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CONTROL OF RESPIRATION IN PROTEOLIPOSOMES CONTAINING CYTOCHROME aa_3

I. STIMULATION BY VALINOMYCIN AND UNCOUPLER

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Summary

1. Both valinomycin and *p*-trifluoromethoxy carbonyl cyanide phenylhydrazone (FCCP) are required for full release of respiration by cytochrome *c* oxidase-containing proteoliposomes (prepared by sonicating beef heart cytochrome aa_3 in salt solution with 4 parts phosphatidylcholine, 4 parts phosphatidylethanolamine and 2 parts cardiolipin) in the presence of external ascorbate and cytochrome *c*. In the absence of valinomycin the response to FCCP is rather sluggish, as reported by Wrigglesworth et al. (1976) (Abstracts, 10th Int. Congr. Biochem., No. 06-6-230).

2. The K_m for cytochrome *c* in 67 mM, pH 7.4, phosphate buffer with ascorbate as substrate, was 9 μM in both absence and presence of valinomycin and FCCP. Energization thus acts non-competitively towards cytochrome *c* oxidation.

3. The apparent K_m for oxygen is greater in the energized than in the deenergized state; double reciprocal plots of respiration rate versus oxygen concentration are concave downward in the absence of uncouplers, as found with intact mitochondria. Energization thus acts "competitively" towards oxygen.

4. Despite the lack of a functional ATPase system, all the kinetic features of energization found in intact mitochondria can be mimicked in the reconstituted liposomes. This supports the chemiosmotic idea that electrical and perhaps H^+ gradients modify the oxidase activity in reconstituted vesicles.

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Abbreviation: FCCP, *p*-trifluoromethoxy carbonyl cyanide phenylhydrazone.

Introduction

Kimelberg and Lee [1] were among the first to use artificially reconstituted membrane systems as models for mitochondria and submitochondrial particles, and studied the binding and reactivity of cytochrome *c* in such "liposome" preparations. Hinkle and co-workers [2,3] later showed that it was also possible to mimic some of the "respiratory control" effects seen with mitochondria, firstly with the redox pair ascorbate-ferricyanide [2] and subsequently by incorporating the lipoprotein beef heart cytochrome *c* oxidase into liposomes and restoring the characteristic uncoupler sensitivity lost when the enzyme was isolated [3]. With externally added quinol and cytochrome *c* the respiration rate of the system could be increased by a factor of 5 when FCCP plus valinomycin or valinomycin plus nigericin were added. The H^+ : O ratio was very close to 2, with a proton efflux linked to a concomitant uptake of potassium. Ion translocations were only observed in the presence of valinomycin and disappeared upon addition of FCCP. Hinkle [4] has recently shown that the initial flux direction and the pH change of the medium may be a function of the electron donor used, which can be a hydrogen atom donor (quinol), a two electron one proton donor (ascorbate) or a one electron donor (cytochrome *c*).

Jasaitis, Skulachev and co-workers [5,6] have observed that cytochrome oxidase-containing liposomes prepared with internal trapped cytochrome *c* can take up lipophilic anions, when respiring on ascorbate and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. They conclude that such a system creates a membrane potential as a result of electron transfer. Recently they have directly verified this conclusion [7], by measuring the potential across a black lipid membrane to which cytochrome oxidase vesicles were adhered, obtaining values of up to 110 mV.

This paper describes the use of cytochrome *c* oxidase-containing liposomes to probe effects of energization already seen with the mitochondrial system [8]. Using the oxygen diffusion technique of Degn and Wohlrab [9], we have tried to determine whether the reconstituted system shows any consistent differences in uncoupler response when compared to the natural mitochondrial oxidase system. The system containing only cytochrome *c* oxidase and lipid may also permit easier interpretation of such responses than the mitochondrial system, where other cytochromes affect the spectroscopic analysis and in which membrane carriers and porters modify in rather unpredictable ways the transmembrane "energization" steps.

There have been indications that reconstituted proteoliposomes do not always behave like mitochondria. Thus Hinkle et al. [3] found that the combination of nigericin plus valinomycin was a more effective uncoupler than some single proton carriers such as dinitrophenol. Hunter and Capaldi [10] reported that full release of maximal respiration by cytochrome *c* oxidase liposomes required valinomycin in addition to uncoupler, a phenomenon not seen in mitochondria. Wrigglesworth and Nicholls [11] suggested that these differences might be a function of the phospholipid preparation used, with the large valinomycin effects seen with vesicles from crude phospholipid extracts but not in proteoliposomes from purified commercial phospholipids. They postulated different intrinsic potassium exchange capacities among the

different types of liposome. It was therefore important to examine more carefully the responses of the reconstituted system to both uncouplers (FCCP) and ionophores (valinomycin). Part of the work described here was reported at the Tenth International Congress of Biochemistry in Hamburg, 1976 [12].

Materials and Methods

(a) Preparations. Cytochrome *c* oxidase was isolated from Keilin-Hartree beef heart submitochondrial particles essentially according to van Buuren [13]. The final enzyme samples were rapidly frozen by pouring into liquid nitrogen and the resulting small pellets stored in polypropylene tubes at 77 K. The stock cytochrome aa_3 concentration was 160 μM and the purity 10.3 μmol heme *a*/g protein. The ratio $A_{444\text{nm}}$ (reduced)/$A_{424\text{nm}}$ (reduced) was 2.2, indicating little non-reducible heme *a*.

