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Abstract

Changes of H+ gradient at various energy states of mitochondria were studied. There was a close relation between the extent of H+ gradient and the level of ATP formation; the former decreased as a result of ATP synthesis but was not completely abolished. A partial depression of H+ gradient was also observed in the presence of uncouplers of oxidative phosphorylation. The H+ gradient seemed to be more closely related to the ion translocation than ATP formation. In the presence of Ca++ the energy of H+ gradient was utilized in translocating Ca++ rather than synthesizing ATP. These findings further substantiate the chemiosmotic theory of MITCHELL on mitochondrial electron and energy transfer.

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CHANGE OF PROTON GRADIENT IN MITOCHONDRIA AT VARIOUS ENERGY STATES

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According to the chemiosmotic hypothesis of MITCHELL (1-3), there must exist a potential difference between the mitochondrial membranes, or a gradient of concentration in H^+ or some other ions between inside and outside of mitochondria in order to accomplish ATP formation. In a previous paper (4) we have described that there are three kinds of H^+ translocations between inside and outside of mitochondria, namely: (a) H^+ displacement dependent on ATP formation, (b) H^+ transfer dependent on electron transport, and (c) H^+ change dependent on oxidation-reduction of respiratory chain components.

Of these H^+ transfer processes, the second one is related to chemiosmotic H^+ transfer reaction, but to obtain data similar to those observed by MITCHELL (1-3) rat liver mitochondria require treatment with a small amount of Triton X-100 or Ca^{++}. Under the experimental conditions of Mitchell, this H^+ gradient would be discharged without ATP formation due to anaerobiosis. This is because under these conditions mitochondrial Ca^{++}, which accumulated at the expense of respiratory energy, would be released to external medium in exchange of H^+ uptake by mitochondria during the aerobic-anaerobic transition. Introduction of an oxygen pulse would reverse the process, *i. e.*, Ca^{++} would be taken up by mitochondria in exchange of H^+ release.

Treatment of mitochondria with Triton X-100 also caused a discharge of H^+ gradient, but the gradient was restored upon introduction of an oxygen pulse (4). This phenomenon is difficult to explain, but it is possible that in mitochondria treated with a small amount of Triton X-100 the respiration-dependent H^+ transfer may be related to translocation

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of K^+ or some other ions. Therefore, it is considered that the oxygen-dependent H^+ gradient in both cases (*i. e.*, mitochondria treated either Ca^{++} and Triton) is closely related to ion translocation. Even though such a correlation exists, an H^+ gradient, as suggested by the chemiosmotic hypothesis, must occur between the inside and outside of mitochondria in order to achieve ATP formation.

In this study we undertook to determine the extent of H^+ gradient between the inside and outside of mitochondria at various energy states, so as to clarify whether H^+ gradient, indeed, is required to carry out ATP formation. In this report we describe that there is a correlation between ATP formation and H^+ gradient, and that in order to generate ATP the mitochondrial membranes must be intact and capable of maintaining an H^+ gradient between the inside and outside without an energy supply, *e. g.* from respiration or ATP hydrolysis.

MATERIALS AND METHODS

Rat liver mitochondria were isolated in a medium containing 0.33 M sucrose and 1 mM Tris-EDTA (pH 7.4) as decribed previously (5). Proton translocation in mitochondria was studied in a medium (unless otherwise described) containing 0.15 M choline chloride adjusted to pH 7.4 by using a minimum amount of NaOH or HCl. Reactions were carried out at 25° in an 8-ml volume. Oxygen pulse was introduced by injection of either an aliquot of fresh reaction mixture or hydrogen peroxide into the anaerobic incubation mixture; in the latter case catalase was pre-added. Protein was determined by the method of LOWRY *et al.* (6).

Hexokinase, catalase and oligomycin were obtained from Sigma Chemical Company. Other reagents used were of reagent grade.

RESULTS

Respiration dependent proton movement: As shown in Fig. 1, three kinds of proton movements may be recognized: (a) under anaerobic conditions fresh mitochondria took up H^+ through ATP formation; fresh mitochondria also took up a large amount of H^+ upon addition of a small amount of Triton X-100, (b) in the presence of a small amount of Triton X-100, incubated mitochondria released a large amount of H^+, but these protons were readily taken up by mitochondria at the aerobic-anaerobic transitions, and (c) in the presence of a high concentration of Triton X-100, respiration-dependent H^+ uptake was observed. These H^+ changes were dependent on oxidation-reduction states of respiratory chain components, *e. g.* pyridine nucleotides, flavins and cytochromes.

