Title

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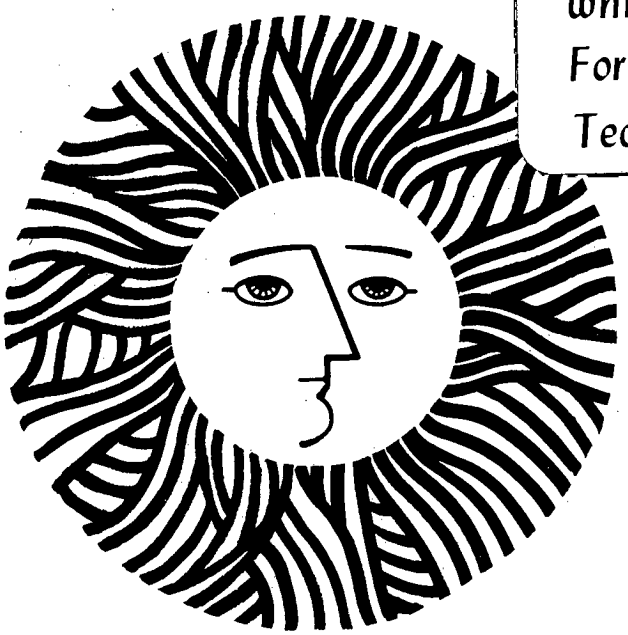
December 1981



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MEMBRANE EFFECTS OF VITAMIN E DEFICIENCY: BIOENERGETIC AND SURFACE CHARGE DENSITY STUDIES OF SKELETAL MUSCLE AND LIVER MITOCHONDRIA*

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SUMMARY

Vitamin E (dl-α-tocopherol) deficiency in rats increased the sensitivity of liver and muscle mitochondria to damage during incubation at various temperatures, irradiation with visible light, or steady state respiration with substrates. In all cases, vitamin E deficient mitochondria exhibited increased lipid peroxidation, reduced transmembrane potential, decreased respiratory coupling, and lower rates of electron transport, compared to control mitochondria. Muscle mitochondria always showed greater negative inner membrane surface charge density, and were also more sensitive to damage than were liver mitochondria. Vitamin E deficient mitochondria also showed slightly more negative inner membrane surface charge density compared to controls. The relationship between greater negative surface potential and increased sensitivity to damage observed, provides for a new and sensitive method to further probe the role of surface charge in membrane structure and function. Implications of these new findings for the well known human muscle myopathies and those experimentally induced by Vitamin E deficiency in animals, are discussed.

INTRODUCTION

The biological role of vitamin E (dl-α-tocopherol) has been extensively discussed. Vitamin E has been shown to act as a powerful antioxidant in the lipid matrix where it is located (1). Vitamin E also seems to be required as a structural component and can provide stability for membranes containing polyunsaturated fatty acids (2). In addition, vitamin E has been proposed to act as a catalytic or regulatory agent in intermediary metabolism (3). However, at present no detailed hypothesis exists which provides a comprehensive explanation for the many effects of vitamin E. Since oxidative damage to lipids and proteins will naturally lead to alteration of membrane structure and function, the characterization of such effects may lead to a fuller understanding of the physiological role of vitamin E.

The effect of vitamin E deficiency on tissue oxidation has been the subject of several earlier studies. Excessive rates of respiration from skeletal muscle have been repeatedly demonstrated in nutritional muscular dystrophy produced by deprivation of vitamin E (4). Although some authors have suggested that the increased oxygen consumption might be due to higher ATPase activities in muscle tissue, they were unable to demonstrate any difference in ATPase activity between muscle and liver homogenates, during the early or late stages of vitamin E deficiency (5). There has been some evidence that vitamin E deficiency uncouples oxidative phosphorylation in muscle homogenates (6). A recent review of the literature reporting on the enzymatic activities of animals deficient in or supplemented with vitamin E shows different authors claiming contradictory results, thereby adding to the overall state of confusion (7,8).

We chose to focus our attention on the protective role of vitamin E against membrane damage. Previous work from our laboratory (9-11) has

clearly demonstrated the role of oxidative and photooxidative damage in the mechanisms of inhibition of electron transport, and uncoupling of rat liver mitochondria: Flavin containing dehydrogenases (e.g., succinate dehydrogenase) were shown to be the most sensitive components of inner mitochondrial membranes to photooxidative damage, when incubated at temperatures of 10° C or less. The present study investigates damage to membrane integrity and structure under conditions (temperature of 26° C, $\pm$ illumination, shorter times of incubation) where succinate dehydrogenase is only slightly inactivated. Preparations of liver and muscle mitochondria from control and vitamin E deficient animals have been used in order to elucidate the differential sensitivity of these membranes to oxidative mechanisms of damage.

METHODS

Animals - Male "Long Evans" rats were purchased from Simonsen Laboratories (Gilroy, CA) weighing approximately 100 gm each.

Diets - Two different diets were purchased from BioServ Inc. (Frenchtown, NJ). Bio-Mix #1331 had less than 1 IU of Vitamin E/Kg and Bio-Mix #1332 had 21 IU of Vitamin E/Kg. During a period of approximately 100 days, a group of 8 rats were fed Bio-Mix #1332 and were denoted control animals. Another group of 8 rats were fed Bio-Mix #1331 and were denoted vitamin E deficient animals (E-). The severity of vitamin E deficiency in E- rats was assessed by a standard blood hemolysis test (12). The E- animals exhibited 93 $\pm$ 3% hemolysis, compared to 10 $\pm$ 3% hemolysis for controls.

Mitochondrial Preparations - Liver mitochondria were prepared according to established procedures (13). The isolation medium used was 150 mM mannitol, 75 mM sucrose, 1 mM Tris buffer and 1 mM EDTA (pH 7.4). For the last centrifugation, the mitochondria were washed in 0.25 M sucrose and finally resuspended in this medium at a concentration of approximatley 70 mg protein/ml. Muscle mitochondria were prepared by grinding, trypsin incubation, and homogenization as previously described (14). They were washed twice in 0.25 M sucrose and resuspended in this medium at a concentration of approximately 35 mg/ml. Protein concentrations were determined by a Biuret method (15).

Illumination Conditions - Incubation of dark and light samples was as previously described (10). The light source was a battery of 300 W quartz iodide lamps covered by a 400 nm cut-off filter (Corning #3389). The net light intensity as measured by a LI-COR LI-185 Radiometer was 17 mE $cm^{-2}sec^{-1}$. For studies of light or dark incubation at 26° C, mitochondrial suspensions

(10 ml at 10 mg/ml) were placed in 50 ml Erlenmeyer flasks and slowly shaken in a water bath at 26° C. Dark samples were incubated under the same conditions, but inside flasks covered with aluminum foil.

Absorption spectra of the mitochondrial suspensions at 0.4 mg protein/ml were measured in a Dual beam spectrophotometer between 400 nm and 750 nm.