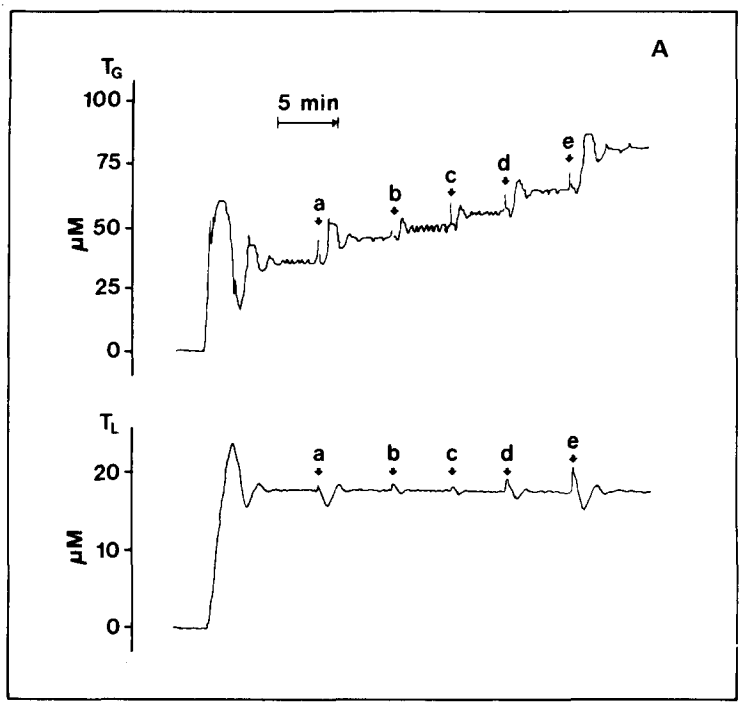
Cytochrome aa_3-containing proteoliposomes were prepared as follows: 15 mg phosphatidylcholine, 15 mg phosphatidylethanolamine and 7.5 mg cardiolipin in chloroform/methanol were pipetted into a test tube, the solution dried under nitrogen, and the test tube evacuated for 15 min to remove any remaining solvent. 1.5 ml cold 67 mM potassium phosphate/1 mM EDTA, pH 7.4, was added to the dried phospholipids and either:

alternative a, 30 μl 160 μM cytochrome aa_3 were added and the mixture vigorously agitated using a "whirli-mixer" until all the phospholipids were dispersed to form a thick suspension of multilamellar liposomes, or

alternative b, the phospholipids were first dispersed by the "whirli-mixer" and after complete dispersion 30 μl 160 μM cytochrome aa_3 were added.

The dispersion was then sonicated with a Branson Sonifier Model B12 equipped with a microtip placed very close to the bottom of a thick-walled test tube containing the dispersion, and surrounded by a beaker connected to a device circulating ice-cold water. The temperature of the exposed solution never exceeded 12°C, although it may have been higher near the tip. The dispersion was sonicated for 15 min at 60 W; the almost clear solution was centrifuged (IEC B60, 8°C) at 180 000 × *g* for 15 min, and the supernatant was transferred to a test tube and placed on ice. The very hard precipitate contained both a small amount of liposomes and titanium dust lost from the microtip during sonication. Except for Table I all the data presented in this paper were obtained with liposomes showing "respiratory control" values of 5 or more (see Fig. 2 and associated text) and prepared according to alternative b.

(b) Materials. Sodium deoxycholate, Tween 80 and ascorbic acid (neutralized with NaOH and stored at −18°C as a 1 M solution) were obtained from Merck. Valinomycin (stored as ethanolic solution), cytochrome *c* (type VI horse heart) and morpholinopropane sulphonic acid were from Sigma Chemical Co. Purified lipids (grade I egg yolk phosphatidylcholine, grade I egg yolk phosphatidylethanolamine, and grade I beef heart cardiolipin) were from Lipid Products, Inc., Nutfield Nurseries, Surrey, U.K. Phospholipids in chloroform/methanol were stored in glass tubes, being flushed with nitrogen before reclosing. Crude soya bean phospholipids were obtained as "asolectin" from Associated Concentrates, Inc., Long Island, N.Y., U.S.A. Trifluoromethoxy



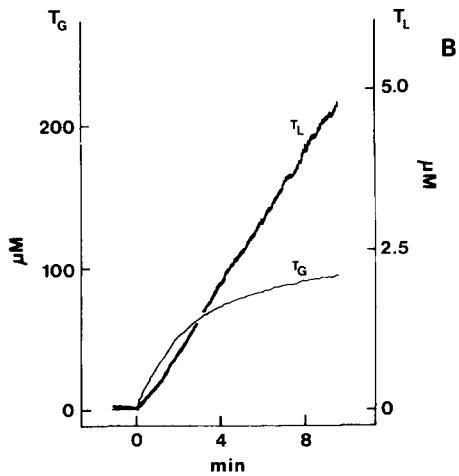


Fig. 1. (A) Recordings of T_G and T_L during an experiment with feed-back control on T_G to give a constant T_L. Before arrow a, the medium contained 58.3 nM liposomal cytochrome aa_3, 2.0 μM cytochrome *c*, 22.2 mM sodium ascorbate, 67 mM potassium phosphate, 1 mM EDTA, pH 7.4, 30°C. At the arrows the cytochrome *c* concentration was increased to: (a) 4.0 μM, (b) 5.0 μM, (c) 6.67 μM, (d) 10.0 μM and (e) 20.0 μM. (B) Recordings of T_G and T_L during an experiment with feed-back control on T_G to give a linear increase with time in T_L. The medium contained 14.6 nM liposomal cytochrome aa_3, 44.4 μM cytochrome *c*, 22.2 mM sodium ascorbate, 1.1 μg/ml valinomycin, 5.6 μM FCCP, 67 mM potassium phosphate, 1 mM EDTA, pH 7.4, 30°C.

carbonyl cyanide phenylhydrazone (FCCP), stored as a methanolic solution, was a gift from Dr. P.G. Heytler of DuPont Co.

(c) Methods. Oxygen uptake was measured using the open respirograph system of Degn and Wohlrab [9] with the addition of the on-line computer system described by Petersen et al. [14]. The computer was programmed to perform two kinds of experiment:

(i) the oxygen tension of the liquid (T_L) was kept at a constant, preset value (the "oxygen-clamp" method). Fig. 1A shows an experiment where proteoliposomes are "titrated" with cytochrome *c*. The oxygen tension of the gas phase (T_G), which is set by the computer, regulates the T_L. When the latter has reached its steady-state concentration at the particular cytochrome *c* level, the computer stores 90 determinations of T_G and T_L at a rate of one determination/s, from which an averagae velocity is calculated. Errors in blank systems were less than 5 nM/s and the "blank" ascorbate autoxidation velocity was approx. 15 nM/s. The autoxidation rate is subtracted from the total rate to give the enzymic rates.

