of uncouplers. No distinction could be made between the latter two mechanisms in the work described (Hitchens & Kell, 1982*a*), but helpful discussions of what is known concerning the mode of action of uncouplers may be found in a number of recent reviews (Hanstein, 1976; McLaughlin & Dilger, 1980; Terada, 1981).

The purpose of the experiments reported here is to distinguish between the above models of *uncoupling* by SF 6847 (i.e. 'shuttling' versus 'co-operative' models) within the context of the apparently localized energy *coupling* that is evident in chromatophores of *Rps. capsulata* N22. By using double-inhibitor titration techniques we conclude that the uncoupling of multiple photophosphorylation sites by single SF 6847 molecules is best explained through the ability of SF 6847 molecules to shuttle rapidly between localized (un)coupling sites.

Experimental

Biological material

The growth under photoheterotrophic conditions of *Rps. capsulata* N22 and the preparation of chromatophores therefrom were carried out as previously described (Hitchens & Kell, 1982*a*).

Photophosphorylation

This was measured as described by Hitchens & Kell (1982*a*), a modification of the method of Nishimura *et al.* (1962) being used.

Chemicals

These were obtained from previously described sources (Hitchens & Kell, 1982*a*) Compound SF 6847 was generously given by Dr. Y. Nishizawa (Sumitomo Chemical Co., Osaka, Japan).

Results

Fig. 1 is a diagrammatic representation showing some possible outcomes of double-inhibitor titrations in which (*a*) represents a titration of the rate of photophosphorylation by illuminated chromatophores with an uncoupler such as SF 6847. Two traces (*b*) and (*c*) predict possible types of outcome of similar titrations with the same uncoupler, using chromatophores from the same preparation in which the rate of ATP synthesis in the absence of uncoupler is decreased to 50% by inhibiting cyclic electron flow with an appropriate titre of the electron-transport inhibitor antimycin A. We consider possible outcomes both for a model in which energy coupling is strictly localized between particular electron-transport chains and H^+-ATP synthase enzymes (e.g. Hitchens & Kell, 1982*a*; Venturoli & Melandri, 1982) and for a model in which delocalization of free-energy transfer is complete at the level of the whole chromatophore.

Trace (*b*) of Fig. 1 would be obtained, we predict, if energy coupling were fully localized, if the amount of SF 6847 that is bound to the chromatophores depends solely on the amount added and if uncoupling occurs at a fixed number (>1) of coupling sites for each uncoupler bound. Such a result could also be interpreted according to the chemiosmotic model if it is assumed that the relationship between the rate of ATP synthesis and the magnitude of the bulk-phase electrochemical proton potential difference (Δp) is such that a decrease in the rate of electron transport is accompanied by appropriate changes in the magnitude of Δp which fortuitously lead to a decrease in the rate of photophosphorylation that, for a given uncoupler concentration, parallels the number of active electron-transport chains. Many recent data (e.g. Baccarini-Melandri *et al.*, 1977) would suggest that this latter idea is unlikely to prove correct.

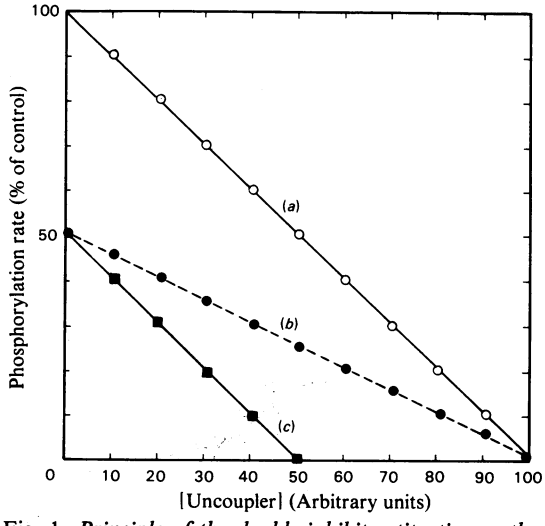

Fig. 1. *Principle of the double-inhibitor titration method for distinguishing between competing models of uncoupling in bacterial chromatophores*