Electron Transport - Rates of oxygen uptake were determined in a Rank oxygen polarograph (Rank Bros., Cambridge, England). Assays were performed at 37° C with 1 mg protein/ml in a medium, adapted (14) from that of Dow (16), consisting of 15 mM KCl, 0.4 mM NAD^+, 45 mM sucrose, 12 mM mannitol, 5 mM $MgCl_2$, 7 mM EDTA, 0.2% bovine serum albumin, 20 mM glucose, 30 mM K^+PO_4 and 25 mM Tris (pH 7.4). Substrates were used at the following concentrations: 20 mM glutamate, 10 mM pyruvate + 2.5 mM malate, 10 mM succinate + 4 µM rotenone. State III and IV rates of oxyen consumption and respiratory control ratios (RCR = state III rate of O_2 consumption/state IV rate of O_2 consumption) were determined according to Chance & Williams (17), with 0.2 mM ADP. The uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) was used at 1 µM. Dicyclohexylcarbodiimide (DCCD, 1 µM) was employed as a blocking agent for the passage of protons across the ATP synthetase complex when measuring the effects of steady state respiration.

Succinate dehydrogenase activity was measured with phenazine methosulfate as intermediate electron acceptor and dichlorophenolindophenol as final acceptor (18).

Lipid Peroxidation - Malondialdehyde produced during lipid peroxidation was determined as follows:

To 0.2 ml of mitochondrial suspension (equivalent to 2 mg of protein) 0.8 ml of double-distilled water was added, followed by 2 ml of the stock reagent 15% (w/v) trichloroacetic acid; 0.375% (w/v) thiobarbituric acid and 0.25 N hydrochloric acid. Lastly, 15 µl of a 2% (w/v) ethanolic solution of

butylated hydroxytoluene was added to abolish any metal-catalyzed auto-oxidation of lipids during heating with the thiobarbituric reagent. The mixture was heated for 15 minutes in a boiling water bath and, after cooling, the flocculent precipitate was removed by centrifugation at 1000 x g for 10 minutes. The absorbance of the sample was determined at 535 nm against a blank that contained all the reagents minus the mitochondria. The malon-dialdehyde concentration of each sample was calculated using an extinction coefficient of $1.56 \times 10^5 \cdot M^{-1} \cdot cm^{-1}$ (19).

The detection of lipids containing conjugated dienes was performed as follows:

One milliliter of mitochondrial suspension (equivalent to 10 mg protein) was mixed thoroughly with 5.0 ml of chloroform-methanol (2:1), followed by centrifugation at 1000 x g for 5 min to separate the phases. Of the lower chloroform layer, 3ml were recovered using a syringe and taken to dryness in a test tube at 45° C under a stream of nitrogen. The lipid residue was dissolved in 1.5 ml of cyclohexane and the absorbance at 233 nm determined against a cyclohexane blank. The molar extinction coefficient used was $2.52 \times 10^4 \cdot M^{-1} \cdot cm^{-1}$ (19).

Surface Potentials - Changes in the electrical surface potential of mitochondria were determined from changes in partitioning between the aqueous and membrane environment of the positively charged paramagnetic amphiphile 4-(decyldimethylammonium)-1-oxyl-2,6,6-tetramethyl piperidine bromide (CAT_{10}) and the uncharged spin label 2,2-dimethyl-5,5-methylheptyl-N-oxazolidinyloxyl (2N9). Equal volumes of mitochondrial suspensions (10 mg protein/ml) and of a solution of 0.25 M sucrose, 10 mM KPO_4 (pH 7.5), 150 µM CAT_{10} (or 2N9) and 0.5 mM potassium ferricyanide, were mixed and allowed to equilibrate for about 3 minutes. The electron paramagnetic resonance

(EPR) spectra of such suspensions were recorded with a Varian E-109E spectrometer.

The partition was defined as the ratio of spin label concentration in the aqueous medium to the concentration on the membrane. These concentrations could be readily determined from the EPR spectra of the spin probes (Figure 1) (20,21). Earlier studies have demonstrated that the mitochondrial outer membrane contributes relatively little to the total binding of such spin probes, compared to the inner membrane (21). Changes in membrane surface potential were estimated as a function of the zero time partitioning of the probes. The absolute value of the surface potential was not determined, since this would require measurement of the partition of CAT_{10} between the mitochondrial membranes and the aqueous environment at high and zero ionic strength; both of which are technically impossible due to loss of mitochondrial integrity.

Transmembrane Electrical Potentials - Changes in transmembrane potential established by substrate energized, mitochondria, were calculated using a tetraphenyl phosphonium electrode (22). Calculations of transmembrane potentials assumed that tetraphenyl phosphonium distribution between mitochondria and medium followed the Nernst equation and that the mass conservation law applied. Mitochondrial volume was assumed to be 1 µl/mg protein and final volume was 3 ml. Mitochondrial concentration was 1 mg/ml, tetraphenyl phosphonium concentration of the medium was 5 µM and tetraphenyl phosphonium concentration in the electrode was 10 µM.

RESULTS

Inactivation of Electron Transport

All mitochondrial samples showed no change in succinate dehydrogenase activity after 3 hours of incubation in the dark, and even the activity of illuminated samples had decreased by at most 30% (compared to zero time values), in agreement with earlier results (10). Significant differences between control and E- samples, and liver and muscle samples, could not be ascribed to differences in light absortion, since aerobic suspensions of liver and muscle mitochondria displayed similar absorption spectra. Muscle mitochondrial suspensions absorbed about 10% less light than liver mitochondria in the whole visible range from 430-760 nm. Between 400 and 430 nm, where flavins absorb strongly, both suspensions absorbed comparable amounts of light. Whether differences between liver and muscle mitochondria reflect the *in vivo* state, or were caused by isolation procedures was not ascertained.

For the liver mitochondria, in both dark and illuminated samples, state IV rates of respiration remained fairly constant during the first two hours of incubation decreasing sharply after that. In the case of muscle mitochondria, for both dark and illuminated samples, state IV rates of respiration increased quite substantially during the first hour of incubation, decreasing dramatically thereafter (data not shown). Similar rates of state III respiration were obtained by the addition of ADP or FCCP. At zero time, state III rates of respiration were similar for control and E- liver mitochondria but were lower in E- muscle mitochondria than control muscle mitochondria. All samples showed a steady decrease in state III rates of respiration during incubation (data not shown).

Respiratory control ratios during incubation ($\pm$ illumination) were always significantly lower in the E- samples compared to controls, except

in the case of liver mitochondria kept in the dark (Figure 2).

The levels of malondialdehyde produced during incubation (Figure 3). reveal that zero time levels were always greater in muscle mitochondria than in liver mitochondria (for both control and E- samples). Initial rates of formation and the final concentrations of malondialdehyde present, were always larger in illuminated samples. Throughout the incubation, the levels of malondialdehyde were consistently higher in E- samples; furthermore, differences between the concentrations of malondialdehyde found in E- and control samples increased with time of incubation. In general, when malondialdehyde levels increased above about 4-8 nmoles/mg protein, mitochondria were uncoupled. The measurement of lipids containing conjugated dienes (Table I) indicated that conjugated diene levels increased with time of incubation and were higher in the E- samples.

