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1 **Electron transfers amongst flavo- and hemo- proteins:**

2 **Diffusible species effect the relay processes, not protein-protein binding.**

3

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33 **Abstract**

34

35 Hitherto, electron transfers (ET) between redox proteins are believed to occur via donor-
36 acceptor binding and diffusible reactive species are deemed as deleterious side-products in
37 such systems. Herein, ET from cytochrome P450 reductase (CPR, an animal membrane
38 flavoprotein) and horseradish peroxidase (HRP, a plant hemoprotein) to cytochrome *c* (Cyt.
39 *c*, a soluble animal hemoprotein) was probed under diverse conditions, using standard assays.
40 ETs in CPR-Cyt. *c* system were critically inhibited by cyanide, sub-equivalent levels of polar
41 one-electron cyclers like- copper ions, vitamin C / Trolox and superoxide dismutase. In the
42 presence of lipids, inhibition was also afforded by amphipathic molecules- vitamin E,
43 palmitoyl-vitamin C and the membrane hemoprotein, cytochrome b_5. Such non-specific
44 inhibitions (by diverse agents in both aqueous and lipid phases) indicated that ET
45 transfer/relay was effected by small diffusible agents, whose lifetimes are shortened by the
46 diverse radical scavengers. When CPR was retained in a dialysis membrane and Cyt. *c*
47 presented outside in free solution, ETs were still observed. Further, HRP (taken at nM levels)
48 catalyzed oxidation of a phenolic substrate was significantly inhibited upon the incorporation
49 of sub-nM levels of Cyt. *c*. The findings imply that CPR-Cyt. *c* or HRP-Cyt. *c* bindings were
50 not crucial for ETs. Further, fundamental quantitative arguments (based on diffusion /
51 collision) challenge the erstwhile protein-protein binding assisted ET hypothesis. It is proven
52 beyond reasonable doubt that mobile and diffusible electron carriers (ions and radicals) serve
53 as "redox-relay agents" in the biological ET models/setup studied.

54

55 **Keywords:** electron transfer; reductase; heme protein; cytochrome *c*; redox enzyme;

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59
Introduction

60

61 Redox-active enzymes mediate electron transfer (ET) reactions amongst diverse molecules
62 and molecular assemblies [1]. Such catalytic processes are ubiquitous and they form the
63 foundations of key metabolic and energy transduction steps, essential to all life forms. Unlike
64 other enzymes, oxidoreductases show significant laxity in specificity and are known to be
65 associated with the generation of "physiologically disruptive" diffusible reactive oxygen
66 species (DROS) and diffusible radicals [2, 3].

67

68 Variants of heme and flavin cofactor moieties are tethered to the apoprotein of redox
69 enzymes in various modalities, which in turn affect their reactivity and selectivity [4, 5]. These
70 enzymes form the mainstay of several electron transfer processes in aerobic cellular systems.
71 Historically, the donor-acceptor protein pair of flavoenzyme cytochrome P450 reductase
72 (CPR) and hemoprotein cytochrome *c* (Cyt. *c*) have been employed to study biological ETs [6-
73 8]. The currently accepted mechanism of ET between these two enzymes vouches for a direct
74 protein-protein complex formation, followed by long-distance electron tunneling [6-8]. While
75 investigating heme-enzyme reaction systems, we had revealed several unusual
76 activations/inhibitions and interpreted them by invoking upon the roles of diffusible species
77 generated within the milieu [9-17]. Recently, we had alluded to the role of cyanide-based
78 diffusible radicals to explain the inhibitions of heme-enzyme activities at low cyanide
79 concentrations, in a regime where cyanide binding with the heme center would be a low
80 probability event [18]. We have also found that the reaction cycle of the bi-enzymatic system of
81 liver microsomal cytochrome P450 (CYP) and CPR can be explained without entailing
82 protein-protein complexations between the two enzymes [11, 13, 17] (and unpublished results).
83 Furthermore, analysis of elementary kinetics data gave us an intuitive idea that the "protein-

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84 protein binding-based ET hypothesis" appeared probable only at very high concentrations of
85 proteins involved. These findings and arguments persuaded us to challenge the mechanism of
86 ET in the fundamental *in vitro* CPR-Cyt. *c* model. This work reveals that small diffusible
87 species (radicals/ions) serve as relays for facilitating ETs between CPR and Cyt. *c* under
88 routine assay (and thereby, physiological) conditions.
89

90 **Materials and Methods**

91

92 Peroxidase (HRP, horseradish, P6782), Catalase (C9322) Cu-Zn Superoxide Dismutase
93 (SOD, bovine, S2515), Cyt. *c* (horse heart, C2506) and reduced glutathione were purchased
94 from Sigma-Aldrich (USA). Human recombinant CPR was procured from Invitrogen
95 (currently, ThermoFisher P2309) and cytochrome b_5 (Cyt. b_5) was a purified laboratory
96 preparation. Dilauroylphosphatidyl choline (DLPC) was purchased from Avanti Lipids. All
97 other chemicals (of analytical grade, certified to be >98% purity) were procured from reputed
98 chemical manufactures like Alfa-Aesar and Lancaster (USA), SRL and Loba-Chemie (India),
99 etc.
100

101 Flavin-based CPR quantification was done using a molar extinction coefficient of 21,200 at
102 550 nm [19]. For Cyt. *c*, the Soret band absorption was used to determine oxidized heme (ε =
103 106,000 $M^{-1}cm^{-1}$ at ~410 nm) and the emergence of α band at 550 nm was used for reduced
104 Cyt. *c* estimation, with a molar extinction coefficient of 27,600 [20, 21]. Generally, the reactions
105 were carried out in aerated (open) cuvettes, using potassium phosphate buffer, pH 7.4, at 27
106 °C. Absorption spectra and time-course data were obtained using a UV-Visible
107 spectrophotometer interfaced with a computer. To an appropriately made up reaction mixture,
108 a suitable volume of NADPH stock was added and mixed to start the reaction. For

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109 determining the effect of ionic strength on reaction rates, the reactions contained potassium
110 phosphate buffer at various molarities, [Cyt. *c*] = 20 μM, [NADPH] = 20 μM, and [CPR] = 1
111 nM. For anaerobic reactions, the protein mixtures and NADPH solutions were flushed and
112 maintained separately under argon for an hour, prior to NADPH addition by syringe. Specific
113 reaction conditions are detailed in the appropriate figure legends. All data reported herein are
114 average values (with standard deviations) of duplicates/triplicates.