(*a*) A titration curve is established of the effect of uncoupler on the rate of phosphorylation by the membrane-vesicle preparation under study (○). Then the membranes are allowed to react with sufficient quantities of either an inhibitor of electron transport (here, antimycin A) or a covalent H^+-ATP synthase inhibitor (here, DCCD) to decrease the rate of phosphorylation by 50%. Two anticipated types of outcomes of a subsequent titration with uncoupler are shown (●, ■); (*b*) a given concentration of uncoupler inhibits the residual rate of phosphorylation to the same degree as in the absence of the electron transport or H^+-ATP synthase inhibitor, so that the titre of uncoupler required for full uncoupling is unchanged (●); (*c*) a given concentration of uncoupler inhibits the residual rate of phosphorylation to a greater degree than that found in the absence of added electron-transport or H^+-ATP synthase inhibitor, such that the titre of uncoupler required to give full uncoupling is reduced by, say, 50% (■). For further detailed discussion, see the text.

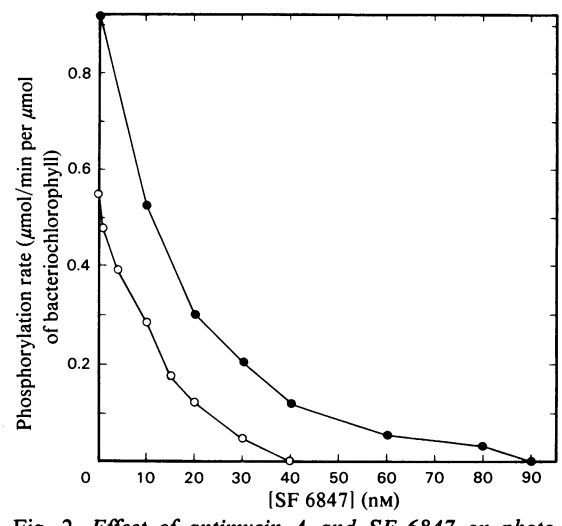

Fig. 2. *Effect of antimycin A and SF 6847 on photophosphorylation by chromatophores of Rps. capsulata N22*

The rate of photophosphorylation was measured as described in the Experimental section, under conditions of saturating illumination, at pH 7.8 and 25°C, in 6ml of a reaction medium containing 3mM-KH_2PO_4, 10mM-magnesium acetate, 30mM-potassium acetate, 0.2mM-sodium succinate, 1.5mM-sodium ADP, 5μM-P^1,P^5-diadenosine pentaphosphate, 800μg of carbonic anhydrase and chromatophores corresponding to a concentration of bacteriochlorophyll of 20μM (Hitchens & Kell, 1982*a*). The uncoupler SF 6847 was added to the concentrations indicated (●, ○) and, where indicated (○), antimycin A (0.7μM) was also present.

Full uncoupling by concentrations of uncoupler corresponding to <1 molecule of SF 6847 per electron-transport chain might also be explained by a model in which uncoupler molecules shuttle rapidly between coupling sites in illuminated chromatophores. The predicted type of trace, if such a mechanism takes place, whether coupling is localized *or* delocalized, is shown in Fig. 1, trace (*c*), and the data from an actual titration of photophosphorylation with SF 6847 in the presence or absence of antimycin A is displayed in Fig. 2. The experimental data evidently follow the pattern of uncoupling predicted for a shuttle type of mechanism of uncoupling. Thus full uncoupling is achieved at a lower concentration of SF 6847 when the rate of photophosphorylation in the absence of uncoupler has been partially reduced by restricting electron flow than when no electron-transport inhibitor has been added. A similar observation was made for succinate-driven phosphorylation in rat-liver mitochondria (Terada & van Dam, 1975). In terms of a localized energy-coupling model, this result implies that an uncoupler molecule can act to uncouple only those sites that are unaffected by antimycin A and are thus 'energized'. In other words, although *coupling* is localized at these sites, shuttling between several such sites by an uncoupler molecule occurs within the time necessary for a complete turnover of the photophosphorylating system, and by slowing the rate of electron transport with antimycin A, a given uncoupler molecule can successfully uncouple a greater number of coupling sites than is the case when antimycin A is absent.