(ii) The oxygen tension of the liquid was linearly increased with time from zero to 5 μM (Fig. 1B), and $1/v_R$ versus $1/T_L$ was continuously plotted on a X-Y recorder [19].

Spectrophotometry was carried out with a Cary 118C double-beam spectrophotometer or a Perkin-Elmer 356 dual-wavelength instrument. Extinction coefficients of 27 $mM^{-1} \cdot cm^{-1}$ ($\epsilon^{red\text{-}ox}_{605-630}$) for cytochrome aa_3 and 21 $mM^{-1} \cdot cm^{-1}$ ($\epsilon^{red\text{-}ox}_{550-540}$) for cytochrome *c* were used. Turnover numbers are expressed in electrons per s per cytochrome aa_3.

Results and Discussion

Fig. 2 illustrates the responses to FCCP and valinomycin of the steady-state system of Eqn. 1,

$$\text{ascorbate} \rightarrow \text{cytochrome } c \text{ (external)} \rightarrow \text{liposomal } aa_3 \rightarrow O_2 \qquad (1)$$

measured by following the steady-state reduction of added cytochrome *c* at 550 nm. The effective "respiratory control" ratio (the ratio of the maximal enzyme turnover number in the presence of both uncoupler (FCCP) and ionophore (valinomycin) to the maximal turnover number in the absence of either) can be calculated from such data by the method described for "mitoplasts" (outer membrane-stripped mitochondria) by Petersen et al. [15]. As the rate of electron transfer (v) from ascorbate is proportional to $[c^{3+}]$, and as the maximal turnover of the enzyme is proportional to $v \cdot [c]_{total}/[c^{2+}]$, the change in this maximal turnover number ("respiratory control" ratio) is given by: $[c^{2+}]_4[c^{3+}]_{3u}/[c^{3+}]_4[c^{2+}]_{3u}$, where subscript "4" indicates concentration in the controlled state and subscript "3u" concentration in the state stimulated by FCCP and valinomycin [15]. The typical experiment of Fig. 2 gives a respiratory control ratio of 6, as obtained with mitoplasts under the same conditions [15]. Such a ratio is also seen when oxygen uptake by such proteoliposomes is measured polarographically in the presence of ascorbate and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (experiment not shown). As with intact mitochondria, the effect of valinomycin on the respiration rate (and

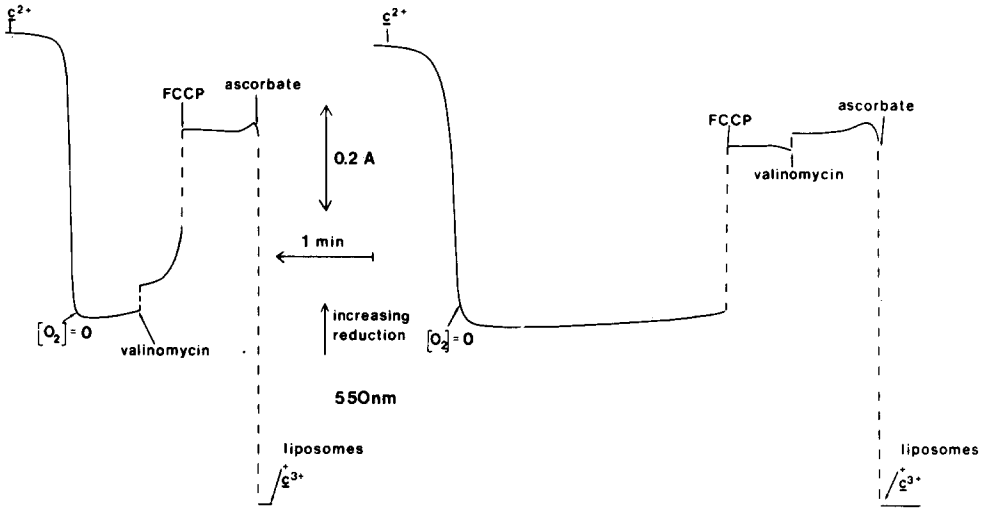


Fig. 2. The effect of FCCP and valinomycin on the steady-state reduction of cytochrome *c*. The medium contained 72 nM liposomal cytochrome aa_3, 44 μM cytochrome *c*, 5.5 mM sodium ascorbate, 3.6 μM FCCP, 0.44 μg/ml valinomycin, 67 mM potassium phosphate, 1 mM EDTA, pH 7.4, 22°C. Cary 118C spectrophotometer, measurements at 550 nm.

hence the steady-state reduction level of cytochrome *c*) is very small in the absence of FCCP (Fig. 2, right hand trace). The response to FCCP addition is however faster in the presence of valinomycin (right hand trace) than in the latter's absence (left hand trace). A similar effect has been reported by Wrigglesworth et al. [16].

Fig. 3 shows the Lineweaver-Burk plots for ascorbate-reduced cytochrome *c*

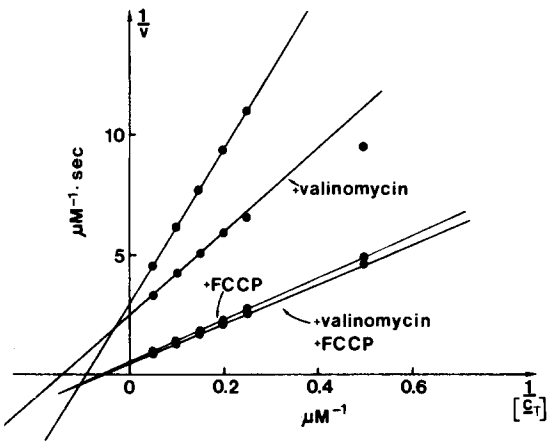


Fig. 3. Reciprocal plots of the cytochrome *c* concentration versus the respiration rate by liposomal cytochrome oxidase. The effects of valinomycin and FCCP. The medium contained 22 mM sodium ascorbate, 29.3 nM liposomal cytochrome aa_3, 2.2 μM FCCP, 0.53 μg/ml valinomycin, 67 mM potassium phosphate, 1 mM EDTA, pH 7.4, 30°C and varying amounts of cytochrome *c*. The respiration rates were measured in separate experiments using the "oxygen-clamp" mode at 17.7 μM O_2.