115

116 **Results**

117

118 **Elementary kinetics.** It can be noted from the positive controls in Figures 1A and 1C that
119 initial product formation rate was practically constant over a protracted time period (for very
120 low, ~nM level CPR concentrations and μM levels of Cyt. *c*) and owing to this, the Cyt. *c*
121 reduction assay is highly reproducible. Practically, zeroth order dependence (into micromolar
122 levels of Cyt. *c* and NADPH) was seen in the control reactions. This is quite akin to the
123 kinetic profiles observed in chloroperoxidase (CPO) catalyzed chlorination reactions, which
124 is mediated via diffusible species [9]. Figure 1A shows that 10 to 20 mM level of cyanide
125 completely causes cessation of ETs to Cyt. *c*. At similar conditions, incorporation of azide
126 caused only ~46% inhibition. The relatively higher efficiency of cyanide (as inhibitor) may
127 be explained by considering its higher redox potential. The initial absorbance values and final
128 yields of reduced Cyt. *c* were traced at higher amounts of reactants (CPR, Cyt. *c* and
129 NADPH). The results indicate that even nM to μM levels of cyanide significantly lowered
130 productive reaction cycles (Figure 1B). [Though the traces have similar slopes, the initial and
131 end-point absorbance values are the points of interest here.] At higher concentrations of CPR,
132 we would expect higher levels of radicals [11] and therefore, a greater inhibitory effect by
133 cyanide. Figure 1B ratifies this consideration. Further, the inhibition of Cyt. *c* reduction by

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134 cyanide was due to its impact on CPR's activity per se, and not owing to cyanide's oxidation
135 or binding of Cyt. *c* (Figure S1a). In fact, in the absence of CPR (but in the presence of
136 NADPH), cyanide reduced Cyt. *c* slightly, under the assay conditions (Figure S1b). This
137 points out the role of cyanide-based radicals in the milieu. Also, though initial rates were not
138 significantly altered by lowering NADPH concentration from 40 to 4 μM (in the controls),
139 incorporation of 100 nM cyanide increased the extent of inhibition from 13 % to 36 % in the
140 test reactions (not shown). This signifies that NADPH also plays an electron moderator's role
141 in the reaction system (quite similar to peroxide's role in CPO reactions [9]), rather than merely
142 serving as the electron donor to CPR. A positive reaction control in the routine assay is
143 shown in Figure 1C and it is compared with the time profile of the reaction with the inclusion
144 of small amounts of copper ions. These types of rate determination with model assays (as
145 shown in the positive controls of Figure 1A and 1C; but not like those shown in Figure 1B
146 where CPR is in excess) were employed for the determination of pseudo-first order rates, for
147 profiling the dose-response curves.
148
149 **Dose response profiles obtained upon the incorporation of various ions, molecules and**
150 **enzymes.** Incorporation of catalytic amounts of Cu-Zn SOD lowered the reduction rates of
151 Cyt. *c* (Figure 2A). The inhibitory effect was observed even with the free solution of heat-
152 denatured SOD. When copper sulphate and zinc sulphate were included at 100 μM
153 concentration, the latter afforded only ~20% inhibition; whereas, the former gave ~95%
154 inhibition. The inhibitory effect of Cu^{2+} ions (an efficient one-electron cycler) was confirmed
155 with other salts like acetate and nitrate (data for the latter is shown in Figure 2A). At
156 equivalent concentrations (10 μM each Cu^{2+} and Cyt. *c*), copper ions shunted away more than
157 80% of the electrons from CPR. This outcome cannot be explained by a CPR-Cyt. *c* binding
158 process. Furthermore, copper ions retained the inhibitory effect well into sub-equivalent

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159 concentrations and did not give convergence or meaningful global K_i values when plotted for
160 non-linear regression analysis of dose-response. Figure 2A also shows that addition of
161 glutathione and catalase (all two-electron redox-active agents) did not significantly diminish
162 reduction rates at sub-micromolar concentrations. (The assay with the inclusion of catalase is
163 given in Figure S2). The effect(s) of incorporation (at submicromolar concentrations) of two
164 redox-active vitamins (organic molecules) in CPR catalyzed reduction of Cyt. *c* was studied
165 and the results are shown in Figure 2B. While the hydrophobic Vitamin E (Vit. E) yielded
166 very low concentration-dependent effects, Vitamin C (Vit. C, a smaller and very highly water
167 soluble molecule) showed highly concentration-dependent effects and caused profound
168 inhibition at nM concentration ranges. To further explore this effect, derivatives of the two
169 vitamins were incorporated (at submicromolar concentrations) in the reaction mixture and the
170 results are given in Figure 2C. Trolox (a water-soluble derivative of Vit. E) showed highly
171 concentration-dependent inhibitory effects, quite similar to Vit. C. This was in stark contrast
172 to the effect of L-ascorbic acid palmitate (LAP, an amphipathic derivative of Vit. C), which
173 showed very little effect, quite akin to Vit. E. As noted with copper or cyanide ions, the polar
174 redox-active organic molecules did not show the anticipated sigmoidal inhibitory dose-
175 response profile.
176
177 **Effect of lipids, in conjunction with the incorporation of vitamins/derivatives or**
178 **cytochrome b_5, on electron transfers between CPR and cytochrome *c*:** Selected vitamins
179 and their derivatives were included at supra-micromolar concentrations in the presence of
180 lipids, in order to probe if partitioning effects could perturb the ET reaction. The results are
181 shown in Figure 3. At lower concentrations of the additive, the presence of lipids alleviated
182 inhibitions. Particularly, the hydrophobic (or amphipathic) vitamin / derivative was seen to
183 activate the ETs marginally. At high concentrations of the additives, the presence of lipids

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184 lowered the inhibition efficiency of the water soluble vitamin C or water soluble derivative of
185 vitamin E (Trolox). In contrast, the presence of lipids yielded higher inhibitions with excess
186 amounts of the hydrophobic vitamin E or hydrophobic derivative of vitamin C (LAP). That
187 is, at higher concentrations, hydrophobic additives showed more profound inhibitory effect in
188 the presence of lipids and the water-soluble additives were more efficient (at inhibition) in the
189 absence of lipids. This showed that ET phenomena can be rate-inhibited in both CPR-lipid
190 microenvironment and Cyt. *c*-aqueous microenvironment.

