ON THE MECHANISM OF THE INTERACTION OF

UBIQUINONE WITH MITOCHONDRIAL

UBIQUINOL-CYTOCHROME c

REDUCTASE

By

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1982

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
May, 1989

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CHAPTER I

INTRODUCTION

An Overview of Energy Conservation in Mitochondria

Aerobic organisms obtain most of their energy for cell metabolism and biosynthesis through the citric acid cycle and the electron transport system through mitochondria. NADH and succinate generated by citric acid cycle are oxidized in the electron transfer chain. The free energy liberated in these oxidations is partially conserved in the phosphoanhydride bonds of ATP. These reactions occur within discrete multienzyme respiratory chain complexes located in the inner mitochondrial membrane. These multienzyme respiratory chain complexes are: NADH-ubiquinone reductase (Complex I), succinate-ubiquinone reductase (Complex II), ubiquinol-cytochrome c reductase (Complex III) and cytochrome c oxidase (Complex IV).

NADH and succinate donate electrons to NADH-ubiquinone reductase and succinate-ubiquinone reductase respectively. Electron transfer proceeds from NADH-ubiquinone reductase and succinate-ubiquinone reductase to coenzyme Q. Reduced coenzyme Q reduces, in turn, cytochrome c via ubiquinol-cytochrome c reductase. Reduced cytochrome c is then oxidized by cytochrome c oxidase, which finally reduces oxygen to water. The oxidation of NADH is coupled to the phosphorylation of three moles of ADP and succinate to two moles of ADP (Fig. 1).

In 1961 Mitchell (1) proposed a chemiosmotic hypothesis, in which an electrochemical gradient across the membrane provides the driving force for phosphorylation of ADP. The eletrochemical gradient is created by asymmetric ejection of
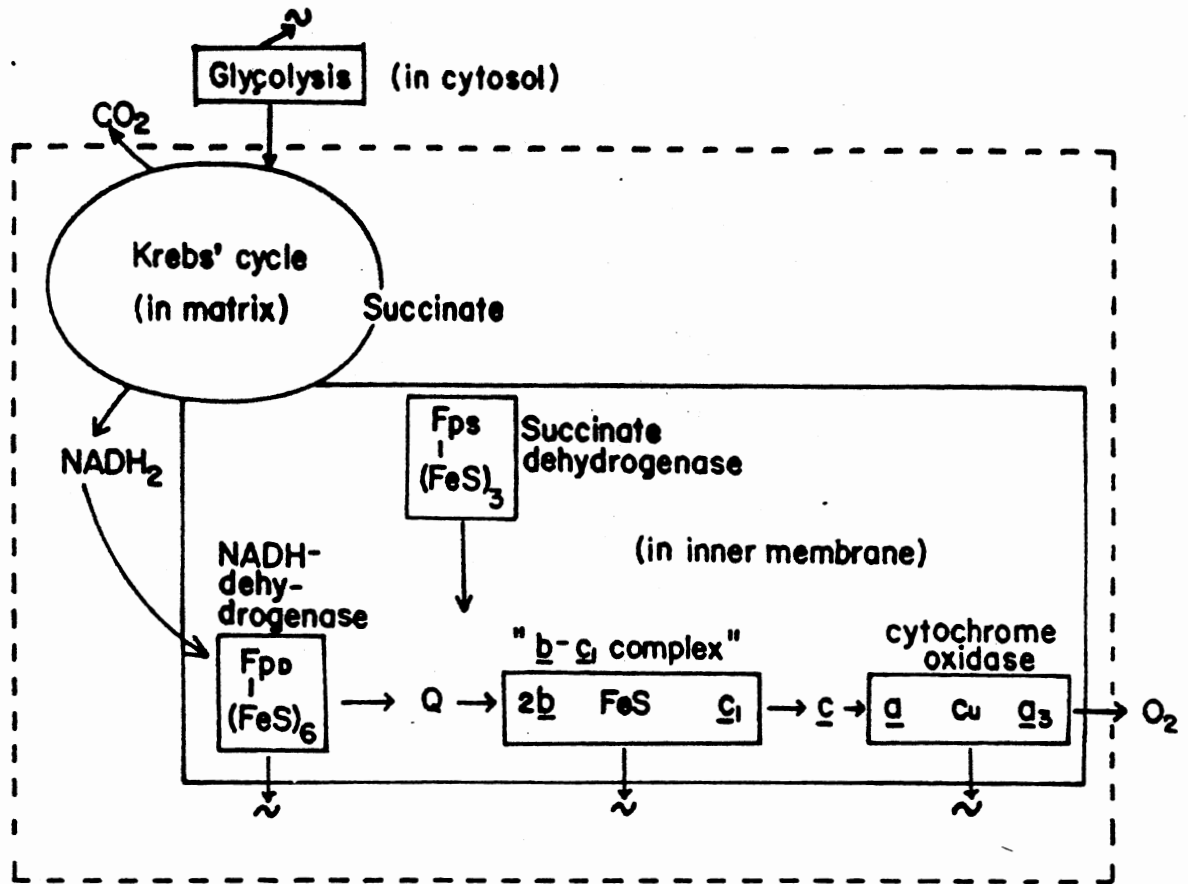
Fig. 1.   Flow of Electrons in Respiration Chain and the Sites of ATP Synthesis

protons into the intermembrane space. The driving force for gradient formation is the energy released from the redox reactions catalyzed by the respiratory chain complexes. The driving force has two components (2): pH gradient (ΔpH) and membrane potential (ΔY). The sum of these two terms is called the proton motive force (ΔP). The relationship of ΔpH, ΔY and ΔP can be described by the following formula analogous to the Nernst equation.

$$\Delta P = \Delta Y - 2.303 \ (RT/F) \ \Delta pH$$

The term ΔP, whose unit is volts, corresponds to E'; ΔY is analogous to E_0'; and the term -2.303 (RT/F) ΔpH is the Nernst potential, also in volts, that is generated by the ejection of protons into the intermembrane space.

In the chemiosmotic hypothesis the proton gradient is directly coupled to ATP synthesis. The membrane sector of the mitochondrial ATPase is a conduit for protons and the flow of protons back into the mitochondria through ATPase alters the active site of the ATPase leading to ATP synthesis (Fig 2) (3).

It has been demonstrated that the three coupling sites of the respiratory chain at NADH-ubiquinone reductase, ubiquinol-cytochrome c reductase and cytochrome c reductase can each generate an electrochemical potential across the membrane. Ubiquinol-cytochrome c reductase of the mitochondrial respiratory chain catalyzes electron transfer from ubiquinol to cytochrome c which is coupled to ATP synthesis. In agreement with the chemiosmotic theory of energy coupling, ubquinol-cytochrome c reductase catalyzes the electrogenic translocation of protons across the membrane during the transfer of electron down to the complex IV. The overall reaction is as follows:

$$QH_2 + 2 \text{ cyt. } c^{3+} + 2 H^+(M \text{ side}) \longrightarrow Q + 2 \text{ cyt. } c^{2+} + 4 H^+(C \text{ side})$$
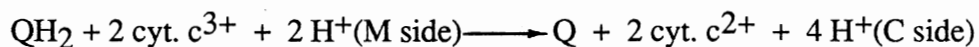
As seen in the above reaction, four protons appear on the cytoplasmic side of the inner mitochondrial membrane with the transfer of 2 electrons. Two of the four protons
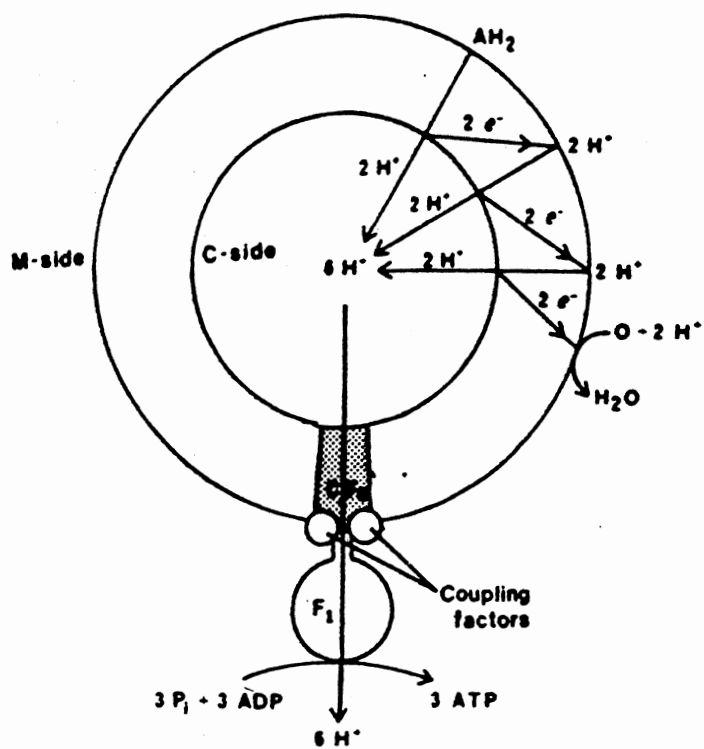
Fig. 2. Flow of Protons through Coupling Factors in a
Submitochondrial Particle

are from the oxidation of QH_2 by cytochrome c, the other two from the electronegative, matrix side of the inner mitochondrial membrane. Apparently ubiquinol-cytochrome c reductase not only functions as an electron carrier, but also appears to be involved directly in transmembrane proton pumping. Although much progress on the structure studies of ubiquinol-cytochrome c reductase has been achieved, the mechanism of proton and electron transfer in this complex is still far from being undersdood. The role of ubiquinones, especially the protein-bound form, in electron and proton transfer reactions in bovine heart mitochondrial ubiquinol-cytochrome c reductase is the primary concern of this thesis.

The Chemical Composition of Ubiquinol-cytochrome c Reductase of Bovine Heart Mitochondria

Ubiquinol-cytochrome c reductase of bovine heart mitochondria, the segment of energy coupling site 2, is a multiprotein complex, which is composed of more protein subunits than ubiquinol-cytochrome c reductase of chromatophores and b_6f complex of chloroplasts. It consists of 11 subunits, 3 of which are carrying the redox centers. Certain amounts of phospholipids and ubiquinone are contained in fully active isolated ubiquinol-cytochrome c reductase. In the past 25 years since the first reported purification and description of ubiquinol-cytochrome c reductase (161), much effort has been made in the purification of the enzyme, isolation and identification of the individial subunits, as well as the arrangement of each subunit in the respiratory chain. Although further study of composition and function of the reductase is needed, the outline about the chemical composition is clear.

Preparations of the mitochondrial ubiquinol-cytochrome c reductase obtained by different procedures are similar in composition. It usually contains 8 nmoles of cytochrome b and 4-5 nmoles of cytochrome c_1 per mg. protein (4). The high purity ubiquinol-cytochrome c reductase was prepared (5) from succinate-cytochrome c

reductase. The composition of the high purity ubiquinol-cytochrome c reductase is shown in Table I.

The lipid content in the mitochondrial preparations depends on what kind of solubilizing detergents is used in the process. If bile salts are used, about 0.2 mg. lipid per mg. protein is retained, which corresponds to about 50 lipid molecules per cytochrome c_1 (5-8). The major phospholipids are cardiolipin, phosphatidylethanolamine and phosphatidylcholine present in a molar ratio of about 10:20:20 (9).

Subunit Structure

The subunit structure of ubiquinol-cytochrome c reductase of bovine heart mitochondria has been extensively studied. When ubiquinol-cytochrome c reductase is subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the Weber and Osborn system, seven bands are found with apparent molecular weights of 53, 50, 37, 30, 28, 17, and 15 Kd (8). The 37 and 17 Kd subunits were assigned to be associated with b cytochrome, the 30 Kd subunit was cytochrome c_1, whereas the 28 Kd subunit was iron-sulfur protein. In 1986 Schagger et al. (10) separated all the 11 subunits of ubiquinol-cytochrome c reductase using a high resolution SDS-PAGE system and suggested an assignment of each polypeptide(Fig.3). In 1988 based on the N-terminal analysis of each subunit, Gonzalez-halphen et al. proposed a new assignment of the 11 subunits (54). According to their assignment, bands VII and VIIIa in Fig. 3 should be the same protein because they have an identical N-terminal GRQFG. These two bands on the gel are eletrophoretic variants of the same subunit. It might be due to incomplete reduction of the disulfide bonds. These two bands were designated as VIIa and VIIb. Since there VIIIa was absent in Fig. 3, VIIIb was assigned as VIII. However, there is some controversy concerning the assignment and the functions of the small polypeptides. For instance, subunit VI in figure 3 was reported as Q-binding protein (QPc) (158), but

TABLE I

THE COMPOSITION OF HIGH PURITY UBIQUINOL-CYTOCHROME c REDUCTASE

Component	Concentration (nmol/mg protein)
cytochrome b	10 - 10.5
cytochrome c_1	5.7 - 6.0
iron-sulfur protein	6.6 - 7.2
ubiquinone	2-3
phospholipids	250

band	protein	kDa
I	core protein I	49
II	core protein II	47
III	cytochrome b	43.7
IV	cytochrome c_1	27.9
V	iron-sulfur protein	25
VI	Q-binding protein	13.4
VII	11 kDa protein	11
VIII	hinge protein	9.2
IX	DCCD-binding protein	8.0
X	7.2 kDa protein	7.2
XI	6.4 kDa protein	6.4
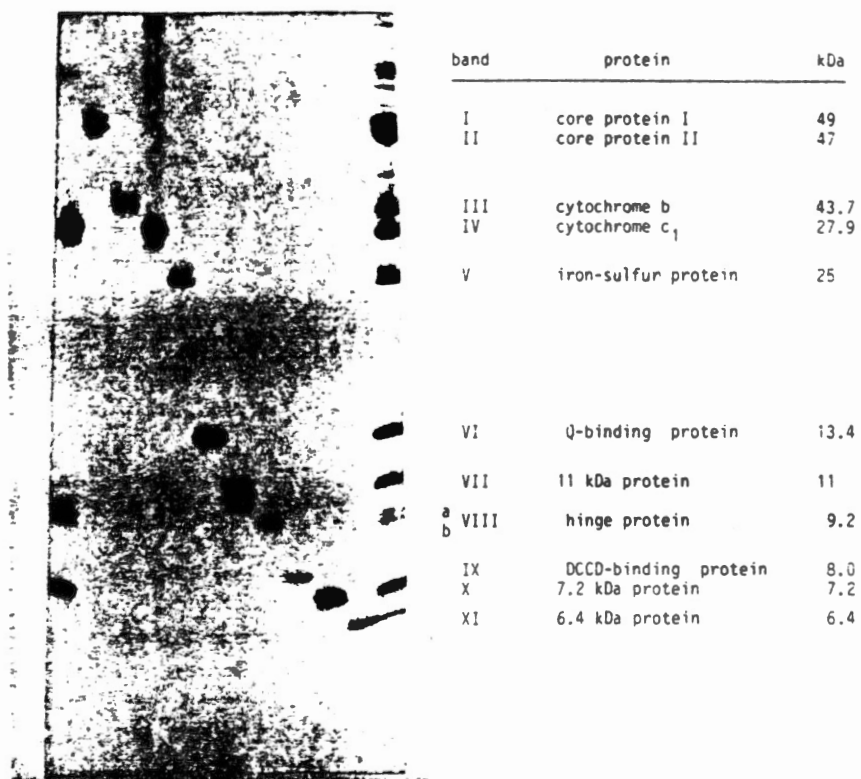
Fig. 3. SDS-PAGE of the Subunits of Ubiquinol-Cytochrome c Reductase from Beef Mitochondria. Outer left lane, c_1 subcomplex; outer right lane, ubiquinol-cytochrome c reductase isolated in Triton X-100; middle lanes, corresponding isolated subunits.

actually it is not QPc. The real QPc is subunit VII (chapter II). The function of subunit VI has not been confirmed.

Redox Components

There are five redox components in ubiquinol-cytochrome c reductase of beef heart mitochondria. They are two b cytochromes (b_{566}, b_{562}), cytochrome c_1, iron sulfur protein, and ubiquinone. Cytochrome b, c_1 and iron sulfur protein are present in the ratio of 2:1:1. The amount of ubiquinone is variable in different preparations of ubiquinol-cytochrome c reductase. However, it was reported that when the ratio of ubiquinone to cytochrome c_1 was 1:1, the highest activity was observed (117).

Cytochrome b. Cytochromes b are thermodynamically heterogeneous. There are at least two b cytochromes with different midpoint potentials and absorption spectra present in ubiquinol-cytochrome c reductase (12). The one with midpoint potential of +93 mV corresponds to a component with an α-peak at 562 nm. Another one with midpoint potential of +34mV corresponds to a component with a α-peak at 566 nm (13). The potential of both cytochromes b exhibit phospholipid dependence (141) and pH-dependence in the physiological range(14). Cytochrome b has been isolated from beef heart mitochondria(15,16). The isolated protein shows only a single α-peak at 562 nm. The molecular weight of isolated cytochrome b (30-37kd) estimated by electrophoresis is not reliable because it was reported that the detergent binding property of the protein is abnormal. Also it is difficult to calculate the molecular weight from the heme content, as heme is easily lost during purification in the presence of detergent. The molecular weight calculated from the sequence of the gene is 42,540 (157). EPR studies of the cytochrome b of ubiquinol-cytochrome c reductase (17) have indicated that both b type hemes are presebt at low spin status. The effect of externally added dysprosium complexes on the EPR spin relaxation of the two heme g=3.8 (b_L) and g=3.4 (b_H) signals was studied

(55). The results of the EPR study of the hemes, together with the structure prediction derived from the amino acid sequences of cytochrome b (123,124), have led to the proposal that the two hemes are sandwiched between the membrane-spanning helices II and V (or IV), and that the Fe-Fe distance is 21Å (Fig.4) (55).

Cytochrome c_1. Cytochrome c_1 is a distinct functional component of ubiquinol-cytochrome c reductase. Cytochrome c_1 of beef heart mitochondrial ubiquinol-cytochrome c reductase has been isolated by several groups (18, 162, 160). The absorption spectrum of the reduced cytochrome c_1 possessed maxima at 552.5, 530, 522.5, 512, 417, 317 and 276 nm, and the oxidized form at 558, 522, 411, 355 and 276 nm. Isolated cytochrome c_1 contains 25 nmoles heme per mg of protein and shows two bands in SDS-PAGE with apparent molecular weights of 30 and 15 Kd, respectively (18). The c type heme is covalently attached to the 30 Kd subunit. The 15 Kd protein subunit is the so called hinge protein, the function of which is still not very clear. The midpoint potential of cytochrome c_1 is between +225 and +245mV(18-20). It is almost identical to the potential of cytochrome c and appears to be significantly more negative then that of the iron sulfur protein (+280 mV). In iron sulfur protein depleted ubiquinol-cytochrome c reductase cytochrome c_1 is oxidizable by cytochrome c plus cytochrome c oxidase indicating that c_1 is a terminal electron acceptor in ubiquinol-cytochrome c reductase and electron donor for cytochrome c (21-23).

Iron Sulfur Protein. The iron sulfur protein of ubiquinol-cytochrome c reductase was first discovered in mitochondria by Rieske (25). It has been isolated in a reconstitutively active form from beef heart mitochondria (26,27). It is a 25 kD polypeptide containing an iron-sulfer cluster of the 2Fe-2S type. Its midpoint potential has been determined to be +280 mV. Its reduced form is paramagnetic and exhibits an electron paramagnetic resonance (EPR) spectrum with a central resonance at g=1.90 (27).
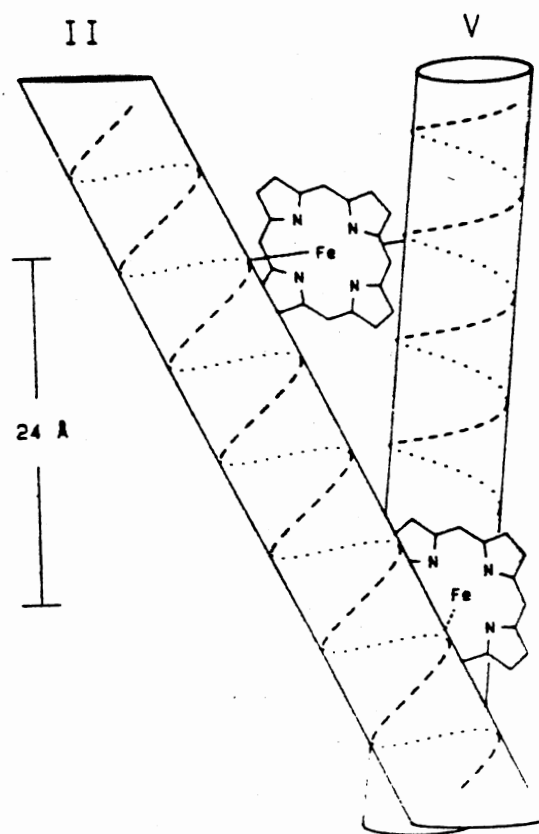
Fig. 4. Location of the Two b Type Cytochromes in
Ubiquinol-Cytochrome c Reductase

This EPR spectrum can be affected by the redox status of ubiquinone (28,32) and quinone-like inhibitors (33-37).

The function of iron-sulfur protein in electron transfer in ubiquinol-cytochrome c reductase has been confirmed by examining oxidation-reduction reactions of cytochrome b and c_1 in the presence and absence of iron-sulfur protein and antimycin. In the presence of antimycin iron-sulfur protein is required for the reduction of both cytochrome b and c_1. In the absence of antimycin the reduction of cytochrome b does not require iron-sulfur protein, but the reduction of cytochrome c_1 does. These finding provide an evidence in support of a branched cycle pathway of electron transfer in ubiquinol-cytochrome c reductase.

Ubiquinone. Ubiquinone (UQ) is the only small lipophilic molecule in the mitochondrial respiratory chain, which is oxidized and reduced through reactions which unequivocally involve net release and uptake of protons. The structure (Fig. 5.) which is obviously related to its function is the benzoquinone ring which can undergo oxidation-reduction. Also the ring substituents can give ubiquinone additional properties which are important to its biological function. The length and the flexibility of the alkyl side chain can also affect the activity of ubiquinone as electron acceptor, donor and mediator in electron transfer reactions (82). Ubiquinone occurs in about tenfold molar excess compared to the individual cytochromes. This fact has led to the idea that Q is a mobile component (38-40) which can move freely within phospholipid bilayer and shuttles electrons among the electron-transfer complexes. Based on this idea, the hypothesis of ' pool function of Q' has been proposed (41). The amount of ubiquinone contained in ubiquinol-cytochrome c reductase varies with different preparations. It was reported (4) that in preparations of mitochondrial ubiquinol-cytochrome c reductase, up to one equivalent of ubiquinone per cytochrome c_1 is carried along if Triton X-100 is avoided during purification. Even highly purified preparations of ubiquinol-cytochrome c
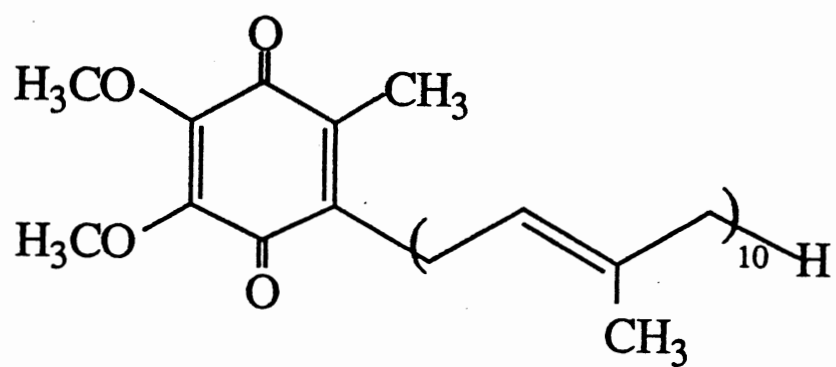
Fig. 5. The Structure of Ubiquinone (UQ$_{10}$)

reductase still contain significant amounts of ubiquinone which can not easily be removed Q (44). The redox potential of the ubiquinol-ubiquinone overall reaction and of the two half reactions in ethanolic solution at pH 7 were determined [E'(QH$_2$/Q)=70 mV), E'(QH$_2$/Q$^-$)=380 mV, E'(Q$^-$/Q)=-240 mV] (4). Binding of ubiquinone to the ubiquinol-cytochrome c reductase complex might change the redox potential span between the two half reactions, as well as the potential of the overall reaction.

<u>Topological Arrangement of the Subunits</u>. The topographical arrangement of the subunits in ubiquinol-cytochrome c reductase has been extensively studied and several techniques have been used in these studies. The techniques employed are crosslinking, radioactive labeling (45-51) and paramagnetic probe (52, 53).

In 1988 Gonzalez-halpgen et al proposed a topological arrangement of the whole complex based on their own and other's experiments (Fig. 6) (54). It was found that cytochrome b was labeled 20 fold more heavily than other subunits when ubiquinol-cytochrome c reductase was incorporated into membrane and treated with 3-(trifluoromethyl)-3-(m-[^{125}I]iodophenyl)diazirine. This indicates that cytochrome b is the predominent bilayer intercalated part of ubiquinol-cytochrome c reductase. Studies by protease digestion and crosslinking experiments confirm that iron-sulfur protein is on the cytoplasmic side of the membrane and peripherally located, possiblly bound to cytochrome b through hydrophobic interaction. Cross-linking data indicate hinge protein and subunit X are near neighbors of cytochrome c$_1$. Two core proteins and subunit VI were proved to be neighbors and located on the M side by cross-linking and protease digestion experiments.

The excess ubiquinone is largely situated in the hydrophobic membrane core and called ubiquinone pool which functions as a redox buffer (55).
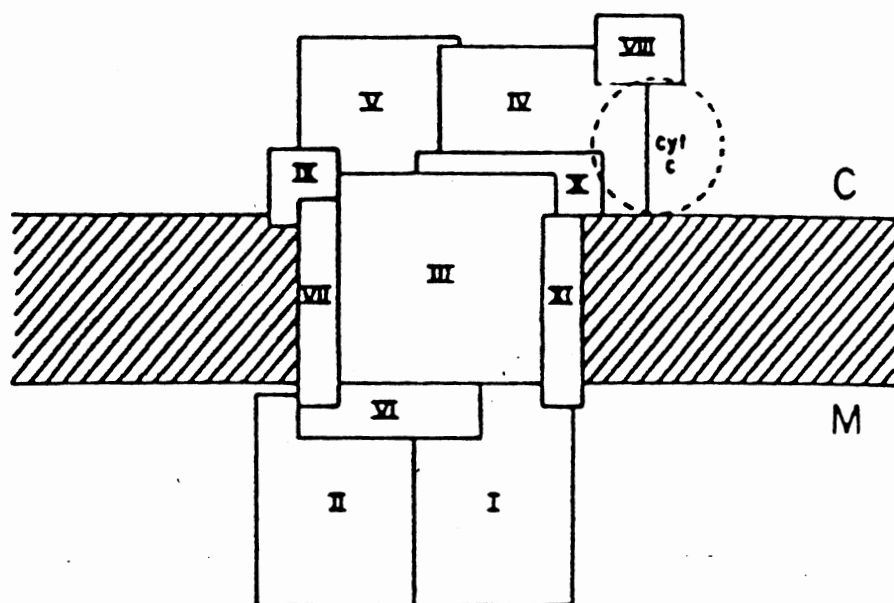
Fig. 6. Topography of Ubiquinol-Cytochrome c Reductase in
    Mitochondrial Electron Transfer Membrane. M and C refer to
    matrix and cytoplasmic side of the inner mitochondrial
    membrane. For the assignment of each subunit, see Fig. 3.

Proposed Mechanism of Electron Transfer

in Ubiquinol-Cytochrome c Reductase

Because of experimental expendiency, electron-transfer processes in ubiquinol-cytochrome c reductase have been studied principally in terms of the redox components of the complex, namely cytochrome b, c_1 and the iron-sulfur center. Due to their visibility by spectroscopic methods coupled with electrochemical measurements, the electron-transfer relationships among these subunits have been studied extensively. Several hypotheses have been proposed to explain the experimental pheomena concerning the electron and proton transfer in ubiquinol-cytochrome c reductase. These hypotheses are: protonmotive ubiquinone cycle (Q cycle) originally proposed by Mitchell (42,43) and later modified by Trumpower (56,57); cytochrome b cycle (b cycle) proposed by Wikstrom and Krab (58); semiquinone cycle (SQ cycle)-- a modified b cycle proposed by Wikstrom and Saraste (59); double Q cycle proposed by Slater and his co-workers (87). At present the Q cycle mechanism is received a greatest attention because it can explain a number of phenomena related to electron transfer in ubiquinol-cytochrome c reductase, such as two distinct ubisemiquinone radicals, the oxidant induced cytochrome b reduction,and the stoichiometry of vectorial proton translocation with an H^+/e^- ratio of 2.

