Proton Translocation By

Cytochrome c Oxidase

reconstituted into

Proteoliposomes

Stephen G. Shaughnessy, B.Sc. (Hon.)

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St. Catharines, Ontario

ABSTRACT

Cytochrome c oxidase inserted into proteoliposomes translocates protons with a stoichiometry of approximately 0.4-0.6 H^+/e^- in the presence of valinomycin plus pottasium. The existance of such proton translocation is supported by experiments with lauryl maltoside which abolished the pulses but did not inhibit cyt. c binding or oxidase turnover. Pulses with K_3FeCN_6 did not induce acidification further supporting vectorial proton transport by cyt.aa_3. Upon lowering the ionic strength and pulsing with ferrocytochrome c, H^+/e^- ratios increased. This increase is attributed to scaler proton release consequent upon cyt.c-phospholipid binding. Oxygen pulses at low ionic strength however did not exhibit this large scaler increase in H^+/e^- ratios. A small increase was observed upon O_2 pulsing at low ionic strength. This increase was KCN and FCCP sensitive and thus possibly due to a redox linked scaler deprotonation. Increases in the H^+/e^- ratio also occurred if pulses were performed in the presence of nonactin rather than valinomycin.

The fluorescent pH indicator pyranine was internally trapped in aa_3 containing proteoliposomes. Internal alkalinization, as monitored by pyranine fluorescence leads to a ΔpH of approximately 0.35 units, which is proportional to electron flux. This internal alkalinization was also DCCD sensitive, being inhibited by approximately 50%. This 50% inhibition of internal alkalinization supports the existance of vectorial proton transport.

ACKNOWLEDGEMENTS

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Literature Review and Introduction

A)   Cytochrome c Oxidase and Artificial Membranes
      1. Cytochrome c Oxidase

Cytochrome c oxidase (Ferrocytochrome c; O_2 Oxidoreductase; EC 1.9.3.1; also Cytochrome Oxidase or Cyt.aa_3) is the terminal electron acceptor in the mitochondrial respiratory chain. It functions reducing dioxygen to water as in eq.1.

eq. 1. $O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O$
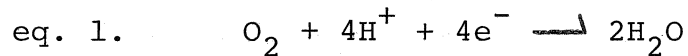
Cytochrome c oxidase of the aa_3 type is found not only in eukaryotic mitochondrian but also in gram positive and photosynthetic prokaryotes (Sone et.al.,1983; Sone and Yanagita ,1982; Ludwig and Schatz, 1980 and Ferguson, 1982).
      Electrons are transferred from cytochrome c, located on the C-side of the mitochondrial membrane via cytochrome c oxidase to molecular oxygen (see Papa,1976). The enzyme spans the membrane completely (Malmstrom,1979).
      Cytochrome c oxidase isolated from yeast contains at least 7 subunits (Poyton and Schatz, 1975). Tracy and Chan (1972) gave molecular wieghts for the corresponding 7 subunits of beef heart enzyme using electrophoresis in highly cross-linked polyacrylamide gels containing sodium dodecyl sulfate and urea. The molecular weight values were: I,44600; II, 22700; III,23500; IV, 16900; V,9400; VI, 7600; VII4300. YU and Yu (1977) reported that the subunits were present in 1:1 ratios. In addition to these 7 subunits, there appear to be at least five further subunits in beef heart preparation (Capaldi,1983). Three of these subunits, which appear to be associated with cytochrome c oxidase, are re fered to as a,b, and c,(Capaldi,1983). These polypeptides may not be essential to oxidase function,but may play a regulatory role ,(Capaldi et.al.1983). The largest subunits I,II,and III appear to be essential for electron transfer and the proton pumping function of cyt. aa_3.

The subunits II, VI, and VII are located on the
outer (C) side of the mitochondrial membrane, subunit IV on
the inner (M) side while subunits I and V are inaccessible
and thus probably located in the membrane interior (Frey et.
al., 1978; Malmstrom, 1979; Capaldi et.al. 1983).

Cytochrome $\underline{c}$ oxidase contains two hemes and two copper atoms
(Malmstrom, 1979). The two hemes are designated cytochrome a
and a_3 and the copper Cu_A and Cu_B. The hemes a_3 and a may
be contained in subunits I and II respectively, whereas
subunit II may contain the copper (Winter et. al., 1980, but
see Malmstrom, 1979). All four redox centers are one electron
carriers. Cytochrome a_3 and Cu_B form a binuclear center for
O_2 reduction (Tweedle et. al., 1978).

2. Liposomes

One of the earliest concepts of lipid-protein structural
association was the Danielli-Davson model (1943). These
authors envisioned a membrane with a lipid bilayer center coated
on either side with protein. This model was revised by Singer
and Nicholson (1972) when they presented the fluid mosaic
model. This model assumed that phospholipids exist in bilayers,
but it differed from previous models in two ways. Firstly,
membrane components were regarded as capable of lateral diffusion
in the plain of the membrane and secondly, the existence of
proteins which completely spanned the membrane (integral proteins)
was proposed. In 1965 Bangham et. al. showed that phospholipid
aqueous suspensions spontaneously formed closed multilamellar
vesicles, capable of trapping ions. These first liposomal
vesicles were multilamellar (MLV) (Bangham et. al., 1965).
To prepare (M L V) a hand suspension of lipid films, is
dried down by solvent evaporation, into saline or buffer
solution (Nicholls, 1981; Szoka and Papahadjopoulos, 1980).
Hydration time and method of resuspension is important in
determining the size and average number of lamelae per lipsome
(Szoka and Papahadjopoulos, 1980). These vesicles typically

have large internal volumes, but consist of bilayers contained
within bilayers. Their diffusion properties are thus complex
(Nicholls, 1981), but their relatively large internal volume
makes them suitable for internal trapping of substances.

Two other types of vesicular suspension may be prepared.
The first is referred to as small unilamellar vesicles (S U V).
These are prepared by sonicating M L V by a probe or bath
type sonicator (Szoka and Papahadjopoulos, 1980). These
vesicles consist of a single bilayer, forming optically clear
suspensions, with diameters between 215 and 500 Å (Nicholls,
1981; and Szoka and Papahadjopoulos, 1980). Due to the high
radius of curvature in these vesicles there is a higher
percentage of phospholipid in the outer monolayer (approx.
60 - 70%) as compared to the inner monolayer. These vesicles
typically have a internal trap volume of $0.2\mu l$ to $1.5\mu l/\mu mole$
of lipid (Nicholls, 1981; Szoka and Papahadjopoulos, 1980).

The second class of unilamellar vesicles is referred to
as large unilamellar vesicles (L U V). Deamer and Bangham,
1976, formed L U V by injecting ether solutions into a variety
of lipids suspended in warm aqueous solutions. These vesicles
were osmotically active, unilamellar and had an internal trap
volume ten times that of sonicated and handshaken preparations
(Deamer and Bangham, 1976). Papahadjopoulos et. al., (1975)
introduced a calcium fusion technique for the preparation of
L U V (Szoka and Paphadjopoulos, 1980). Upon the addition of
calcium to S U V , fusion occurs, resulting in the formation
of large cylindrical, multilamellar structures in a "swiss
role" configuration. Upon EDTA addition however, large vesicles
are formed. This method however, is restricted to mixtures of
phospholipids containing a majority of acidic phospholipids
(Szoka and Papahadjopoulos, 1980).

In 1972, Hinkle et. al. first used the cholate dialysis
method to incorporate cytochrome oxidase into soybean
(asolectin) phospholipid vesicles. In this method cholate is
used to solubilize cytochrome aa_3 and dialysis is used to slowly
remove the cholate. The parameter referred to as "respiratory
control" was examined and found to be 3 - 5 fold. This parameter

reflects membrane integrity and is defined as the rate of respiration (eq. 1) in the presence of protonophores divided by the rate of respiration in their absence.

Further work was performed by Racker in order to define optimal conditions for oxidase reconstitution (Carrol and Racker,1977). Cytochrome $\underline{c}$ oxidase when reconstituted into vesicles spans the membrane and can orientate itself in two ways. In one orientation heme a faces the external medium of the vesicle and thus can accept electrons from added cytochrome $\underline{c}$. The second orientation is in the opposite direction with heme a facing the internal mileau of the vesicle. In this orientation cytochrome $\underline{c}$ cannot donate electrons to heme a directly. Carroll and Racker,(1977) described a method for determining the orientation of reconstituted oxidase (uncoupled activity measured polaragraphically was compared to activities in the presence of 3% tween 80 which gives total activity) and found that approximately 50% of the enzyme orientated itself right-side out. Oxidase orientation can also be determined spectrophotometrically using ascorbate and cytochrome $\underline{c}$ to determine those molecules with heme a facing externally and ascorbate,cytochrome $\underline{c}$ and TMPD to determine total oxidase present,(Proteau et.al. 1983).

3. Membrane Permeability

The hydrophobic core of phospholipid bilayers creates a barrier to charged species. Thus, most cations and anions are relatively impermeant to membranes. Small uncharged species such as O_2, H_2O, and CO_2 are however, permeable to bilayers as are a number of low molecular weight neutral species such as ammonia and acetic acid. These neutral weak acids or bases can therefore be used in the estimation of pH gradients across membranes (Berry and Hinkle,1983).

Hauser et.al. (1973) studied the mechanism of Na^+ and Cl- escape from phosphatidylcholine and phosphatidylserine small unilamellar vesicles at 4°C. Chloride escaped approximately 3 orders of magnitude faster than sodium at acid pH. The authors believed that protons and chloride associate at the lipid water interface diffusing across as a neutral species. Sodium can simply diffuse across the bilayer or be released

as a consequence of vesicle rupture. This
would occur more frequently at higher temperatures. The rate
of escape was given in terms of a permeability coefficient
measured in dimensions of cm/s. Permeability coefficients
for sodium were approximately 10^{-14} - 10^{-15} cm/sec. while
for chloride the permeability coefficient ranged from 10^{-11}-
10^{-13} cm/sec. at high pH.

Nicholls and Miller (1974) agreed with Hauser et. al.
(1973) stating that Cl could diffuse across the membrane as a
neutral species associated with protons. However, this was
observed as being linked to OH- counter ion exchange. They
also gave evidence for the rate of Cl- diffusion being greater
than K+ diffusion.

Nicholls and Deamer (1980) followed the collapse of
small pH gradients in large unilamellar vesicles (0.1 - 0.2
pH units) with a glass electrode. The H+/OH- permeability
was found to be several orders of magnitude greater than that
of other monovalent ions (ie. 10^{-4} cm/sec.) and is insensitive
to lipid composition. They propose that protons and hydroxyls
permeate the bilayer via hydrogen bond exchange with associated
water molecules within the lipid bilayer.

Cafiso and Hubbell (1983) created transmembrane pH
gradients across phospholipid vesicular bilayers and monitored
their collapse using the EPR spectrum of phosphonium ion spin
labels. Membrane permeability of H^+ was found to be 5 ± 2 x
10^{-7} cm/sec. The differences between the values of Cafiso and
Hubbell and those found by Nicholls and Deamer may reflect the
differences in the extent of the established H^+ gradient.

Kaufmann and Silman (1983) working with soyabean lecithin,
synthetic diphytanoylphosphatidylcholine and dioeoylphoshatyl-
cholate found evidence in support of pH-dependent ion channel
formation at acid pH, (pH 3.5). Upon further lowering of the
pH below pH 3.5, a restoration of the low conductance state
was observed (as seen at neutral pH). They suggest that the
observed channel formation involves a protonation of the lipid
head groups within the critical pH range; these in turn create
a destabilization in the bilayer forming ion channels.

Internally trapped pH probes have also been used to study proton permeability (see pyranine section below). Membrane permeability is a reflection of the inherent properties of phospholipid bilayers and thus may be complex in nature.

4. Cytochrome c Binding to Liposomes

Cytochrome c binds externally to negatively charged liposomes. Binding to positively charged liposomes does not seem to occur. Nicholls and Malviya (1973), found no evidence for egg phosphatitidycholine binding cytochrome c. However, binding occurred on the addition of 12% negatively charged amphiphile to the vesicles. Phosphatidic acid seems to bind cytochrome c more effectively than other negatively charged lipids. Each cytochrome c molecule is associated with four phosphatic acid molecules compared to other negatively charged lipids such as eight cardiolipin, ten phospatidylserine, or twelve dicetyl phosphate molecules (see Nicholls, 1974). This increased binding of cytochrome c to phosphatidic acid presumably reflects its potential double negative charge. Cannon and Erman (1980), at low ionic strength (.003M) found a binding stoichiometry between cytochrome c and asolectin vesicles of 15 ± 2 phospholipid/Cyt. c (mole ratio).

Binding of cytochrome c may affect liposomal properties. The binding of cytochrome c to liposomes caused aggregation of the vesicles (Nicholls and Malviya, 1973) which decreased upon the reduction of cytochrome c. This demonstrated that ferro-cytochrome c binds less tightly than does ferricytochrome c (Nicholls and Malviya, 1973; Nicholls, 1974). The effects on the lipid bilayer itself have also been investigated. Nicholls and Malviya (1973) found no effect on the membrane transition temperature upon cytochrome c binding. Kruijff and Cullis (1980) studied the interaction of cytochrome c and cardiolipin containing liposomes with P-NMR and freeze fracture.Cytochrome c binds specifically to cardiolipin inducing an HII configuration, destroying the local bilayer structure. Singer (1978) found increased sodium permeability above the transition temperature

in agreement with Papahadjopoulos et. al. (1971, 1973).

Ionic strength affects the binding of Cytochrome c to liposomes (Nicholls and Malviya, 1973). Cannon and Erman (1980) found equilibrium association constants for the binding of cytochrome c to asolectin vesicles ranging from 2.2×10^6 to $1.8 \times 10^3 M^{-1}$ between 0.02 and 0.1M ionic strength respectively.

Bound ferricytochrome c retains its characteristic spectrum, while the rate of ascorbate reduction declines. Free cytochrome c is reduced at a rate of $k = 150 M^{-1} s^{-1}$, while bound cytochrome c is reduced at a rate of $k < 10 M^{-1} s^{-1}$ (Nicholls and Malviya, (1973). Cannon and Erman (1980) however, found as increase in the rate of cytochrome c reduction upon binding to asolectin vesicles by pteridine derivatives (2900 to 16000 Ms).

Thus upon cytochrome c binding to phospholipid vesicles both the properties of cytochrome c and the vesicles may be altered. The binding of cytochrome c to phospholipid vesicles is believed to be mainly electrostatic in nature, limited primarily to the vesicles surface (Nicholls, 1974), and thus affected by ionic strength. Papahadjopoulos et. al. (1975) however, found evidence for surface binding followed by partial penetration (hydrophobic interaction) and/or deformation of the membrane. In support of this cytochrome c binding drastically decreases both Tc (transition temperature) and ΔH (enthalpy of transition) of the lipid as well as increasing membrane permeability. Teissie (1981) also found evidence for cytochrome c penetration of phospholipid monolayers.

B)   Proton Translocation in Mitochondria and pH Gradients
     1. Generation of Proton Gradients by the Mitochondrion

Before Mitchell (1961) proposed his Chemiosmotic Theory, the coupling of electron transfer to ATP synthesis was explained in terms of the chemical hypothesis (Slater, 1953). The later hypothesis proposed that there was a high energy intermediate, denoted by "$\sim$", ("squiggle"), which was formed at energy conserving sites in the respiratory chain. Its formation depended upon changes in the redox state of the respiratory chain components. The high energy intermediate ($\sim$) could then

be utilized to form ATP from ADP and Pi (Slater, 1953).

In 1961, however, Mitchell proposed the chemiosmotic hypothesis in which it was suggested that electron transfer by mitochondria, bacteria and chloroplasts is coupled to ATP synthesis by means of a proton electo chemical gradient ($\Delta\mu H^+$), according to Mitchell and Moyle, (1967); Berry and Hinkle, (1983).

eq. 2. $$\frac{\Delta\mu_{H+}}{F} = \Delta\Psi - z\Delta pH$$

where $\Delta\mu_{H+}/F$ = proton motive force

$\Delta\mu_{H+}$ = electrochemical difference of H+ activity

F = Faraday constant

$\Delta\Psi$ = membrane potential

ΔpH = the pH difference across the membrane

Z = is a constant numerical factor (2.3RT/F = 60)

The generation of a sustained proton electrochemical gradient necessitates the existence of sites along the mitochondrial respiratory chain which translocates protons across the inner mitochondrial membrane as well as separating charge across the membrane.

Proton translocation can not be calculated stoichiometric-ally during the steady state, however, it is possible to detect the proton translocation (calculated as $H^+/2e^-$ or H^+/O ratios) by the respiratory chain that occurs upon the initiation of respiration (see Fig. 7). This can be achieved by two methods: (a) by a known and precise addition of oxygen to an anerobic mitochondrial or reconstituted oxidase system and monitoring proton movement via a pH meter (Mitchell and Moyle, 1967; Brand et. al., 1976; Moyle and Mitchell, 1978, Wrigglesworth and Nicholls, 1982) or (b) by the addition of substrate to an

aerobic, substrate-depleted mitochondrial or reconstituted system (Proteau et. al., 1983; Sigel and Carfoli, 1980; Papa et. al., 1980; Casey et. al., 1979).

In mitochondria the latter method may be used to determine the specific points of H^+ translocation along the respiratory chain and their respective $H^+/2e^-$ ratios (Papa et. al., 1980). The oxygen pulse method can be used to determine specific sites of proton translocation only in conjunction with specific inhibitors (Sigel and Carafoli, 1978); however, this is not the case with reconstituted systems (see section B3b).

2. Stoichiometry of Proton Translocation by the Mitochondrial Respiratory Chain

The stoichiometry of proton release by the mitochondrion has been the subject of some debate. The chemiosmotic theory as proposed by Mitchell, organized the respiratory chain into redox loops in which transmembrane hydrogen carriers alternated with pure election carriers spanning the membrane as shown in Fig. 1. Such a mechanism necessitates the translocation of two protons for each pair of electrons transferred through a redox loop (Mitchell, 1961; Papa, 1976). In this scheme, cytochrome c oxidase functions only as the final arm in the third redox loop, carrying electrons to molecular oxygen, and thus has no proton translocating functions.

Thus, Mitchell and Moyle (1967, 1968), proposed that 6 protons are released per two electrons flowing through the respiratory chain from NAD-linked substrates and that 4 protons are released per pair of electrons flowing to oxygen from succinate (Papa et. al., 1980).

These values were, however, challenged by Brand et. al. (1976) when they demonstrated that H^+/O quotients obtained in O_2 - pulse experiments were increased by the presence of (N-ethylmaleimide or Mersalyl) inhibitors of the H^+/Pi symportor. The rapid uptake of H^+ with endogenous Pi lost from the mitochondria during anaerobic preincubation may lead to an underestimation of the true H^+/O ratios. Upon blocking

FIGURE 1

Looped Respiratory
Chain:
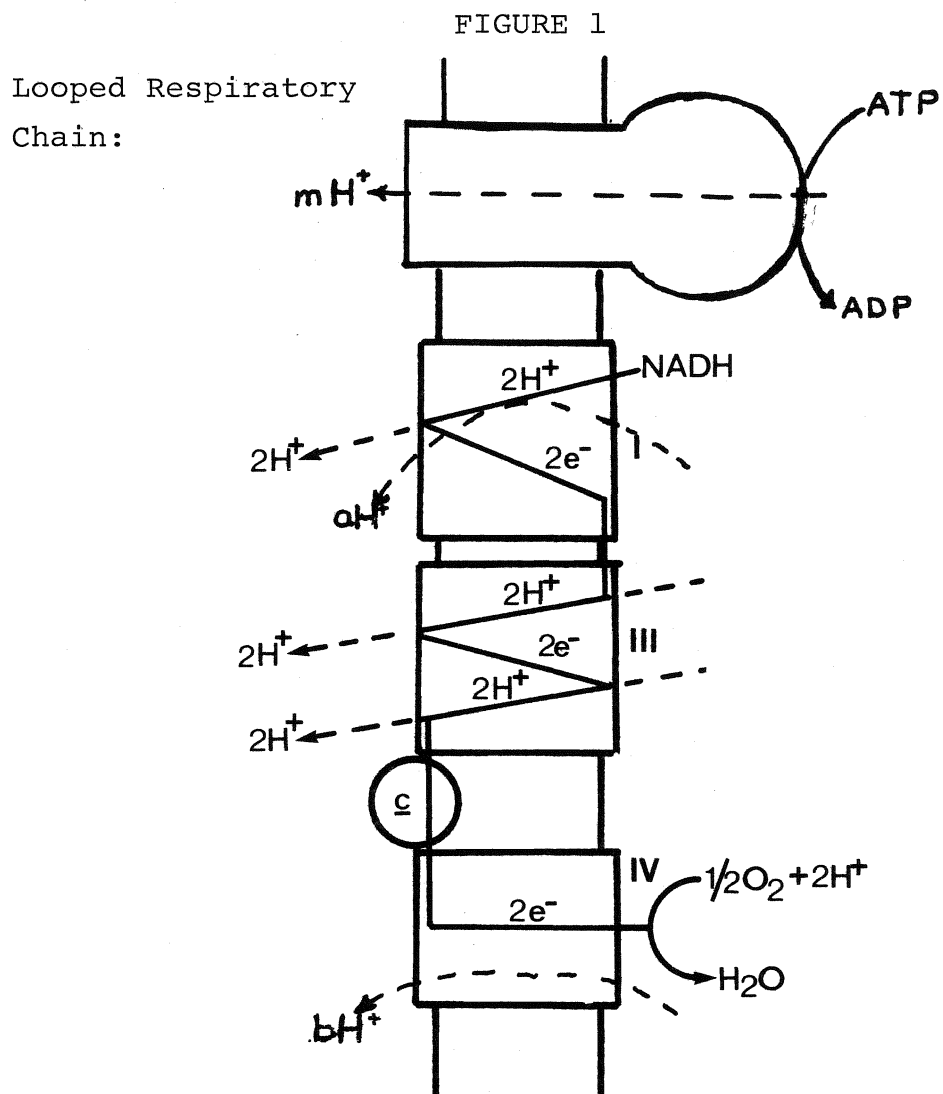
Demonstrates transmembrane hydrogen carriers alternating
with electron carriers. Modified from D. Nicholls, 1982.

i) according to Mitchell (see text)
   m=2, a=0, b=0, H^+/O (NADH)=6.0

ii) according to Brand et.al. (see text)
   m=3, a=1 , b=2, H^+/O (NADH)=9.0

the H^+/Pi symportor H^+/O quotients were raised from 4 to 6 and from 6 to 9 with succinate and NADH linked substrates respectively. Moyle and Mitchell (1978) dismissed these results on the basis that the addition of external phosphate to mitochondrial suspensions did not seem to increase H^+/O quotients. They thus concluded that NEM acts only by stimulating NADPH oxidation in respiratory pulses increasing the H^+/O quotient.

The increased H^+/O ratio was not accountable by Mitchell's direct loop mechanism of H^+ release as originally proposed. Therefore, a conformational pump mechanism was proposed. This also allowed for the possibility that cytochrome c oxidase pumped protons and did not function solely as an electron carrier in a mitchellian loop (Wikstrom, 1977). Wikstrom, (1977) thus proposed that the redox activity of the oxidase led to a strained configuration (low spin ferric heme) which upon relaxation (to high spin) is coupled to electrogenic proton translocation across the membrane. However, as will be seen below, the proton pumping function of cytochrome c oxidase is still in question.

3. Proton Translocation by the Cytochrome c Oxidase Complex

(a) Translocation in Antimycin inhibited Mitochondria

The proton pumping capability of cytochrome c oxidase is still debated. This activity has been studied in antimycin-inhibited mitochondria (Wikstrom, 1977, 1984; Wrigglesworth and Nicholls, 1982; Sigel and Carafoli, 1978; Papa et. al., 1980) as well as in phospholipid vesicles reconstituted with cytochrome c oxidase (Proteau et. al., 1983; Wikstrom and Saari, 1977). The later system eliminates the problem of complexes other than IV contributing protons upon pulsing as well as eliminating the problem of endogenous hydrogenated reductants which upon their oxidation release protons, contributing to the calculation of the H^+/O ratio, (Wrigglesworth and Nicholls, 1982; Papa et. al., 1983a and b). The later process of proton release is a "scalar" event, in which proton observance occurs on the same side of the membrane on which it is released. The former

process of proton release is a "vectorial" event, inferring directionality, with proton observance occurring on the opposite side of the membrane to which it was released. The pumping of protons by cytochrome c oxidase involves a vectorial process.

Proton pumping activity by cytochrome c oxidase has been observed in antimycin A inhibited mitochondria to give $H^+/2e^-$ ratios of 1.6 (Siegel and Carafoli, 1978), with ascorbate and TMPD as substrate, and 2.0 (Wikstrom, 1977) using ferrocyanide as substrate. However, Papa et. al., (1980, 1983b) discounted proton pumping by cytochrome c oxidase in antimycin inhibited mitochondria using ascorbate - TMPD induced proton pulses. Acid production was in fact observed in addition to that due to the oxidation of ascorbate to dehydroascorbate ($0.24\ H^+/e^-$) eq. 3, step 1 below. This proton release was attributed to the oxidation of endogenous hydrogenated reductants. Since the scalar uptake of protons, in the presence of FCCP in the formation of water by cyt. c oxidase was less than that expected by the amount of oxygen utilized, ie. H^+/O ratio less than 2, (see eq. 1 above). This would occur if there was proton release from endogenous hydrogenated reductants (Mitchell and Moyle, 1983).

Lehninger et. al., (1982,1981,1978)and Azzone et.al.(1979) represents the other extreme as far as the stoichiometry for proton translocation is concerned. Lehninger reports $H^+/2e^-$ ratios of approximately 4 for cytochrome c oxidase (as well as the other proton translocating complexes - 1978) in rat liver mitoplasts (mitochondria with the outer membrane removed) and mitochondria. It has, however, been suggested that these high $H^+/2e^-$ ratios are due to the use of a slow responding Clark Oxygen Electrode in estimating electron flux through the cytochrome oxidase complex, thus causing an overestimation of the true $H^+/2e^-$ ratio (see Lehninger, 1981). However, similar results have been obtained with the use of a fast responding oxygen electrode as well as a dual-wave length spectrophotometer following ferrocytochrome c oxidation (Lehninger, 1982). Lehninger explains the low $H^+/2e^-$ ratios obtained by other investigators as being due to experimental conditions. He showed that

$H^+/2e^-$ ratios increase from 2 to 4 as ferrocyanide concentration increases from 6 to 8 mM. Thus previous experiments which used low ferrocyanide concentrations ($<$ 1mM) underestimated the true $H^+/2e^-$ ratios (Wikstrom and Saari, 1977). Low K^+ concentrations as well as the methods of calculation have also been cited as possible sources of error (Lehninger et. al., 1981, 1982).

(b) Translocation in reconstituted Phospholipid Vesicles

In order to eliminate the contribution of protons by endogenous substrates cytochrome c oxidase can be inserted into phospholipid vesicles (Wrigglesworth and Nicholls, 1982; Hinkle et. al., 1972). Proteau et. al. (1983), using ferrocytochrome c induced pulses found H^+/e^- ratios approaching +1 in the presence of valinomycin. Casey et. al. (1979), found ratios of 0.9 H^+ released per ferrocytochrome c molecule oxidized. Siegel and Carafoli(1980) found stoichiometries between 0.58 and 0.75 H^+/e^- when using asc/TMPD induced proton pulses.

Mitchell and Moyle (1983) discount these results on the basis that in the presence of FCCP the net alkinization attributable to the uptake of one H^+ per electron according to step 2, eq. 3.

$$\text{eq. 3.} \quad 1...2AH^- + 4c^{3+} \rightarrow 2A + 4c^{2+} + 2H^+_{out}$$
$$\underline{2...4c^{2+} + O_2 + 4H^+_{in} \rightarrow 2H_2O + 4c^{3+}}$$
$$1 + 2...2AH^- + O_2 + 4H^+_{in} \rightarrow 2A + 2H_2O + 2H^+_{out}$$
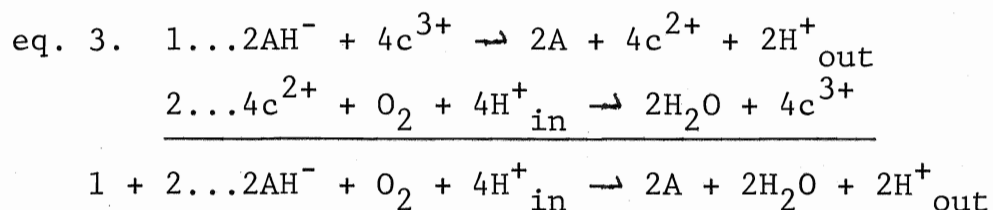
is less than expected stoichiometric aly. This, as mentioned, could occur if scalar protons were being released upon pulsing. Mitchell and Moyle, (1983) thus proposed that proton release occurs due to a redox-linked deprotonation of a complex between cytochrome c and the vesicles phospholipid possibly phosphatidyl-serine.

Papa et. al., (1983) agreed with Mitchell and Moyle in this view stating that the observed acidity seen upon pulsing results from a rupture of protonated salt bridges in the Oxidase-Lipid complex, as the enzyme changes to its active

state and under goes changes in tertiary and quaternary
structure. In support of this, Papa et. al. (1983a) showed
that proton ejection, induced by ferrocytochrome c pulses,
at low ionic strength increased to a value of $2H^+/e^-$, and
was essentially insensitive to FCCP. Thus Papa (1983a)
proposed that the proton release observed at high ionic
strength is what remains of a scalar deprotonation which at
low ionic strength is maximal.

   4.   The Effects of N, N-dicyclohexylcarbodimide (DCCD)
        and the Involvement of subunit III

The effects of DCCD in blocking proton translocation is
not specific for cytochrome c oxidase. The mitochondrial
ATPase is composed of two multiprotein subunits referred to as
F1 and FO. The F1 or coupling factor lies on the surface of
the inner membrane extending into the matrix of the mitochondria.
This is connected to the FO multiprotein subunit which spans
the inner membrane. If the mitochondrial membrane is depleted
of F1, a pH gradient can no longer be maintained, however,
proton conductivity still occurs through FO, which acts as a
proton channel. This proton conductivity can be inhibited by
DCCD, due to the covalent binding of DCCD to a glutamate
residue in the 5.4 K.D. FO subunit (Jagow and Engel, 1980).

In addition to this Lenaz et. al. (1983) found evidence
for DCCD inhibiting proton translocation by the bc1 complex
in beefheart submitochondrial particles. 35µM DCCD was
sufficient to allow half inhibition of Ubiquinol-cyt. c
reductase activity as well as the formation of a succinate-
driven transmembrane pH gradient (monitored by 9-Aminocridine),
without inhibition of succinate oxidation. 150µM DCCD
completely inhibited the latter.

Clejan and Beattie (1983) found that DCCD inhibited
proton translocation by over 60% in reconstituted yeast bc1
complex with only a minimal decrease in cyt. c reductase
activity. DCCD treatment of the bc1 complex reduced antimycin
sensitivity as well as affecting the red shift which occurs

upon antimycin binding. It was therefore proposed that DCCD interacts with cytochrome b in complex III.

Nalecz et. al. (1983) agreed with Clejan and Beattie (1983) in postulating that DCCD binds to cyt. b. However, they found that DCCD inhibited electron flow and proton translocation equally (ie. the H^+/e^- ratio was unaffected) in the reconstituted system.

DCCD in addition to acting as the above sites of inhibition may also increase nonspecifically the H^+ permeability of membranes. Beattie and Villalobo (1982) found that the mitochondrial membrane (yeast) became more permeable to protons (ie. $t_{\frac{1}{2}}$ = 32 sec in DCCD-treated SMPs vs 143 sec in untreated), upon passive HCl additions.

DCCD has also been used to demonstrate the possible proton pumping functions of cytochrome c oxidase. Casey et. al. (1980) demonstrated that ferrocytochrome c induced proton release is inhibited by prior treatment of cytochrome c oxidase with DCCD. They also claimed that the site of inhibition was subunit III of cytochrome oxidase. Mitchell and Moyle (1983) and Papa et. al. (1983a) however, explained such DCCD inhibition as being due to the reaction of DCCD with a carboxyl group (phospholipid), involved in the formation of protonated salt bridges, possibly of phosphatidylserine (Mitchell and Moyle, 1983) or of glutamic residues (Papa et. al. 1983a). Evidence of such a mechanism of action lies in the fact that DCCD reacts with phosphatidylserine in cristae membrane vesicles from mitochondria. A test of this hypothesis as proposed by Mitchell and Moyle is that varing the phosphatidylserine content in cytochrome c oxidase vesicles should affect the extent of the DCCD sensitive redox-linked proton ejection. Casey and Azzi (1983) however, found that the extent of DCCD inhibition did not vary as phosphatidylserine content was varied from 0 to 55%.