191

192 In another experiment, ET from CPR (2-50 nM) to Cyt. b_5 (1-5 μM) was monitored in the
193 presence of varying concentrations of lipid (0-100 μg/ml). Although CPR consumed
194 NADPH, Cyt. b_5 spectral signature (Soret and α-β bands) remained unchanged in these
195 concentration regimes, within 10 minutes of mixing the reaction components (Figure 4). This
196 is when a much lower amount of CPR was enough to give reduction of even lower amounts
197 of Cyt. *c* (Figure 4, the lower three traces). These observations showed that CPR to Cyt. b_5
198 electron-transfers / "complexations" in the lipid phase are relatively inefficient or short-lived.

199

200 In yet another experiment, the effect of inclusion of Cyt. b_5 (a hydrophobic cytochrome, with
201 approximately 200 mV lower redox potential, in comparison to Cyt. *c*) was investigated on
202 CPR-Cyt. *c* ET and the results are given in Table 1. (If the text that follows in this paragraph
203 seems confusing, the reader is advised to study the data and make the interpretations
204 directly.) Increase in lipids almost always lowers ET rates (except at 20 nM Cyt. b_5, for both
205 high and low Cyt. *c*, when lipid was raised from 10 to 40 μg/ml). At low Cyt. *c*, for a given
206 Cyt. b_5, increasing lipid (from 0 to 400 μg/ml) and Cyt. b_5 (from nil to 40 nM) lowers ET
207 gradually and predictably. At high Cyt. *c*, for a given Cyt. b_5, increasing lipid (from 0 to 400
208 μg/ml) and Cyt. b_5 (from nil to 40 nM) lowers ET in a somewhat unpredictable but not highly

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209 significant manner. For a given lipid concentration and high Cyt. *c* concentration, addition of
210 Cyt. b_5 does not majorly perturb rates. For a lower Cyt. *c* concentration, presence of lipids in
211 lesser amounts lowered rates upon increasing Cyt. b_5 incorporation. Whereas, upon
212 increasing Cyt. b_5 concentrations and in the presence of lipids in greater amounts, the rates
213 were relatively higher. Further, for a given lipid and Cyt. *c* concentration, there exists an
214 optimum concentration level of Cyt. b_5; at low lipid- 5 nM Cyt. b_5 (regardless of Cyt. *c* conc.)
215 and at high lipid- either 5 or 20 nM Cyt. b_5. Presence of low or high amounts of Cyt. b_5
216 retains steady ET rates when low concentrations of Cyt. *c* is taken, irrespective of lipid
217 content. From 0 to 100 µg/ml lipid, the efficiency of increase in ET rate by ten-fold increase
218 in Cyt. *c* concentration remains around 2.4 ± 0.4 (when compared in analogous systems, with
219 similar amounts of Cyt. b_5). At higher lipid (400 µg/ml) and at any Cyt. b_5 concentrations,
220 enhancement in ET rate was lower with ten-fold increase of Cyt. *c* concentration.
221
222 **Effect of ionic strength and separation of the redox proteins by dialysis membrane.** The
223 effect of ionic strength was probed with respect to the ETs between the two proteins.
224 Reactions were performed in distilled water (D/W) and at various ionic strengths (and the raw
225 data are given in Figure S3a). The rates of Cyt. *c* reduction in the first five minutes of
226 incubation gave pesudo-first order (s^{-1}) values of 10.9 ± 0.2 in a pure aqueous medium
227 (devoid of added buffering ions) and 15.1 ± 0.6, 24 ± 0.2, 27.1 ± 0.9, 32.1 ± 0.5 and 30.8 ±
228 0.5 s^{-1}, respectively, for 25 mM, 100 mM, 200 mM, 250 mM and 500 mM strengths of
229 potassium phosphate buffer. Initial rates increase for up to 250 mM and then become slightly
230 lower at the 500 mM range. However, when compared to higher ionic strengths (250 & 500
231 mM), the yield of reduced Cyt. *c* is slightly higher at ~15 minutes of reaction time with 100
232 mM ionic strength. This indicates that- (i) higher ionic strength is deleterious to enzyme
233 stability and/or (ii) there could be competitive interactions of ions with multiple species in the

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234 milieu, which could in turn be dependent on temporally evolving concentration terms of
235 reaction components. The important roles played by high ionic concentrations signify the role
236 of mobile conducting species in water. A similar effect of diffusible species and excess ions
237 was noted in the reaction milieu of CPO-catalyzed chlorinations [9], which tended to make the
238 reaction approach zeroth order, with respect to the acceptor molecule. In order to investigate
239 the effect of physical separation of Cyt. *c* and CPR, the latter was confined in a dialysis
240 tubing, thus preventing direct protein-protein complexation. From the raw data (Figure S3b),
241 initial Cyt. *c* reduction rates for various reactions were calculated, as specified in Table 2. In
242 the D/W system, the separated reaction setup gave 14.6% activity of the positive control;
243 whereas the buffered & separated reaction gave 5.4% activity of the respective positive
244 control (after the values of negative control reaction rates were deducted from rates for both
245 positive and test reactions). Under these reaction conditions, reaction in D/W slightly
246 enhanced the yield of reduced Cyt. *c* at longer incubation times (in positive control and in test
247 reactions). These outcomes (very reproducible, as shown in Figure S4) cannot be explained
248 by considering ionic strength as a mere requirement for optimal protein-protein
249 complexation.
250
251 **Effect of inclusion of cytochrome *c* on redox reactions mediated by peroxidases- insight**
252 **into electron transfer between hemoproteins:** We have already shown that low
253 concentrations of a redox-active protein like Cyt. *c* could enhance heme-enzyme
254 catalyzed peroxidations [16, 17]. (Cyt. c could not affect any peroxidations on its own merit
255 under the concentration ranges studied herein.) When HRP-Gua reactions were studied with
256 the incorporation of Cyt. *c* at pH 9, product formation was enhanced at most concentration
257 ranges used. At pH 6, HRP-Gua reactions were inhibited, profoundly so at even sub-
258 equivalent concentration ranges (Figure 5A). When we used a stipulated concentration of

