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CATALYTIC ACTIVITY OF CYTOCHROMES *c* AND c_1 IN MITOCHONDRIA AND SUBMITOCHONDRIAL PARTICLES

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SUMMARY

1. Beef heart mitochondria have a cytochrome $c_1 : c : aa_3$ ratio of 0.65 : 1.0 : 1.0 as isolated; Keilin-Hartree submitochondrial particles have a ratio of 0.65 : 0.4 : 1.0. More than 50 % of the submitochondrial particle membrane is in the 'inverted' configuration, shielding the catalytically active cytochrome *c*. The 'endogenous' cytochrome *c* of particles turns over at a maximal rate between 450 and 550 s^{-1} during the oxidation of succinate or ascorbate plus TMPD; the maximal turnover rate for cytochrome *c* in mitochondria is 300–400 s^{-1}, at 28°–30°C, pH 7.4.

2. Ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylene diamine added to antimycin-treated particles induces anomalous absorption increases between 555 and 565 nm during the aerobic steady state, which disappear upon anaerobiosis; succinate addition abolishes this cycle and permits the partial resolution of cytochrome c_1 and cytochrome *c* steady states at 552.5–547 nm and 550–556.5 nm, respectively.

3. Cytochrome c_1 is rather more reduced than cytochrome *c* during the oxidation of succinate and of ascorbate+*N,N,N',N'*-tetramethyl-*p*-phenylene diamine in both mitochondria and submitochondrial particles; a near equilibrium condition exists between cytochromes c_1 and *c* in the aerobic steady state, with a rate constant for the $c_1 \rightarrow c$ reduction step greater than 10^3 s^{-1}.

4. The greater apparent response of the c/aa_3 electron transfer step to salts, the hyperbolic inhibition of succinate oxidation by azide and cyanide, and the kinetic behaviour of the succinate-cytochrome *c* reductase system, are all explicable in terms of a near-equilibrium condition prevailing at the c_1/c step. Endogenous cytochrome *c* of mitochondria and submitochondrial particles is apparently largely bound to cytochrome aa_3 units in situ. Cytochrome c_1 can either reduce the cytochrome *c*-cytochrome aa_3 complex directly, or requires only a small extra amount of cytochrome *c* to carry the full electron transfer flux.

Abbreviations: TMPD, *N,N,N',N'* tetramethyl-*p*-phenylene diamine; FCCP, trifluoromethoxycarbonylcyanide phenyl hydrazone. EGTA, ethyleneglycol-*bis* (β-amino ethyl ether) *N,N'* tetraacetic acid.

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INTRODUCTION

The membrane location, and the quantitative catalytic activity, of cytochrome *c* in mitochondria and endogenous cytochrome *c* in submitochondrial particles, are not precisely known. I have suggested (see ref. 1) that mitochondrial cytochrome *c* is largely bound to cytochrome aa_3 and that the reaction between cytochromes c_1 and *c* is rather rapid, perhaps involving a direct oxidation of c_1 by the *c*-aa_3 complex, as originally proposed by Orii et al. [2]. Alternative models have been put forward [3, 4] in which the oxidation of cytochrome *c* by the aa_3 system does not involve a defined complex, and in which the oxidation of *c* by *a* is faster than the oxidation of c_1 by *c*.

The creation of the cytochrome oxidase vesicle, or proteoliposome [5, 6], both with external cytochrome *c* and internal cytochrome *c* as cofactor [7], a practical consequence of the theoretical advances made by Mitchell [8] and Bangham [9], demands a reexamination of the behaviour of cytochrome *c*, and the membrane components with which it interacts, in both mitochondria and submitochondrial particles. Is the catalytic action of cytochrome *c* in the proteoliposome kinetically similar to that in its natural membrane milieu?

In the mitochondrial membrane the response of cytochrome *c* to ascorbate plus *NNN'N'*- tetramethyl-*p*-phenylene diamine (TMPD) can be compared with the response to a substrate such as succinate. The kinetics and spectroscopy of the TMPD-*c* interaction have been outlined by Mustafa and his coworkers [10, 11], and reexamined with the soluble oxidase by Kimelberg and Nicholls [12]. To obtain tentative values for the kinetic constants involved, the present experiments extend these studies and attempt (a) to allow for, or eliminate, the kinetic and spectroscopic anomalies that sometimes occur in the presence of TMPD [13], and (b) to distinguish the behaviour of cytochromes c_1 and *c* by appropriate spectroscopic criteria (cf. ref. 14, 15).

A preliminary version of this paper was communicated at the International Symposium on Electron Transfer Chains and Oxidative Phosphorylation, Fasano (Bari, 1975) [16].

MATERIALS AND METHODS

Keilin-Hartree type submitochondrial particles from beef heart were prepared as described previously [17]. Cytochrome *c*-reconstituted preparations were prepared by incubating normal particles ($10\ mg \cdot ml^{-1}$) with high concentrations (0.5–1.0 mM) of horse heart cytochrome *c* at a low ionic strength (10 mM phosphate or Tris, pH 7.3–7.6), followed by a brief (12×5 s bursts) sonication, setting 1.0 (Branson Probe sonifier B-12). Uncombined (exogenous) cytochrome *c* was then removed by dilution five-fold with 0.1 M phosphate pH 7.4, centrifugation at $10^5 \times g$ for 30 min followed by two washings with 0.1 M phosphate. Particles treated in a similar way without sonication accumulated almost as much extra cytochrome *c*, but retention was erratic.

Rat liver mitochondria were prepared according to Myers and Slater [18], with some media according to Chappell and Hansford [19]. Cytochrome *c*-deficient rat liver mitochondria were made using the method of Jacobs and Sanadi [20]. Rat

heart and beef heart mitochondria were made according to Chappell and Hansford [19] or to Tyler and Gonze [21]. Digitonin particles were prepared from beef heart mitochondria by the procedure of Elliott and Haas [22].

Cytochrome aa_3 was isolated from beef heart following the modified deoxycholate method of van Buuren [23]. Cytochrome *c* was Sigma Type VI horse heart, used without further treatment.

Deoxycholate particles were obtained by treating Keilin-Hartree particles (approx. 10 mg · ml^{-1} protein) with 1 % deoxycholate solutions [24] immediately before use. Such deoxycholate particles were diluted out for oxygen electrode assay at least 80-fold, giving a residual deoxycholate concentration $\cong$ 0.0125 %.