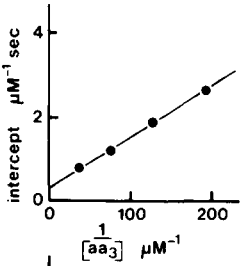
oxidation at a fixed oxygen concentration of 17.7 μM ("oxygen-clamp" method). Except for the possible case with valinomycin alone (curve b), straight lines are obtained intersecting in the 3rd quadrant. Energization is slightly anti-competitive towards cytochrome *c* oxidation, due to the finite rate of reduction by ascorbate; energization will be purely non-competitive at an infinite rate of reduction. A similar type of "inhibition" has been found with "mitoplasts" [15]. Turnovers at infinite cytochrome *c* concentration were measured as 40 s^{-1} in the absence and 240 s^{-1} in the presence of the ionophores FCCP and valinomycin.

Using the open electrode system under the conditions of Fig. 1B (linear gradients in oxygen concentrations), the kinetics of the reaction of the vesicles with oxygen were then measured at fixed concentrations of cytochrome *c*. Fig. 4A shows the computer-derived reciprocal plots of $1/v_r$ vs. $1/[O_2]$ for several enzyme concentrations in the presence of FCCP and valinomycin. Straight lines are obtained, intersecting in the 2nd quadrant. When the cytochrome *c* concentration is raised (Fig. 4B), parallel plots are obtained, in the same way as with the isolated oxidase [14]. From the intercept plot (inset, Fig. 4A) a maximal turnover of 290 s^{-1} is obtained, and from the slope of this plot a value of $6.25 \cdot 10^7\ M^{-1} \cdot s^{-1}$ for the second-order velocity constant in the reaction of liposomal oxidase with molecular oxygen (k_1). From Fig. 4B (inset), the K_m for oxygen under these conditions at an infinite concentration of cytochrome *c* is 1.1 μM as compared to 0.95 μM for the isolated enzyme reported by Petersen et al. [14].

When the liposomal aa_3 oxygen kinetics are examined in the "energized" state (i.e. in the absence of FCCP and valinomycin), the Lineweaver-Burk plots are no longer linear (Figs. 5A and 5B). At all the enzyme concentrations employed, the plots are concave downward (Fig. 5A), and indicate apparent k_1 values of less than $10^7\ M^{-1} \cdot s^{-1}$, one order of magnitude less than in the uncoupled case (Fig. 4). This result is qualitatively and quantitatively similar to those obtained with intact mitochondria [8] and with "mitoplasts" [15]. When the cytochrome *c* concentration is raised under "energized" conditions (Fig. 5B), an approximately parallel set of "concave downward" curves are obtained. Although cytochrome aa_3 is energized, its catalytic mechanism can still be described by a kinetic model of the "ping-pong" type (cf. inset plots, Fig. 5B).

Fig. 6 compares the oxygen kinetics of the four types of system under identical conditions. Both the fully energized (a) and valinomycin-supplemented systems (b) show non-linear kinetic behaviour. Addition of FCCP to either system (c, d) produces linear plots. Plots obtained in the presence of valinomycin (b, d) are almost parallel to those in its absence. Thus valinomycin may affect the maximal turnover (at infinite oxygen concentration), but probably not the behaviour at low oxygen levels.

The effect of "asolectin" on the oxygen kinetics of the solubilized enzyme (in 0.5% Tween 80) is shown in Figs. 7A (minus asolectin) and 7B (plus asolectin). While the apparent K_m values for oxygen are almost identical (0.8 and 0.9 μM, respectively) the apparent molecular activities are markedly different; "asolectin" raises the turnover of the enzyme from 130 or 290 s^{-1} (inset). The latter value is identical to the activity of liposomal cytochrome aa_3,


