BBA 46001

ELECTRON TRANSPORT IN MAMMALIAN NUCLEI

II. OXIDATIVE ENZYMES IN A LARGE-SCALE PREPARATION OF BOVINE LIVER NUCLEI

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(Received February 13th, 1970)

SUMMARY

1. A large-scale preparation of highly purified bovine liver nuclei is studied enzymatically.

2. Trace activities of succinate oxidase and NADPH–cytochrome *c* reductase are ascribed to slight mitochondrial and microsomal contamination, respectively.

3. Rotenone, antimycin A, piericidin A-insensitive NADH–cytochrome *c* reductase, NADH oxidase, cytochrome *c* oxidase, glucose-6-phosphatase, and Mg^{2+}-stimulated ATPase are shown to be endogenous to nuclei.

4. The O_2 consuming nuclear NADH oxidase differs from its mitochondrial counterpart in its greater resistence to histone inhibition, its susceptibility to inhibition by deoxyribonuclease, its dependency on cytochrome *c* for activity, and the 10-fold higher concentration of exogenous cytochrome *c* needed for maximum O_2 consumption.

5. The nuclear cytochrome *c* oxidase system cannot oxidize tetrachlorohydroquinone in contrast to the mitochondrial cytochrome *c* oxidase system.

6. The nuclear preparations lack coenzyme Q, a characteristic component of the electron transport system of inner mitochondria membrane.

7. The NADH/cytochrome *c* oxidase ratio is 1.3 for the nuclear oxidases and 0.59 for mitochondrial oxidases.

8. The NADH oxidases of nuclei, outer mitochondrial membrane and microsomes are discussed.

INTRODUCTION

One of the difficulties in identifying an electron transport system in nuclei is the enormous size of cell nuclei relative to other cellular organelles. Oxidative enzymes may represent only a very small percentage of the total nuclear protein. This results in relatively low specific activities for oxidative enzymes, which may then be attributed to mitochondrial contamination.

In previous studies, REES and co-workers[1,2] reported the presence of oxidative

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate.

enzymes in rat liver and kidney nuclei isolated in 0.25 M sucrose solutions and further proposed an electron transport system in nuclei. CONOVER AND SIEBERT[3] then concluded that the oxidative enzymes were due to contamination. However, PENNIALL and collaborators[4–6] using nuclei highly purified by centrifugation through dense sucrose solutions, reported NADH oxidase and cytochrome *c* oxidase in rat liver nuclei and the localization of the NADH oxidase in a nucleoli fraction. More recently oxidative enzymes have been demonstrated in nuclei from rat liver[7], mouse liver[8], bovine liver[10,12] and shown to be highly concentrated in nuclear membrane fractions[7,9,10,12].

In order to more effectively demonstrate the presence of an electron transport system, we have developed a method for preparing nuclei from bovine liver in useful enough quantities for further biochemical fractionation studies[11]. Correlated chemical, enzymic and electron microscopic studies indicated that the nuclei from this large-scale preparation were of high purity. This paper deals with the description of nuclear oxidative enzymes and their comparison with mitochondrial oxidases. A portion of these studies have appeared elsewhere in preliminary form[10,12].

METHODS

Nuclei on a large scale, mitochondria and microsomes were prepared from a bovine liver homogenate according to BEREZNEY *et al.*[11]. Protein was estimated by a modified biuret procedure of YONETANI[13]. Coenzyme Q was determined according to CRANE AND DILLEY[14]. NADH–cytochrome *c* reductase, NADPH–cytochrome *c* reductase and succinate–cytochrome *c* reductase were assayed by following the reduction of cytochrome *c* at 550 mμ at 30° according to the assay system of ERNSTER *et al.*[15]. Succinate–cytochrome *c* reductase was assayed at a succinate concentration of 5 mM. Succinate–phenazine methosulfate (PMS) reductase was assayed spectrophotometrically by coupling PMS reduction to 2,6-dichlorophenolindophenol (DCIP) as described by KING[16]. The assays were performed at a fixed PMS concentration of 0.3 mM. Glucose-6-phosphatase and ATPase were measured by determining the amount of inorganic phosphate released over a given time interval. The assay system for glucose-6-phosphatase was according to SWANSON[17]. The assay mixture for Mg^{2+}-stimulated ATPase was that of ERNSTER *et al.*[15]. Incubation for 10 min at 30° was used for both assays. Inorganic phosphate was determined by the isobutanol–benzene extraction procedure of Martin and Doty as modified by LINDBERG AND ERNSTER[18].