Penttila and Wikstrom (1981) gave further support for proton translocation by the oxidase and the involvement of subunit III. They removed subunit III from the isolated enzyme with the use of Triton X-100. The modified enzyme when reconstituted into phospholipid vesicles showed no

difference in electron transfer activity, or main optical properties (respiratory control decreased due to increased proton permeability); however, there was a loss of redox-linked proton ejection. At the same time potassium uptake in the presence of valinomycin went from a K^+/e^- ratio of approx. 2 to a K^+/e^- ratio of approx. 1 (see also Penttila, 1983). The value of 1 K^+/e^- would be expected due to the uptake of 1 H^+/e^- forming water, (see eq. 3 above and eq. 4 below).

$$\text{eq. 4.} \quad 1...2AH^- + 4\underline{c}^{3+} \rightarrow 4\underline{c}^{2+} + 2A + 2H^+_{out}$$

$$2...4\underline{c}^{2+} + O_2 + 4H^+_{in} \rightarrow 2H_2O + 4\underline{c}^{3+}$$

$$3... \quad nH^+_{in} \rightarrow nH^+_{out}$$

$$1 + 2 + 3...2AH^- + O_2 + (4+n)H^+_{in} \rightarrow 2A + 2H_2O + (2+n)H^+_{out}$$
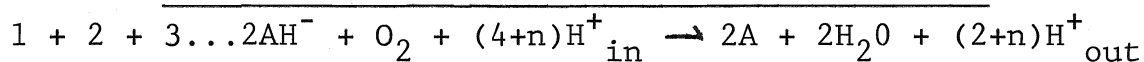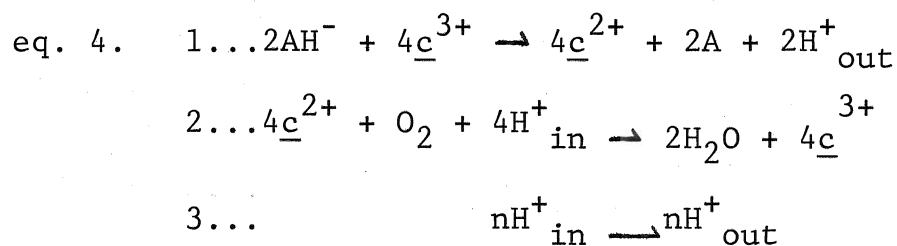
Further work on the involvement of subunit III was performed by Chan and Freedman (1983) who used specific antibodies. These antibodies included an anti-oxidase type (primarily anti-subunits II & IV) and an anti-III type. Proton pulse experiments in the presence of "anti-oxidase" showed no specific effects, but "anti-III" inhibited proton pumping ($>75\%$) without inhibiting electron transfer. Thus it appears that subunit III may be involved in cytochrome $\underline{c}$ oxidase H^+ pumping activity as shown by experiments involving its removal, its inhibition by DCCD and its complexation by anti-III antibodies.

5. The pH Indicator Pyranine

(a) Behavior and Properties of Pyranine in Liposomes

The pH gradient which cytochrome $\underline{c}$ oxidase establishes accross membranes has been followed by the use of pH indicators. Wikstrom (1977) followed the establishment of ΔpH in mitochondria with the pH indicator Neutral Red in SMP (Wikstrom and Saari, 1977) with the pH indicator Phenol Red.

A relatively new pH indicator Pyranine (8-Hydroxy-1,3,6,-pyrenetrisulfonate) has now been characterized (Kano and Fendler,1978; Clement and Gould,1981a). Pyranine is a fluorescent probe of pH,with the amount of 510nm fluorescence excited at 450nm reflecting the concentration of the unprotonated form of the 8-hydroxy group (pka=7.2) and hence the medium pH (Clement and Gould,1981a). The Pyranine polyanion (Clement and Gould 1981a;Kano and Fendler,1978) is a very hydrophilic molecule and binds only slightly to membranes containing negative phospholipids (eg.asolectin). It is thus largely free within the vesicles internal mileau,after external pyranine has been removed via a G-25 Sephadex column.

Clement and Gould (1981a) found that upon the addition of HCl to alkaline asolectin vesicles,there was a biphasic decrease in pyranine fluorescence. A fast initial component was believed to represent an electrically uncompensated H+ influx. The slower valinomycin sensitive phase is believed (Clement et.al.1981a) to represent potassium counterion proton exchange in which the rate of proton influx depends upon K+ counterion movement decreasing the membrane potential. Thus valinomycin accelerated the slow phase of H+ influx in the presence of potassium. The high H+ permeability of the fast phase is in contrast to Nichols and Deamer (1980) and the results found in this thesis and may represent the external binding of pyranine. In addition these results may indicate that valinomycin may at high concentrations act as an uncoupler.

Clement and Gould (1981b) showed that the incorporation of bulky substituted molecules (eg.butylated hydroxytoluene, butylated hydroxyanisole, and p-di-tert-butylbenzene increased the slow phase (rate) of H+ influx in the presence of valinomycin 4-6 fold. These molecules seemed to exert a synergistic effect upon valinomycin mediated K+ transport. Biegel and Gould (1981) found that the passive addition of HCl to alkaline preincubated asolectin vesicles did not show the characteristic biphasic response when followed in a rapid mixing spectrofluorimeter. Under these conditions, a monophasic change in fluorescence was observed, which is accelerated by valinomycin.

The useful pH range of entrapped pyranine is 6.0 to 8.5.

Bell et. al., (1983) incorporated 1 naphthal-3-6-disulphonate (pka=8.2) into vesicles together with pyranine, thereby increasing the pH sensitivity in more alkaline regions. In this way it was possible to follow active internal pH changes in reconstituted bacterialrodopsin proteoliposomes (Bell et. al., 1983).

Damiano et. al., (1984) also studied pH changes in E.coli with entrapped Pyranine. Changes in the internal pH during the oxidation of D-Lactate or succinate by the E.coli respiratory chain were followed. The observed alkalinization upon substrate addition to the E.coli was nigericin and FCCP sensitive. ΔpH generation was also studied passively by Damiano (1984) following the imposition of an outward acetate diffusion gradient. This led to a transient alkalinization of the internal space of E.coli which was proportional to the acetate gradient imposed.

C) Ionophores in Bioenergetic Systems

1. Electrogenic Mobile Ionophores - K^+ Transport

Ion transport is electrophoretic, if the ion is trans-ported in response to a preexisting membrane potential. Electrophoretic movement of ions may lead to a dissipation of the existing membrane potential.

Valinomycin and nonactin represent this class of ionophores. Lauger (1972) showed that valinomycin has a specificity of $Rb^+ > K^+$, $Cs^+ >> Na^+$, Li^+. Potassium, valinomycin mediated, transport was greater than sodiums by a factor of 10^3. Nonactin shows a selectivity of K^+, $Rb^+ > Cs^+ > Na^+$, (Eisenman and Horn, 1983).

Valinomycin and nonactin are produced by microorganisms and possess antibiotic properties. Valinomycin is a depsipeptide and is composed of alternating amino acids and -hydroxyaminoacids (Lauger, 1972). The structure of the valinomycin-K^+ complex (Fig. 2) has been described (Lauger, 1972). The oxygen atoms of the six ester carbonyl groups form an octahedral cage surrounding the potassium, thus replacing

FIGURE 2

Valinomycin - K+ complex:
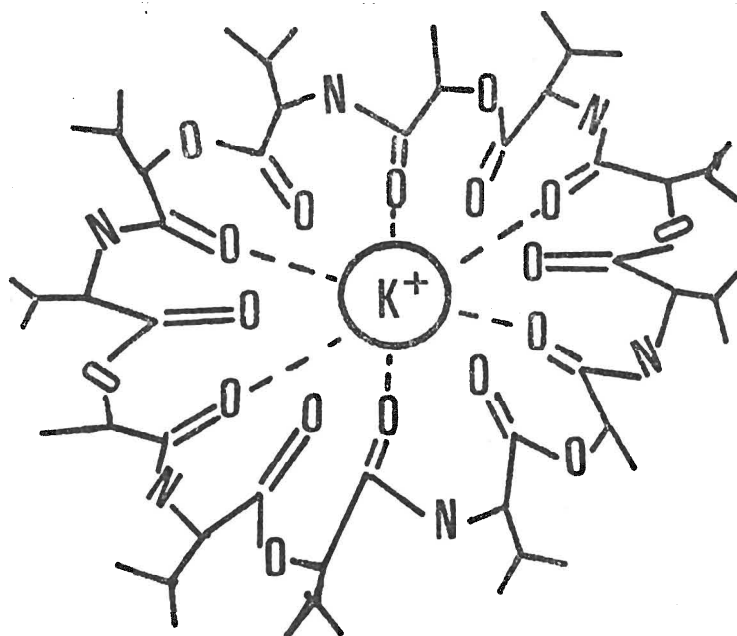
from Reed (1979)

the complexed ions hydration shell. The exterior of the
shell is strongly hydrophilic. Nonactin however, is a cyclic
compound which contains four ether and four ester bonds (see
Lauger, 1972; Reed, 1979). The ion is stabilized within the
molecule by the eight oxygen atoms of the ether and esters.

Both nonactin and valinomycin, in uncomplexed form, are
neutral carriers. Upon complexation with cations these neutral
ionophores become charged species. Both the uncomplexed and
complexed forms of these carriers are capable of diffusion
across the membrane. Thus upon cation complexation the carrier
and cation will diffuse across the membrane in response to the
existing membrane potential. The complex then dissociates,
releasing the ion into the aqueous phase on the opposite
side of the membrane and the free carrier diffuses back across
the membrane completing the cycle (Lauger, 1972; Reed, 1979).

The usefulness in bioenergetic systems of these two
ionophores lies in their dissipation of membrane potentials.
As previously mentioned, this is a requirement for the
observation of proton pumping by respiratory chain components.
Both valinomycin (Krab and Wikstrom, 1978) and nonactin
(Sarti et. al., 1983), have been used to study proton pumping
activity by cytochrome $\underline{c}$ oxidase.

2. Protonophores; Ionophores which conduct H^+

There is a long list of molecules which conduct protons
across biological membranes. These include FCCP (Trifluoro-
methoxy carbonylcyanide), CCCP(carbonyl cyanide m-chloro-
phenylhydraxone), S-13 (5-chloro-3-tert-butyl-2-chloro-4-
nitrosalicylanilide), DNP (2, 4-dinitrophenol), and gramicidin.

The mechanism of action of protonophores which uncouple
respiration from ATP synthesis was originally explained in
terms of the chemical hypothesis. In accordance with this,
Slater (1953), proposed that the action of the classical
uncoupler dinitrophenol could involve the promotion of hydrolytic
cleavage of the high energy intermediate. The high energy
intermediate would then no longer be available for ATP formation
(see also Borst and Slater, 1961; Chance et. al., 1963).

Several authors (Sanadi, 1968; Kurup and Sanadi, 1968; Wilson and Azzi, 1968; Margolis et al., 1967) found that an apparent stoichiometric relationship existed between uncoupler concentration and the amount of respiratory chain complexes present at 100% uncoupling. This would not be expected if uncouplers functioned solely as proton conductors, across the membrane as proposed in the Chemiosmotic Hypothesis.

Sanadi (1968, Kurup and Sanadi, 1968) with the use of thymol blue absorbance as an indication of 100% uncoupling, found that only one FCCP molecule per respiratory chain (or cyt. b molecule) was required at sites II and III, but as many as 14 were required for 100% uncoupling at site I. Katre and Wilson (1977) found a stoichiometry of 1.6 ± 0.3 molecules/cytochrome a with N_3CCP as an uncoupler, following release of respiratory control as a measure of uncoupling. Wilson and Azzi (1968) and Wilson, (1969) found that with the uncoupler S-13, 1.05 ± 0.06 molecules/cyt. a resulted in full uncoupling (full ATPase activity). They believe that this was due to a specific uncoupling at the cytochrome oxidase site, which resulted in uncoupled respiration at all 3 sites by equilibration through X∼I. Margolis et. al. (1967) found that under slightly different conditions with the uncouplers FCCP and CCCP, only one molecule of uncoupler was required for as many as 27 potential uncoupling sites. They concluded that these uncouplers bound to the high energy intermediates and not directly to coupling sites. Thus, although evidence was supposedly obtained for the involvement of stoichiometric amounts of uncoupler, the values of the stoichiometry varied between different groups.

Specificity and reversibility of uncoupler binding have also been studied. Evidence had been obtained showing uncoupler binding to proteins which lent support to the chemical coupling hypothesis (Hanstein and Hatefi, 1974abc). Other investigators who support the chemiosmotic hypothesis, have found evidence for the localization of uncouplers in the phospholipids of their membrane systems (Terada, 1975, Hemker, 1962; Bakker et. al., 1974).

Hanstein and Hatefi, (1974ab) used the structural analogue of 2,4-dinitrophenol, NPA (2-azido-4-nitrophenol) in order to localize the site of uncoupler binding. The uncoupler NPA when irradiated with visible or U.V. light covalently binds to neighbour molecules. It was found that 40% of NPA was associated with protein (M.W. = 20,000 to 30,000) and not with mitochondrial lipid. The authors concluded that uncoupling was achieved by binding to the high energy intermediate, dissipating its energy.

Hemker (1962) used a series of alkyl substituted 2,6-dinitrophenols of various lipid solubilities to study the effect this had on their uncoupling action. He showed that the degree of stimulatory action (ATPase activity) found at a given concentration is dependent upon the lipid solubility of the compound. The result was that a larger concentration of a less lipid soluable compound was needed to give the same effect as a small concentration of a more lipid soluable compound. These conclusions support the chemiosmotic hypothesis.

Terada (1975) studied the spectral properties of SF 6847 (a potent uncoupler) in an aqueous medium, in the presence of liposomes(negative, neutral, positive), ethanol, bovine serum albumin, mitochondria and phospholipid depleted mitochondria. In aqueous solution SF 6847 shows two absorption peaks at 365 and 456nm. These peaks vary characteristically in the presence of liposomes, depending on the surface charge. It was found that such variations in the presence of mitochondria mimic the changes in the presence of neutral liposomes or ethanol. The spectra did not resemble those in the presence of positive, negative liposomes, bovine serum albumin, or phospholipid depleted mitochondria. It was therefore, concluded (Terade, 1975) that the uncoupler SF 6847 was most likely localized in the phospholipid moiety of the mitochondrial membrane. This lent support for the chemiosmotic hypothesis where uncouplers act as protonophores translocating protons across the mitochondrial membrane by-passing the ATPase.

Two different mechanisms of proton conduction are represented by FCCP and Gramicidin: mobile carriers (FCCP) or channel formers (Gramicidin). Channel forming ionophores transport ions at a greater rate but with less selectivity than do mobile carrier ionophores.

Gramicidin A is a linear molecule, 15 amino acids long, which dimerizes in the membrane. The dimer forms a channel approx. 3nm long and 0.4nm in internal diameter which spans the membrane (Reed, 1979). Falcone and Hadler,(1968a) using ATP dependent, Gramicidin induced mitochondrial swelling, found a selectivity of cations of $Rb^+ = Cs^+ = K^+ = Li^+$ $>NH_4+ = Na^+$. Like some other channel forming ionophores Gramicidin induced shows poor selectivity. Gramicidin also transports protons (Reed, 1979) at high rates. Never the less, Henderson, et. al.,(1969) found that upon further addition of mobile uncoupling agents at low Gramicidin concentrations a stimulation of H^+ transport occurred. A comparison between proton and cation selectivity was not addressed in this study.

FCCP functions as a mobile proton carrier (Benz and McLaughlin, 1983; McLaughlin and Dilger, 1980). A model for uncoupling may be proposed as follows: Across the membrane exists a membrane potential; in the case of reconstituted proteoliposomes this is negative inside. FCCP exists in a deprotonated form (anion) and a protonated form (neutral). Under the influence of the membrane potential the anionic form of FCCP (A-) will diffuse across the membrane to the outer surface (+). This movement increases the concentration of A- on the outer surface. The increased concentration "pushes" the reaction (eq. 5) to the right.

eq. 5 $$A^- + H^+ \rightleftharpoons HA$$

Thus, a proton is taken up by the anionic form of FCCP from the outer aqueous phase forming the undissociated acid HA. As the concentration of HA increases a concentration gradient builds and HA diffuses across the membrane to the inner face, At the inner face of the membrane the depletion of A- causes a reversal of the above reaction releasing a proton into

the inner aqueous phase. Thus a proton has been translocated across the membrane.

FCCP has been used in bioenergetic systems to monitor the scalar reaction (eq. 1), of cytochrome c oxidase both in reconstituted systems (Krabb et. al., 1978), and in mitochondria (Papa et. al., 1980). In conjunction with valinomycin it will abolish $\Delta\mu H^+$ completely.

Complex formation occurs between FCCP and valinomycin when used in conjunction in the presence of potassium. O'Brien et. al., (1978) gave spectrophotometric and circular dichromism evidence for a 1:1:1 complex formation both in aqueous and in nonpolar solvents. In the presence of valinomycin and K+ the absorption spectrum of FCCP (at 380nm) is significantly changed and there is also a large induced circular dichroism signal. It is possible that it is the ionic form of FCCP which forms the complex, (O'Brien et. al., 1978). Other uncouplers such as SF 6847 also seem to form a ternary complex with valinomycin plus potassium (Yamaguchi et. al., 1978; Yoshikawa and Terada, 1982). This uncoupler-valinomycin complex can then lead to electroneutral exchange of K+/H+ (Yamaguchi et. al., 1978), as with Nigericin.

3. Electroneutral Ionophores - (K+/H+ exchange)

Nigericin catalyzed H+/cation exchange with a selectivity for cations as follows: K+, Rb+ > Na+ > Cs+ > Li+ (Henderson et. al., 1969). The process is electroneutral.

Nigericin is a linear molecule with heterocyclic oxygen containing rings together with hydroxyl groups (Reed, 1979). In the membrane it forms a cyclic structure similar to that of valinomycin, with five oxygen atoms binding the metal ion (Reed, 1979). Nigericin upon binding a cation dissociates a proton, and forms a neutral complex capable of diffusion across the membrane. It is also permeant in its protonated, uncomplexed form.

Nigericin in bioenergetic systems may be used in conjunction with valinomycin to uncouple electron transport from ATP generation (Montal et. al., 1970).

4. Voltage Gated Ionophores-Alamethicin

Alamethicin is a cyclic polypeptide of molecular weight approximately 1700. This antibiotic's primary structure is known (Payne et. al., 1970). Alamethicin shows selectivity for K+ > Rb+ > Cs+,Na+ (Reed, 1979). Fox et. al., (1982) proposed models for channel formation by this ionophore based on the crystal structure of alamethicin in nonaqueous solvent.

The uniqueness of the alamethicin channel is that its conductance is strongly voltage and electrolyte concentration-dependent increasing e-fold for every +5mV of applied voltage (Mueller and Rudin, 1968).

Alamethicin conductance also depends strongly on the concentration of the ionophore (Gordon and Haydon, 1972) suggesting that the alamethicin ion channel consists of 6 or more molecules (Fox and Richards, 1982).

II   Summary and Problems

As indicated above, the major problem to be resolved experimentally is whether or not cytochrome c oxidase functions as a proton translocator. Several authors (Papa et. al., 1983a; Mitchell and Moyle, 1983), believe that cytochrome c oxidase does not translocate protons vectorially. According to these workers, the protons which appear in the external medium upon O_2 or ferrocytochrome c induced pulsing are a result of a redox-linked deprotonation of cytochrome c-lipid complex salt bridges or of oxidase-lipid complexes.

Papa et. al., (1983a) gave evidence for scalar proton release at low ionic strength with ferrocytochrome c induced pulses. An increase in the extent of the redox-linked acidification was observed upon ferrocytochrome c pulsing, which was essentially FCCP insensitive. This was interpreted as being the result of a scalar deprotonation, which also occurs at high ionic strength but is maximal at low ionic strength. Were these results interpreted correctly, however, and if so, does this also occur with oxygen induced proton pulses?

Further debate concerning redox-linked proton release is found in the inhibition of oxygen or ferrocytochrome c induced pulsing by DCCD. Both Papa et. al., (1983a) and Mitchell and Moyle, (1983) believe that DCCD modifies the salt bridges involved in the scalar release of protons and not by affecting oxidase function as suggested by Casey et. al., (1980). If this is the case, will DCCD affect the formation of a pH gradient if this is followed internally by an internally trapped pH indicator?

A further concern of this study was to examine the effects of certain ionophores on oxidase turnover and proton translocating functions. A requirement for collapsing agents (eg. valinomycin and nonactin) in order to observe proton translocation has been clearly demonstrated, (Krab and Wikstrom, 1978; Sarti et. al., 1983). Quantitation and comparison of these requirements has been less closely documented. These effects were therefore further characterized.

III Materials and Methods

A)   Preparation of Cytochrome c Oxidase from Beef Heart

The beef heart was obtained fresh from a local slaughter house and kept on ice (1hr. max.) until the isolation of cytochrome c oxidase was inititated. Isolated beef heart cytochrome c oxidase was prepared according to Kuboyama, Yong and King, (1972) with tween 80 substituting for emasol and stored in 100mM NaPi pH 7.4, 0.25% tween 80 at -70°C (see flow chart, Fig. 6 for the preparative sequence). Typical concentrations obtained were 230-240μM cytochrome aa$_3$.

B)   Protein and Heme Determination

Protein content of the isolated oxidase was determined by the following biuret method (Jacobs et. al., 1956): 0.01 to 0.15 ml samples (isolated oxidase) were mixed with 0.1 ml 10% (w/v) sodium deoxycholate and 0.05 ml 30% H_2O_2. After incubation at room temperature for 2 to 3 minutes the volume was adjusted with distilled water to 1.0ml and 4.0ml biuret reagent (1.5gm/L cupric sulphate, 6.0g/L Na^+/K^+ tartrate, 3% NaOH, stored in the dark) was added. The samples remained in the dark at room temperature for at least 2 hours. Absorbances were measured on a Bauch and Lombe Spectronic 20 spectrophotometer (Analitical Systems Division, Scarbourgh, Ontario), using bovine serum alumin as a standard.

Cytochrome c oxidase concentration was determined spectrophotometrically using a Du-7 UV-Vis Scanning Spectrophotometer (Beckman Instruments Inc., Irvine, California, U.S.A.); a $\Delta\Delta$E value of 27mM at 605 minus 630nm, reduced minus oxidized was used (Proteau et.al. 1983).

C)   1.   Preparation of Cytochrome c Oxidase Containing Phospholipid Vesicles

Reconstitution was performed essentially as Racker, (1973). 0.5g of dry asolectin (40% L - phosphatidylcholine, Sigma type IV-S was dispersed in 10.0ml of 50mM potassium

phosphate buffer (unless otherwise stated below), pH 7.4, by rapid shaking on a mechanical mixer. Cytochrome c oxidase was then added (0.1ml stock) to give a final phospholipid/ protein weight ratio of approx. 80:1. The mixture was gently handshaken, placed in a container surrounded by ice and sonicated to clarity (10-15 min.) with a Heat-system Ultrasonics model W375 sonicator set on the pulsed mode at 30% duty cycle. The solution was then centrifuged at 15,000 rpm for 10min. (in a IEC B-20A centrifuge), and any pellet was disgarded. Proteoliposomes were stored at 4°C.

2. Preparation of Cytochrome c Oxidase Proteoliposomes Containing Pyranine

Pyranine containing vesicles were prepared as above but the asolectin was suspended in 10ml of 5mM Tricine/Mes buffer, pH 7.4, containing 100mM choline chloride, and 5mM KCl. Pyranine (8-Hydroxy-1,3,6,-pyrene-trisulfonic Acid, Trisodium Salt) was added (2mM) before mechanical mixing. Vesicles were allowed 24 hours for equilibration prior to use and the external pyranine was removed by passing through a G-25 sephadex column, (approx. 1.7cm-dia., 16cm-long), equilibrated with the internal medium of the vesicles.

D) Polargraphic Assay System

Oxidase turnover was followed using an oxygen electrode (Yellow Springs Instrument Company, Yellow Springs, Ohio, U.S.A.) maintained at constant temperature using a Haake temperature bath (Model F4391), and a polarizing box and converter (Brock University) and recorded on a Perkin-Elmer recorder (model 56, Hatachi, Tokyo, Japan). The total volume of the reaction vessel was 4.14ml. Reagents were added using Hamilton syringes; and were mixed upon addition by a magnetic stirring bar in the reaction vessel. The vessel was placed on a Thomas-Magnematic stirrer (model 14, Arthur H. Thomas Company, Philadelphia, Pennsylvania, U.S.A.) maintained at a constant rate by a rheostat (Omite Manufacturing Company, Stokie, Illinous, U.S.A.) Fig. 3.

FIGURE 3

Oxygen electrode system:
A: water bath
B: rheostat
C: magnetic stirrer
D: stir bar
E: reaction vessel temperature bath
F: reaction vessel
G: oxygen electrode
H: polarizing box and converter
I: recorder

FIGURE 3
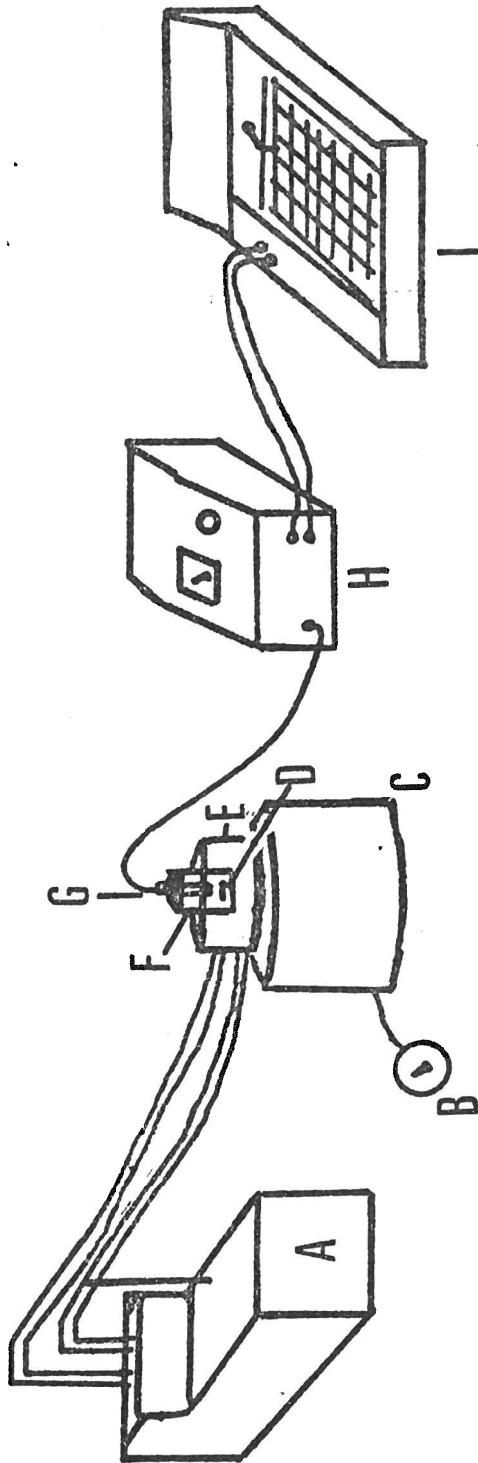
E)   PH and pk+ Electrode System

Vesicles were passed down a G-25 Sephadex column, at room temperature at a rate of approx. 1-2 ml/min. (dimensions as above) to remove external buffer prior to studing proton translocation activity of cytochrome c oxidase. The column had been previously equilibrated with 50mM K_2SO_4, 50mM Na_2SO_4 plus 100mM Mannitol and 35 mM Sucrose depending on the medium (Ionic Strength) required to observe proton translocation in the pH electrode system.

PH changes were monitored in a thermostatically controlled glass chamber (8ml) fitted with a magnetic stirrer, by using a glass pH electrode, Fig. 4, (Type G2222 B or C, Radiometer, Copenhagen, Denmark), connected to a digital pH meter (pH M64) and recorder (REA 100, Radiometer, Copenhagen, Denmark). A Calmel double bridge reference electrode (K701, Radiometer) was used. The primary salt bridge contained saturated KCl while the secondary salt bridge contained saturated NaCl (for high ionic strength pulsing) or half saturated NaCl for low ionic strength studies in order to cut down noise levels.

A potassium ion electrode (F231 2K, Radiometer) was also fitted in the vessel, (Fig. 4) connected to a separate digital pH meter and recorder as above and to the same reference electrode.

Hamilton syringes were used for reagent additions. PH changes were monitored between pH 7.3 to 7.5.

For O_2-induced proton translocation an air saturated medium was prepared by bubbling filtered air through a solution which was identical to that on the outside of the ves icles (ie. 50mM K_2SO_4), for two hours prior to pulsing. The reaction mixture containing the ves icles was bubbled with Nitrogen for 5-10 min. prior to pulsing to achieve an anerobic solution. Preparation of reduced Cytochrome c for ferrocytochrome c induced pulsing is described below.

FIGURE 4

Reaction vessel for following pH and pk changes:

A: reference electrode
B: pH electrode
C: potassium electrode
D: reaction vessel

F)   Fluorimetry System

The excitation of pyrine fluorence at 460nm was observed (Clement & Gould, 1981a) using PM tube protected by an Oriel Long Pass filter (num. 5129, above 480nm) in a converted Gilford single beam spectrophotometer (2400 from Gilford Instruments Co., Oberlin, Ohio, U.S.A.), to which a Kraayenhoff curvette system was fitted. The Gilford tungston lightsource and monochromator provided the exciting light and the Hamamatsu photomultiplier was mounted at 90° to the light beam. Fig. 5. Ports exist in the reaction vessel (approx. 2.7ml) for the placement of an oxygen and pH electrodes. Stirring is achieved by a constant overhead stirrer.

Constant temperature (30±1°C) was maintained by a Berton P.I.D. temperature controller (model 215). Results were recorded on a Cole-Palmer 3 channel recorder (model 8373-30, Cole Palmer Instrument Company, Chicago, Illinois).

Additions were made into the reaction curvette with Hamilton Syringes.

G)   Column Chromatography

In order to estimate the effects of lauryl (dodecyl) maltoside on cyt. $\underline{c}$ binding to aa_3 containing phospholipid vesicles two G-100 Sephadex columns were employed. One column was eluted with 5mM KPi and the other with 5mM KPi plus 0.03% Maltoside. The column containing only 5mM KPi was then loaded (on top) with 1.5ml of vesicles (aa_3=2.95μM) and 0.1 ml of cyt. $\underline{c}$ (14mg/ml). The second column containing 0.03% multoside was loaded as the first, but 30mg of multoside was also present to start. The columns were then run simultaneously and 1.1ml fractions were collected using a Model MF mini-Escargot fraction collector, (Gilson Medical Electronics, Inc., Middleton, Wisconsin).

The fractions were then assayed using a Du-7 UV-Vis scanning spectrophotometer (Beckman Instrument Inc., Irvine, California, in order to determine aa_3 and cyt. $\underline{c}$ concentrations in each fraction.

FIGURE 5

Fluorimetry System

A: recorder

B: polarizing box and converter

C: oxygen electrode

D: monochromator power supply

E: power source (PM tube, stirrer, temperature controller)

F: monochromator

G: photomultiplier tube (fluorescence)

H: photomultiplier (absorbance)

I: overhead stirrer

J: filter

K: ports for water bath

FIGURE 5

H)   Reagents

Horse heart cytochrome c (Sigma type VI) was prepared at a stock concentration of 14mg/ml. Ferrocytochrome c was prepared by reduction with sodium ascorbate acid (Sigma No. A-7631) overnight. The reduced cytochrome c was then passed down a G-25 column to remove the ascorbate.

T.M.P.D. (stock 0.15M) was obtained from Aldrich Chem. and FCCP (stock 1mM) was obtained from Dupont (Wilmington, Delaware, U.S.A.). Valinomycin (stock 1mg/ml) was obtained from Sigma (No. V-0627, St. Louis, Missouri, U.S.A.).

Lauryl-B-D-Maltoside (dodecyl) was obtained from Calbiochem (La Jolla, California). Sodium Dithionite was obtained from B.D.H. Chemicals Ltd., Poole, England. All other chemicals were of analytical grade.

All buffers were stored at room temperature except 5mM K_2SO_4, 100mM Mannitol, 35mM Sucrose which was stored at 4°C. Stock solutions of cyt. c, TMPD, Ascorbate, Valinomycin and FCCP were kept frozen.

FIGURE 6

Flow Chart for Oxidase Isolation

Beef Hearts - connective tissue and fat removed

↓

Mince

↓

Put into 5mM NaPi
  Buffer pH 7.4

↓

Wring out ——————————————————→ Blood

↓

Mince

↓

Homogenize in 20mM
NaPi Buffer pH 7.4

↓

Centrifuge
(3700 r.p.m. for 15min) ——————→ Discard precipitate
                                 (cell membranes...)

↓

Acidify supernatant
  (pH 5.6)

↓

Precipitate [to borate phosphate
     Buffer (for SMPs)]

↓

Add Na cholate final conc. 1%. Add
solid $(NH_4)_2SO_4$ to 25% sat. (pH to 8.0)

↓

Stand for 1. hour

↓

Add $(NH_4)_2SO_4$ to 35% sat.

↓

Centrifuge (13,000 r.p.m. for 20min.) ——→ Disgard supernatant
                                            (antimycin A-sensitive
                                            cyt. c reductase)

↓

PPT to 0.1M NaPi. Add Na cholate-
final concentration 2%

↓

Add $(NH_4)_2SO_4$ to 25% sat. pH 7.4-7.8

↓

FIGURE 6 - con't.

Incubate 10 - 12 hours.

Centrifuge (13,000 r.p.m. for
20 minutes)

Greenish Brown snt.   Add 100mM   PPT for NADH dehydrogease
NaPi pH 7.4 with 1.5% cholate
        Add $(NH_4)_2SO_4$

Fractioniate with $(NH_4)_2SO_4$ and
differential centrifugation
disgarding tan prec.

Take supernantant to 37%
$(NH_4)_2SO_4$. spin.

Green precipitate taken up in
100mM NaPi pH 7.4 with 1% Tween 80

IV Theoretical and Data Analysis

Fig. 7 demonstrates the appearance of a typical O_2 induced proton pulse. The extent of acidification is corrected for proton backflow by extrapulation from the decay phase to the point of O_2 addition as seen in Fig. 7. This acidification is represented by n. The s value represents the scalar alkalinization (eq. 1) due to the reduction of oxygen by cytochrome c oxidase.

The reciprocal of the values of B, and A seen in the accompanying trace (HCl pulse), represent the total buffering capacity and external buffering capacity respectively.