Q-cycle

The pathway of electron and proton transfer in ubiquinol-cytochrome c reductase predicted by the protonmotive Q-cycle is shown in Fig. 7. There are two reaction centers, o center and i center, for ubiquinone to be oxidized and reduced. Ubiquinol is oxidized at the o center to ubisemiquinone radical with one electron going to iron-sulfur protein en route to cytochrome c_1, two protons are released to the C side of the membrane. The ubisemiquinone radical thus formed reduces cytochrome b-566 (b_L). The electron from reduced cytochrome b-566 is then transferred to cytochrome b-562 (b_H), which in turn reduces ubiquinone at i center to ubisemiquinone radical. The ubisemiquinone radical
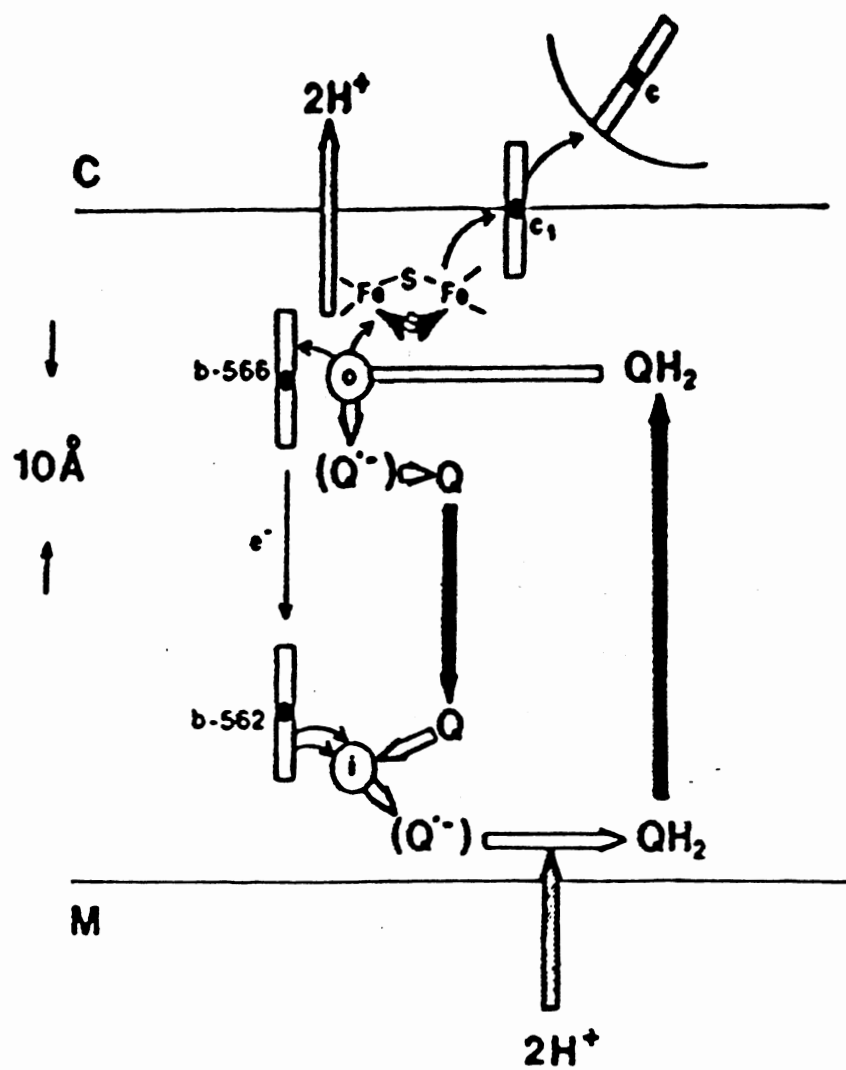
Fig. 7. Protonmotive Q Cycle Mechanism for Electron Transfer
through Ubiquinol-Cytochrome c Reductase

produced then gets one electon from succinate dehydrogenase and picks up two protons from matrix side to become ubiquinol. In the whole cycle for one electron going through ubiquinol-cytochrome c reductase, two protons are translocated from the M side to the C side of the innermitochondrial membrane.

<div align="center">Inhibitors of Ubiquinol-Cytochrome c Reductase</div>

A number of specific inhibitors have long been known to inhibit electron transport through ubiquinol-cytochrome c reductase (Table II). They inhibit the reduction of ubiquinone or the oxidation of ubiquinol by blocking of different redox components. Studies of the inhibitory natures of these inhibitors provide a valuable approach to the elucidation of the mechanism of electron transfer in ubiquinol-cytochrome c reductase. According to the Q cycle mechanism, there are three inhibition sites in ubiquinol-cytochrome c reductase as shown in Fig. 8. Accordingly, the three classes of inhibitors have been classified: (a), antimycin-like inhibitors, including antimycin, funiculosin and 2-n-heptyl-4-hydroxyquinolin-N-oxide(HQNO); (b), MOA-type inhibitors, characterized by a reactive methoxyacrylate moiety in the molecule, including myxothiazol, mucidin and stigmatellin; and (c), quinone-like inhibitors including 5-Undecy1-6-Hydroxy-4.7-dioxybenzothiazole (UHDBT), undecylhydroxynaphthoquinone and heptadecylmercaptohydroxyquinolinequinone (73).

Antimycin

Antimycin is a very potent inhibitor of ubiquinol-cytochrome c reductase and the most extensively studied inhibitor of respiration . A one to one ratio of amtimycin to cytochrome c_1 completely abolishes the enzymatic activity of ubiquinol-cytochrome c reductase (83). It causes a red-shift of the alpha peak of cytochrome b-562 by 1.5 nm, without affecting the peak of b-566. It causes the line shift of b-562 EPR spectrun without changing the EPR spectrum of b-566 (64). It was also found that antimycin

TABLE II

INHIBITORS OF ELECTRON TRANSFER IN MITOCHONDRIAL UBIQUINOL-CYTOCHROME c REDUCTASE
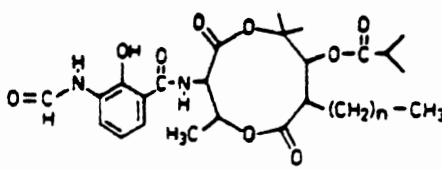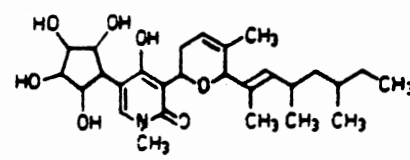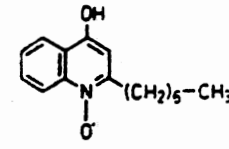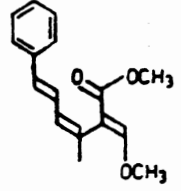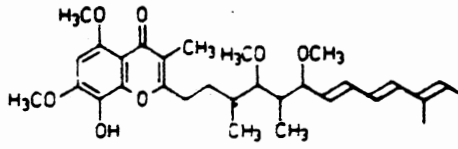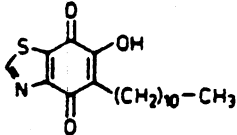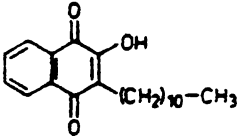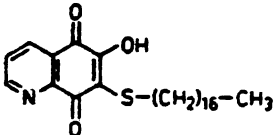
Inhibitor	Structure	Formular	Ref
Antimycin		$C_{28}H_{40}N_2O_9$	144,145
Funiculosin		$C_{27}H_{41}NO_7$	146,147
HQNO (heptylhydroxyquinoline-*N*-oxide)		$C_{16}H_{21}NO_2$	148
Myxothiazol		$C_{25}H_{33}N_3O_3S_2$	149,150
Strobilurin A (mucidin)		$C_{16}H_{18}O_3$	151
Stigmatellin A		$C_{30}H_{42}O_7$	152

TABLE II (Continued)

UHDBT (undecylhydroxydioxobenzothiazole)		$C_{18}H_{23}NO_3S$	111,153
UHNQ (undecylhydroxynaphthoquinone)		$C_{21}H_{28}O_3$	154
HMHQQ (heptadecylmercaptohydroxyquinoline quinone)		$C_{26}H_{39}NO_3S$	155
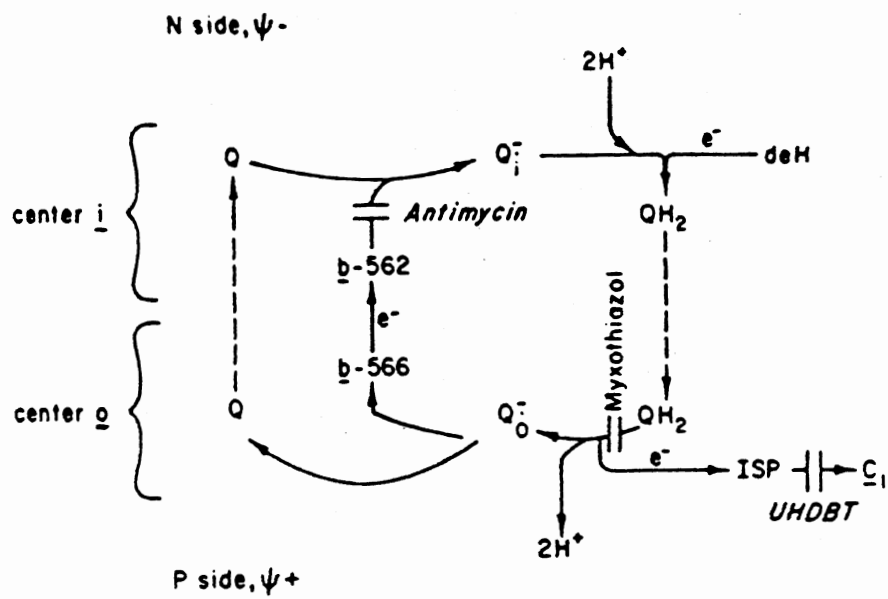
Fig. 8. Inhibitory Sites in Ubiquinol-Cytochrome c Reductase
According to Q Cycle Mechanism

inhibits the oxidation of cytochrome b and stimulates the oxidant-induced reduction of b

cytochromes (65). These results together with the observation of the abolishment of the

EPR signal of the ubisemiquinone radical by antimycin indicate that antimycin binds to

center i or in its proximity and therefore blocks electron transfer between b-562 and the

Q/QH couple (43), it is possibly that antimycin lowers the stabilization of

ubisemiquinone by b-562 (11) or a closely associated peptide (66).

Myxothiazol

Myxothiazol is an antibiotic which blocks mitochondrial respiration in ubiquinol-

cytochrome c reductase segment of the respiration chain (67). The doses required for 50%

inhibition of enzymatic activity are 1 mol myxothiazol per mole of cytochrome c_1 (4). It

blocks the reduction of cytochrome b via an antimycin-insensitive pathway and causes a

red shift of the α-peak of cytochrome b-566 (68). When antimycin and myxothiazol are

added together to ubiquinol-cytochrome c reductase,the two b-heme centers are completely

shielded against electrons. This result seems to match the probability of a linear sequence

of the two heme b centers in the path of electron flow with two ubiqunone binding sites,

one for each heme center (68) (Fig 9). However, further study (69) found that

myxothiazol had effects on the reduction of cytochromes b and c_1 by succinate or

ubiquinol which were identical to those caused by removal of the iron-sulfer protein from

ubiquinol-cytochrome c reductase. Also, it inhibited the reduction of iron-sulfer protein

and shifts the g_x resonance of the EPR spectrum of the iron-sulfer protein from 1.79 to

1.76. Therefore it was suggested that myxothiazol induces a conformational change in

cytochrome b that is transmitted to a quinone binding site, which consists of peptide

domains from both cytochrome b and iron-sulfur protein (69).
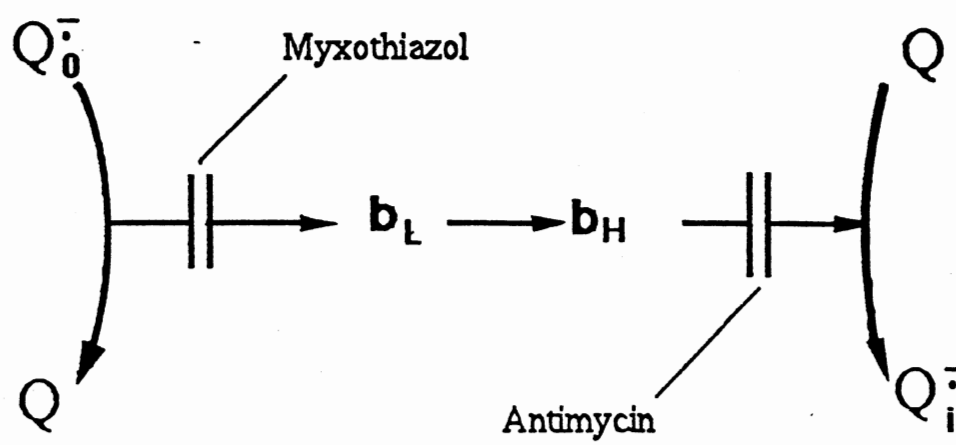
Fig. 9. Linear Arrangement of the Two b Hemes in Ubiquinol-Cytochrome c Reductase

5-Undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT)

UHDBT was first intrduced by Folkers et al (70) as an inhibitor of mitochodrial respiration and identified as an inhibitor of electron transfer in bc_1 segment of mammalian mitochondria by Trumpower and Haggerty(71). Addition of UHDBT to ubiquinol-cytochrome c reductase inhibits the oxidant induced reduction of cytochrome b (89). UHDBT increases the midpoint redox potential of the Rieske iron-sulfur protein from +280 to +350 mV at pH 7.2 and alters its EPR characterestics. An upfield shift of g_z (1.80 to 1.76) and a downfield shift of g_x (2.02 to 2.03) were observed (36). Since UHDBT raises the midpoint redox potential of iron-sulfur protein to at least +350 mV, that is much higher than the potential of cytochrome c_1 (+225-+245 mV), therefore it was proposed that UHDBT inhibits the electron transfer in ubiquinol-cytochrome c reductase by its binding to iron-sulfur protein (36). It inhibits the oxidation of the reduced iron-sulfur protein by ferricytochrome c_1, but not the reduction of the oxidized iron-sulfur protein by ubiquinone (36).

Ubiquinone-binding Proteins

Because of the uneven distribution of ubiquinone among the four complexes, the implication of specific Q-protein interaction in the mitochondrial electron-transfer chain appeared as early as the begining of 1960, when the electron transfer chain was resolved into four complexes. However, the existance of an ubiquinone-proein complex as the active entity in electron transfer chain was not advanced until 1981 (44). The first evidence of the existance of specific ubiquinone-binding proteins in ubiquinol-cytochrome c reductase was deduced from the repeated observation of a 1:1 stoichiometry between ubiquinone and cytochrome c_1 in fully active isolated succinate-cytochrome c reductase (117). When the concentration of Q is lower than that of cytochrome c_1, a stimulation of the activity of succinate-cytochrome c reductase was observed upon the addition of exogenous Q (86). If Q were merely dissolved in the phospholipid phase of succinate-

cytochrome c reductase, a fixed stoichiometry between ubiquinone and cytochrome c_1 would not be expected. The introduction of the "proton motive Q cycle" mechanism of electron transfer in ubiquinol-cytochrome c reductase (42) and the acceptance of the chemiosmotic theory (1) of energy coupling have given further support to the idea of the existance of Q-binding proteins in ubiquinol-cytochrome c reductase.

A significant advance in the study of Q-binding proteins in ubiquinol-cytochrome c reductase was the detection of ubisemiquinone radical (62, 88). The concentration of the ubisemiquinone radical as high as 60% of the total Q present in ubiquinol-cytochrome c reductase has been detected at pH 9.0. The formation of the ubisemiquinone radical in ubiquinol-cytochrom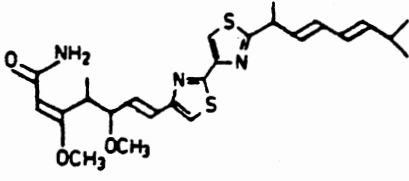e c reductase was attributed to the presence of a Q-binding protein because: (1), in the free state or in phospholipid micells, ubisemiquinone radical is very unstable. It will undergo dismutation immediately and one would not expect to detect it at neutral or slightly alkaline conditions. (2), digestion of the ubiquinol-cytochrome c reductase with trypsin abolished the formation of the ubisemiquinone radical.

Direct evidence of the existance of Q-binding proteins in ubiquinol-cytochrome c reductase came from an experiment on the interaction of arylazido ubiquinone, 2,3-dimethoxy-5-methyl-6-{10'-[4-(azido-2-nitroanilino-propionoxy)]-decyl}-1,4-benzoquinone (Q_0C_{10} NAPA), with mitochondrial ubiquinol-cytochrome c reductase, where two polypeptides with M_r=37,000 and 17,000 were photolabeled by arylazido ubiquinone (61). However the photoaffinity group is on the isoprenyl side chain, and the ability of Q-binding proteins to stablize the ubisemiquinone radical indicate that the binding of Q to protein may be involved more with the benzoquinone ring rather than the isoprenyl side chain. Therefore, the Q-binding proteins need to be further elucidated.

The introduction of the concept of Q-binding protein into electron transfer complexes has changed the conventional thinking about Q as a free molecule participating in electron transfer to the theory that Q functions as a Q-protein complex. Its function depends on both Q and Q-binding protein. The actual functional entity of Q is the Q-

protein complex, rather than Q alone. Therefore, elucidation of electron transfer and energy coupling mechanisms requires an understanding of the function of Q-binding proteins, the Q-protein interaction and the interaction between unbound Q and Q-protein complexes.

CHAPTER II

INTERACTION AND IDENTIFICATION OF UBIQUINONE-BINDING PROTEINS IN UBIQUINOL-CYTOCHROME c REDUCTASE BY AZIDO-UBIQUINONE DERIVATIVES

The existence of specific ubiquinone (Q)-binding proteins in the mitochondrial and photosynthetic electron transfer complexes has been established through biochemical studies (44). The Q-binding proteins in the mitochondrial cytochrome bc_1 region have been detected by EPR measurement, using the ability to stablize ubisemiquinone radicals (88, 62). Recently, a photoaffinity labeling study using an arylazido-Q derivative has identified two polypeptides, with M_r=37,000 and 17,000 (61), in the highly purified ubiquinol-cytochrome c reductase, that are photolabeled by the reagent. Whether both proteins have individual Q-binding sites or both form a common Q-binding site remains to be elucidated. Evidence for the existence of two distict Q-binding sites in the cytochrome bc_1 region is available (87, 92).

One ambiguity of studies using an arylazido-Q derivative to identify the Q-binding protein is due to the location of the photoactivatable group on the Q molecule. In the arylazido-Q molecule, the photoactivable group is located on the part of the Q molecule where structural requirements for the electron transfer reaction are much less specific (157). A nonspecific labeling of complex III (161) with arylazido-phospholipid has been reported (93). This problem can now be resolved through the use of azido-Q derivatives. Among various azido-Q derivatives (94) synthesized for this work, 3-azido-2-methyl-5-methoxy-6-geranyl-1,4-benzoquinone (3-azido-2-methyl-5-methoxy-Q_2) and its hydrogenated derivative, 3-azido-2-methyl-5-methoxy-6-(3',7'-dimethyloctyl)-1,4-

benzoquinone (3-azido-2-methyl-5-methoxy-Q_{2s}) possess the best properties for use as photoaffinity labels in identification of those Q-binding protein(s) for which no isolation procedure has yet been developed, and for study of the interaction between Q and protein and/or between Q and phospholipids. These azido-Q derivatives have the ability to restore partial (12%) succinate-cytochrome c reductase activity to the Q- and PL-depleted succinate-cytochrome c reductase in the absent of light, and show complete binding to the Q-binding sites after photolysis (95). The relative ease of preparation of these azido-Q derivatives in radioactive form greatly aided our use of these azido-Q derivatives to unambiguously identify the Q-binding proteins in this segment of the electron transfer chain.

Here we report the detailed photoaffinity labeling conditions, identification of the Q-binding proteins in the cytochrome bc_1 region, and the effect of phospholipids and inhibitors on the Q-binding and Q:protein interaction.

Experimental Procedures

Beef heart submitochondrial particles (107), succinate-Q reductase (107), and a highly purified ubiquinol-cytochrome c reductase (96) were prepared and assayed according to reported methods. The Q- and PL-depleted ubiquinol-cytochrome c reductase was prepared by repeated ammonium sulfate precipitation in the presence of sodium cholate and glycerol (137). The depleted enzyme was dissolved in 50 mM phosphate buffer, pH 7.4, containing 10% glycerol and 1mM EDTA. Determination of protein concentration (98) was carried out according to the reported methods. Synthesis of oxidized and reduced form of $Q_0(CH_2)_{10}Br$ and Q_2 and their quantitative analysis were carried out according to the method developed in Dr. Yu's laboratory (106).

Cholate, deoxycholate, antimycin A, and n-heptyl-4-hydroxyquinoline-N-oxide (HQNO) were purchased from Sigma. N,N'-Diallyltartardiamide (DATA) was obtained from Kodak and Insta-Gel from United Technologies Packard. 5-n-undecyl-6-hydroxy-

4,7-dioxobenzothiazole (UHDBT) was obtained from Dr. B. L. Trumpower, Dartmouth Medical School. Other chemicals were obtained commercially at the highest purity available.

Photolysis

The Q- and PL-depleted ubiquinol-cytochrome c reductase was diluted with 50 mM phosphate buffer, pH 7.4, containing 0.25% sodium cholate and 1 mM EDTA to a protein concetration of about 3 mg/ml. 3-Azido-2-methyl-5-methoxy-Q_{2s} in 95% ethanol was added. The final concentration of ethanol in the mixture was kept lower than 5% to prevent denaturation of the enzyme. The azido Q-treated sample was incubated at 0°C for 20 min in the dark then illuminated as in the previous work (61). For activity assay, the photolyzed samples were reconstituted with phospholipid and incubated at 0°C for 1 h prior to activity determination. The time course study of the incorporation of radioactivity of azido-Q into protein was conducted as reported (61).

Determination of the Distribution of ^{3}H-Radioactivity among the Subunits of Ubiquinol-cytochrome c Reductase

The photolyzed samples were diluted with 50 mM phosphate buffer, pH 7.4, to 10 ml and centrifuged at 120,000 x g for 5 h. The precipitates were collected and resuspended with 0.8 ml of water. The free Q, or the residual phospholipid-bound Q, was extracted from the protein by the method of Bligh and Dyer (99). The protein solution (0.8 ml) was mixed with 2 ml of methanol and 1 ml of chloroform, and incubated at room temperature for 30 min with occasional shaking. After incubation, 1 ml of H_2O and 1 ml of chloroform were added and the mixture was kept at room temperature for another 10 min with occasional shaking. The chloroform layer was separated from the aqueous layer by centrifugation and removed. The aqueous layer was extracted once more

with 1 ml of chloroform and the chloroform layer was removed. The methanol in the aqueous layer was evaporated under a stream of nitrogen gas before the solution, which contains the photolyzed protein, was subjected to lyophilization. the lyophilized sample was suspended in 0.2 ml of 20 mM K_2HPO_4 and shaken gently at room temperature for two h. SDS (5 mg/mg protein) and ß-mercaptoethanol (1%) were added and the protein was incubated at 37 °C for 2 h before it was subjected to SDS-PAGE. The SDS-polyacrylamide gel columns were prepared either by the method of Weber and Osborn (121) or by the modified method substituting the cross-linker bisacrylamide, in the Weber and Osborn system, with a cleavable cross-linker, N,N'-diallytartardiamide (DATA) (100, 101). The buffer used for gel column and electrophoresis was 0.1 M Na/Na phosphate bufer, pH 7.0. Electrophoresis was performed at 8 mA for each gel column. Each gel was loaded about 30 µg of protein. After electrophoresis, the gels were stained and destained according to the reported method (121). The gels were sliced according to the stained protein bands. About three gels were used for each sample. When the Weber and Osborn gel system was used, the gel slices were hydrolyzed with 6 N NH_4OH according to the method described previously (61). When the SDS-DATA gel system was used, the gel slices were completely dissolved by incubation in 0.7 ml of 3% periodic acid at room temperature for 1 h. 10 ml of Insta-Gel counting fluid was added and radiactivity determined.

Results and Discussion

Biological Properties of 3-Azido-2-methyl-5-methoxy-6-(3',7'-dimethyloctyl)-1,4-benzoquinone (3-azido-2-methyl-5-methoxy-Q_{2S})

Preliminary studies showed that the azido-Q derivatives synthesized in Dr Yu's laboratory all partially restorate the enzymatic activity of Q- and PL-depleted succinate-

cytochrome c reductase in the dark and all abolish the restored activity upon photolysis. 3-Azido-2-methyl-5-methoxy-Q_2 is superior to the other azido-Q derivatives for study of the Q:protein interaction and identification of the Q-binding proteins because of its ability to completely inactivate the photolyzed samples against reactivation by other active Q-derivatives. This azido-Q derivative was made into ^{14}C- or ^{3}H-radioactive form through methylation and used in the early stage of this investigation. Although 3-azido-Q_2 has properties similar to that of 3-azido-2-methyl-5-methoxy-Q_2, the poor yield in the synthesis made subsequent preparation of the radioactive form difficult. It was further observed that hydrogenation of the geranyl group of 3-azido-2-methyl-5-methoxy-Q_2 to 3-azido-2-methyl-5-methoxy-6-(3', 7'-dimethyloctyl)-1,4-benzoquinone (3-azido-2-methyl-5-methoxy-Q_{2s}) did not alter the biological properties, compared to the unhydrogenated parent compound. Table III compares the biological activities of 3-azido-2-methyl-5-methoxy-Q_2 and 3-azido-2-methy-5-methoxy-Q_{2s}. When these two azido-Q derivatives were added to the Q- and PL-depleted ubiquinol-cytochrome c reductase in the absence of light, both derivatives restored about 12% of the reconstitutive activity to the depleted enzyme (relative to that restored by $Q_0C_{10}Br$ which restored over 90% of the activity of undepleted reductase). These azido-Q derivatives had no effect on the ubiquinol-cytochrome c reductase activity without photolysis when reduced $Q_0C_{10}Br$ or Q_2 was used as substrate. The reconstituted activity is the ability of ubiquinol-cytochrome c reductase to reconstitute with succinate-Q reductase to form succinate-cytochrome c reductase. The reconstitution was routinely performed by addition of Q analogues to the Q- and PL-depleted ubiquinol-cytochrome c reductase followed by addition of phospholipid and excess succinate-Q reductase. The ubiquinol-cytochrome c reductase activity of the preparation was measured in the presence of 50 μM reduced form of Q_2 (Q_2H_2). Since Q_2H_2 is the substrate for the ubiquinol-cytochrome c reductase assay, no difference in ubiquinol-cytochrome c reductase was observed when the Q- and PL-depleted ubiquinol-cytochrome c reductase was treated , in the absence of light, either with

TABLE III

BIOLOGICAL ACTIVITY 3-AZIDO-2-METHYL-5-METHOXY-Q_2 AND 3-AZIDO-2-METHYL-5-METHOXY-Q2s

Q derivatives	Enzymatic activities					
	Succinate-cytochrome c (reconstituted)			Ubiquinol-cytochrome c		
	Before hν	After hν	Inactivation	Before hν	After hν	Inactivation
	$\mu mol\ c$ reduced/min·mg		%	$\mu mol\ c$ reduced/min·mg		%
None	0.96	0.89	7.3	87.2	81.7	6
$Q_0C_{10}Br$	17.51	16.28	7.1	87.5	81.4	7
3-Azido-2-methyl-5-methoxy-Q_2	3.05	0.61	80.0	87.1	5.2	94
3-Azido-2-methyl-5-methoxy-Q_{2s}	3.10	0.58	81.3	87.0	5.2	94

Two-tenth-ml aliquots of the Q- and PL-depleted ubiquinol-cytochrome c reductase in 50 mM phosphate buffer, pH 7.4, containing 0.25% sodium cholate and 1 mM EDTA were mixed with 5 μl of 5 mM alcoholic solution of Q analogues and incubated at 0 °C for 10 min in the dark. Fifty-μl aliquots were withdrawn and mixed with 5 μl of asolectin micellar solution (10 mg/ml in H_2O) and reconstituted with 80 μl of succinate-Q reductase (3 mg/ml) in 20 mM Tris-succinate, pH 8.0, containing 0.1% deoxycholate to form succinate-cytochrome c reductase. After 1 h incubation, the mixture was diluted with 50 mM phosphate buffer, pH 7.4, containing 0.5% sodium cholate and the succinate-cytochrome c and ubiquinol-cytochrome c reductase activities were assayed. The remaining enzyme-Q mixture were subjected to photolysis at 0 °C for 20 min. After photolysis, aliquots were withdrawn and reconstituted with asolectin and succinate-Q reductase and the activities of both reductases were assayed. Reduced Q_2, 50 μM, was used as substrate for the ubiquinol-cytochrome c reductase activity assay.

a Q-derivative or azido-Q derivative followed by addition of phospholipid. Addition of only phospholipids to the Q- and PL-depleted ubiquinol-cytochrome c reductase also showed the same ubiquinol-cytochrome c reductase activity. However, photolysis of these azido-Q treated-enzyme complexes completely abolished, not only the reconstitutive activity, but also the ubiquinol-cytochrome c reductase activity. These results suggest that the low reconstituted activity obtained by addition of these two azido-Q derivatives to the Q- and PL-depleted ubiquinol-cytochrome c reductase is a result of the low electron transfer ability of these azido-Q derivatives, and not of incomplete binding of the Q-binding sites by these azido-Q derivatives.

The complete inactivation of the ubiquinol-cytochrome c reductase activity upon photolysis of this azido-Q derivative treated samples indicates that all the binding sites were covalently linked to the azido-Q derivatives, preventing access of Q_2H_2. Thus the degree of azido-Q binding could be directly indicated by assaying the QH_2-cytochrome c reductase activity after photolysis.