When incubated on ice in the dark, only E- muscle mitochondria showed a large decrease in RCR, during a 3 hour period (Figure 4). The decrease in RCR could be almost totally ascribed to increases in state IV rates of respiration, indicative of uncoupling. State III rates of respiration did not change significantly during the incubation for either control or E- samples. Attempts were made to alter this pattern of respiratory uncoupling in E- samples by addition of the antioxidant butylated hydroxytoluene (BHT) or the sulfydryl agent (reduced) glutathione (Figure 5). During three hours of incubation, BHT slowed somewhat the rates at which the RCR decreased due to uncoupling of state IV respiration. Both reagents, however, lowered somewhat the levels of malondialdehyde produced.

Uncoupling During Steady State Respiration

The effect of prolonged steady state respiration on the coupling of mitochondria was also studied. Mitochondria (1 mg/ml) were incubated in an open Rank Oxygen Polarograph at 22° C for various lengths of time in the

presence of pyruvate + malate and DCCD. These suspensions were not illuminated. The O_2 concentration in the medium remained approximately 200 μM. At various times during incubation, the oxygen polarograph was capped to enable measurement of basal rates of respiration. Maximal rates of respiration were generated by the addition of FCCP and the respiratory control index (RCI = uncoupled rate of O_2 consumption/basal rate of O_2 consumption) calculated. It is clear that the control samples always exhibited higher RCI than did E- samples (Figure 6). Furthermore, after 30 minutes of incubation, the only sample that was almost completely uncoupled (RCI = 1.2) was the E- muscle. All the others still showed a RCI of 2 or higher. Compared to control samples, the E- samples of muscle mitochondria lost their RCI faster than did E- liver mitochondria.

Surface Potentials

Differences in surface potential arising from the outer surface of the inner membrane, of muscle and liver mitochondria were observed at zero time, and during incubation (± illumination) at 26° C (Figure 7). Differences in partitioning of the 2N9 probe between the various samples was negligible, implying that hydrophobic interactions with amphipathic probes were not altered.

Surface potential measurements revealed that the surface potential of muscle mitochondria was always substantially more negative than that of liver mitochondria. We also observed that E- samples exhibited slightly more negative surface potentials than did control samples, both for muscle and liver mitochondria; these differences were, however, not always significant.

Transmembrane Potentials

Changes in transmembrane potential generated during respiration, as a function of incubation time (± illumination) at 26° C are shown in Figure 8. For muscle mitochondria, pyruvate-malate was employed as substrate.

In the case of liver mitochondria, transmembrane potentials generated by this substrate were small and succinate was used instead. The results consistently showed that control mitochondria developed a larger transmembrane potential than did E- mitochondria, that muscle mitochondria were more readily inactivated than were liver mitochondria, that when samples were illuminated their capacity to generate transmembrane potentials decreased more readily than that of dark samples, and that when E- samples were illuminated loss of transmembrane potential was more rapid than for controls.

DISCUSSION

Vitamin E deficiency has been clinically implicated in muscular and neuromuscular diseases and in the sensitivity of lung, liver, red blood cells, and platelets to damage. Animal models of vitamin E deficiency have proved extremely useful in the study of many of these phenomena.

a) Muscle versus liver mitochondria susceptibility to damage.

It appears that the effects of vitamin E deficiency in rats include marked increases in mitochondrial sensitivity to damage. This is particularly conspicuous in the case of muscle mitochondria and may well provide a clue as to why vitamin E deficiency causes nutritional muscular dystrophy (24,25) and why, in human clinical studies, it has been implicated in neuromuscular myopathies (see articles by M.A. Guggenheim et al. and D.P.R. Muller et al. in this volume). The direct effects of vitamin E on membrane structure have always been hard to assess owing to its low molar ratio in relation to phospholipids (about 1:200 for the inner mitochondrial membrane). The important physiological role of dl-α-tocopherol in protecting inner mitochondrial membranes, may arise partially through its antioxidant properties, although its structural stabilizing properties may also be important. Since lipid peroxidation may strongly affect structure and permeability, these two aspects may be closely related.

b) Surface potentials.

The observation of increased sensitivity of mitochondria with greater negative surface potential to damage, and of small changes in membrane surface charge density accompanying vitamin E deficiency, provides a new and sensitive method with which to further investigate the effect of vitamin E deficiency and surface charge on membrane structure and function.

In accord with previous results from our laboratory (9-11), the inactivation effects are enhanced by visible light irradiation. These alterations

are always more pronounced in the mitochondrial samples with more negative surface potentials (i.e., muscle vs. liver and E- vs. control).

The inter-relationship between more negative surface potentials and greater sensitivity to damage may arise from differences in the ionic nature of binding of extrinsic proteins to membranes (see article by S.S. Shapiro et al. in this volume) and from changes in the interaction between intrinsic components in membranes (see article by M. Sayare et al. in this volume). The effect is particularly clear in the case of muscle vs. liver mitochondria, but is also found for E- mitochondria compared to controls. These differences are maintained during the course of incubation ± illumination and although small, are consistent.

Whether lipid peroxidation causes the loss of membrane functional integrity (respiratory control, transmembrane potential) or whether functional losses facilitate lipid peroxidation, is not known at present. It is found that, under certain conditions, antioxidants slow down each process.

c) Temperature effects.

The effect of temperature in the overall inactivation also appears to be important. Earlier experiments performed with liver mitochondria at 10° C showed that the respiratory control ratio was still high after 12 hours of incubation in the dark, and that loss of state III respiration followed succinate dehydrogenase inactivation during illumination (10). In the present study, we show that when incubated at 26° C, both liver and muscle mitochondria (± illumination) loose their respiratory control, and state III rates of respiration decrease by 75% within 3 hours. Since succinate dehydrogenase is only 30% inactivated even in the light, decreased rates of substrate dehydrogenation cannot explain the loss of overall electron transport. It appears possible that decreased state III rates are precipitated by diminished electron transport between respiratory components in the disrupted inner

membranes. Clearly, at higher temperatures (26° C), modifications of the electron transport chain and/or the structural integrity of the membrane are more severe than at 10° C. The lack of protective effect of reduced glutathione suggests that sulfhydryl groups are probably not involved in the mechanisms of inactivation. The increased perturbations to membrane integrity at higher temperatures could afford an explanation to some of the damage we observe in muscle following exercise (26), since it is well known that during physical activity the temperature of muscle tissue increases significantly (27). Moreover, the decreased levels of latency for cellular enzymes and in respiratory control of mitochondria, and the increased levels of lipid peroxidation and stable tissue radicals, which accompany vitamin E deficiency in rats (26), may account for the lower exercise endurance capacity of these animals.

d) Steady-State respiration.