The calculation for n values in terms of H^+/e^- ratios were performed as follows:

eq. 6a)

$$n\ (H^+/e^-) = \frac{\dfrac{A}{[HCl]} \times n}{[O_2] \times 4}$$

Where $n\ (H^+/e^-)$ represents the number of pumped protons per electron used to reduce molecular oxygen via cytochrome c oxidase. The factor 4 arises since 4 electrons are required to reduce one molecule of oxygen. The term $[O_2]$ represents the oxygen concentration in a known amount of unbuffered medium used to induce pulsing. Thus, if $100\mu l$ of air saturated (approx. $230\mu M\ O_2$ at $30°C$) medium is injected to induce proton translocation, eq. 6a) revises to:

eq. 6b)

$$n\ (H^+/e^-) = \frac{\dfrac{A}{[HCl]} \times n}{92\ (neq/e^-)}$$

The alkinization seen following the acidification is calculated in terms of s (H^+/e^-) as seen in eq. 7:

eq. 7.

$$s \ (H^+/e^-) = \frac{\frac{B}{[HCl]} \ x \ s}{[O_2] \ x \ 4}$$

where B represents the total buffering capacity.

$T\frac{1}{2}$ values were also calculated as seen in Fig. 7 and reflect the decay rate of the observed acidificatin.

FIGURE 7

Appearance of a typical O_2 pulse: calculations.

    3.5ml of proteolipomsome (aa_3 conc. = .398μM)
    3.5ml of 50mM K_2SO_4, 0.11mM cyt. c,
    0.25mM ascorbate, 1.25μg/ml valinomycin,
    1.25μM FCCP. Temperature = 28°C
    pH = 7.4

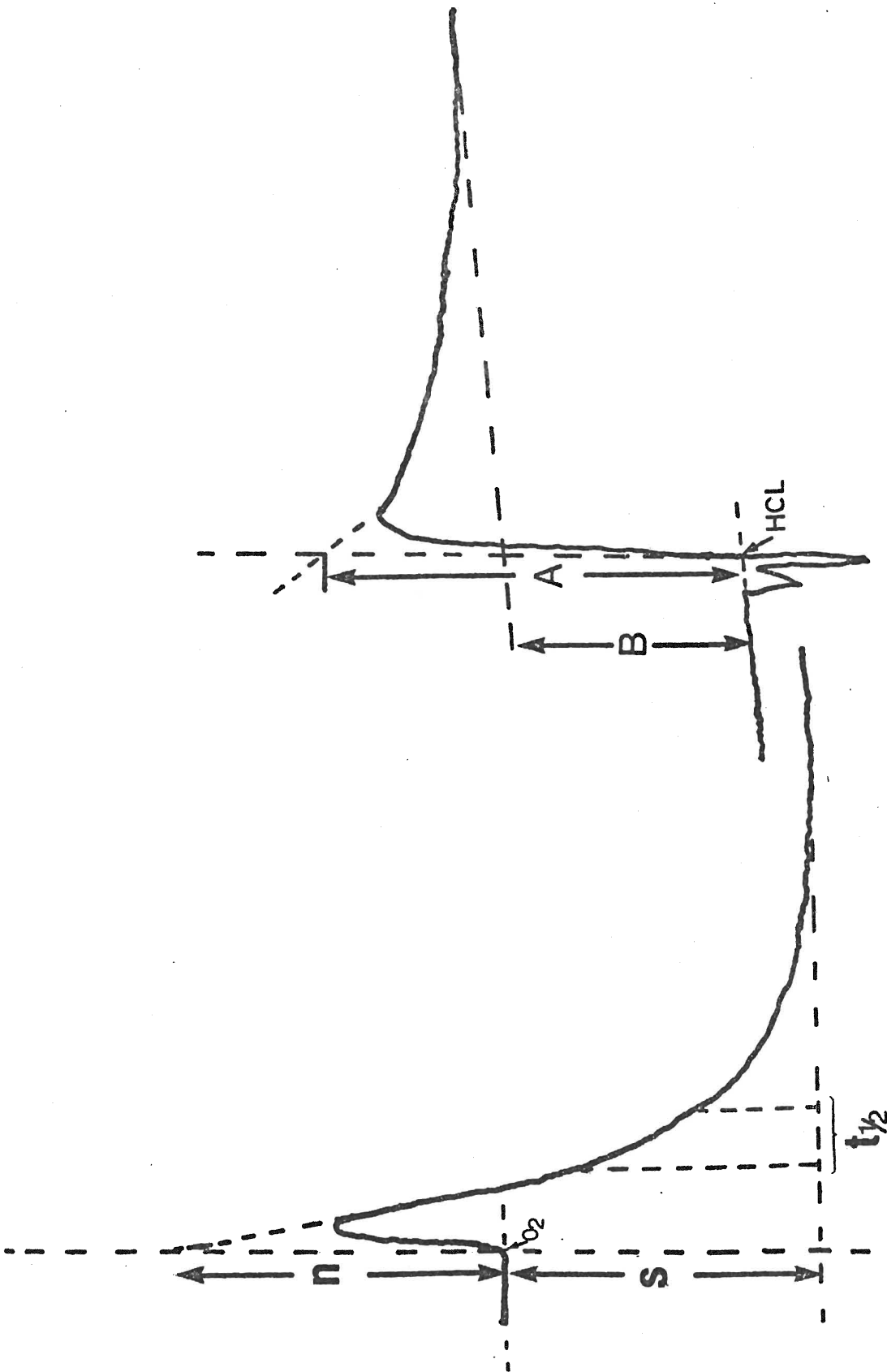   n = acidification, ie. pumped protons

    s = internal alkalinization due to the scalar
        reaction of cyt. aa_3.

    A = reciprocal of external buffering capacity

    B = reciprocal of total buffering capacity

    $t\frac{1}{2}$ = the decay of acidification (time)

FIGURE 7

V RESULTS

A) Is Proton Translocation a Scaler or Vectorial Process?

   1.Effects of Detergents on Activity:

If proton ejection is a scaler event then the lysis of aa$_3$ containing vesicles by a detergent (while maintaining oxidase turnover and cytochrome c binding to the phospholipid) should not effect the observed proton translocation. The effects of three detergents; lauryl maltoside,DOC, and Triton X-100, on cytochrome c oxidase turnover were examined polarographically, (Fig. 8 to 10). For a given detergent, concentration (%) was plotted against turnover number (μeqe$^-$/aa$_3$/sec). This was per-formed in the absence of ionophores,in the presence of valino-mycin, and in the presence of valinomycin plus FCCP. Conditions for the assays are given in the figure legends.

Fig.8 shows the effects of triton X-100 on oxidase turn-over. In the presence of val. and FCCP, there is an inhibition of turnover which is maximal at 0.025% triton X-100; above this level no stimulation of turnover is seen upon ionophore addition. At lower triton concentrations the addition of iono-phores leads to an enhancement of oxidase turnover.

The effect of DOC on oxidase turnover is more complex, Fig.9. Ionophore addition at DOC levels below 0.05% leads to a stimulation of oxidase turnover. Above this concentration a slight inhibition is observed upon ionophore addition. Maximal turnover (180s^{-1}) in the presence of valinomycin and FCCP how-ever remains relatively constant throughout the concentration range of DOC tested.

The effect of lauryl maltoside addition on oxidase turnover was stimulatory throughout the concentration range tested. Turnover increased approx. 190 to 330s^{-1} in the presence of valinomycin plus FCCP. Above 0.025% maltoside, there was little effect on oxidase turnover upon addition of ionophores. At maximum maltoside concentrations (0.125%) there was almost two fold stimulation of oxidase turnover, (Fig.10).

The effects of these three detergents were further explored by varying cytochrome c concentration at three different

FIGURE 8

The effects of Triton X-100 on Cytochrome $\underline{c}$ Oxidase
Turnover in reconstituted vesicles:

Ordinate shows oxidase turnover in $\mu eq.e^-/sec$
Abscissa shows Triton concentration

13.2nM vesicular aa_3, 4.9mM ascorbate, 0.18mM
TMPD, 12.2μM cytochrome $\underline{c}$, 1.22μg/ml valinomycin,
1.22μM FCCP. Total volume = 4.1ml,
medium - 50mM KPi pH- 7.4

$\triangle$: control (+triton)

$\square$: Triton, + valinomycin

$\bigcirc$: Triton, valinomycin, + FCCP

FIGURE 8
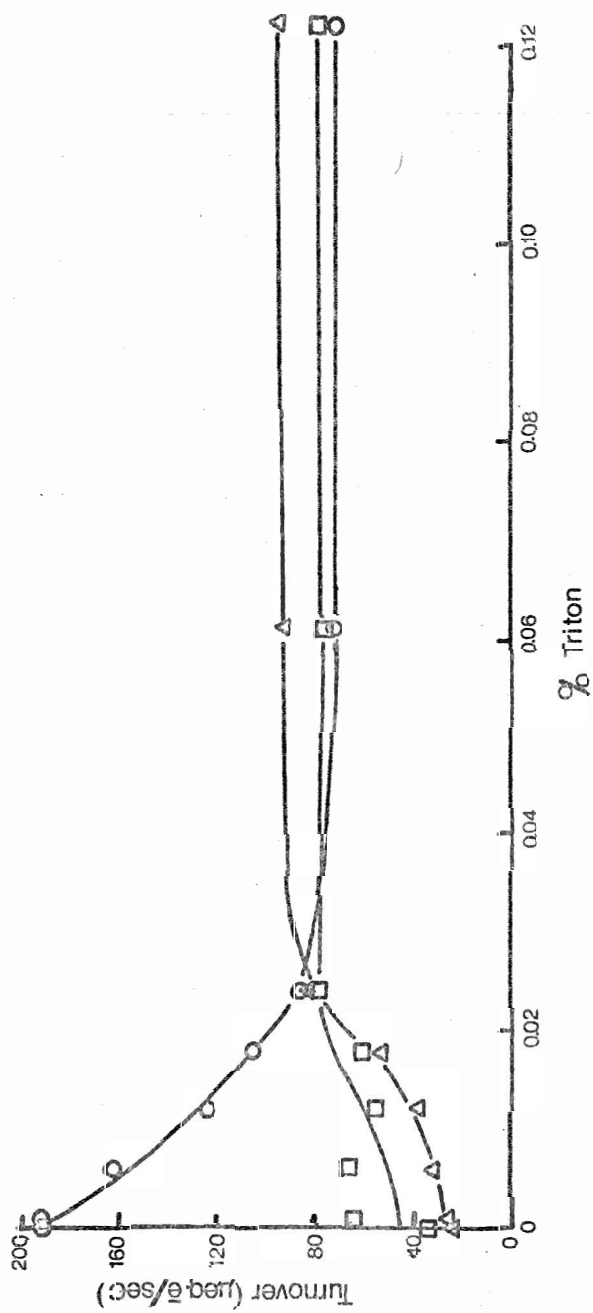
FIGURE 9

The Effects of Deoxycholate on Cytochrome c Oxidase
Turnover in Reconstituted Vesicles:

Ordinate shows oxidase turnover in $\mu eq.e^-$/sec
Abscissa shows DOC concentration

13.2nM vesicular aa_3, 4.9mM ascorbate, 0.18mM
TMPD, 12.2μM cytochrome c, 1.22 μg/ml valinomycin,
1.22μM FCCP. Total volume = 4.1ml. Medium- 50mM
KPi pH= 7.4.

▲: control (+DOC)
☐: DOC, + valinomycin
●: DOC, valinomycin, + FCCP

FIGURE 9
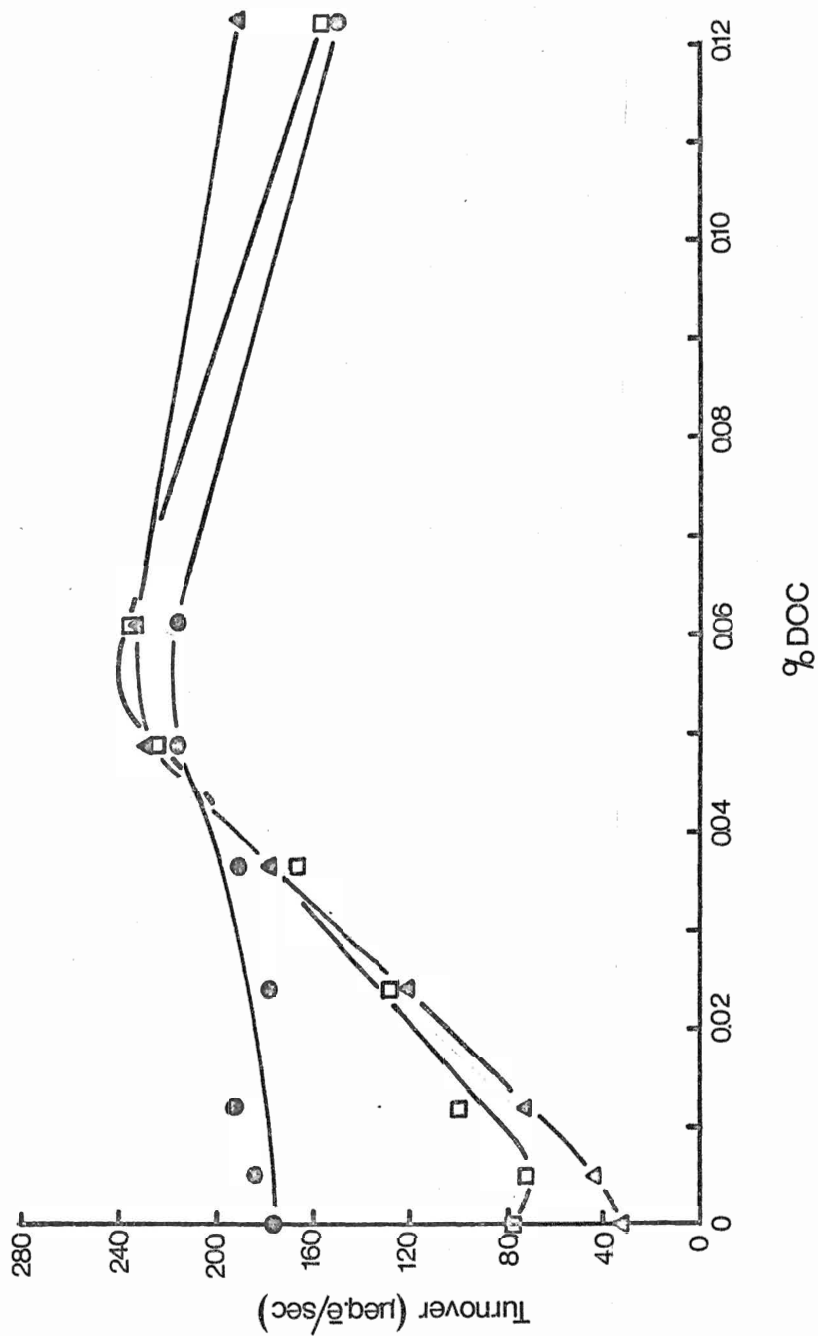
FIGURE 10

The Effect of Lauryl Maltoside on Cytochrome c
Oxidase Turnover in Reconstituted Vesicles:

Ordinate shows oxidase turnover in $\mu eq e^-$/sec
Abscissa shows lauryl maltoside concentration

13.2nM vesicular aa_3, 4.9mM ascorbate, 12.2μM
cytochrome c, 0.18mM TMPD, 1.22μg/ml valinomycin
1.22μM FCCP. Total volume = 4.1ml. Medium- 50mM
KPi pH- 7.4

△: control (+maltoside)
▨: maltoside, +valinomycin
◉: maltoside,valinomycin, +FCCP

FIGURE 10
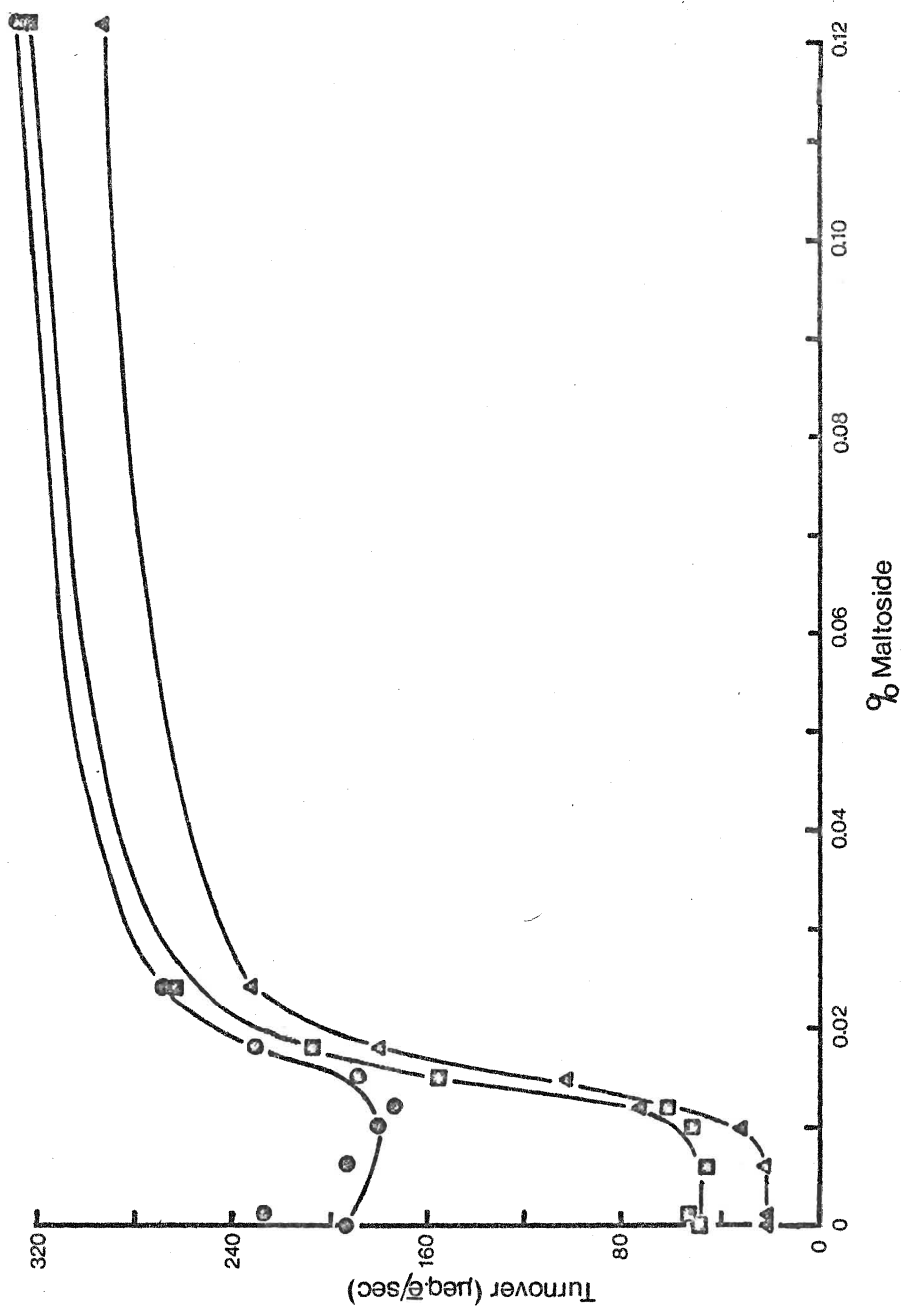
concentrations of detergent. For each of the detergents, experiments were performed at 0.0%, the concentration where ionophore addition showed no further stimulation (0.024% - for Triton & Maltoside, 0.049% - for DOC), and 0.122% (the maximum detergent concentration tested).

At increasing concentrations of Triton X-100, (Fig. 11), Vmax decreased, by as much as 4.5 fold at 0.122% Triton. The apparent Km for cytochrome c also decreased 3 fold at 0.122% Triton.

DOC however, (Fig. 12), in agreement with the earlier polaragraphic assays had little effect on either the Km or Vmax of the reaction with only a slight decrease in the Vmax at 0.122% DOC.

Lauryl Maltoside, (Fig. 13), gave an approx. two fold increase in Vmax at higher concentrations, (ie. 0.122%), agreeing with the earlier poloragraphic measurements, and a slight increase in Km. The two fold increase in Vmax observed with Lauryl Maltoside presumably reflects increased substrate accessibility since approx. 50% of the oxidase is in the opposite orientation (heme a faces inside) across the membrane.

2. Effects of Detergents on Cyt. c Binding

In the experiment shown in Fig. 14, mixtures of cytochrome c oxidase-containing vesicles and cytochrome c were passed through sephadex G-100 columns (as described in Methods) in the absence and presence of Lauryl Maltoside. The cytochrome c oxidase concentration (determined spectro-photometrically), was used as an indication of vesicle concentration. The cytochrome c concentration following reduction was measured at 550-540. The extent of cytochrome c binding could then be estimated.

Figs. 14a & b plot fraction number (abscissae) versus aa_3 concentration on the left axis and cytochrome c concentration on the right (ordinates). In both cases, (± Lauryl Maltoside) only two bands were detected; the first

FIGURE 11

Linweaver Burk Plot for Cytochrome $\underline{c}$ Oxidase in the presence of Triton X-100.

Ordinate show $1/v$ (where v = oxidase turnover in $ueq.e^-/sec$)

Abscissa shows $1/s$ (where s = cyt. $\underline{c}$ conc.)

13.2nM of aa_3, 4.9mM ascorbate, 0.18mM TMPD, 1.22µg/ml valinomycin, 1.22µM FCCP, Total volume = 4.1ml, medium - 50nM KPi pH - 7.4.

○: control (no Triton)

□: 0.024% Triton

△: 0.122% Triton

FIGURE 11
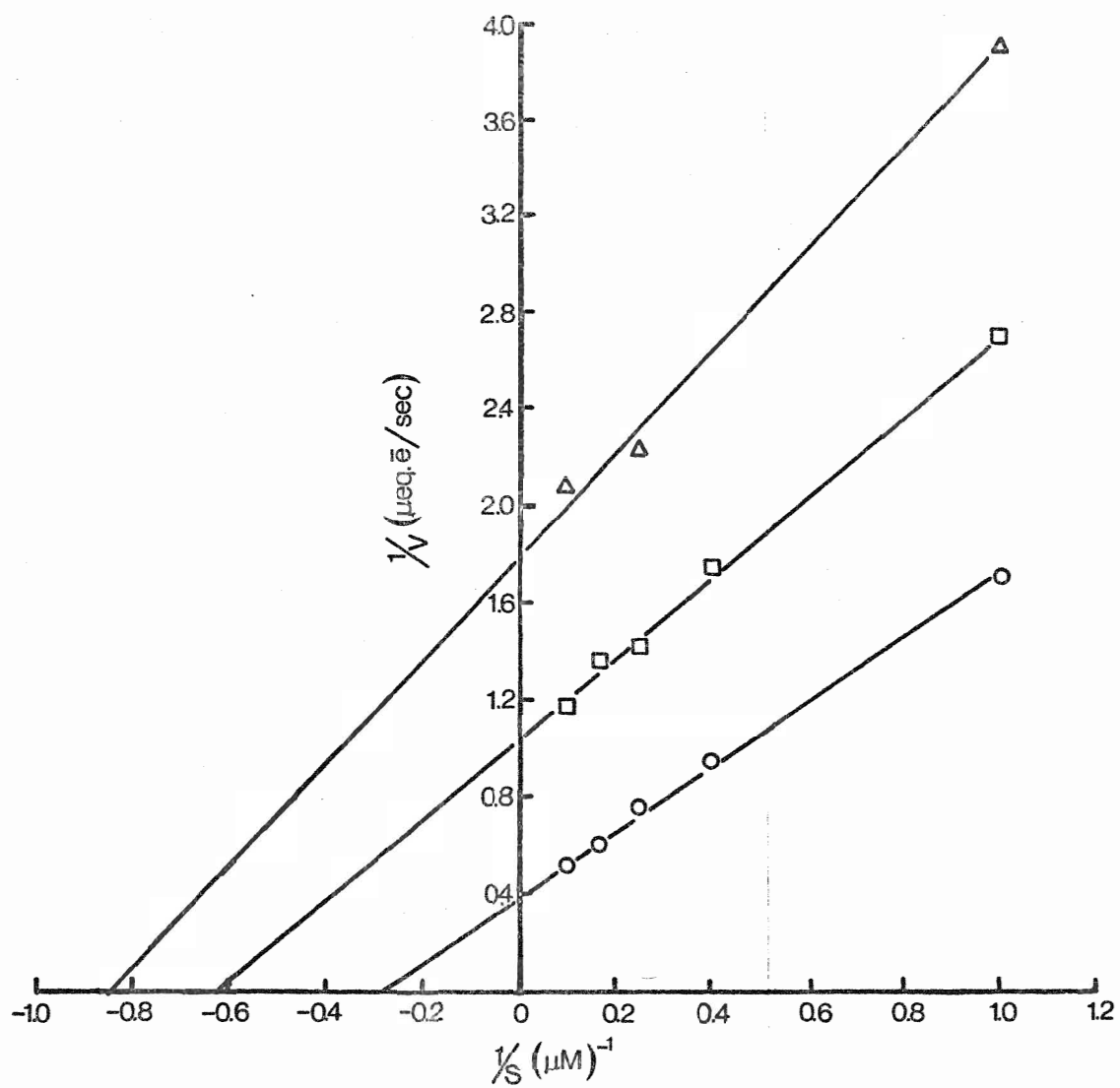
FIGURE 12

Linweaver Burk Plot for Cytochrome $\underline{c}$ Oxidase in the
presence of Deoxycholate:

Ordinate shows $1/v$ (where v = oxidase turnover
in ueq.e^-/sec)

Abscissa shows $1/s$ (where s = cyt. $\underline{c}$ conc.)

13.2nM of aa_3, 4.9mM ascorbate, 0.18mM TMPD,
1.22 µg/ml valinomycin, 1.22 µM FCCP.
Total volume = 4.1ml, medium - 50mM KPi
pH - 7.4.

△:   control (no DOC)
○:   0.049% DOC
▢:   0.122% DOC

FIGURE 12
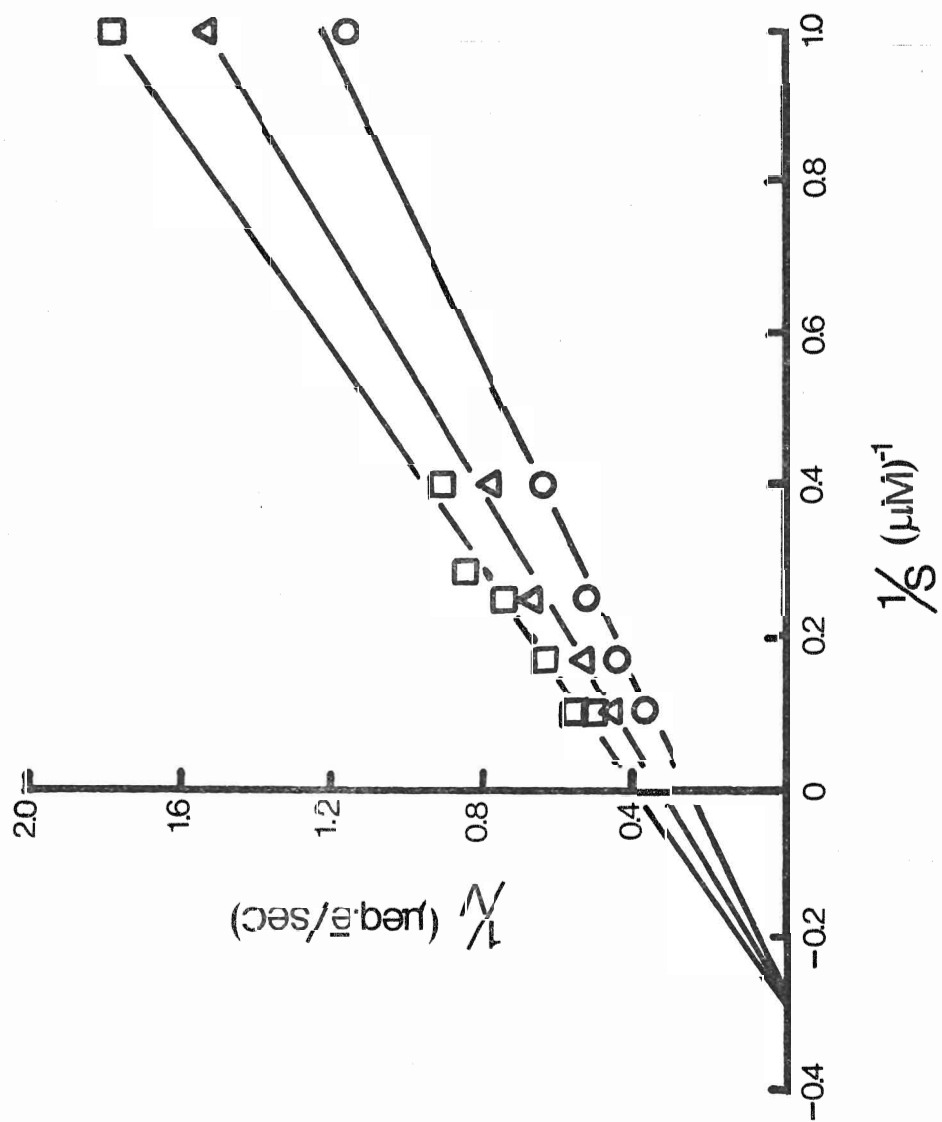
FIGURE 13

Linweaver Burk Plot for Cytochrome $\underline{c}$ Oxidase in the
presence of Lauryl Maltoside:

Ordinate shows $1/v$ (where v = oxidase turnover
in $uneq.e^-/sec$)

Abscissa shows $1/s$ (where s = cytochrome $\underline{c}$
concentration)

13.2nM of aa_3, 4.9mM ascorbate, 0.18mM TMPD,
$1.22\mu g/ml$ valinomycin, $1.22\mu M$ FCCP.
Total volume = 4.1ml, medium = 50mM KPi
pH - 7.4.

O: control (no maltoside)

□:   0.024% maltoside

△:   0.122% maltoside

FIGURE 13
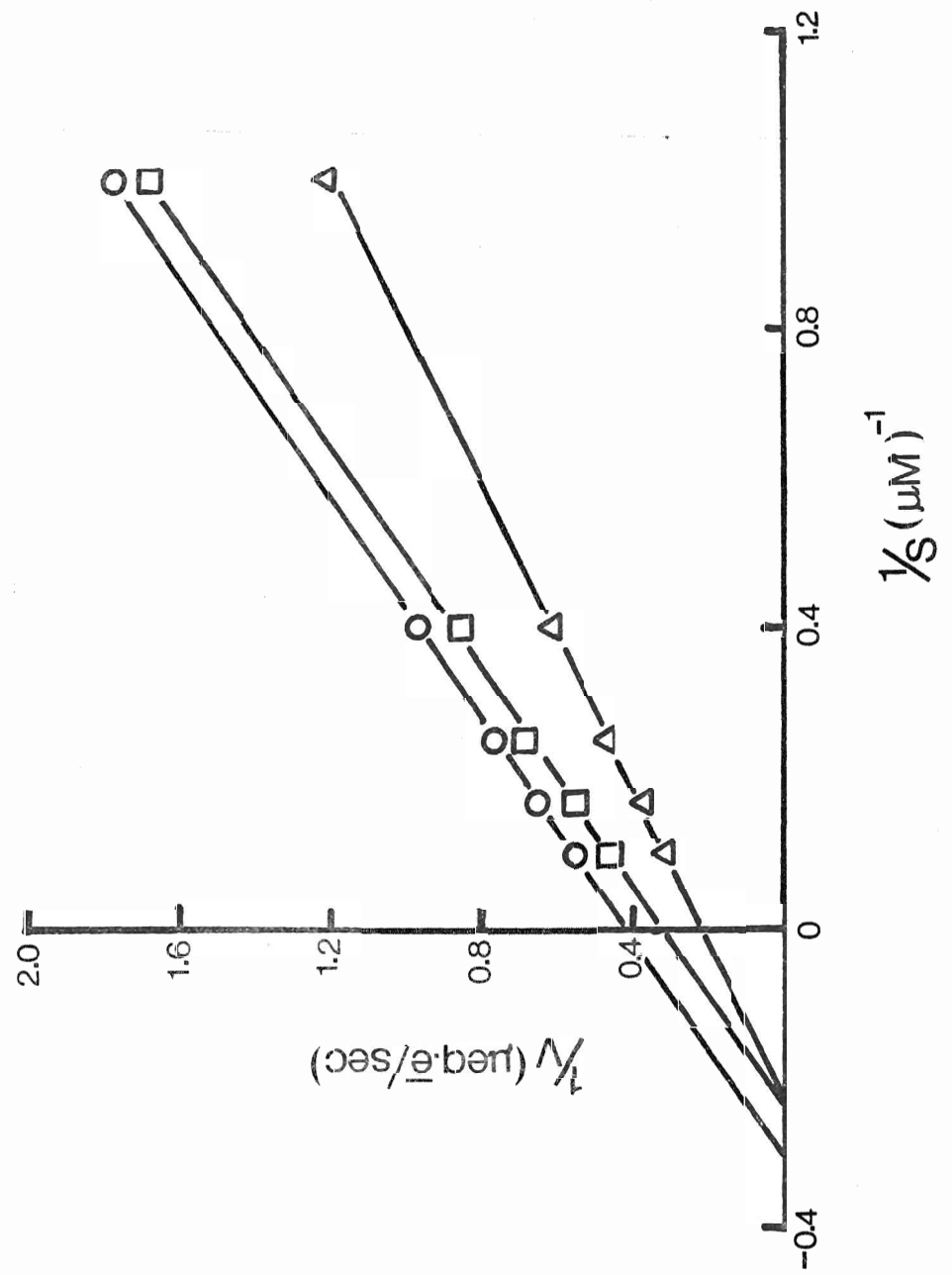
FIGURE 14a & b

Effect of Lauryl Maltoside on Cytochrome c - Proteo-
liposome binding: column chromatography.

  Abscissa shows fraction number.
  Ordinate shows oxidase concentration (right
  axis), cytochrome c concentration (left axis).

  a)   1.5ml of proteoliposomes (2.95μM aa$_3$)
  plus 0.1ml cytochrome c (14mg/ml) containing
  30 mg maltoside placed on a G-100 column
  equilibrated with 0.03% maltoside, 5mM KPi,
  Each fraction had a volumn of 1.1ml.

  b)   1.5ml of proteoliposomes (2.95μM aa$_3$)
  plus 0.1 ml cytochrome c (14mg/ml) was placed
  on a sephadex G-100 column equilibrated with
  5mM KPi. Each fraction had a volume of 1.1ml.