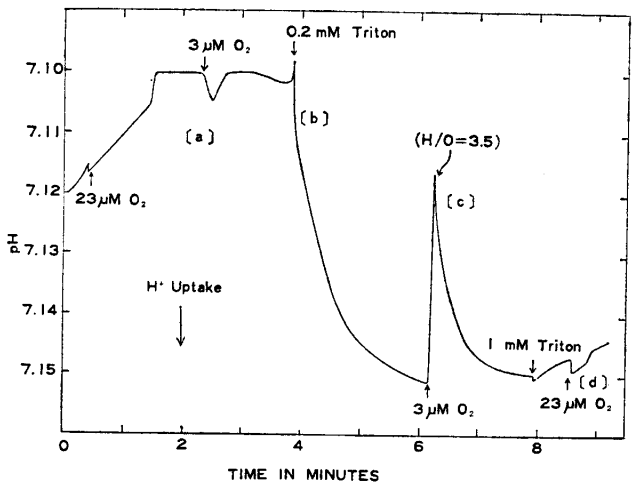
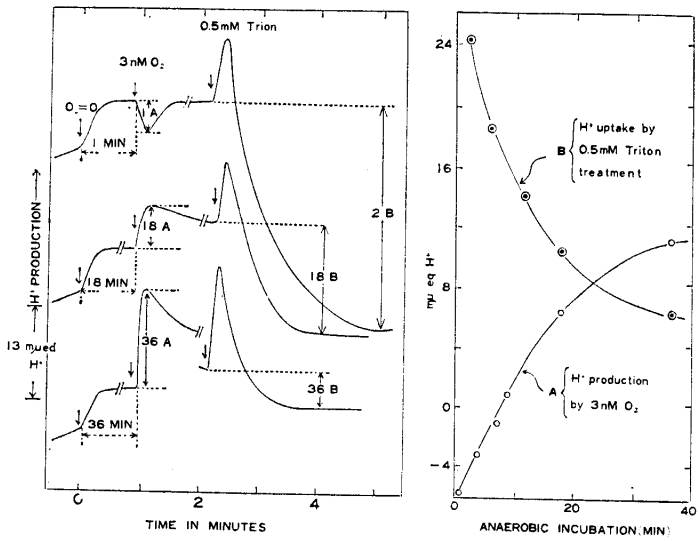


Figure 1. Profile of H^+ change induced by oxygen pulse under different conditions of mitochondria membrane. Mitochondria (1.5 mg protein/ml) was incubated in a medium of 150 mM choline chloride, 2 mM succinate and 20 units of catalase, pH 7.4. Oxygen pulse was introduced by injection of either an aliquot of fresh reaction mixture or hydrogen peroxide into the anaerobic incubation mixture. Different conditions for mitochondrial membrane were brought about by adding various concentrations of Triton X—100. The following explanations are pertinent: (a) intact mitochondria under anaerobic conditions take up H^+ upon receiving an oxygen pulse but depending upon the occurrence of ATP formation; (b) intact mitochondria under anaerobic conditions take up a large amount of H^+ upon treatment with a small amount of Triton X—100, which disrupts H^+ gradient; (c) impaired mitochondria release a large amount of H^+ upon receiving an oxygen pulse, but the gradient is readily destroyed by the aerobic-anaerobic transition; and (d) disrupted mitochondria produced by treating with a large amount of Triton X—100 take up H^+ upon receiving an oxygen pulse depending upon the reduction of respiratory components.

Proton gradient in mitochondria: When mitochondria were incubated under anaerobic conditions, H^+ uptake upon treating with a small amount of Triton X-100 gradually decreased with incubation time. This indicates that H^+ gradient between the inside and outside of mitochondria decreases as permeability of mitochondria to H^+ increases. The following observations support this assumption. When the oxygen pulse was introduced to a suspension of mitochondria incubated for various time lengths under anaerobic conditions, H^+ uptake which initially occurring in fresh mitochondria was reversed to H^+ release. This release of H^+ increased with the time of incubation (Figs. 2A and B). We also found that H^+ gradient of fresh mitochondria upon treatment with a small amount of Triton X-100 depended upon protein concentrations of mitochondria. As shown in



(*A*) (*B*)

Figure 2 *A* and *B*. Relationship between H^+ production following oxygen pulse and H^+ uptake following Triton X—100 treatment for various incubation time under anaerobic conditions. *A*—Mitochondria (3.5 mg protein/ml) were incubated in a medium of 250 mM sucrose. Ten mM KCl, 10 mM $MgCl_2$ and 3 mM succinate, pH 7.4. Oxygen pulse was 6 mμ atoms/ml. Anaerobic incubation was carried out for various time periods. *B*—Values plotted were obtained from the experiments of Fig. 2A.