In terms of a chemiosmotic model in which energy *coupling* is fully delocalized over the whole chromatophore membrane, an appropriate relationship must again be assumed to exist between the rate of electron transport, the concentration of uncoupler, the magnitude of Δp and the rate of photophosphorylation, although it seems qualitatively

reasonable, in terms of such a model, that uncouplers should uncouple more efficiently at lower rates of electron transport.

That the type of data obtained, using this type of approach, are not unique to the uncoupler SF 6847 is evidenced by the fact that very similar types of data are also obtained using the somewhat less potent uncoupler FCCP (Fig. 3). It was also observed in complementary experiments (results not shown) that the titre of antimycin A required to inhibit the rate of photophosphorylation completely was decreased by approx. 50% when a concentration of SF 6847 sufficient to inhibit the rate of photophosphorylation in the absence of antimycin A by approx. 50% was also present.

Double-inhibitor titrations were also performed on bacterial chromatophores that had been allowed to react with sufficient of the covalent H^+-ATP synthase inhibitor DCCD to decrease the rate of photophosphorylation to approx. 50% of that displayed by membranes that had not been so treated. Under these conditions, the rate-limiting reaction is notionally (Hitchens & Kell, 1982*a*) associated with the turnover of the H^+-ATP synthase enzymes themselves, rather than with the electron-transport reaction or degree of membrane 'energization'. Assuming that energy coupling is in fact essentially fully localized (Hitchens & Kell, 1982*a*; Venturoli & Melandri, 1982), there are again two general possible outcomes of a titration with SF 6847 under these sets of conditions. These are again represented by traces (*b*) and (*c*) of Fig. 1, where, in this example, approximately half of the H^+-ATP synthase enzymes have been inhibited by DCCD. Trace (*b*) may be explained simply, as before, by co-operative ('substoichiometric') uncoupling occurring at a fixed number of sites, regardless of the energetic status of these sites and dependent only upon the number of bound uncoupler molecules. Still assuming a 'localized' model of energy coupling, the simplest explanation for a trace such as trace (*c*) of Fig. 1, obtained when the rate of photophosphorylation in the absence of uncoupler is decreased with an H^+-ATP synthase inhibitor, is analogous to that advanced to account for the data observed when the rate of phosphorylation in the absence of uncoupler is reduced with an electron-transport inhibitor (Figs. 2 and 3). Thus uncoupler molecules are visualized as moving rapidly between the localized coupling sites, which remain after addition of the H^+-ATP synthase inhibitor, so that, during a complete turnover of the residual phosphorylation apparatus, a given uncoupler molecule can successfully uncouple a greater number of coupling sites than is the case when the H^+-ATP synthase inhibitor is absent.

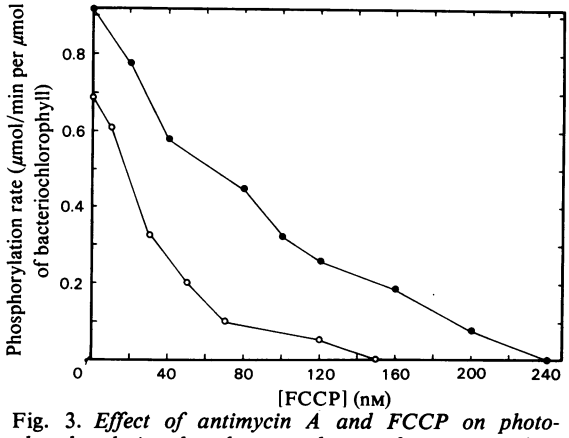

Fig. 3. *Effect of antimycin A and FCCP on photophosphorylation by chromatophores of Rps. capsulata N22*

Photophosphorylation was measured as described in the legend to Fig. 2. FCCP was added to the concentrations indicated (●, ○), and where indicated antimycin A (0.9 μM) was also present (○).