Illumination Time-dependent azido-Q Uptake by
Protein and Loss of Enzymatic Activity

Fig. 10 shows the photolysis time-dependent binding of the radioactive [3H]azido-Q derivative to the ubiquinol-cytochrome c reductase protein and inactivation of the azido-Q-treated ubiquinol-cytochrome c reductase. When the azido-Q-treated and PL-depleted ubiquinol-cytochrome c reductase was subjected to varying times of photolysis at 0-2 °C with long wavelength UV light, the ubiquinol-cytochrome c reductase activity, measured after reconstitution with phospholipid, decreased concurrently with increased [3H]azido-Q derivative uptake by protein over time. The maximal inactivation was observed when the azido-Q-treated sample was illuminated for 20 min. Illumination beyond 20 min did not further inhibit the activity, although the azido-Q uptake by the enzyme complex continued to increase, at a slower rate. A control sample, treated with the same amounts of solvent
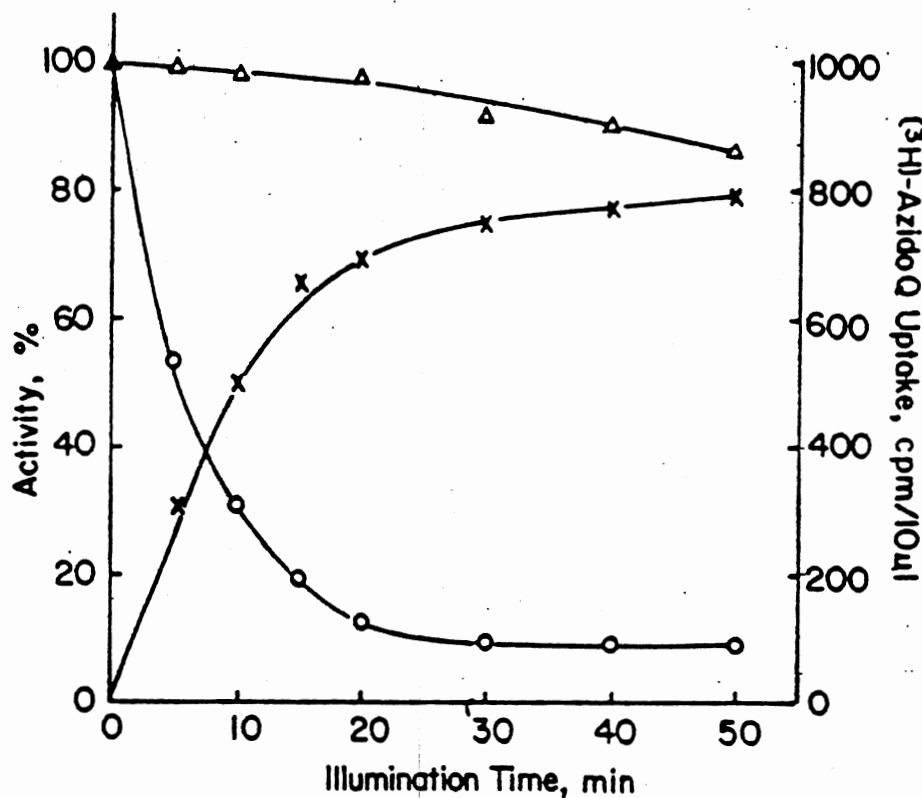
Fig. 10. Effect of Illumination Time on the Binding of Azido-Q Derivative to Ubiquinol-Cytochrome c Reductase and on Activity Inhibition. Five µl of 95% ethanol or of [^{3}H]3-azido-2-methyl-5-methoxy-Q_{2s} (13.4 mM in 95% ethanol, specific radioactivity 500 cpm/nmol) were diluted with 0.2 ml of 50 mM phosphate buffer, pH 7.4, containing 0.25% sodium cholate and 1 mM EDTA in the dark, then mixed with 50 µl of the Q- and PL-depleted ubiquinol-cytochrome c reductase (12 mg/ml, 120 µM cytochrome b) in 50 mM phosphate buffer, pH 7.4, containing 10% glycerol and 1 mM EDTA. Photolysis was carried out under the conditions described under " Experimental Procedures." At the times indicated, 10-µl aliquots were withdrawn and mixed with 50 µl of 50 mM phosphate buffer, pH 7.4, containing 0.5% sodium cholate, 20% glycerol, and 0,05 mg of asolectin micellar solution. Ubiquinol-cytochrome c reductase activity of the control (Δ) and the azido-Q treated (O) samples were assayed after incubation at 0 °C for 1 h. Ten-µl aliquots were also withdrawn from the azido-Q-treated sample at the given time intervals for the determination of radioactivity incorporation (x).

but no azido-Q, was placed beside the azido-Q-treated sample during photolysis. Little loss of activity occurred in the illuminated control sample. A plot of the logarithm of inactivation versus illumination time (not shown) yields a nearly linear curve, indicating that the inactivation resulting from photolysis follows first order kinetics. The kinetics of azido-Q uptake by ubiquinol-cytochrome c reductase, however, was more complex, as two different rates were involved, one of which was 10 times faster than the other. The rapid uptake seemed to correlate with the inactivation and was complete in 20 min of photolysis. The slower uptake was probably due to nonspecific binding of azido-Q derivative to protein. Thus, a 20-min illumination time was chosen for the identification of the Q-binding protein and study of the Q:protein interaction.in ubiquinol-cytochrome c reductase. The kinetics of azido-Q uptake by protein and inactivation are quite similar to those observed with the arylazido-Q derivative (61), although the inactivation was more complete with the azido-Q derivative.

Identification of the Q-binding Protein(s) in
Ubiquinol-Cytochrome c Reductase

Since the uptake of azido-Q derivative by ubiquinol-cytochrome c reductase upon photolysis was correlated with the enzymatic inactivation, it is reasonable to assume that the azido-Q derivative is bound specifically to the Q-binding site(s). Thus, the distribution of the covalently bound azido-Q among the subunits of ubiquinol-cytochrome c reductase after SDS-PAGE indicates the specific Q-binding protein in this enzyme complex. Fig.11 shows the ^{3}H-radioactivity distribution among subunits of ubiquinol-cytochrome c reductase under various conditions. Two SDS-polyacrylamide gel system were used: System A, the Weber-Osborn gel system, exactly as described for identification of the Q-binding protein in ubiquinol-cytochrome c reductase with the arylazido-Q derivative $(Q_0C_{10}NAPA)$ (61); and System B, the SDS-DATA gel system, which is a modification of the Weber-Osborn gel system. The Weber-Osborn cross-linker, bisacrylamide, was

replaced with a cleavable linker, N,N'-diallytartardiamide (DATA) (101). The advantage of using the SDS-DATA gel system is that the gel slices can be completely dissolved in 2% periodic acid and used for radioactivity determination without further treatment. The SDS-DATA gel system has been successfully used for identification of the Q-binding protein in a bacterial reaction center (100). As shown in Fig 11, the electrophoretic pattern of ubiquinol-cytochrome c reductase obtained from the SDS-DATA gel system was similar to that obtained from the Weber-Osborn gel system, with seven major protein bands observed in each. Although the electrophoretic mobility of each subunit, relative to cytochrome c in the SDS-DATA gel system was different from that in the Weber-Osborn gel system, the distribution of radioactivity among the subunits of ubiquinol-cytochrome c reductase was the same in those two gel systems (see panels i and ii of Fig. 11, A and B). One can assume that the protein subunits of ubiquinol-cytochrome c reductase shown in the SDS-DATA gel correspond to those observed in the Weber-Osborn gel system. The former is preferred for study of the Q-binding proteins in ubiquinol-cytochrome c reductase.

Results of photoaffinity labeling experiments are often complicated by the radioactivity observed near the dye front of the gel column resulting from free Q, phospholipid-Q, or detergent-Q adducts. It is especially important that the electrophoresis data be completely devoid of this radioactivity interference when the Q-binding site is in a small molecular weight protein. In the previous study of interaction of an arylazido-Q derivative with ubiquinol-cytochrome c reductase (61), it was observed that the radioactivity interference resulting from free Q and PL-bound Q in the gel column can be removed by prolonged destaining of the gel in the acetic acid-methanol solution. However, this usually required more than two weeks, with constant changes of destaining solution, to completely eliminate the interference. Introduction of an organic solvent extraction step successfully eliminated this interference. Fig. 11 shows the distribution patterns of

radioactivity after (i) and before (ii) the extraction step and in the extract (iii) in SDS-bisacrylamide and SDS-DATA gel systems.

To ensure that the organic solvent extraction of the photolyzed sample removed no protein-Q complex but only free Q, Q-PL, or Q-detergent adducts, the chloroform extract of the photolyzed samples were collected, dried, redissolved in a small amount of alcohol and assayed for protein. When the alcohol solution was mixed with the untreated, depleted ubiquinol-cytochrome c reductase and subjected to SDS-PAGE, the radioactivity was found mainly on the dye front of the gel column (see panel iii of Fig. 11, A and B). The very low amount of radioactivity found with the protein confirmed that all the [^{3}H]azido-Q in the chloroform extract had been photoactivated and covalently linked to some small molecules in the system and was no longer able to react with protein. This result also indirectly substantiated the observation that the radioactivity located in the protein bands of panel i and ii resulted from the covalent linkage between azido-Q and protein subunits and not a nonspecific hydrophobic interaction between photolyzed Q molecule and protein during SDS-PAGE. It should be mentioned that the extraction method described under " Experimental Procedure" for the removal of the non-protein bound azido-Q derivative does not cause protein aggregation, a common phenomenon after organic solvent extraction, as no protein remained on the top of the gel column after SDS-PAGE.

Panels i and ii of Fig. 11 clearly show that the two proteins with M_r=37,000 and 17,000 are responsible for specific Q-binding in ubiquinol-cytochrome c reductase. These results are identical to those obtained from a previous study in this laboratory, using the arylazido-Q derivative, $Q_0C_{10}NAPA$, as the labeling reagent. In addition to the M_r=17,000 and 37,000 subunits, a significant amount of radioactivity was also found in the M_r=30,000 protein, which was not labeled when $Q_0C_{10}NAPA$ was used. Whether the observed radioactivity on the M_r=30,000 subunit resulted from an abnormal electrophoretic mobility of the M_r=37,000 protein or from an actual partial labeling of the
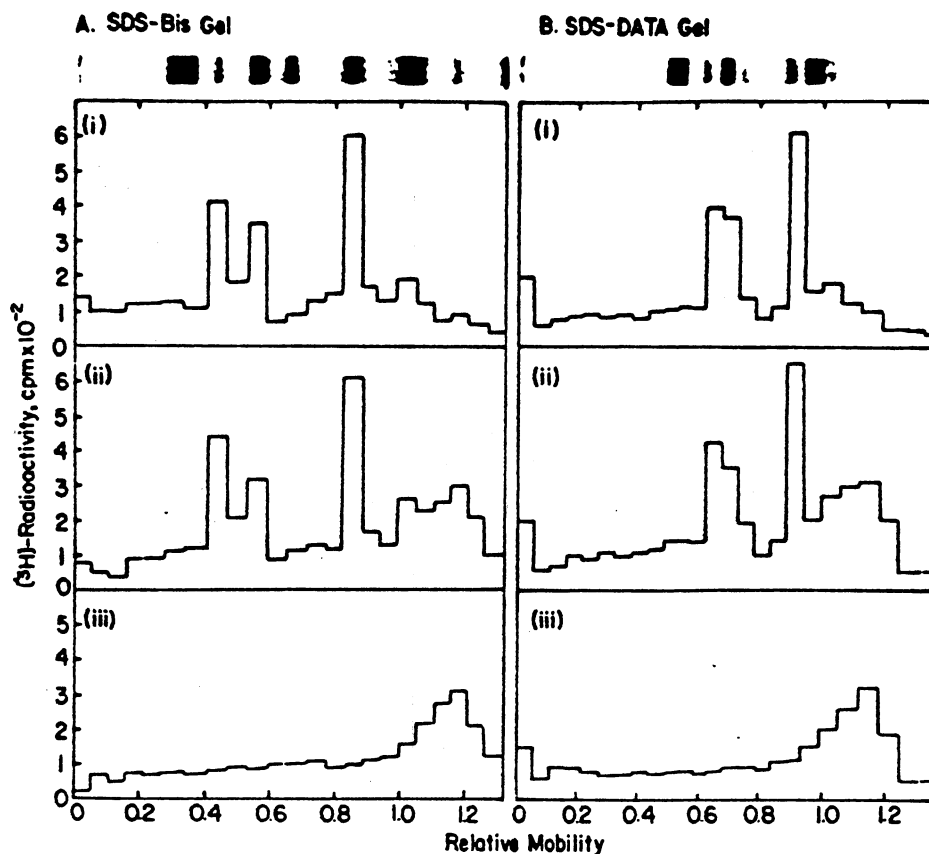
Fig. 11. Effect of Organic Extraction of the Azido-Q Derivative Treated Sample on the Radioactivity Distribution among the Subunits of Ubiquinol-Cytochrome c Reductase in the Weber-Osborn Gel and the SDS-PAGE Gel Systems. Four-tenth ml of the Q- and PL-depleted ubiquinol-cytochrome c reductase (3.1 mg/ml) was treated with 10 μl of 6.3 mM ethanolic solution of [^{3}H]3-azido-2-methyl-5-methoxy-Q_{2s} (4000 cpm/nmol). The mixture was photolyzed and diluted to 10 ml with 50 mM phosphate buffer, pH 7.4. The protein was collected by centrifugation and suspended with 0.5 ml of H_2O. The sample was used for the SDS-PAGE with or without subject to organic solvent extraction step according to the procedure described under "Experimental Procedures," using Weber-Osborn gel (A) and SDS-DATA gel (B) systems. Panels i and ii represent sample with and without organic solvent extraction, respectively, and panel iii represent a mixture of untreated ubiquinol-cytochrome c reductase and the organic extract of azido-Q-treated sample.

M_r=30,000 protein remains to be verified experimentally. The electrophoretic abnormality of the M_r=37,000 protein in ubiquinol-cytochrome c reductase has been documented (44,102). In fact, in some reports, the M_r=37,000 subunit was completely absent from the subunit structure of ubiquinol-cytochrome c reductase or complex III on SDS-PAGE. The M_r=37,000 subunit of ubiquinol-cytochrome c reductase has been purified and identified as one of the b cytochrome proteins (82). The M_r=17,000 protein has been identified as another b cytochrome protein (82). The M_r=17,000 protein has an electromobility faster than the Rieske iron-sulfur protein but slower than the cytochrome c_1-associated protein in the SDS-PAGE. It is not the same protein recently isolated and assumed by Wang and King to be QPc (103). The protein they assumed to be the Q-binding protein in the cytochrome b-c_1 region has the same electrophoretic mobility as the cytochrome c_1 associated small molecular weight polypeptide (M_r=15,000 or less), and was not labeled either with the radioactive azido-Q or arylazido-Q derivative. The nature of this protein (103) remains unclear, but is likely to be a Q:PL:protein mixture rather than a specific Q-binding protein.

Effect of the Redox State of the Azido-Q Derivative on the Binding to Ubiquinol-Cytochrome c Reductase

It has been suggested that the binding affinity of reduced Q to ubiquinol-cytochrome c reductase is stronger than that of the oxidized form of Q (85). It is, therefore, of interest to see whether the reduced azido-Q derivative binds ubiquinol-cytochrome c reductase better than does the oxidized azido-Q derivative. When the depleted ubiquinol-cytochrome c reductase was treated with different redox forms of azido-Q derivative at various concentrations, a 50% inactivation after photolysis was observed when 2 mol of azido-Q/mol of cytochrome b in the depleted enzyme complex were used, regardless of the redox state of the azido-Q derivative (see Fig. 12). The maximal inactivation by either redox state of the azido-Q derivative was almost the same.
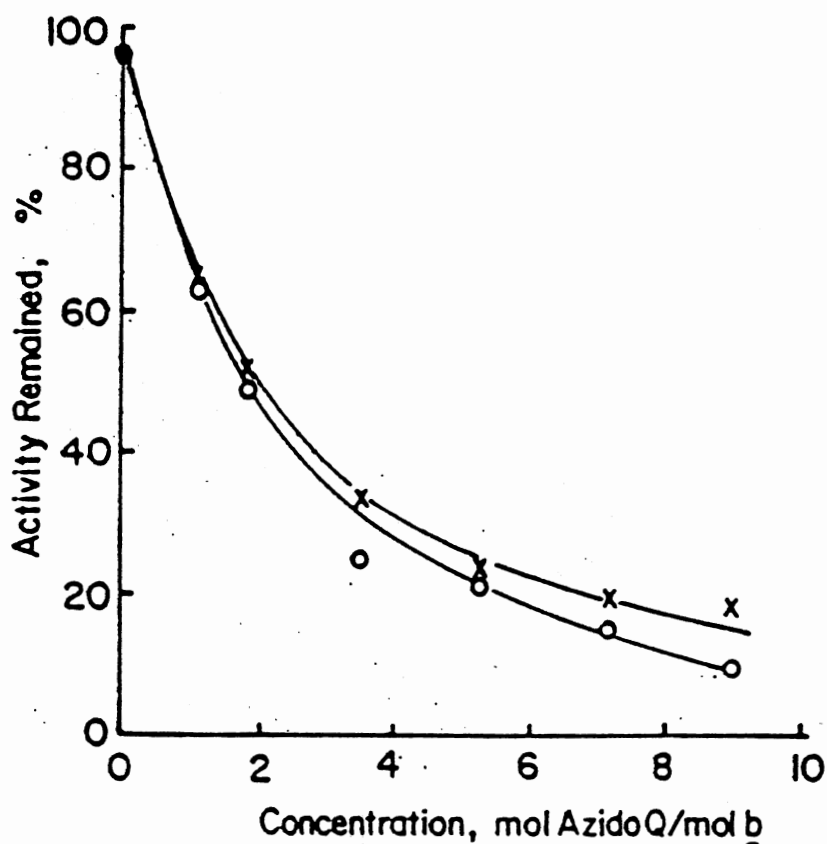
Fig. 12. Effect of the Redox State of the Azido-Q Derivative on the Binding to Ubiquinol-Cytochrome c Reductase. Indicated amounts of 3-azido-2-methyl-5-methoxy-Q_{2s} in 0.2 ml of 50 mM phosphate buffer, pH 7.4, containing 0.25% cholate and 1 mM EDTA were reduced by 0.43 M succinate, in the presence of succinate-Q reductase (10 μl, 1.6 mg/ml) in the dark at room temperature. After reduction, the activity of succinate dehydrogenase was inhibited by the addition of oxalacetate (5 μl, 1.6 mg/ml) and the mixture were placed in the ice bath and mixed with 60 μl of Q- and PL-depleted ubiquinol-cytochrome c reductase (O). When the oxidized azido-Q (x) was used, 0.2-ml aliquots of buffer containing the indicated amount of azido-Q were mixed with the depleted enzyme complex without pretreatment. Ten-μl aliquota were withdrawn from each sample before and after photolysis, reconstitution with asolectin, and assayed for ubiquinol-cytochrome c reductase activity. The per cent activity remaining after photolysis was based on the activity obtained before photolysis of each sample.

The results indicate that no significant difference in the Q-binding affinity between the oxidized and reduced forms of azido-Q exists in the cytochrome b-c_1 region of the electron transfer chain.

In the above experiment, the reduced form of the azido-Q derivative was generated by reduction of the oxidized azido-Q derivative with a catalytic amount of succinate-Q reductase and succinate. The formation of the reduced azido-Q was confirmed by thin layer chromatography after extraction of the azido-Q from the system. After the reduction was complete, the enzymatic activity of succinate-Q reductase was stopped by the addition of oxalacetate before mixing with depleted ubiquinol-cytochrome c reductase, to prevent a slow reduction of the depleted enzyme by the added succinate-Q reductase. To control for possible complications resulting from the addition of succinate and oxalacetate to the system, succinate and oxalacetate were also added to the system with oxidized azido-Q derivative. No difference in the inactivation effect of the oxidized azido-Q derivative after photolysis was found between the systems with and without succinate and oxalacetate.

Since more than one specific Q-binding subunit was detected in ubiquinol-cytochrome c reductase by the azido-Q derivative, the observed equal degree of inactivation by the oxidized and reduced forms of the azido-Q derivative after photolysis might result from the different contributions of the Q-binding proteins to the azido-Q binding. To clarify this point, the radioactivity distribution among subunits of the enzyme complex treated with oxidized and reduced forms of the azido-Q derivative was investigated, and the results were compared. An identical distribution of ^{3}H-radioactivity among the subunits of ubiquinol-cytochrome c reductase was obtained for the reduced and oxdized azido-Q derivative treated samples.

Effect of Phospholipids on the Inactivation of Ubiquinol-

Cytochrome c Reductase by Azido-Q Derivative

after Photolysis

The requirement for phospholipids, in addition to Q, for restoration of enzymatic activity and stabilization of the ubisemiquinone radical (44, 88) in ubiquinol-cytochrome c reductase, suggests an intimate relationship between the phospholipids and Q in this segment of the electron transfer chain. The reported observation (117) that only by adding Q prior to addition of phospholipids to the Q- and PL-depleted succinate-cytochrome c reductase can the enzymatic activity be completely restored, further comfirms the role of phospholipids in the Q:protein interaction. A similar phospholipid effect was observed with azido-Q derivative binding to ubiquinol-cytochrome c reductase. As indicated in Table IV, when the azido-Q derivative was added to the Q- and PL-depleted ubiquinol-cytochrome c reductase prior to the addition of phospholipids, a greater inactivation after photolysis was observed than in the sample mixed with phospholipids before addition of azido-Q derivative. This result suggests that at least one of the Q-binding sites can be easily masked or modified by phospholipids when Q is absent. It is possible that when photolysis of the azido-Q-treated enzyme complex is carried out in the presence of phospholipids, some of the photoproduced nitrenes may covalently link to phospholipids close to the Q-binding site, resulting in less inactivation of the ubiquinol-cytochrome c reductase. When phospholipids and azido-Q were premixed and then added to the depleted enzyme, only slightly less inactivation was observed after photolysis than with addition of azido-Q prior to phospholipids. This suggests that the binding of azido-Q to the Q-binding site is some what stronger than the binding of phospholipids to the Q-binding site. These results differ from those obtained by activity restoration using Q_6 mixed first with phospholipids, and then added to the depleted enzyme complex (117). In this case, only 30% of the activity was restored when Q_6 and phospholipids were mixed before being added to the depleted enzyme, compared to the activity restored by addition

TABLE IV

EFFECT OF ADDITION SEQUENCE OF PHOSPHOLIPIDS AND AZIDO-Q TO THE DEPLETED UBIQUINOL-CYTOCHROME c REDUCTASE (dQCR) ON THE AZIDO-Q BINDING AFFINITY

Treatments	Ubiquinol-cytochrome c reductase		
	Before hν	After hν	Inactivation
	$\mu mol\ c$ reduced/min·mg		%
1. dQCR + azido-Q	91.5	11.9	87
2. dQCR + azido-Q + PL	92.3	24.0	74
3. dQCR + PL + azido-Q	92.2	51.6	44
4. dQCR + (PL + azido-Q)	92.2	29.5	68

0.23-ml aliquots of the depleted ubiquinol-cytochrome c reductase, 1.16 mg/ml, in 50 mM phosphate buffer, pH 7.4, containing 0.25% sodium cholate and 1 mM EDTA were mixed with 1) 4 μl of 3-azido-2-methyl-5-methoxy-Q_{2a} (6.2 mM); 2) 4 μl of 3-azido-2-methyl-5-methoxy-Q_{2a}; 3) 25 μl of asolectin micellar solution (10 mg/ml in H_2O); and 4) 29 μl of a mixture of 3-azido-2-methyl-5-methoxy-Q_{2a} and asolectin (0.85 mM azido-Q and 86 mg/ml of asolectin). After incubation at 0 °C for 20 min in the dark, 25 μl of asolectin solution was added to 2, and 4 μl of 3-azido-2-methyl-5-methoxy-Q_{2a} was added to 3, and the samples were incubated in the dark for another 20 min. After incubation, a 0.1-ml aliquot was withdrawn from each sample and diluted with 0.5 ml of 50 mM phosphate buffer, pH 7.4, containing 0.5% sodium cholate and 10% glycerol for activity measurement. Sample 1 was diluted in the same buffer, but containing 0.11 mg of asolectin. The rest of the solutions were subjected to photolysis for 20 min at 0 °C. After photolysis, aliquots were withdrawn, diluted exactly as were those withdrawn before photolysis, and ubiquinol-cytochrome c reductase activity was assayed.

of Q_6 prior to the addition of phosphlipids. One explanation for this difference is that the photoaffinity labeled Q, once activated by photolysis, becomes covalently linked to the Q-binding protein and can not be displaced by the competing phospholipids. Thus the binding competition during photolysis favors azido-Q against phospholipids.

The phospholipid masking or competition at the Q-binding site can be overcome by exess (substrate level) Q_2H_2 as the Q-depleted but phospholipid-sufficient enzyme showed full activity when assayed with Q_2H_2.

Effect of Phospholipids on the Distribution of Azido-Q
Binding among Subunits of Ubiquinol-cytochrome
c Reductase

The Q- and PL-depleted ubiquinol-cytochrome c reductase was reconstituted with phospholipid in the absence of azido-Q derivative and subsequently replenished with the azido-Q derivative at various time intervals. The amount of azido-Q derivative binding to the Q-binding sites decreased, as indicated by the decreased inactivation upon photolysis. as the time of phospholipids incubation with the depleted enzyme increased (see Fig.13 A). The decreased azido-Q derivative binding to the Q-binding site, or the decreased inactivation upon photolysis, resulting from prolonged incubation of phospholipids with the depleted enzyme before addition of azido-Q, could be explained as either an increase in enzyme aggregation by phospholipids upon incubation or, more probably, a gradually increasing occupation (masking) of the Q-binding sites by phospholipids. This masking effect could also result from a protein conformational change upon incubation with phospholipids in the absence of Q, thus changing the affinity of Q-binding. The fact that the Q-depleted but PL-sufficient sample shows full ubiquinol-cytochrome c reductase activity when Q_2H_2 is used as substrate in the assay mixture, supports this deduction. As the amount of azido-Q added to the Q-depleted but PL-sufficient ubiquinol-cytochrome c reductase was increased, an increase in inactivation after photolysis was observed. This
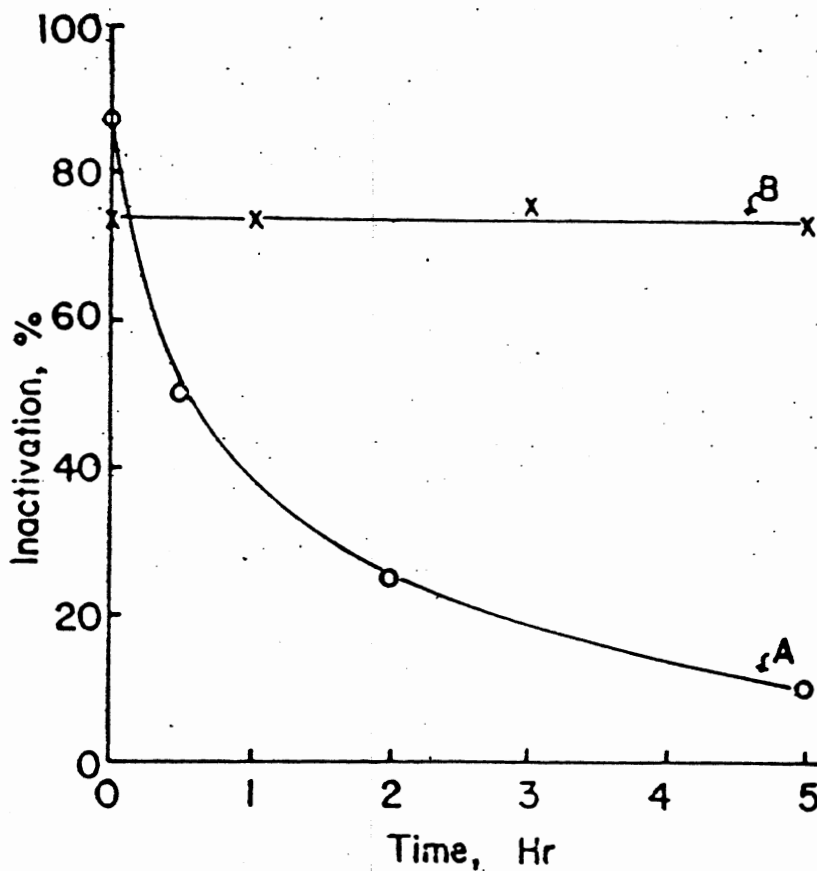
Fig. 13. Effect of Phospholipid Incubation Time on the Inactivation of the
Q- and PL-depleted Ubiquinol-Cytochrome c Reductase by
Azido-Q derivatives after Photolysis. A, Five-tenth ml of the
Q- and PL-depleted ubiquinol-cytochrome c reductase (8.5
mg/ml) was treated with 100 µl of asolectin micellar solution
(10 mg/ml in H_2O) and incubated at 0 °C. At the Times
indicated, 45-µl aliquots of the sample were withdrawn and
mixed with 0.15 ml of 50 mM phosphate buffer, pH 7.4,
containing 0.25% sodium cholate, 1 mM EDTA and 0.2 mM
3-azido-2-methyl-5-methoxy-Q_{2s}. Ten µl aliquots were
withdrawn before and after photolysis, and assayed for
ubiquinol-cytochrome c reductase activity. B, Two-tenth ml
of the depleted reductase (87 µM cytochrome b) were mixed
with 0.9 ml of 50 mM phosphate buffer, pH 7.4, containing
0.25% sodium cholate, 1 mM EDTA, and 0.2 mM azido-Q
derivative. After incubation at 0 °C for 5 min, the mixture was
mixed with 54 µl of asolectin micellar solution (10 mg/ml in
H_2O). At the times indicated, 0.2-ml aliquots were withdrawn,
photolyzed, and assayed for activity.

also supports the observation that replenishing Q to the Q-depleted, PL-containing ubiquinol-cytochrome c reductase requires a higher concentration of Q than in the absence of phospholipids.