The effect of steady state respiration on uncoupling of mitochondria (28) may be related to the well known superoxide anion generation during state IV respiration (29). This possibility is especially interesting since E- mitochondria are substantially more sensitive to damage during steady state respiration than are control mitochondria. Such findings are consistent with an antioxidant role for vitamin E in the mitochondrial membrane. An abstract of some of the studies reported in this work has already appeared (30).

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ACKNOWLEDGEMENTS

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TABLE I

MITOCHONDRIAL CONCENTRATIONS OF CONJUGATED DIENES BEFORE AND AFTER ILLUMINATION AT 26° C

Mitochondria		Conjugated Diene Concentration (nmoles/mg protein)	
		0 Time	2 h light
Liver	Control	0.19	0.28
	E Deficient	0.41	0.50
Muscle	Control	0.19	0.56
	E Deficient	0.49	0.99

FIGURE LEGENDS

FIGURE 1. Electron paramagnetic resonance spectrum and partition of a CAT probe in the presence of membranes.

FIGURE 2. Effects of light and dark incubation at 26° C on mitochondrial respiratory control ratios. Standard deviation for respiratory control ratios was $\pm$ 0.1.

FIGURE 3. Effects of light and dark incubation at 26° C on mitochondrial malondialdehyde concentrations. Standard deviation for malondialdehyde concentrations was $\pm$ 0.2 nmoles/mg.

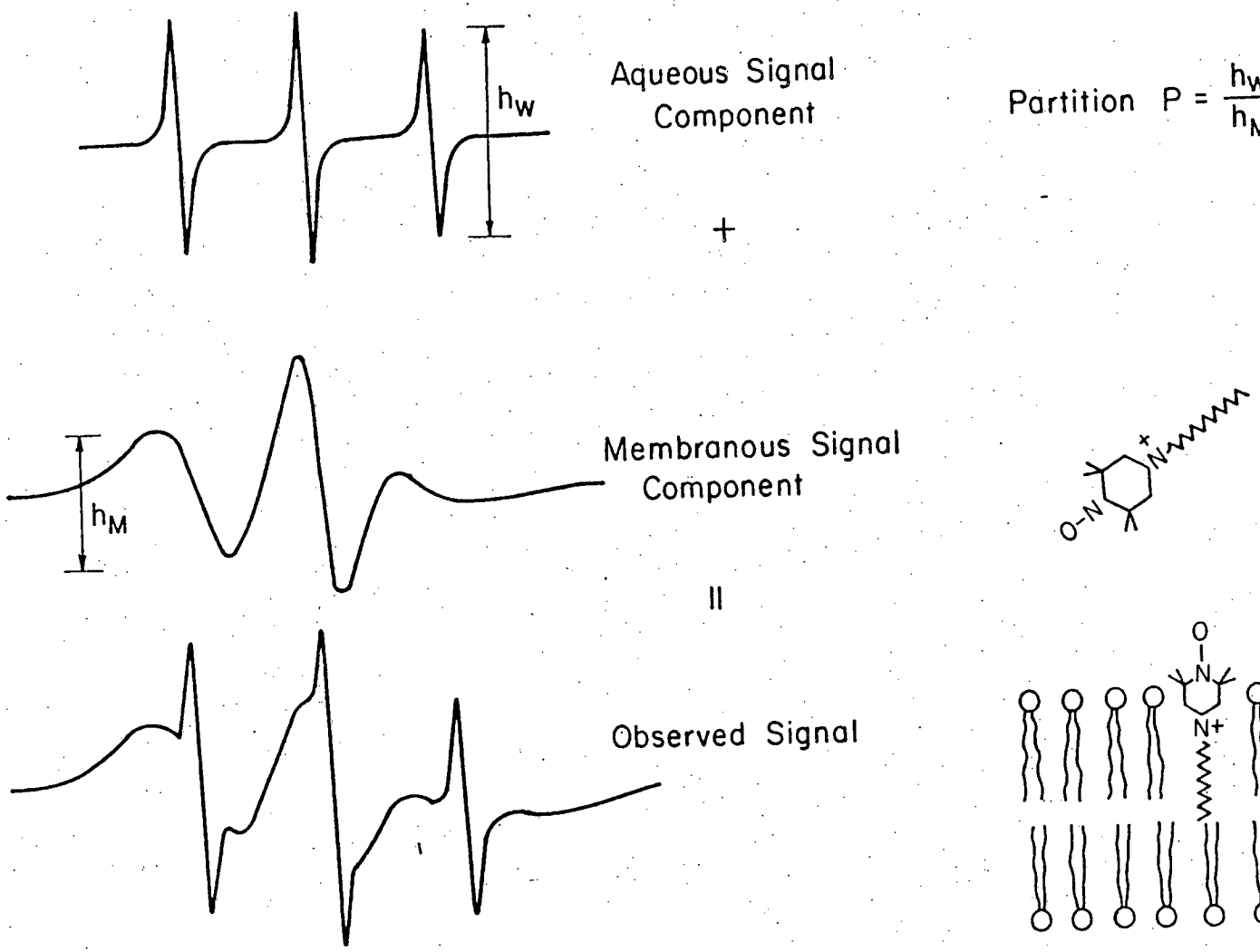
FIGURE 4. Effects of dark incubation at 5° C on muscle mitochondrial state III and IV respiration, and respiratory control ratios. Standard deviation for respiration rates was $\pm$ 5 nmoles O_2/mg·min and for respiration control ratios was $\pm$ 0.1.

FIGURE 5. Effects of dark incubation at 5° C with antioxidants on muscle mitochondrial respiratory control ratios, and malondialdehyde concentrations. Butylated hydroxytoluene was used at 2 μM and glutathione (reduced) concentration was 1 mM. Standard deviation as in Figures 1 and 2.

FIGURE 6. Effects of steady state respiration at 22° C on muscle mitochondrial respiratory control indices. Mitochondria were incubated with pyruvate-malate and DCCD for varying periods. Uncoupled rates were obtained following the addition of FCCP. Standard deviation in respiratory control index was $\pm$ 0.2.

FIGURE 7. Effects of light and dark incubation at 26° C on mitochondrial surface charge density (surface potential). Standard deviation in surface charge density was $\pm$ 6%.