Potassium ascorbate solutions (1 M) were prepared by neutralization of L-ascorbic acid (Merck, G. F. R.) and stored frozen. TMPD solutions were made from *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (BDH Chemicals) immediately before use. Potassium succinate solutions (1 M) were prepared by neutralization of succinic acid (Merck, G. F. R.). Rotenone was from BDH Chemicals, antimycin solutions were made up in ethanol from Sigma Co. antimycin. Trifluoromethoxycarbonylcyanide phenyl hydrazone (FCCP) was the gift of Dr. P. G. Heytler, Du Pont Co. Cholic acid (sodium salt) and deoxycholic acid (sodium salt) were from Merck, EDTA and ethyleneglycol-*bis*(β-amino ethyl ether) *N,N'*-tetra-acetic acid (EGTA) from Sigma, Tween-80 from Bie and Berntsen A/S, Copenhagen.

Spectrophotometry was carried out (standard 1 cm, 3 ml cells) with Perkin-Elmer Hitachi 356 (dual wavelength) or Cary 118C (split beam-scanned spectra) instruments at temperatures from 27–32 °C. Oxygen electrode measurements used a Radiometer Clark type electrode with teflon membrane coupled to a Keighley amplifier and Servoscribe recorder. The total reaction volume was 3.8 ml in a 10 ml beaker held at 30 °C.

Cytochrome aa_3 concentration is determined by ΔEmM (red-ox) 605–630 nm = 27; cytochromes $c+c_1$ by ΔE_{mM} (red-ox), 551–540 nm = 20; and cytochromes *c* and c_1 separately (see caveats in text) by ΔE_{mM} (red-ox), 550–556.5 nm = 19.7 and 552.5–547 nm = 15.6, respectively. Protein concentrations were obtained according to Gornall et al. [25].

RESULTS

Table I lists the calculated cytochrome *c* and c_1 concentrations in the four beef heart preparations obtained as given above. The only significant variation is in measured cytochrome *c* concentration. In agreement with Vanneste [15], intact beef heart mitochondria have a $c : aa_3$ ratio (where 1 aa_3 = 2 haem *a*) close to 1.0. Digitonin particles, which have a membrane orientation similar to that in intact mitochondria [26, 27], show a cytochrome *c* content not much less than the parent mitochondria. Keilin-Hartree particles, with a partially inverted configuration [24], retain about 0.4 equivalents of cytochrome *c* per mole cytochrome aa_3. This cytochrome *c* cannot be removed by salt washing, and may be associated with 'inaccessible' (inward-facing) cytochrome oxidase units. Thus maximal turnover of intact particles with ascorbate and added cytochrome *c* is 180 s^{-1} (electrons/s per aa_3), 40 % of the value (450 s^{-1}) for deoxycholate-treated particles. Succinate oxidase activity without added cytochrome *c* is about 60 % of that with exogenous cytochrome *c*

TABLE I

CONCENTRATIONS OF CYTOCHROMES *c* AND c_1 RELATIVE TO CYTOCHROME aa_3 IN PREPARATIONS OF MITOCHONDRIA AND SUBMITOCHONDRIAL PARTICLES

Preparation	Concentration (μmol/μmol aa_3)***		
	c	c_1	$c+c_1$
1. Beef heart mitochondria*	1.05	0.6	1.6
2. Beef heart digitonin particles*	0.65–0.80	0.6	1.25
3. Beef heart (Keilin-Hartree) particles*	0.3 –0.4	0.65	1.1
4. Beef heart (cytochrome *c*-repleted) particles*	0.6 –1.5	0.65	1.2–2.2
5. Rat heart mitochondria*	0.85	0.45(?)	1.30
6. Rat liver mitochondria**	0.85–1.5	0.65–1.0	$\approx$ 2.0

* Approx. 0.5 μmol cytochrome aa_3/g protein.
** Approx. 0.2 μmol cytochrome aa_3/g protein.
*** By measurements at 550–556.5 nm (cytochrome *c*), 552.5–547 nm (cytochrome c_1) and 551–540 nm (cytochromes $c+c_1$) – see text and methods.

present. Evidently about 40 % of the electron transfer chains have the mitochondrial configuration and 60 % the inverted configuration. The latter have 2/3 of a full complement of cytochrome *c* (sufficient for full succinate oxidase activity).

Reconstitution by sonication with excess cytochrome *c* (Methods) can give particles with $c : aa_3$ ratios between 1.5 and 2.0, as found in other mitochondria, including rat liver [14] and yeast [28] preparations. Calculation based upon the spectra of isolated cytochromes *c* [29] and c_1 [30] shows that in mixtures, cytochrome c_1 may be measured at 552.5–547 nm where there is little contribution from cytochrome *c*, while the latter may be measured at 550–556.5 nm, where there is little contribution from cytochrome c_1. The extinction coefficients cited (Methods) give the same total concentration of cytochromes *c* plus c_1 as is obtained using the conventional ΔE_{mM} of 20 at 551–540 nm. Additions of cytochrome *c* give no changes at 552.5–547 nm but proportionate changes at 550–556.5 nm.

When cytochrome *b* is reduced, estimates of cytochrome *c* concentration using the 550–556.5 nm pair become more difficult. Approximately 1/3 of the 562–575 nm absorbance must be added to the 550–556.5 nm value to obtain the true cytochrome *c* contribution. In succinate steady state experiments, only the 551–540 and 552.5–547 nm values are therefore used.