When the azido-Q derivative was added to the Q- and PL-depleted ubiquinol-cytochrome c reductase first, followed by phospholipids, the amount of inactivation after photolysis was nearly independent of the incubation time of phospholipids in the reconstituted system (see Fig 13 B). This suggests that once the azido-Q derivative is bound to the Q-binding site, it can not be removed or displaced by phospholipids, and, indirectly, suggests that phospholipids facilitate the Q-binding, as supported by the fact that stabilization of the ubisemiquinone radical is phospholipid-dependent (101).

Since two subunits in ubiquinol-cytochrome c reductase were heavily labeled with [3H]azido-Q derivative upon photolysis, it is of interest to see whether the decreased azido-Q uptake by ubiquinol-cytochrome c reductase in the presence of phospholipids results from a decrease of the azido-Q binding by a specific Q-binding subunit or by both Q-binding subunits. Fig. 14 compares the ^{3}H-radioactivity distribution patterns, after photolysis and SDS-PAGE, among subunits of the complex from samples treated with various phospholipid incubation times. When phospholipids were preincubated with the depleted enzyme complex for 0, 1, and 5 h before the addition of [3H]azido-Q derivative, the inactivation after photolysis was 83, 50, and 10%, respectively. The ^{3}H-radioactivity incorporation at the M_r=17,000 protein decreased as the inactivation after photolysis decreased. This result demonstrates that the M_r=17,000 protein in ubiquinol-cytochrome c reductase is a Q-binding protein, with a Q-binding site easily masked by phospholipids in the absence of Q. By contrast, the radioactivity incorporation at the M_r=37,000 or 30,000 protein was relatvely constant with varying phospholipid incubation, indicating that this Q-binding site is less affected by phospholipids. Although the incorporation of [3H]azido-Q derivative at the M_r=37,000 or 30,000 protein showed no correlation with the inactivation after photolysis, the constant incorporation of azido-Q into this protein makes it unlikely
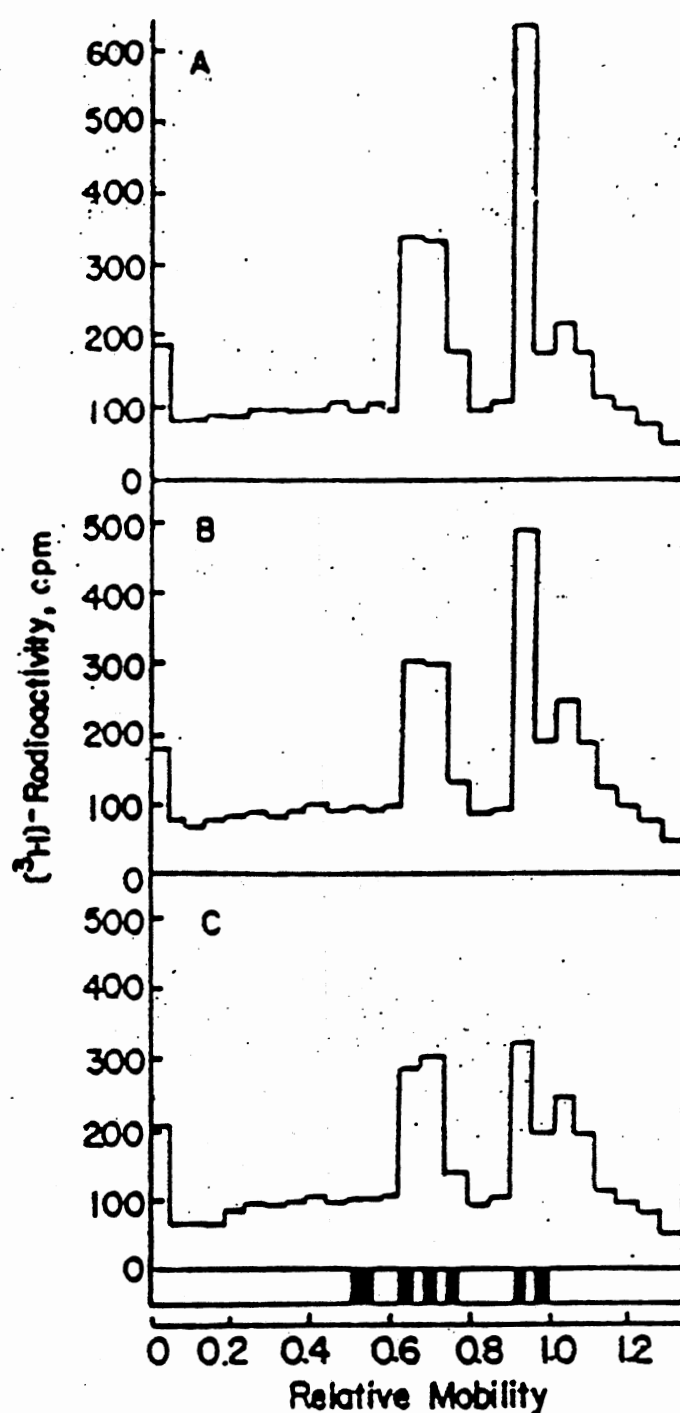
Fig. 14. Effect of Phospholipid Incubation Time before Addition of Azido-Q Derivative on the Distribution of [^{3}H]-radioactivity among Subunits of Ubiquinol-cytochrome c Reductase. The Experimental conditions were as described for Fig. 13A, except [^{3}H]3-azido-2-methyl-5-methoxy-Q_{2S} was used. A, Zero time incubation, 18% activity remained after photolysis; B, 1-h incubation, 50% activity remained after photolysis; and C, 5-h incubation, 90% activity remained after photolysis. The conditions for photolysis and preparation of samples for SDS-PAGE were as described for Fig. 11B, panel i.

that this is a nonspecific uptake. It is not clear at present whether or not the covalently linked azido-Q molecule in the Q-binding site of the M_r=37,000 protein is still functionally active, that is , whether or not displacement or rotation of the Q molecule in this Q-binding site is required during the redox reaction. Although two different Q-binding sites in ubiquinol-cytochrome c reductase have been documented through inhibitor studies (92) further clarification of the M_r=37,000 protein as a Q-binding protein is needed. One possible approach to this problem is to identify the Q-binding site, particularly the amino acid residues involved at the Q-binding site. Investigation of this aspect is currently under way in Dr. Yu's laboratory.

Effect of Inhibitors on the Binding of Azido-Q Derivative
to Ubiquinol-cytochrome c Reductase

The inhibitory effect of antimycin A and UHDBT on ubiquinol-cytochrome c reductase has been well established (104) both with intact enzyme complex and the Q- and PL-depleted form. The reversal of antimycin A (105) or UHDBT (69) inhibition by addition of Q derivarives has also been claimed or implied. It is, therefore, of interest to see whether the azido-Q derivative can reverse the inhibitory effect of antimycin A or especially, that of UHDBT, which contains a quinone group and has been suggested to be a quinone analogue. Addition of the azido-Q derivative to the Q- and PL-depleted ubiquinol-cytochrome c reductase either before or after addition of antimycin A or UHDBT did not alter the inhibitory action of these compounds, compared to the enzyme samples to which no azido-Q derivative was added. The distribution of the ^{3}H-radoactivity among the subunits of the enzyme upon photolysis and SDS-PAGE was practically identical in the presence or absence of these inhibitors, indicating that the Q-binding sites in ubiquinol-cytochrome c reductase are probably not the inhibitor binding site(s) of antimycin A or UHDBT, even though UHDBT has often been referred to as a Q

analogue. The same results were obtained when the arylazido-Q derivative was the photoaffinity labeling reagent (61).

It is well established (104) that HQNO inhibits electron transfer in the cytochrome b-c_1 segment of the mitochondrial electron transfer chain by inhibiting the oxidation of ubiquinol. This inhibition can be reversed by addition of Q derivatives (105). Whether or not the binding site of HQNO is the same as the Q-binding site, however, is not established. The effect of Q on inhibition by HQNO must be studied in succinate-cytochrome c reductase, intact or reconstituted, since HQNO is not inhibitory in the ubiquinol-cytochrome c reductase assay. Fig. 15 shows the concentration-dependent reversal of the HQNO inhibition by Q derivatives. When $Q_0C_{10}Br$ at a concentration of 3 mol/mol cytochrome b, was added to the HQNO-treated succinate-cytochrome c reductase, 87% of the original succinate-cytochrome c reductase activity was restored. When the azido-Q derivative, at 1.5 mol/mol cytochrome b, was added to the HQNO-treated sample, about 67% of the original succinate-cytochrome c reductase activity was restored. However, when duroquinol (2,3,5,6-tetramethyl-1,4-benzoquinone) was used, no restoration of the HQNO-inhibited activity was observed. In the absence of inhibitor, a 10-20% activation of intact reductase by addition of $Q_0C_{10}Br$ was observed. This is due to a partial deficiency of Q in the enzyme preparation resulting from the isolation procedure. The amount of $Q_0C_{10}Br$ required for this activation was very low: less than 1 mol/mol cytochrome b. No detectable increase of activity in intact reductase was observed in samples treated with azido-Q derivative, even when high concentrations of azido-Q derivative were used. This is probably due to the low electron transfer efficiency of the azido-Q derivative. When the succinate-cytochrome c reductase samples which were inhibited by HQNO and reactivated by the azido-Q derivative were subjected to photolysis, no decrease of the succinate-cytochrome c reductase activity or ubiquinol-cytochrome c reductase activity was observed. These results can be explained in several ways: (a) the HQNO is probably not bound to the Q-binding site and the reversal of the HQNO
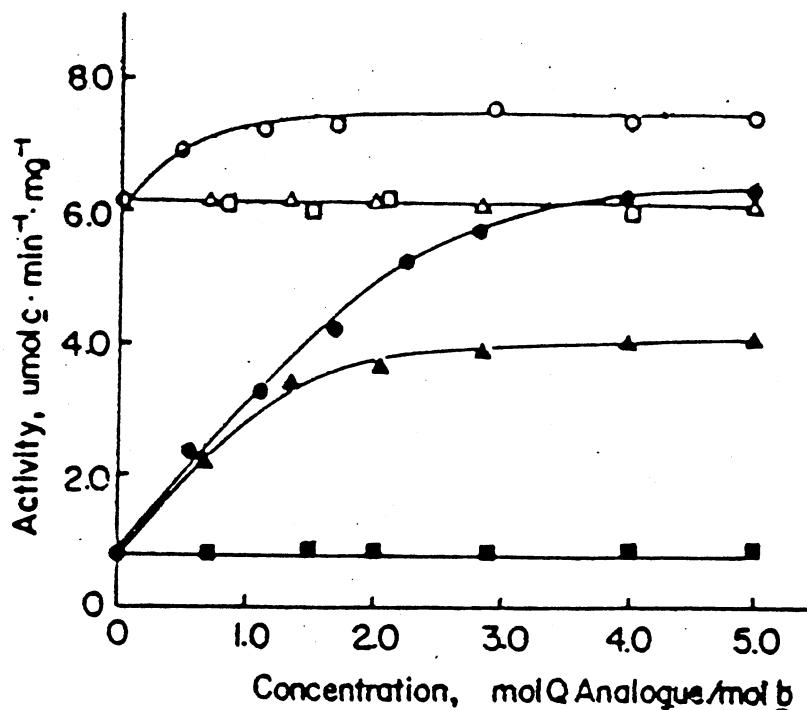
Fig. 15. Restoration of the HQNO-inhibited Activity by Q
Derivatives. 4.2-ml aliquots of intact succinate-
cytochrome c reductase, 1.1 mg/ml (4.5 μM
cytochrome b), in 50 mM phosphate buffer, pH
7.4, containing 0.4% sodium cholate and 3%
ammonium sulfate were mixed with 21 μl of
95% ethanol (O,Δ, □) or 21 μl of 29.4 mM
HQNO in 95% ethanol (●,▲, ■). Aliquots of
0.2 ml were withdrawn from the control and
HQNO-treated samples and mixed with 5 μl of
ethanolic solution containing the indicated
concentration of $Q_0C_{10}Br$ (O,●), 3-azido-2-
methyl-5-methoxy-Q_{2s} (Δ,▲), or duroquinol
(□,■).After incubation in the dark for 20 min,
the succinate-cytochrome c reductase activity was
assayed.

inhibition by Q analogue does not involve direct displacement of HQNO from the Q-binding site; (b) the binding of HQNO to the Q-binding site is Q concentration-dependent and native Q binds better than does azido-Q derivative; or (c) the HQNO is bound to a Q-binding site which requires Q for activity but does not require physical movement of the Q molecule, so that even a covalently linked Q (after photolysis) is still functionally active. If the last were true, then HQNO may be bound to the Q-binding site of the $M_r=37,000$ protein subunit. This is suggested by the observation that intact ubiquinol-cytochrome c reductase, when photolyzed with azido-Q, incorporated azido-Q into the $M_r=37,000$ protein, but underwent no loss of activity. More experiments are needed before the mode of action of HQNO is clear.

CHAPTER III

THE NATURE OF THE INHIBITION OF 4,7-DIOXOBEN-
ZOTHIAZOLE DERIVATIVES ON MITOCHONDRIAL
UBIQUINOL-CYTOCHROME C REDUCTASE

The indispensability of ubiquinone (Q) in mitochondrial and photosynthetic

electron transfer has been established (157, 60, 56, 63) and its possible role in proton

translocation suggested (56, 63). However, the reaction mechanisms of Q in electron

transfer and proton translocation are not yet understood and are currently the subjects of

intensive investigation (73,74). A powerful tool for studying the reaction mechanism of Q

is the use of specific inhibitors (75, 76), particularly Q-like compounds such as 5-n-

undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) (153, 35),

dibromomethylisopropyl-1,4-benzoquinone (DBMIB) (77-80), and other quinone

derivatives (75). Molecules structurally similar to Q may mimic the function of Q and

compete with it for binding sites, thus interfering with Q-mediated electron transfer

reactions.

UHDBT was first introduced as a specific inhibitor in the NADH or succinate

oxidase of yeast mitochondria by Roberts et al. (153). Lately it has been used extensively

in studying mammalian mitochondrial (71, 84) and bacterial photosynthetic electron

transfer systems (35). This inhibitor is reported to block electron transfer from ubiquinol

to cytochrome c and to cause alteration of the EPR characteristics of Rieske's iron-sulfur

protein.

Although the inhibition mechanism of UHDBT is not known, it has been shown to

raise the midpoint redox potential (pH 7.0) of the iron-sulfur cluster of ubiquinol-

cytochrome c reductase from 280 to 350 mV (35, 36). The fact that UHDBT has a quinone-like structure and is capable of altering the EPR characteristics of iron-sulfur protein has led some investigators (4, 81, 85, 90) to propose that UHDBT binds to Rieske's iron-sulfur protein and imply that Rieske's iron-sulfur protein is responsible for Q binding in ubiquinol-cytochrome c reductase. However, since the inhibitory effect of UHDBT is not reversed by exogenous Q, Q and UHDBT may not bind to the same site. Furthermore, recent photoaffinity labeling studies of Q binding proteins using arylazido- and azido-Q derivatives (61, chapter two) do not support the same site proposal because the iron-sulfur protein of the complex was not labeled by azido-Q, and the labeling pattern of the Q binding protein was not altered when UHDBT was present in the system.

To delineate the nature of UHDBT inhibition and to help to understand Q-mediated electron transfer, we have synthesized a series of 4,7-dioxobenzothiazole derivatives and compared their inhibitory efficacies and their ability to alter the EPR spectra of iron-sulfur protein in ubiquinol-cytochrome c reductase. By placing an azido group in the molecule, we were able to locate the binding site of this inhibitor after illumination. Herein we report the synthesis and properties of several 4,7-dioxobenzothiazole derivatives and that the binding site of UHDBT in ubiquinol-cytochrome c reductase using 6-azido-5-{1', 2'-[^{3}H] undecyl}-4,7-dioxobenzothiazole ([^{3}H] 6-azido-UDBT).

Experimental Procedures

Materials

Benzothiazole, 2-amino-6-methoxybenzothiazole, and other organic chemicals used in the syntheses were from either Aldrich or Eastman; cytochrome c and silica gel, 230-400 mesh, from Sigma; silica G thin layer plates, 250 micron, and HPTLC plates from Analtech. The Si-5 micropack HPLC column was from Varian. 2,3-Dimethoxy-5-methyl-6-geranyl-1.4-benzoquinone (Q_2) and its reduced form (Q_2H_2) were synthesized

in our laboratory as previously reported (106). Other chemicals were of the highest purity commercially available.

Enzyme Preparations

Ubiquinone-cytochrome c reductase and its Q- and phospholipid (PL)-depleted preparations (107) were prepared and assayed as previously reported. Protein was estimated by the Lowry method (108) using crystalline bovine serum albumin as the standard. Phosphorus was determined by the method reported by Dubin (143).

Spectral Measurements

The absorption spectra were measured in a Cary spectrophotometer, model 219, at room temperature. NMR spectra were measured using either a Hitachi Perkin-Elmer Model R24B or a Varian XL-300 NMR spectrometer. Mass spectra were measured with a high resolution CEC21-110 B Mass Spectrometer with Nora data acquisition system. IR spectra were done with a Perkin-Elmer Grating Infrared Spectrophotometer, model 457. EPR measurements were done with a Brucker ER-200D spectrometer. The detailed conditions and instrument settings are given in the legend of Fig. 23.

Synthesis of 4,7-Dioxobenzothiazole Derivatives

For simplicity of presentation the syntheses of 4,7-dioxobenzothiazole derivatives are divided into two groups: those with substituents at the 2-position of the 4,7-dioxobenzothiazoles and those without. The 2-substituted derivatives include 2-bromo- and 2-azido-5-decyl-6-methoxy-4.7-dioxobenzothiazole, and 2-bromo- and 2-azido-5-decyl-6-hydroxy-4,7-dioxobenzothiazoles; the 2-unsubstituted derivatives include 6-bromo- and 6-azido-5-decyl (or undecyl)-4,7-dioxobenzothiazoles.

Synthesis of 2-Substituted Derivatives

The essential steps are outlined in Scheme 1.

Synthesis of 2-Bromo-6-methoxybenzothiazole (II). Synthesis of compound II from compound I was carried out by the method of Erlenmeyer and Ueberwasser (109). Fifteen grams of 2-amino-6-methoxybenzothiazole and 75 ml of 85% H_3PO_4 were placed in a three-necked flask equipped with a mechanical stirrer and a thermometer. The mixture was warmed to and maintained at 40°C until a clear solution was obtained. The solution was then cooled to -10 °C, and 21 ml of conc. HNO_3 was added. The temperature of this mixture was maintained at –12 °C to –8 °C during the addition of HNO_3. Six grams of $NaNO_2$ in 7.5 ml of H_2O were added in 1-ml portions with a pipette to the bottom of the flask. After the addition of $NaNO_2$, the mixture was stirred for 10 min and then poured slowly into 300 ml of HBr solution (48%) containing 22.5 g of CuBr at 0°C. After the evolution of N_2 ceased, the mixture was stirred for 15 minutes, diluted with three volumes of H_2O, neutralized with 5 N NH_4OH, and extracted with benzene. The extract was dried over anhydrous Na_2SO_4, and benzene was removed by distillation. The residue was purified by the silica gel column chromatography (2.5 x 10 cm, 230-400 meshe), using ether-hexane (1:1) as the eluting solvent. The product, 2-bromo-6-methoxybenzothiazole, was collected as a purple fraction. The solvent was removed and compound II was crystalized from CH_3OH-H_2O as purple needles. The yield was 56%. 2-Bromo-6-methoxybenzothiazole has a m.p = 56-7°C; ^{1}H NMR ($CDCl_3$): 3.88 (s, 3H), l7.08 (q.1H), 7.28 (d, 1H), 7.88 (d, 1H).

Synthesis of 2-Bromo-6-methoxy-4.7-dioxobenzothiazole (III). Synthesis of compound III from compound II was carried out essentially according to the method of Braude & Fawcett (110). Five grams of 2-bromo-6-methoxybenzothiazole (II) were dissolved in 40 ml of 95% acetic acid and placed in a 100 ml three necked flask equipped with a mechanical stirrer and thermometer. To this solution, 5 g of chromium trioxide

SCHEME 1

THE SYNTHESIS OF 2-SUBSTITUTED DERIVATIVES
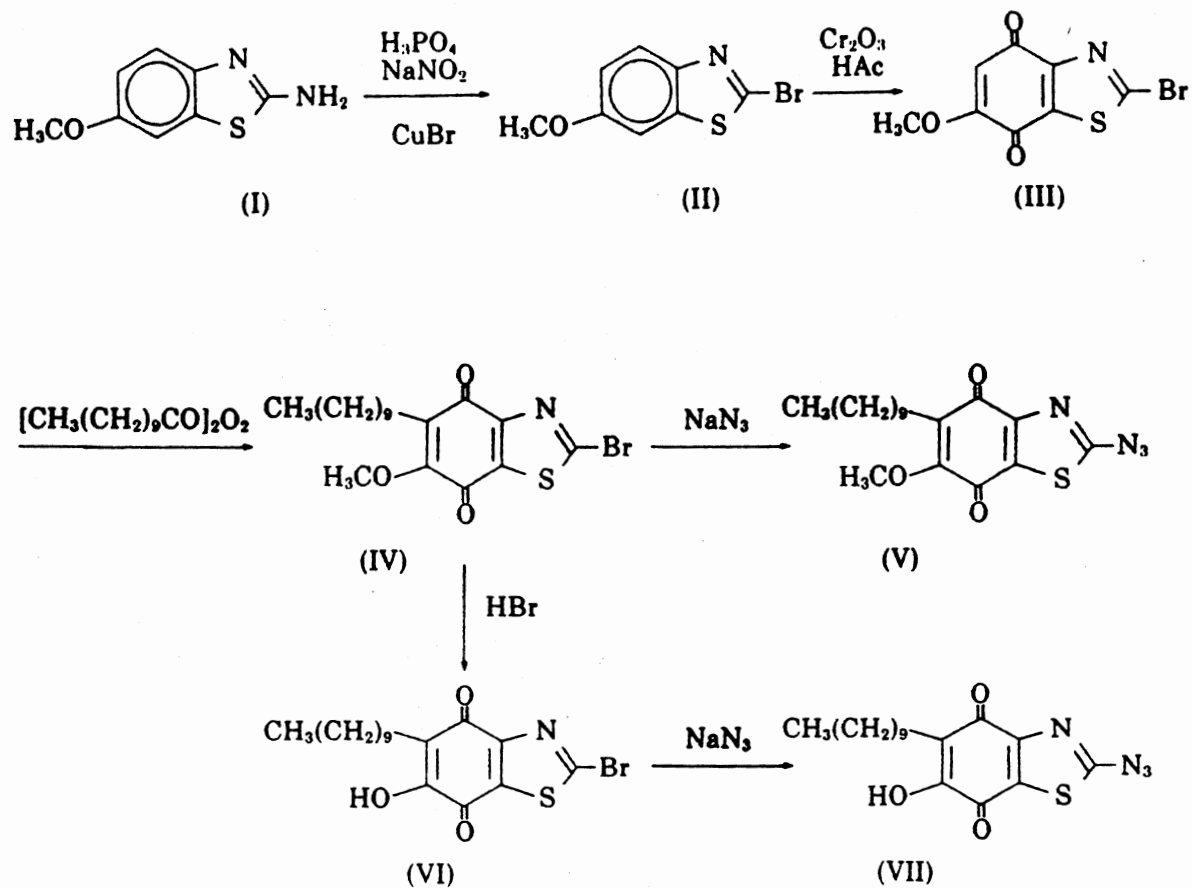
were added in small portions at 20°C. The reaction mixture was stirred at 20°C until it became dark green (about 30 hrs), poured into 120 ml of H_2O, and extracted three times with ether. The extract was dried over anhydrous Na_2SO_4. After the removal of ether the residue was separated by a silica gel column (2.5 x 10 cm, 230-400 meshes) using hexane-ether (1:1) as the eluting solvent. The yellow fraction was collected, dried, and subsequently crystalized from absolute ethanol to yield 50 mg of yellow needle crystals of 2-Bromo-6-methoxy-4.7-dioxobenzothiazole. The yield was 1%. Compound III shows ^1H NMR ($CDCl_3$): 3.94 (s, 3H), 6.06 (s, 1H); IR 1680 cm^{-1}, 1660 cm^{-1}.

Synthesis of 2-Bromo-5-decyl-6-methoxy-4.7-dioxobenzothiazole (IV).
Synthesis of compound IV from Compound III was carried out according to the method of Friedman et. al. (111). To fifty mg of 2-bromo-6-methoxy-4,7-dioxobenzothiazole (III) in 5 ml of glacial acetic acid were added 150 mg of diundecanoyl peroxide and stirred for 5 hrs at 95°C. The mixture was extracted three times with ether after cooling to room temperature. The extract was washed, in sequence, with H_2O, 1 N HCl, 0.5 M $NaHCO_3$, and H_2O before being dried over anhydrous Na_2SO_4. After the removal of ether the residue was separated by TLC developed with benzene-ethanol (95:5). 8 mg of 2-bromo-5-decyl-6-methoxy-4.7-dioxobenzothiazole (IV) were obtained. $R_f = 0.79$; ^1H NMR ($CDCl_3$): 0.88 (t, 3H), 1.26 (m, 14H), 1.50 (m), 2.55 (t, 2H), 4.11 (s, 3H); IR: 2950 cm^{-1} (s), 2860 (s), 1750 cm^{-1} (s), 1720 cm^{-1} (s); UV 95% EtOH: oxi., 295, 248 nm, red., 260 nm.

Synthesis of 2-Azido-5-decyl-6-methoxy-4,7-dioxobenzothiazole (V). NaN_3 (60 mg) were added to 4 mg of 2-bromo-5-decyl-6-methoxy-4,7-dioxobenzothiazole (IV), dissolved in a solution containing 1.5 ml of EtOH, 1.5 ml of acetone and 0.4 ml of H_2O, and stirred for 5 hours at room temperature in the dark. After the mixture was concentrated , it was applied to a TLC plate and developed with hexane-ether (3.5:1). The compound with a $R_f = 0.79$ was collected, eluted with ether, dried to yield 2 mg of yellow

oil of 2-azido-5-decyl-6-methoxy-4,7-dioxobenzothiazole. UV $^{95\% \text{ EtOH}}$: oxi., 308, and 272 nm; red., 250 nm. ^{1}H NMR (CDCl3): 0.85 (t, 3H), 1.25 (m, 14H), 1.45 (m, 2H), 2.52 (t, 2H), 4.08 (s, 3H).

Synthesis of 2-Bromo-5-decyl-6-hydroxy-4,7-dioxobenzothiazole (VI).

Synthesis of compound VI from compound IV was carried out by a modification of the method reported by Clarke et .al (112). Five mg of 2-bromo-5-decyl-6-methoxy-4,7-dioxobenzothiazole (IV) in 10 ml of glacial acetic acid were treated with 10 ml of hydrobromic acid (48%) and stirred for 5 hrs at 45°C. The mixture was poured into 200 ml of H_2O and extracted twice with ether. The extract was washed twice with H_2O and dried over anhydrous Na_2SO_4. After the solvent was removed, the residue was dissolved in 1 ml of acetone and applied to a TLC plate developed with hexane-ether (1:1). The compound with a $R_f = 0.70$ was collected, eluted with ether, and dried to yield 4 mg of yellow oil of 2-bromo-5-decyl-6-hydroxy-4,7-dioxobenzothiazole. UV $^{95\% \text{ EtOH}}$: oxi., 295, 251 nm; red., 261 nm; IR: 1720 cm^{-1} (s). ^{1}H NMR (CDCl3): 0.90 (t, 3H), 1.29 (m, 12H), 1.52 (m, 2H), 1.65 (m, 2H), 2.58 (t, 2H).

Synthesis of 2-Azido-5-decyl-6-hydroxy-4,7-dioxobenzothiazole (VII). To 2.4 mg of 2-bromo-5-decyl-6-hydroxy-4,7-dioxobenzothiazole (VI) in a solution containing 400 μl of EtOH, 400 μl of acetone, and 200 μl of glacial acetic acid, was added 20 mg of NaN_3 in 200 μl of H_2O. After stirring for 10 hrs at room temperature the mixture was applied to a TLC plate and developed with hexane-ether (3.5:1). The band with a $R_f = 0.72$ was collected, eluted with ether, and dried to yield 1 mg of yellow oil of 2-azido-5-decyl-6-hydroxy-4,7-dioxobenzothiazole. The yield was 40 %,. Compound VII has UV $^{95\% \text{ EtOH}}$: oxi, 312 and 260 nm; red., 264 nm ; IR, 2250 cm^{-1}. ^{1}H NMR (CDCl3): 0.88 (t, 3H), 1.25 (m, 14H), 1.52 (m, 2H), 2.55 (t, 2H).

Synthesis of 2-Unsubstituted Derivatives

The essential steps involved in the synthesis of 2-unsubstituted derivatives, 6-bromo- and 6-azido-5-decyl (or undecyl)-4,7-dioxobenzothiazole, are outlined in Scheme 2.