intercept μM⁻¹ sec
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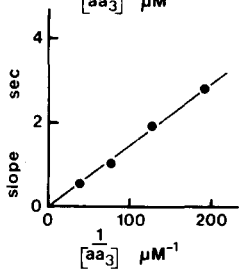
1/[aa3] μM⁻¹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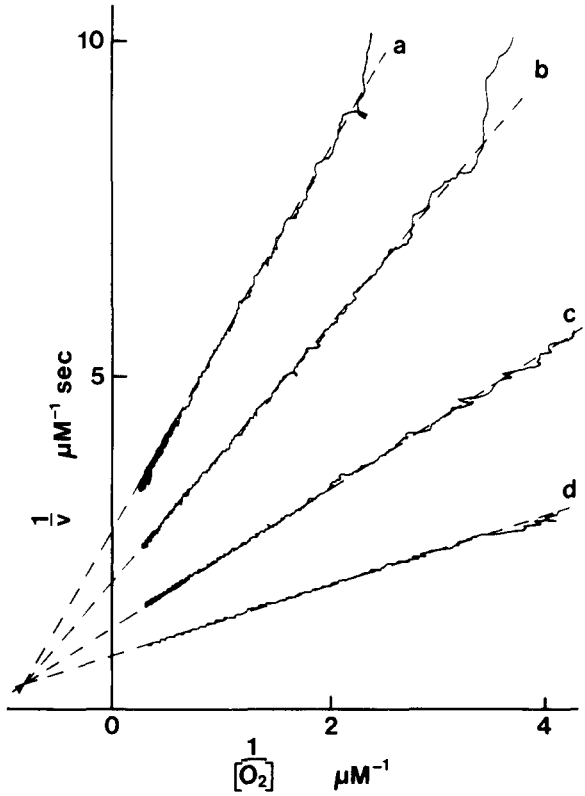
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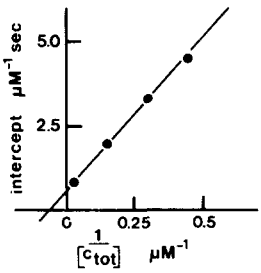
intercept μM⁻¹ sec
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2.5
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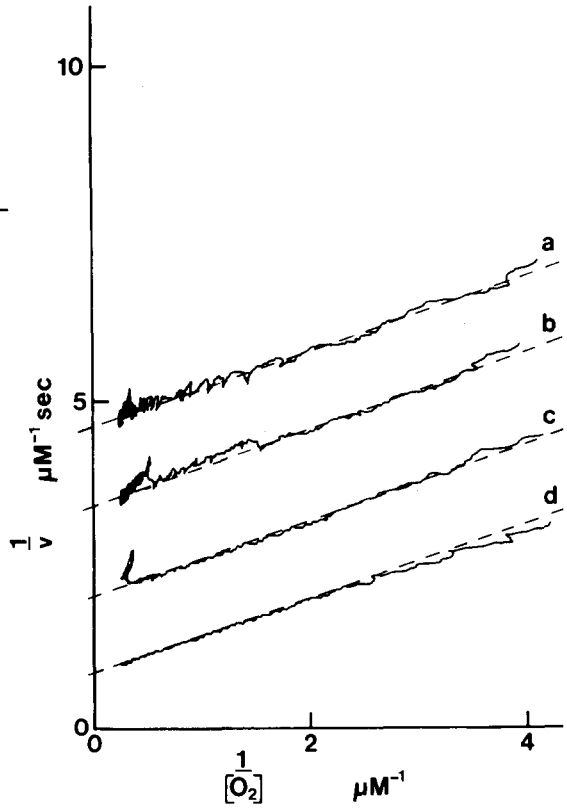
0.25
0.5
1/[ctot] μM⁻¹

B


10
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1/v μM⁻¹ sec
1/[O2] μM⁻¹

TABLE I

EFFECTS OF SONICATION AND DEOXYCHOLATE TREATMENT ON MAXIMAL TURNOVERS OF LIPOSOMAL CYTOCHROME OXIDASE

Proteoliposome preparation		Maximal turnover		Percent outward-facing molecules ***
		Intact vesicles	Sodium deoxycholate-treated †	
Multilamellar *	(a) †	10	109	9
	(b) †	93	132	70
Unilamellar **	(a)	120	115	~100 ?
	(b)	275	235	~100 ?

* Assay carried out in 'oxygen clamp' mode (see Methods) with 22 mM sodium ascorbate, 44.4 μM cytochrome *c*, 17.7 μM oxygen, 67 mM potassium phosphate, pH 7.4, 1 mM EDTA, 30°C.

** Assay carried out in 'oxygen clamp' mode with 22 mM sodium ascorbate, varying amounts of cytochrome *c*, 26.7 nM (intact vesicles) or 21.3 nM (sodium deoxycholate-treated vesicles) liposomal cytochrome aa_3, 4.4 μM FCCP, 1.33 μg/ml valinomycin, 67 mM potassium phosphate, 1 mM EDTA, pH 7.4, 30°C. With sodium deoxycholate-treated vesicles 0.5% Tween 80 and 1% asolectin were included in the assay medium. Maximal turnovers are obtained by extrapolating double reciprocal plots to infinite cytochrome *c* concentrations.

*** See text for difficulties involved in this calculation.

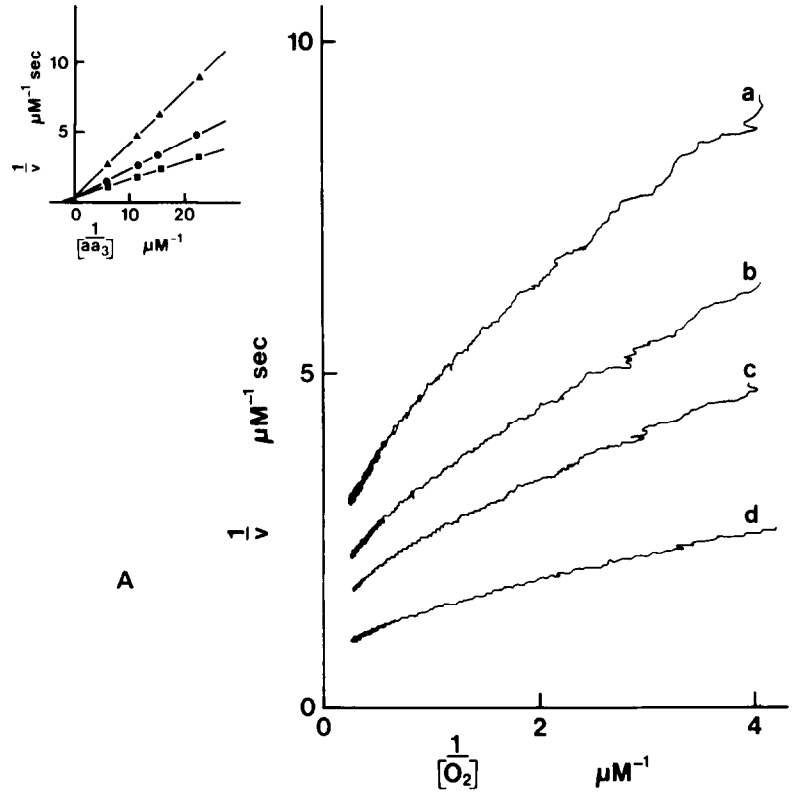
† See Methods for the preparation of 'type a' and 'type b' proteoliposomes. For deoxycholate treatment, 125 μg liposomal phospholipids were mixed with 125 μg sodium deoxycholate in a total volume of 625 μl.

but in this case the apparent K_m for oxygen is 0.8 μM.

