in the absence of knowledge of the kinetics of the individual steps in coupling and uncoupling, we can therefore state that if the number of competing acceptors (uncoupler molecules) exceeds the number of ATP synthase molecules then we always have the possibility of apparent pool behaviour. This will be exacerbated as the turnover number of the ATP synthases is decreased, such that if we need a ratio of 10 uncouplers to 1 ATP synthase to effect uncoupling of that synthase we will always obtain behaviour indistinguishable from pool behaviour, whatever the reality (Rich, 1984).

In our own photophosphorylation work (cited above), we confined ourselves to situations in which the titre for full uncoupling did not exceed approx. $\frac{1}{3}$ the number of redox chains or ATP synthases present. In the work of Van der Bend *et al.* (1985), the (extrapolated) titres for full uncoupling (which was not in fact obtained) corresponded to more than 30 times the number of F_0F_1 molecules present.

Thus the claim of Van der Bend *et al.* (1985), using superstoichiometric uncouplers and poorly coupled co-reconstituted systems, that the observation of apparent pool behaviour in their titration experiments not only might provide a means of distinguishing localized from delocalized coupling, but indicated delocalized coupling, is erroneous.

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A model system for delocalized chemiosmotic coupling exhibited the features thought diagnostic of localized coupling

Quite a few experimental observations (Westerhoff *et al.*, 1984; Ferguson, 1985) are not readily understandable

in terms of the 'delocalized' chemiosmotic coupling model (Mitchell, 1981). Whereas it is appropriate to develop alternative views, the experimental observations should also be scrutinized both with respect to systematic experimental errors (e.g. Woelders *et al.*, 1985) and with respect to their interpretation. At issue here is the interpretation of the uncoupler-inhibitor (ui) and inhibitor-inhibitor (ii) titrations.

We shall discuss all the experiments as if dealing with oxidative phosphorylation: one inhibits some 50% of the electron-transfer chains (ui1, ii1) or 50% of the H^+ -ATPases (ui2, ii2) and examines how this affects the potency by which (ui) an uncoupler or (ii1) an inhibitor of the H^+ -ATPases, or (ii2) an inhibitor of the electron-transfer chains, inhibits oxidative phosphorylation. For delocalized coupling, Hitchens & Kell's (1982a) rule of thumb for ii experiments has been that the potency be reduced; for ui experiments, that the potency of the uncoupler not be increased. Baum *et al.* (1971, 1978), Hitchens & Kell (1982a,b, 1983a,b) and we (Westerhoff *et al.*, 1983a,b) found that experimental results in actual energy-coupling systems conflicted with these rules of thumb. This has been taken as evidence against delocalized coupling.

We (Van der Bend *et al.*, 1985) undertook checking the operational validity of these rules of thumb in an experimental model system which we assumed to have delocalized chemiosmotic coupling: bacteriorhodopsin and yeast H^+ -ATPase reconstituted into liposomes (Van der Bend *et al.*, 1985). In experiments of type ii, the inhibitors did not reduce each other's potency. The above rules of thumb would lead us to the conclusion that the model system for delocalized chemiosmotic coupling would operate in a localized fashion. By such *reductio ad absurdum* we proved that, for the experiments of the types ii and ui1, the rules of thumb are not by themselves sufficient to build a case against chemiosmotic coupling. The case ui2 was special in that the results obtained in our delocalized model system did conform to the rule of thumb: contrary to the observations in the more physiological systems, inhibition of H^+ -ATPases did not increase the potency of the uncoupler. Consequently, the rule of thumb for the experiment of type ui2 was the only one not compromised by our experimental results, and we granted that this type of experiment might still discriminate between delocalized and localized coupling.

Bacteriorhodopsin, reconstituted with H^+ -ATPase in liposomes, has been characterized experimentally [for a review, see Westerhoff & Van Dam (1986)]. The rate of ATP synthesis ($-J_p$) should depend on the activity of the H^+ -ATPase (L_p), the activity of bacteriorhodopsin (L_v) and the proton permeability of the membrane (L_H) as follows:

$$-J_p = A_v \cdot L_p \cdot L_v / (L_p + L_v + L_H)$$

(A_v is a constant). Since the system is fairly leaky towards protons (cf. below), this equation predicts our experimental results if indeed our co-reconstituted system were delocalized. This reassures us that there is no inconsistency between our experimental results and our *a priori* assumption that, in the co-reconstituted system, energy coupling was delocalized.