NOTE: #1The second band; free cytochrome c occurred
     after fraction number 40.
   #2 Binding is presumed to occur both to the
     phospholipid as well as aa$_3$. Column chrom-
     atography for aa$_3$ minus phospholipid was not
     performed.

FIGURE 14a

aa₃ Conc. (μM)

Cyt.c Conc. (μM)

Fraction No.
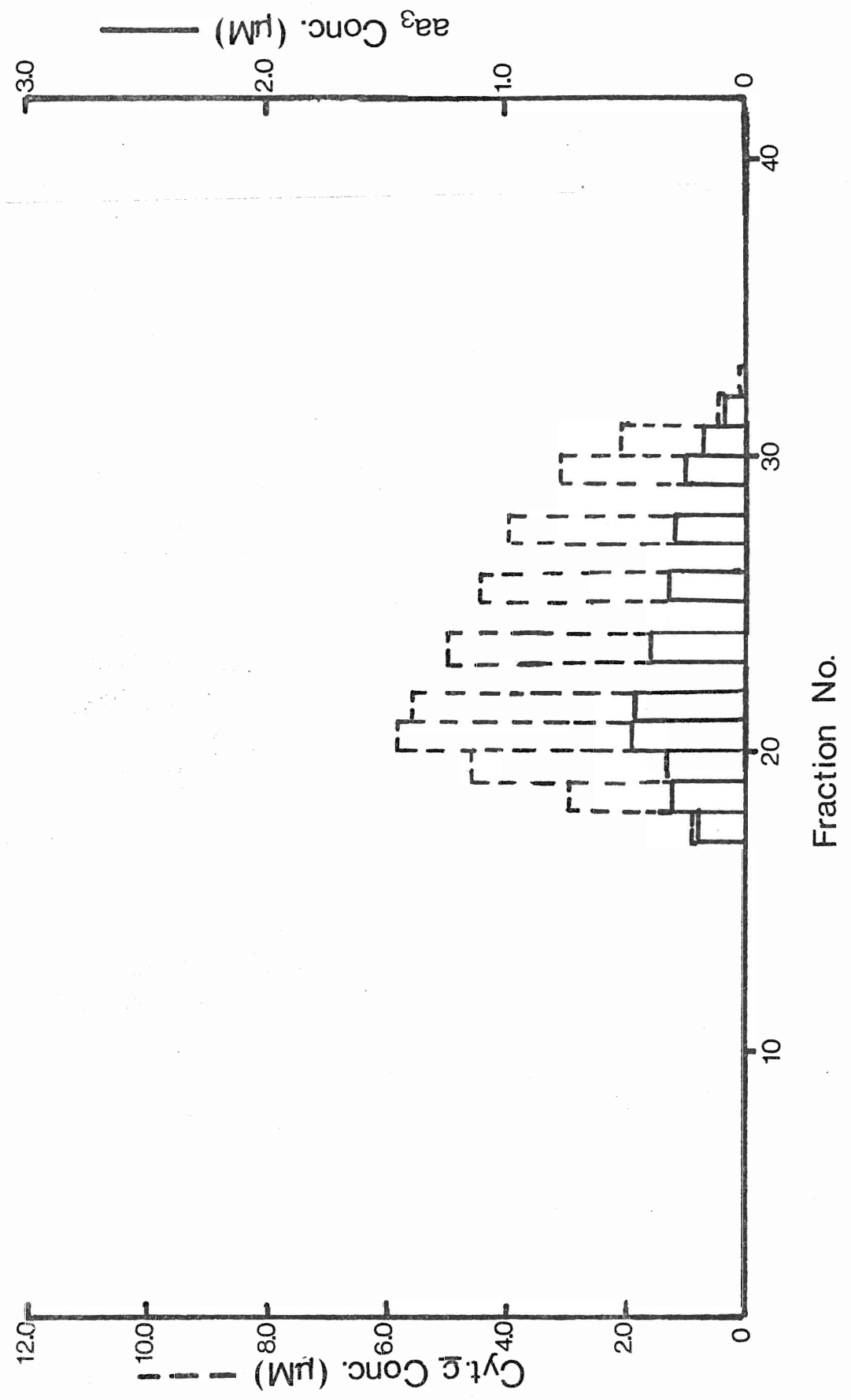
FIGURE 14 b
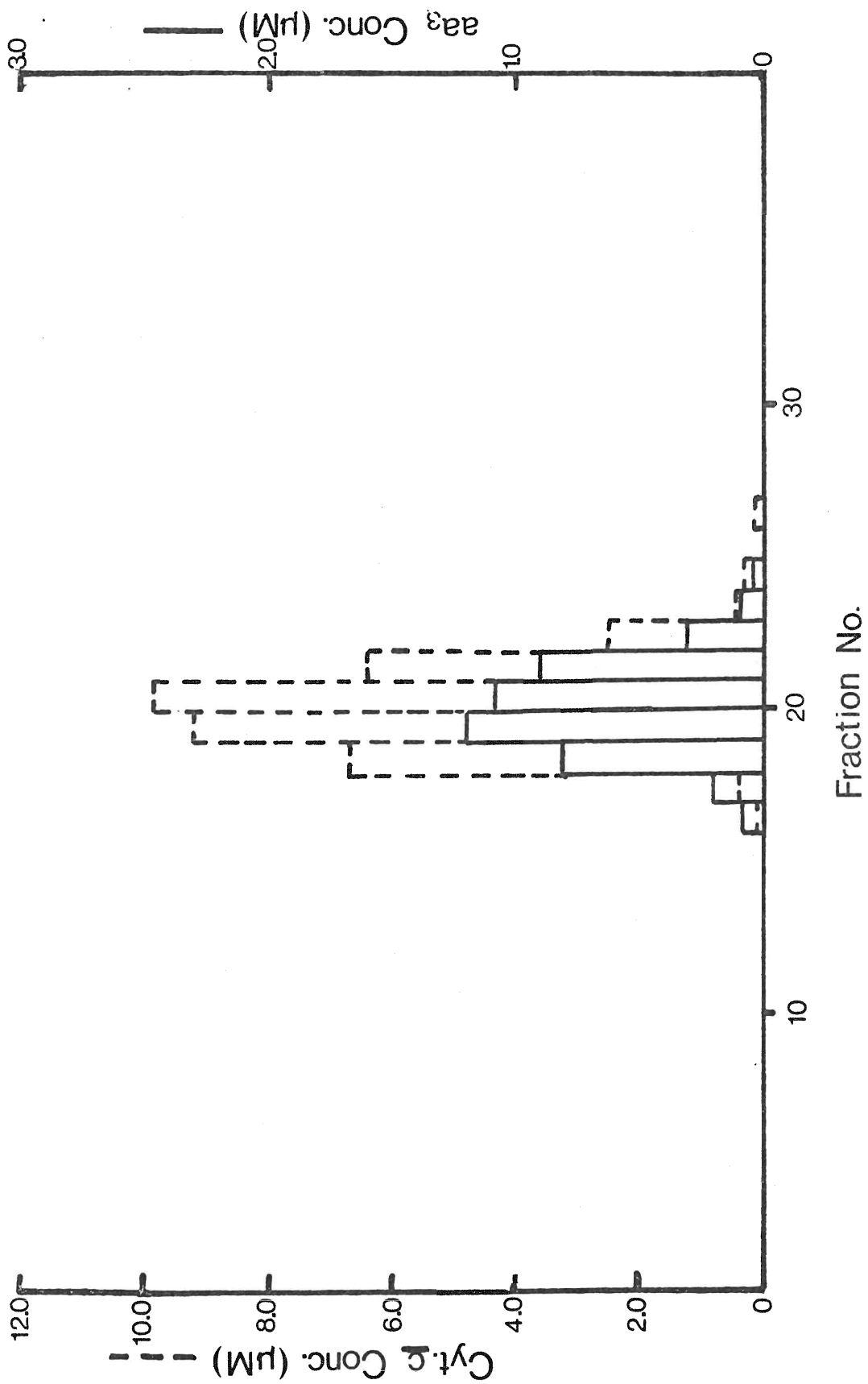
contained both cytochrome $\underline{c}$ oxidase and cytochrome $\underline{c}$, while the second contained only free cytochrome $\underline{c}$. The cytochrome $\underline{c}$: aa_3 ratio in the first band, (in the presence of Maltoside) was = 10.45 whereas in the untreated vesicles, the Cyt. $\underline{c}$: aa_3 ratio was = 6.87.

3. Effects of Detergent on H+ Pulses

The effect of Lauryl Maltoside on oxygen induced proton ejection was also examined. The external acidification upon O_2 pulsing is calculated in terms of n values (H+/e$^-$) and the alkalinization due to the scalar reaction of cytochrome $\underline{c}$ oxidase is calculated as s values. T½ values for the collapse of the acidification seen upon O_2 pulsing were calculated in addition to n and s values. (Fig. 15 A & B).

At high concentrations of Lauryl Maltoside proton release is eliminated and only scalar reaction of cytochrome $\underline{c}$ oxidase is observable, (s values), in the external medium. The extent of this alkalinization is not increased in the presence of valinomycin plus FCCP. Thus, the value of n is zero but the value of s is constant. However, at lower concentrations of Maltoside, the initial acidification is still observable although reduced when compared to pulses in it's absence, (Fig. 15A trace b versus trace a).

The rate of collapse of this acidification in the presence of Maltoside remains relatively constant when compared to the control. Only a low concentration of FCCP increases the collapse of acidification (while not decreasing the initial acidification), Fig. 15A trace d. The acidification observed in the presence of Maltoside is eliminated in the presence of high FCCP, (Fig. 15A trace c). The values for n, s and t½ for Fig. 15A is summarized in Fig. 15 B.

The ordinates in Fig. 15 B are n and s values in terms of H+/e$^-$ ratios on the left hand axis and t½ values in seconds on the right. In the presence of 0.125% Maltoside proton release is still detectable (n = 0.28 H+/e$^-$), although less than that of the control (n = 0.40 H+/e$^-$).

FIGURE 15 A

The Effects of Lauryl Maltoside on Proton Translocation by Cytochrome c Oxidase: traces.

a)   3 ml proteoliposomes (aa_3 = 0.35μM)
      3 ml medium (45mM K_2SO_4 pH - 7.4)
      0.125mM cyt. c, 1.25 μg/ml valinomycin

b)   a + 0.125% Maltoside

c)   b + 1.25μM FCCP (high)

d)   a + .125μM FCCP (low)

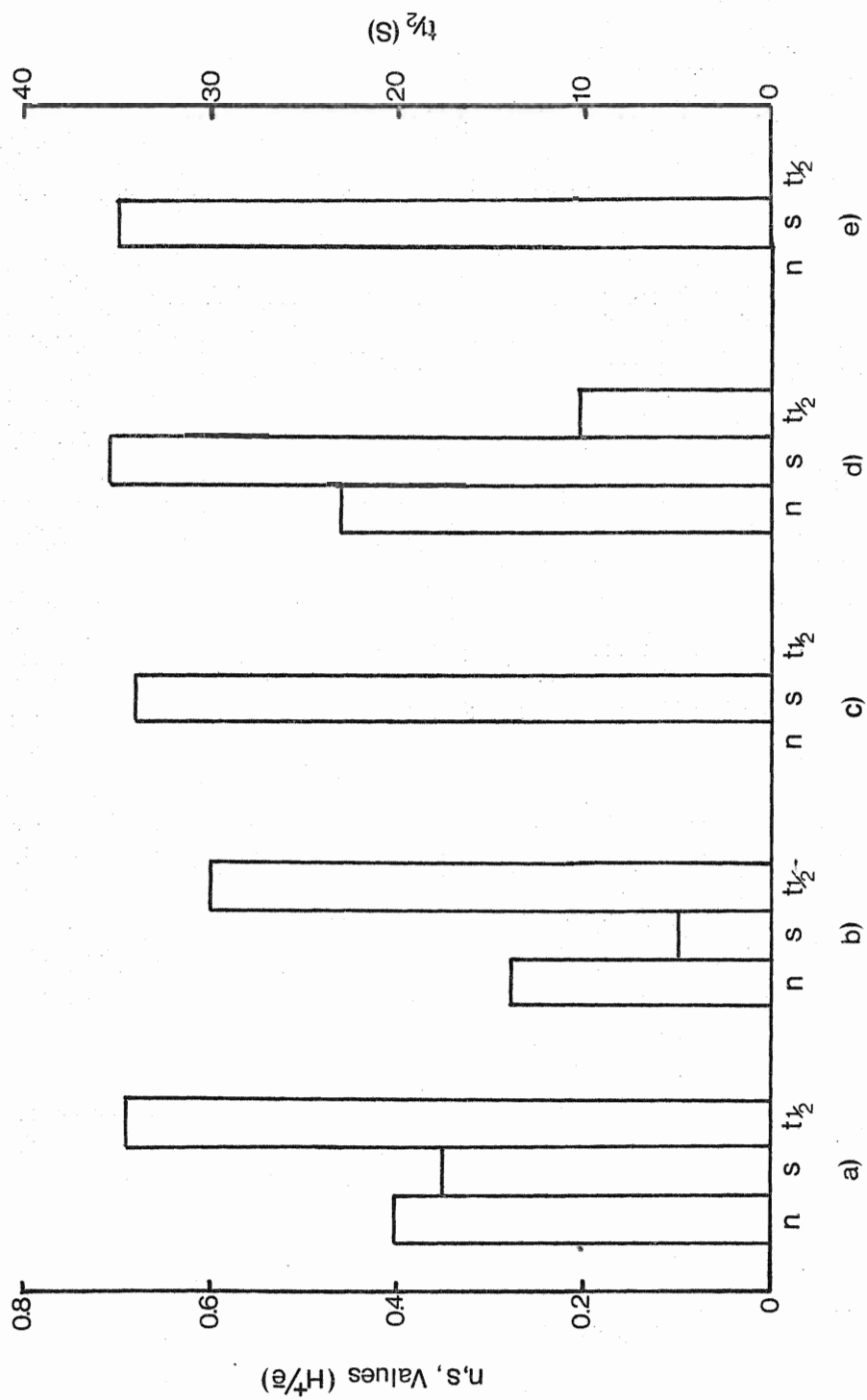e)   a + 1.25μM FCCP (high)

FIGURE 15 A

FIGURE 15 B

The Effects of Lauryl Maltoside on Proton Trans-
location by Cytochrome $\underline{c}$ Oxidase; H+/e$^-$ ratios,
t½ values.

Ordinate represents H+/e$^-$ values - left axis,
t½ values - right axis.

Conditions are as found in Figure 15 A.

FIGURE 15 B

The Decay half times (t½) of the acidification remained essentially unaffected when compared to the control, with values of 30sec and 34.5sec respectively. In contrast, upon titrating in a low concentration of FCCP (d) the t½ values decreased to 9.0 and 11.5sec, (t½ average = 10.2sec), in the control experiment (a), with no decrease in the acidification (n) value.

4. Ferricyanide Effects upon Proton Release

To examine the possibility that proton release is a scalar process involving changes in phospholipid or protein salt bridges, linked to the oxidation of ferrocytochrome c (Mitchell and Moyle, 1983; Papa et. al., 1983a) the effects of $K_3Fe(CN)_6$ addition on proton release was examined.

After progressive additions of $K_3Fe(CN)_6$ to a mixture of proteoliposomes containing aa_3, cytochrome c and ascorbate, proton release due to the scalar chemical reaction of $K_3Fe(CN)_6$ with ascorbic acid diminishes until essentially no proton release occurs, Fig. 16. At this point, proton release induced by O_2 pulses still occurs. Thus $K_3Fe(CN)_6$ pulses do not induce proton release.

The plot in Fig. 17 also demonstrates that proton release by cytochrome c oxidase in the presence of ferrocytochrome c does not occur upon addition of $K_3Fe(CN)_6$. Ascorbate was titrated into the reaction mixture until no further proton release due to the oxidation of ascorbate to dehydroascorbate by cytochrome c (eq. 3) occurred, (ie. all cyt. c is reduced). At lower levels of ascorbate H+ release was maximal and showed a linear correlation between ascorbate added and proton appearance. The extrapolation of this linear part of the curve leads to a line which allows for the release of protons by the oxidation of excess ascorbate (not used to reduce cyt. c) by $K_3Fe(CN)_6$.

The addition of $K_3Fe(CN)_6$ at the point where ascorbate addition gives no further appearance of protons, produces proton release. However, this extra proton release was a

FIGURE 16

Effect of $K_3Fe(CN)_6$ on proton translocation by
Cytochrome <u>c</u> oxidase: trace.

3.5ml of 50mM K_2SO_4, 3.5ml proteoliposomes
(0.67μM aa_3), 1ml cytochrome <u>c</u> (14mg/ml),
1.25μg/ml valinomycin, 1.25μM FCCP, 0.125mM
ascorbate, 12.5μM $K_3Fe(CN)_6$ pulses. Pulses
are shown in sequence.

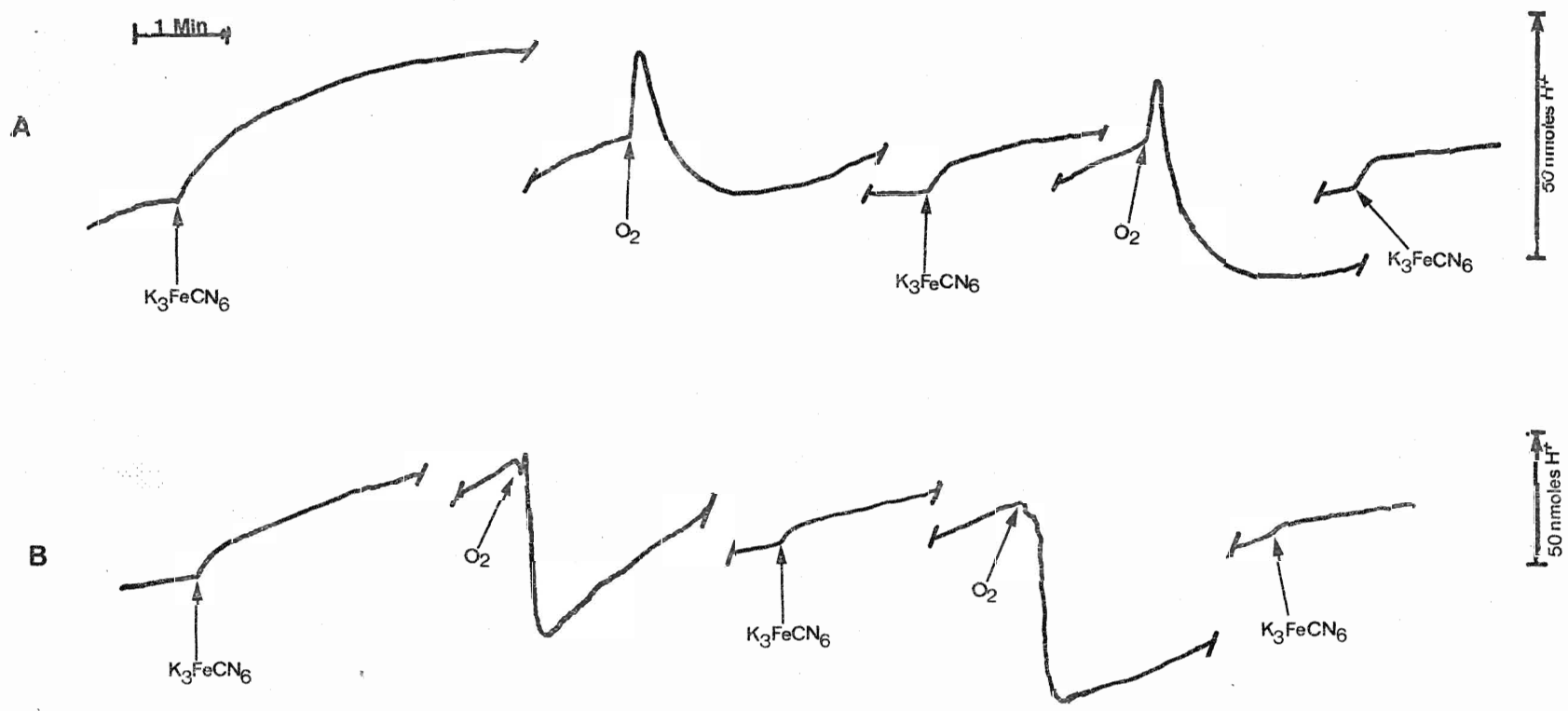A) + Valinomycin

B) + Valinomycin, + FCCP

FIGURE 16

FIGURE 17

Proton Release by $K_3Fe(CN)_6$ pulses in the presence of aa_3 containing Proteoliposomes:

Conditions: same as in Figure 16, except
$K_3Fe(CN)_6$ pulse - 0.313mM

O: H+ released due to ascorbate addition

□: H+ released due to $K_3Fe(CN)_6$ pulse

---: theoretical proton release due to ascorbate (excess) oxidation

FIGURE 17
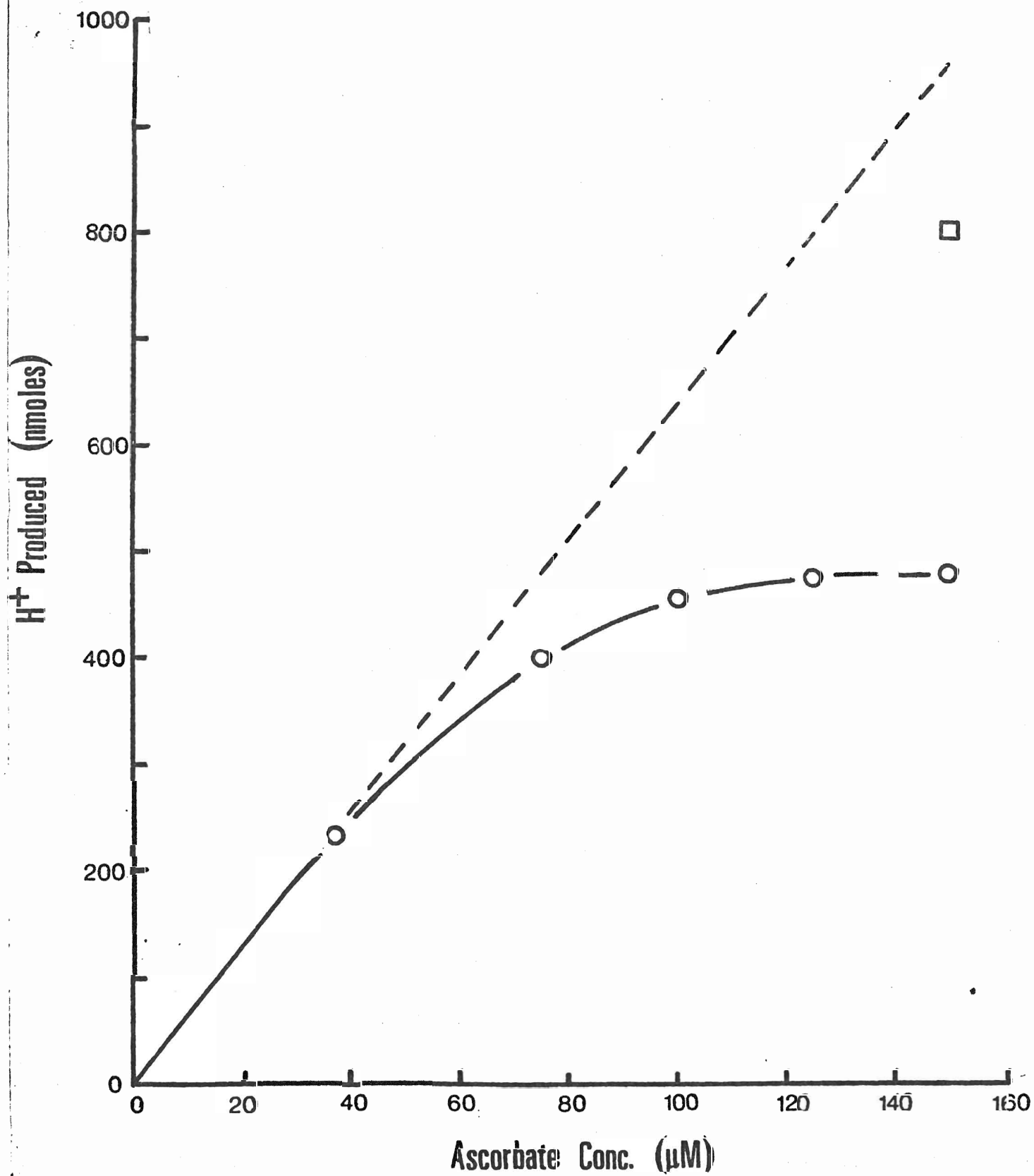
result of the reaction shown in equation 3. This is support-
ed by the fact that the proton release produced by $K_3Fe(CN)_6$
addition lies below the extrapolated line for proton release
due to the oxidation of excess ascorbate.

B)   Effects of Low Ionic Strength on Proton Ejection

        1.   Ferrocytochrome c induced Proton Release

        Papa et. al., (1983a) reported that the acidification
seen upon pulsing proteoliposomes containing aa_3 with
ferrocytochrome c is a result of a scalar process. Papa,
and his co-workers were able to demonstrate that ferro-
cytochrome c pulses at low ionic strength show a much larger
acidification (2.0 H+/e⁻), than at high ionic strength,(Hir kle 1978).
However, this acidification is FCCP insensitive. They
postulated that the proton release observed at high ionic
strength is what remains of a scalar deprotonation, which
at low ionic strength is maximal. As will be seen below,
this is an oversimplification of their results.
        The appearance of ferrocytochrome c induced pulses at
high and low ionic strength is demonstrated in Fig. 18. As
can be seen, the rather modest proton pulses observed at
high ionic strength (A, upper trace), increase in size at
low ionic strength (B, upper trace). Some H+ release is
still observed in the absence of aa_3 at low ionic strength
as shown in Fig. 18C. In the presence of FCCP as well as,
valinomycin (lower traces), pulse appearance also changes
with ionic strength. At high ionic strengths proton uptake
is observed upon ferrocytochrome c oxidation but at low ionic
strengths, both in the absence and presence of enzyme, there
is an acidification observed upon ferrocytochrome c addition.
This acidification declines to the base line, (lower traces,
B & C).

FIGURE 18

The appearance of ferrocytochrome c pulses performed
at low ionic strength in the presence and absence
of aa_3.

    Conditions as in Table 1, p.   following.

    A. High ionic strength proteoliposomes

    B. Low ionic strength proteoliposomes

    C. Low ionic strength liposomes

Upper traces: + valinomycin

Lower traces: + valinomycin + FCCP
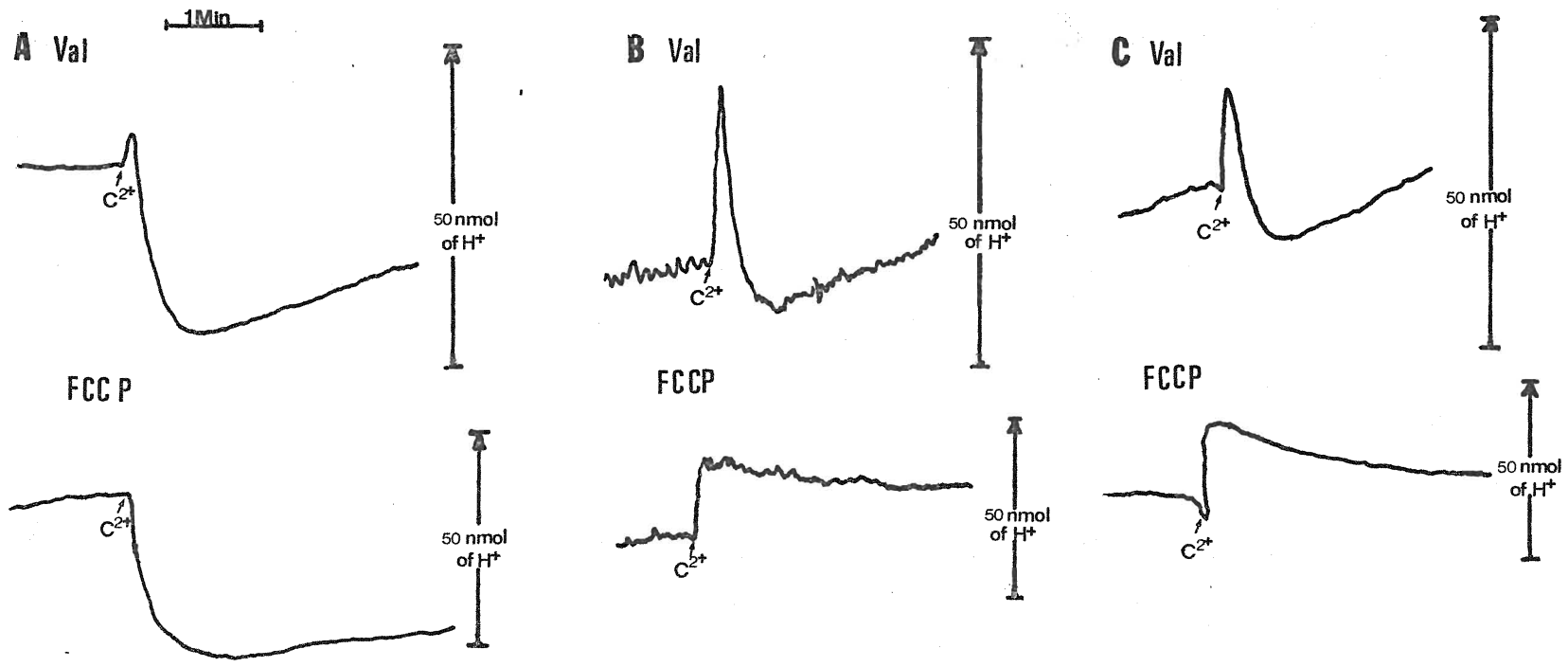

46 nmole c^{2+} additions as indicated.

FIGURE 18

57

Table 1 summarizes the n and s values obtained in the presence of valinomycin, and in the presence of valinomycin plus FCCP, for proteoliposomes at both high and low ionic strengths, as well as for liposomes at low ionic strength. In agreement with the results of Papa (1983), if ferrocytochrome c pulsing of proteoliposomes is carried out at low ionic strength, an increase in the acidification is observed. The average n value rises from 0.18 to 0.72 $H+/e^-$. Only part of the increased acidification however, was FCCP sensitive. The average decreased not to zero but to 0.40 $H+/e^-$, in the presence of FCCP at low ionic strength. Thus 55% of the acidification was FCCP insensitive. The FCCP-insensitive acidification correlated with the acidification seen when enzyme free liposomes at low ionic strength were pulsed with ferrocyto-chrome c. Thus, the FCCP insensitive portion of the ferrocyto-chrome c induced proton release does appear to be due to a scalar deprotonation of phospholipid upon reduced cytochrome c addition to the reaction mixture.

Table 2 shows that only a portion of the ferrocytochrome c - induced acidification at low ionic strength is azide sensitive. Proteoliposomes were pulsed at low ionic strength with ferrocytochrome c in the presence of valinomycin plus FCCP and the n values calculated. In the second experiment, proteoliposomes from the same vesicle preparation were pulsed with ferrocytochrome c in the presence of valinomycin alone at low ionic strength. This was then repeated in the presence of azide. The acidification (n value) decreased upon azide addition to 0.70 from 1.15. Thus, at low ionic strength ferrocytochrome c induced proton release includes acidification which occurs at high ionic strength and is FCCP and azide sensitive and the acidification which occurs only at low ionic strength (in the presence or absence of enzyme) and is FCCP and azide insensitive.

The FCCP and azide-insensitive Ferrocytochrome c induced proton release also occurs in the absence of cytochrome c oxidase. This acidification mimics the true vectorial process by showing a collapse following the initial proton ejection. This final

TABLE 1

The Effect of Low Ionic Strength on Ferrocytochrome
c-induced Pulses:

$T = 30°C$ pH approx. 7.4

$\bar{n}$ = average n value $\bar{s}$ = average s value

A) 3.5ml proteoliposomes (aa_3 conc. = 0.639μM),
    4.5ml of 50mM K_2SO_4, 1.25μg/ml valinomycin,
    1.25μM FCCP, 25μl ferrocytochrome c pulse
    (1.83 mM).

B) 3.5ml proteoliposomes (aa_3 conc. = 1.416μM),
    4.5ml of 5mM K_2SO_4, 100mM Mannitol, 35mM Sucrose,
    1.25μg/ml of valinomycin, 1.25μM FCCP, 25μl
    ferrocytochrome c pulse (1.83 mM).

C) 3.5ml liposomes, 4.5ml of 5mM K_2SO_4, 100mM
    Mannitol, 35mM sucrose, 1.25 μg/ml of valinomycin,
    1.25μM FCCP, 25μl ferrocytochrome c pulse
    (1.83 mM).

TABLE 1

Vesicles and Medium	Conditions	$\overline{H^+/e}$	
		$\overline{n}$	$\overline{s}$
A Proteo-liposomes: High ionic strength	+ VAL	0.18 ± 0.06	0.77 ± 0.44
	+ FCCP	0.0	0.87
B Proteo-liposomes: Low ionic strength	+ VAL	0.72 ± 0.11	0.44 ± 0.06
	+ FCCP	0.40 ± 0.03	< 0*
C Liposomes: Low ionic strength	+ VAL	0.30 ± 0.04	0.47 ± 0.06
	+ FCCP	0.37 ± 0.06	< 0*

* Pulses did not return to baseline.ie. values of approx. -.3 were obtained and were often variable.