FIGURE 8. Effects of light and dark incubation at 26° on mitochondrial transmembrane electrical potential. Standard deviation in transmembrane electrical potential was $\pm$ 5%.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h_M
Membranous Signal Component
=
Observed Signal
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N+
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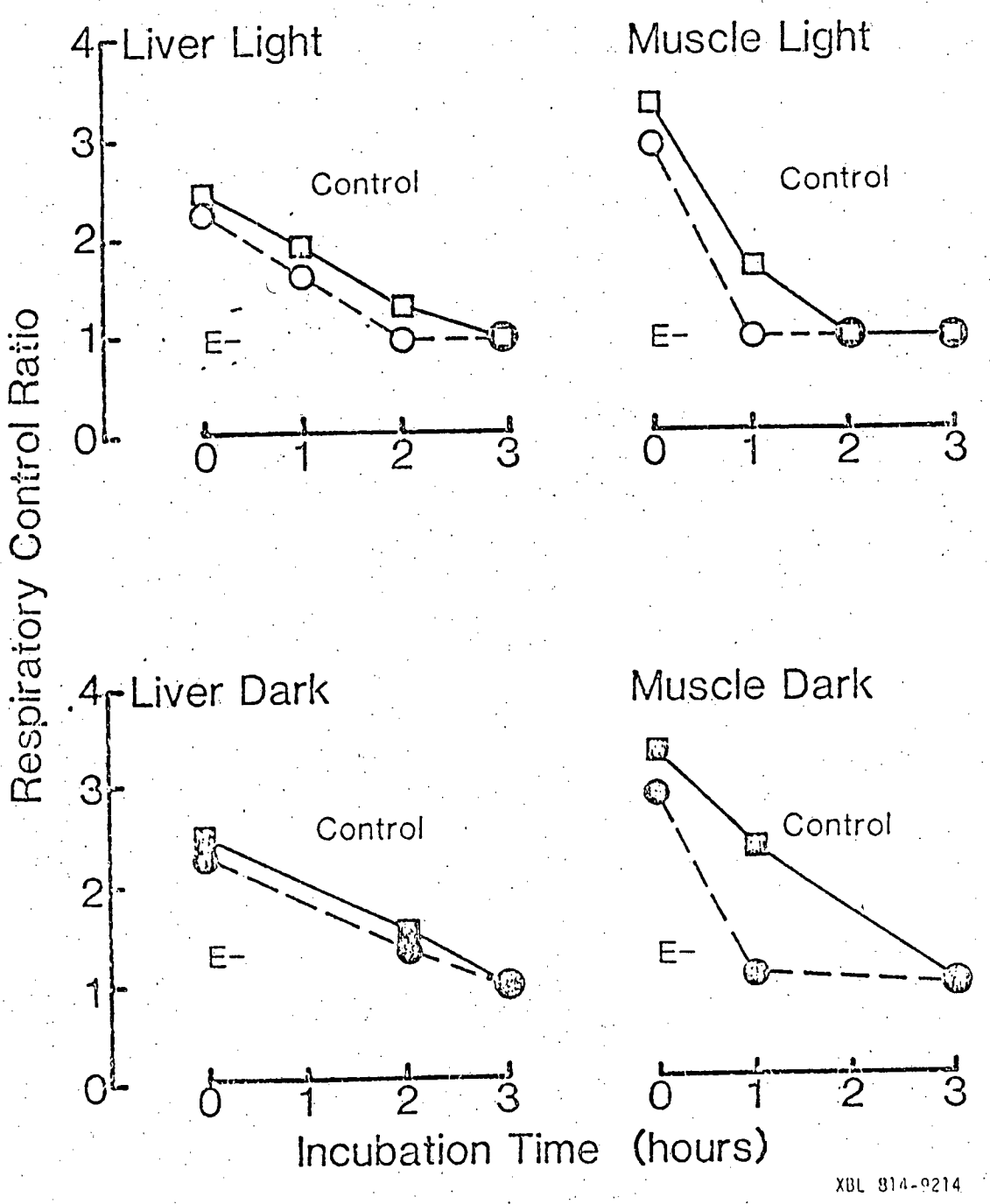
Figure 1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Muscle Light
Liver Dark
Muscle Dark
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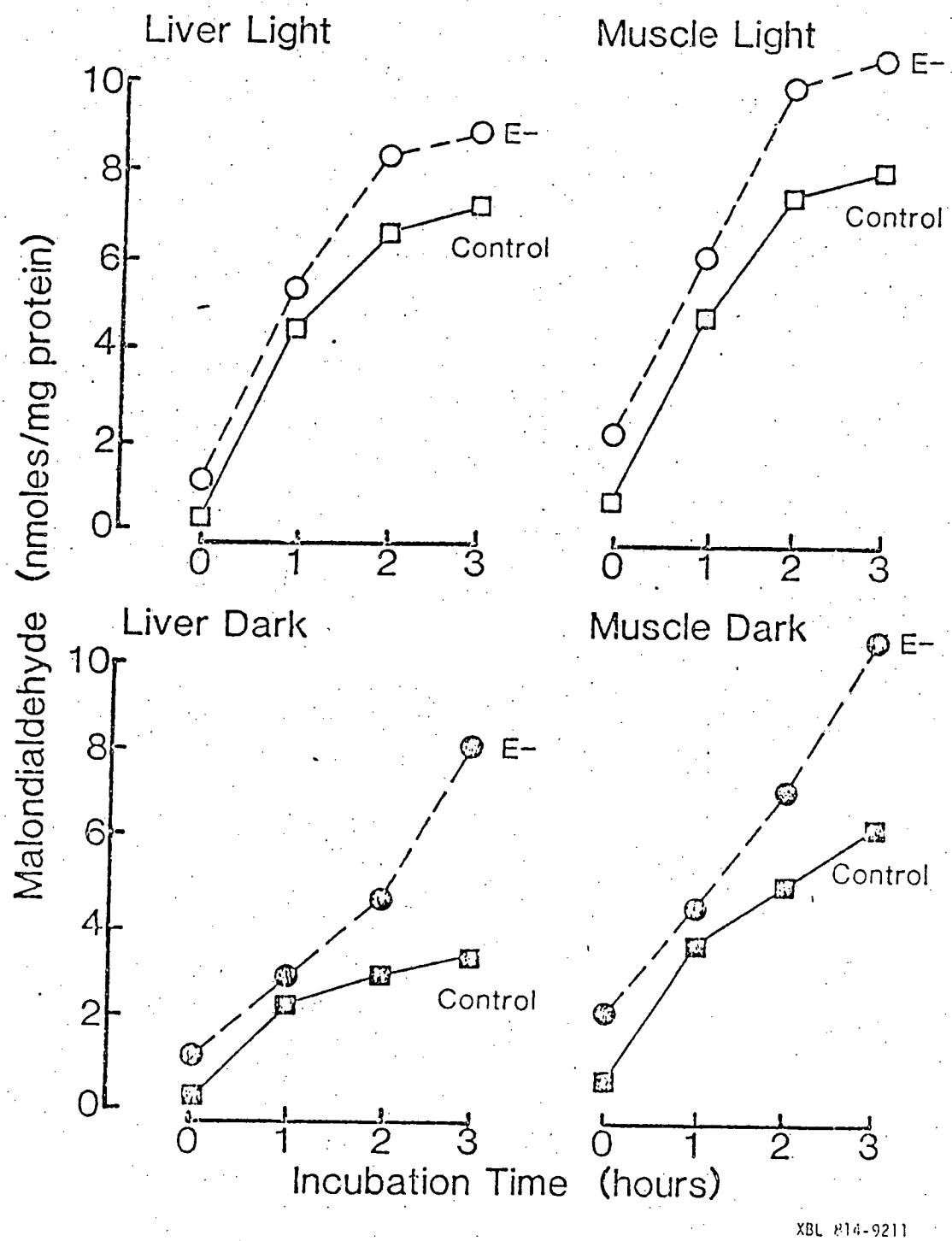
Figure 2