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259 Cyt. *c* (10 nM) while keeping peroxide and HRP at identical concentrations (Figure 5B), it
260 was seen that the effect was dependent on the nature of the substrate. While ABTS
261 peroxidation was significantly enhanced by Cyt. *c* and TMPD peroxidation was marginally
262 inhibited, the product formation from phenolics Gua and Pyr were significantly inhibited by
263 >25 % of control.
264

265 **Discussion**

266

267 Heme iron in Cyt. *c* is hexacoordinated. Though the axial methionyl ligation in Cyt. *c* could
268 be displaced to give a cyanide-coordinated protein species, it occurs significantly only at very
269 high concentrations (0.1 M) of cyanide [22]. The inhibitions by low concentrations of diverse
270 species such as- metal ions, cyanide/azide anions, enzymes and organic molecules etc.,
271 reported in the current manuscript, cannot be attributed to such displacement of coordination
272 spheres. CPR is not known to "bind" cyanide and is known to transfer electrons to a wide
273 variety of enzymes (heme oxygenase, fatty acyl elongase, squalene monooxygenase,
274 cholesterol reductase etc.), several cytochromes (hundreds of P450 isozymes, b_5, *c* etc.) and a
275 bevy of natural organics and synthetic dyes. It is difficult to envisage that CPR has
276 topological and electrostatic binding complementarities with all of these redox active
277 molecules (particularly negatively charged ions and small organic molecules). If that were the
278 case, we should not have found critical dependence on ionic strength and maverick
279 modulations by enzymes/small organic molecules/metal or inorganic ions. The present work
280 does not challenge the existence or solved crystal structures of complexes between various
281 redox proteins (like cytochrome P450 and CPR). However, it is doubtful that these
282 complexes hold any significance in the physiological or *in vivo / in vitro* assay scenarios. Till
283 date, we find no direct/conclusive evidence for donor (CPR) - acceptor (Cyt. *c*) complexes'

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284 contributory role in ETs in dilute aqueous solutions or low mobility scenarios (like- within
285 phospholipid membranes).
286
287 The diverse diffusible ionic or molecular or radical species (and enzymes like SOD) can
288 inhibit or enhance the electron shuttling mechanism between CPR and Cyt. *c*. This finding is
289 along the lines that we had demonstrated in our recent work on heme peroxidase catalysis [18].
290 Therefore, the promiscuity of CPR and non-conformity to classical dose-response profiles is
291 explained by the intermediary role of diffusible species. Similar effects are afforded by
292 molecules that have comparable molecular parameters (as exemplified by the parameters for
293 solubility and partitioning, log P and log D, Figs. 2B & 2C). Partitioning effects are clearly
294 evident in the data shown in Table 1 and Figures 2 & 3. It can be seen from Figure 3 that in
295 the absence of lipid, an excess of the polar molecule ($\geq$ 10 μM of Vitamin C being a salient
296 example; Trolox is relatively less polar and therefore, requires a greater excess, ~1 mM) is
297 more efficient in inhibiting CPR-Cyt. *c* reaction. In contrast, when lipid is added, excess of
298 the hydrophobic/amphipathic molecules (LAP and and Vitamin E) are more effective in
299 inhibiting the CPR-Cyt. *c* ET process. This is because the addition of lipid partitions CPR
300 solely into the lipid phase and therein, the excess of redox active hydrophobic molecules have
301 greater sway to access and dissipate the electrons. Secondary catalytic activity mediated by
302 additive-based radicals alone can explain why- (a) 100 nM levels of Vit. C or Trolox do not
303 inhibit ET, but 1 nM levels of these molecules inhibit ET significantly (Figures 2B and 2C).
304 & (b) sub-equivalent levels (1 - 100 pM) of Vit. C or Trolox significantly perturb (activate or
305 inhibit) the catalysis by nM concentrations of CPR (Figures 2B & 2C). Increasing the
306 concentration of a radical scavenger not only promotes its competition with Cyt. *c;*
307 additionally, the scavenger's catalytic ability to dissipate the electrons unproductively could
308 also be affected (because they collide more frequently amongst themselves, and collapse).

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309 Also, the redox active molecule/ion/radical species may (catalytically) reduce Cyt. *c* on its
310 own merit, which could explain some of the activations observed [14-17]. Since small amounts
311 of water-soluble and highly mobile radical cyclers (but not hydrophobic ROS scavengers)
312 critically affect ETs between CPR-Cyt. *c*, the rate limiting / essential process in these regimes
313 (in the absence of lipids) is understood to be 'diffusion-based' in water. These inferences are
314 corroborated and complemented/supplemented from our own studies involving the CPR-CYP
315 catalytic system (both hydrophobic proteins, co-localized in the phospholipid membrane
316 interface) [17]. In these systems (subjected to diffusion in the phospholipid microenvironment),
317 the reactions were drastically inhibited by sub-micromolar concentrations of the amphipathic
318 / hydrophobic and bulky DROS scavengers, and not the soluble vitamins or derivatives [17].
319

320 Table 1 shows that the radicals generated by CPR (in the lipid phase) need to reach Cyt. *c* (in
321 the aqueous milieu) and for the same, there is an optimal lipid / Cyt. b_5 paradigm. If excess
322 lipid or Cyt. b_5 was present, the probability increases that the electrons are sequestered or
323 dissipated to water formation. If the amount is optimal, the ET is optimally phased and
324 smooth. The concept that "protein-protein collisions lead to binding or result in conformation
325 changes of redox proteins and therefore, enhancement of catalytic activity" [23, 24] makes little
326 justice to scientific logic or experimental data. If the erstwhile paradigm were true, then an
327 increase in Cyt. b_5 should only give an increase in ET in all CPR-Cyt. *c* ETs or higher
328 concentrations of Cyt. b_5 should only enhance all CYP reactions. But this is not seen. In
329 contrast, in spite of possessing a favorable redox potential (with respect to CPR) and being
330 co-localized in the lipid environment, Cyt. b_5 does not get noticeably reduced (Figure 4). This
331 shows that protein-protein collisions are a relatively slow phenomenon in lipid membranes
332 (and/or that Cyt. b_5 cannot be the stable/terminal electron acceptor in the system), with
333 respect to the mechanistic phenomena involved in the kinetics of ET.