TABLE 2

The Effect of Azide on ferrocytochrome-induced pulsing
at low ionic strength:

| | Conditions | $H^+/\bar{e}$ | |
		$\bar{n}$	$\bar{s}$
A	+VAL,+FCCP	0.58 ± 0.01	<0 *
B	+VAL	1.15 ± 0.08	<0 *
C	+AZIDE	0.70 ± 0.01	<0 *

* Pulses did not return to baseline.

A) 3ml proteoliposomes (aa$_3$ conc. = 0.92µM),
5ml of 5mM K_2SO_4, 100mM mannitol, 35mM sucrose,
1.25µg/ml of valinomycin, 1.25µM FCCP, ferrocytochrome
c pulses - 33.3 neq.ē.

B) conditions as in (A), but in absence of FCCP.

C) conditions as in (B), but in presence of 1.25µM azide.

alkalinization was observed to proceed to, or below, the baseline (the original pH before a pulse) depending on the magnitude of the initial acidification. In addition it was found that the extent of the return alkalinization declined with successive pulses.

Fig. 19a and b demonstrates this phenomenon. The X-axis represents the order in which pulsing was performed. The left hand axis represents proton release to the external medium, upon ferrocytochrome c pulsing at liposomes, given as $H+/e^-$ ratios. The right hand axis represents the collapse to the baseline, given as % realkalinization. 100% realkalinization is taken as total collapse of acidification to the baseline. Fig. 19a and b show that the addition of H_2O_2 in the presence of catalase restores the collapse of acidification.

In the experiment of Fig. 19a, the medium was first bubbled with Nitrogen and then pulsed with successive ferrocytochrome c additions; under these conditions the % realkalinization began to decrease immediately. Under the aerobic conditions in Fig. 19b, a decrease was not observed until after the seventh ferrocytochrome c addition. The collapse of acidification seen following proton release therefore depends on the presence of oxygen in the reaction medium.

2.   Oxygen Induced Proton Release

The traces in Fig. 20 represent the appearance of a typical oxygen induced proton pulse at high and low ionic strength. As with ferrocytochrome c induced pulses at high ionic strength, there is an initial acidification followed by a realkalinization, (Fig. 20 trace A). Oxygen pulses at low ionic strength lead to an acidification of the external medium, slightly greater than that found at high ionic strength (Fig. 20 trace B). However, unlike ferrocytochrome c pulses at low ionic strength O_2 induced pulses lead to realkalinization and no acidification is seen in the presence of FCCP [Fig. 20 trace B (lower trace)]. Fig. 20 trace C, demonstrates that an increase in acidificaion is observed upon

FIGURE 19 (A & B)

The Effect of Oxygen on the Realkalinization
following Ferrocytochrome $\underline{c}$ pulsing at low ionic
strength:

A)   Under partially aerobic initial conditions,
     (the starting solution was first bubbled with
     Nitrogen): 3.5ml liposomes, 4.5ml of 5mM
     K_2SO_4, 100mM mannitol, 35mM sucrose, 1.25µg/ml
     valinomycin, 0.435mg of catalase was used
     (34000 units/mg).

B)   Under initially aerobic conditions: other
     conditions as in (A).

FIGURE 19A

FIGURE 19B

O %Realkalinization

Pulse Number

n.Value (H+/e)
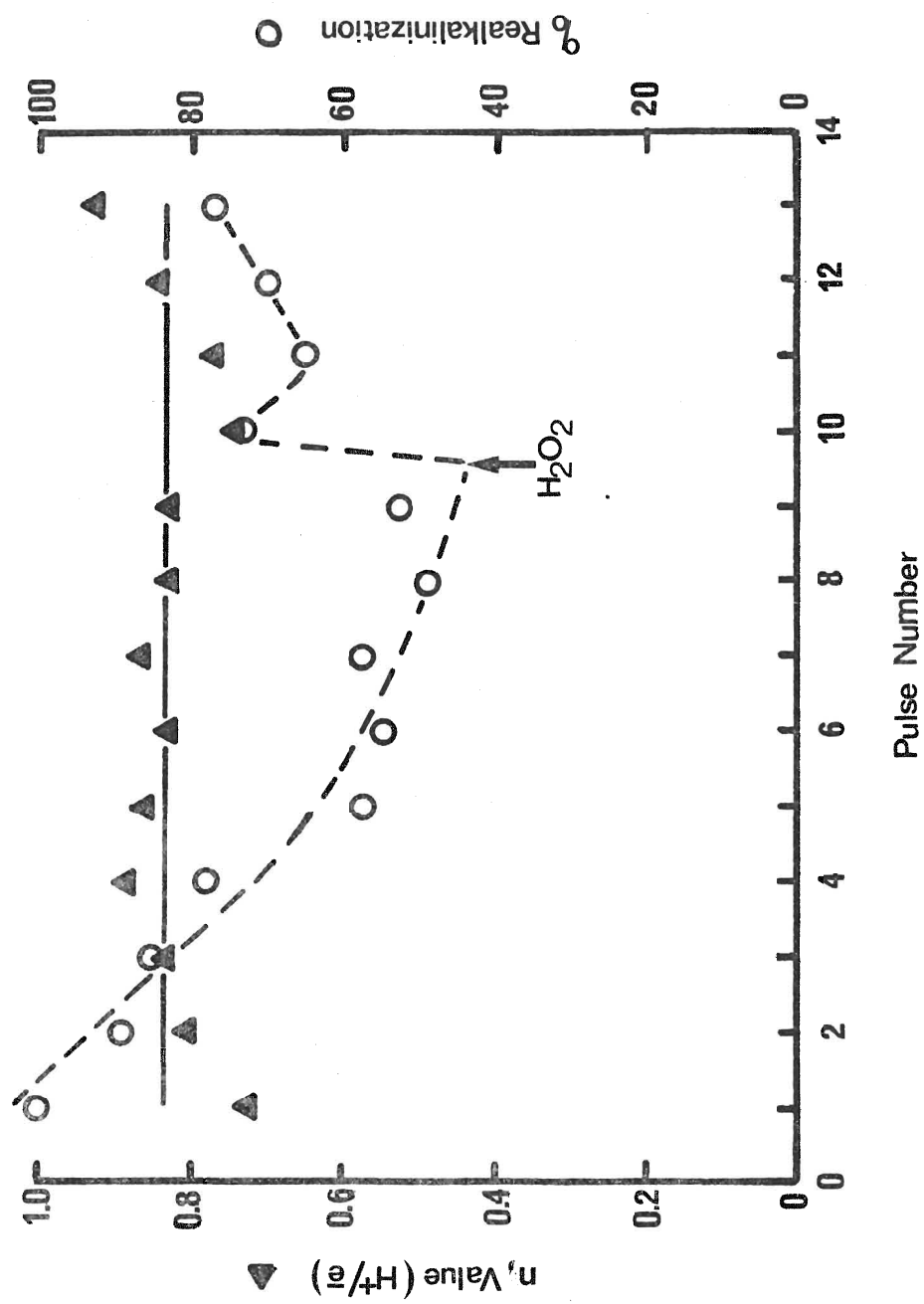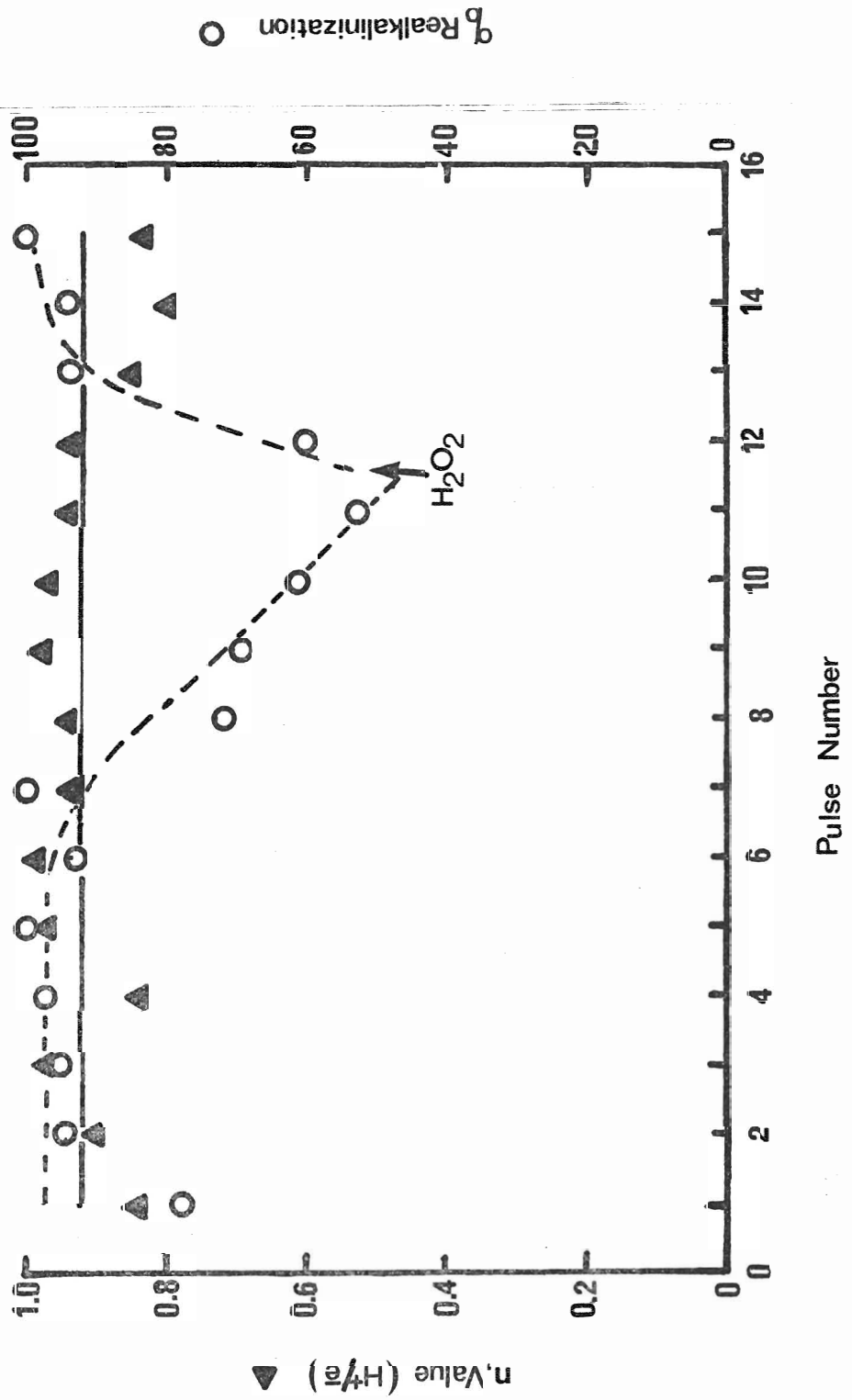
FIGURE 20

The appearance of O_2 induced pulses at high and low
ionic strengths:

A)   High cyt. c, low ascorbate, high ionic strength:
     ie. 4ml of 50mM K_2SO_4, 3ml proteoliposomes
     (aa_3 conc. = 0.593µM), 0.111mM cyt. c, 0.125mM
     ascorbate, 1.25µg/ml of valinomycin, 1.25µM FCCP.

B)   High cyt. c, low ascorbate, low ionic strength:
     ie. 3ml of proteoliposomes (aa_3 conc. = 0.528µM),
     4 ml of 5mM K_2SO_4, 100mM mannitol, 35mM sucrose,
     0.111mM cyt. c, 0.125mM ascorbate, 1.25µg/ml of
     valinomycin, 1.25µM FCCP.

C)   Low cyt. c, high Asc/TMPD, low ionic strength:
     ie. 3ml of proteoliposomes (aa_3 conc. = 0.528µM),
     4ml of 5mM K_2SO_4, 100mM mannitol, 35mM sucrose,
     0.995µM cyt. c, 5.0mM ascorbate and 93.75µM TMPD.

     upper traces - +valinomycin
     lower traces - +valinomycin + FCCP

FIGURE 20
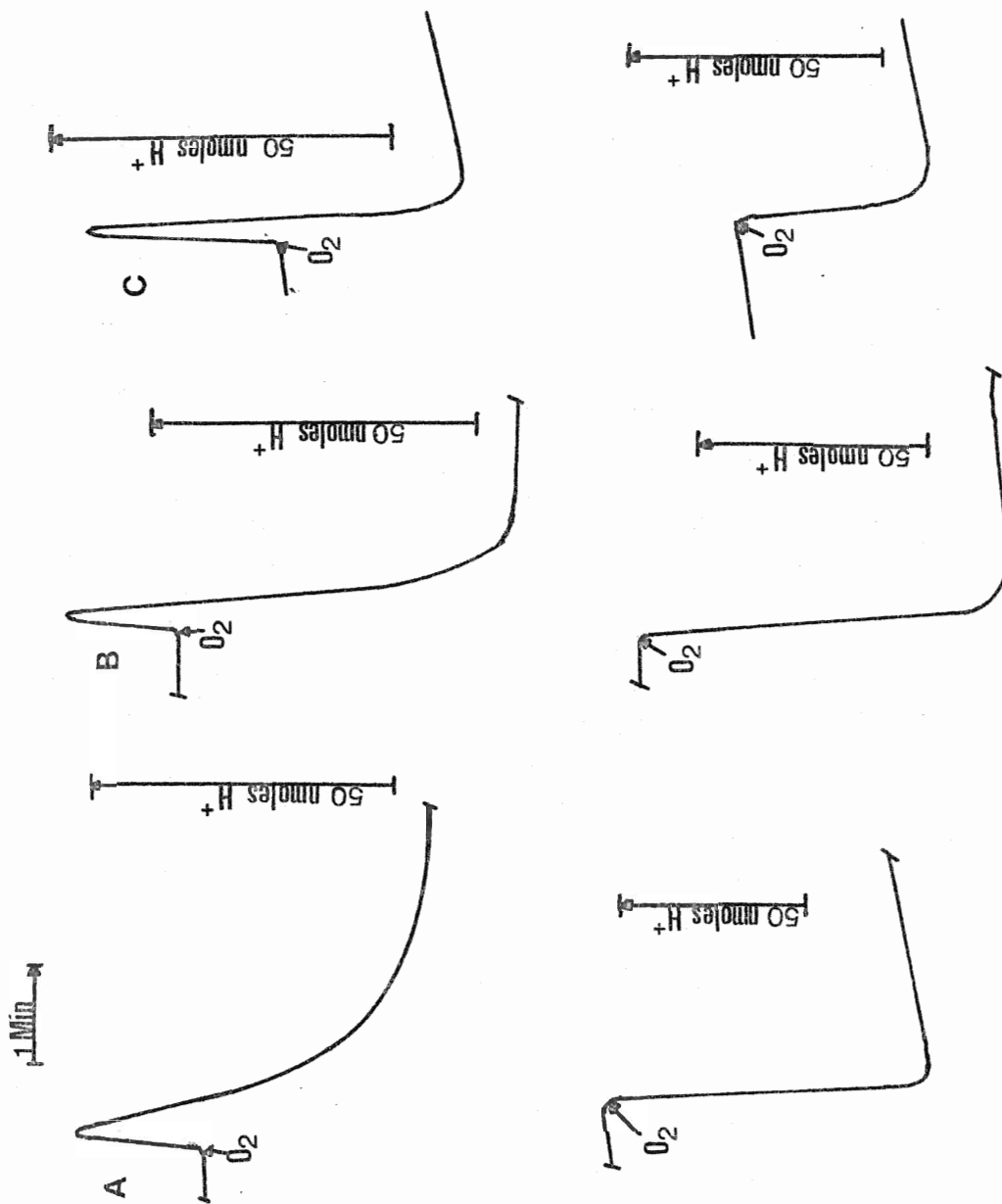
O_2 pulsing at low ionic strength in the presence of high ascorbate and TMPD. This is reflected in a decrease of the realkalinization which occurs in the presence of FCCP [Fig. 20 trace C (lower trace)].

The values obtained are summarized in Tables 3 and 4. However, the magnitude of this effect although greater than that at high ionic strength is much smaller than observed with ferrocytochrome c pulsing; the apparent n value increases from 0.38 to 0.45 (Table 3) and 0.50 to 0.60 H+/e in table 4. The data in table 3 and 4 were from different vesicle preparations. The further increase obtained in the presence of excess ascorbate and TMPD is a result of proton release upon their oxidation (eq. 3).

The increase in acidification seen upon oxygen induced pulsing at low ionic strength is KCN sensitive. In the experiment summarized in Table 5, a low concentration of FCCP was added in order to observe the alkalinization which follows proton release upon O_2 induced pulsing. As can be seen, the average n value rose from 0.24 at high ionic strength to 0.37 H+/e$^-$ at low ionic strength, upon pulsing with oxygen. No acidification was observed in the presence of KCN at low ionic strength. Unlike ferrocytochrome c induced pulses at low ionic strength with liposomes, oxygen pulsing fails to cause any scalar proton release. The magnitude of proton release by oxygen pulses at low ionic strength is also dependent upon the presence of potassium in the external medium, Table 6. The average n value decreased from 0.49 to 0.31 upon replacing the external medium (5mM K_2SO_4) with 5mM Na_2SO_4. It may be concluded that the increase in acidification at low ionic strength with O_2 pulses is not a scalar process.

TABLE 3

The Effect of Low Ionic Strength on Oxygen induced
Proton pulsing:

Expt. No.	H^+/e^- Ratio		+FCCP
	+VAL		
	$\bar{n}$	$\bar{s}$	$\bar{s}$
A	$.384 \pm 0.03$	$.761 \pm 0.11$	1.02 ± 0.01
B	$.450 \pm 0.02$	$.872 \pm 0.07$	$.897 \pm 0.02$
C	$.685 \pm 0.03$	$.465 \pm 0.02$	$.465 \pm 0.04$
D	$.530 \pm 0.06$	$.491 \pm 0.07$	$.452 \pm 0.01$

Conditions: as in Fig. 20 for A), B), and C).

D)   3ml of proteoliposomes (aa_3 conc. = 0.474µM),
     4ml of 5mM K_2SO_4, 100mM mannitol, 35mM sucrose,
     0.111mM cyt. c, 0.125mM ascorbate, 1.25µg/ml
     valinomycin, 1.25µM FCCP, 93.75µM TMPD.

TABLE 4

The Effect of Low Ionic Strength on Oxygen Induced Pulses:

Medium	Conditions	H^+/e		$t\frac{1}{2}$ (secs)
		n	s	
A High Ionic Strength	**+VAL**	0.50	0.45	36
	+VAL,+FCCP	0.0	0.91	<3
B Low Ionic Strength	**+VAL**	0.60	0.45	18
	+VAL,+FCCP	0.0	0.57	<3

A)   3.5ml of proteoliposomes (aa_3 conc. = 0.388µM), 3.5ml 60mM K_2SO_4, 0.11mM cyt. c, 0.25mM ascorbate, 1.25µg/ml valinomycin, 1.25µM FCCP.

B)   Conditions are as in (A), except 3.5ml of 5mM K_2SO_4 plus 135mM sucrose was used instead of 50mM K_2SO_4.

TABLE 5

The Effect of KCN on Oxygen Induced Pulses at Low Ionic Strength:

	Conditions	$\bar{n}$	$\bar{s}$
A High Ionic Strength	+VAL	0.24	<0*
	+FCCP (Low)	0.24±0.01	0.25 ±0.1
	+KCN	0.00	0.00
B Low Ionic Strength	+VAL	0.40±0.02	0.28±0.07
	+FCCP (Low)	0.36±0.01	0.41±0.01
	+KCN	0.00	0.00

∗   Pulse did not return to baseline.

A)   3ml of proteoliposomes (aa$_3$ conc. = 0.494µM), 4ml of 50mM K_2SO_4, 0.11mM cyt. c, 0.125mM ascorbate, 1.25µg/ml valinomycin, 0.125µM FCCP, 1.25mM KCN

B)   3ml of proteoliposomes (aa$_3$ conc. = 0.49µM), 4ml of 5mM K_2SO_4, 100mM mannitol, 35mM sucrose, all other conc. are as in (A).

TABLE 6

The Effect of Potassium on Oxygen Induced pulses at Low
Ionic Strength:

	Conditions	$\frac{H^+/e}{\bar{n}}$	$\bar{S}$	ΔK^+
A	+VAL	0.49±0.03	0.49±0.06	N.D.
5mM K_2SO_4	+VAL,+FCCP	0.0	0.68±0.09	0.0
B	+VAL	0.31±0.04	0.55±0.03	1.98
5mM Na_2SO_4	+VAL,+FCCP	0.0	0.88±0.04	0.0

A) 3.5ml proteoliposomes (aa_3 conc. = 0.583μM),
3.5ml of 5mM K_2SO_4, 135mM sucrose, 0.11mM cyt. c,
0.125mM ascorbate, 1.25μg/ml valinomycin,
1.25μM FCCP.

B) As in (A) except vesicles were passed down a G-25
column equilibrated with 5mM Na_2SO_4, 135mM sucrose
and the medium used was 5mM Na_2SO_4, 135mM sucrose.

C) Ionophore Effects on Proteoliposome Kinetics:
   1. Effects on Cytochrome c Oxidase Turnover

As indicated on page 26 if proton ejection is a vectorial event, certain characteristic ionophore effects are to be anticipated, thus these effects were further characterized.

Figure 21 illustrates the effects of increasing nigericin concentration in the presence and absence of valinomycin on the rate of proteoliposome respiration. Valinomycin stimulation of respiration was limited, but nigericin alone gave a four-fold stimulation and valinomycin plus excess nigericin leads to full stimulation. 50% of the maximal respiratory stimulation occurred at a nigericin concentration of 0.015 µg/ml, both in the presence and absence of valinomycin (1.22µg/ml).

Nigericin is an electroneutral K^+/H^+ exchanger (Section I, C3). A comparison was also made between the effects of the electrogenic K^+ ionophores, valinomycin and nonactin. These results are summarized in the bar graphs in Figure 22. The vertical-axis represents the percent maximal stimulation by ionophore addition measured polaragraphically. The bars in Figure 22 are grouped together in order of ionophore addition in each experimental case. Case # 1, shows that valinomycin addition releases respiration by approx. 24% of that obtained with valinomycin plus FCCP. In Case # 2, the addition of nonactin leads to a 40% release of respiration which was not further increased by valinomycin addition. But valinomycin stimulation of respiration is increased by nonactin addition (Case # 3). Nigericin addition leads to as much as 80% release of respiration, (Case # 4) which upon nonactin addition attains 100% release, (cf. Fig. 21).

A third class of ionophore is the voltage-gated type (Section I, C4). Fig. 23 shows the effect of alamethicin addition on turnover of proteoliposomal cytochrome c oxidase.

Alamethicin addition alone released respiration by between 65.0 and 70.0% (experiments - #1, #4, & #6). Upon valinomycin addition to an alamethicin stimulated system respiration was inhibited (experiment #1). Conversely, if valinomycin was present prior to alamethicin addition (experiment #2)

FIGURE 21

Respiration rates in the presence of Ionophores:
Nigericin stimulation.

   10µl of proteoliposomes (aa$_3$ conc. = 13.17nM),
   4.9mM ascorbate, 12.2µM cyt. $\underline{c}$, 0.18mM TMPD,
   plus 1.22 µg/ml valinomycin and/or 1.22µM FCCP
   where indicated. Medium - 50mM KPi pH 7.4
   Temperature - 28°C, Volume - 4.1ml.

   O: + Nigericin

   △: + Nigericin + valinomycin

   ☐: + Nigericin + valinomycin + FCCP

NOTE: half closed symbols represent runs performed
      the following day. These show no uncoupling
      'trend' with increasing [nigericin].

A:nigericin stimulation

FIGURE 22

The Effect of Nonactin on Oxidase Respiration:

The ordinate represents % maximal stimulation of oxidase respiration calculated as:

$$\frac{\left(V_{ionophore} - V_{control}\right)}{\left(V_{FCCP} - V_{control}\right)} \times 100$$

10µl of proteoliposomes (aa$_3$ conc. = 10.58nM), 4.9mM ascorbate, 0.18mM TMPD, 12.2µM cyt. c. Medium - 50mM KPi, pH - 7.4. Temp. 28°C, Vol. 4.1ml.

      □: nonactin
      ⊞: valinomycin
      ▨: FCCP
      ■: nigericin

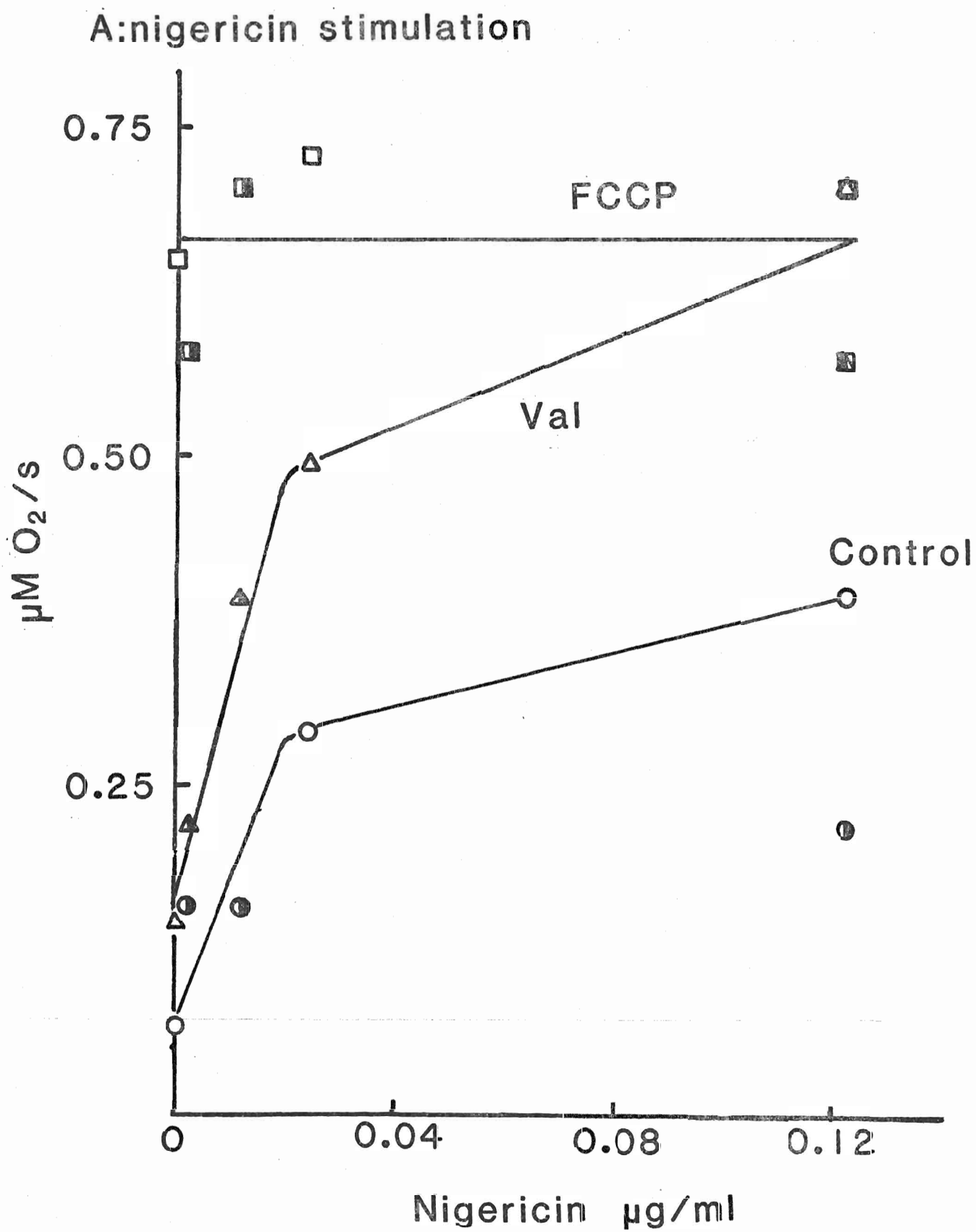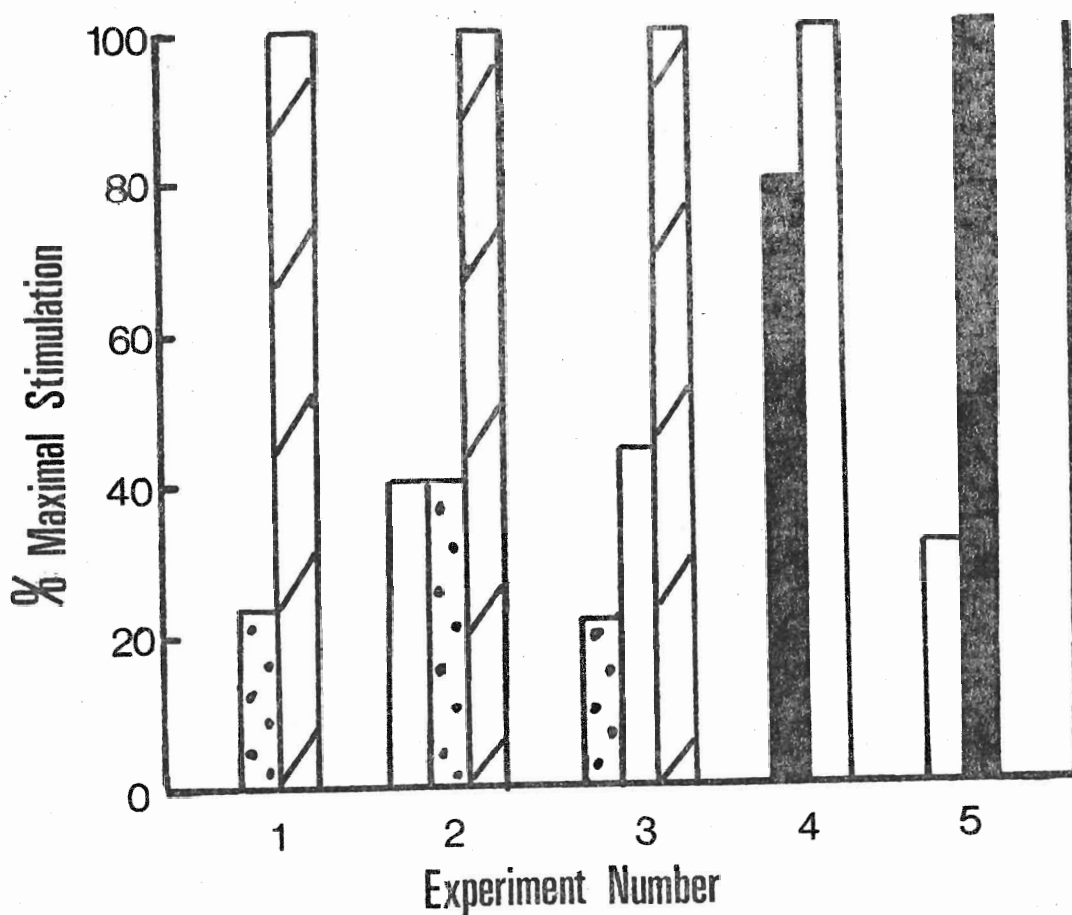FIGURE 23   73

The Effect of Alamethicin on Oxidase Respiration:
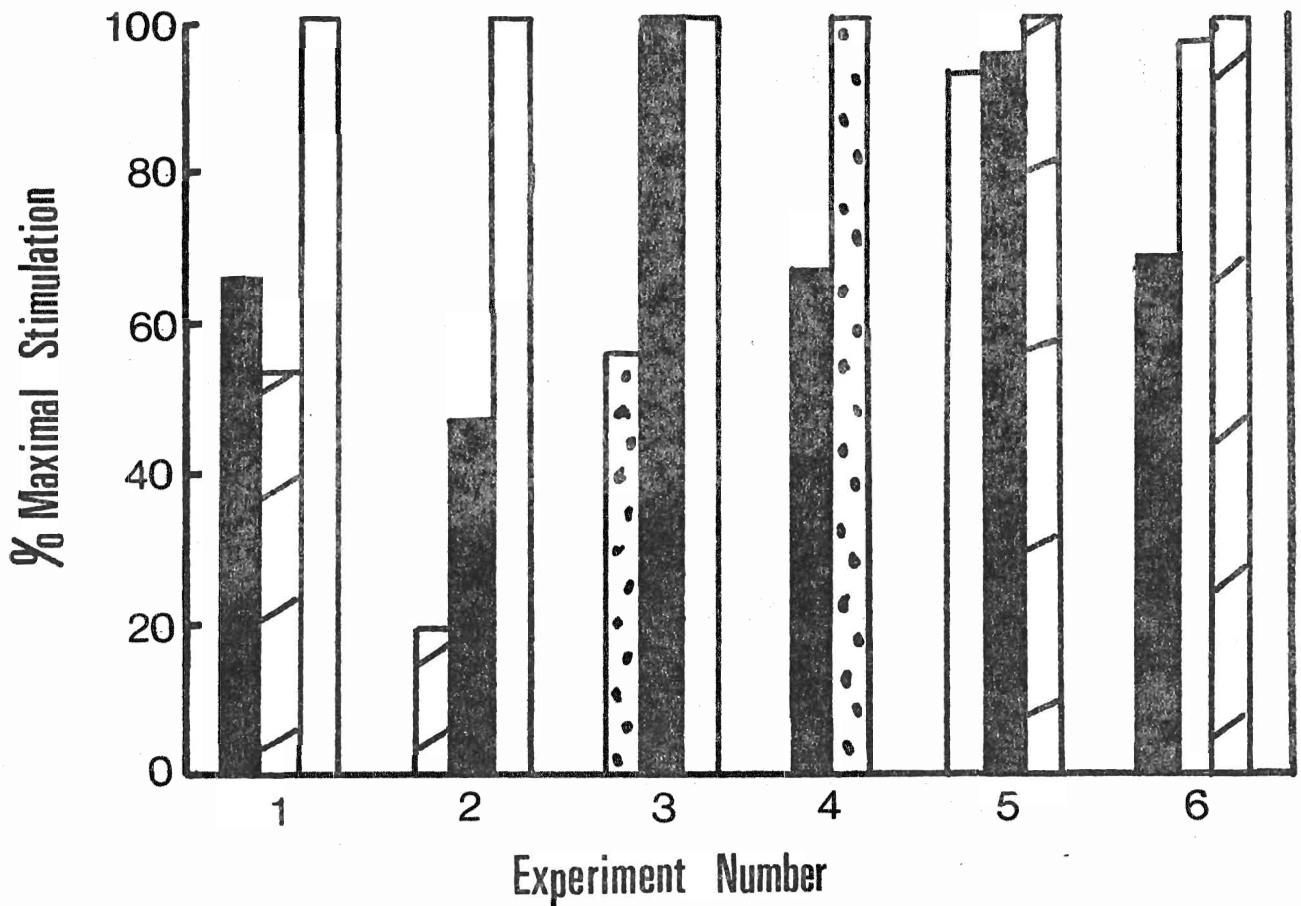
The ordinate represents % maximal stimulation of
oxidase respiration calculated as in Fig. 22.