Liver Light
Muscle Light
Liver Dark
Muscle Dark
E-
Control
Malondialdehyde (nmoles/mg protein)
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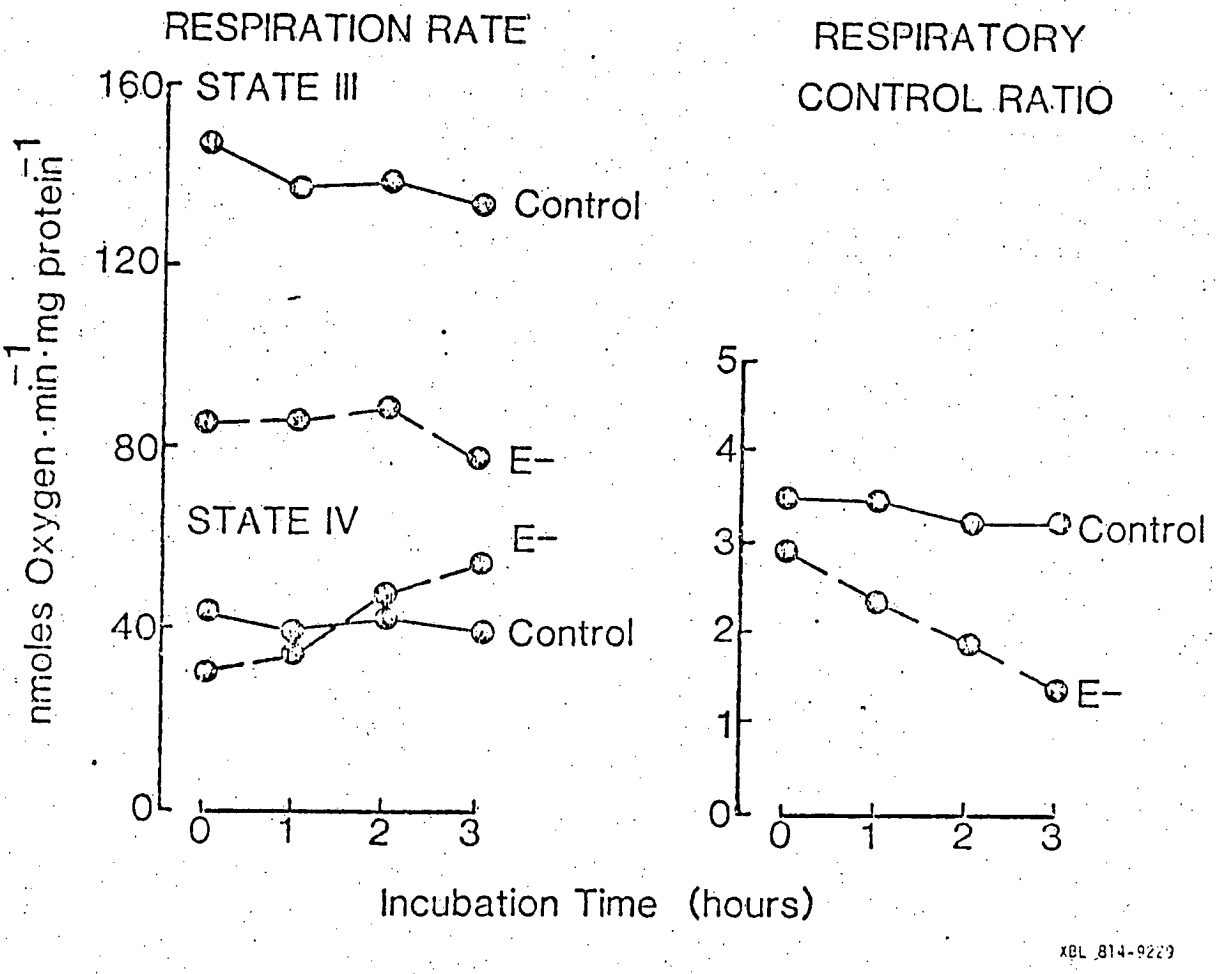
4
6
8
10
XBL 814-9211

Figure 3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E-
STATE IV
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Control
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RESPIRATORY
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4
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2
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0
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E-
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Incubation Time (hours)
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Figure 4

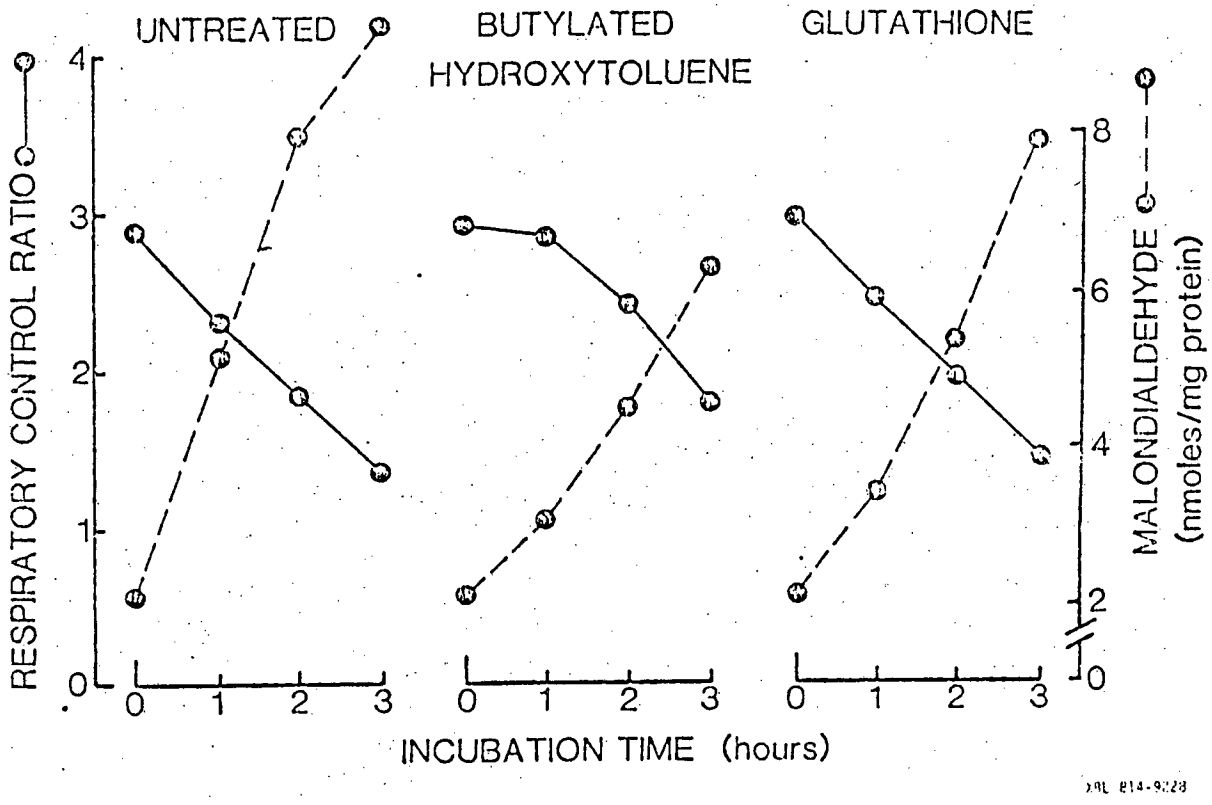
UNTREATED
BUTYLATED HYDROXYTOLUENE
GLUTATHIONE
RESPIRATORY CONTROL RATIO
MALONDIALDEHYDE (nmoles/mg protein)
INCUBATION TIME (hours)
0
1
2
3
4
6
8