Fig. 1 compares the responses of submitochondrial particles on the addition of succinate or ascorbate plus TMPD as substrate. As previously observed by King [13], the TMPD response (Fig. 1A), even in the presence of antimycin, does not represent a simple aerobic/anaerobic transition like the succinate response (Fig. 1C). But if succinate is added after antimycin, the ascorbate-TMPD system behaves in an 'orthodox' manner (Fig. 1B). The spectroscopic responses at a number of wavelengths (Fig. 1, D and E) show that in the presence of antimycin but absence of succinate a broad absorption peak centred at about 563 nm develops during the steady state (Fig. 1D, (a)) and disappears slowly at anaerobiosis (cf. (b) and (c)). When nearly all the cytochrome *b* is reduced with succinate (Fig. 1E, (d)), these anomalous effects

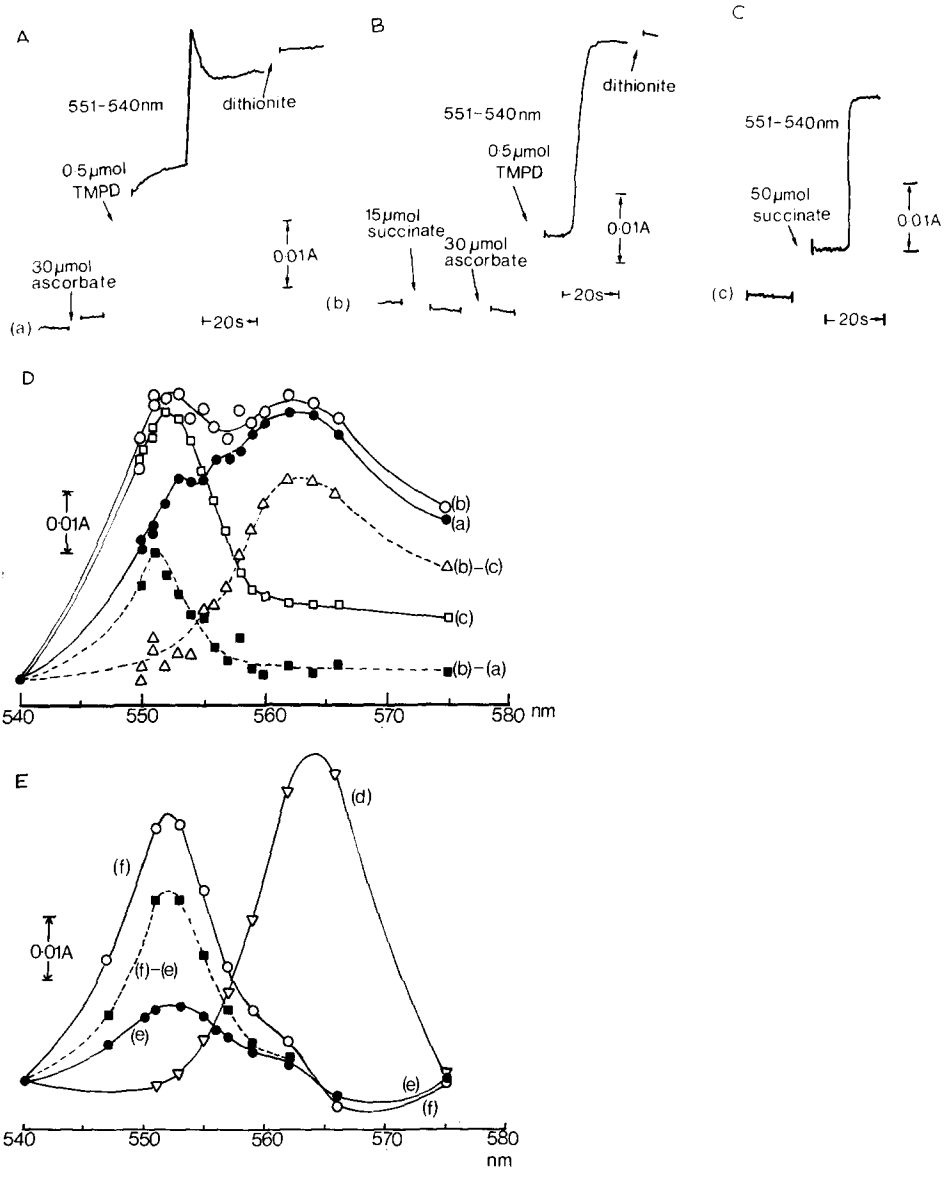

Fig. 1. Reduction of cytochromes *c* and c_1 by ascorbate plus TMPD and by succinate. (A) Steady state changes induced by 10 mM ascorbate, 0.2 mM TMPD in the presence of 2 μg/ml antimycin. Submitochondrial particles (2.4 μM aa_3 or 5 mg protein/ml) in 225 mM mannitol, 75 mM sucrose, 10 mM potassium morpholinopropane sulphonate, 1 mM EDTA, pH 7.4, 27 °C. (B) Steady state changes as in (A), but with preaddition of 5.7 mM succinate. (C) Steady state changes with 11 mM succinate alone. Submitochondrial particles (1.5 μM aa_3 or 3 mg protein/ml) in 225 mM mannitol, 75 mM sucrose, 4 mM Tris · Cl, pH 7.4, 27 °C. (D) Difference spectra of components reduced in (A), in the absence of succinate: (a) final steady state spectrum; (b) initial spectrum on anaerobiosis; (c) final anaerobic spectrum. (E) Difference spectra of components reduced in (B), in the presence of succinate: (d) species reduced by succinate; (e) steady state induced by ascorbate and TMPD; (f) anaerobic spectrum (less succinate-induced reduction). Dual wavelength measurements.