10µl of proteoliposomes (aa_3 conc. = 10.58nM),
12.2µM cyt. $\underline{c}$, 0.18mM TMPD, 4.9mM ascorbate.
Medium - 50mM KPi pH 7.4, volume - 4.1ml.

■: alamethicin

▨: valinomycin

□: FCCP

▨: nigericin

the release of respiration was only 47%, although full release (100%) was achieved upon FCCP addition.

Addition of nigericin plus alamethicin leads to full release of turnover as shown in experiments 3 and 4 respecitively. Alamethicin also does not affect stimulation by FCCP (experiments 5 and 6).

The alamethicin stimulation of respiration seen is concentration dependent, as shown in Fig. 24. Within this admittedly narrow range of alamethicin concentration there is no evidence of ionophore cooperativity.

2. Ionophore Effects on the Apparent Proton Pumping Activity of Cytochrome c Oxidase

Fig. 25 illustrates the effect of valinomycin concentration, in the presence of excess potassium, on the magnitude of cytochrome c oxidase-induced proton translocation. The ordinate represents the acidification observed upon O_2 pulsing, given as $H+/e^-$ ratio (n value). The abscissa represents the concentration (µg/ml) of valinomycin present in the reaction medium. The $H+/e^-$ ratios increase linearly as valinomycin concentration increases , up to a concentration of approx. 0.4 µg/ml where the $H+/e^-$ ratio is maximal. This compares with the standard value of 1.25 µg/ml valinomycin normally used in other studies of proton translocation (sections A & B above). Fig. 26, however, shows that the realkalinization typically observed following the initial proton appearance, given in terms of 's' values, does not reach a maximum until valinomycin concentration is greater than 2 µg/ml.

The experiment repeated in order to compare the effects of 50mM Na_2SO_4 and 50 mM K_2SO_4 as shown in Figures 27 and 28. Little difference was found between the n ($H+/e^-$) values in 50mM K_2SO_4 and in 50mM Na_2SO_4, (Fig. 27 & 28). The pulses obtained in K_2SO_4 appear at lower valinomycin concentrations (0.0 to 0.2 ug/ml) than those in Na_2SO_4. The maximal $H+/e^-$ values obtained in Na_2SO_4 was approximately 0.25 $H+/e^-$.; the maximal value obtained in K_2SO_4 approximated 0.29 $H+/e^-$.

FIGURE 24

The Effect of Alamethicin Concentration on Oxidase Respiration:
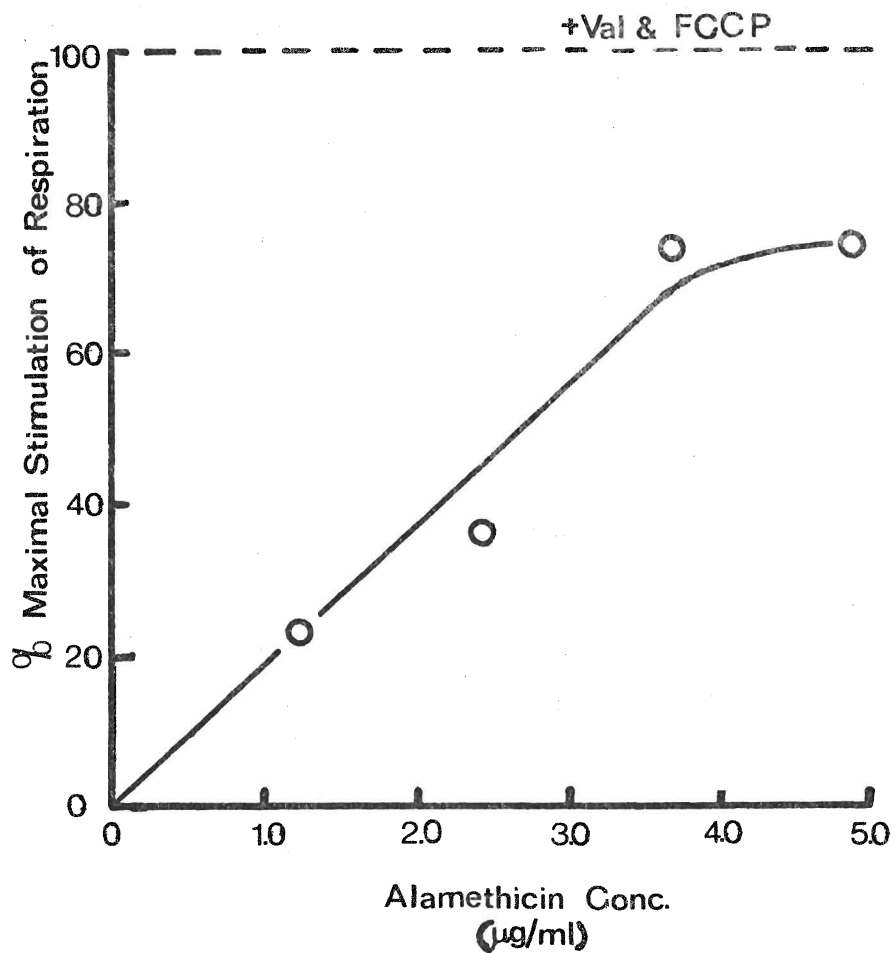
Ordinate show % maximal stimulation of respiration
as calculated in Fig. 22.

Ordinate gives alamethicin concentration in µg/ml.

All concentrations are as in Fig. 23 except for
alamethicin concentration which was varied as
indicated.

FIGURE 25

The Effect of varying Valinomycin Concentration on
Oxygen-induced Proton Pulsing: n values.

3.5 ml of proteoliposomes (aa_3 conc. = $0.412\mu M$),
3.5 ml of 50mM K_2SO_4, 0.11mM cyt. c., 0.250mM
ascorbate plus $1.25\mu g/ml$ valinomycin.
pH: 7.3 - 7.4. Temp. 28°C. Total vol. - 8 ml.

n values (ordinate) calculated from traces as in
Fig. 7.

FIGURE 25

n Values (H⁺/e⁻) vs. Valinomycin Conc. (µg/ml)

FIGURE 26   77
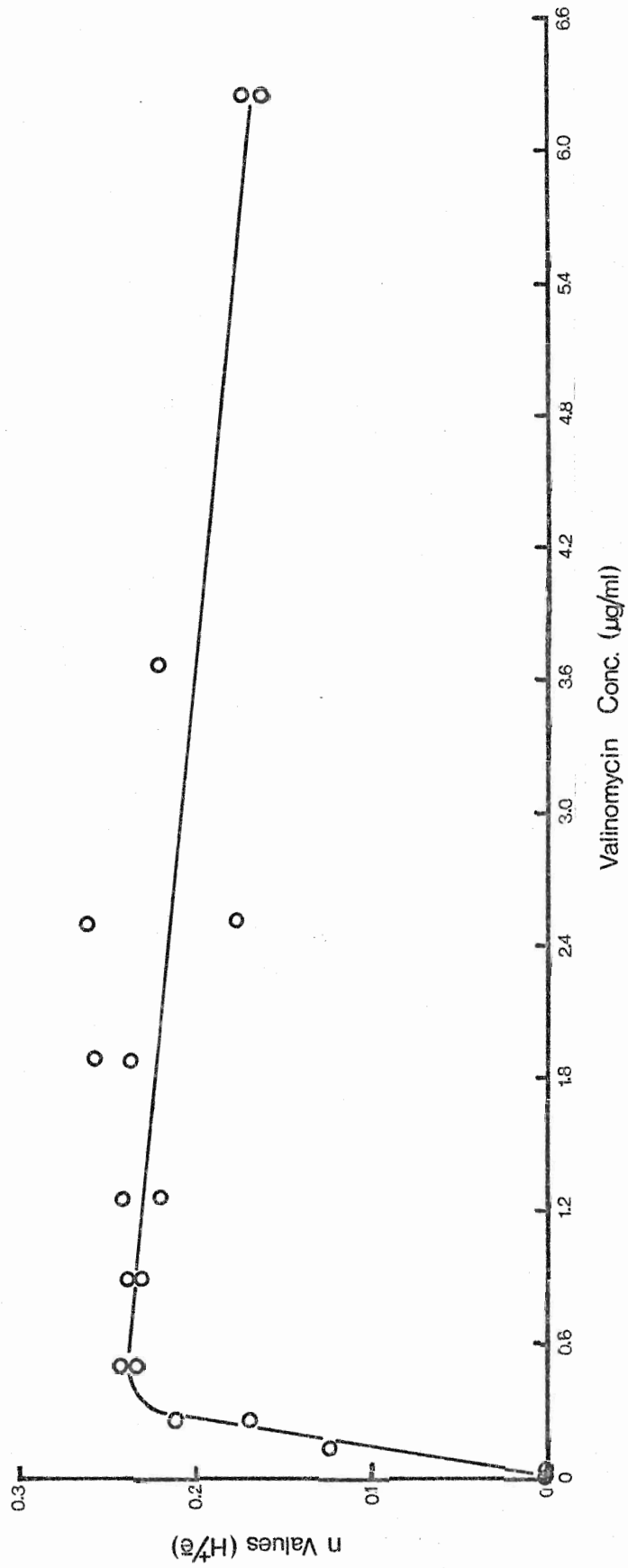
The Effect of Varying Valinomycin Concentration on Oxygen-
Induced Proton Pulsing: s values.
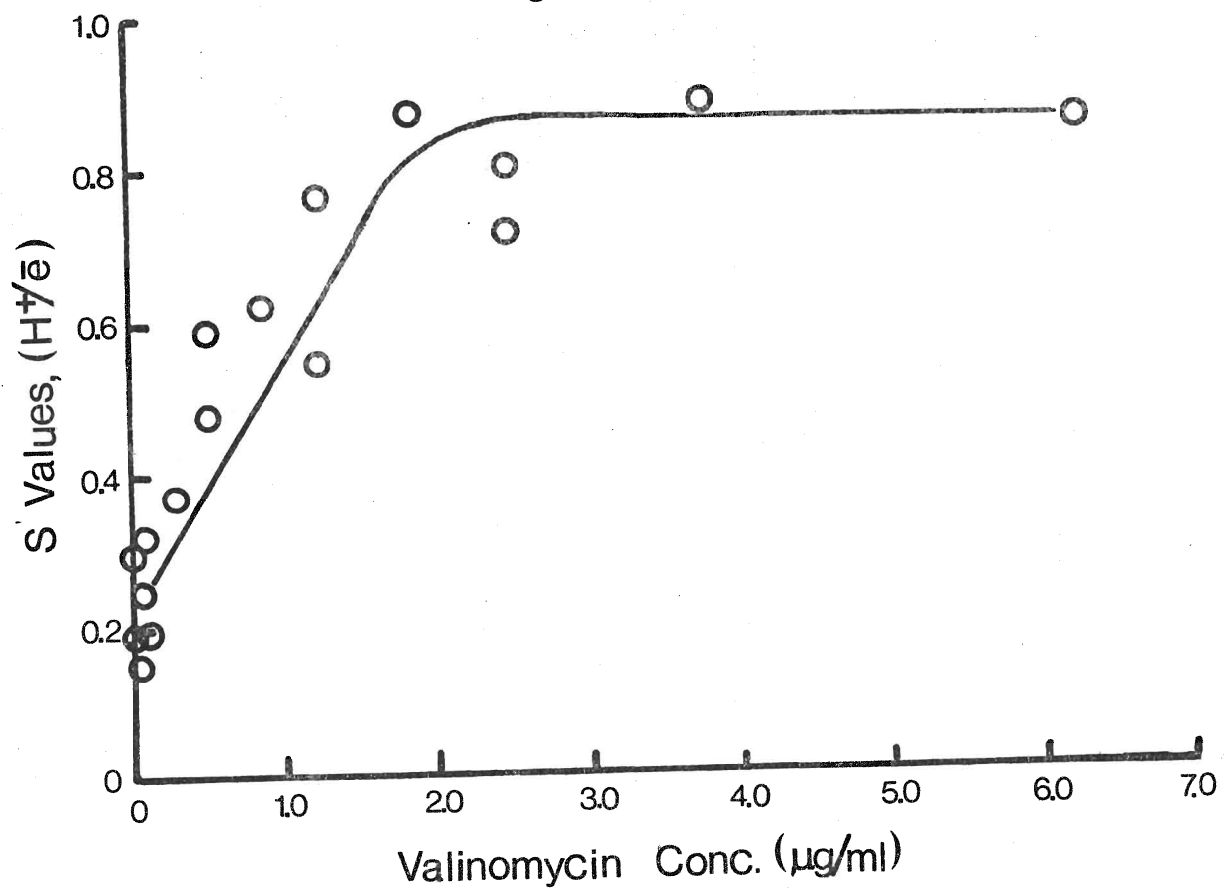
Conditions are as found in Fig. 25 plus 1.25µM FCCP.

s values (ordinate) calculated from traces as in

Fig. 7.

FIGURE 27   78

The Effect of Valinomycin on Proton Translocation by
Cytochrome $\underline{c}$ Oxidase: K_2SO_4 external medium.
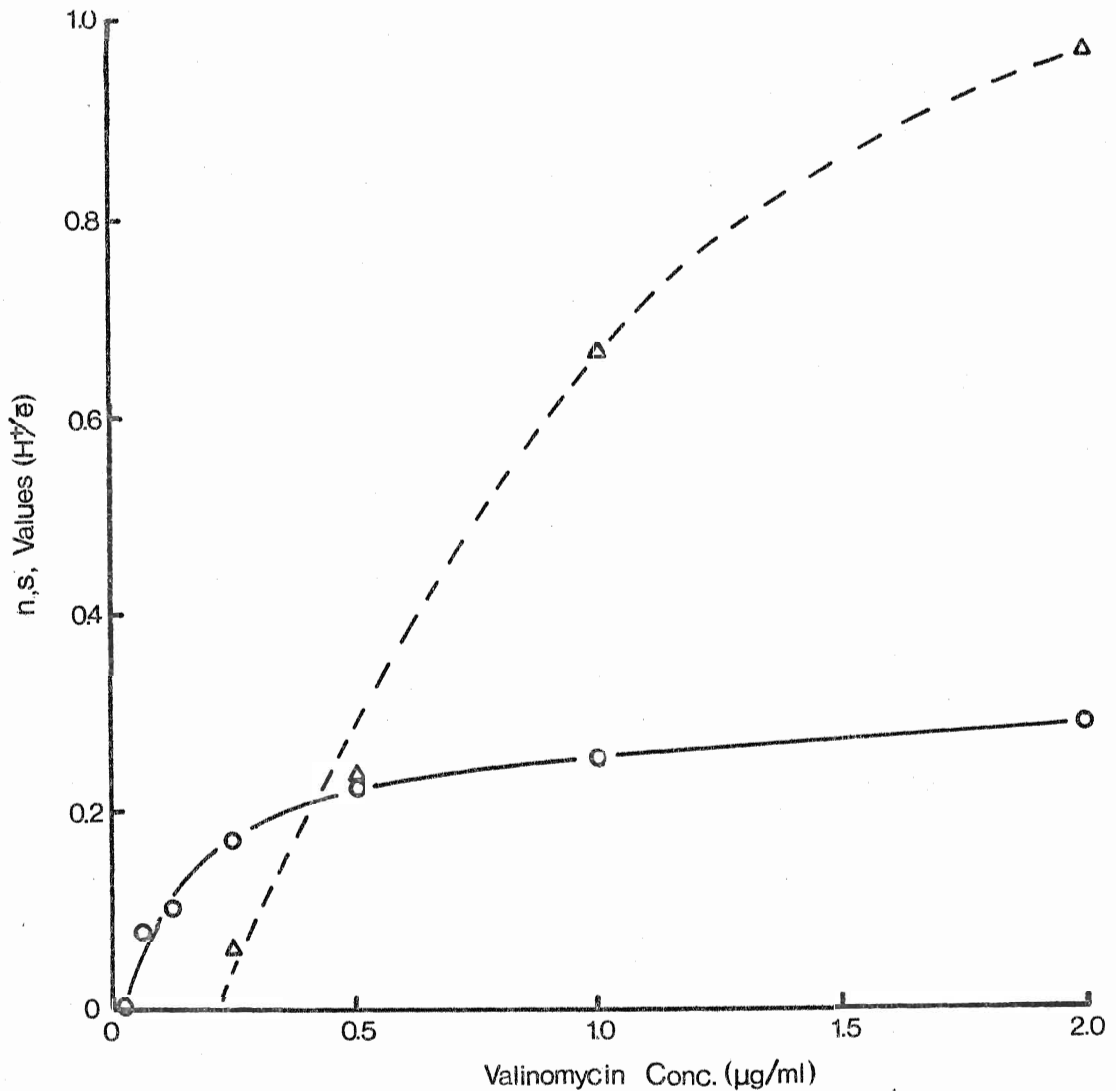
Ordinate show n and s values in terms of H+/e⁻ values.
Abscissa shows valinomycin conc. in μg/ml.

3.5ml of proteoliposomes[*] (aa₃ conc. = .412μM),
3.5ml of 50mM K_2SO_4, 0.12mM cyt. $\underline{c}$, 0.125mM
ascorbate, 1.25μM FCCP.

    ○: n values

    △: s values

    ■: s value in presence of FCCP

* Internal medium 50mM potassium phosphate.

FIGURE 28   79

The Effect of Valinomycin Concentration on Proton Translocation by Cytochrome c Oxidase: Na_2SO_4 as external medium.
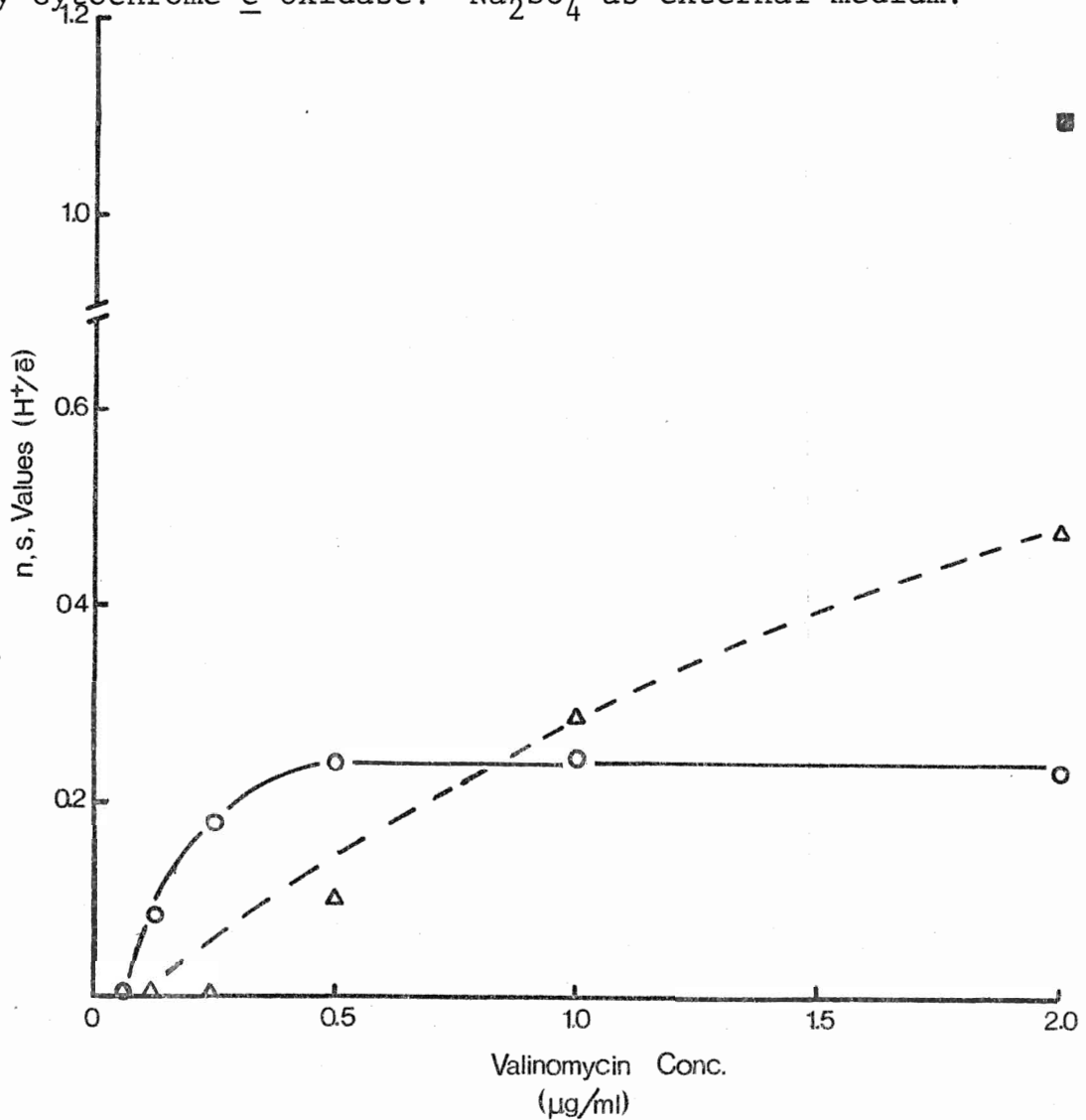
Ordinate shows n and s values in terms of $H+/e^-$ ratios. Abscissa shows valinomycin concentration in µg/ml.

3.5 ml of proteoliposomes,* (aa_3 conc. = 0.397µM), 3.5 ml of 50mM Na_2SO_4 0.12mM cyt. c, 0.125mM ascorbate, Temperature - 28°C, pH = 7.3 - 7.4

    O: n values

    △:   s values

    ■:   s value in presence of 1.25µM FCCP

*   Internal medium 50mM potassium phosphate.

The s values (alkalinization) in K_2SO_4 were substantially larger than in Na_2SO_4 at any given valinomycin concentration, reaching a maximum of 0.97 H+/$\bar{e}$, which was increased to 1.04 upon FCCP addition. The maximum s value obtained in Na_2SO_4 was 0.48 H+/e^- (at 2.0 μg/ml valinomycin) which increased to 1.1 upon FCCP addition, (Fig. 28). It may be noted that, as the vesicles were prepared in 50mM potassium phosphate in each case, a small amount (0.1 - 0.2mM) of K+ ions is always present in the incubation medium.

Oxygen induced proton pulses (n values) were also obtained with nonactin instead of valinomycin in the presence of 50mM K_2SO_4. These were substantially larger in magnitude than those obtained with equivalent concentrations of valinomycin and 50mM K_2SO_4. Fig. 29, shows that the maximum n value obtained was 0.33 H+/e^- at 1.0 μg/ml nonactin. Nonactin was also substantially more effective at lower concentrations ($<$ 0.5 μg/ml) than was valinomycin in allowing the detection of proton translocation.

The measured s values also increased at a substantially greater rate with increasing nonactin levels compared with valinomycin; further addition of ascorbate (▲) to the reaction mixture dramatically reduced the magnitude of the alkalinization according to eq. 3, (Fig. 29). FCCP addition increased the s value to 0.89 (hydroxyl ions per electrons).

D.   Internal Alkalinization of Proteoliposomes

1.   Characterization:

In an attempt to monitor the internal alkalinization which occurs in proteoliposomes during cytochrome c oxidase activity, the pH indicator pryanine (8-hydroxy-1.3,6-pyrenetrisulfonate) was entrapped in proteoliposomes according to the method of Clement and Gould (1981a), as described in Chap. III (pg. 28).

FIGURE 29   81

The Effects of Nonactin on Proton Translocation by Cytochrome
c Oxidase:
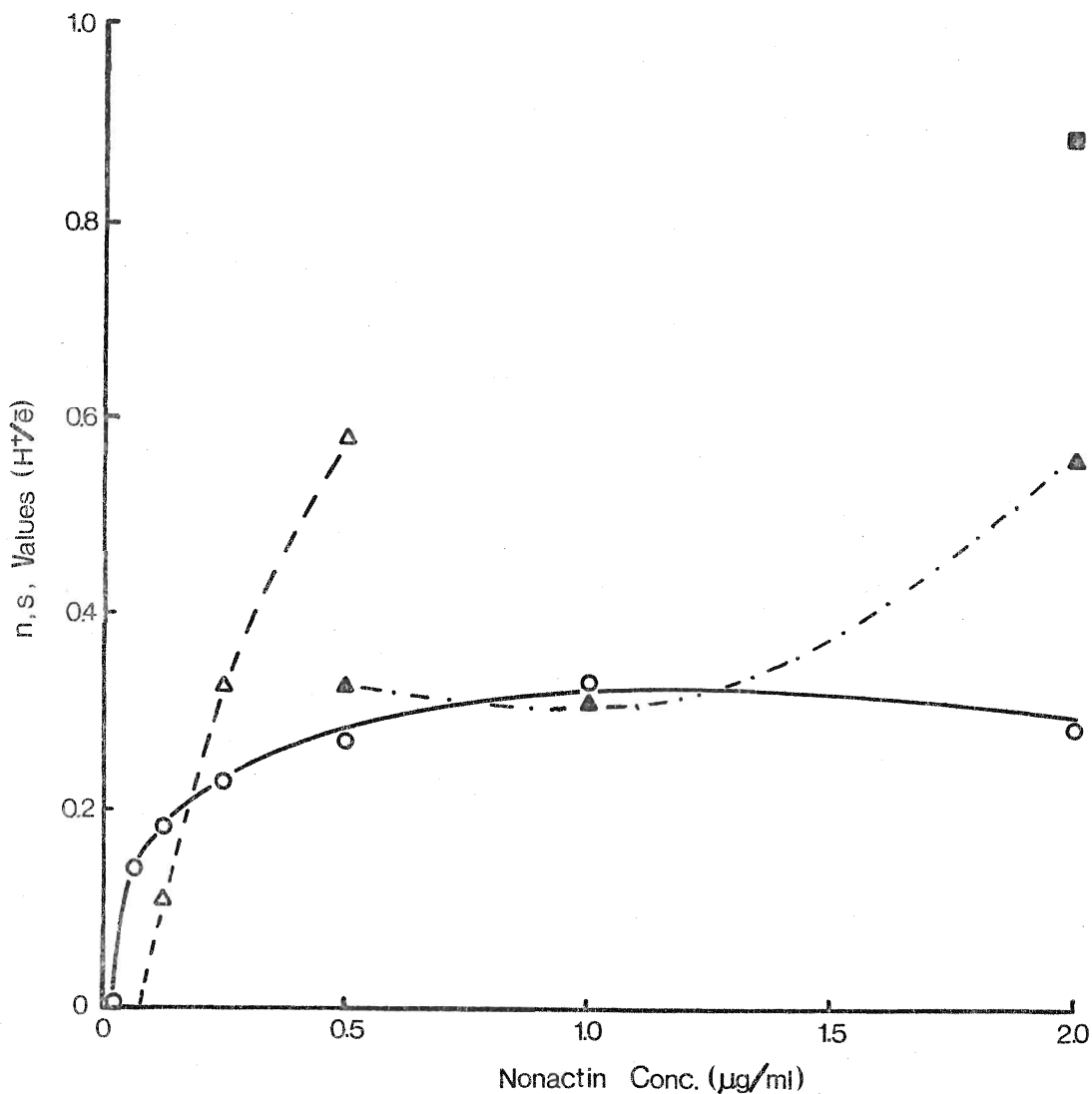
Ordinate shows n and s values in terms of H+/e⁻ ratios

Abscissa shows nonactin concentration in µg/ml

3.5 ml of proteoliposomes, (aa$_3$ conc. = .412µM),
3.5 ml of 50mM K_2SO_4, 0.12mM cyt. c, .125 mM ascorbate,
pH = 7.3 - 7.4, Temp. - 28°C, Volume - 8 ml.

O :   n values
△ :   s values (.125mM ascorbate)
▲ :   s values (.250mM ascorbate)
■ :   s values in the presence of 1.25µM FCCP

Fig. 30, illustrates the chromatographic separation of external pyranine (upper band), from proteoliposomes containing internally-trapped pyranine (lower band), as they pass down a Sephadex G-100 column. As can be seen the separation is quite distinct, indicating that little pyranine remains loosely bound externally to the vesicles but highly bound pyranine could be present externally.

The internal alkalinization in such vesicles induced by respiratory activity is then monitored by pyranine fluorescence. Fig. 31, shows the changes in internal pyranine fluorescence (ΔF) upon activation of cytochrome $\underline{c}$ oxidase turnover by addition of ascorbate, TMPD, and cytochrome $\underline{c}$ to an aerobic suspension of pyranine containing vesicles.

In Fig. 31, trace (a), the control, there is an immediate internal alkalinization upon oxidase activation, which collapses upon anerobiosis. In the presence of valinomycin [trace (b)] the initial rate of alkalinization is greater, as is its' collapse upon anerobiasis. Traces (c) and (d) show the alkalinization occurring in the presence of nigericin and FCCP respectively. Although there is a continuous alkalinization, this does not collapse upon anerobiosis. In the presence of these ionophores a pH gradient cannot be formed, and the observed effects are due to bulk pH change. The rate of this bulk alkalinization in the presence of FCCP is greater than that observed in the presence of nigericin, but is substantially less than that occurring internally either in the control or valinomycin stimulated systems.

The results presented in Fig. 32 show the internal and external alkalinizations which occur either in the presence of valinomycin [trace (a)] or in the absence of ionophores [trace (b)]. Upon anerobiosis [trace (a)], or the addition of FCCP, [trace (b)], there is a collapse of the pH gradient, represented by the ΔpH of x. The generation of this gradient is a combined function of the proton pumping and electron translocation activities of cytochrome $\underline{c}$ oxidase. The bulk alkalinization due to the overall cytochrome $\underline{c}$ oxidase reaction is given by the final ΔpH of y [traces (a) and (b)].

FIGURE 30

Separation of External Pyranine from Internally-trapped
Pyranine:

    Vesicles were passed down a G-25 Sephadex column
    equilibrated with 5mM Tricine/MES buffer, pH - 7.4,
    100mM Choline chloride and 5mM KCl at room temperature,
    at a rate of 1ml/min.

FIGURE 31

The Effects of Ionophores on Internal Alkalinization as
Monitored by Pyranine Fluorescence:

> 0.2ml proteoliposomes, 14.8mM ascorbate,
> 0.26mM TMPD, 3.7μM cyt. c, additions as
> indicated. 2.5ml of 5mM tricine/MES,
> pH - 7.5, total volume = 2.7ml.

a)   Control: (no other additions)

b)   1.9μg/ml of valinomycin.

c)   1.9μg/ml of nigericin.

d)   1.9μM FCCP

NOTE: ΔF is given in % of total fluorescence where
100% ΔF equals 3.25 pH units at pH 9.1,
(A.P.Singh personal communication). However,
fluorescence is only linear with ΔpH between
7.0 and 8.0 where these experiments were
conducted.