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334

335 The observation that ETs occurred even after physical separation of the two proteins confirms
336 the inference that protein-binding is not obligatory for electron transfer. It is demonstrated
337 from our works that CPR initiates a radical reaction [11], which is subsequently relayed to Cyt.
338 *c*. Oxygen is not obligatory for this step (as we have noted that electron transfers to Cyt. *c*
339 occurs even in anoxic conditions) and ionic conductivity facilitates this relay step. Redox-
340 active additives can enhance/lower this process. The concentration of reduced Cyt. *c* goes up
341 in the milieu, only because this species is more stable (owing to the dissipation of electron
342 into the heme/apoprotein and owing to the high redox potential) than the other "transiently
343 reducible" components within the reaction system. As of now, it is not facile to characterize
344 the exact nature of diffusible species involved, for the following simple reasons- (i) the
345 diffusible species are short-lived, (ii) their effective concentrations would be in sub-μM to
346 supra-pM ranges and (iii) these species can be envisaged to be in dynamic equilibrium with
347 diverse species in aqueous milieu, and each pathway could lead to chaotic outcomes. Till
348 date, there are no physical or chemical probes available to pinpoint the dynamics in such
349 systems accurately/precisely.

350

351 The argument that- "The physically separated reaction setup could only give ~10-15% of
352 control's activity and therefore, the primary route is via protein-protein complexation." is
353 misplaced. This is because radicals like superoxide have high auto-collapse (dismutation)
354 rates, with second order rate constants of 10^5 M^{-1} s^{-1} [25]. If an 'electron-relaying species' does
355 not encounter a molecule of Cyt. *c* in its immediate vicinity, the electron is taken away by
356 other interactive components in the milieu, leading to two electron processes and "non-
357 productive" water formation. Such a scenario is also involved in the CPO reaction milieu,
358 where, the reactive intermediate is consumed by peroxide itself, if a suitable substrate is not

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359 present [9]. Therefore, separation by the membrane drastically decreases Cyt. *c* reduction rates
360 in comparison to the freely mixed system.
361
362 Calculations and experimentations show that the diffusion rate of a small molecule like
363 oxygen in water (diffusion coefficient, D = 2000 $\mu m^2/s$) is ~ 10^2 to 10^3 times faster than the
364 diffusion rates of a protein in cytoplasm (D = 20 to 2 $\mu m^2/s$) and ~ 10^4 to 10^5 times the rate of
365 diffusion of a protein on phosopholipid membranes (for which, D is approximately 0.2 to
366 0.02 $\mu m^2/s$) [26]. Optimized ET rates between CPR and Cyt. *c* afforded pseudo-first order rates
367 of 32 s^{-1} or above. This would mean an overall second order rate constant of ~ 10^7 M^{-1} s^{-1}
368 (when considering dependence on Cyt. *c* into micromolar ranges) or > 10^{10} M^{-1} s^{-1} (with
369 dependence on nM levels of CPR as the rate determining component, in the reaction assays).
370 The latter value is apparently erroneous because the second order diffusion limitations of
371 small molecules in water is ~ 10^9 M^{-1} s^{-1}. If we multiply it by the concentration of CPR, we
372 get the collision frequency- which is 1 per second, about an order short of the actually
373 observed rates. When considering that we are dealing with two dilute and bulky proteins that
374 are housed/distributed micro-heterogeneously (nM levels of CPR housed in lipids with µM
375 levels of Cyt. c in aqueous phase), we can never envisage such super-efficient collisions of
376 bulky proteins. If we factor in the lower mobility of bulky proteins stationed in the plasma
377 membranes and/or cytoplasm, the rates afforded by protein-protein complexation mediated
378 ET would fall short of the observed rates by five to seven orders of magnitude! On the
379 contrary, if we consider that the reaction occurs between diffusible radical species and Cyt. *c*,
380 then the higher concentration of the much faster species at an instant would result in a lower
381 second order rate calculation or more facile collision frequencies. We can now see that it is
382 improbable that collisions between proteins occurring in lipid phase (a low energy
383 environment) could ever account for the electron transfer phenomenon under study. There is

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384 little reason to argue that CPR would give electrons only to another protein [11]. Also, only the
385 presence and obligatory involvement of significant amounts of highly active diffusible
386 species alone can explain how low amounts of redox-active proteins (like cytochromes *c* and
387 b_5) can significantly modulate ET rates (between proteins) and impact oxidation efficiency of
388 several small molecules (in reactions mediated by heme proteins). At the acidic pH ranges we
389 studied, HRP-Gua reactions were inhibited with sub-nM levels of Cyt. *c* (Figure 5A), when
390 HRP was taken at 1 nM concentration. Two bulky proteins at nanomolar levels cannot collide
391 with enough frequency to bring about an alteration of electron transfer rates in the timescales
392 carried out in our experiment. Cyt. *c* is an animal protein and HRP is a plant protein and there
393 is no rhyme or reason for any affinity binding between the two either. Even if there was a 1:1
394 or 1:2 tight binding under these conditions (assuming the most improbable scenario!), it
395 should only knock out 1 - 2 % of activity, and cannot give such high inhibition. Therefore,
396 the shunting of electrons between HRP-Cyt. *c* must occur via diffusible species. As a logical
397 consequence, the one-electron transfers must occur via the route - CPR to diffusible species
398 to Cyt. *c*. This deduction is confirmed by the fact that Cyt. *c* inhibited HRP mediated electron
399 abstractions from guaiacol (Figure 5A) in a rather maverick concentration-dependent fashion,
400 quite akin to Vit. C or Trolox inhibiting ET in the CPR-Cyt. *c* couple (Figure 2B and 2C).
401 This showed that the ET phenomenology in such systems is generic and not specific. This
402 electron relay is quite similar to the well-established processes in aqueous electrochemistry
403 wherein ions relay the charges under an applied potential or when two half-cells of varying
404 redox potentials are connected via a salt-bridge. In the "biological" relay studied herein, there
405 is a lot of room for uncoupling too (to form water), which quenches the "transfer" process.
406 This is why the receiving element must be located close by to the donating element, which is
407 effectively achieved by having them co-immobilized on phospholipid membranes or by
408 solubilizing them homogeneously in the aqueous phase.