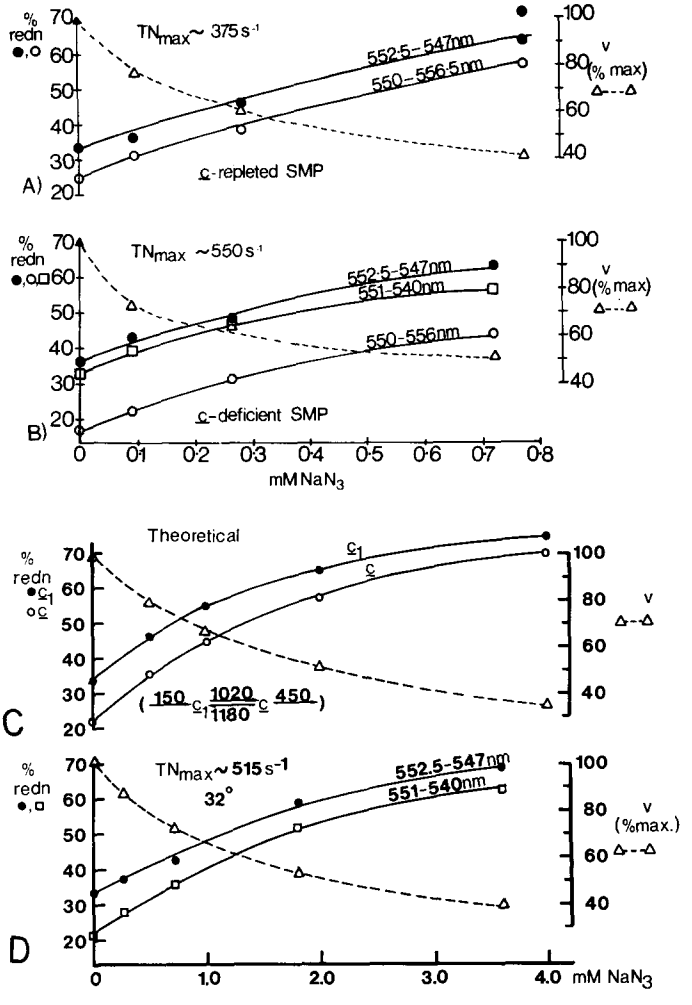

Fig. 2. Effect of azide on catalytic activity and steady states of cytochromes c and c_1. (A) Cytochrome c-repleted particles containing 0.8 μM cytochrome c_1, and 1.4 μM bound cytochrome c, with 10 mM ascorbate, 0.2 mM TMPD as substrates. (B) Normal particles containing 0.8 μM cytochrome c_1 and 0.7 μM endogenous cytochrome c, with 10 mM ascorbate, 0.2 mM TMPD as substrates. (C) Theoretical plot for bracketed system, according to Eqn. 5 (text). (D) Experimental data for partially cytochrome c-repleted particles (approximately equal amounts of c and c_1) ,with 11 mM succinate as substrate. 225 mM mannitol, 75 mM sucrose, 4 mM Tris medium pH 7.5 at 30 °C. ●–●, steady state at 552.5–547 nm (cytochrome c_1); □–□, steady state at 551–540 nm (cytochromes $c+c_1$); ○–○, steady state at 550–556.5 nm (cytochrome c); △–△, respiration rate (as % maximal).

are negligible and the changes seen (cf. (e) and (f)) reflect only reduction of cytochromes c and c_1.

Fig. 1, D and E suggest that the 550–553 nm peak in the initial steady state is red-shifted compared to the anaerobic peak. Cytochrome c_1 (α-peak at 553 nm) is probably more reduced in the steady state than cytochrome c (α-peak at 550 nm); conversely, more cytochrome c is involved in the subsequent anaerobic transition

TABLE II

STEADY STATE REDUCTIONS AND MAXIMUM TURNOVER NUMBERS FOR CYTOCHROME *c* IN MITOCHONDRIA AND SUBMITOCHONDRIAL PARTICLES

System	Steady state % reduction[a]		TN (s^{-1})[b]		TN_{max} (s^{-1})[c]	
	ascorbate −TMPD[d]	succinate[d]	ascorbate +TMPD	succinate	ascorbate +TMPD	succinate
1. Beef heart mitochondria	21	(15)[a]	48	53	230	350
2. Beef heart particles	26	(18)	101	75	390	415
3. Rat heart mitochondria	(24)	(8)	60	14	250	175
4. Rat liver mitochondria	(18)	(15)	51	44	285	295

[a] Cytochrome *c* reduction measured at 550–556.5 nm or (bracketed figures) estimated from cytochrome c_1 reduction at 552.5–547 nm and total ($c+c_1$) reduction at 551–540 nm (see text).
[b] μequivalent electrons · s · μmol cytochrome *c*.
[c] TN × 100/(% reduction of cytochrome *c*), see text.
[d] 10 mM ascorbate+0.2 mM TMPD or 11 mM succinate. 27 °C, 0.225 M mannitol, 0.075 M sucrose and 4 mM Tris medium, pH 7.4. 5 mM phosphate, 0.6 μM FCCP and 0.4 μM rotenone added in expts. 1, 3 and 4. Mitochondrial concentration approx. 2 mg · ml^{-1} protein.

(Fig. 1D, (b)-(a), and E (f)-(e)). The reduction levels are a function of the relative rates of oxidation and reduction. If the oxidation rate is progressively slowed by addition of the terminal inhibitor azide the steady states can be plotted as a function of inhibitor concentration (Fig. 2). Both with normal particles (2B), and with cytochrome *c*-repleted systems (2A) the steady state reduction of cytochrome c_1 is higher than that of cytochrome *c*. An intermediate response is observed at 551–540 nm (Fig. 2B).

For a physiological substrate such as succinate, all the electrons passing to oxygen must traverse the c_1/c couple. Fig. 2C shows the calculated flux and steady state changes for a system involving a dissociation constant (pH 7.6) for azide and cytochrome *c* oxidase of 0.5 mM and the indicated velocity constants for cytochrome c_1 reduction, cytochrome *c* oxidation, and cytochrome c_1/cytochrome *c* interaction. The resulting curves are of the same general form as those obtained experimentally (2D).

How are turnover numbers, such as those used to construct Fig. 2C, determined? Table II lists the maximal turnover observed for cytochrome *c* oxidation during the ascorbate+TMPD or succinate-induced steady states, according to the equation:

$$TN_{\substack{max \\ (s^{-1})}} = 4v/[c^{2+}]_{\text{steady state}} \qquad (1)$$

where *v* is in mol $O_2 \cdot l^{-1} \cdot s^{-1}$ and $[c^{2+}]$ steady state (mol · l^{-1}) equals total [cytochrome *c*] · 100/(% steady state reduction). The higher values seen are close to the maximal activity of cytochrome aa_3 in submitochondrial particles treated

with deoxycholate. At pH 7.4, 30° C, and infinite cytochrome *c* concentration, cytochrome aa_3 turns over at 450 s^{-1}.

The rate of cytochrome c_1 reduction (150 s^{-1}) is derived from the overall rate of the succinate oxidase system (75–100 s^{-1} with respect to cytochrome aa_3) divided by the steady-state concentration of oxidized cytochrome c_1.

The rates of cytochrome c_1 and cytochrome *c* oxidoreduction are obtained by a rather more speculative procedure [16]. From data such as those in Fig. 2 one may calculate a dimensionless constant 'Γ' such that:

$$\Gamma = [c_1^{2+}]_{ss}[c^{3+}]_{ss}/[c_1^{3+}]_{ss}[c^{2+}]_{ss} \tag{2}$$

where $[x]_{ss}$ indicates the concentration of the given component in the steady state. When the net flux is zero, $\Gamma = K_{eq}$. If all the reducing equivalents go via this route, we have:

$$\frac{v[c_1]}{[c_1^{3+}]_{ss}[c^{2+}]_{ss}} = k_3\Gamma - k_{-3} = k' \tag{3}$$

where k' is the apparent rate constant, k_3 and k_{-3} are the true forward and reverse rate constants and $k_{-3}/k_3 = K_{eq}$. Table III lists the values of Γ and k' obtained from Fig. 2D. These values are consistent with $k_3 = 1000\ s^{-1}$ and $k_{-3} = 1250\ s^{-1}$.