FIGURE 31

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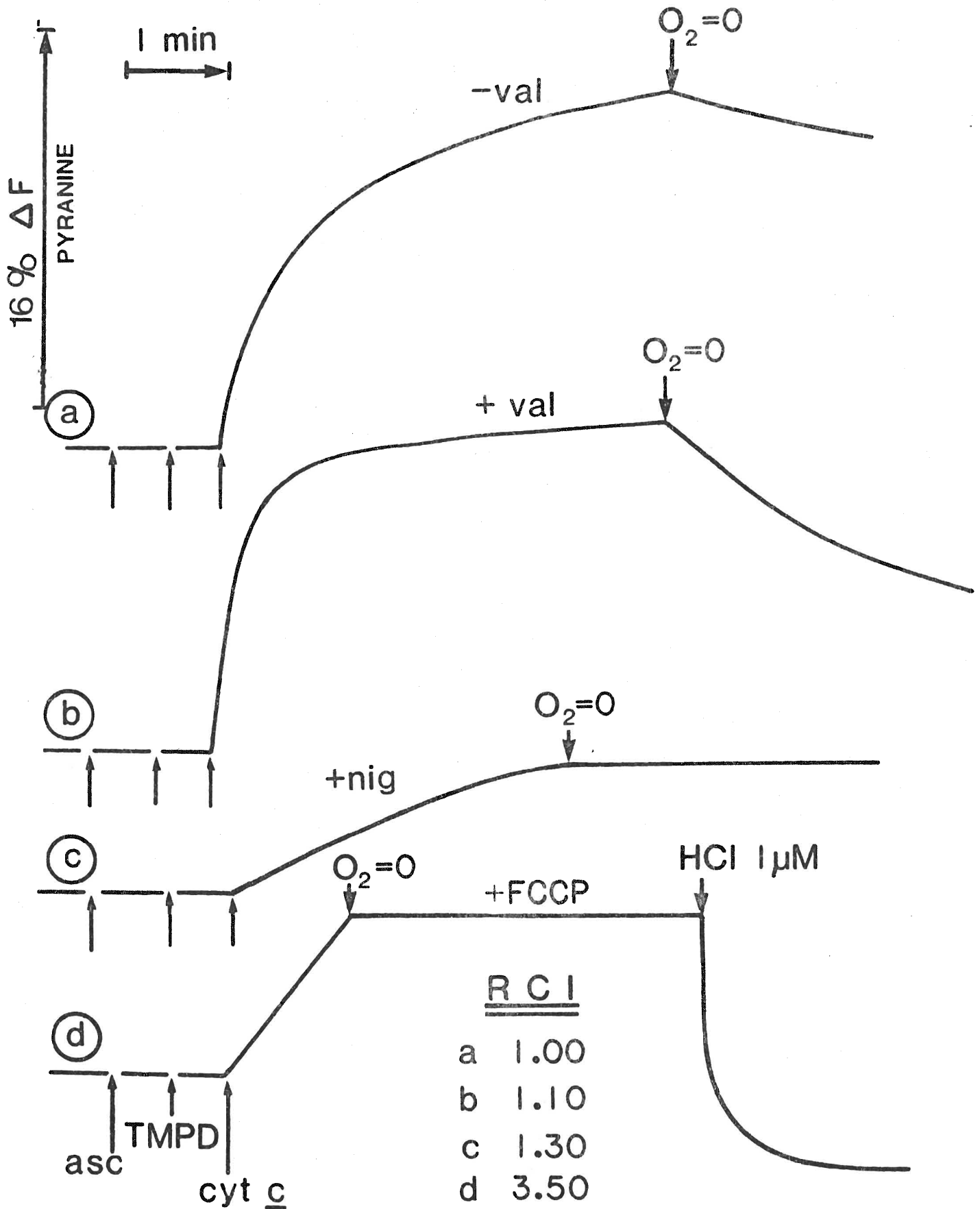# Effect of val, nig and FCCP on pyranine fluorescence
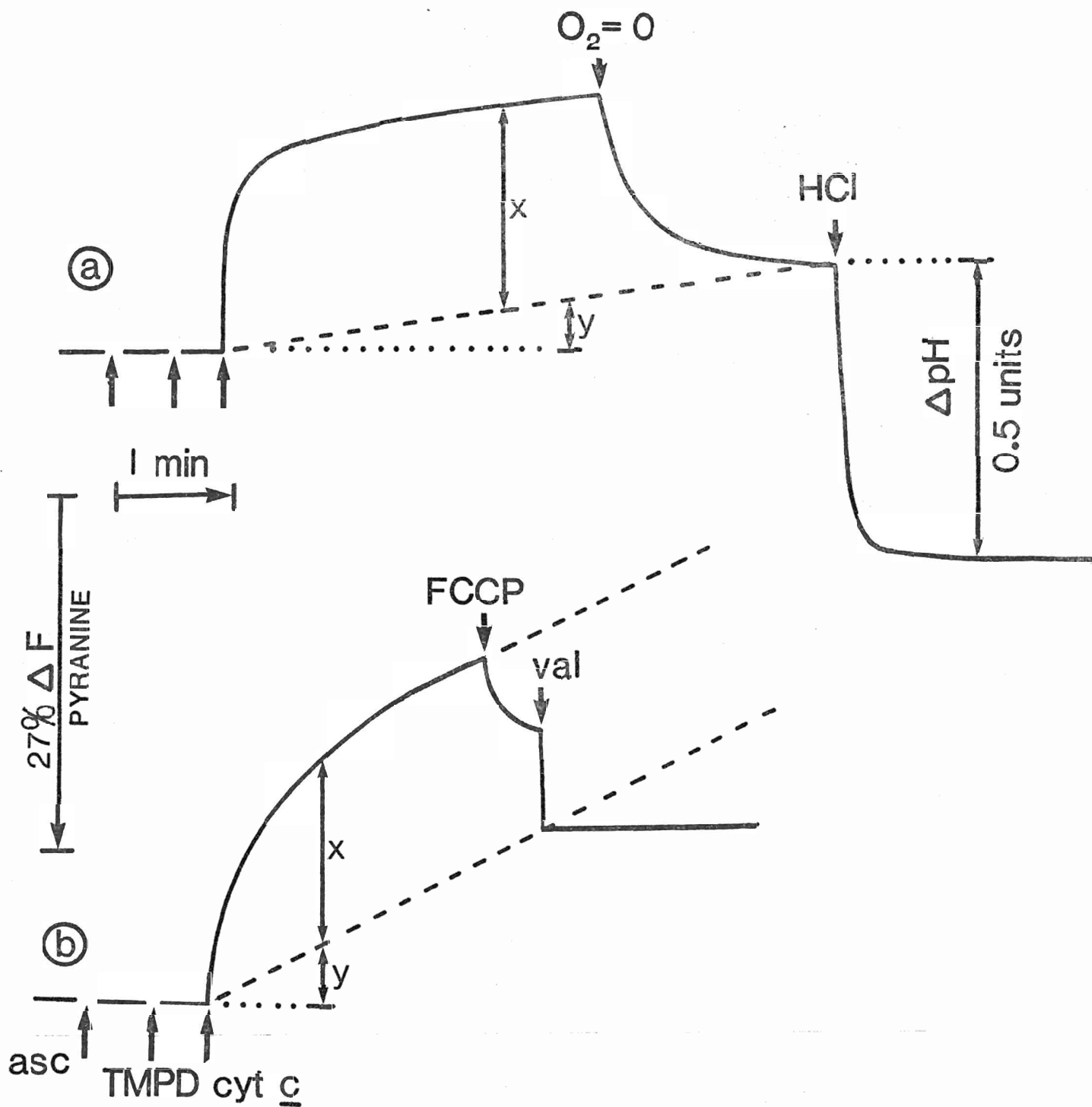
FIGURE 32

Characterization of the Internal Alkalinization as monitored by Pyranine Fluorescence:

> 0.2ml vesicles in 5mM tricine/MES, pH 7.5. Other conditions and reagent concentrations as in Fig. 31.

a) +valinomycin (1.9µg/ml)

b) Control: 1.9µM FCCP and 1.9µg/ml valinomycin added as indicated.

FIGURE 32

From the passive and active responses of the indicator a
calculation of the magnitude of the pH gradient formed by
cytochrome c oxidase can be made as shown in Fig. 33. A pH gradient
of approx.0.35 pH units was estimated assuming vesicle homogenity.

The extent of alkalinization as monitored by pyranine
fluorescence is proportional to the electron flux through
cytochrome c oxidase. In the experiments of Fig. 34, oxygen
uptake measured polaragraphically was used as a measure of
electron flux through cytochrome c oxidase. Fig. 34 (a),
shows that a linear correlation exists between fluorescence
change (steady state) and the proportion on uninhibited oxidase
(monitored simultaneously). The ordinate represents the % of
the control (ΔF_{ss} value) obtained in absence of KCN. The
abscissa represents the percent oxidase uninhibited by KCN
as measured polaragraphically. Thus 100% (X-axis) represents
the maximum rate of oxygen uptake in the absence of KCN and
100% (Y-axis) represents the maximum magnitude of alkalinization
(ΔF_{ss}) observed in the absence of KCN.

In Figure 34 (b), electron flux was varied by altering
the ascorbate concentration. The Figure shows that a linear
relationship also exists between the initial rate of fluoresence
($\Delta F/sec$), and oxygen uptake, as measured polargraphically.
As ascorbate concentration was increased a proportional
increase in the rate of initial fluorescence change and oxygen
uptake was observed. From the data in Fig. 34 (b), $H+/e^-$
ratios for internal alkalinization may be calculated. In order
to do so, however, the initial rate of fluorescence change
must be converted into the initial rate of proton disappearance
(OH^-, production). This is possible only if the relationship
between pH change and fluoresence change is known ($\Delta pH/\Delta F$)
as well as the internal buffering ($B = \Delta H+/ pH$). The former
can be calculated experimentally while the latter depends upon
trap volume and the concentration of internal buffering species
(see appendix 1). When this calculation was performed as
outlined in appendix 1 an $H+/e^-$ ratio of approximately 0.38
$H+/e^-$ was found for the data in Fig. 34. However, due to the

FIGURE 33

Calculation of $\triangle$pH as monitored by Pyranine Fluoresence:

0.2ml proteoliposomes, 14.8mM ascorbate,
0.26mM TMPD, 3.7μM cyt. c. 2.5ml of 5mM
tricine/MES, pH - 7.5, total volume - 2.7ml.

FIGURE 33

$$\Delta pH = \frac{pH\,initial - pH\,final}{Z} \times X$$
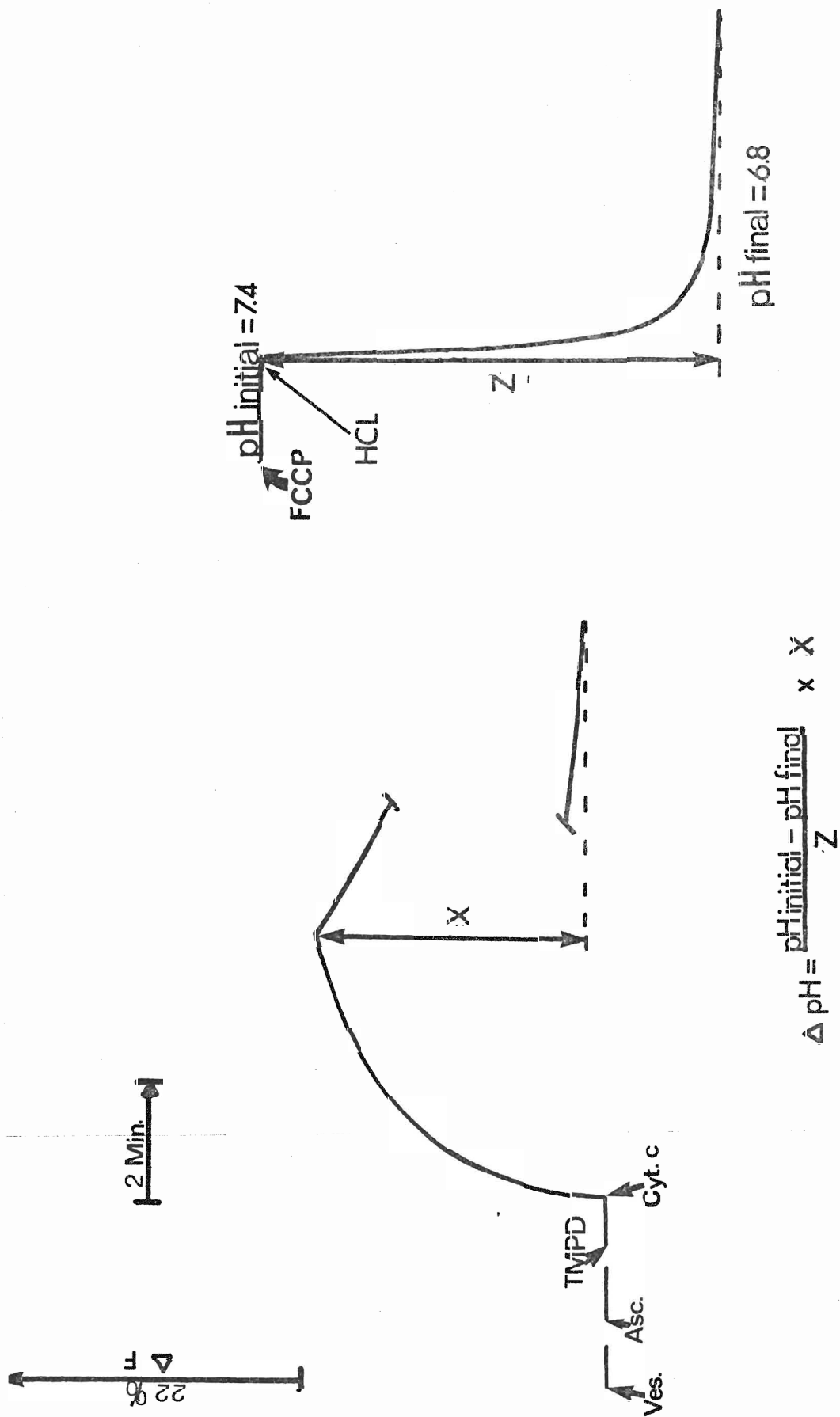
FIGURE 34

The Effect of electron flux through Cytochrome c Oxidase
on the internal alkalinization as monitored by Pyranine
fluorescence.

     0.2ml vesicles, 2.5ml tricine/MES, pH - 7.4,
     3.7μM cyt. c, 1.9μg/ml valinomycin.

a)   14.8mM ascorbate, 0.26mM TMPD.
     KCN was used to alter electron flux (0 - 36μM KCN)

b)   ascorbate was used to alter electron flux.
     TMPD was not present.

a)

ΔF_{SS} (%)

Oxidase Respiration Rate (%)

b)

$\Delta F/sec$

Oxidase Turnover (neq.e/sec)

FIGURE 34

inherent uncertainties in such a calculation, this is not
compelling evidence against proton translocation. The latter
remains unproven. But by the variation of substrate and of
inhibitor concentrations, it was demonstrated that both the
magnitude (ΔF_{ss}) and the initial rate of internal fluorescence
change ($\Delta F/min$) are proportional to electron flux.

2.   DCCD (dicyclohexylcarbodiimide) Effects Upon
      Internal Alkalinization

The effect of dicyclohexylcarbodiimide (DCCD) on the
initial rate of internal alkalinization and on the steady state
magnitude of the alkalinization within the vesicles upon
initiation of oxidase activity was then studied using the
fluoresence of entrapped pyranine. The internal alkalinization
depends on two processes: a) oxidase mediated uptake of
protons while reducing molecular oxygen to water, and b)
the proton pumping function of cytochrome c oxidase.

Cytochrome c oxidase containing vesicles were preincubated
with DCCD (final concentrations as shown in Fig. 35) overnight
at 4°C. Fig. 35 shows the effect of DCCD preincubation on the
initial rate of internal alkalinization in the absence of
valinomycin (circles), and on the steady state magnitude of
internal alkalinization (squares). The triangles represent the
effect of DCCD on oxidase turnover as measured polarographically.
The Y-axis expresses the above parameters in terms of percentage
of the control (ie. with DCCD absent), where control values
are arbitrarily set at 100%. The X-axis represents the final
concentration of DCCD, with which the vesicles were preincubated
at 4°C overnight.

Figure 35 (b) shows the results of an anologous experiment
carried out in the presence of valinomycin, using the same
batch of vesicles but on the following day. Figs. 35 (a) and
(b) demonstrate that the initial rate of internal alkalinization
decreases with increasing DCCD concentration. There is a
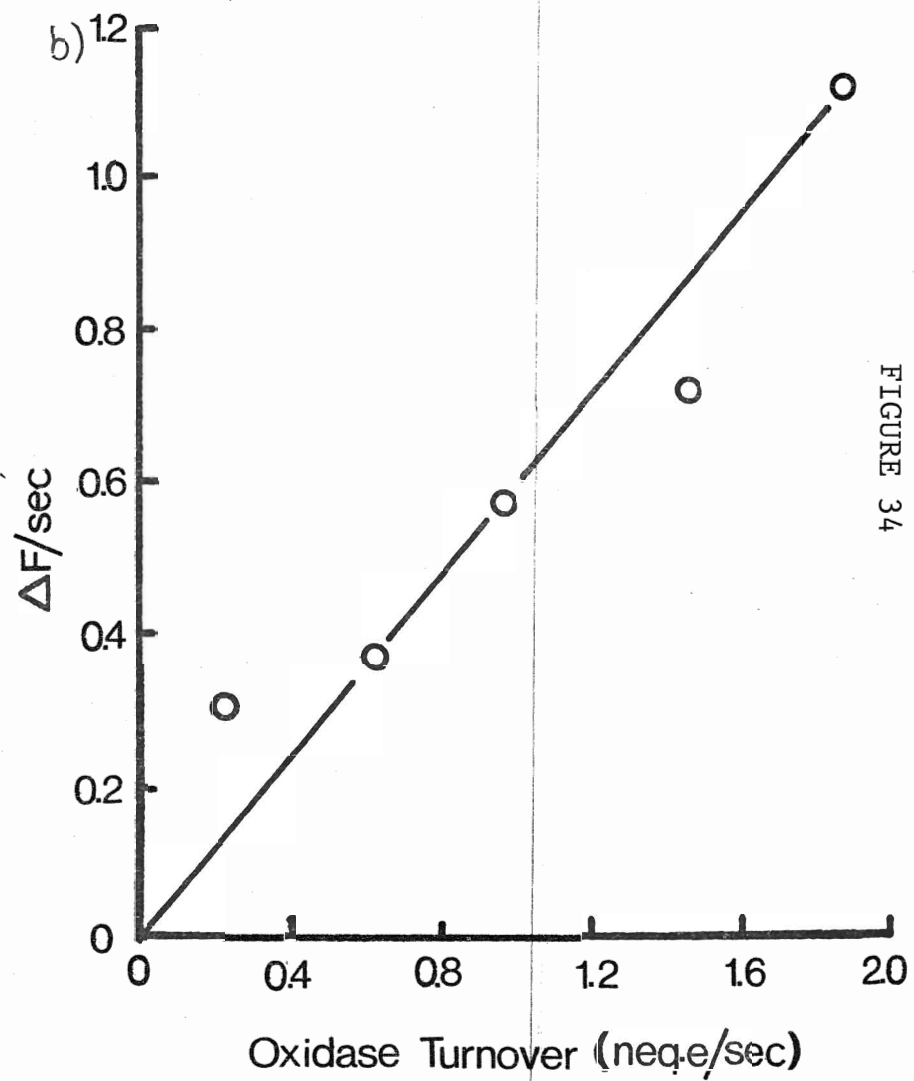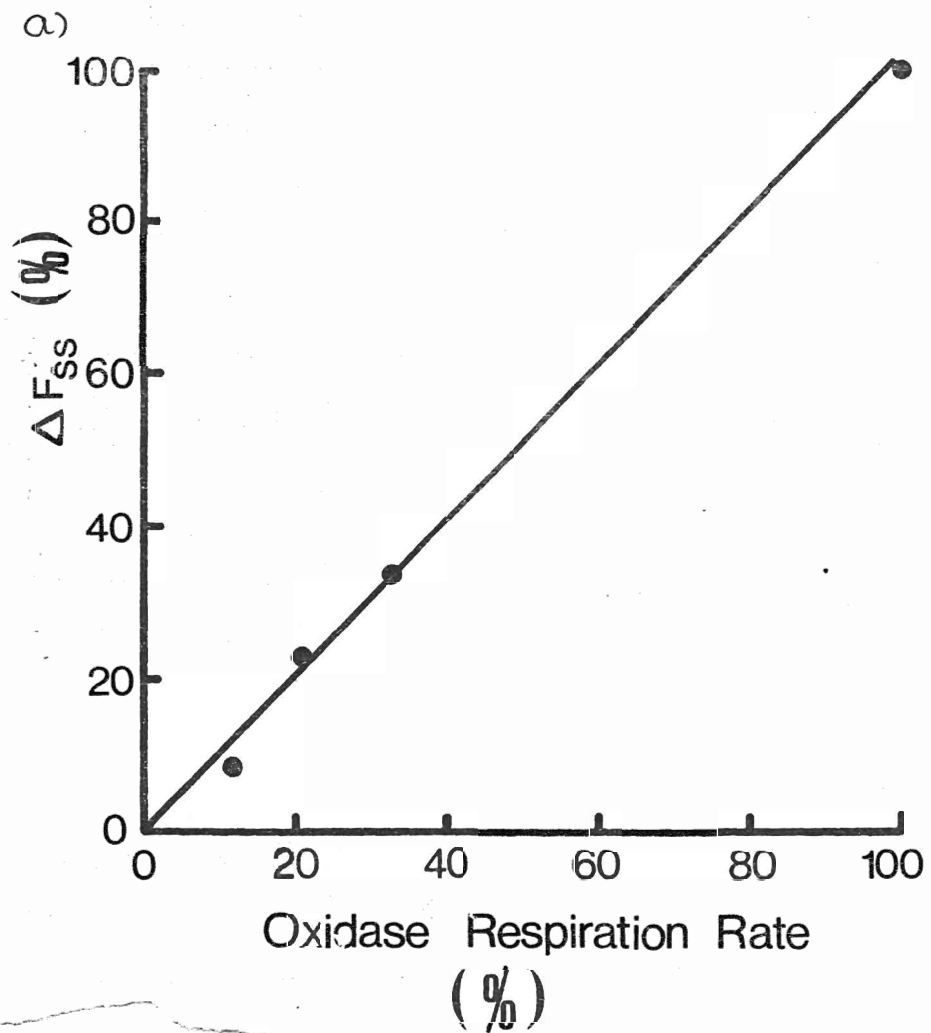stimulation of oxidase turnover at lower DCCD concentrations
which then decreases below the control value at higher DCCD

FIGURE 35

The Effect of DCCD on Internal Alkalinization as monitored
by Pyranine fluoresence:

Conditions: (A - D)

0.3ml of a 1:3 dilution of vesicles, 14.8mM
ascorbate, 0.26mM TMPD, 3.7µM cyt. c, 1.9µg/ml
valinomycin (B and D only), 2.4ml tricine/MES
pH - 7.4.

A: The effect of DCCD in the absence of
     valinomycin on the steady state magnitude
     of internal alkalinization (▱), the
     initial rate of internal alkalinization(o)
     and rate of respiration, (△).

B: The effect of DCCD in the presence of
     valinomycin on the steady state magnitude
     of internal alkalinization (▢), the
     initial rate of internal alkalinization(⟡)
     and rate of respiration, (△).

C: The effect of DCCD in the absence of
     valinomycin on the steady state magnitude
     of internal alkalinization divided by the
     rate of respiration, (△) and the initial
     rate of internal alkalinization divided
     by the rate of respiration, (◐).

D: The effect of DCCD in the presence of
     valinomycin on the steady state magnitude
     of internal alkalinization divided by the
     rate of respiration, (◯) and the initial
     rate of internal alkalinization divided
     by the rate of respiration, (△).

FIGURE 35 (A)   90

(Δ)

% of Control

DCCD Conc. (mM)

FIGURE 35 (B)   91

DCCD   Conc. (mM)
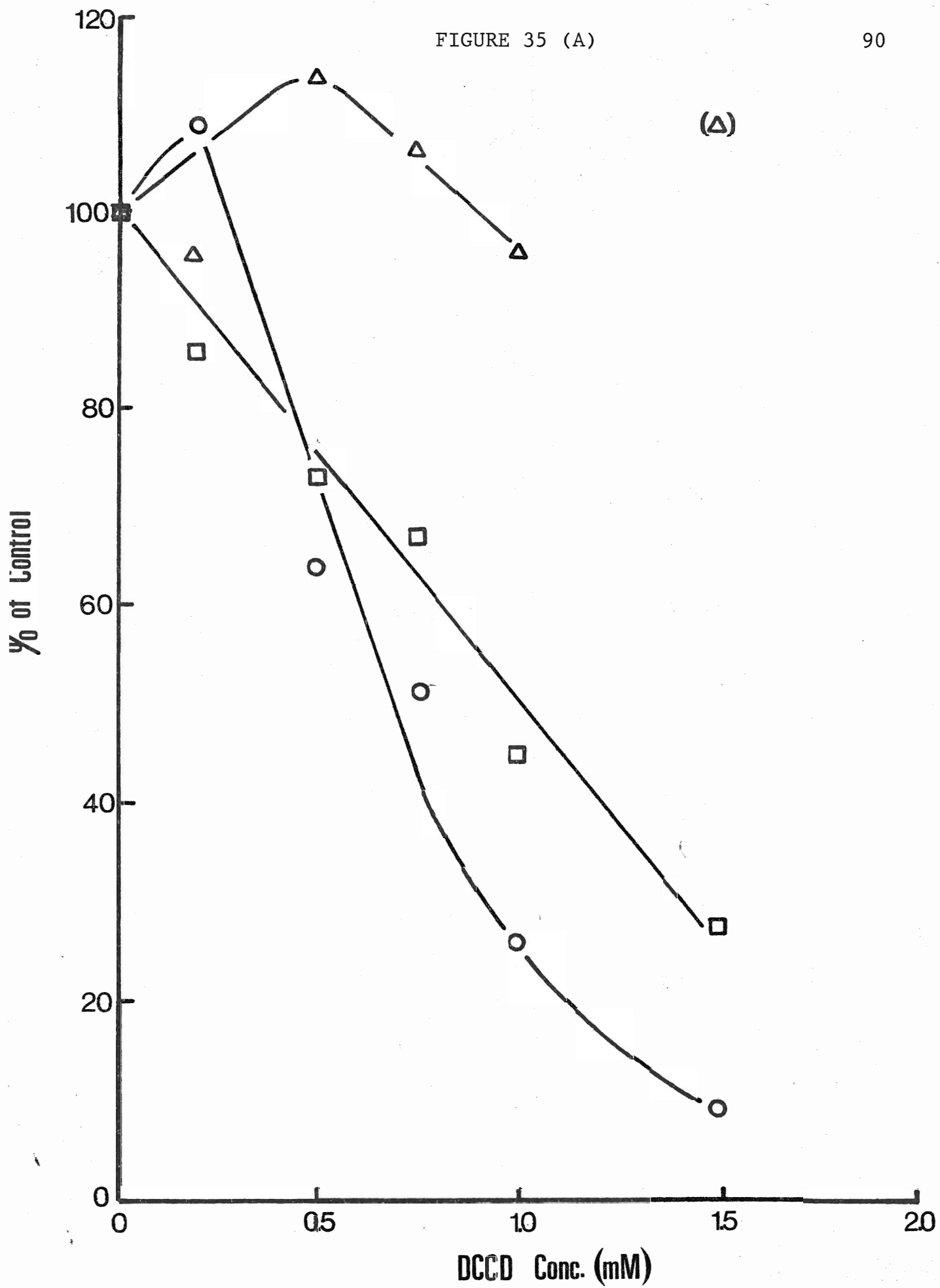
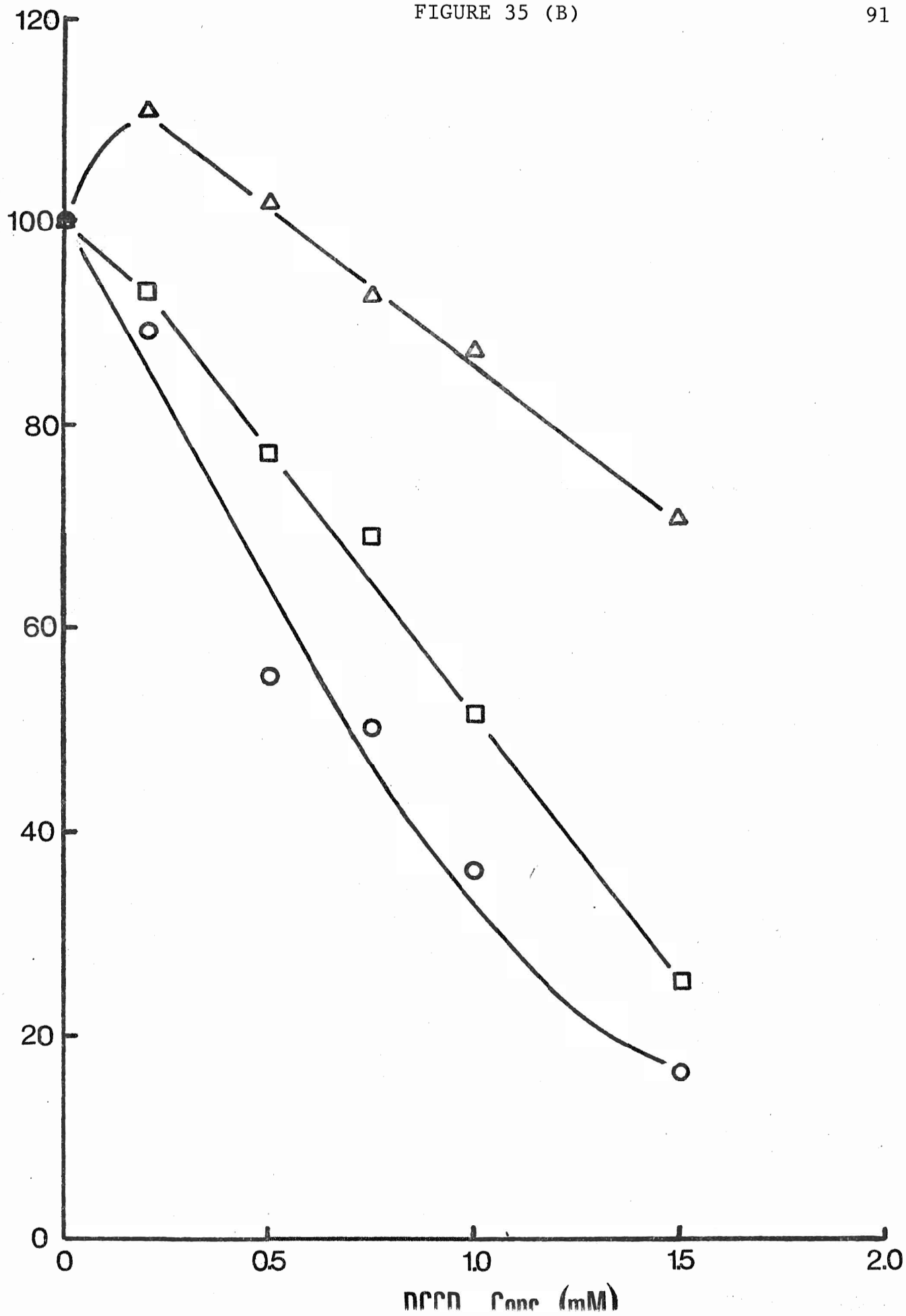FIGURE 35 (C)   92
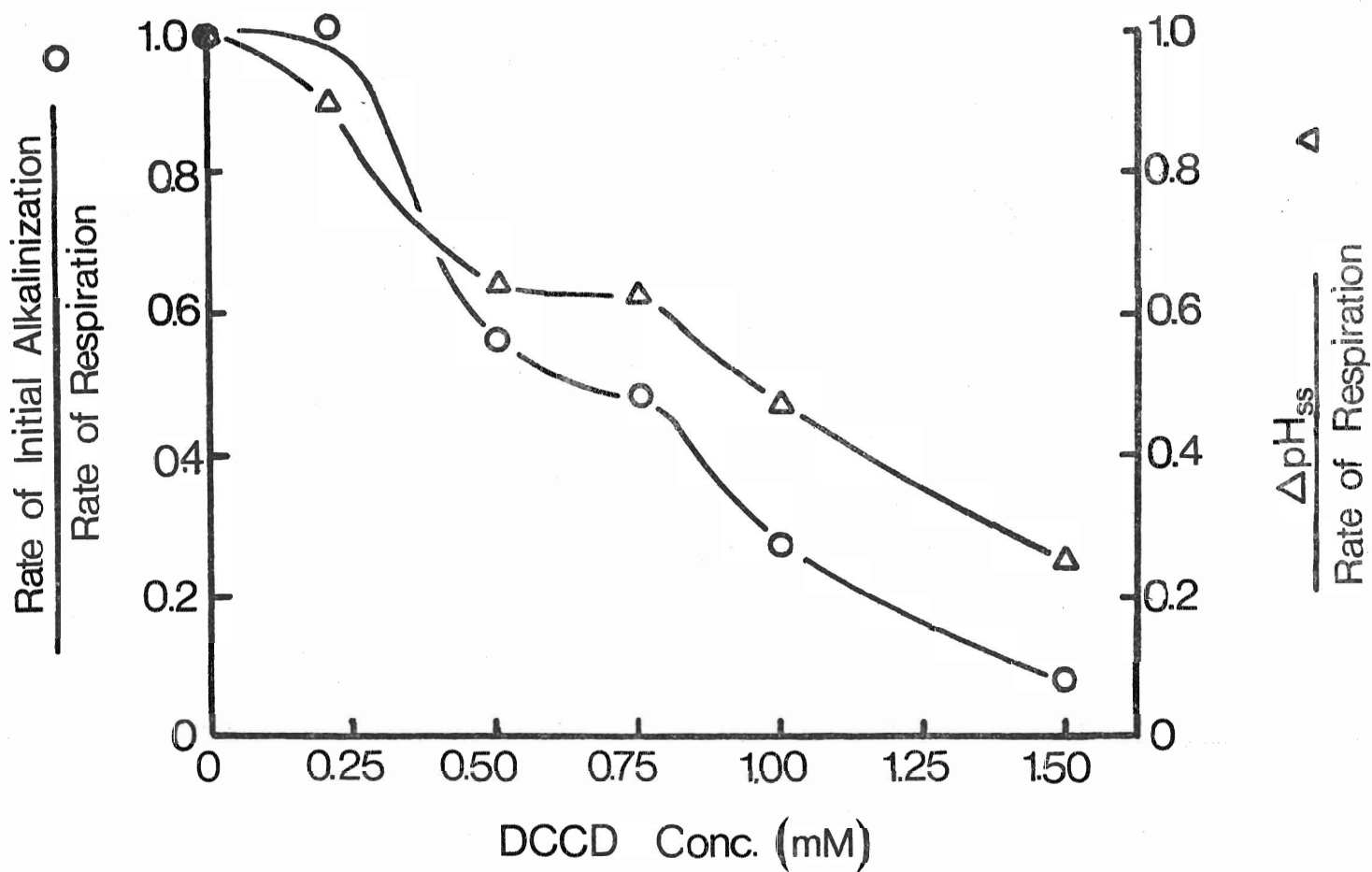
FIGURE 35 (C)

FIGURE 35 (D)
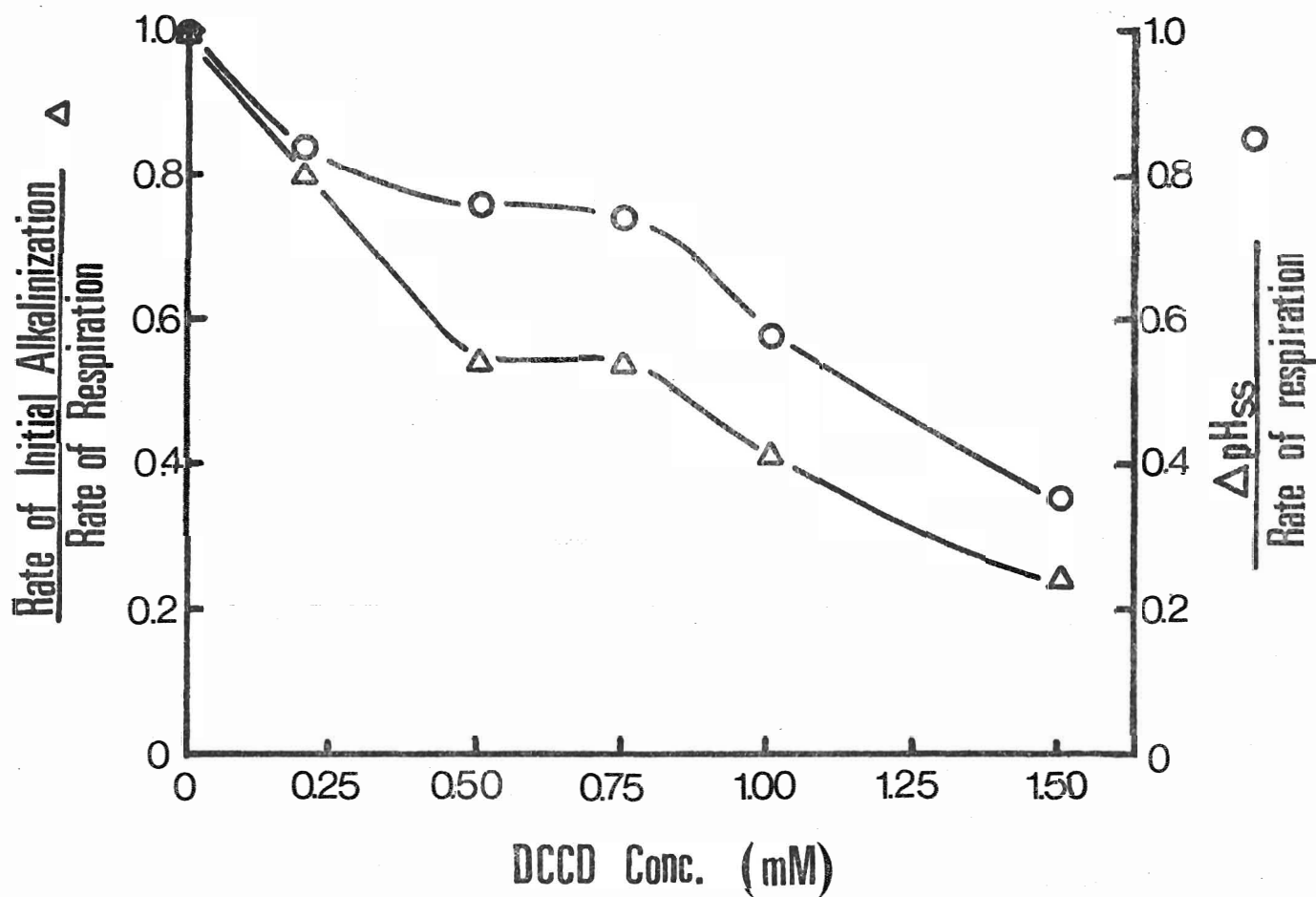
DCCD concentrations. In the absence of valinomycin the initial rate of internal alkalinization decreases by approximately 70% before oxidase turnover begins to become inhibited. The experiments carried out in the presence of valinomycin show an approximate 40% inhibition of alkalinization before oxidase turnover is inhibited.