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409

410 The aesthetic argument- "Life could not have evolved with biological ET based on diffusible
411 species (via a radical or one-electron process), because such a process would be chaotic."
412 does not hold merit. Life emerged from chaos and therefore, its fundamental signature is
413 written at the basis of life sustaining process of energy transduction (through ET). In
414 mitochondrial membranes, ubiquinone (a soluble small molecule) is known to play
415 indispensable role as a two-electron shuttling agent. Therefore, it is not obligatory that one-
416 electron transfer in cells is exclusively mediated via a protein-protein transaction. It has
417 already been demonstrated that enzymatic redox reactions mediated by diffusible species
418 could be highly selective and reproducible [9]. We have shown that both theoretical
419 considerations and experimental findings clearly point out that the erstwhile aesthetic
420 considerations are misplaced here. Though the diffusible species mediate electron transfer,
421 they do it in an efficient and highly reproducible manner (the impeccably small standard
422 deviations in the rate calculations are a testimony to the same). It is proven beyond
423 reasonable doubt that ET phenomena in CPR-Cyt. *c* (Cyt. b_5) systems is mediated through a
424 relay of small molecules/ions/radicals. The projections we had made earlier that DROS
425 serves as the electron transfer agent between diverse CYPs and CPR is hereby ratified [11-13, 17].
426 Therefore, this work lends solid support to the '*murburn*' hypothesis [27] proposed to explain
427 CYP+CPR mediated metabolism of xenobiotics in liver microsomes. Also, we can now
428 understand how Cyt. b_5 can serve as a "transient buffer" of one-electron equivalents in a
429 CYP-CPR reaction system. The findings reported herein serve to explain the promiscuity of
430 redox enzymes like CPR and further explicate the physiological toxicity of low amounts of
431 agents like cyanide (species that are lipid soluble and have high redox potentials / mobility;
432 [18]). The findings usher in a new paradigm in cellular redox biochemistry. Now, the diffusible
433 radicals and ROS species cannot be seen merely as redox signaling molecules or

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434 manifestation of patho-physiological states alone. They are now understood as an obligatory
435 requirement of routine oxidative metabolism in such membrane systems involving
436 heme/flavin proteins.

437

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442

443 **References**

444

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497 # Tables (accompanied by respective legends)

498

499

500 Table 1:

		DLPC									
		(0 µg/ml)		(10 µg/ml)		(40 µg/ml)		(100 µg/ml)		(400 µg/ml)	
No.	**Cyt. *c*→** **Cyt. b_5↓**	*2 µM*	**20 µM**	*2 µM*	**20 µM**	*2 µM*	**20 µM**	*2 µM*	**20 µM**	*2 µM*	**20 µM**
1	**0 nM**	*8.7* *±0.3*	**18.0** **±0.3**	*7.1* *±0.3*	**17.0** **±0.2**	*6.6* *±0.2*	**15.6** **±0.3**	*4.7* *±0.2*	**12.5** **±0.4**	*4.5* *±0.2*	**10.2** **±0.1**
2	**5 nM**	*9.6* *±0.2*	**20.7** **±0.3**	*7.7* *±0.1*	**19.0** **±0.4**	*7.4* *±0.2*	**16.6** **±0.5**	*5.5* *±0.3*	**15.2** **±0.2**	*5.7* *±0.2*	**10.9** **±0.2**
3	**20 nM**	*7.3* *±0.3*	**17.6** **±0.4**	*6.6* *±0.1*	**17.4** **±0.2**	*7.0* *±0.1*	**18.0** **±0.4**	*5.9* *±0.1*	**14.3** **±0.3**	*5.1* *±0.1*	**11.2** **±0.2**
4	**40 nM**	*6.7* *±0.1*	**17.5** **±0.3**	*5.8* *±0.1*	**16.6** **±0.4**	*5.8* *±0.2*	**14.5** **±0.3**	*5.8* *±0.2*	**11.7** **±0.3**	*5.6* *±0.3*	**9.2** **±0.3**

501

502 **Table 1: Effect of Cyt. b_5 and DLPC on CPR mediated reduction of Cyt. *c*:** The values
503 given are given in the unit nmoles of Cyt. c reduced by nmole of CPR per second. The
504 reaction conditions were: 100 mM potassium phosphate buffer (pH 7.4), [Cyt. *c*] = 2 or 20
505 µM, [NADPH] = 20 µM, [DLPC] = 0, 10, 40, 100 and 400 µg/ml, [Cyt. b_5] = 5, 20 and 40
506 nM, [CPR] = 2 nM. The total reaction volume was 1 ml, temperature ~26 ºC. The upper row
507 values are the rates and the lower row values are the standard deviations.

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512 Table 2:

Setup	Rate (in D/W, s^{-1})	Rate (in buffer, s^{-1})
Positive control	4.98 ± 0.02	12.83 ± 0.11
Test reaction	0.82 ± 0.03	0.79 ± 0.02
Negative control	0.11 ± 0.01	0.1 ± 0.01

Table 2. **Initial rates calculated from CPR-Cyt. *c* separation experiment, as shown in Figure S2b**: The positive control had: 1 nM CPR, 20 µM Cyt. *c*, 20 µM NADPH in D/W or 100 mM potassium phosphate (pH 7.4). The respective negative controls in D/W or buffer lacked CPR. The test reactions (in D/W & buffer) comprised of two physically separated portions- 1 nM CPR was taken in the dialysis tubing (~10 kD cutoff value, from Spectra/Por, integrity verified for its non-permeability to protein) and placed in the free solution reservoir surrounding the tubing, which contained 20 µM Cyt. *c* (at the identical concentration of 20 µM NADPH in both portions). All samples were gently stirred and aliquots were drawn from the reservoir for spectral analysis.

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540
Legends to Figures

541

542 **Fig. 1**. **Elementary kinetics and time course profiles**: In Figures 1A and 1B, the effect of
543 incorporation of cyanide on Cyt. *c* reduction rates (as observable by time profiles) is shown.
544 In Figure 1A, the initial conditions were- 2 nM CPR, 2 μM Cyt. *c* and 20 μM NADPH. The
545 kinetic traces in Figure 1B were acquired with 5 nM CPR, 3.5 μM Cyt. *c* and 900 μM
546 NADPH, incorporating cyanide at different concentrations. In Figure 1C, the conditions
547 were: [CPR] = 2 nM, [Cyt. *c*] = 10 μM, [NADPH] = 50 μM.