Fig. 8 shows the effect of varying the ionophore (Fig. 8A) or uncoupler concentration (Fig. 8B) on the respiration rates of cytochrome aa_3-proteoliposomes. In the presence of FCCP (whether added before or after the liposomes start respiring) the reciprocal plot of $1/(v-v_0)$ vs. 1/[valinomycin] is linear, with a "K_m" for valinomycin of 0.1 μM (Fig. 8A), a value five times higher than that reported by Hunter and Capaldi [10]. However, the latter value was obtained in the presence of nigericin. Moreover, this value declines as the oxidase concentration (17.5 nM) in the assay is decreased. As with valinomycin in the absence of FCCP, FCCP in the absence of valinomycin (Fig. 8B) shows complex kinetics. But in the presence of valinomycin, the reciprocal plots are again linear, and indicate an apparent "K_m" of 0.1 μM for FCCP. All these ionophore effects are also abolished in the presence of Tween 80.

Table I lists the maximal turnover numbers obtained with the two types of proteoliposome (alternatives a and b) described in the Methods section, both before and after sonication. Maximal turnovers were elicited according to

Fig. 4. Reciprocal plots of the oxygen concentration versus the respiration rate by liposomal cytochrome oxidase in the presence of valinomycin and FCCP. (A) The effect of varying the enzyme concentration. (a) 5.8 nM, (b) 8.75 nM, (c) 14.6 nM and (d) 29.3 nM liposomal cytochrome aa_3. The medium contained 22.2 mM sodium ascorbate, 44.4 μM cytochrome *c*, 0.44 μg/ml valinomycin, 0.57 μM FCCP, 67 mM potassium phosphate, 1 mM EDTA, pH 7.4, 30°C. Insets: Slope and intercept plots of A. (B) The effect of varying the cytochrome *c* concentration. (a) 2.2 μM, (b) 3.3 μM, (c) 6.7 μM and (d) 33.3 μM cytochrome *c*. The medium contained 22.2 mM sodium ascorbate, 29.3 nM liposomal cytochrome aa_3, 0.44 μg/ml valinomycin, 0.57 μM FCCP, 67 mM potassium phosphate, 1 mM EDTA, pH 7.4, 30°C. Inset: Intercept plot of B.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$\frac{1}{[O_2]}$ μM^{-1}
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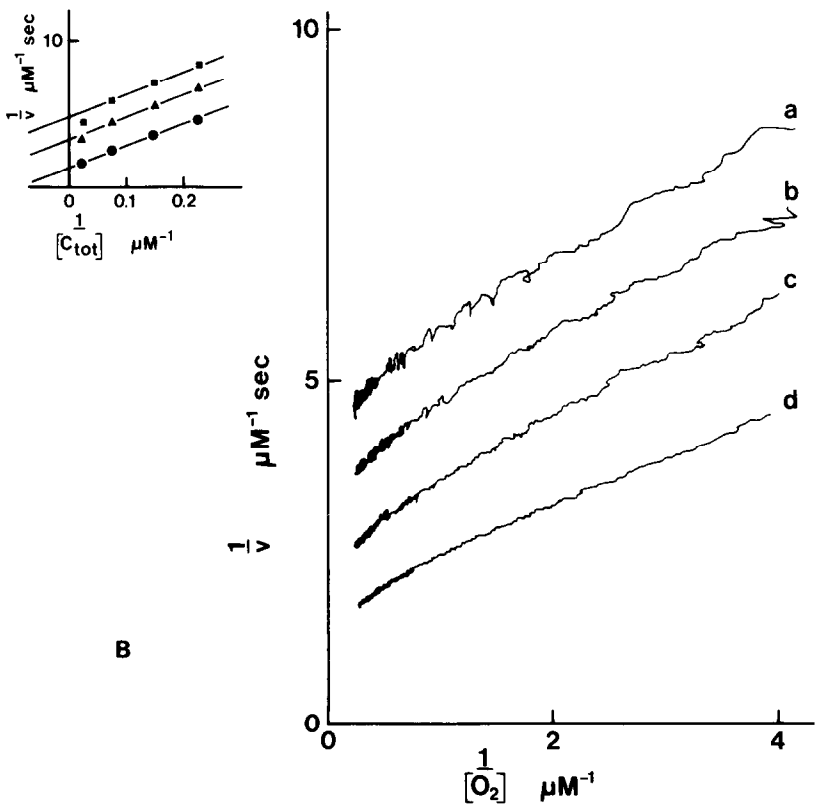
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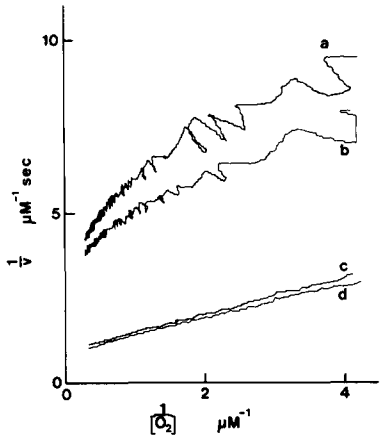

Fig. 6. The effects of valinomycin and FCCP on reciprocal plots of the oxygen concentration against the respiration rate by liposomal cytochrome oxidase. (a) proteoliposomes with no further additions, (b) plus 1.1 μg/ml valinomycin, (c) plus 5.6 μM FCCP, (d) plus 1.1 μg/ml valinomycin and 5.6 μM FCCP. The medium contained 22.2 mM potassium ascorbate, 44.4 μM cytochrome *c*, 46.7 nM liposomal cytochrome aa_3, 67 mM potassium phosphate, 1 mM EDTA, pH 7.4, 30°C.

the method of Smith and Camerino [17], who treated submitochondrial particles with 1% deoxycholate for a brief period, and diluted the treated particles into the appropriate assay systems. Using this method, Nicholls [18] has reported that 60% of the oxidase molecules in Keilin-Hartree submitochondrial particles are inward-facing with respect to their cytochrome *c* reaction sites, and only 40% outward-facing. Table I shows that although the multilamellar liposomes show a latency of oxidase activity toward sodium deoxycholate treatment, the sonicated ones do not. While the unilamellar proteoliposomes prepared according to alternative b have a maximal turnover close to the value of the isolated oxidase in the presence of "asolectin", the a type seem to have lost some activity during sonication.