Synthesis of 6-Bromo-4, 7-dioxobenzothiazole (XIV). 6-Bromobenzothiazole (XI) was prepared from benzothiazole (VIII) by nitration, reduction, and diazotization according to the procedure reported by Boggust et al (113). 7-Amino-6-bromobenzothiazole (XIII) was synthesized from 6-bromobenzothiazole (XI) by the procedure reported by Haddock et. al (114). To 2.7 g of 7-amino-6-bromobenzothiazole (XIII) dissolved in 450 ml of 3.44 N H_2SO_4 was added 60 ml of 10% $Na_2Cr_2O_7$, over 1.5 hrs, at 3°C. After stirring at room temperature for 10 hrs the dark brown precipitate was removed by filtration and the filtrate was extracted three times with ether. The extract was washed three times with H_2O and dried over anhydrous Na_2SO_4. 0.2 g of yellow plate crystals of 6-bromo-4,7-dioxobenzothiazole were obtained after ether removal and crystallization from EtOH. The product has m.p.= 188-189°C, ^{1}H NMR (CDCl$_3$): 7.47 (s, 1H), 9.16 (s, 1H).

Synthesis of 6-Bromo-5-decyl (or undecyl)-4,7-dioxobenzothiazole (XV or XVI). To twenty mg of 6-bromo-4,7-dioxobenzothiazole (XIV) in 4 ml of glacial acetic acid were added 60 mg of diundecanoyl peroxide (or dilauroyl peroxide). After stirring at 95°C for 13 hrs the mixture was extracted three times with ether. The extract was washed, in sequence, with H_2O, 1 N HCl, 0.5 M $NaHCO_3$, and H_2O twice before being dried over anhydrous Na_2SO_4. After ether removal the residue was applied to a thin layer plate and developed with hexane-ether (1:1). The band with a $R_f = 0.43$ (XV or XVI) was collected and eluted with ether to yield 1.6 mg of XV (or XVI). XV has ^{1}H NMR (CDCl$_3$): 0.88 (t, 3H), 1.27 (m, 12H), 1.43 (m, 2H), 1.57 (m, 2H), 2.84 (t, 2H), 9.12 (s, 1H); UV ^{EtOH}: 285 and 230 nm. XVI has ^{1}H NMR (CDCl3): 0.91 (t, 3H), 1.29

SCHEME 2

THE SYNTHESIS OF 2-UNSUBSTITUTED DERIVATIVES

(VIII)   (IX)   (X)

(XI)   (XII)

(XIII)   (XIV)

(XV)   R = decyl   (XVII)   R = decyl

(XVI)   R = undecyl   (XVIII)   R = undecyl
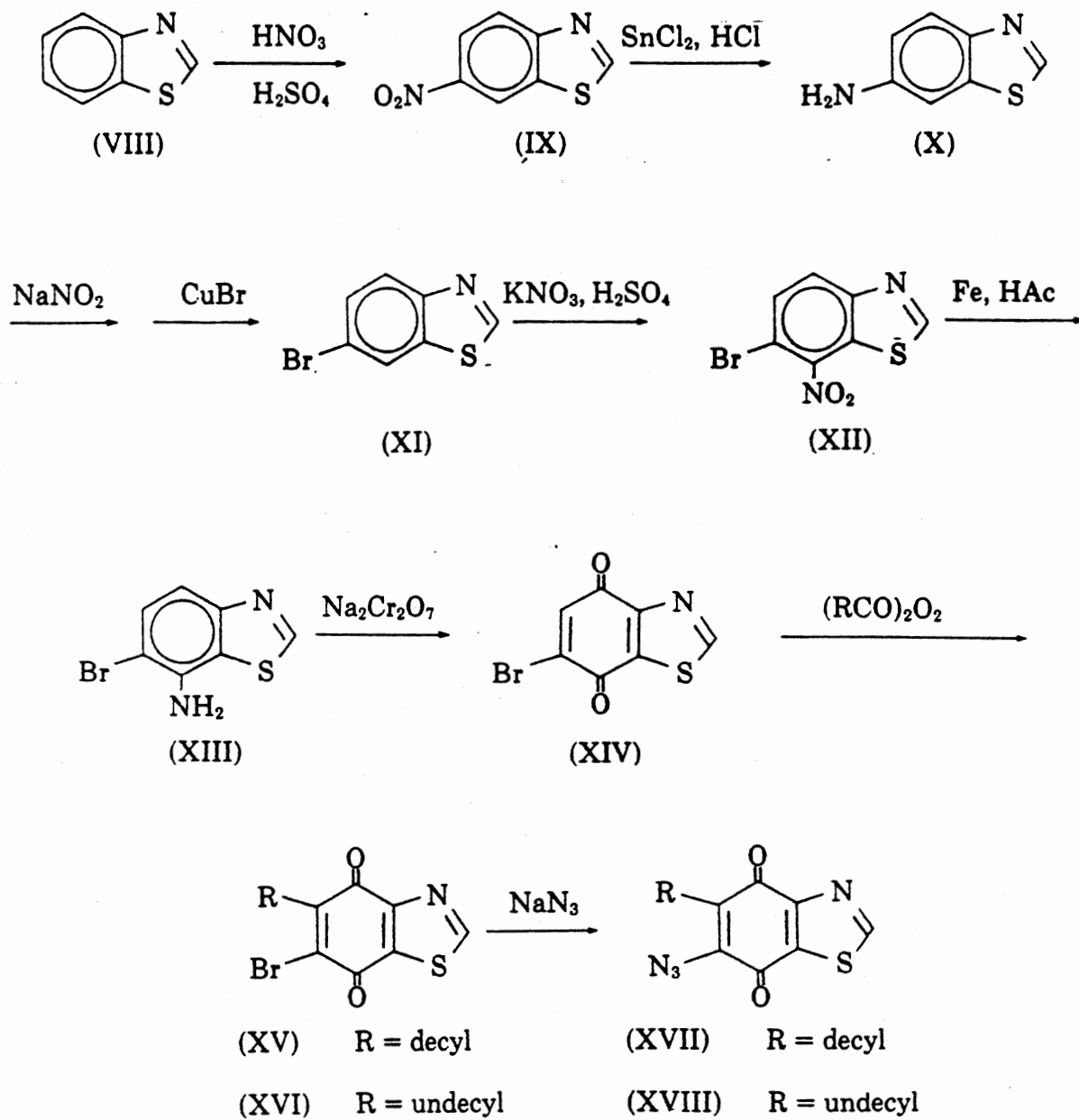
(m, 14H), 1.46 (m, 2H), 1.60 (m, 2H), 2.88 (t, 2H), 9.14 (s, 1H); UV ^{EtOH}: 290, 231 nm.

Synthesis of 6-Azido-5-decyl (or undecyl)-4,7-dioxobenzothiazole (XVII or XVIII). To 1.6 mg of 6-bromo-5-decyl-4,7-dioxobenzothiazole (or 6-bromo-5-undecyl-4,7-dioxobenzothiazole) in a solution containing 200 μl of ethanol, 200 μl of acetone, and 70 μl of water was added 1 mg of sodium azide. After stirring at room temperature in the dark for 12 hrs, the mixture was applied to a thin layer plate and developed with hexane-ether (1:1). The band with a $R_f = 0.39$ (XVII or XVIII) was collected and eluted with ether to yield 0.8 mg of 6-azido-5-decyl (or undecyl)-4,7-dioxobenzothiazole. ^{1}H NMR of XVII (CDCl$_3$): 0.75 (t , 3H), 1.25 (m, 12H), 1.47 (m, 2H), 1.56 (m, 2H), 2.57 (t , 2H), 9.12 (s, 1H). ^{1}H NMR of XVIII (CDCl3): 0.86 (t, 3H), 1.26 (m, 14H), 1.47 (m, 2H), 1.56 (m, 2H), 2.57 (t, 2H), 9.11 (s, 1H).

Isolation of 6-N$_3$-UDBT Bound Phospholipid

Ubiquinol-cytochrome c reductase or its delipidated preparation, 1.5 mg/ml, in 50 mM Na/K phosphate buffer, pH 7.4, containing 10% glycerol and 0.5% sodium cholate was incubated with a five-molar excess 6-azido-5-{1', 2'-[^{3}H]-undecyl}-4,7-dioxobenzothiazole {[^{3}H]-6-N3-UDBT} in the dark for 10 min. at 0 °C. Under these conditions, about 40% of the enzyme activity is inhibited when intact ubiquinol-cytochrome c reductase is used; about 75% of the reconstituted activity is inhibited when delipidated ubiquinol-cytochrome c reductase is used and assayed after reconstitution with phospholipid (asolectin) and Q. The [^{3}H]-6-N3-UDBT-treated sample was transferred to a photolysis apparatus made of two quartz glasses sandwiched by a Teflon ring and illuminated with a long UV light at 0 °C for 10 min (chapter two). The photolyzed complex was then extracted twice with a chloroform-methanol (2:1) mixture to remove phospholipid from its protein fraction. All the radioactivity was found in the extract which

was dried, applied to a thin layer plate and developed with ether to remove non-phospholipid materials. Three parallel control experiments were run: (a) [^{3}H]-azido-UDBT in the same buffer and photolyzed in the absence of reductase; (b) reductase in the same buffer photolyzed in the absence of [^{3}H]-azido-UDBT; and (c) asolectin in the same buffer photolyzed with [^{3}H]-azido-UDBT. The phospholipid fraction was collected, dried, dissolved in chloroform-methanol (2:1), and applied to a Si-5 Micropak HPLC column. The column was eluted with a gradient formed from a mixture of hexane:isopropanol (97:3) and a mixture of hexane:isopropanol (60:40). One-ml fractions were collected and [^{3}H]-radioactivity was determined.

Identification of Fatty Acids of the Azido-UDBT
Linked Phospholipid

150 µg of the [3H]-azido-UDBT linked phospholipid fraction obtained by HPLC were dried, dissolved in 4 ml Na-dried benzene, and added to 0.4 ml of HCl-CH$_3$OH and 0.5 ml of 2,2-dimethoxyl-propane. The mixture was kept at room temperature overnight, after which the solvents were removed with a stream of nitrogen. The methyl esters of the fatty acids were identified by capillary gas chromatography, Tracor 560 (115)

Identification of the Head Group of the
Azido-UDBT Linked Phospholipid

The head group of phospholipid was determined by the method of Latyshev and Vaskovsky (116) with slight modifications. To 10 µg of the [^{3}H]-azido-UDBT linked phospholipid in 40 µl of carbon tetrachloride were added, in sequence, 380 µl of ethanol, 33 µl of H$_2$0, and 12.5 µl of 1 N NaOH. The mixture was incubated at 37 °C for 20 min, treated with 20 µl of ethyl formate to neutralize the excess alkaline, and incubated for another 5 min at 37 °C. The solution was dried with a N$_2$ stream, and the residue was dissolved in 300 µl chloroform-isobutanol (2:1, v/v) saturated with H$_2$0 and partitioned

with 300 μl of water saturated with chloroform-isobutanol (2:1) by shaking. The H_2O layer was separated from the chloroform-isobutanol layer by centrifugation, collected, and concentrated to 50 μl. Three-μl of this solution, along with glycerophosphoryl choline, glycerophosphoryl ethanolamine and diglycerophosphoryl glycerol hydrolized from standard phospholipids, were applied to a 5 x 5 cm High Performance TLC-HL plate and developed with MeOH : CH_3COCH_3: $CHCl_3$: H_2O : NH_4OH (10:4:4:1:2, v/v). The plate was air dried, sprayed with $HClO_4$, and placed on a hot plate at a surface temperature of 250 -300°C until the plate became white. After the plate was cooled to room temperature, it was sprayed with Malachite Green and kept it in a desiccator. The background became orange and the spots with phosphate became bright green.

Results and Discussion

Synthesis, Structure, and Properties of
4,7-Benzothiazole Derivatives

The 4,7-dioxobenzothiazole derivatives used in this study were synthesized by two general routes: route I for compounds having the substituent at the 2-position of the 4,7-dioxobenzothiazole ring, such as 2-bromo- and 2-azido-5-decyl-6-methoxy (or hydroxy)-4,7-dioxobenzothiazole, and route II for compounds possessing no substituent at the 2-position of the ring, such as 6-bromo-and 6-azido-5-decyl (or undecyl)-4,7-dioxobenzothiazole (Schemes 1& 2). The overall yields are low because no effort was made to maximize the yield of each synthetic step. However, they can be improved if reaction conditions are carefully chosen. In general, the synthetic procedures reported here are satisfactory since only a small amount of the compound is needed for the study.

An attempt to synthesize 2-bromo-6-methoxy-4,7-dioxobenzothiazole from 2-bromo-6-methoxybenzothiazole through nitration, reduction and oxidation (scheme 3) was

SCHEME 3

AN UNSUCCESSFUL PATHWAY OF THE SYNTHESIS OF
2-BROMO-6-METHOXY-4,7-DIOXOBENZOTHIAZOLE
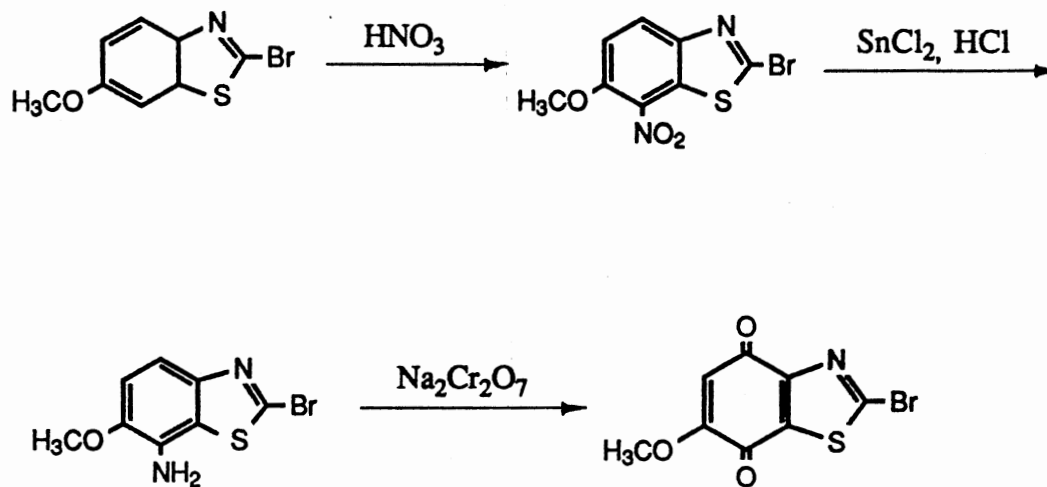
made. It was not successful. The bromine at position 2 is subjected to hydrogenolysis when the nitro group at position 7 is reduced (Fig. 16).

Table V summarizes the chemical structure, UV and NMR spectral properties, and inhibitory efficacies of these 4,7-dioxobenzothiazole derivatives. 5-Undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) shows a UV absorption peak at 285 nm with a shoulder at 248 nm in 95% ethanol. Replacing the 5-undecyl group with decyl group (DHDBT) causes no change in the UV absorption characteristics. Replacing the 6-hydroxyl group with a bromo group (6-Br-UDBT) causes a 5 nm red-shift of the peak with a 15 nm blue-shift of the shoulder absorption. Replacing the 6-hydroxyl group with a methoxyl group(UMDBT) or an azido (6-N_3-UDBT) causes a red-shift of both the peak and shoulder absorptions. It should be noted that the spectral shift caused by the change of the 6-hydroxyl of DHDBT is the same as those observed with UHDBT. Introducing a bromo atom or an azido group to the 2-position of DHDBT molecule also results in a red-shift of both the peak and shoulder absorptions.

As expected, photolysis of azido derivatives, such as 2-azido-5-decyl-6-hydroxy-4,7-dioxobenzothiazole (2-N_3-DHDBT) or 6-N_3-UDBT, results in a drastic decrease in the UV absorption intensity. Fig. 17 shows the absorption spectra of 6-N_3-UDBT and its photolyzed products in 95% ethanol.

All these 4,7-dioxobenzothiazole derivatives show inhibitory effects on ubiquinol-cytochrome c reductase with different potencies. When purified ubiquinol-cytochrome c reductase is treated with a 10-fold excess of UHDBT per mole of cytochrome b, about 90% of the enzymic activity is lost. Replacing the 6-hydroxyl of UHDBT with a bromo or with an azido group decreases the inhibition potency by 10% and 50%, respectively, indicating that the 6-hydroxyl is not a structural requirement for inhibition.

Although introducing a bromo in the 2-position of DHDBT molecule causes a decrease in inhibition potency by 10%, subsequently replacing the 6-hydroxyl with a methoxy increases the inhibition efficacy to the same level as that of UHDBT. Introducing
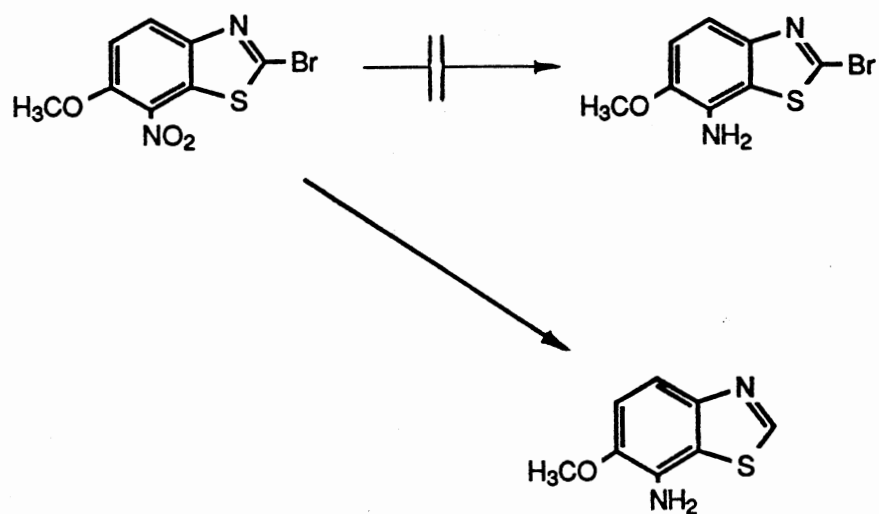
Fig. 16. Bromine Was Removed with Reduction of the Nitro Group

TABLE V

STRUCTURE, SPECTRAL, AND INHIBITORY PROPERTIES OF 4,7-DIOXOBENZOTHIAZOLE DERIVATIVES

General Structure
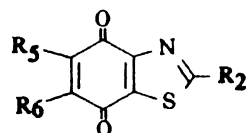
Compound	R2	R5	R6	UV 95% EtOH max	NMR	Inhibition* %
UHDBT	H	$CH_3(CH_2)_{10}H$	OH	285, 246	0.90 (t), 1.26 (m), 2.59 (t), 9.14 (s).	90
DHDBT	H	$CH_3(CH_2)_9H$	OH	285, 246	0.88 (t), 1.25 (m), 1.52 (m), 2.59 (t), 9.14 (s).	80
6-Br-UDBT	H	$CH_3(CH_2)_{10}H$	Br	290, 231	0.91 (t), 1.29 (m), 1.46 (m), 1.60 (m), 2.88 (t), 9.14 (s)	80
6-Br-DDBT	H	$CH_3(CH_2)_9H$	Br	285, 230	0.88 (t), 1.27 (m), 1.43 (m), 1.57 (m), 2.84 (t), 9.12 (s)	75
6-N$_3$-UDBT	H	$CH_3(CH_2)_{10}H$	N_3	305, 258	0.86 (t), 1.26 (m), 1.47 (m), 1.56 (m), 2.57 (t), 9.11 (s).	42
6-N$_3$-DDBT	H	$CH_3(CH_2)_9H$	N_3	305, 259	0.75(t), 1.25 (m), 1.47 (m), 1.56 (m), 2.57 (t), 9.12 (s)	40
UMDBT	H	$CH_3(CH_2)_{10}H$	CH_3O	289, 253	0.88 (t), 1.26 (m), 1.45 (m), 2.56 (t), 4.10 (s), 9.06 (s)	85
2-Br-DHDBT	Br	$CH_3(CH_2)_9H$	OH	295, 251	0.90 (t), 1.29 (m), 1.52 (m), 1.65 (m), 2.58 (t).	80
2-Br-DMDBT	Br	$CH_3(CH_2)_9H$	CH_3O	295, 248	0.88 (t), 1.26 (m), 1.50 (m), 2.55 (t), 4.11 (s)	90
2-N$_3$-DHDBT	N_3	$CH_3(CH_2)_9H$	OH	312, 260	0.88 (t), 1.25 (m), 1.52 (m), 2.55 (t).	90
2-N$_3$-DMDBT	N_3	$CH_3(CH_2)_9H$	CH_3O	308, 272	0.88 (t), 1.25 (m), 1.43 (m), 2.52 (t), 4.10 (s).	64

* 10 moles 4,7-dioxobenzothiazole derivatives per mole of cytochrome *b* were used.
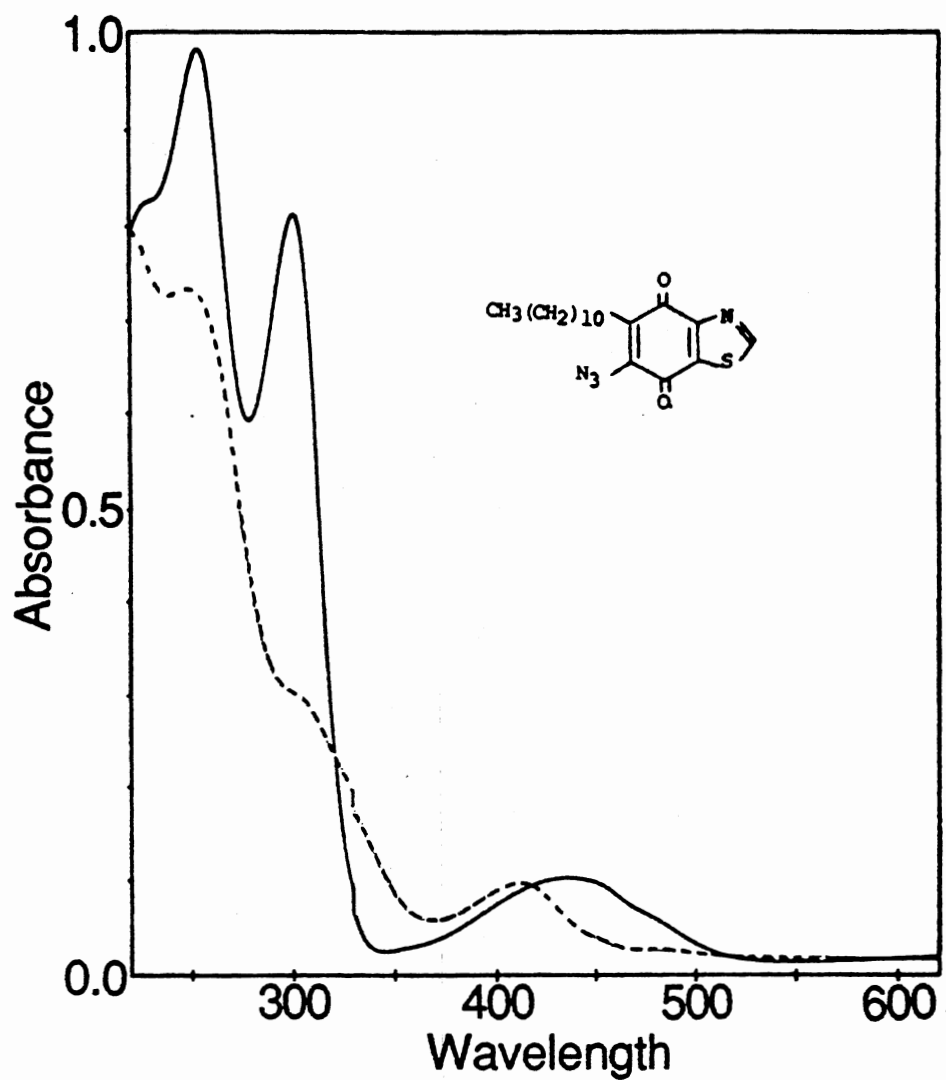
Fig. 17. Absorption Spectra of 6-Azido-UDBT. 50 μM of 6-azido-
UDBT in 95% ethanol was used. Solid line (—)
represents oxidized form; broken line (- -)represents
sample being illuminated with long UV light for 10 min.
at 0 °C.

an azido group to the 2-position of DHDBT does not decrease the inhibitory potency, whereas introducing an azido at the 2-position of DMDBT decreases inhibition by 21%. These results indicate that an unsubstituted 2-position is not necessary for UHDBT inhibition. It should be mentioned that the data given in Table 3 for the inhibition of ubiquinol-cytochrome c reductase by these derivatives are the maximum inhibitions obtained All these derivatives show maximum activity inhibition when 10 moles of the derivative per mole of cytochrome b. are used.

Effect of pH on the Inhibitory Efficiency of
 4,7-Dioxobenzothiazole Derivatives

It has been reported (71) that inhibition of mitochondrial ubiquinol-cytochrome c reductase by UHDBT is pH dependent. Maximum inhibition was observed when ubiquinol-cytochrome c reductase was reacted with UHDBT at a pH around 6.5; little inhibition was observed when the reacting pH was above 7.5. Since the pK_a for the 6-hydroxyl of UHDBT is 6.5 (71), the observed pH-dependency was attributed to the dissociation state of the 6-hydroxyl of UHDBT. That is, the non-dissociated (protonated) form of 6-hydroxy of UHDBT is essential for inhibition. One way to find out whether or not dissociation of the 6-hydroxyl group is responsible for the pH-dependent inhibition observed with beef heart mitochondrial ubiquinol-cytochrome c reductasde is to study the pH effect of derivatives bearing a nondissociable group at the 6-position, such as 6-Br-UDBT. Fig 18 shows the results of such a study using 6-Br-UDBT and UHDBT. When beef heart mitochondrial ubiquinol-cytochrome c reductase at various pH's is treated with 6-Br-UDBT, the percent inhibition decreases as the pH increases, just as when UHDBT is used. Obviously pH dependent inhibition is not due to the dissociation of the 6-hydroxyl group. Rather, it must be due to a change which occurs in the enzyme molecule. Very likely the binding of UHDBT involves a group having a pK_a of around 6.5 in the enzyme
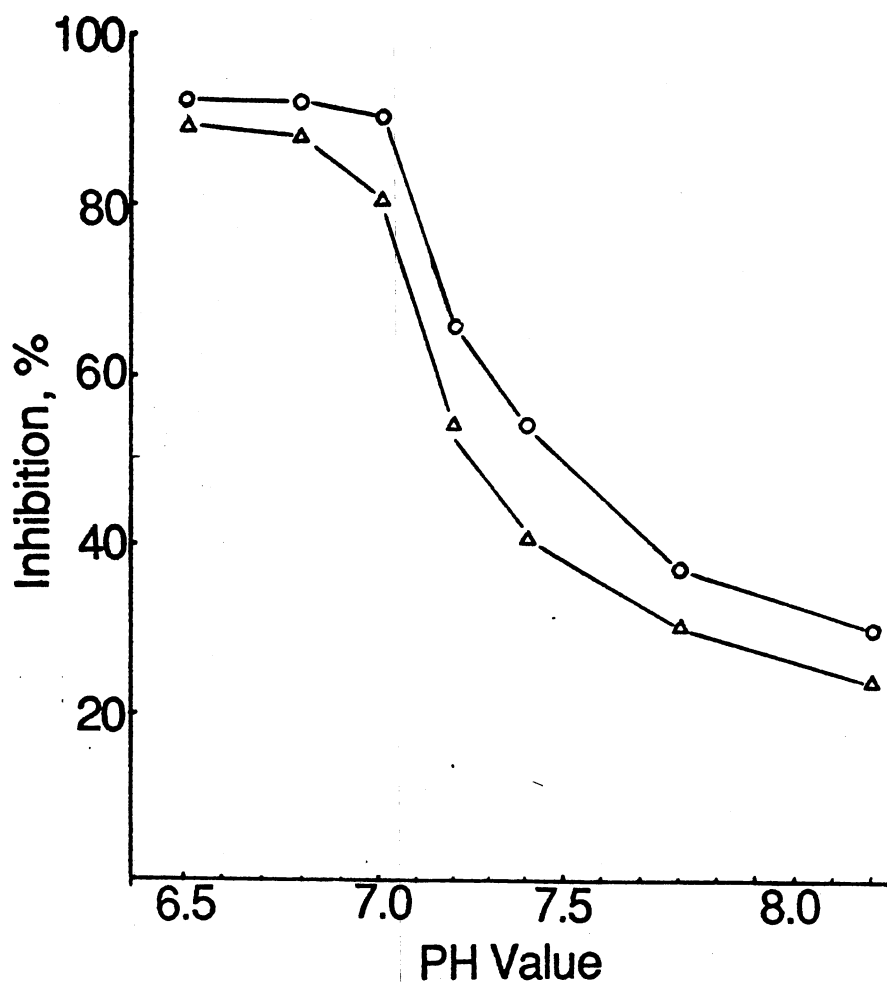
Fig. 18. Effect of pH on the Inhibitory Efficacy of UHDBT and 6-Br-
UDBT. 0.2 mlaliquots of ubiquinol-cytochrome c reductase, 2
mg/ml (20 μM of cytochrome b), in 50 mM phosphate buffer
containing 10% glycerol and 0.5% sodium cholate at indicated
pH's were added UHDBT (-o-) or 6-Br-UDBT (-Δ-). The
molar ratio of inhibitor to cytochrome b used was 10:1. The
mixture wasincubated at 0 °C for 20 min. before the enzymatic
activity was assayed. ThepH of the assay mixture used
corresponds to that of the incubating solution.

complex, such as a histidyl residue, or is affected by a dissociable group with a pk_a of 6.5 located close enough to induce the change of the inhibitor binding site or domain.