TABLE III

VALUES OF Γ AND k' AT DIFFERENT RATES OF ELECTRON TRANSFER TO OXYGEN

Conditions other than table footnotes as in legend to Fig. 2. Note: $\Gamma \to 1.25$ as $k' \to 0$. Γ is proportional to k' with a slope of 1000 s^{-1} in both (a) and (b).

(a)* Steady states in presence of succinate (Fig. 2D)

Azide (mM)	Flux (s^{-1}) (v)	% reduction		$([c_1^{2+}]\,[c^{3+}])/([c_1^{3+}]\,[c^{2+}])$ (Γ)	$v[c_1]/([c_1^{3+}]\,[c^{2+}])$ (k')
		cytochrome c_1	cytochrome *c*		
0	102	33	20	1.97	761
0.27	88	36	24	1.78	573
0.72	72	45	33	1.66	397
1.8	58	58	47	1.56	294
3.2	40	68	59	1.48	212

(b)** Steady states in presence of ascorbate+TMPD (Fig. 2A)

Azide (mM)	Flux (v s^{-1})	% reduction		$([c_1^{2+}]\,[c^{3+}])/([c_1^{3+}]\,[c^{2+}])$ (Γ)	$v([c_1]+[c])/([c_1^{3+}]+[c^{3+}])\,[c^{2+}]$ (k')
		cytochrome c_1	cytochrome *c*		
0	94	34	25	1.55	271
0.096	77	36	31	(1.25)	168
0.28	63	46	39	1.33	95
0.78	41	63	57	1.28	30

* (a) 0.8 μM cytochrome c_1, 0.8 μM cytochrome *c*

** (b) 0.75 μM cytochrome c_1, 1.4 μM cytochrome *c*

When ascorbate+TMPD is the electron donor system, Γ would always equal K_{eq} if TMPD reduced only endogenous cytochrome *c*. The figures given in Table III show that this assumption is probably false. A slightly less simplified model assumes that TMPD reduces both cytochromes c_1 and *c* at similar rates. We then have [16] Eqn. 4 in place of Eqn. 3:

$$\frac{v([c]+[c_1])}{([c_1^{3+}]+[c^{3+}])_{ss}[c^{2+}]_{ss}} = k_c\Gamma - k_{-c} = k' \qquad (4)$$

Table III also lists the values of Γ and k' for the ascorbate-TMPD system. Eqn. 4 gives similar values for k_c and k_{-c} to those given by Eqn 3 in the case of the succinate data from Fig. 2A. Much higher Γ values derived from Fig. 2B seem to reflect the existence of reduced cytochrome c_1 species not equilibrating rapidly with cytochrome *c*, and hence more reduced than the remainder. Perhaps the inward-facing c_1 units equilibrate with endogenous *c* but the outward-facing units require added cytochrome *c* before they can behave in the same way.

Fig. 3 shows similar steady state observations with intact but uncoupled beef heart mitochondria. In such mitochondria the spectral "anomalies" (Fig. 1) are not seen, and steady states with ascorbate and TMPD can be obtained both in presence (a, b) and absence (c, d) of antimycin. Cytochrome c_1 is more reduced during the steady state (a, c) and cytochrome *c* is the predominant component reduced upon anaerobiosis (b-a, d-c). As with particles, turnover can also be determined according to Eqn. 1 in the aerobic succinate-reduced steady state. Although the

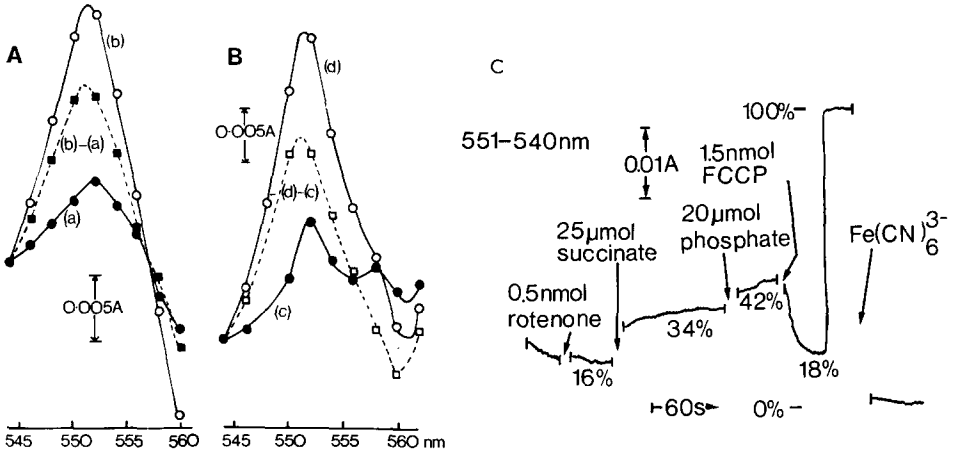

Fig. 3. Steady state changes in intact beef heart mitochondria. (A) Spectra of cytochromes reduced by ascorbate plus TMPD in presence of 0.6 $\mu g \cdot ml^{-1}$ antimycin. Beef heart mitochondria (1.2 μM cytochrome aa_3, 1.0 μM cytochrome *c*, 0.8 μM cytochrome c_1) plus 0.12 μM FCCP, oxidizing 11 mM ascorbate+185 μM TMPD, in 225 mM mannitol, 75 mM sucrose, 10 mM morpholinopropane sulphonate, 1mM EDTA pH 7.4, 27 °C. (B) As in A without antimycin addition. (a), (c) ●–● steady state spectrum; (b), (d) ○–○ anaerobic spectrum; ■–■, □–□ components reduced on transition to anaerobiosis. (C) Steady state reduction of cytochromes *c* and c_1 during succinate oxidation. Beef heart mitochondria (1.5 μM cytochrome aa_3, 1.4 μM cytochrome *c*, 0.95 μM cytochrome c_1), as in A. Measurements made at 551–540 nm following addition of 0.19 μM rotenone, 9 mM succinate, 7 mM phosphate, and 0.6 μM FCCP. Full reduction by dithionite, full oxidation by ferricyanide.