It might be expected that cytochrome *c* added externally should indeed react with only part of the liposomal oxidase. But the actual proportion of the latter which is catalytically active is not easily determined. Random incorporation of a bipolar molecule should result in 50% of the enzyme facing outwards to react with cytochrome *c*. Racker [19] has reported, on the basis of kinetic experiments, that with cholate-treated and dialysed vesicles, 80%

Fig. 5. Reciprocal plots of the oxygen concentration versus the respiration rate by liposomal cytochrome oxidase in the absence of valinomycin and FCCP. (A) The effect of varying the enzyme concentration. (a) 44 nM, (b) 65.5 nM, (c) 87.5 nM and (d) 175 nM liposomal cytochrome aa_3. The medium contained 22.2 mM sodium ascorbate, 44.4 μM cytochrome *c*, 67 mM potassium phosphate, 1 mM EDTA, pH 7.4, 30°C. Inset: Reciprocal plots of the enzyme concentration versus the respiration rate at varying fixed oxygen concentrations. 0.25 μM (▲), 1.0 μM (●) and 4.0 μM (■) oxygen. (B) The effect of varying the cytochrome *c* concentration. (a) 4.4 μM, (b) 6.7 μM, (c) 13.3 μM and (d) 44.4 μM cytochrome *c*. The medium contained 22.2 mM sodium ascorbate, 87.5 nM liposomal cytochrome aa_3, 67 mM potassium phosphate, 1 mM EDTA, pH 7.4, 30°C. Inset: Reciprocal plots of the cytochrome *c* concentration versus the respiration rate at varying fixed oxygen concentrations: 0.26 μM (■), 1.0 μM (▲) and 4.0 μM (●) oxygen.

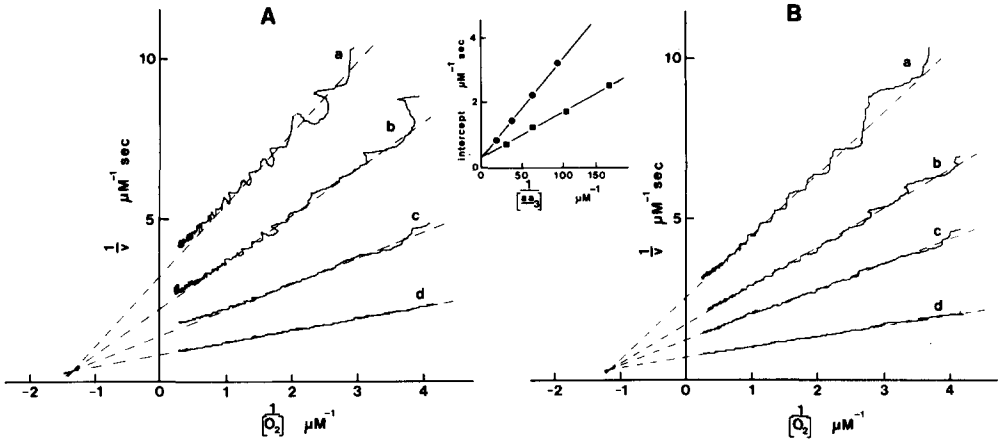

Fig. 7. Reciprocal plots of the oxygen concentration versus the respiration rate by solubilized cytochrome oxidase in the presence and in the absence of asolectin. (A) The effect of varying the enzyme concentration in the absence of asolectin. (a) 10.7 nM, (b) 16.0 nM, (c) 26.7 nM and (d) 53.3 nM solubilized cytochrome aa_3. The medium contained 22.2 nM sodium ascorbate, 44.4 μM cytochrome *c*, 67 mM potassium phosphate, 1 mM EDTA, 0.5% Tween 80, pH 7.4, 30°C. (B) The effect of varying the enzyme concentration in the presence of asolectin. (a) 6.4 nM, (b) 9.6 nM, (c) 16.0 nM and (d) 32.0 nM solubilized cytochrome aa_3. The medium contained 22.2 mM sodium ascorbate, 44.4 μM cytochrome *c*, 67 mM potassium phosphate, 1 mM EDTA, 0.5% Tween 80, 1% asolectin, pH 7.4, 30°C. Inset: Intercept plot of data from A (•) and B (▪).

of the cytochrome aa_3 units are oriented in such a way as to permit reaction with added, external, cytochrome *c*. A similar configuration for the sonicated vesicles might be deduced from the data in Table I, which show that deoxycholate treatment, effective in the release of latent activity in submitochondrial particles [17,18] and in hand-shaken (multilamellar) vesicles, has little effect on the activity of sonicated proteoliposomes. However, this conclusion is complicated by the occurrence of lipid activation, which is probably most effective when cytochrome aa_3 is located in a membrane. Such an effect will cause an overestimation of the number of outward-facing molecules. Although the activity of proteoliposomes prepared by "alternative method b" is identical with that of the isolated oxidase in the presence of "asolectin", a significant difference between the apparent K_m values for oxygen is observed. This indicates either an increase in the maximal turnover of proteoliposomal cytochrome aa_3 or a decrease in k_1, the velocity constant for the reaction between the oxidase and molecular oxygen [8]. If the former is correct, calculation indicates a real turnover of 400 s^{-1} for 70% outward-facing cytochrome aa_3 molecules, close to the activity seen with "mitoplasts" [15]. Direct observations of the reducibility of cytochrome aa_3 in proteoliposomes by Wrigglesworth [20] indicate that the proportion of outwardly accessible molecules is between 50 and 60%.