Figure 5

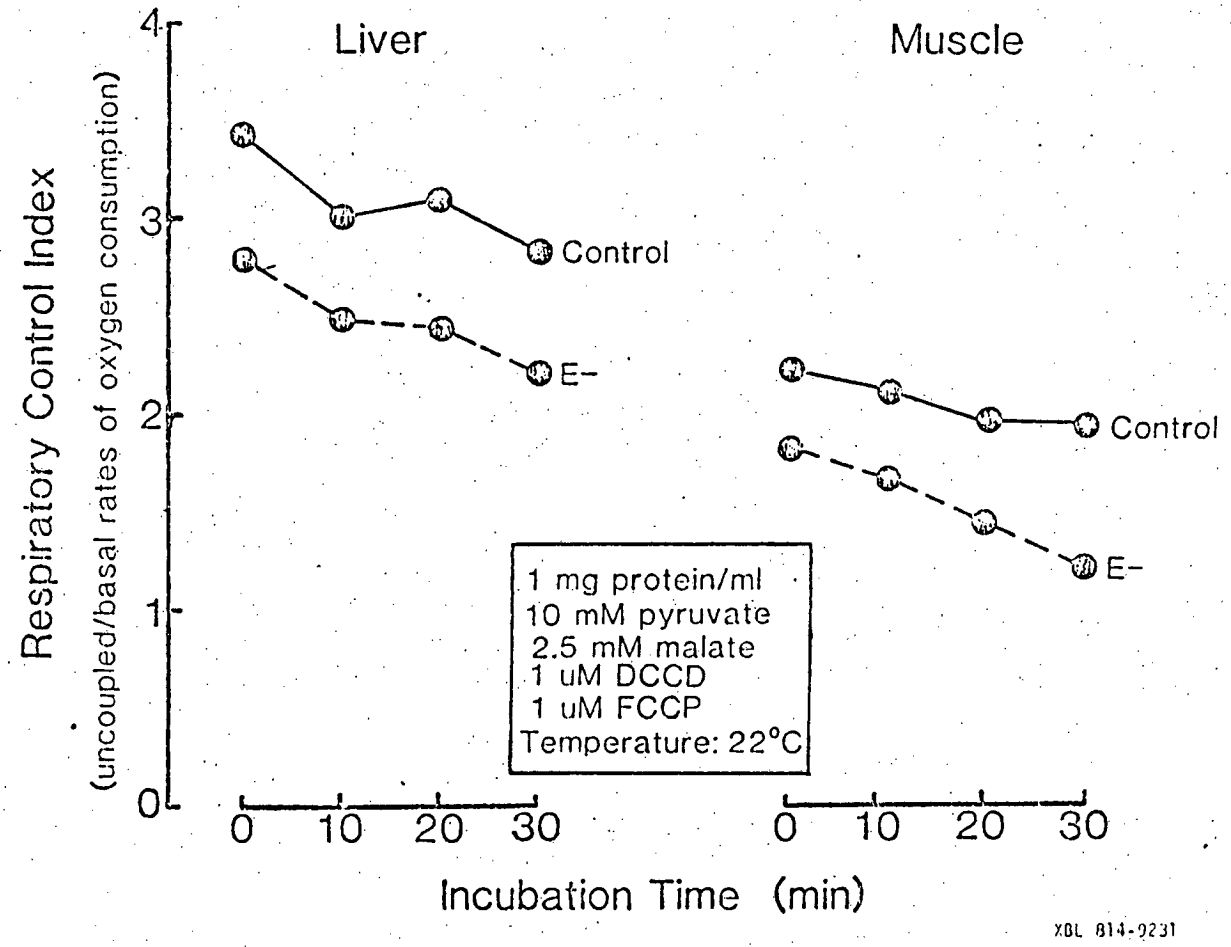
Liver
Muscle
Respiratory Control Index
(uncoupled/basal rates of oxygen consumption)
Control
E-
Control
E-
1 mg protein/ml
10 mM pyruvate
2.5 mM malate
1 uM DCCD
1 uM FCCP
Temperature: 22°C
0
1
2
3
4
0
10
20
30
0
10
20
30
Incubation Time (min)
XBL 814-9231