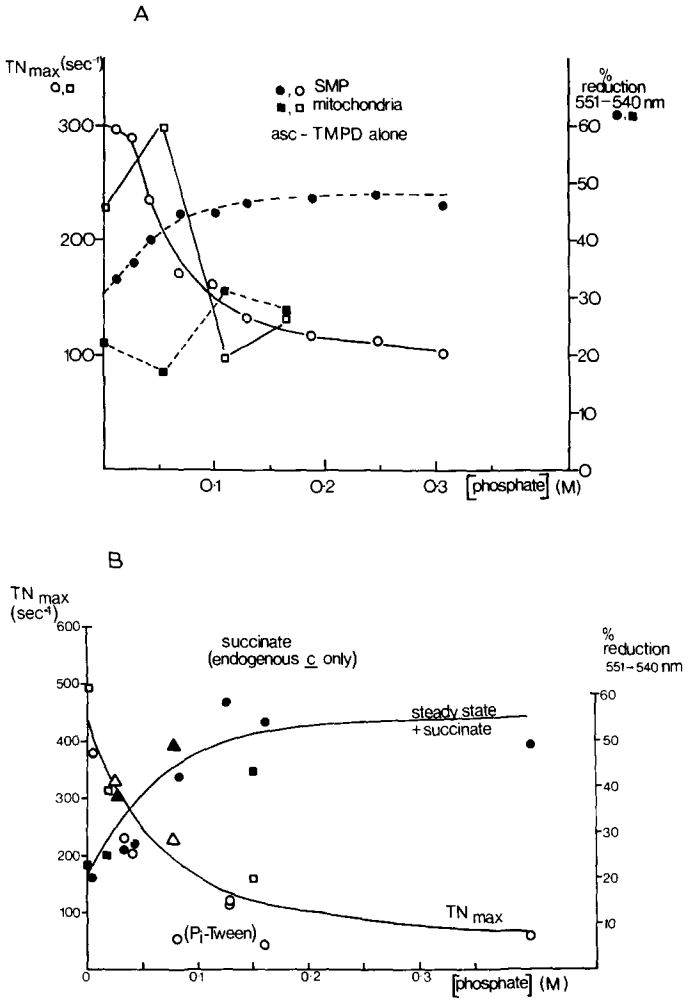

Fig. 4. Cytochrome $c+c_1$ steady states and estimated turnovers during respiration of rat liver mitochondria and of beef heart particles as a function of phosphate concentration. (A) Oxidation of ascorbate plus TMPD. ○–○, maximal turnover of submitochondrial particles (rate of respiration/[cytochrome c^{2+}], Eq. 1 in text); □–□, maximal turnover of mitochondria; ●–●, % reduction at 551–540 nm (particles); ■–■, % reduction at 551–540 nm (mitochondria). Phosphate buffer concentrations as indicated, with isotonicity maintained at 308 mosm up to 0.1 M phosphate level by appropriate additions of 225 mM mannitol, 75 mM sucrose, 4 mM Tris, 1 mM EGTA medium, pH 7.35, 28 °C. Submitochondrial particle data in presence of 7 mM succinate, 11 mM ascorbate, 0.18 mM TMPD, 7 μg/ml antimycin. Mitochondrial data in presence of 0.36 μM rotenone and 0.6 μM FCCP. N.B. Mitochondria in the higher phosphate concentrations are probably swollen but here we are measuring uncoupled rates only. (B) Oxidation of succinate (beef heart submitochondrial particles). ●, ▲, ■, % Reduction at 551–540 nm; ○, △, □, maximal turnover (as in A). Phosphate concentrations at pH 7.2–7.4, 26 °C, as indicated, varying tonicity. 11 mM succinate, 0.8-1.5 μM cytochrome aa_3 (0.15–1.3 μM cytochrome c). Differently shaped symbols identify different experiments.

measurements are facilitated by the higher level of cytochrome *c* (Table I), Fig. 3C shows that accurate steady state values are more difficult to determine. Very different uncoupled steady state estimates are obtained using the rotenone-inhibited state and the ferricyanide-oxidized state as reference levels. Values in Table II are based on the latter.

The maximal turnover of isolated cytochrome aa_3, or of deoxycholate-treated particles, is a function of ionic strength [31]. The optimal phosphate buffer concentration is between 0.05 M and 0.1 M at pH 7.3, 30 °C. As shown by Keilin and Hartree [32], low activities in the 0 to 0.05 M range may be due to poor particle dispersion, overcome by high (non-specific) protein concentrations. However, inhibitory effects of high ionic strength seem to be truly 'molecular' in origin, and are seen in both cytochrome oxidase [31, 33] and succinate oxidase activities [32, 34]. Fig. 4A compares the effects of increasing ionic strength (with osmolarity kept constant at 310 mosm up to 0.1 M phosphate) on ascorbate+TMPD oxidase activity of beef heart particles and rat liver mitochondria. As ionic strength rises, the steady state reduction level at 551–540 nm increases, and the calculated turnover (Eqn. 1) declines sharply. Similar changes are observed at lower protein concentrations using

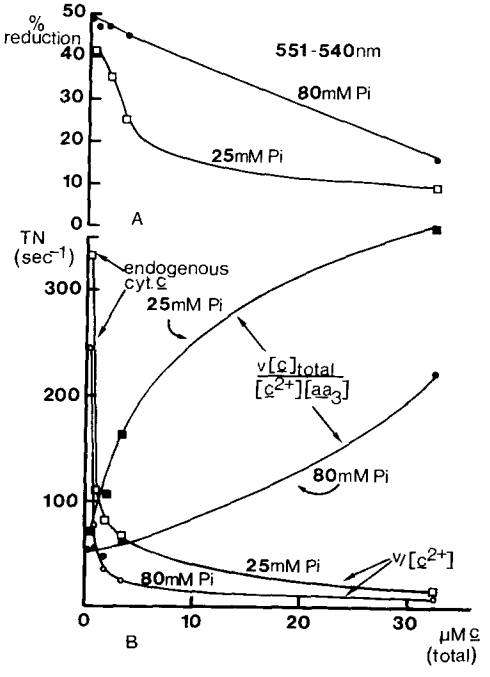

Fig. 5. Cytochrome $c+c_1$ steady states and calculated turnovers as a function of cytochrome *c* concentration. Succinate oxidation by beef heart submitochondrial particles (1.3 μM cytochrome aa_3), in 80 mM phosphate pH 7.4 (closed symbols) or 25 mM phosphate pH 7.2 (open symbols). 11 mM succinate as substrate. (A) % reduction at 551–540 nm: ●–●, 80 mM phosphate; □–□, 25 mM phosphate. (B) estimated turnovers (see text): ● and ○, 80 mM phosphate; ■ and □, 25 mM phosphate. Cytochrome *c* concentration equals total of added (exogenous) and endogenous. Reaction medium contained 0.11 M sucrose, 12.5 mM borate and 2 mM EDTA, plus indicated levels of sodium/potassium phosphate at 27 °C.