Succinate oxidase, NADH oxidase, tetrachlorohydroquinone oxidase and cytochrome *c* oxidase were measured polarographically at 38° on a Gilson oxygraph utilizing a Clark electrode covered with a Teflon membrane. Succinate and NADH oxidase were assayed in a total volume of 1.8 ml containing 66.7 μmoles of phosphate at pH 7.5 in the absence or presence of cytochrome *c* as indicated[10]. Cytochrome *c* oxidase was assayed according to the procedure of SUN AND CRANE[19]. Tetrachlorohydroquinone oxidase was determined according to MACHINIST AND CRANE[20]. Monoamine oxidase was assayed spectrophotometrically according to SCHNAITMAN *et al.*[21].

MATERIALS

NADH, NADPH, cytochrome *c*, ATP, succinic acid, antimycin A, rotenone and histone were purchased from Sigma Chemical Co. Glucose-6-phosphate and deoxy-

ribonuclease were obtained from Cal Biochem. Polyacrylic acid was obtained from K and K Laboratories, tetrachlorohydroquinone from Eastman Organic Chemicals Corporation and protamine as protamine sulfate from Nutritional Biochemical Corp. Piericidin A was kindly provided by Dr. Karl Folkers. All other chemicals were of reagent grade. Glass-distilled water was used exclusively.

RESULTS

Estimation of mitochondrial and microsomal protein contamination in nuclear fractions

Because of the finding of oxidative enzymes in nuclear preparations[10,12], we have extended our previous study of mitochondrial contamination to include additional enzymatic assays as well as using coenzyme Q as a chemical marker for mitochondria. The mitochondrial marker enzyme, succinate oxidase, was measured by means of O_2 consumption, cytochrome *c* reduction (succinate–cytochrome *c* reductase) and phenazine methosulfate reduction (succinate dehydrogenase). The results presented in Table I show excellent correlation among the three assays and an average of 1.4 % mitochondrial protein. This result also suggests that histone inhibition is not masking mitochondrial succinate oxidase. If this were the case, the oxidase would be lower than the cytochrome *c* reductase or dehydrogenase activity, due to the reported inhibitory action of histones on cytochrome *c* oxidase[22].

The very low coenzyme Q content in the nuclear fractions correlates well with the low succinate oxidase activities. Furthermore, the fractions contained no tetrachlorohydroquinone oxidase in the presence or absence of protamine. In contrast, mitochondrial fractions displayed tetrachlorohydroquinone oxidase activity which was 267 % stimulated by protamine (Table I). No outer mitochondrial membrane was detected in the nuclear fractions using monoamine oxidase as an outer membrane marker. Microsomal contaminating protein averaged 1.1 % when estimated by the microsomal enzyme NADPH–cytochrome *c* reductase.

Enzymes endogenous to nuclei

The activity of an enzyme in the nuclear preparations due to mitochondrial or microsomal contaminating protein is calculated by multiplying the percentage protein contamination in the nuclear fraction by the activity of the particular enzyme in the contaminating fraction. Net nuclear activities are then obtained by subtracting the activities due to these contaminating proteins.

The results of such analysis reported in Table I indicates that only 16.3 % of NADH–cytochrome *c* reductase, 8.7 % of NADH oxidase, 17.3 % of a cytochrome *c* oxidase activity, 23.6 % of Mg^{2+}-stimulated ATPase and 6.9 % of glucose-6-phosphatase can be accounted for by mitochondrial and/or microsomal contamination.

Since the nuclear NADH–cytochrome *c* reductase was insensitive to rotenone, antimycin A and piericidin A, it appears to resemble microsomal NADH–cytochrome *c* reductase. The characteristic NADH : NADPH–cytochrome *c* reductase ratio of 20–50 in microsomal fractions becomes 200–400 in the nuclear preparations reflecting the only trace amount of NADPH–cytochrome *c* reductase in the nuclear preparations. The nuclear ATPase also resembles the microsomal ATPase by its insensitivity to oligomycin. This is in contrast to mitochondrial ATPase which is 90–95 % inhibited by oligomycin. The cytochrome *c* oxidase activity was 100 % inhibited by KCN (1 mM), NaN_3 (1 mM) or by CO.