The steady state magnitude of internal alkalinization, (□ , A & B) also decreases with increasing DCCD concentration. In the absence of valinomycin the magnitude of the internal steady state alkalinization decreases by almost 50% before oxidase turnover begins to become inhibited. In the presence of valinomycin the magnitude of the steady state alkalinization decreased by 25% before inhibition of oxidase turnover occurred. Thus there appears to be a portion of the internal alkalinity change other than that due to the scalar reaction of cytochrome c oxidase (eq. 1) which is DCCD sensitive, presumabley cytochrome c oxidase proton pumping function.

Fig. 35 (C) and (D) are replots of the data found in 35 (A) and (B) respectively. The left hand axis represents the % rate of initial alkalinization (of control values) divided by the % rate of respiration, also that of the control. The right hand axis represents the % extent of alkalinization ($\triangle pH_{ss}$) divided by the % rate of respiration. DCCD concentration is given on the X-axis.

In both presence and absence of valinomycin the curves show biphasicity, plateauing between 0.50 and 0.75 mM DCCD. The second phase is due to nonspecific effects of DCCD at high concentrations, including loss of respiratory control. In both systems, there is an approximate 50% inhibition of the rate of internal alkalinization.

3.   Oxygen Pulses Induced Proton Release,
      Monitored by Pyranine Fluoresence

O_2 pulse --induced pH changes were also monitored using
internally trapped pyranine. Fig. 36 demonstrates the appearance
of O_2 pulse induced alkalinization as monitored by pyranine
fluorescence. In the presence of valinomycin (trace B) the
pulses were typically 80% larger (more alkaline) than in its
absence (A). In the presence of valinomycin plus FCCP no
change in internal alkalinity was observed (trace C).

As a result of proton translocation by cytochrome c
oxidase, the $H+/e^-$ stoichiometry for the internal alkalinization
in the presence of valinomycin would be expected to be greater
than 1. One proton taken up per electron would be due to the
scalar reaction of cytochrome c oxidase and the uptake to
at least one further proton would be due to proton trans-
location by the oxidase. Typical $H+/e^-$ values were, however,
found to average 0.10 in the presence of valinomycin (appendix 2).

The presumed underestimation of the $H+/e^-$ ratio may be due
to the inherent problems in calculating internal buffering
capacity. However, the experiments demonstrate qualitatively
that the internal monitoring of oxygen-pulse induced
alkalinization is possible by this method.

FIGURE 36

Oxygen induced Proton pulses as monitored internally by
Pyranine Fluorescence:

0.2ml of proteoliposomes, 2.5ml 50mM K_2SO_4,
pH = 7.4, 14.8mM ascorbate, 3.7µM cyt. c,
1.9µM FCCP, 1.9µg/ml valinomycin. Pulse size -
23 neq e^- (25µl O_2-air saturated).

NOTE: △F is given in % of total fluorescence where
100% △F = 3.25 pH units at pH 9.1. However,
fluorescence is only linear with △pH between
7.0 and 8.0 where these experiments were
conducted.

FIGURE 36
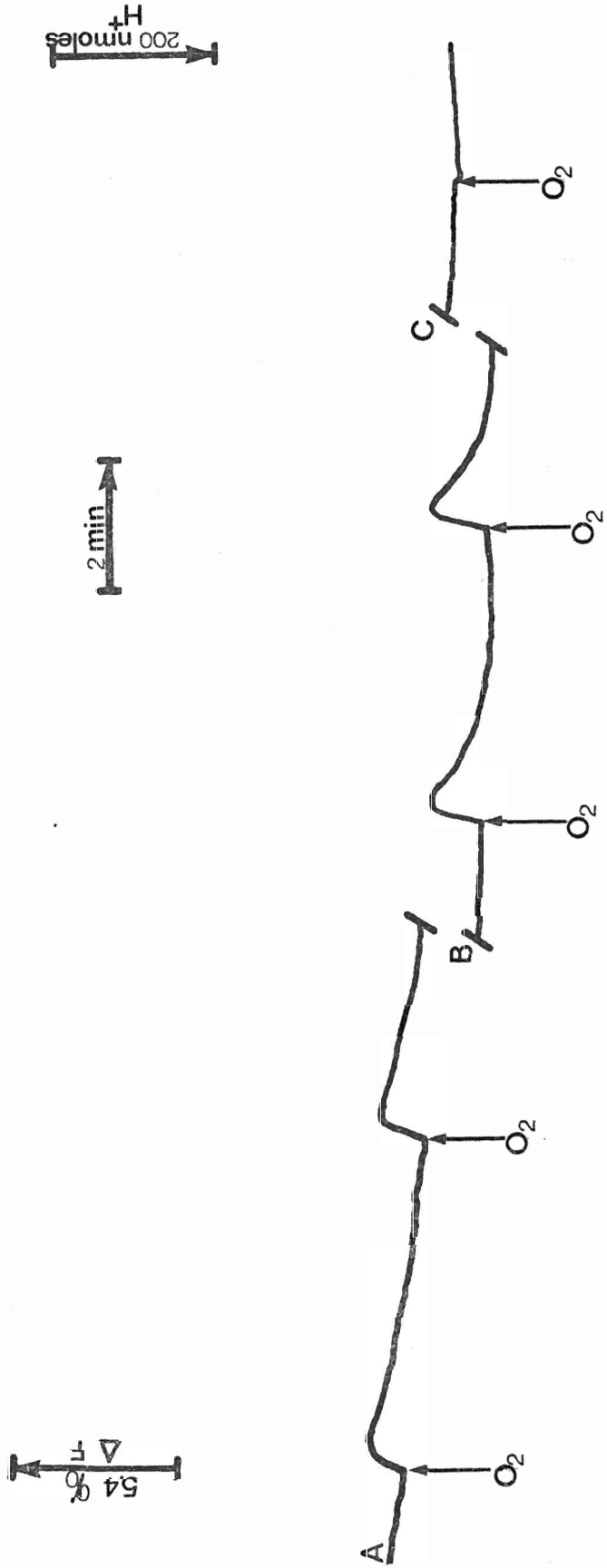
Evidence relating to the vectorial transfer of protons includes data from five sources:

    A)   Detergent lysis of vesicles;

    B) $K_3Fe(CN)_6$ treatment of c^{2+} plus vesicles;

    C)   Low Ionic Strength O_2 and c^{2+} Pulses;

    D)   Ionophore effects

    E)   Pyranine monitoring of $\triangle$pH.

A)   Effects of Lauryl Maltoside on Proton Pulses, Oxidase Turnover and Cytochrome c Binding:

Proteoliposomal membrane rupture occurs upon Lauryl Maltoside addition. Fig. 10 indicated that such membrane rupture occurs at maltoside concentrations greater than 0.025%. Above this concentration ionophore additions have no effect. Upon membrane rupture, oxidase molecules in the inverted orientation which were previously inaccessible to cytochrome c may now bind cyt. c, increase oxygen uptake, and thus stimulate enzyme turnover. Such enzyme molecules comprise approximately 50% of the total in these vesicles. The apparent binding of cytochrome c to the vesicles also increases upon maltoside addition, reflecting the additional exposure of the inner membrane to the externally added cytochrome c.

Although lauryl maltoside does not inhibit the oxidase, Triton X-100, and DOC do, as was shown in Figures 8 & 9. This inhibition is probably due to the stripping of the surrounding phospholipids from the oxidase, (Nicholls et. al., 1980). In addition, Triton X-100 may affect cyt. c binding to the oxidase (Fig. 11).

Lauryl maltoside addition also affects the pattern of O_2-induced proton pulsing (Fig. 15). The decreased H+/e$^-$ ratios and constant t½ values which occur upon lauryl maltoside addition, support the concept of vectorial proton translocation by the oxidase. The data indicate the existence of two oxidase populations in the presence of lauryl maltoside. Some oxidase is in intact vesicles for which proton translocation is still observable and for which the proton permeability of the membrane

is unaltered, (ie. the t1/2 values are unchanged). The rest of
the enzyme is in vesicles which have lysed and which cannot con-
tribute to the observed H+/e ratios but can further decrease the
H+/e ratio. If the observed proton release upon pulsing were in-
deed a scaler event as Mitchell and Moyle (1983a) suggested,
lauryl maltoside addition,which does not effect oxidase turnover
or cytochrome c binding,would not be expected to affect the ob-
served H+/e⁻ ratios.

B) Protons are not Released upon $K_3Fe(CN)_6$ Additions:

Upon the oxidation of cyt.c in the presence of proteoliposomes
by $K_3Fe(CN)_6$ no proton release is observable other than that
attributable to the oxidation of ascorbic acid. The proton re-
lease observed upon oxidation by O_2 via the oxidase must there-
fore be a purely vectorial process.

C) The Effects of Low Ionic Strength on Proton Pulses:

Papa et.al.,(1983a) reported that the scaler proton release
using ferrocytochrome c pulses at low ionic strength, exceeded
in magnitude the observed proton release at high ionic strength,
(Hinckle 1977). The results obtained in this present study indi-
cate that the observed acidity seen upon ferrocytochrome c

pulsing at low ionic strength is a result of two processes: a
scalar process (eq.8), and a vectorial process (eq.9).

$$\text{eq. 8:} \quad c^{2+} + XH \longrightarrow c^{2+}\text{-}X + H+$$

$$\text{eq. 9:} \quad nH^+_{in} \longrightarrow nH^+_{out}$$
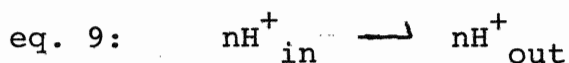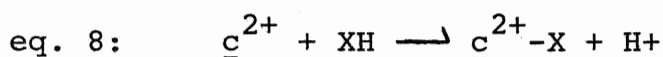
Only the vectorial process (eq.9) occurs at high ionic
strength. Table 1 demonstrated that   proton release is observed
with ferrocytochrome c induced pulses at low ionic strength
even in the absence of enzyme. This occurs as a consquence of
the scalar process of eq.8. The acidification seen in the presence
of oxidase is greater than in its absence and is

attributable to the combination of eqs. 8 & 9. At high ionic
strength the acidification seen upon pulsing is assigned only
to eq.9.

Possible canidates for X in eq.8 are the negatively char-
ged phospholipids phosphatidyl serine (as previously proposed
by Mitchell and Moyle,1983) or phosphatidyl inositol probably
found in asolectin.

The collapse of acidification at low ionic strength mimics
that seen at high ionic strength. This realkalinization is due
primarily to proton requilibration across the membrane,involving
both the pumped protons and those taken up as cytochrome c
oxidase reduces molecular oxygen. But in the absence of enzyme,
as indicated by the data in Fig.19A and B, a lipid mediated auto-
xidation reaction (eq.10) can also induce alkalinization.

eq.10: $2c^{2+}-X + 1/2O_2 + 2H^+ \longrightarrow 2c^{3+}-X + H_2O$
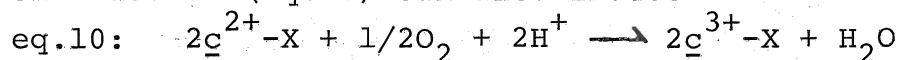
This process occurs only at low ionic strength. Upon
successive ferrocytochrome c pulses, the extent of realkalinization
declines but may be restored upon H_2O_2 addition in the presence of
catalase,restoring O_2 to the system. Under these conditions the
lipids undergo a lipid autoxidation.

In contrast to the results with ferrocytochrome c pulses,
the acidification induced by O_2 pulses at low ionic strength is
almost entirely due to vectorial proton release by cytochrome c
oxidase. No proton release is observed in the absence of enzyme
or in the presence of inhibited enzyme (Table 5). With O_2-induced
pulses there is an excess of ferrocytochrome c in the reaction
medium prior to pulsing. Thus all of the enzyme sites and most
of the phospholipid sites for cyt.c binding are occupied and the
reaction of eq.8 does not occur. But these pulses do show a slight
increase in H^+/e^- ratios at low ionic strength. This could be a
reflection of the additional binding of cytochrome c which can
occur upon oxidation due to the higher affinity of the ferric
form of the cytochrome (Nicholls et.al.,1980).

This increase in the apparent value of "n" at low ionic
strength is also seen with ferrocytochrome c induced pulses. If
the acidification known to be due to cyt.c binding is

subtracted from the overall acidification in the presence of enzyme, the apparent "n" value is still greater than that observed at high ionic strength. This difference is of a similar magnitude to that found with oxygen induced pulses when values at low and high ionic strengths are compared.

D)   The Effects of Ionophore Addition on Oxidase Turnover
        and Proton Translocation:

Sarti et. al., (1983) have used the ionophore nonactin instead of valinomycin to collapse $\Delta \Psi$ in proton pumping proteoliposomes. The present study indicates that the H+/e$^-$ ratios obtained in the presence of nonactin (cf. Fig. 27 vs. 29) are somewhat greater and occur at lower ionophore concentrations than those obtained with valinomycin. This enhancement of the H+/e$^-$ ratio may indicate that nonactin collapses $\Delta \Psi$ more effectively than does valinomycin. Thus the $\Delta \Psi$ created by oxidase turnover is not necessarily always collapsed fast enough for all of the protons translocated to be observed. Casey et. al., (1984) found that the decay kinetics of such proton pulses were much slower than would be expected from observations of passive decay kinetics. This was attributed to insufficient charge equilibration in the presence of low levels of valinomycin and was corrected for by increasing valinomycin concentration or by replacing K+ with tetraphenyl phosphonium. Fig. 22, shows that nonactin alone stimulated proteoliposomal respiration to a greater extent than did valinomycin alone. Valinomycin addition may not fully collapse $\Delta \Psi$ within the time scale of this experiment, (1 - 2 min.).

The s values for the realkalinization seen following proton release into the external medium often do not reach the expected value of 1H+/e$^-$. Mitchell and Moyle (1983) take this as evidence for scalar proton release (see section $\text{I}8$). The evidence in Figs. 26 and 27, indicate that much higher concentrations of valinomycin than which is typically used is required to observe "s" values equal to one. In order to allow sufficient K+ exchange to compensate for proton influx an excess of valinomycin is needed (cf. Casey et. al., 1984)

The inhibition of Alamethicin stimulation of respiration by valinomycin can be explained by the fact that this ionophore forms a voltage gated channel, (Mueller and Rudin, 1968). Upon valinomycin addition, alamethicin stimulation of respiration becomes inhibited due to a "closing" of the alamethicin channels. This could thus be a useful future tool in monitoring the effects of various ionophores in collapsing $\Delta \psi$. The concentration dependence for alamethicin stimulation of respiration may indicate the formation of channels consisting of several molecules of the ionophore, in accordance with the findings of Gordon and Haydon, (1972), although cooperativity was not seen in these experiments.

E)   Evidence From Studies Using Pyranine-Entrapped Vesicles:

Mitchell and Moyle, (1983) suggest that DCCD inhibits proton release by binding to some of the specific components involved in the cyt. c - phospholipid salt bridges, eg. phosphatidyl serine (see literature review section). If this were indeed occurring, DCCD should have no effect upon internal alkalinization. If, however, DCCD inhibits proton translocation then the inhibition of a proton pump with a stoichiometry of 1 H+/e$^-$ would lead to a 50% inhibition of internal alkalinization. The results in Fig. 35 (c) & (d), show that this is indeed the case. 50% inhibition of ΔF_{ss} occurs upon incubation at higher DCCD concentrations. This occurs since the transfer of one electron to molecular oxygen leads to the scalar uptake of one proton and to the translocation of one proton. Upon inhibition of proton translocation only the scalar uptake of one proton occurs.

An apparent contradiction occurs however, the resolution of which is not intuitively obvious. In the absence of valinomycin proton translocation is not observed upon pulsing. Then why is an approximate 50% inhibition of the initial rate of internal alkalinization observed upon DCCD preincubation in the absence of valinomycin? This anomaly can be explained in the following terms.

We assume that in the absence of valinomycin proton translocation still occurs. One proton is extruded externally for every electron used in the reduction of molecular oxygen. Due to the absence of valinomycin a membrane potential is created which requires the influx of two positive charges in compensation. The two permeant species are potassium and protons with the latter the most permeant. Therefore, in the absence of valinomycin, in order for proton translocation not to be observed, at least one proton must rapidly influx for every pumped proton but the remaining equilibration may involve up to one potassium ion. In order for internal alkalinization (monitored by pyranine fluoresence) to be observed, less than two protons must influx for every pumped proton with a stoichiometry of $1H+/e^-$. The actual ratio of H+ influx/K+ influx depends on the ratio of their permeability coefficients.

In the presence of DCCD, however, proton pumping has been inhibited and thus only one positive charge is required internally to compensate for the passage of one electron across the membrane to molecular oxygen. Therefore, half as many protons are required as in the absence of DCCD (as well as half as many potassium ions, maintaining the original influx ratio). Since half as many potassiums move in and pumping is inhibited, the internal alkalinization is reduced by $\frac{1}{2}$ even in the absence of valinomycin. The difference between protons taken up to reduce oxygen and influxed protons is reduced by 50% compared to the uptake in the absence of DCCD.

The variable stoichiometries (H+/e$^-$ ratios $\angle$ 1) obtained throughout this study in the presence of valinomycin can also be explained in terms of this model, Fig. 37. Upon valinomycin addition, potassium becomes the more permeant ion. Thus if one H+/e$^-$ is translocated by cytochrome oxidase and the ratio of proton influx to potassium influx is less than one then, depending on the compensatory proton influx, variable stoichiometries can be obtained. For example, if 1 proton is translocated out, and 0.6 protons (and therefore 1.4 potassium) move inward in compensation, a proton pump with a stoichiometry of 0.4 H+/e$^-$ would be observed, even though the actual ratio is 1H+/e$^-$.

Moreover, an internal alkalinity of only 1.4 OH-/e$^-$ would be observed.

The concept of vectorial proton translocation by cyt. c oxidase is further supported by evidence from O_2-induced internal alkalinization pulses followed by pyranine fluorescence, Fig. 36. Pulses in the presence of valinomycin were 80% larger than in its absence. This would be expected if valinomycin is required to collapse $\Delta\psi$ as previously discussed. However, it has been reported by Proteau et. al., (1983) that H+/e$^-$ ratios increase in magnitude as oxidase turnover increases. These workers found that upon successive ferrocytochrome c pulses, there was a decline in the H+/e$^-$ ratios, which was attributed to the inhibitory effect of the ferricytochrome c produced on oxidase turnover. Upon valinomycin addition, oxidase respiration also increases and this may also induce an increase in the H+/e$^-$ ratios.

FIGURE 37

Model for Proton translocation by cyt. aa_3 - containing proteoliposomes:

    A)   ion transfer in control system - $^-$DCCD

    B)   ion transfer in presence of DCCD

In each case the consequences of vectorial transfer of one electron from cytochrome c to oxygen are shown. For the purpose of illustration, it is assumed that the effective rapid backflow of protons is four times that of potassium ions (ie. valinomycin is absent or rate-limiting).

FIGURE 37   104

A/ −DCCD

$1/4 \, O_2 + H^+$

$1/2 \, H_2O$

$1.0 \, H^+$

e^-

$1.6 \, H^+$

$0.4 \, K^+$

B/ +DCCD

$1/4 \, O_2 + H^+$

$1/2 \, H_2O$

e^-

$0.8 \, H^+$

$0.2 \, K^+$
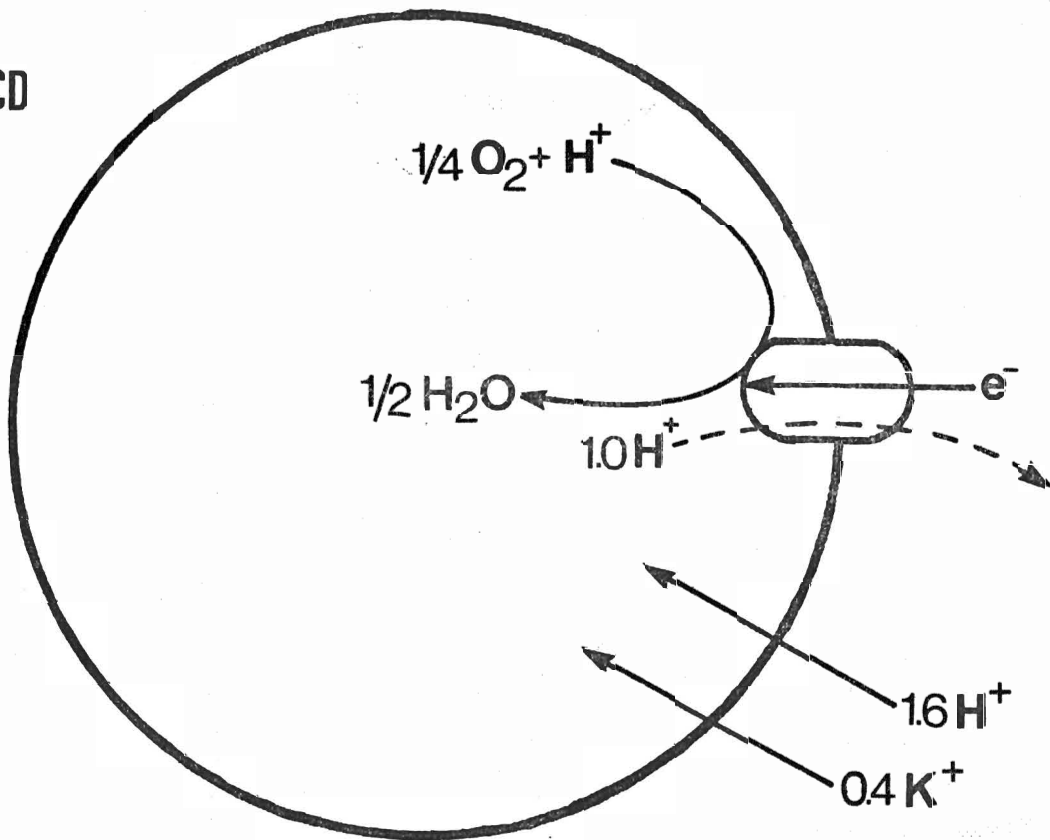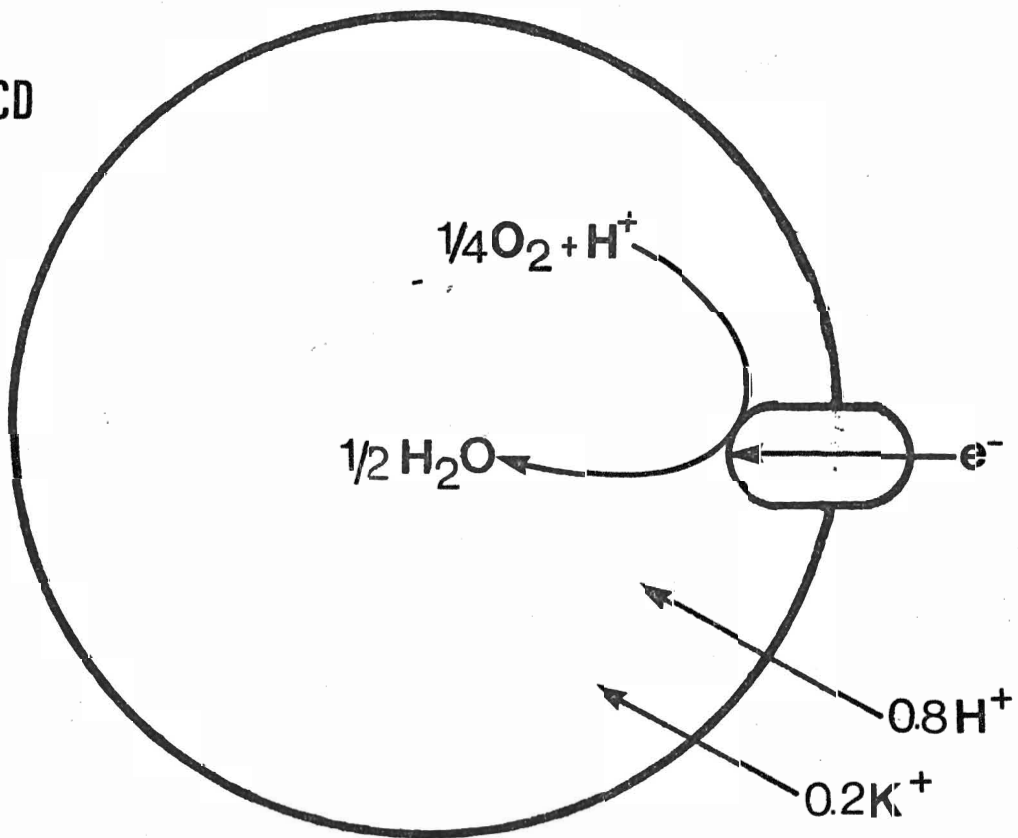
VII Conclusions

This study supports the idea of vectorial proton transport by cytochrome $\underline{c}$ oxidase. The scalar release of protons occurs only at low ionic strength with ferrocytochrome $\underline{c}$ pulses. The vectorial transport of protons was examined both by studying external proton appearance with a pH electrode as well as by studying the internal disappearance of protons using entrapped pyranine. These data suggest, as discussed previously, that the acidification seen upon pulsing occurs according to the model presented in Fig. 37. This model can account for the observed stoichiometries of proton translocation, and the effects of DCCD, valinomycin, and maltoside on proton pulses, presented in this study.

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IX APPENDIX 1

Calculation of H+/e$^-$ ratios from observed internal
pH changes.

In order to calculate the H+/e$^-$ ratio from Fig. 34 (b),
the following equation must be used:

$$H+/e^- = \frac{\Delta F}{neq.e^-} \quad X \quad \frac{\Delta pH}{\Delta F} \quad X \quad \frac{neq. H+}{\Delta pH}$$

A) The term ΔF/neq.e$^-$ comes directly from Fig. 34 (b)
and equal to the slope.

$$\therefore \quad \frac{1.12 \ \Delta F/sec. - .367 \ \Delta F/sec.}{1.859 neq.e^-/sec - 0.617 neq.e^-/sec}$$

$$= \frac{.753 \ \Delta F/sec}{1.242 neq.e^-/sec.}$$

$$= .606 \ \Delta F/neq.e^-$$

B) The term ΔpH/ ΔF reflects the pH change that a
given change in fluorescence represents. This value
is obtained by taking the initial pH, adding a given
aliquat of HCl (calculating ΔF_{ss}), and then taking
the pH in the presence of FCCP after the addition. The
curve of pH vs fluorescence is nonlinear. However,
approximate linearity is found between pH 7.0 - 7.8.
This pH change gives a ΔF of 43 units.

$$\therefore \quad \frac{\Delta pH}{\Delta F} = \frac{7.8 - 7.0}{43} = \frac{.8}{43} = .0186 \text{ for .2 ml of}$$

vesicles in a
volume of 2.7 ml,
(equilivant to .037
for .1 ml)

APPENDIX 1 con't

C) The term neq.H+/pH or $\triangle$H+/$\triangle$pH reflects the internal buffering (Bi).

The value of $\triangle$H+/$\triangle$pH was calculated from passive HCl additions to 3.5ml vesicles which were followed externally by a pH electrode, giving traces such as that in Fig. 7; with values in Table below.

[HCl] addition	Bt	Bo
50nmoles H+	13.4	15.9

$$Bt = Bi + Bo$$

$$\frac{1}{13.4} = Bi + \frac{1}{15.9}$$

$$Bi/Bt = \left(\frac{15.9 - 13.4}{15.9 \times 13.4} \right) \times 13.4$$

$$= 2.5/15.9$$

As indicated in the above Table the addition of 50nmoles HCl led to an acidification corresponding to 15.9 units (external buffering) followed by a realkalinization (due to internal buffering) of 2.5 units. This latter corresponded to a pH change of .0134 pH units.

$\therefore$ to calculate the nmoles H+ taken up internally:

$$\frac{Bi}{Bt} = \frac{Xnmoles\ H+}{50nmoles\ H+} = \frac{2.5}{15.9}$$

$\therefore$ X $= 50 \times 2.5/15.9$

$= 7.86$nmoles H+, corresponding to a pH change of 0.0134 pH units

$\therefore$ Bi $= 586.7$nmoles H+/pH units

APPENDIX 1 con't

D) The internal volume of 3.5 ml of vesicles (assuming a standard trap volume for these vesicles of 1 µl/mg) is given by:

If 10 ml of vesicles contain 0.5gm. lipid, then 3.5 ml of vesicles contain X gm. lipid.

Where $X = \dfrac{0.5 \ x \ 3.5}{10} = 175$ mg

∴ the trap volume of .175g phospholipid
= 175 µl

The trap volume for 0.1 ml vesicles (in Fig. 34 (b) corresponds to:

$Y = \dfrac{0.5 \ x \ 0.1}{10}$

Y = 5 mg.

∴ the trap volume of .005 phospholipid = 5 µl.

Thus, to calculate $\triangle$H+/ $\triangle$pH for the vesicles of Fig. 34 (b), we assume that it is proportional to the $\triangle$H+/ $\triangle$pH of vesicles with a total trap volume of 175µl.

$$\frac{586.7 \text{ nmoles H+}}{175 \text{ µl}} = \frac{Z}{5 \text{ µl}}$$

Z   = 16.76 neq.H+/pH unit

APPENDIX 1 con't

$$\therefore \quad H+/e^- = \frac{\Delta F}{neq.e^-} \quad X \quad \frac{\Delta pH}{\Delta F} \quad X \quad \frac{neq.H+}{\Delta pH}$$

$$= \frac{.606 \, \Delta F}{neq.e^-} \quad X \quad \frac{.037 \, \Delta pH}{\Delta F} \quad X \quad \frac{16.76 \, neq.H+}{pH \; unit}$$

$$= \underline{.38 \; H+/e^-}$$

APPENDIX 2

Upon the addition of 23 neq.e$^-$ of O_2 a ΔF of 3.6 units occurred.

A) $\quad \therefore \quad \dfrac{\Delta F}{neq.e^-} = \dfrac{3.6 \, \Delta F}{23 \; neq.e^-}$

$\qquad\qquad\qquad = .157$ fluorescence units/nanoequivalent.

B) $\quad \dfrac{\Delta pH}{\Delta F} = .037$ for 0.1 ml vesicles

$\therefore$ .0186 for 0.2 ml vesicles

C) $\quad$ 0.2 ml of vesicles were used in this run which therefor involved 10µl trap volume.

$\therefore \quad \dfrac{\Delta H+}{\Delta pH} = \dfrac{586.7 \; X \; 10}{175}$

$\qquad\qquad = 33.53$ neq.H+/pH unit

$$H+/e^- = .157 \; x \; .0186 \; x \; 33.53$$
$$= .0989$$
$$\simeq \underline{.10 \; H+/e^-}$$