the oxygen electrode technique; under such conditions the activating effect of low phosphate levels [32] is seen as well. Fig. 4A indicates that mitochondria (taken to slightly hyperosmotic phosphate concentration levels) show a similar, albeit less marked trend.

Succinate-induced steady states and turnovers are also modified by increasing phosphate concentration, as shown in Fig. 4B. Because the steady state reduction level rises as turnover falls, the decline in maximal turnover is a steeper function of ionic strength than is the overall activity [34]. The maximal turnover in 'non-ionic' media is identical with the turnover of cytochrome aa_3 (400–500 s^{-1}), but at 0.1 M phosphate and above the turnover is less than the activity of isolated oxidase under the same conditions. Addition of exogenous cytochrome *c* completely restores the 'missing' activity, both when measured polarographically and spectrophotometrically. Fig. 5 illustrates the effect of increasing added cytochrome *c* concentration at high and low phosphate concentrations on (i) the steady state reduction level, (ii) the turnover calculated according to $v/[c^{2+}]$ (Eqn. 1), and (iii) the turnover calculated in terms of the product of $v/[c^{2+}]$ and the ratio of total $[c]$ to total $[aa_3]$ present. The latter 'turnover' at high cytochrome *c* concentrations rises to a value similar to that given by Eqn. 1 at the lowest (endogenous) cytochrome *c* concentrations. At the same time the cytochrome *c* steady state reduction falls from the endogenous (40 %–50 %) value to the characteristic exogenous (7–15 %) value, along a curve (Fig. 5A) whose shape is controlled by the K_m for cytochrome *c* (≈ 2.5 μM in 25 mM phosphate, 16 μM at 80 mM phosphate). Freshly prepared particles, at low concentrations (≈ 0.05 μM aa_3) in 67 mM phosphate buffer with excess cytochrome *c* ($\geqslant 30$ μM), show succinate oxidation rates between 60 and 100 electrons $\cdot$ s^{-1} (cytochrome $aa_3)^{-1}$ and steady state reduction levels between 7 and 10 %. The corresponding values of $v[c]/[c^{2+}][aa_3]$ lie between 600 and 1000 s^{-1}, in excess of the maximal oxidase turnover. Under these conditions endogenous cytochrome *c* is probably more reduced than exogenous cytochrome *c* (unlike the possible situation illustrated in Fig. 5B). If 40 % of the chains are outward-facing and 60 % inward-facing, then a turnover of 100 s^{-1} at 10 %(exogenous) reduction will mean that 18 % ($0.4 \cdot 450 \cdot 0.1$) of the flux is carried externally and 82 % ($0.6 \cdot 450 \cdot 0.3$) internally. The suggested greater endogenous (30 %) than exogenous (10 %) cytochrome *c* reduction may reflect a lack of equality in the proportionate 'scrambling' of the reductase and oxidase parts of the membrane (see Discussion).

DISCUSSION

Truly cytochrome *c*-deficient submitochondrial particles, prepared according to Tsou [35], require only a small amount of added cytochrome *c* to develop full succinate oxidase activity [1, 36]. Although the particles described here are only partially *c*-deficient, they show a characteristic response to added cytochrome *c*. This could imply the presence of a mixed population of vesicles, some fully deficient, and others fully active. However, the homogeneous response of all the cytochromes on the transition to anaerobiosis [37] indicates that deficient and cytochrome *c*-containing regions can communicate with each other at a rapid (but catalytically incompetent) rate. The relative sizes of the immediately accessible and deoxycholate-released cytochrome *c* oxidase activities suggest that the cytochrome *c* containing

regions are those with an inverted configuration, and the cytochrome *c* deficient regions those which retain the original mitochondrial configuration, and hence lose cytochrome *c* in ionic media. Single vesicles containing regions of both types could arise as a result of fusion processes during preparation.

The absorption increases in the region of the cytochrome *b* α-peak in antimycin-inhibited submitochondrial particles during the ascorbate plus TMPD-induced steady state, which disappear following anaerobiosis, may be related to the aerobic cytochrome b_T reduction seen by Erecińska et al. [38] in intact mitochondria, and analysed by Berden and Wikström [39] in terms of a coenzyme Q-semiquinone dependent cytochrome *b* reduction. Alternatively a membrane-bound or trapped form of Wurster's blue (TMPD radical), accessible to the succinate dehydrogenase system but not to ascorbate, may be involved. The absence of a similar effect in intact mitochondria (Fig. 3) suggests that either (a) cytochrome *b* accessibility or redox potential may differ, or (b) a different membrane orientation may prevent radical trapping or binding.

The estimated value of $K_{eq} = 1.25$ for the cytochrome c_1-*c* equilibrium (Table III) indicates a redox potential for endogenous cytochrome *c* about 5 mV more negative than that of c_1; this result simply confirms the titration data which show no significant difference between the potentials of c_1 and bound *c* [40, 41]. The rate of succinate oxidation via the c_1/c system obeys the (rather restrictive) triple equation [5]:

$$v = k_4[c_1^{3+}] = k_3[c_1^{2+}][c^{3+}] - k_{-3}[c_1^{3+}][c^{2+}] = k_2[c^{2+}][aa_3] \quad (5)$$

(cf. Figs. 2C and 2D), where k_3 and k_{-3} must both exceed $10^3\ s^{-1}$. The assumption that TMPD reduces cytochromes *c* and c_1 at similar rates (Table III) also gives a reasonable fit for the ascorbate-TMPD data. The rate constant k_5 in Eqn. 6,

$$v = k_5[\text{TMPD}]([c_1^{3+}] + [c^{3+}]) \quad (6)$$

has a calculated value of $4.5 \cdot 10^5\ M^{-1}\ s^{-1}$ at pH 7.4, 30 °C, while the reduction of cytochrome *c* in solution under the same conditions occurs with a rate constant not greater than $5 \cdot 10^4\ M^{-1}\ s^{-1}$ [12].