Effect of 4,7-Dioxobenzothiazole Derivatives on the EPR
Characteristics of Iron Sulfur Protein

It was reported (36) that inhibition of ubiquinol-cytochrome c reductase by UHDBT was accompanied by a down-field shift of the g_z (2.02 - 2.03) and up-field shift of the g_x (1.80 - 1.76) of Rieske's iron sulfur protein. The g's of iron sulfur protein in ubiquinol-cytochrome c reductase treated with these 4,7-dioxobenzothiazole derivatives are summarized in Table VI. Like UHDBT, inhibition by 4,7-dioxobenzothiazole derivatives is accompanied by an up-field shift of the g_x of iron sulfur protein in the reductase. The correlation between inhibition and the effect on the g_x of iron sulfur protein makes these derivatives, especially those bearing azido groups, good tools for studying the UHDBT action (binding) site.

Interaction between Azido-Derivatives and
Ubiquinol-Cytochrome c Reductase

From Table V it is clear that among the three azido derivatives, 2-azido-DHDBT has the highest inhibitory potency (90%) on ubiquinol-cytochrome c reductase in the dark. However, when this sample is subjected to illumination at 0 °C for 10 min, there was no inhibition. There were at least two plausible explanations; (a) the formation of a covalent linkage between the reductase and 2-azido-DHDBT upon illumination alters the structure of the inhibitor such that it loses its inhibitory function; (b) the photolysis process causes molecular rearrangement of 2-azido-DHDBT to a tetrazole isomer (see Fig. 19) which does not inhibit. In fact when ubiquinol-cytochrome c reductase was incubated with 2-azido-DHDBT which has been stored at 4 °C overnight or with 2-azido-DHDBT which has been subjected to photolysis, no inhibition is observed, indicating that the conversion

TABLE VI

EFFECT OF 4,7-DIOXOBENZOTHIAZOLE DERIVATIVES ON ENZYMATIC ACTIVITY AND EPR CHARACTERISTIC OF UBIQUINOL-CYTOCHROME c REDUCTASE

Derivatives Added	Concentration Used XUDBT/cyt. b	Inhibition, % intact	(delipiated)*	EPR Signal of ISP g_z	g_y	g_x
None	0	0	(0)	2.018	1.890	1.801
UHDBT	10	90	(95)	2.025	1.890	1.775
6-Br-UDBT	10	82	(90)	2.025	1.944	1.775
6-N$_3$-UDBT	10	40	(80)	2.020	1.890	1.765

* The activity was determined after the XUDBT derivative-treated delipidated ubiquinol-cytochrome c reductase was reconstituted with phospholipid (0.5 mg micelle asolectin per mg protein) and incubated at 0 °C for 20 min.
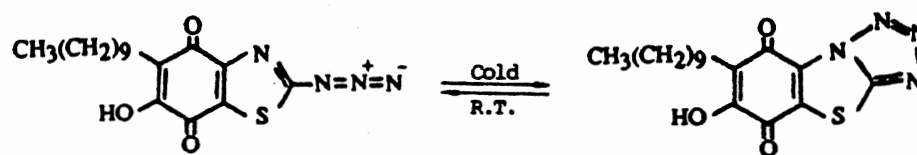
Stability	Stable at RT	Stable at Cold
Photosensitivity	+	-
Inhibitory Effect	+	-
UV Absorption (nm)	312, 260	295, 284

Fig. 19. Azido-Tetrazole Tautomerism of 2-Azido-DHDB

of 2-azido-DHDBT to a tetrazole isomer occurs in the cold or upon photolysis. Therefore, 2-azido-DHDBT and 2-azido-UHDBT are not suitable compounds for probing the UHDBT binding site by photoaffinity labeling.

6-Azido-UDBT is the least potent inhibitor of ubiquinol-cytochrome c reductase; it only inhibits 40% of the activity when incubated with intact ubiquinol-cytochrome c reductase in the dark. Illumination of the 6-azido-UDBT-treated sample does not alter the extent of inhibiton. Surprisingly, when a PL- and Q-depleted ubiquinol cytochrome c reductase (about 90% of PL and 95% of Q are removed) is treated with a 10-fold excess of 6-azido-UDBT per mole cytochrome b followed by reconstitution with Q and phospholipid, about 80% of the reconstituted activity is inhibited (see Table V), indicating that the PL in the reductase hinders the binding of 6-azido-UDBT to its binding site. Fig. 20 shows the titration of the Q- and PL-depleted ubiquinol-cytochrome c reductase with varying concentrations of 6-azido-UDBT and UHDBT. The ubiquinol-cytochrome c reductase activity is measured after the treated sample is reconstituted with asolectin. The inhibition behavior of 6-azido-UDBT is similar to that of UHDBT. A 50% inhibition is observed when two moles of 6-azido-UDBT per mole of cytochrome b are used. This is only a slightly higher ratio than what UHDBT needs to reach the same level of inhibition. 6-Azido-UDBT is thus suitable for photoaffinity labeling studies.

Correlation between 6-Azido-UDBT Uptake by Phospho-
lipid and Inhibition of Enzymatic Activity

Fig. 21 shows the concentration-dependent incorporation of radioactive [3H]-6-azido-UDBT into ubiquinol-cytochrome c reductase phospholipid and inhibition of 6-azido-UDBT-treated ubiquinol-cytochrome c reductase. When intact or PL- and Q-depleted ubiquinol-cytochrome c reductase is treated with increasing concentrations of [3H]-6-azido-UDBT and subjected to illumination for 10 min at 0°C, the ubiquinol-cytochrome c reductase activity decrease correlates with the increase in [3H]-6-azido-
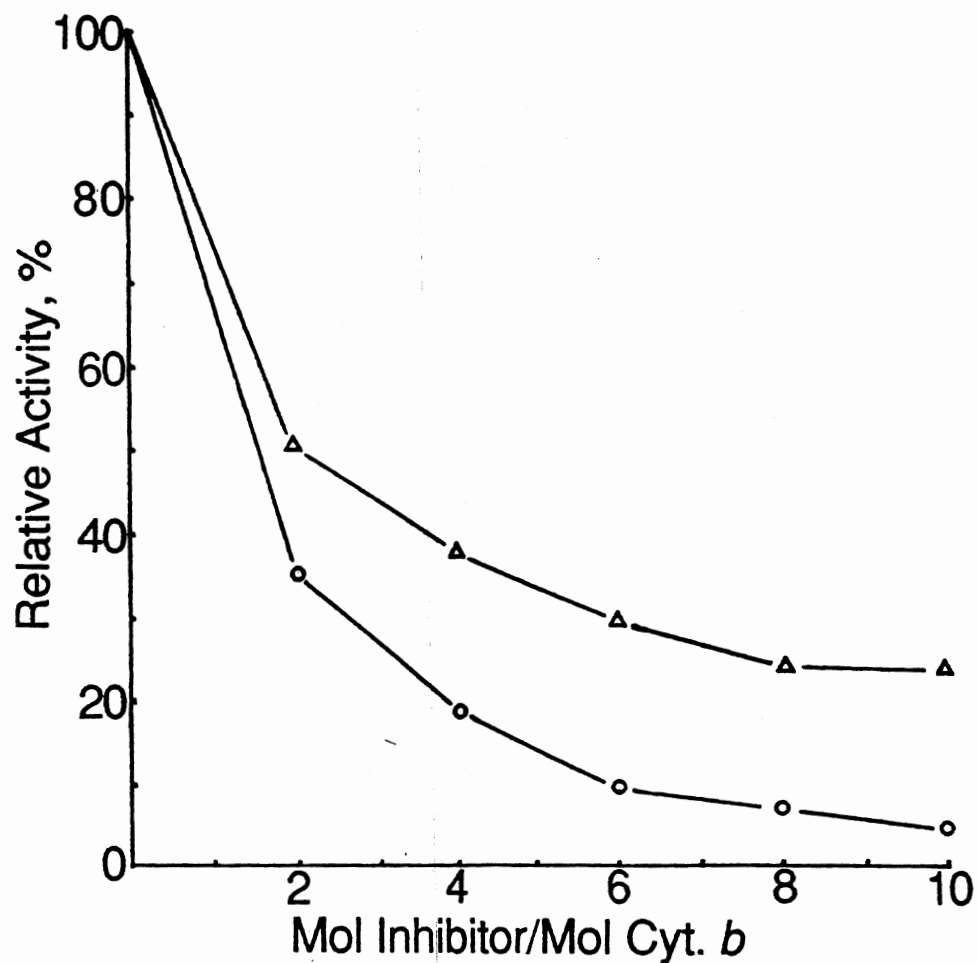
Fig. 20. Concentration-dependent Inhibition of Ubiquinol-cytochrome
c Reductase by UHDBT and 6-azido-UDBT. 0.2 ml
aliquots of the Q- and PL-depleted ubiquinol-cytochrome c
reductase, 1 mg/ml (10 μM cytochrome b), in 50 mM
Na/K phosphate buffer, pH 7.4, containing 10% glycerol
were added 10 μl of 95% ethanol containing indicated
concentration of UHDBT (-o-) or 6-azido-UDBT (-Δ-).
After incubation at 0 °C for 10 min., 10 μM of
micelleasolectin (10 mg/ml) were added. Ubiquinol-
cytochrome c reductase activity was assayed after the
mixture was incubated for 20 min. at 0 °C. The assay
mixture contains 50 μM Q_2H_2.
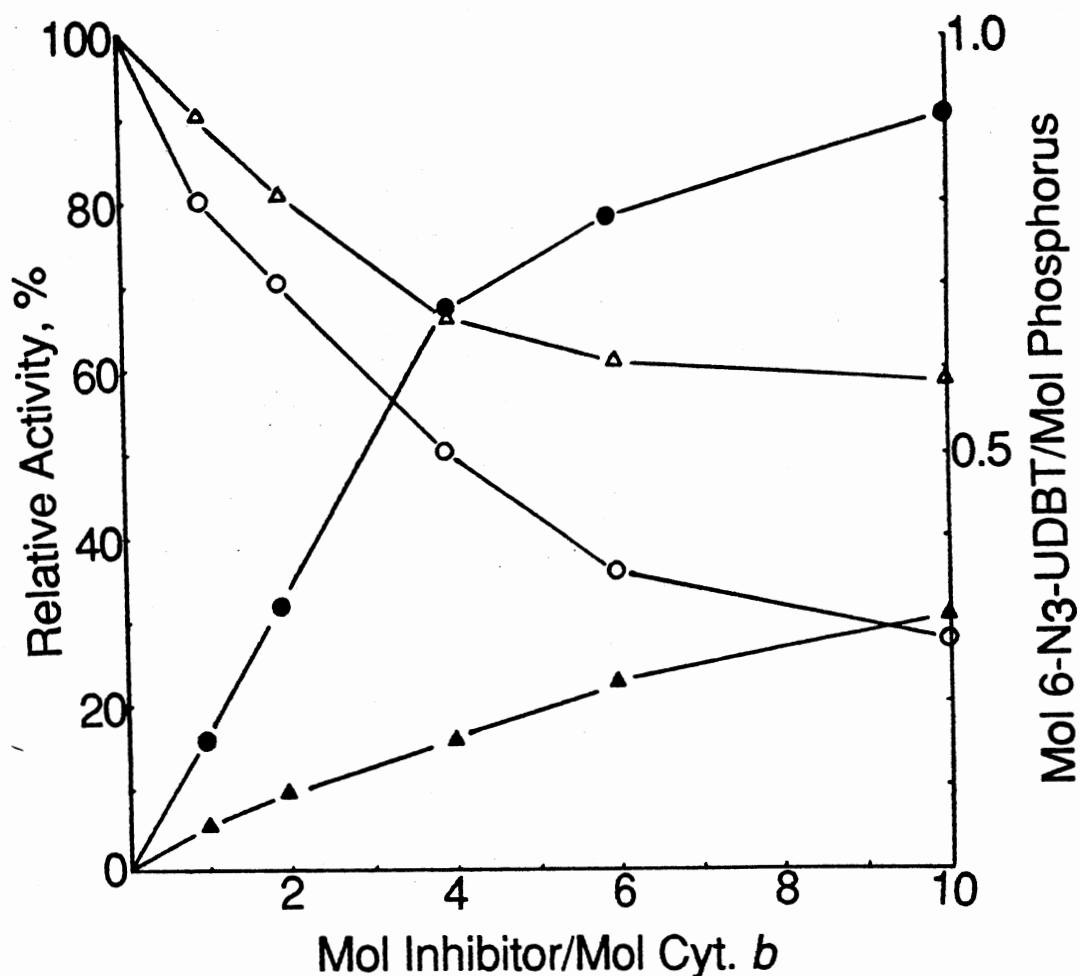
Fig. 21. Correlation between 6-Azido-UDBT Incorporation and Activity Inhibition. To 0.2 ml aliquots of intact (Δ,$\blacktriangle$) and delipidated (O,$\bullet$) ubiquinol-cytochrome c reductase, 2 mg/ml, were added 10 μl of 95% ethanolic solution containing indicated concentration of [^{3}H]-azido-UDBT. After incubation at 0 °C for 20 min, they were illuminated with a long UV light for 10 min at 0 °C. Twenty-μl aliquots were withdrawn from the azido-UDBT treated intact reductase samples, diluted with 400 μl of 50 mM phosphate buffer, pH 7.4, containing 0.5% sodium cholate, and assayed for ubiquinol-cytochrome c reductase activity (Δ); 20 μl aliquots were withdrawn from the azido-UDBT treated delipidated reductase samples, reconstituted with 2 μl of micelle asolectin (10 mg/ml) and assayed the activity (-O-) after incubation at 0 °C for 20 min. The remaining solutions were extracted three times with 1 ml of chloroform: methanol (2:1), and spotted on a TLC plate. The plate was developed with ether to remove the free form, and detergent adduct of photolyzed [^{3}H]-azido-UDBT. After developed with ether, the spot at the original place was scrapped from the plate, eluted with chloroform:methanol (2:1), and phosphorus and radioactivity determined. Curves with solid triangles (-$\blacktriangle$-) and solid circle (-$\bullet$-) represent mole of 6-azido-UDBT uptake per mole phosphorus in the intact and delipidated reductase, respectively.

UDBT uptake by phospholipid. No radioactivity is found in the protein of ubiquinol-cytochrome c reductase. It should be noted that when PL-and Q-depleted reductase is used, the activity is measured after the azido-UDBT treated sample is reconstituted with phospholipid and Q. 6-Azido-UDBT gives greater inhibition and radioactivity incorporation per mole of phosphorus when used with PL- and Q-depleted reductase than with intact reductase. This suggests that UHDBT is bound to phospholipid(s) that are tightly bound to the ubiquinol-cytochrome c reductase protein. The PL-and Q-depleted preparation still contains 10% of the phospholipid, the majority of which was reported to be cardiolipin (117).

Binding of 6-Azido-UDBT to Cardiolipin of
Ubiquinol-Cytochrome c Reductase

Fig. 22 shows the radioactivity distribution in the HPLC chromatogram of phospholipid obtained from photolyzed, [^{3}H]-6-azido-UDBT-treated ubiquinol-cytochrome c reductase. When analyzed by HPLC using a Micropak Si 5 column eluted with a gradient formed from hexane-isopropanol (97 :3) and hexane-isopropanol (60:40), most of the radioactivity is found in fraction 46. The peak height as well as that radioactivity in fraction 46 increases as the percent inhibition by [^{3}H]-6-azido-UDBT increases (data not shown), indicating that the UHDBT binding site is located in this fraction.

The fraction containing radioactivity was shown to be a phospholipid-UDBT adduct by identification of phosphorus and fatty acid. When the fraction was methyl esterified and subjected to HPLC analysis under the same conditions, the peak was split and eluted earlier. The head group of this phospholipid fraction was identified as diglycerophosphorylglycerol. 6-Azido-UDBT was linked to the acyl group and not to the head group of the cardiolipin molecule because when the radioactivity-containing phospholipid fraction was subjected to methyl esterification and then partitioned with
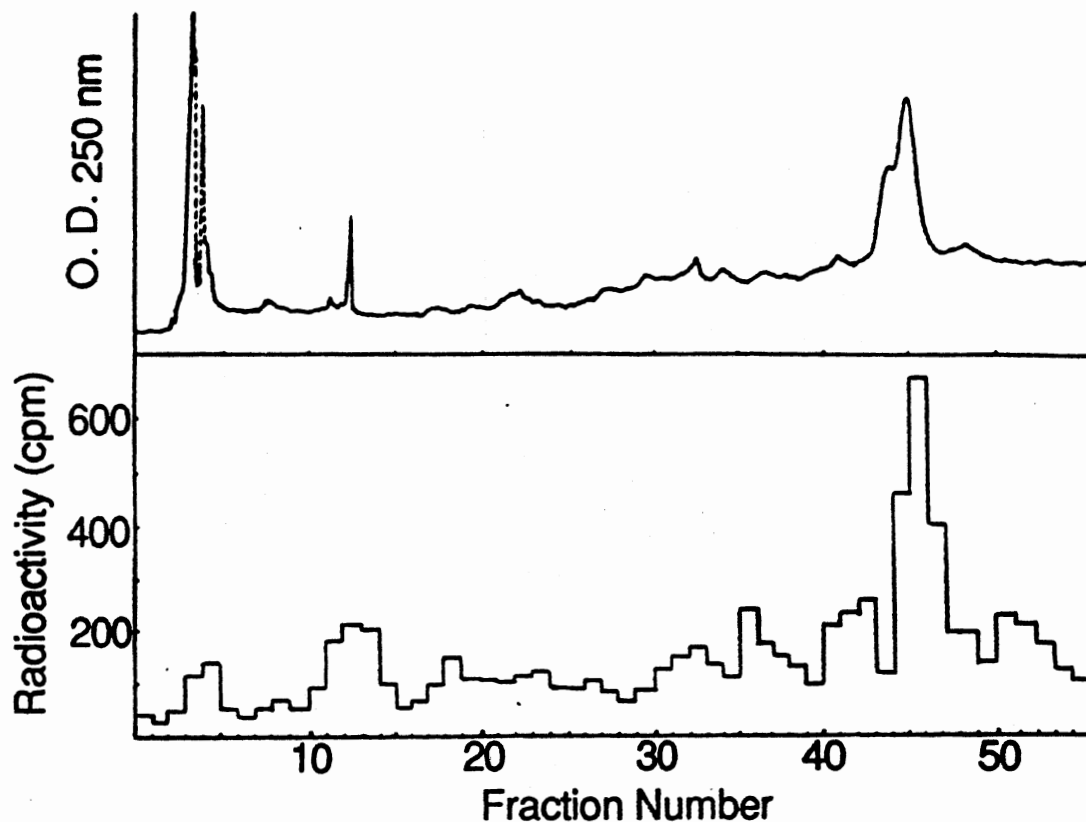
Fig. 22. [3H]-radioactivity Distribution on the HPLC Chromatogram of Ubiquinol-
Cytochrome c reductase Phoapholipids. Ubiquinol-cytochrome c reductase, 1
mg/ml (10 μM cytochrome b), in 50 mM K/K phoaphate buffer, pH 7.4,
containing 10% glycerol and 0.5% sodium cholate, was treated with [3H]-
azido-UDBT in 95% ethanol at 2 moles inhibitor per mole cytochrome b.
After incubation for 20 min at 0 °C, the [3H]-azido-UDBT treated sample was
subjected to illumination with long UV light for 10 min at 0 °C. The
illuminated sample was extracted twice with chloroform:methanol (2:1). The
extract was dried, applied to a TLC plate developed with ether to remove non
phospholipid materials. The phospholipid was collected, dried, dissolved in
chloroform:methanol (2:1), and applied to a Micropak Si-5 HPLC column.
The column was eluted with a linear gradient formed from hexane-isopropanol
(97:3) and hexane-isopropanol (60:40) at a flow rate of 1 ml/min. One-ml
fractions were collected and radioactivity determined.

ether, the radioactivity was found in the ether layer. The purified [^{3}H]-azido-UDBT linked cardiolipin fraction had an azido-UDBT to phosphorus ratio of 1:2, indicating that only one of the four fatty acyl groups was linked to 6-azido-UDBT since one diphosphatidyl glycerol molecule contains four fatty acyl groups and two phosphates. Identification of the fatty acyl group which binds to 6-azido-UDBT has been hindered by the complexity of the photochemistry of the azido compound. When the fatty acyl ester, prepared from [^{3}H]-azido-UDBT-linked cardiolipin, was analyzed by gas chromatography, a rather complicated chromatogram was observed. Two peaks with retention times corresponding to normal C16 and C18 methyl esters were detected. Apparently these are not covalently linked with [^{3}H]-6-azido-UDBT, but they were fatty acyl groups of cardiolipin, because the methyl ester of a azido-UDBT-linked fatty acyl group would be expected to have different chromatographic behavior.

Isolated, fully active mitochondrial ubiquinol-cytochrome c reductase contains about 0.2 mg phospholipid per mg protein. About 90% of the phospholipid and 95% of the Q_{10} present in the preparation can be removed by repeated ammonium sulfate fractionation in the presence of cholate. The resulting delipidated preparation shows no ubiquinol-cytochrome c reductase activity. Addition of phospholipid (asolectin) to the delipidated preparation completely restores the enzymatic activity, indicating that phospholipids which are removed by this delipidation procedure are important, but not structurally indispensable, since they can be replaced. Removal of the residual phospholipid from the deplipidated preparation requires more drastic conditions and results in an irreversible denaturation, indicating that phospholipids present in reconstitutively active, delipidated reductase are in a specific environment and structurally indispensable. The detection of 6-azido-UDBT-linked cardiolipin in PL-and Q-depleted reductase and the fact that UHDBT inhibits ubiquinol-cytochrome c reductase activity of delipidated reductase assayed after reconstitution with Q and PL indicate that the UHDBT-perturbed cardiolipin molecule is structurally indispensable to reductase and is tightly

associated with the reductase protein. The involvement of phospholipid in antimycin binding to yeast ubiquinol-cytochrome c reductase has been reported (118).

Although at present the identity of the protein subunit(s) of ubiquinol-cytochrome c reductase which is associated with this specific cardiolipin is not known, it is reasonable to assume that such a protein subunit(s) requires phospholipid for structural integrity. Among the components of reductase, cytochrome b is the most phospholipid requiring protein. It needs phospholipid for both functional activity and structural integrity. When intact ubiquinol-cytochrome c reductase phospholipid is mostly removed by the repeated cholate-ammonium sulfate fractionation, the spectral and redox properties of cytochrome b (though not cytochrome c_1 or Reiske's iron sulfur protein) are seriously altered. These altered spectral and redox properties are restored upon replenishment with phospholipid. Complete removal of phospholipid from the delipidated preparation causes the irreversible denaturation of cytochrome b. So far, all the isolated cytochrome b preparations contain no phospholipid and are functionally inactive. On the other hand, isolated cytochrome c_1 (119) or iron sulfur protein (120), contains no phospholipid but does maintain the same redox properties, indicating that these two proteins do not depend on phospholipid to maintain their structure. This, however, does not rule out the functional requirement of phospholipid for these proteins. These facts, together with the structural similarity between UHDBT and quinone, suggest that this specific cardiolipin is associated with the Q-binding proteins (b cytochrome and/or a $Mr = 9, 507$ dalton subunit 2) of ubiquinol-cytochrome c reductase and may represent part of the Q-binding site or domain. Perturbation of this cardiolipin molecule by binding to UHDBT alters the Q binding domain, causing the inhibition of ubiquinol-cytochrome c reductase. In this case, the observed alteration of the EPR characteristics of iron sulfur protein in ubiquinol-cytochrome c reductase treated with UHDBT may result from a secondary effect exerted by a third component whose structure depends on the presence of phospholipid. This possibility is further supported by the results of EPR studies of iron sulfur protein in PL

sufficient - and deficient-ubiquinol-cytochrome c reductases treated with UHDBT. As shown in Fig. 23, the EPR spectrum of iron sulfur protein in UHDBT-treated, PL-sufficient ubiquinol-cytochrome c reductase resembles the EPR spectrum of iron sulfur protein in the delipidated reductase. The addition of UHDBT to delipidated reductase does not cause further alteration of the EPR spectrum of iron-sulfur protein. This phospholipid-dependent UHDBT effect is similar to that observed with 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone (DBMIB) except that the effect of DBMIB on EPR characteristics of iron sulfur protein in PL-sufficient reductase is more profound (80). The effect of DBMIB is believed to exert through a PL-dependent component, such as b cytochrome or the Q-binding proteins. The UHDBT effect probably results from the change of phospholipid environment of the Q-binding proteins (cytochrome b and/or the Mr= 9.5 Kd subunit) through the binding of UHDBT to its action site (cardiolipin), indirectly causing a conformational change in a nonphospholipid dependent component (iron sulfur protein) and showing a discernible change in the EPR characteristics of the iron sulfur cluster.

Since UHDBT inhibition is accompanied by a change in the EPR characteristics of iron sulfur protein, it is also possible that this specific cardiolipin is associated with the iron sulfur protein of ubiquinol-cytochrome c reductase. This possibility is, however, less likely because isolated, reconstitutively active iron sulfur protein (120, 27) does not require phospholipid for structural integrity and has EPR characteristics (80, 27) similar to those of the iron-sulfur protein in delipidated ubiquinol-cytochrome c reductase. No EPR spectrum alternation is observed upon treatment of isolated iron sulfur protein with UHDBT, regardless of the additions of phospholipid made before or after the treatment.
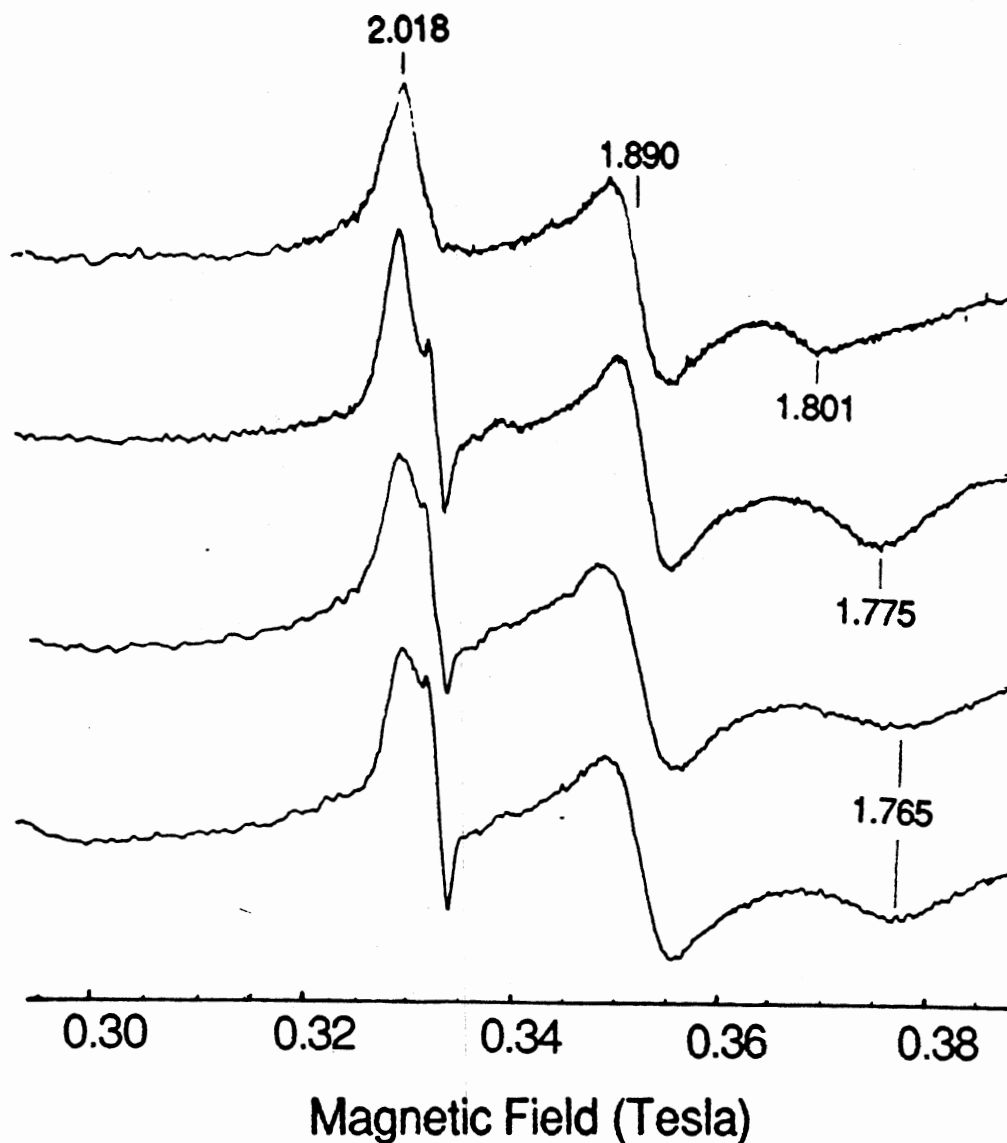
Fig 23. Effect of UHDBT on the EPR spectra of Iron-Sulfur Protein of Intact and Delipidated Ubiquinol-Cytochrome c Reductase. 0.3 ml aliquots of ascorbate reduced ubiquinol-cytochrome c reductase, 20 mg/ml, in 50 mM Tris-acetate buffer, pH 7.8, containing 10% glycerol were treated with 10 μl of 95% ethanol containing (a) none, (b) 10 fold molar excess of UHDBT. Spectra (c) and (d) are the same as (a) and (b) except delipidated reductase was used. The EPR instrument settings were: modulation frequency, 100 KHz, modulation amplitude, 6.3 gauss; microwave frequency, 9.38 gHz; microwave power, 20 mW; scan rate, 5 gauss/sec. Spectra were recorded at 80 K.