Such titrations may also be considered in terms of models such as those envisaged in the macroscopic chemiosmotic coupling theory, in which the energized intermediate between the reactions of electron transport and phosphorylation is taken to be delocalized over the entire chromatophore membrane in the form of a bulk-phase Δp. It is to be assumed under the above experimental conditions, in the absence of SF 6847, that when an H^+-ATP synthase inhibitor is added, but there is no added electron transport inhibitor, the bulk-phase Δp will not be decreased. The rate-limiting step must be assumed to lie in the terminal stages of photophosphorylation, i.e. at the level of the ATP-synthesizing enzyme itself, when sufficient DCCD is present to decrease the rate of phosphorylation to, say, 50% of that observed in its absence. Each ATP synthase molecule must therefore be responding at its maximum possible rate to a saturating Δp. One would therefore expect (in the context of *delocalized* coupling) that to decrease Δp, by titrating with an uncoupler to a level sufficient to inhibit photophosphorylation completely, would require a similar titre of uncoupler both in the presence and absence of the H^+-ATP synthase inhibitor (trace *b* of Fig. 1). If DCCD could exert some 'coupling' effect upon the native chromatophore preparation (cf. Lee & Ernster, 1965), an idea against which evidence exists (Melandri *et al.*, 1981), the titre of uncoupler required for full uncoupling must be assumed to be *greater* in the presence of DCCD than in its absence.

No ready explanation based on a delocalized energy-coupling model seems possible to account for an *increase* in uncoupling efficiency (trace *c* of Fig. 1) when the rate of phosphorylation in the absence of uncoupler is decreased by addition of an H^+-ATP synthase inhibitor.

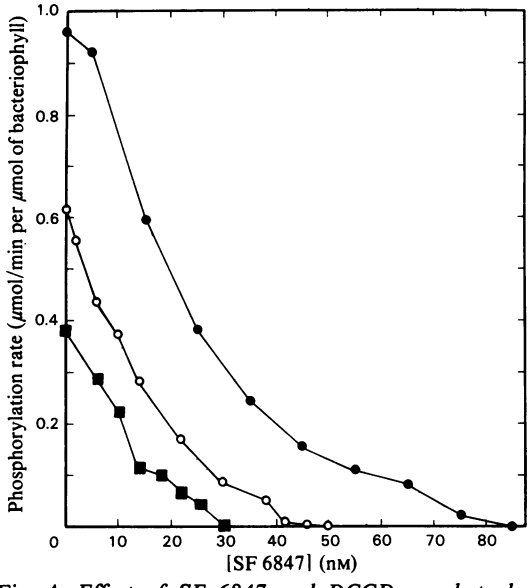

Fig. 4. *Effect of SF 6847 and DCCD on photophosphorylation by chromatophores of Rps. capsulata N22*
Photophosphorylation was measured as described in the legend to Fig. 2. SF 6847 was added to the concentrations indicated (●, ○, ■). In the cases indicated (○, ■) the chromatophores were preincubated with DCCD [18 μM (○) or 30 μM (■)] for 25 min in the reaction vessel, before photophosphorylation was initiated by illuminating the chromatophore suspension.

Fig. 4 displays typical data obtained from such double-inhibitor titrations of photophosphorylation in *Rps. capsulata* chromatophores, using DCCD and SF 6847. The data is comparable with that represented by trace (*c*) of Fig. 1, and is thus apparently explicable solely by models in which free-energy transfer between electron transport and H^+-ATP synthase complexes in these chromatophores is not delocalized over the entire chromatophore membrane (Hitchens & Kell, 1982*a*; Venturoli & Melandri, 1982), and in which uncoupler molecules can shuttle rapidly between these more localized coupling sites.