Figure 6

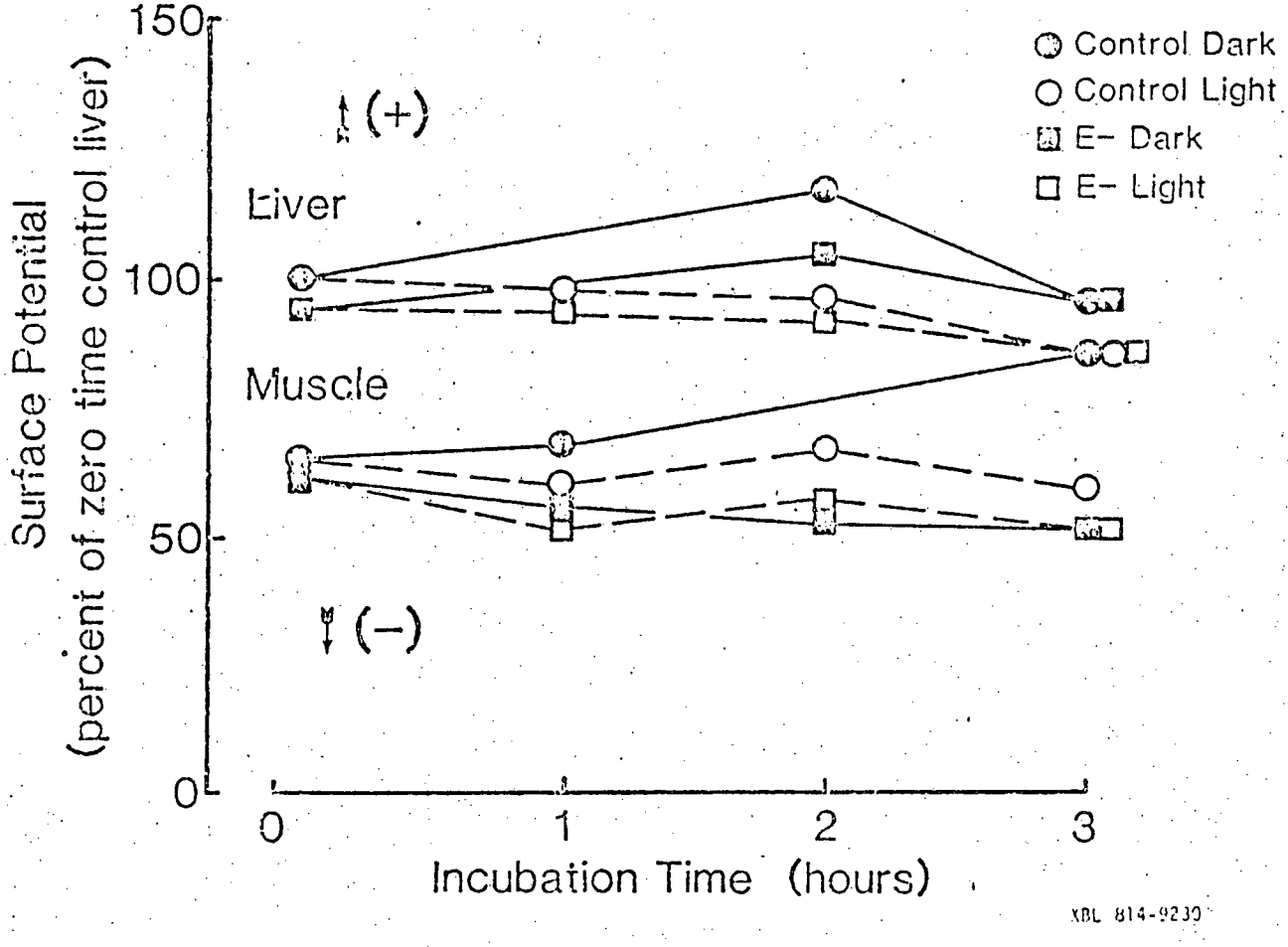
Control Dark
Control Light
E- Dark
E- Light
(+)
Liver
Muscle
(-)
150
100
50
0
Surface Potential
(percent of zero time control liver)
0
1
2
3
Incubation Time (hours)
XBL 814-9230

Figure 7

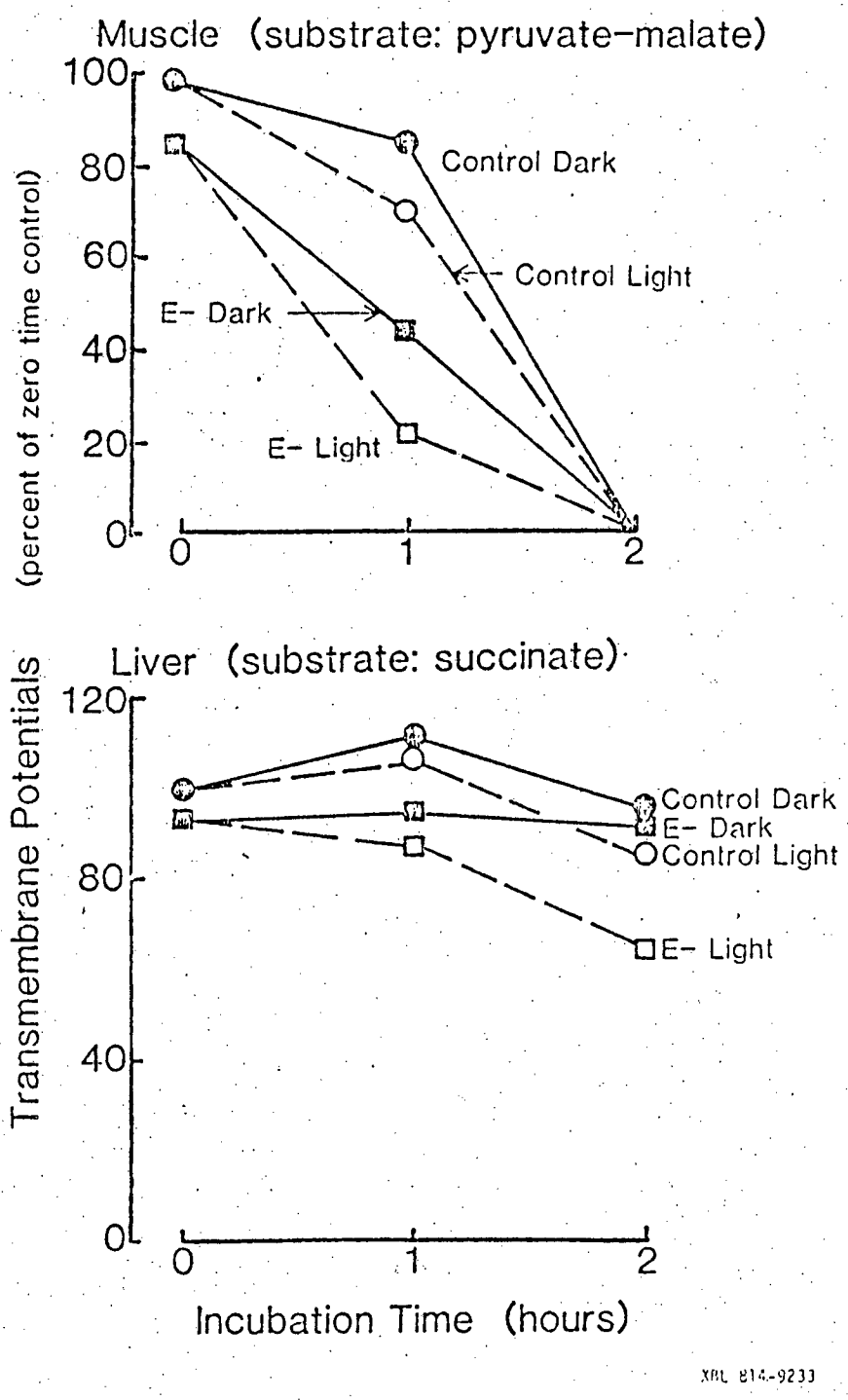
Muscle (substrate: pyruvate-malate)
100
80
60
40
20
0
(percent of zero time control)
Control Dark
Control Light
E- Dark
E- Light
0
1
2
Liver (substrate: succinate)
120
80
40
0
Transmembrane Potentials
Control Dark
E- Dark
Control Light
E- Light
0
1
2
Incubation Time (hours)
XBL 814-9233

Figure 8

This report was done with support from the Department of Energy. Any conclusions or opinions expressed in this report represent solely those of the author(s) and not necessarily those of The Regents of the University of California, the Lawrence Berkeley Laboratory or the Department of Energy.

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