TABLE I

EVALUATION OF ENZYMES IN ISOLATED BOVINE LIVER NUCLEI

The three succinate oxidase assays are averaged in calculating percent mitochondrial protein contamination. NADPH–cytochrome *c* reductase is used for percent microsomal protein contamination. The amount of enzyme activity due to contamination in the nuclei equals percent protein contamination times activity in mitochondria or microsomes. Net nuclear activity is obtained by subtracting these contamination activities. Activities for succinate, NADH, cytochrome *c*, and tetrachlorohydroquinone oxidases are expressed in μmoles O_2 per min per mg protein, cytochrome *c* reductases in μmoles cytochrome *c* reduced per min per mg protein, succinate–PMS reductase in μmoles dichlorophenolindophenol reduced per min per mg protein, monoamine oxidase in A change per 10 min per mg protein, and ATPase and glucose-6-phosphatase in μmoles P_i per 10 min per mg protein. Coenzyme Q is expressed in nmoles/mg protein.

Assay	*Mitochondrial activity*	*Microsomal activity*	*Nuclear activity*	*Mitochondrial or microsomal protein in nuclei* (%)	*Activity in nuclei due to mitochondria* (%)	*Activity in nuclei due to microsomes* (%)	*Net nuclear activity*
Mitochondrial markers							
Succinoxidase	0.18		0.002	1.2	100		0
Succinate–cytochrome *c* reductase	0.28		0.004	1.4	100		0
Succinate–PMS reductase	0.14		0.002	1.5	100		0
Monoamine oxidase (outer membrane)	0.58		0.00	0			0
Tetrachlorohydroquinone oxidase	0.035		0.000	0			0
+ protamine	0.093		0.000	0			0
Coenzyme Q	6.0		0.02	0.33			
Microsomal marker							
NADPH–cytochrome *c* reductase		0.080	0.0009	1.1		100	0
Enzymes endogenous to nuclei							
NADH–cytochrome *c* reductase		4.0	0.27			16.3	0.23
NADH:NADPH cytochrome *c* reductase ratio		49.8	300				
NADH oxidase	0.31		0.049		8.7		0.045
Cytochrome *c* oxidase	0.52		0.042		17.3		0.035
Glucose-6-phosphatase		4.73	0.67			7.9	0.62
Mg^{2+}-stimulated ATPase	1.93	3.75	0.29		9.2	14.4	0.22

The comparison of nuclear and mitochondrial NADH oxidases

Ratio of oxidase activities. In mitochondria the NADH:succinate:cytochrome *c* oxidase ratio is about 1:0.6:1.7, whereas in nuclei it is approx. 1:0.04:0.9. The comparatively very low amount of succinate oxidase in the nuclear fraction suggests its presence as mitochondrial contamination and further rules out the possibility of histone inhibition, which if operative, should also inhibit NADH oxidase and cytochrome *c* oxidase activity in the nuclear fractions. After subtracting oxidase activities due to mitochondrial contamination, the NADH:cytochrome *c* oxidase ratio is 1.3 for nuclei as compared to 0.59 for mitochondrial fractions.

Effect of exogenous cytochrome c. Mitochondria show a considerable NADH activity in the absence of exogenous cytochrome *c*, although adding cytochrome *c* stimulates activity 6–25 times (Table II). In contrast, nuclear NADH oxidase is cytochrome *c* dependent. All the NADH oxidase in the absence of added cytochrome *c*

TABLE II

EFFECT OF CYTOCHROME *c* ON NADH OXIDASE ACTIVITY

Nuclei and mitochondria were assayed in the absence and presence of cytochrome *c* (1.0 mg for mitochondria, 8.0 mg for nuclei). The nuclear NADH oxidase activities were corrected for mitochondrial contamination as described in Table I. Activities expressed in μmoles O_2 per min per mg protein.

Fraction	*NADH oxidase specific activity (−cytochrome c)*	*NADH oxidase specific activity (+cytochrome c)*	*Stimulation due to cytochrome c*
Preparation 1			
Mitochondria	0.020	0.37	18.6-fold
Nuclei	0.000	0.054	Cytochrome *c* dependent
Preparation 2			
Mitochondria	0.059	0.36	6.2-fold
Nuclei	0.000	0.042	Cytochrome *c* dependent

TABLE III

CYTOCHROME *c* STIMULATION OF NADH OXIDASE ACTIVITY IN NUCLEI, MITOCHONDRIA AND A MIXED FRACTION CONTAINING EQUAL PROTEIN AMOUNTS OF NUCLEI AND MITOCHONDRIA

The calculated nuclei–mitochondria activities were obtained by adding the nuclei and mitochondria activities together and dividing by two. Activities are expressed in μmoles O_2 per min per mg protein.