Discussion

The prime purpose of the present work was to effect an experimental distinction between two models of the uncoupling of photophosphorylation, in which individual uncoupler molecules either do or do not move *between* energy-coupling sites during the uncoupling process. We have previously discussed in some detail (Hitchens & Kell, 1982*a*) why we do not consider that problems such as those of possible chromatophore heterogeneity, or of energy 'leaks', might lead to premature conclusions being drawn regarding the nature of energy coupling in our chromatophore system, and we do not reiterate these arguments here.

Although we (Hitchens & Kell, 1982*a*) and others (see Baccarini-Melandri *et al.*, 1981; Venturoli & Melandri, 1982, and references therein) have presented data which strongly suggest that the energy *coupling* process during photophosphorylation by bacterial chromatophores is not delocalized over the entire chromatophore membrane vesicle, no assumptions concerning the nature of the extent of localization of energy coupling are in fact necessary at this stage to distinguish the two general models of *uncoupling* that we consider.

Thus, assuming any energy coupling model, it is to be expected that if uncoupler molecules shuttle between coupling sites during the uncoupling process they will do so more often if the net rate of photophosphorylation in the absence of uncoupler is decreased by limiting the rate of energy input to the system through a restriction of the rate of electron transport. In contrast, if uncoupling at several sites is effected by the simple 'substoichiometric' (see Terada, 1981) *binding* of uncoupler molecules to particular components in the energy-coupling membrane, the degree of uncoupling of the residual phosphorylating activity will depend only on the amount of uncoupler added (Fig. 1). It is clear that the experimental evidence (Figs. 2 and 3) strongly favours the former model of uncoupling, and we conclude that, at least for the protonophorous type of uncoupler, uncoupler molecules act via a 'shuttle' type of mechanism.

We can shed further light on the problem of whether free-energy transfer during photophosphorylation is delocalized over the entire chromatophore membrane (as proposed by the 'macroscopic' chemiosmotic coupling model) or if the free-energy transfer is in fact more microscopic in nature, by using the double-inhibitor titration technique with a combination of an uncoupler and an H^+-ATP synthase inhibitor. Thus, if the net rate of photophosphorylation (in the absence of added uncoupler) is decreased by, say, 50% using the covalent H^+-ATP synthase inhibitor DCCD, it must be assumed, according to a macroscopic chemiosmotic coupling model, both that the *bulk-to-bulk* phase protonmotive force is the maximum achievable by the chromatophore preparation, and that each active H^+-ATP synthase molecule is working at its maximum possible rate in response to this protonmotive force. There is therefore no reason to expect an increase in the efficiency of uncoupling by an uncoupler (i.e. a decrease in the titre of uncoupler necessary for full uncoupling) when the rate of photophosphorylation in the absence of uncoupler is decreased by the addition of a covalent H^+-ATP synthase inhibitor; whatever the presumed theoretical relationship between Δp and the rate of

photophosphorylation (for some kinetic models, see, e.g., Jencks, 1980; Rumberg & Heinze, 1980; McCarthy & Ferguson, 1981), it must be assumed to be monotonic and that uncoupler molecules cannot decrease the Δp formed by electron transport more effectively when an H^+-ATP synthase inhibitor is present than when it is not. However, it is found in practice (Fig. 4) that the rate of photophosphorylation *is* decreased more effectively by the uncoupler when photophosphorylation in the absence of uncoupler is partially restricted by DCCD, and it must be concluded that the 'energized intermediate' between the reactions of electron transport and phosphorylation in these chromatophores is *not* delocalized over the entire chromatophore membrane, for instance as a bulk-to-bulk phase electrochemical proton gradient.

It is concluded, in harmony with the proposals of a number of workers using these and related systems (see, e.g., Ernster, 1977; Kell, 1979; Baccarini-Melandri *et al.*, 1981; Conover & Azzone, 1981; Kell & Morris, 1981; Kell *et al.*, 1981; Storey & Lee, 1981) that free-energy transfer during electron-transport phosphorylation is not delocalized at the level of the intact vesicle.

Very recently (Hitchens & Kell, 1982*b*), we have performed dual-inhibitor titrations in this system with the ATPase inhibitor oligomycin. The data are in close accord with those described in the present paper.

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