548

549 **Fig. 2**. **Effect of diverse additives on the dose response at submicromolar concentration**
550 **ranges**: Rates were calculated for the first 45 seconds. In Figure 2A, the values given for the
551 copper nitrate profile are averaged percentage values (derived from the respective controls) of
552 samples in two setups- with and without 10 μg/ml dilauroyl phosphatidylcholine (DLPC). For
553 all reactions (except glutathione profiling) [CPR] = 2 nM, [Cyt. *c*] = 10 μM, [NADPH] = 50
554 μM & sodium phosphate at 10 mM. For the glutathione containing reactions (and its control),
555 the assays were performed in 100 mM potassium phosphate buffer. The components were-
556 [CPR] = 2 nM, [Cyt. *c*] = 20 μM & [NADPH] = 20 μM. In Figures 2B and 2C, the structure
557 of vitamins and their derivatives are shown. Also, the effects of these molecules on CPR
558 mediated reduction of Cyt. *c* are shown. The reaction contained- [CPR] = 2 nM, [Cyt. *c*] = 20
559 μM, [NADPH] = 20 μM, redox-active vitamins were at 10^{-6} to 10^{-12} M. Under these
560 circumstances, the control rate (without any additive) was 18 ± 0.2 s^{-1}(which is the 100%
561 value depicted by the straight line parallel to X axis).

562

563 **Fig. 3: Influence of vitamins, their derivatives (at supramicromolar concentrations) and**
564 **lipids on reduction of cytochrome *c*:** All reactions were performed at 27±1 ºC with 100

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565 mM potassium phosphate buffer (pH 7.4). The other components were: [Cyt. *c*] = 20 µM,
566 [DLPC] = 10 µg/ml (when present), [NADPH] = 20 µM, redox molecules were at 1, 10, 100
567 and 1000 µM concentrations, [CPR] = 2 nM. The total reaction volume was 1 ml.
568
569 **Fig. 4: Probing the reduction of cytochromes *c* and b_5 with CPR:** The spectra were
570 recorded in 100 mM phosphate buffer of pH 7.4. The concentrations of the reactants were-
571 For [Cyt. *c*] = ~2 µM & CPR = 2 nM; For [Cyt. b_5] = ~4 µM, [CPR] = ~50 nM, For both
572 [NADPH] = 20 µM, after 10 minutes at 26 ± 1 ºC
573
574 **Fig. 5: Incorporation of a redox protein like Cyt. *c* at very low concentrations inhibits**
575 **HRP reactions:** In all reactions, [HRP] = 1 nM, [ABTS]/ [Gua]/ [Pyr]/ [TMPD] = 1 mM and
576 [H_2O_2] = 1 mM. Potassium phosphate buffer (of required pH) was used at 100 mM. **A.**
577 Inhibitions sponsored by Cyt. *c* are shown for HRP mediated peroxidations of different
578 substrates. In the controls, Cyt. *c* does not give any affect on a mixture of peroxide+substrate
579 in the corresponding reaction regimes. **B.** The effects of changing the substrate molecules for
580 specific enzyme-additive combinations were explored (at pH 5) with four different substrates,
581 with the same Cyt. *c* concentration of 10 nM. Substrate-dependent effects can be seen once
582 again. % Activity denotes activity with respect to control, which forms the zero value
583 baseline, or the X axis.
584

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Figure 1:

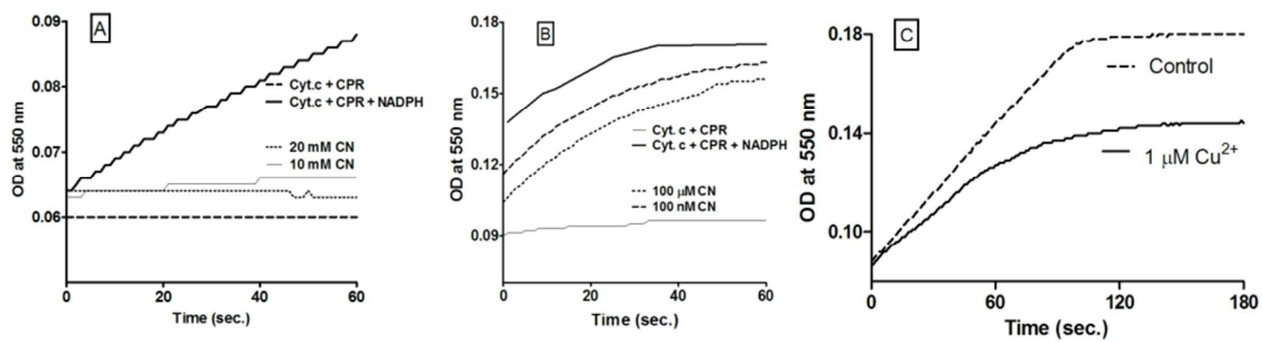

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Figure 2:

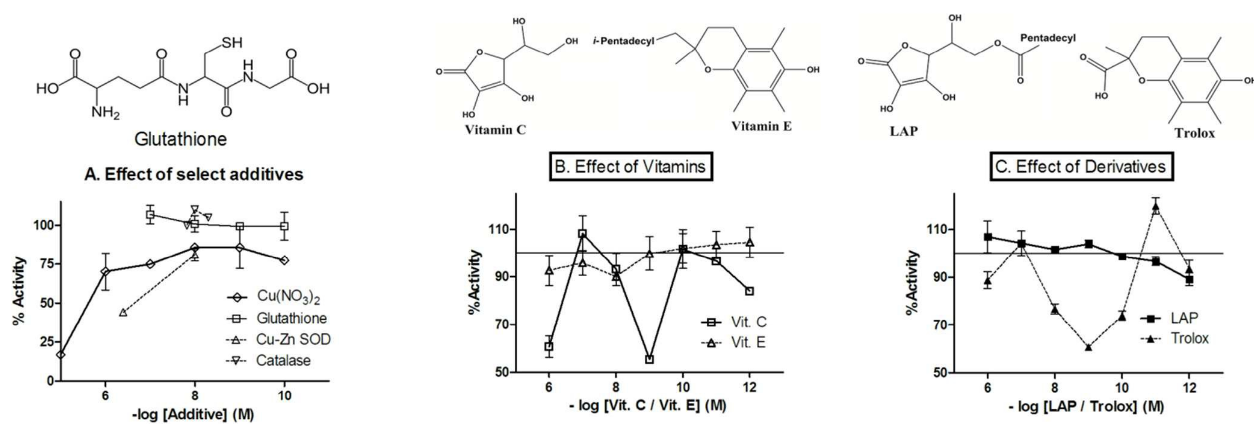

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Figure 3:

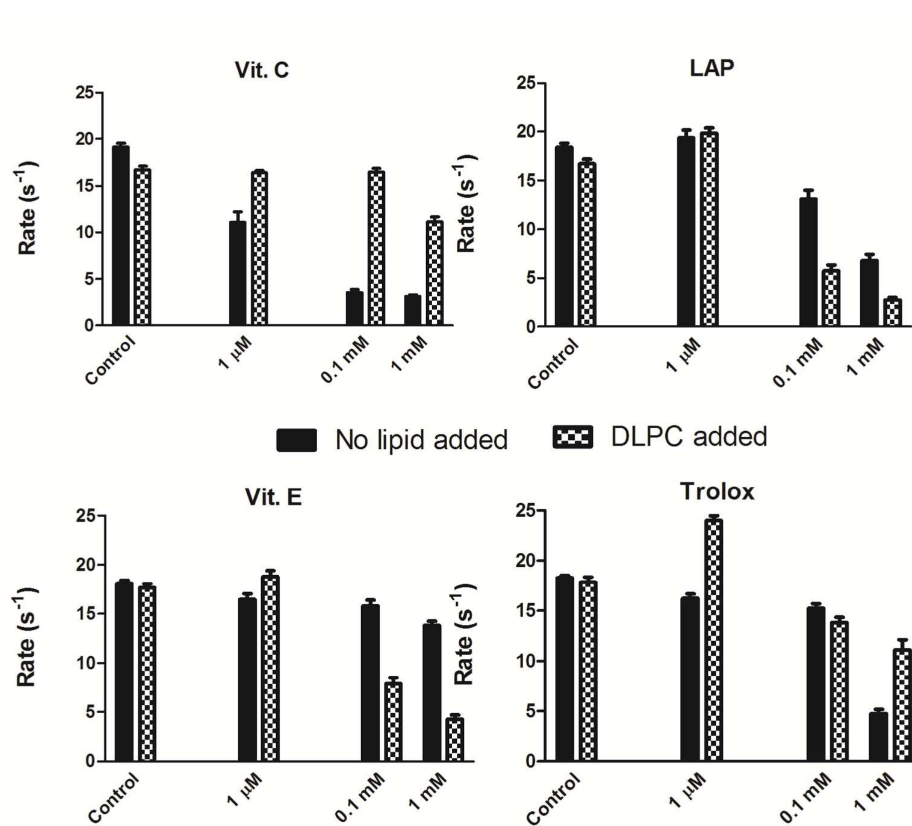

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Figure 4:

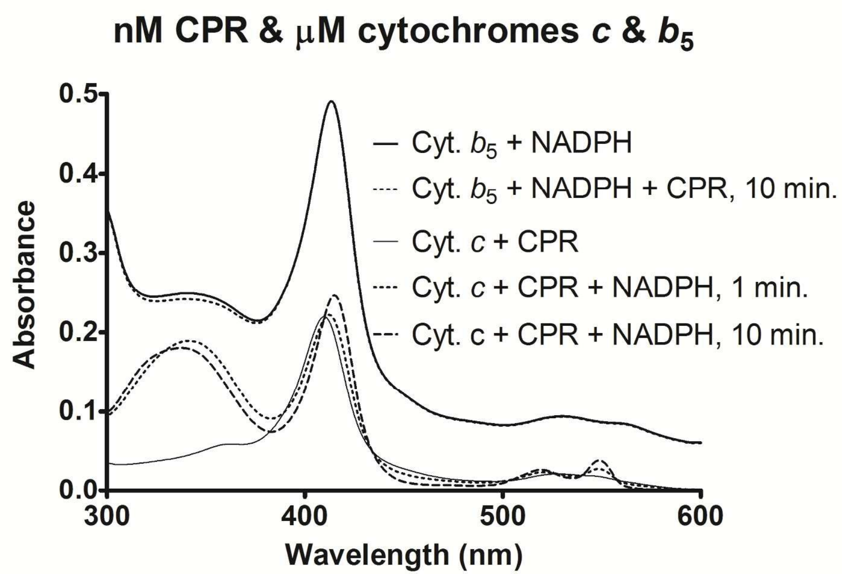

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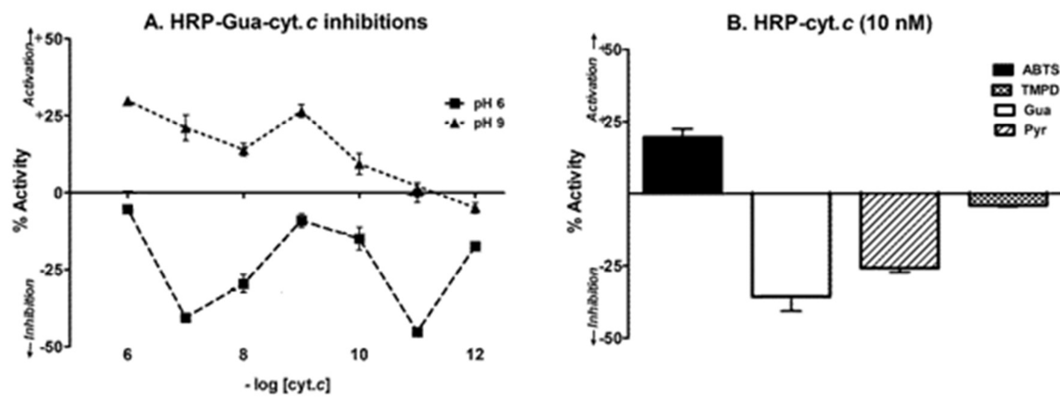
Figure 5:



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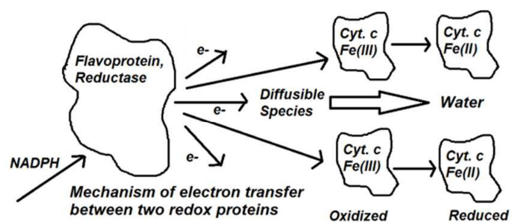
Manoj et al.: TOC graphic and statement

Reductase reduces cytochrome *c* via relays of highly mobile diffusible agents; not by direct binding and inter-protein long-distance electron tunnelling.



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