Addition of cytochrome c (mg)	*NADH oxidase activity*			
	Nuclei	*Mitochondria*	*Observed nuclei–mitochondria*	*Calculated nuclei–mitochondria*
0.1	0.013	0.24	0.12	0.12
0.2	0.022	0.29	0.14	0.16
0.4	0.028	0.34	0.17	0.17
1.0	0.035	0.40	0.23	0.22
2.0	0.045	0.43	0.25	0.24
4.0	0.049	0.40	0.26	0.23
8.0	0.054	0.41	0.27	0.23

can be attributed to mitochondrial contamination (Table II). Nuclear NADH oxidase also needs more than 10 times as much cytochrome *c* per mg protein for maximal activity (Table III). It is possible that the higher concentration of cytochrome *c* needed for maximal activity is due to the initial binding of large amounts of cytochrome *c* into a non-functional state by the nuclei. This possibility was tested by comparing NADH oxidase activities in a mixed fraction of nuclei and mitochondria with the activities in the separate nuclear and mitochondrial fractions. This study, performed at various cytochrome *c* concentrations, shows no appreciable differences between the actual activities of the mixed fraction and activities calculated for the mixed fraction from the separate nuclear and mitochondrial fractions (Table III).

Effect of electron transport inhibitors. Both nuclear and mitochondrial fractions behave similarly in that they are sensitive to rotenone, antimycin A and piericidin A in the absence of cytochrome *c* (Table IV) where all the nuclear NADH oxidase is mitochondrial (Table II). In the presence of added cytochrome *c*, the inhibitors have

TABLE IV

EFFECT OF MITOCHONDRIAL ELECTRON TRANSPORT INHIBITORS IN THE PRESENCE AND ABSENCE OF 4 mg OF CYTOCHROME *c* ON NADH OXIDASE ACTIVITY

Activities are expressed in μmoles O_2 per min per mg protein. 10-μl amounts of each inhibitor were added to the assay mixture to a final concentration as indicated below.

Addition	*Inhibition* (%)			
	Nuclear fraction		*Mitochondrial fraction*	
	−Cytochrome c	*−Cytochrome c*	*−Cytochrome c*	*+Cytochrome c*
Piericidin A (0.28 μg/ml)	72.4	4.5	84.2	8.7
Rotenone (2.77 μM)	73.1	5.2	76.3	6.4
Antimycin A (2.8 μg/ml)	81.2	3.7	78.5	6.4
KCN (1 mM)	100	100	100	100
NaN_3 (1 mM)	100	100	100	100

TABLE V

EFFECT OF DEOXYRIBONUCLEASE ON OXIDASE ENZYMES OF NUCLEI AND MITOCHONDRIA

Nuclei and mitochondria at a protein concentration of 10.0 mg/ml were digested with 50 μg deoxyribonuclease per ml. Digestion was allowed to proceed 1 h at 30°. Digestion was stopped by centrifugation and washing of the obtained pellets two times to get rid of residual deoxyribonuclease and subsequently assayed along with control samples kept at the same temperature for the same time period.

Fraction	*Inhibition* (%)		
	NADH oxidase	*Cytochrome c oxidase*	*Succinoxidase*
Preparation 1			
Nuclei	40.5	41.7	6.5
Mitochondria	5.9	7.2	4.2
Preparation 2			
Nuclei	44.5	50.2	4.5
Mitochondria	3.8	2.8	3.6

much less effect (Table IV). Both fractions in the presence and absence of cytochrome *c* are fully inhibited by CN^- and N_3^-.

Effect of deoxyribonuclease. Deoxyribonuclease has little effect on mitochondrial NADH, succinate, or cytochrome *c* oxidase activity (Table V), while deoxyribonuclease treatment inhibits the nuclear NADH and cytochrome *c* oxidases by some 40–60 %. In contrast, succinoxidase of the nuclear fraction is not inhibited by the deoxyribonuclease treatment. This is to be expected if the succinoxidase is of mitochondrial origin and if the mitochondrial oxidases present in the nuclear fraction are not inhibited by deoxyribonuclease. The inhibiting effect of deoxyribonuclease on nuclear respiratory activity has been previously reported[23], and has been used as a distinguishing characteristic between nuclear respiration and mitochondrial respiration, which is not affected by deoxyribonuclease.