A second problem upon which these results may throw some light is the apparent potassium ionophore requirement for full release of respiration found in some cytochrome aa_3 proteoliposome systems [3,10,11,]. Wrigglesworth et al. [16] have suggested that the valinomycin effect may be a consequence of the existence of net pH gradients in the liposome preparations; FCCP alone

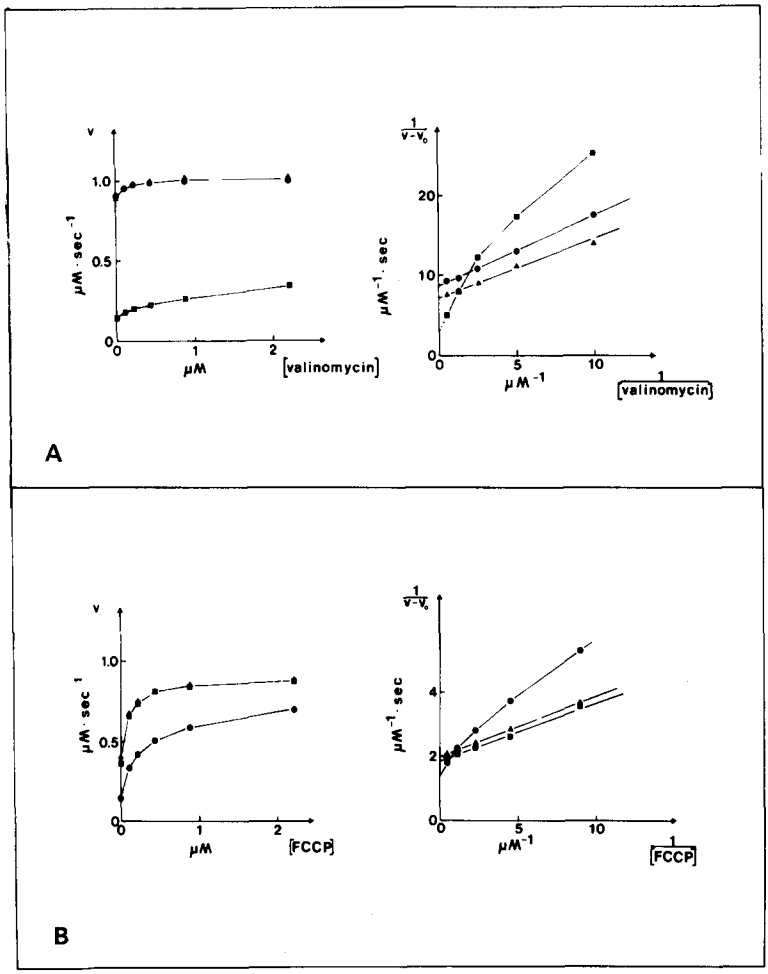

Fig. 8. The respiration rate of liposomal cytochrome oxidase as a function of ionophore or uncoupler concentrations. (A) The effect of varying the valinomycin concentration. The medium contained 22 mM sodium ascorbate, 44.4 μM cytochrome *c*, 17.5 nM liposomal cytochrome aa_3, 67 mM potassium phosphate, 1 mM EDTA, pH 7.4, 30°C. No further additions (■). 4.4 μM FCCP was added before ascorbate and cytochrome *c* (▲). 4.4 μM FCCP was added after the T_L had been clamped for 15 min (●). (▲, ● give rise to superimposed curves in the left hand plot). (B) The effect of varying the FCCP concentration. The medium contained 22 mM sodium ascorbate, 44.4 μM cytochrome *c*, 17.5 nM liposomal cytochrome aa_3, 67 mM potassium phosphate, 1 mM EDTA, pH 7.4, 30°C. No further additions (●). 1.2 μM valinomycin added before cytochrome *c* and ascorbate (■). 1.2 μM valinomycin added after T_L had been clamped for 5 min (▲). (■, ▲ give rise to superimposed curves in the left hand plot). The experiments were performed in the "oxygen-clamp" mode at 17.7 μM oxygen. The value of $(v-v_0)$, plotted in reciprocal form in the two right hand plots A and B, is calculated by subtracting the rate in the absence of the indicated ionophore from the rate at the concentration concerned.

may promote full deenergization only after a long "lag" phase (cf. Fig. 2 above), which these authors [16] attribute to slow equilibration of the net gradients. Fig. 8 shows that although with the present proteoliposomes the effects of valinomycin are quite small, the kinetics of FCCP-induced release of respiration in the absence of valinomycin are more complex than in its presence. This supports the idea that some net gradients are created in the

liposome that do not occur in mitochondria. Preliminary experiments (Hansen, F.B., unpublished results) indicated that highest "respiratory control" ratios (lowest respiration rates in the absence of FCCP and valinomycin) are obtained when potassium is the external and $Tris^+$ the internal cation. It was under these conditions that the most marked stimulation by valinomycin was seen; inward potassium fluxes evidently accompanied deenergization.

The most striking kinetic phenomena reported here are the "non-competitive" effect of energization on the cytochrome *c* kinetics (Fig. 3) and the "competitive" effect of energization, coupled with the induction of non-linear double reciprocal plots, on the oxygen kinetics (Figs. 4 and 5). Such effects have also been observed with intact mitochondria [8] and "mitoplasts" [15], and strongly suggest that the reaction mechanism of cytochrome oxidase is always the same whether the enzyme is present in the artificial or in a natural membrane.

We conclude that cytochrome aa_3-containing proteoliposome systems can be used to mimic successfully the energy-dependent responses of intact mitochondrial systems [8,15], with the exception of the ADP/ATP/phosphate responses, which require functional ATPase and ATP translocator enzymes. Electrical gradients, and perhaps gradients of net proton-motive force, control the oxidase activity in reconstituted vesicles. If the system is a "simple" chemiosmotic one, then the "respiratory control" ratio should be a function of the turnover of the enzyme. Experiments to probe this last question are described in the accompanying paper [21].

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