The more striking conclusion that can be deduced from this equation is, however, that (in contrast with the contention by Dr. Kell) the proper rule of thumb for an experiment of type ui2 demands that, in a delocalized system, an inhibitor of the H^+ -ATPases does not

decrease the potency of the uncoupler. In coming to the opposite conclusion, Hitchens & Kell (1983b) may have overlooked that after elimination of some of the H^+ -ATPases the 'dynamic buffering' of $\Delta\bar{\mu}_{H^+}$ by the H^+ -ATPases will be reduced. At any rate, this conclusion compromises the last surviving double titration as sole criterion to test delocalized chemiosmotic coupling (cf. Westerhoff *et al.*, 1983a,b).

In his letter, Dr. Kell makes two points. We agree with both, but wish to point out that they confirm rather than invalidate our argumentation. The first is that the co-reconstituted systems generally have a rather low rate of ATP synthesis, which is probably caused by a high proton leakage relative to the pumping capacity of the correctly reconstituted H^+ -ATPases and bacteriorhodopsins. For our argumentation, however, it was irrelevant how well the reconstituted system functioned; the only requirement was that its energy coupling be delocalized. We would expect that the loss of factors responsible *in vivo* for localizing the energized protons to their domains would cause the coupling in the reconstituted system to be delocalized and thereby inefficient.

The only scenario, then, in which these conclusions of our recent paper would not be upheld is the one alluded to by Dr. Kell: the co-reconstituted system would be organized in the localized fashion. Protoneural proteins (or corresponding factors) would be present in the H^+ -ATPase preparation and be reconstituted successfully. Against a (not our!) potential contention that the ui2 experiment would indicate that the coupling in the coreconstituted system was delocalized, Dr. Kell anticipates that a localized system may exhibit delocalized (i.e. 'pool') behaviour if any of the factors involved functions as a pool. In Hitchens & Kell's (1983b) view, the uncouplers rapidly shuttle between the H^+ -ATPases, but spend most of their time in uncoupling them (e.g. by removing the local proton). Inhibition of some of the H^+ -ATPases would allow the uncoupler molecules more time to uncouple the other H^+ -ATPases. Clearly, to have inhibitors of the H^+ -ATPase potentiate the uncouplers, all uncoupler molecules should be engaged in the uncoupling act. This implies that the amounts of uncoupler should be substoichiometric. In the co-reconstituted system, we used superstoichiometric amounts [of the same uncoupler that Hitchens & Kell (1983a) used].

We have difficulties with this uncoupling scenario in localized systems: why would inhibition of an H^+ -ATPase cause the uncouplers to spend less time in the corresponding coupling unit? The inhibition ('coupling') of respiration induced (in most, though not all, systems) by inhibition of some of the H^+ -ATPases, tends to be relieved (uncoupled) by the addition of uncouplers, suggesting the opposite. Yet, even if we would accept this uncoupling scenario, the observations really suggest that the coreconstituted system is delocalized: otherwise much less (< 2%) than stoichiometric amounts of uncoupler should have abolished light-driven ATP synthesis. Indeed, the very fact that we had to add superstoichiometric amounts of uncoupler to observe any uncoupling at all

supports our interpretation that the energy coupling in the co-reconstituted system is delocalized. If we were to reconstitute localized coupling into that system, e.g. by carefully adding protoneural (or corresponding) proteins, then we would expect to see an increase in light-driven ATP synthesis that is highly sensitive to substoichiometric amounts of uncouplers.

Where does this leave us? Does this prove that free-energy transduction is organized in a delocalized fashion? Of course not: our contentions merely prove that double-inhibitor experiments plus the rules of thumb as they have been formulated by Hitchens & Kell (1982a, 1983b) cannot serve as sole evidence that energy coupling is organized in a localized fashion. In interpreting this type of experiment one needs additional knowledge of the system [e.g. that proton leakage is insignificant; cf. Hitchens & Kell (1982a)], or [Westerhoff *et al.*, 1983a; Van der Bend *et al.*, 1985; Westerhoff & Van Dam, 1986] an improved formulation of the rules of thumb (the results obtained in our reconstituted system do differ systematically from those obtained in the more physiological systems).

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