Effect of histones. It has been reported that nuclear NADH oxidase is more resistant to histone inhibition than mitochondrial NADH oxidase[5,6]. Table VI shows the effect of various amounts of purified histone on the NADH oxidase of mitochondrial and nuclear fractions. At a histone/protein fraction ratio of 0.56, the mitochondrial NADH oxidase is fully inhibited, while the nuclear NADH oxidase is inhibited to the extent of only 28.9 %. Results with a mixed fraction of nuclei and mitochondria indicates that the nuclear fraction is not interferring with histone inhibition in mitochondria (Table VI).

Blockage and reversal of histone inhibition. Mitochondrial polycationic inhibition of cytochrome *c* oxidase is reversible and can be blocked by the addition of polyanionic molecules such as DNA, polyethylene sulfonate, heparin, and polyacrylic acid[22]. Table VI shows the relative protection which polyacrylic acid gives when various amounts of histone are added to the NADH oxidase assay. Polyacrylic acid acts as an effective blocker of histone inhibition at all levels of histone added in the nuclear, mitochondrial and the mixed mitochondrial nuclear fraction. Reversibility of histone inhibition with polyacrylic acid is presented in Table VII. Once again reversal of histone inhibition appears similar in the mitochondrial, nuclear and mixed fractions.

TABLE VI

INHIBITORY EFFECTS OF HISTONE ON NADH OXIDASE AND THE EFFECT OF POLYACRYLIC ACID ON BLOCKAGE OF HISTONE INHIBITION IN NUCLEI, MITOCHONDRIA AND A MIXED FRACTION CONTAINING EQUAL PROTEIN AMOUNTS OF MITOCHONDRIA AND NUCLEI

Histone added per mg protein fraction	*Inhibition (%)*					
	Mitochondria		*Nuclei*		*Mitochondria and nuclei*	
	NADH oxidase	*NADH oxidase in presence of 1.0 mg polyacrylic acid*	*NADH oxidase*	*NADH oxidase in presence of 1.0 mg polyacrylic acid*	*NADH oxidase*	*NADH oxidase* in presence of 1.0 mg polyacrylic acid*
0.28	82.9	1.4	22.4	9.4	4.2	8.9
0.56	100	1.4	28.9	15.6	75.9	6.2
1.12	100	1.4	61.9	6.2	91.7	6.2
2.24	100	1.4	79.4	6.2	100	6.2

* 1.0 mg polyacrylic acid alone gives 0.0–7.0 % inhibition of nuclear or mixed fractions and 0.0–5.0 % activation of mitochondrial oxidase.

TABLE VII

EFFECT OF POLYACRYLIC ACID ON THE REVERSIBILITY OF HISTONE INHIBITION OF NADH OXIDASE IN NUCLEI, MITOCHONDRIA AND A MIXED FRACTION CONTAINING EQUAL PROTEIN AMOUNTS OF MITOCHONDRIA AND NUCLEI

Histone added per mg protein fraction	*NADH oxidase (% inhibition)*		
	Mitochondria	*Nuclei*	*Mitochondria and nuclei*
0.28	82.9	22.4	4.2
+ 1 mg polyacrylic acid	0.0	4.1	16.9
0.56	100	28.9	75.9
+ 1 mg polyacrylic acid	1.4	0.0	42.1
1.12	100	61.9	91.7
+ 1 mg polyacrylic acid	6.0	21.4	34.3
2.24	100	79.4	100
+ 1 mg polyacrylic acid	7.9	12.2	25.6

DISCUSSION

These studies confirm previous reports of oxidative enzymes in mammalian liver nuclei. Under conditions in which slight contamination with microsomes and mitochondria are carefully evaluated, rotenone, antimycin A, piericidin A-insensitive NADH–cytochrome *c* reductase, NADH oxidase, cytochrome *c* oxidase, glucose-6-phosphatase and Mg^{2+}-stimulated ATPase are shown to be of nuclear origin. Preliminary reports by this laboratory[10,12] have further localized the above enzymes in a nuclear membrane fraction, results consistent with the investigations of ZBARSKY *et al.*[7] and KUZMINA *et al.*[9] on electron transport enzymes in isolated rat liver nuclear membranes.