CHAPTER IV

SYNTHESIS AND BIOLOGICAL PROPERTIES OF FLUORO SUBSTITUTED UBIQUINONE DERIVATIVES

The relevent abundance of ubiquinone compared with other redox components in the inner mitochomdrial membrane has indicated that ubiquinone may function as a homogeneous mobile carrier which shuttles electrons between the complexes. Most of the studies concening the function of ubiquinone have concentrated on a dynamic or kinetic approach (125-130), which support the idea of the pool function of ubiquinone. Success in the identification of the Q-binding proteins in mitochondrial ubiquinol-cytochrome c reductase (61, chapter two and three) indicates that the reduction of ubiquinone or oxidation of ubiquinol in the electron transfer chain requires the participation of specific proteins to provide specific Q-binding sites. It seems that the existence of specific Q-binding sites conflicts with the idea of the pool function of ubiquinone. Although it has been proposed that the bound quinone may be in equilibrium with the pool by very fast association-dissociation, there is some uncertainty over how ubiquinone functions as both a mobile pool and protein-bound prosthetic group. To reconcile these two aspects of the functions of ubiquinone, a new approach to the process of Q-binding to the mitochondrial ubiquinol-cytochrome c reductase by certain spectroscopically labeled ubiquinone derivatives is obviously needed.

Recently, the introduction of the ^{19}F NMR techniques in biochemistry has opened a significant new approaches for highly sensitive investigations of the molecular order and dynamics in model and biological membranes (131), lipid-lipid and protein-lipid

83

interactions (132-134), substrate-enzyme binding (24, 135) and enzyme-enzyme interaction (136). The change of relaxation time T_1 and line broadening can give some information about the ordering of molecules and the interaction between the substrate and enzyme. The use of fluorine as a spectroscopic label has several advantages. First, the sensitivity of ^{19}F NMR is relatively high, 83% of that of ^1H NMR which can meet the requirement of dilute conditions. Second, since ^{19}F is not normally present in biological systems, the spectra are generally simple. Third, the chemical shifts of ^{19}F NMR are large and are much more sensitive to chemical environments which makes it very suitable for use as a probe.

As a qualified biological probe of Q-binding, the labeled analogues of ubiquinone must possess properties that the ubiquinone has, or that they must be competitive inhibitors of ubiquinol-cytochrome c reductase for Q-binding. In order to directly examine the property of Q-binding in ubiquinol-cytochrome c reductase by ^{19}F NMR spectroscopy, three fluoro-ubiquinone derivatives, 2,3-dimethoxy-6-(9'-fluorodecyl)-5-methyl-1,4-benzoquinone (9'FQ), 6-decyl-2-methoxy-5-trifluoromethyl-1,4-benzoquinone (TFQ) and 6-(9'-fluorodecyl)-2-methoxy-5-trifluiromethyl-1,4-benzoquinone (9'FTFQ), have been synthesized and their biological properties have been studied.

<div align="center">Experimental Procedures</div>

Materials

α,α,α-Trifluoro-p-cresol were from Aldrich; cytochrome c from Sigma; silica G thin layer plates from Analtech; 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone (Q_2) and its reduced form (Q_2H_2) were synthesized as previously reported (106).

Enzyme Preparations

Succinate-ubiquinone reductase (107), succinate-cytochrome c reductase (107),

cytochrome c reductase (107) and the ubiquinone-, phospholipid(PL)-depleted succinate-cytochrome c reductase (137), ubiqunol-cytochrome c reductase (107) were prepared as previously reported.

Spectral Measurements

The absorption spectra were measured in a Cary spectrometer, model 219, or DW 2000 spectrophotometer. Both ^{1}H NMR and ^{19}FNMR spectra were measured with a Varian XL-300 NMR spectrometer. The instrument settings for ^{19}F NMR measurement were: spectral width, 39840 Hz; 77° pulse; delay time, 0.65 s; acquisition time, 0.35s. A standard 2 pulse sequence (S2PUL) was used and the data was gathered with a 16K data set. The molecular weight was determined with a TS-250 mass spectrometer.

Synthesis of 2,3-Dimethoxy-6-(9'-fluorodecyl)-5-methyl-1,4-benzoquinone

The total synthesis pathway of 2,3-dimethoxy-6-(9'-fluorodecyl)-1,4-benzoquinone is shown in scheme 4. Since 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q_0) is commercially available, the synthesis simply became the preparation of bis(10-fluoroundecanoyl)peroxide. To synthesize the diacyl peroxide, it was started with 10-undecenoic acid, which underwent hydrogen fluorination of the double bound, conversion of the fluoroundecanoic acid to the acyl chloride and the oxidation of acyl chloride to diacyl peroxide. The procedure for alkylation of Q_0 is a standard method in the preparation of alkylated Q derivatives.

Synthesis of 10-Fluoroundecanoic acid (142). In a 200 ml Teflon container was placed 100 ml of 70% hydrogen fluoride/pyridine solution. To this solution was added 18.5 g of 10-undecylenic acid at 0 °C. The reaction mixture was stirred for 50 minutes at

SCHEME 4

THE SYNTHESIS OF 2,3-DIMETHOXY-6-(9'-FLUORODECYL)-5-METHYL-1,4-BENZOQUINONE
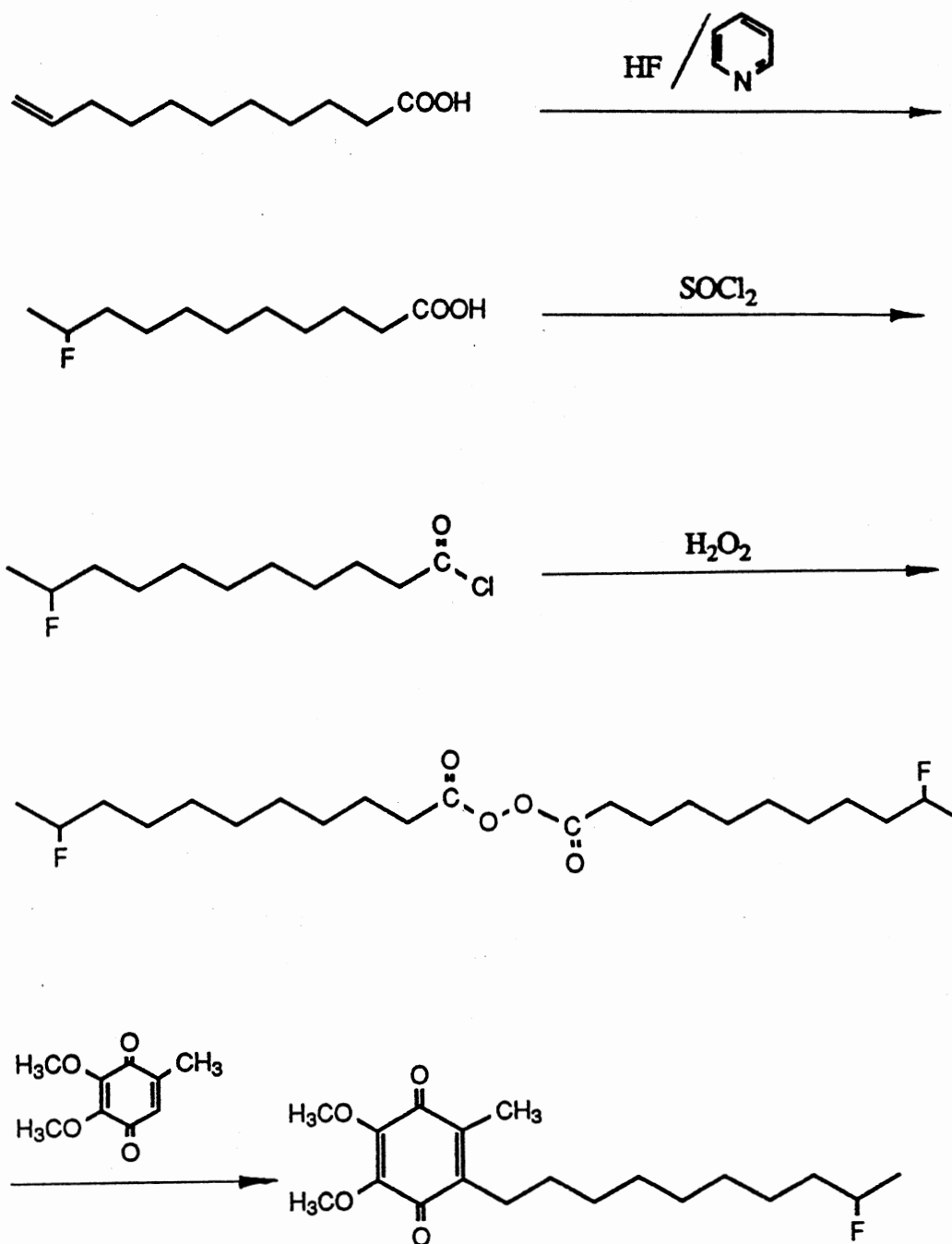
the same temperature, then it was diluted with 200 ml of ice-water and extracted with chloroform. The extract was washed with water and dried over anhydrous sodium sulfate. After the solvent was removed, a pale yellow oil was obtained. The crude product was then purified by two crystallizations with petroleum ether (30-60 °C). 10-Fluoroundecanoic acid (12 g) was obtained. ^{1}H NMR (CDCl$_3$), 0.95 (q,3H), 1.33 (m,8H), 1.45 (m,2H), 1.62 (m,4H), 2.34 (t,2H), 4.64 (m,1H).

Synthesis of Bis(10-fluoroundecanoyl)peroxide.   10-Fluoroundecanoic acid (10 g)was mixed with 20 ml of thionyl chloride and the mixture was refluxed for 1.5 hours. The excess thionyl chloride was then removed by vacuum evaporation. A yellow oil was obtained and transferred to a 250 ml three necked flask equipped with stirrer and thermometer. 150 ml of ether was added to dissolve the oil. The solution was cooled to 0 °C and 15 ml of hydrogen peroxide (65%) was added dropwise at 0 °C with stirring. To this mixture was added 10 ml of pyridine in 2 hours at the same temperature with vigorous stirring. The mixture was stirred another hour at room temperature. The product was extracted with ether. The extract was washed in sequence with water, 1N hydrochloric acid, 5% sodium bicarbonate, water, and dried over anhydrous sodium sulfate. After the ether was removed, 7 g of pale yellow oil was obtained. ^{1}H NMR (CDCl$_3$), 0.91 (q,6H), 1.25 (m,20H), 1.65 (m,8H), 2.39 (t,4H), 4.65 (m,2H).

Synthesis of 2,3-Dimethoxy-6-(9'-fluorodecyl)-5-methyl-1,4-benzoquinone. Q$_0$ (40 mg) was dissolved in 2 ml of benzene in a 10 ml Enrlymeyer flask. To this solution was added 120 mg of bis(10-fluoroundecanoyl) peroxide and refluxed for 3 hours. The product was directly separated by TLC developed with a mixed solvent of hexane and ether (3.5:1). 2,3-Dimethoxy-6-(9'-fluorodecyl)-5-methyl-1,4-benzoquinone (10 mg, R$_f$=0.7) was obtained. It shows UV ^{EtOH} oxid. 276 nm, red. 288 nm; MW.340; ^{1}H NMR (CDCl$_3$), 0.95 (q,3H), 1.31 (m,12H), 1.59 (m,2H), 1.98 (s,3H), 2.45 (t,2H), 3.97 (s,6H), 4.63 (m,1H) and ^{19}F NMR (CDCl$_3$), -99.2 ppm (m) (Ref. TFA).

## Synthesis of 6-Decyl-2-methoxy-5-trifluoromethyl-1,4-benzoquinone

The synthesis of 6-decyl-2-methoxy-5-trifluoromethyl-1,4-benzoquinone was started with commercially available 2,2,2-tifluoro-p-cresol, and the total pathway of the synthesis is shown in scheme 5.

Synthesis of p-Trifluoromethylanisole (138). 2,2,2-Trifluoro-p-cresol (20 g) was dissolved in 200 ml of acetone at room temperature in a 500 ml Erlenmeyer flask. Anhydrous potassium carbonate (18 g) was added into this solution followed by the addition of 28 g (14 ml) of iodomethane with stirring, and the reaction mixture was heated with a hot water bath and allowed to reflux for 6 hours. Following the acetone removal by distillation, the residue was mixed with water and extracted with ether, washed with water and dried over anhydrous magnesium sulfate. The ether was removed by vacuum evaporation and p-trifluoromethylanisole (17.3 g) was obtained by vacuum distillation as a colorless liquid (80°C at 30 mm). Yield, 80%; ^{1}H NMR (CDCl$_3$), 3.84 (s,3H), 6.96 (d,2H), 7.54 (d,2H)

Synthesis of 2-Nitro-4-trifluoromethylanisole. p-Trifluoromethylanisole (17.3 g) was dissolved in 36 ml of carbon tetrachloride in a 250 ml three necked flask equipped with a stirrer and thermometer. The solution was cooled to 5°C and 36 ml of 90% nitric acid was added dropwise at 5 c in 30 minutes. After stirred at 10-15°C for 1 hour, the mixture was then poured onto 400 g of ice and extracted with chloroform. The extract was washed with water and dried over anhydrous magnesium sulfate. After the solvent was removed by vacuum evaporation, the residue (16.5 g) was crystalized from 30-60 c petroleum ether (200 ml). A yellow flat crystal of 2-nitro-4-trifluoromethylanisole (15.4 g) was obtained. Yield, 71%; ^{1}H NMR (CDCl$_3$), 4.04 (s,3H), 7.21 (d, 1H), 7.80 (q,1H), 8.13 (d,1H)

SCHEME 5

THE SYNTHESIS OF 6-DECYL-2-METHOXY-5-
TRIFLUOROMETHYL-1,4-BENZOQUINONE
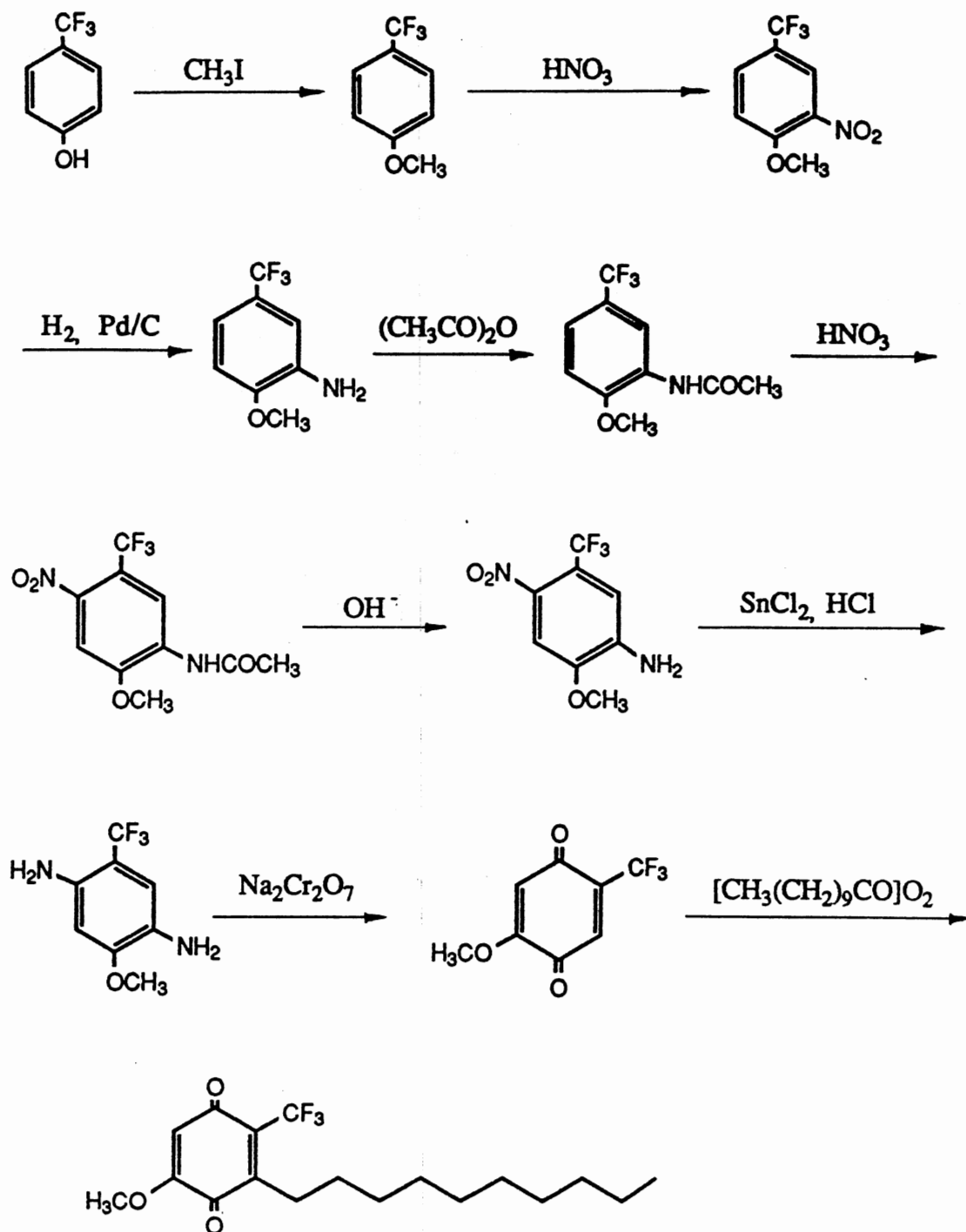
Synthesis of 2-Amino-4-trifluoromethylanisole. 2-Nitro-4-trifluoromethylanisole (5 g) was dissolved in 70 ml of CH_3OH-HCl in 500 ml hydrogenation bottle. 0.5 g of Pd/C catalyst was suspended in the solution. After the oxygen in the reactor was removed, the hydrogenation was started. The reaction was completed when no hydrogen absorption was observed. The methanol was removed by vacuum evaporation. The residue was neutralized with 1 N of sodium hydroxide and extracted with ether. The extract was washed with water and dried over anhydrous magnesium sulfate. After the ether was removed, the residue was crystalized from 80 ml of 30-60°C petroleum ether. 3.5 g of pale yellow flat crystalline 2-amino-4-trifluoromethylanisole was obtained. Yield, 81%; ^{1}H NMR ($CDCl_3$), 3.89 (s,3H), 3.93 (s,2H), 6.82 (d,1H), 6.92 (d,1H), 7.00 (q,1H)

Synthesis of 2-Acetylamido-4-trifluoromethylanisole. 2-Amino-4-trifluoromethylanisole (2 g) was dissolved in 6 ml of acetic anhydride in a 25 ml Erlenmeyer flask. The reaction mixture was refluxed for 1 hour and was mixed with 50 ml of water, extracted with ether. The extract was washed two times with water, 1% of sodium bicarbonate and water subsequently and dried over anhydrous magnesium sulfate. After the ether was removed, 2.3 g of white crystalline 2-acetylamido-4-trifluoromethylanisole was obtained. Yield, 96%; ^{1}H NMR ($CDCl_3$), 2.28 (s,3H), 3.89 (s,3H), 7.09 (d,1H), 7.39 (d,1H), 7.67 (q, 1H), 7.80 (s,1H).

Synthesis of 2-Acetylamido-5-nitro-4-trifluoromethylanisole. 2-Acetylamido-4-trifluoromethylanisole (2.3 g) was dissolved in 75 ml of carbon tetrachloride in a 250 ml three necked flask equipped with stirrer and thermometer. The solution was cooled to 5°C and to it was added 8 ml of nitric acid (95%) dropwise at such a rate to maintain the temperature at 5°C. Then the mixture was stirred 1 hour at 10°c. The aqueous layer was separated and poured onto 200 g of ice. The yellow precipitate was filtered out, washed with water and dried at room temperature. The crude product was suspended in 10 ml of

hot carbon tetrachloride, the insoluble solid was filtered out and washed three times with hot carbon tetrachloride. A white powder of 2-acetylamido-5-nitro-4-trifluoromethylanisole (1.4 g) was obtained. Yield, 52%; ^{1}H NMR (CDCl$_3$), 2.27 (s,3H), 4.04 (s,3H), 7.47 (s,1H), 7.90 (s,1H), 8.97 (s,1H).

Synthesis of 2-Amino-5-nitro-4-trifluoromethylanisole. 2-Acetylamido-5-nitro-4-trifluoromethylanisole (1.4 g) was dissolved in 1.75 ml of Claison alkali (139) (prepared by dissolving 88 g of potassium hydroxide in 63 ml of water, cooling, and diluting to 250 ml with methanol). The reaction mixture was heated on a boiling water bath for 15 minutes. Then 1.75 ml of hot water was added to the mixture and heated for 5 minutes at the same temperature. The product was extracted with ether. The extract was washed with water and dried over anhydrous magnesium sulfate. The ether was removed by evaporation and 1 g of yellow solid product was obtained. Yield, 83%; ^{1}H NMR (CDCl$_3$), 3.98 (s,3H), 4.60 (s,2H), 6.99 (s,1H), 7.55 (s,1H).

Synthesis of 2,5-Diamino-4-trifluoromethylanisole. 2-Amino-5-nitro-4-trifluoromethylanisole (1 g) was dissolved in 10 ml of methanol. To this solution were added 10 ml of concentrated hydrochloric acid and 5 g of anhydrous tin(II) chloride subsequently. The mixture was refluxed for 10 minutes and then made to strongly basic with 20% sodium hydroxide. The product was extracted with ether. The extract was washed with water and dried over anhydrous magnesium sulfate. The ether was removed by vacuum evaporation and 0.8 g of pale yellow solid 2,5-diamino-4-trifluoromethylanisole was obtained. Yield, 92%; ^{1}H NMR (CDCl$_3$), 3.49 (s,2H), 3.79 (s,2H), 3.82 (s,3H), 6.20 (d,1H), 6.78 (d,1H).

Synthesis of 2-Methoxy-5-trifluoromethyl-1,4-benzoquinone (140). 2,5-Diamino-4-trifluoromethylanisole (0.4 g) was mixed with a solution of 2.5 ml of concentrated sulfuric acid in 7.8 ml of water in a 50 ml three necked flask equipped with

stirrer and thermometer. 7.8 ml of benzene was added into the flask. The two layer solution obtainedwas cooled to 8 °C and 0.7 g of sodium dichromate in 1.4 ml of water was added dropwise with vigorous stirring at such a rate as to maintain the temperature not higher than 10 °C. After the addition of sodium dichromate, the mixture was stirred for 2 hours at 10 °C. The product was extracted with ether. The extract was dried over anhydrous magnesium sulfate. After the ether was removed, a dark solid residue was obtained, which was subjected to a TLC separation developed with a mixed solvent of hexane and ether (1:1, V/V). A dark green solid (yellow in solution) of 2-methoxy-5-trifluoromethyl-1,4 benzoquinone (28 mg, R_f=0.3)was obtained. Yield, 7%; ^{1}H NMR (CDCl$_3$), 3.98 (s,3H), 6.05 (s,1H), 7.06 (m,1H); UV^{EtOH} oxid. 244 nm, red. 297 nm.

Synthesis of 6-Decyl-2-methoxy-5-trifluoromethyl-1,4-benzoquinone.

2-Methoxy-5-trifluoromethyl-1,4-benzoquinone (20 mg) was dissolved in 2 ml of benzene in a 10 ml Erlenmeyer flask. 20 mg of diundecanoyl peroxide was added to this solution. The reaction mixture was refluxed for 4 hours. The product (4 mg, R_f=0.5) was directly isolated with TLC developed with a mixed solvent of hexane and ether (1:1 V/V). It shows UV ^{EtOH} oxid. 264 nm, red. 296 nm; MW. 346; and ^{1}H NMR (CDCl$_3$), 0.88 (t, 3H), 1.26 (m,14H), 1.40 (m,2H), 2.72 (t, 2H), 3.84 (s,3H), 5.97 (s, 1H). For ^{19}F NMR study further purification with TLC was needed. The chemical shift of its ^{19}F NMR (CDCl$_3$) is +15.4 ppm (ref. TFA).

Synthesis of 6-(9'-Fluorodecyl)-2-methoxy-5-trifluoromethyl-1,4-benzoquinone

6-(9'-Fluorodecyl)-2-methoxy-5-trifluoromethyl-1,4-benzoquinone was prepared by the alkylation of 2-methoxy-5-trifluoromethyl-1,4-benzoquinone as shown in Scheme 6. Thus, 2-methoxy-5-trifluoromethyl-1,4-benzoquinone (40 mg) was dissolved in 2 ml of benzene. To this solution was added 80 mg of bis(10-fluoroundecanoyl)-peroxide. The

reaction mixture was refluxed for three hours. 6-(9'-Fluorodecyl)-2-methoxy-5-trifluoromethyl-1,4-benzoquinone(10 mg, R_f=0.6) was directly separated by TLC developed with a mixed solvent of hexane and ether (1:1). It shows UV ^{EtOH} oxid. 264 nm, red.296 nm; MW. 364; ^{1}H NMR, 0.95 (q,3H), 1.35 (m,12H), 1.57 (m,2H), 2.52 (t,2H), 3.84 (s,3H), 4.64 (m,1H), 5.96 (s.1H) and ^{19}F NMR (CDCl$_3$), +15.4 ppm (s), -99.2 ppm (m) (Ref. TFA).

Results and Discussion

Synthesis of Fluoroubiquinone Derivatives

The molecule of ubiquinone can be divided into two parts, the benzoquinone ring and the side chain at position 6. The benzoquinone ring is directly involved in the electron transfer reactions and the side chain plays an important role in its function (106). Photoaffinity labeling of ubiquinol-cytochrome c reductase indicated that two protein subunits (M_r 1700 and 37000) were heavily labeled when either azido-Q (Chapter II) or arylazido-Q (61) was used. Although the location of the photoactivable azido group in the arylazido-Q is more than 15 carbons away from the benzoquinone ring, it labeled the same protein subunits as did the azido-Q in which the azido group is located on the benzoquinone ring. To study the interaction between ubiquinone and ubiquinol-cytochrome c reductase, as well as the difference of the functions of ubiquinone ring and the side chain in the interaction with ubiquinol-cytochrome c reductase, two kind of fluoro-Q derivatives, one with the fluoro group on the ubiquinone ring and another with the fluoro group on the side chain, are needed.

In the synthesis of 9'FQ, most of the work was the preparation of bis(10-fluoroundecanoyl)peroxide and the yield was relatively high. However, in the reaction of the conversion of 10-fluoroundecanoic acid to the acid chloride careful control of the reaction temperature is essential. When the reaction temperature was higher than 100 °C,

SCHEME 6

THE SYNTHESIS OF 6-(9'-FLUORODECYL)-2-METHOXY-5-TRIFLUOROMETHYL-1,4-BENZOQUINONE
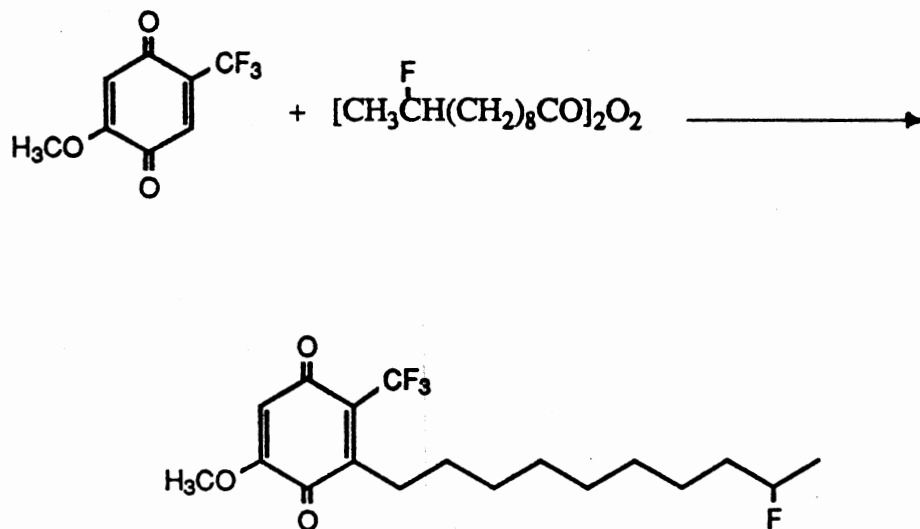
elemination of hydrofluoride was observed. In contrast, if the temperature is too low, the acid could not be completely converted to acid chloride . The unreacted acid could be oxidized to peracid by hydrogen peroxide in the next reaction step, which would destroy Q_0 in the reaction of the alkylation of Q_0. Therefore, using a gentlly boiling water bath for the chlorination is recommended.

Most of the reactions of the preparation of 2-methoxy-5-trifluoromethyl-1,4-benzoquinone are typical reactions of aromatic compounds. The yields are relatively high except for the oxidation of 2,5-diamino-5-trifluoromethylanisole (10%).

The yield of the alkylation of 2-methoxy-5-trifluoromethyl-1,4-benzoquinone was not very high. Usually, to carry out the alkylation reaction of Q_0, an excess of diacyl peroxide should be used (3:1 mol/mol) to get a higher yield. However, in our case excess diacyl peroxide could not be used. If excess diacyl peroxide was used, 3,6-didecyl-2-methoxy-5-trifluoromethyl-1,4-benzothiazole would be produced. Fortunately, the unreacted 2-methoxy-5-trifluoromethyl-1,4-benzoquinone could be recovered. Therefore, the yield based on the consumption of 2-methoxy-5-trifluoromethyl-1,4-benzoquinone was higher than 30%.