Near-equilibration between cytochromes c_1 and *c* can explain a number of kinetic features in electron transport. For example, although both the oxidase step [33, 42] and the $c_1 \rightarrow c$ step [43] are ionic strength dependent [1], when the complete succinate oxidase system is examined, an increase in buffer concentration is associated with a marked increase in cytochrome *c* steady state reduction [44]; if the c_1/c step is not rate-limiting, ions affect k_2 (Eqn. 5) more than k_4. Secondly, if succinate oxidase flux is plotted against azide concentration, a normal hyperbolic inhibition curve is obtained, although the K_i is tenfold greater than for the cytochrome *c* oxidase reaction itself [45]. The predicted inhibition curve for a multienzyme system of this kind acting by essentially non-equilibrium steps would be steeper than the hyperbolic form characteristic of a single enzyme unless the component preceding the penultimate component (cytochrome *c*) were largely reduced in the steady state (the 'cushioning' effect). But cytochrome c_1 is not largely reduced in the steady state. The observed hyperbolic inhibition curve must therefore be due to equilibration between c_1 and *c*, and effectively complete reduction of the component lying below cytochrome c_1. Thirdly, equilibration accounts for the cytochrome *c* reductase kinetics [46].

Smith et al. [46] have shown that succinate-cytochrome *c* reductase activity

is characterized by: (a) a very low K_m value for total cytochrome *c*, (b) a zero order phase followed by a first order phase in the time course, and (c) a transition between the two phases dependent only upon the redox state of cytochrome *c*, and not on the total amount of cytochrome *c* present. For the catalytic system of Eqn. 7, the steady state Eqn. 8 applies at cytochrome *c* concentrations above the (proven) low K_m value.

$$AH_2 \xrightarrow{k_4} c_1 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} c \tag{7}$$

$$\frac{[c_1]}{v} = \frac{k_{-3}[c^{2+}]}{k_3 k_4 [c^{3+}]} + \frac{1}{k_4} \tag{8}$$

If the reciprocal of turnover is plotted against the $[c^{2+}]/[c^{3+}]$ ratio, then the intercept on the $[c_1]/v$ axis will represent the rate constant k_4 for reduction of c_1, and the intercept on the $[c^{2+}]/[c^{3+}]$axis will be k_3/k_{-3}, the equilibrium constant for the c_1/c equilibrium. Such plots (cf. [46]) give values between 4 and 6 for k_3/k_{-3} with externally added *c* and cytochrome *c* depleted mitochondria, indicating a redox potential for cytochrome *c* in solution 35 to 45 mV more positive than that of cytochrome c_1. The difference between the apparent potentials of free and bound cytochrome *c* [40] has been discussed previously [1].

The maximal turnover of cytochrome *c* (Eqn. 1) in a number of preparations (Table II) approaches that for isolated cytochrome aa_3 acting on excess cytochrome *c*. Even in rat liver mitochondria, where there is more cytochrome *c* than cytochrome aa_3, rates of up to 300 s^{-1} are found. Chance [47] determined directly a rate of 380 s^{-1} for cytochrome *c* oxidation in such mitochondria at 32 °C. I conclude that at least 80 % of mitochondrial cytochrome *c* is bound to cytochrome aa_3whenever $[c] \leqslant [aa_3]$, and that cytochrome c_1 may reduce the *c*-aa_3 complex directly, as TMPD is capable of doing [12]. If this is true, it predicts considerable movement of complex III in the membrane.

Alternatively, very rapid exchange of cytochrome *c* may be possible across the surface of the membrane. Wohlrab [48] found rapid equilibration between chains at the cytochrome *c* level. If cytochrome *c* forms complexes alternately with cytochrome aa_3 and with cytochrome c_1 [49] no more than 20 % of the cytochrome *c* in beef heart systems can be associated with cytochrome c_1 at any time. The electron transfer rate within the c_1-*c* complex will then be at least 5000 s^{-1}, similar to the oxidation rate of cytochrome *c* by yeast peroxidase [50]. The rate of dissociation of either ferric or ferrous cytochrome *c* from its complex with cytochrome aa_3 is in excess of 2000 s^{-1} [1, 31] and is unlikely to be rate limiting.

The decrease in apparent catalytic activity of endogenous cytochrome *c* at high ionic strengths, accompanied by increasing steady state reduction (Fig. 4) indicates that membrane-linked cytochrome aa_3 in the inverted configuration may have an unusual ionic sensitivity, or that endogenous *c* does indeed dissociate from aa_3 at higher ionic strengths, not into solution (the concentration would rapidly become very high) but to non-specific binding sites of lower salt sensitivity. Even more dramatic effects are seen with cytochrome oxidase-containing liposomes with trapped cytochrome *c* (Wrigglesworth and Nicholls, unpublished), for which the estimated ratio of internal/external activity rises sharply with decreasing buffer concentration.

I conclude that previously measured rate constants for the $c_1 \rightarrow c$ electron transfer step obtained at 555–540 nm [3] were too low, probably due to large contributions from *b*-type cytochromes at this pair of wavelengths. The electron transfer sequence from succinate to oxygen [51] is best fitted by the steps and rate constants given in Eqn. 9:

$$\text{succinate} \xrightarrow[(k_4)]{150\ s^{-1}} c_1 \underset{(k_3 \text{ and } k_{-3})}{\overset{1000\ s^{-1}}{\underset{1250\ s^{-1}}{\rightleftharpoons}}} c \xrightarrow[(k_2)]{450\ s^{-1}} aa_3 \xrightarrow[(k_1)]{10^8} O_2 \qquad (9)$$

Of these constants, k_2 may represent the true rate of intramolecular electron transfer within the *c*-aa_3 complex, but k_3 and k_{-3} are probably much smaller than the intramolecular electron transfer rates for complexes between cytochromes *c* and c_1.

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