The presence of a nuclear glucose-6-phosphatase confirms histochemical studies indicating a concentration of glucose-6-phosphatase on the nuclear envelope[24–26] and is consistent with biochemical studies[8,11] showing the presence of glucose-6-phosphatase in nuclear membrane preparations. It is clear that the nuclear envelope cannot be considered as microsomal contamination because of its specific position surrounding the nucleus where it may not only serve as a barrier between nucleus and cytoplasm but as a regulator of nucleo-cytoplasmic interactions, as well as be intimately involved in various nuclear specific functions, such as DNA replication[27].

Since the nuclear membrane contains glucose-6-phosphatase, its use as a marker enzyme for microsomal contamination is meaningless. Fortunately, however, we have been able to measure microsomal contamination enzymatically using NADPH–cytochrome *c* reductase as a marker enzyme[11]. The point to be stressed in these contexts is not whether nuclear envelope is "microsomal" but that it forms an integral part of the functioning nucleus.

The nuclear NADH oxidase system is distinguished from mitochondrial NADH oxidase by its susceptibility to deoxyribonuclease inhibition, its greater resistance to histone inhibition, its absolute dependency on cytochrome *c* for activity, the some 10 times higher concentration of cytochrome *c* needed for maximum activity, its insensitivity to the electron transport inhibitors rotenone, amytal, antimycin A, and

piericidin A and the absence of coenzyme Q, a component of the electron transport system of the inner mitochondrial membrane. The nuclear NADH oxidase, as well as the cytochrome *c* oxidase, however, are completely inhibited by KCN, NaN_3 or CO and show a similar pattern of blockage and reversal of histone inhibition as the mitochondrial oxidase.

Protamine stimulation of tetrachlorohydroquinone oxidase activity in liver mitochondrial preparations was previously reported by MOURY AND CRANE[28]. MACHINIST AND CRANE[20] postulated that the addition of tetrachlorohydroquinone to protamine results in a model cytochrome *c* compound and that the cytochrome *c* oxidase system is responsible for the oxidation of tetrachlorohydroquinone. Both CONOVER AND SIEBERT[3] and PENNIALL and co-workers[5,6] have reported the absence of cytochromes $a + a_3$ in liver nuclei. Spectral studies in this laboratory, of a nuclear membrane fraction in which the cytochrome *c* oxidase activity is concentrated some 5–7-fold, revealed no cytochrome $a + a_3$[29]. Thus nuclear cytochrome *c* oxidase activity differs from its mitochondrial counterpart in its inability to oxidize tetrachlorohydroquinone in the presence of protamine and an apparent absence of cytochromes $a + a_3$.

One of the unsolved problems concerning the NADH oxidase system in microsomes is the path of electrons after cytochrome b_5. SOTTOCASA[30] has suggested that O_2 is the terminal electron acceptor and the low rate of NADH oxidase in microsomes may be due to control mechanisms in the chain. The investigations of LÉVY *et al.*[31] on the outer mitochondrial membrane NADH oxidase system and ours on nuclear NADH oxidase lend support to this view. CONOVER AND SIEBERT[3] have suggested that electron transport systems in nuclei may be caused by the fortuitous interaction of contaminating enzymes. Our results suggest that the O_2 consuming nuclear NADH oxidase may be a form of a rotenone-insensitive NADH oxidase system which is also found on microsomal and outer mitochondrial membranes. The ability of both nuclear NADH oxidase and outer mitochondrial membrane NADH oxidase[31] to consume O_2 *via* a microsomal-like NADH–cytochrome *c* reductase in the presence of added cytochrome *c*, may be a reflection of specific functional roles played by the nuclear envelope and outer mitochondrial membrane as compared to the endoplasmic reticulum.

ACKNOWLEDGEMENT

The research has been supported by the National Institute of General Medical Science under Grant GM 10741. F.L.C. is supported by Career Grant K6-21,839 and R.B. by Training Grant 5T1 GM1195. The technical assistance of Joan Jackewicz, Chandra Kanta Awasthi and Dennis L. Kambs and the coenzyme Q determinations by Dr. Rita Barr are gratefully acknowledged.

NOTE ADDED IN PROOF (Received September 1st, 1970)

Recently we have detected some tetrachlorohydroquinone oxidase activity in purified nuclear membrane preparations.

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