Biological Properties of Fluoroubiquinone Derivatives

As expected, replacement of a hydrogen with a fluorine on the side chain of ubiquinone did not affect its biological activity too much. 9'-FQ shows relatively high activity as an electron acceptor, donor and mediator assayed with succinate-ubiquinone reductase (SQR) (Fig. 24), ubiquinol-cytochrome c reductase (QCR) (Fig, 25) and ubiquinone and phospholipid depleted succinate-cytochrome c reductase (dSCR) (Fig. 26), respectively. The activity of the ubiquinone derivatives with fluorine on the benzoquinone ring is changed significantly. Both 6-decyl-2-methoxy-5-trifluoromethyl-1,4-benzoquinone and 6-(9'-fluorodecyl)-2-methoxy-5-trifluoromethyl-1,4-benzoquinone have activity as electron acceptors (Fig, 24), but have no activity as electron donors and

mediators at all. The reason is probably that the trifluoromethyl group, as a strong

electron withdrawing group, makes the benzoquinone ring very electron deficient and

increases its midpoint potential (about 50 mV higher than that of Q_2). Therefore, these

two fluoro ubiquinone derivatives can be reduced, but are hard to be oxidized. As a

result, they can only serve as electron acceptors for SQR (Fig. 24) and do not show any

activity as electron donors and mediators for QCR and succinate-cytochrome c reductase

(SCR). Interestingly, they show inhibitory properties on dSCR (Fig. 27) and QCR (Fig.

28). The maximum inhibition can be reached if 36 fold excess fluoroubiquinone (to

cytochrome b) is incubated with dSCR (85% inhibition for SCR and 70% for QCR). If

intact SCR was used, 60% activity was inhibited indicating that the binding of between the

intrinsic Q and the enzyme is stronger than that between these two fluoro-Q and the

enzyme. Kinetic studies indicate that these two fluoroubiquinones are competitive

inhibitors (Fig.29). As a competitive inhibitor, it must bind to the same site(s) as the

ubiquinone does. It is obvious that these two fluoroubiquinones are qualified candidates

as molecular probes to monitor the process of Q binding in ubiquinol-cytochrome c

reductase. Of course 9'-FQ is also a good candidate because it is biological active.
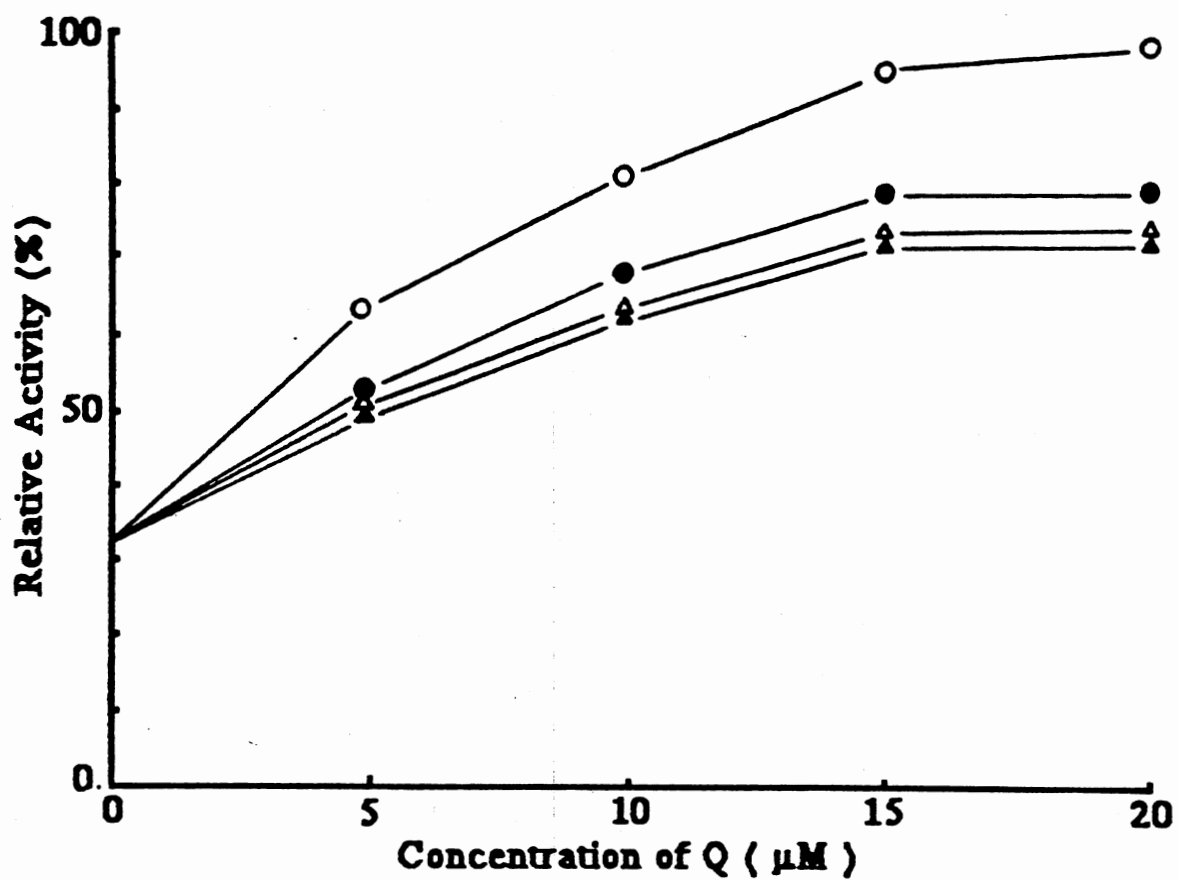
Fig. 24. Fluoro-Q Derivatives Function as Electron Acceptors. 3 μl of succinate-ubiquinone reductase (1 mg/ml) and different amounts of Q_2 (-O-), 9'FQ (-O-), TFQ (-Δ-), and 9'FTFQ (-Δ-) as indicated in the figure were used for SQR activity assay. All the activities were related to the maximum SQR activity when Q_2 was used.

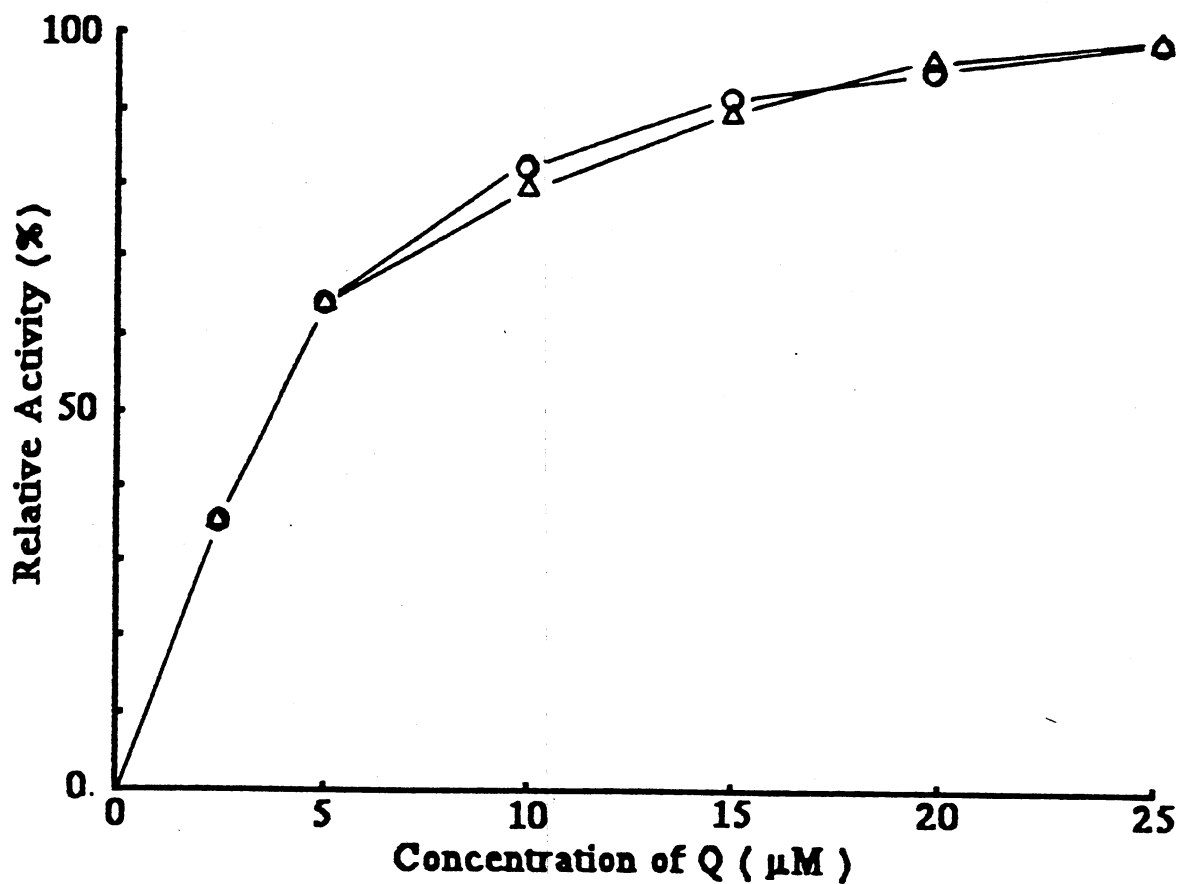Fig. 25. 9'FQ Functions as Electron Donor Assayed with QCR. The QCR activity was
assayed with different amount of $Q_0C_{10}Br(H_2)$ (-O-) and 9'FQ (-Δ-). The
activities were related to the maximum QCR activity when $Q_0C_{10}Br(H_2)$ was
used.
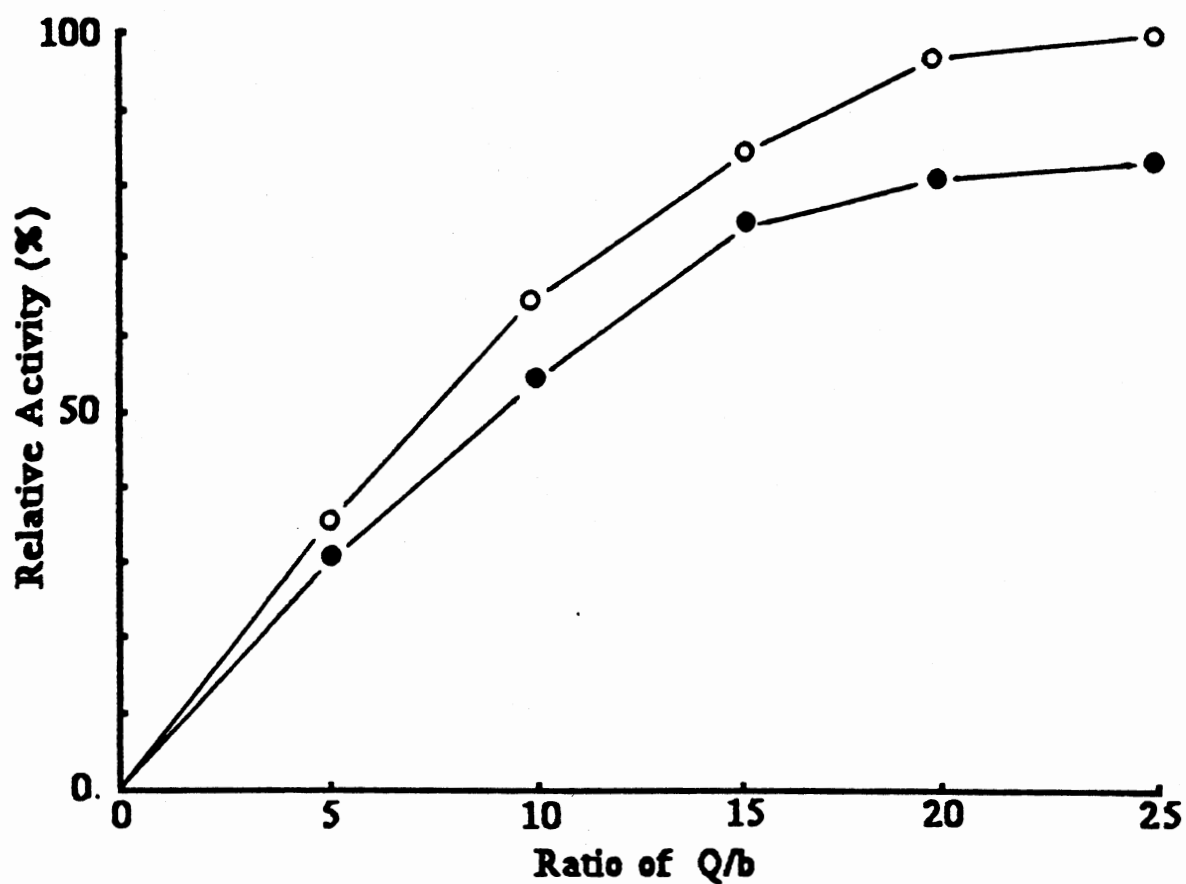
Fig. 26. Fluoro-Q Derivatives Function as Electron Mediators. 100 μl aliquots of d-SCR (1.4 mg/ml, in 50 mM K/K phosphate buffer containing 10% glycerol and 0.5% sodium cholate, pH 7..4) was incubated with different amount of Q_2 (-o-) and 9'FQ (-o-) in 5 μl ehtanol as indicated in the figure for 10 min. at 0 °C. To each of these mixtures were added 7 μl of asolectin (10 mg/ml) and then incubated for 60 min. at 0 °C. 3 μl aliquots of each sample was used for SCR activity assay.
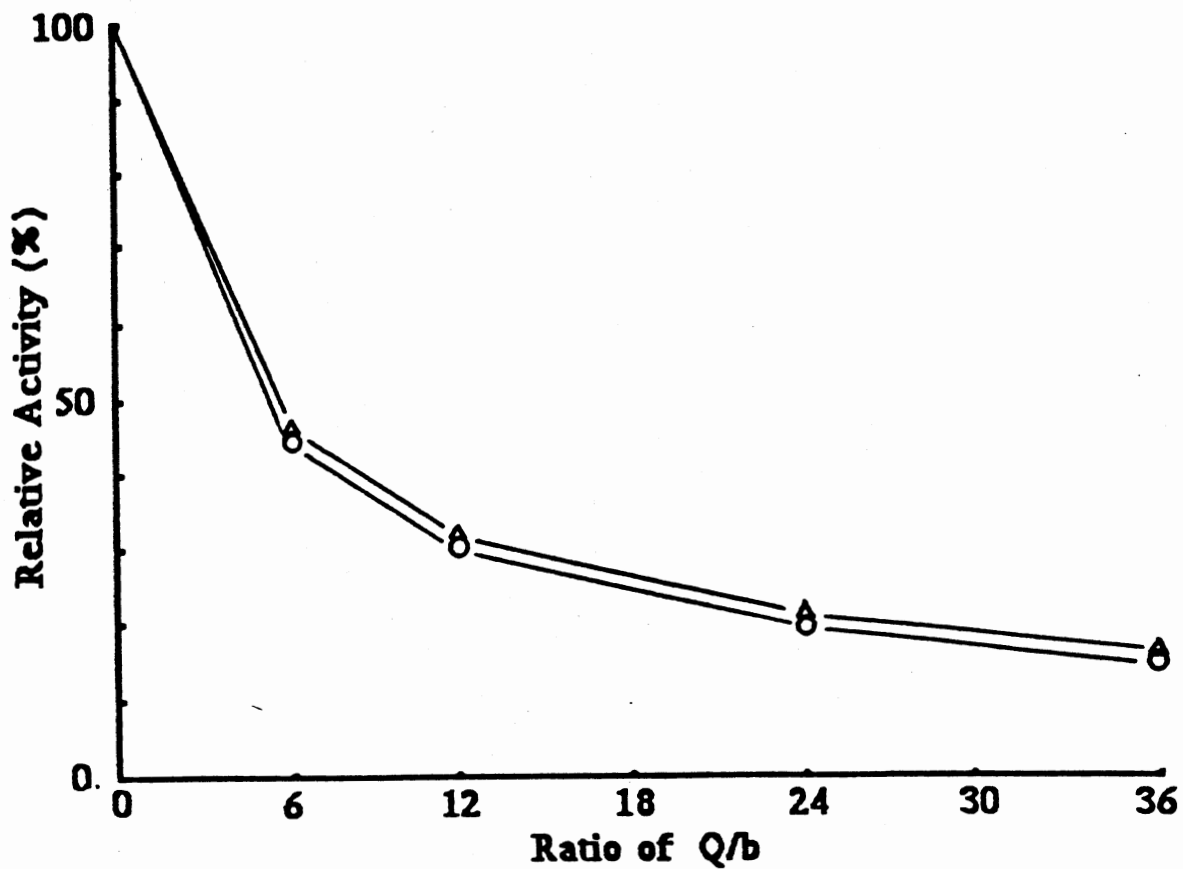
Fig. 27. The Inhibition of Reconstituted SCR by TFQ (-Δ-) and 9'FTFQ (-o-). 100 μl aliquots of d-SCR (2 mg/ml, in the same buffer as in that of fig. 23) were incubated with different amount of fluoro-Q in 3 μl of ethanol as indicated in the figure for 10 min. at 0 °C and then incubated with 3 μl of 5 mM Q2 for 10 min. at the same temperature. To each sample was added 10 μl of asolectin (10 mg/ml). 3 μl aliquots of each sample were used for SCR activity assay.
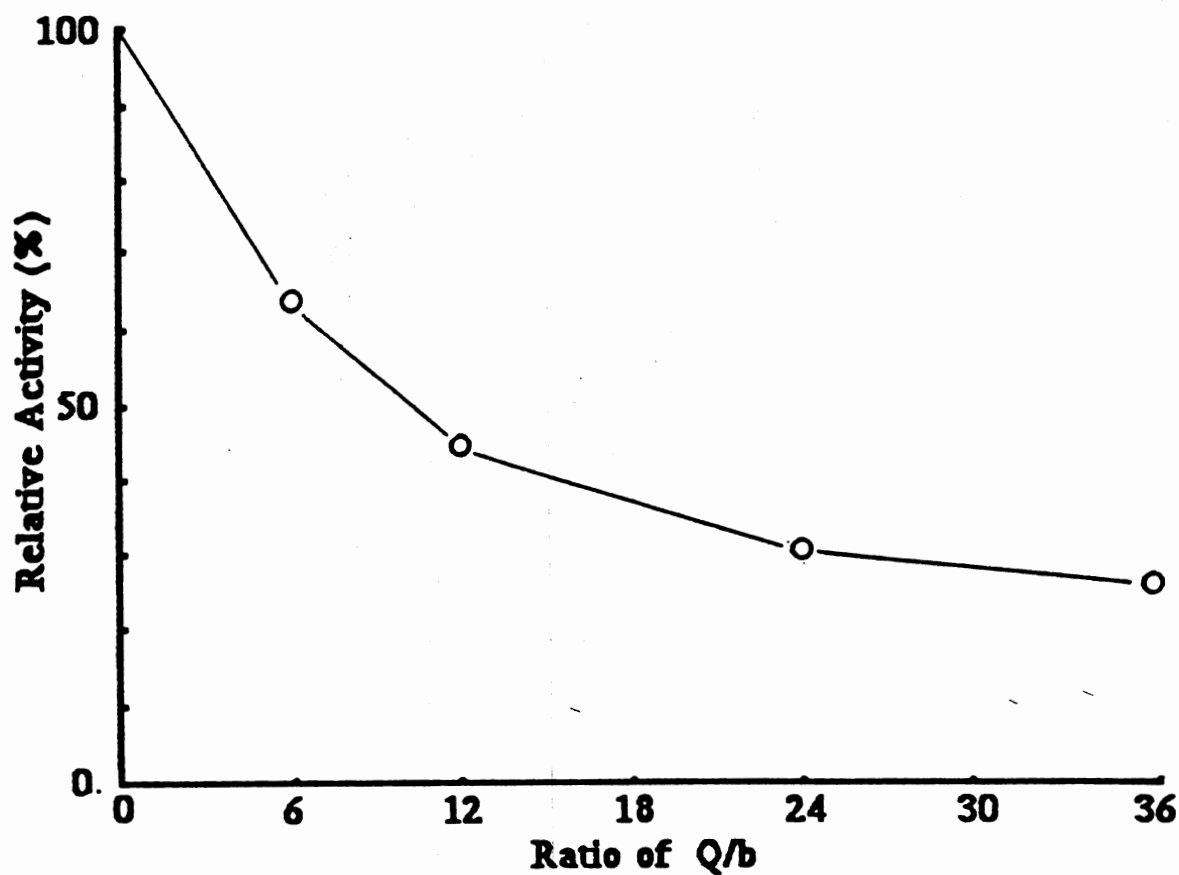
Fig. 28. The Inhibition of QCR Activity by TFQ Assayed with Reconstituted SCR. 100
μl aliquots of d-SCR (2 mg/ml, in the same buffer mentioned before) were
incubated with different amount of the TFQ in 3 μl of ethanol as indicated in
the figure for 10 min. at 0 °C and then incubated with 3 μl of Q2 (5 mM in
ethanol) for 10 min.at the same temperature.To each sample was added 10 μl
of asolectin and incubated for 60 min. at 0 °C. 1 μl aliquots were used for
QCR activity assay.
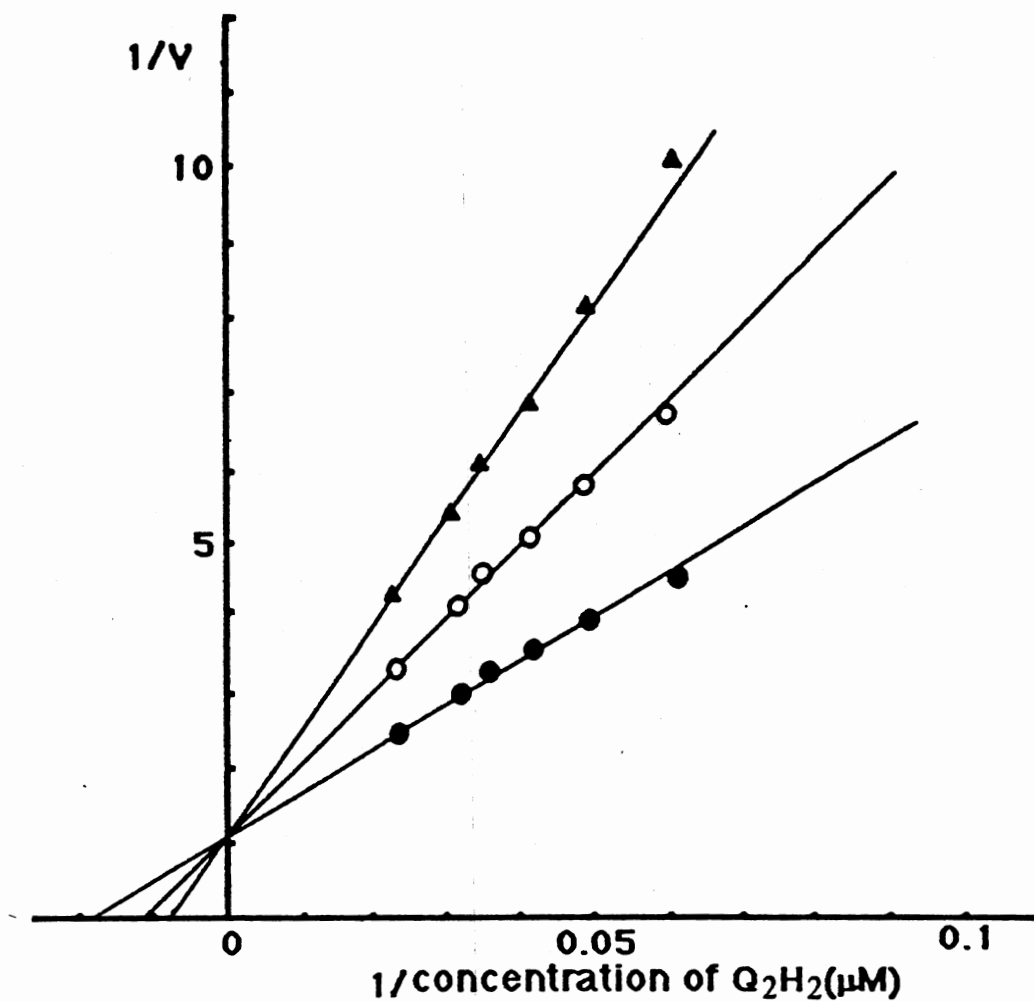
Fig. 29. Double Reciprocal Plot of the Oxidation Rate of Q_2H_2 Catalyzed by QCR
at the Presence of TFQ. The reaction valocity was measured when
different amount Q_2H_2 was used without TFQ (-o-) or with 5 nmoles
of TFQ (-o-) or with 10 nmoles of TFQ. TFQ and Q_2H_2 were
dissolved in the assay mixture first. 3 μl QCR (0.04 mg/ml) was used
for QCR activity assay.

CHAPTER V

SUMMARY AND CONCLUSIONS

Ubiquinone (Q), especially its protein-binding form, plays an essential role in electron transfer and proton translocation in ubiquinol-cytochrome c reductase. Understanding of the interaction of ubiquinone with proteins of ubiquinol-cytochrome c reductase has become the key point for studying the mechanism of energy conservation in site II. One of the best ways for studying the interaction between ubiquinone and its binding proteins is the use of ubiquinone analogues or its Q-like inhibitors with reporting group which can be monitored or detected during or after reaction with ubiquinol-cytochrome c reductase. The interactions of ubiquinol-cytochrome c reductase with photoactivable 3-azido-2-methyl-5-methoxy-6-(3',7'-dimethyloctyl)-1,4-benzoquinone, and with photoactivable Q-like inhibitor, 6-azido-5-undecyl-4,7-dioxobenzothiazole, were tested. Some ^{19}F NMR spectroscopically detectable fluoro substituted ubiquinone derivatives have been synthesized.

2-Azido-2-methyl-5-methoxy-6-(3',7'-dimethyloctyl)-1,4 benzoquinone (azido-Q) was found to be suitable for the study of specific interactions between ubiquinone (azido-Q) and proteins. It showed partial efficiency in restoring activity to the Q- and treated reductase, however, became completely inactivated upon photolysis and the inactivation was not reversed by addition of ubiquinone derivatives. Two protein subunits with M_r=37,000 and 17,000 were fond to be heavily labeled when depleted ubiquinol-cytochrome c reductae was treatd with [^{3}H]azido-Q followed by photolysis and SDS-PAGE. The amount of radioactive labeling of the M_r=17,000 subunit was proportional to the degree of inactivation and was affected by the presence of phospholipid. The

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radioactive labeling fo the M_r=37,000 subunit, however, showed no correlation with the degree of inactivation. Since the radiolabeling at the M_r=17,000 subunit was affected by phospholipid and correlated with the enzymatic activity, this subunit is probably the Q-binding protein in this enzyme complex. The distribution of radioactivity among the subunits of ubiquinol-cytochrome c reductase was not affected by the presence of antimycin A, 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) or n-heptyl-4-hydroxyquinoline-N-oxide, suggesting that the binding site(s) of the inhibitors were not the Q-binding site(s).

Further study of the inhibitory effect of a series of synthesized UHDBT derivatives on ubiquinol-cytochrome c reductase found that the 6-hydroxyl and 2-hydrogen groups of UHDBT are not structural requirements for inhibition and 6-azido-5-(1',2'-^{3}H-undecyl)-4,7-dioxobenzothiazole ([^{3}H]6-azido-UDBT) is suitable for the study of the UHDBT-binding in ubiquinol-cytochrome c reductase. When the photolyzed, [^{3}H]6-azido-UDBT treated ubiquinol-cytochrome c reductase was subjected to organic solvent extraction, no radioactivity was found in the reductase protein. Rather, the radioactivity was located in the phospholipid fraction which was identified to be a [^{3}H]azido-UDBT-cardiolipin adduct. This suggests that the inhibition by UHDBT is due to perturbation of a specific cardiolipin molecule in ubiquinol-cytochrome c reductase. Since UHDBT and 6-azido-UDBT also inhibit the enzymatic activity of delipidated reductase (10% of the original lipid remained) assayed after reconstruction with ubiquinone and phospholipid, and the [^{3}H]azido-UDBT was also covalently linked to the cardiolipin molecule after the azido-UDBT treated delipidated ubiquinol-cytochrome c reductase was photolyzed, the UHDBT perturbated cardiolipin molecule is structurally indispensable to the reductase and tightly bound to the reductase proteins, most likely the Q-binding proteins.

Three fluoro substituted ubiquinone derivatives, 2,3-dimethoxy-6-(9'-fluorodecyl)-5-methyl-1,4-benzoquinone, 6-decyl-2-methoxy-5-trifluoromethyl-1,4-benzoquinone and 6-(9'-fluorodecyl)-2-methoxy-5-trifluoromethyl-1,4-benzoquinone,

have been synthesized. As expected, 2,3-dimethoxy-6-(9'-fluorodecyl)-5-methyl-1,4-benzoquinone was a ubiquinone analogue and possessed all the biological properties that Q_2 had. The other two fluoro-Q derivatives, however, showed potent inhibition on ubiquinol-cytochrome c reductase and kinetic study revealed that they were competitive inhibitors. Since they were active analogues of ubiquinone or ocmpetitive inhibitors for ubiquinone, all the three fluoro-Q derivatives are valuable probes for Q-binding.

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