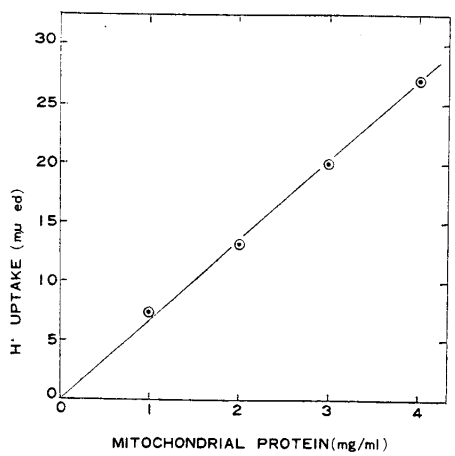


Figure 3. Relationship between mitochondrial protein concentrations and H^+ uptake following Triton X—100 treatment. Mitochondria were incubated in choline chloride medium (as of Fig. 1) and upon anaerobiosis H^+ uptake was measured following addition of 3 mM Triton X—100.

Fig. 3, H^+ gradient of fresh mitochondria upon treatment with Triton X-100 manifested a linear relationship with mitochondrial protein concentrations.

Change of proton gradient due to ATP formation: If mitochondrial H^+ gradient represents an energy source for ATP formation, H^+ gradient formed between the inside and outside of mitochondria must be discharged upon addition of ADP and Pi. In order to compare the H^+ gradient of mitochondria before and after the formation of ATP, we performed the three following experiments:

(a) As shown in Fig. 4, H^+ gradient in the presence of Pi and Mg^{++} with or without addition of oligomycin served as the control experiment. Oligomycin always increased H^+ gradient due to its inhibition of both ATP formation and latent ATPase activity.

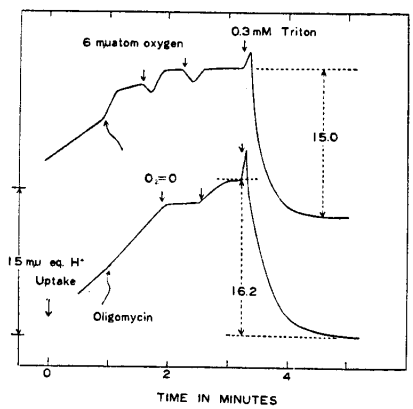


Figure 4. Mitochondrial H^+ gradient under nonphosphorylating conditions. Basic conditions were as in Fig. 1 except for mitochondrial protein (2 mg/ml). The proton release increased slightly due to inhibition of endogenous ATP formation by oligomycin. Concentrations of additions are indicated in the figure.

(b) As shown in Fig. 5, in the presence of ADP, Pi and Mg^{++} mitochondria upon treating with Triton X-100 released H^+ instead of taking up H^+. This was due to stimulation of latent ATPase activity by Triton X-100. Oligomycin inhibited this H^+ production. Actual change of H^+ gradient caused by ATP formation, therefore, could not be detected from this experiment.

(c) To avoid the interference of ATPase activity, glucose-hexokinase system was introduced to trap the ATP formed during incubation. Fig. 6 shows H^+ gradient of mitochondria under conditions of ATP formation

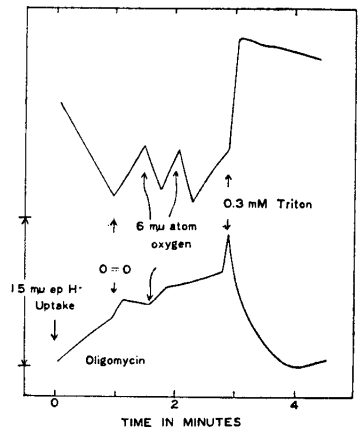


Figure 5. Change of mitochondrial H^+ gradient following Triton X—100 treatment under conditions of ATP formation. Basic conditions were as in Fig. 2. Other additions were 0.3 mM ADP, 0.3 mM phosphate, 1 mM $MgCl_2$ and 1 μg oligomycin/mg protein. Under conditions of ATP formation, ATPase activity was stimulated by Triton X—100 treatment. Therefore, this system was not suitable for measuring H^+ gradient.

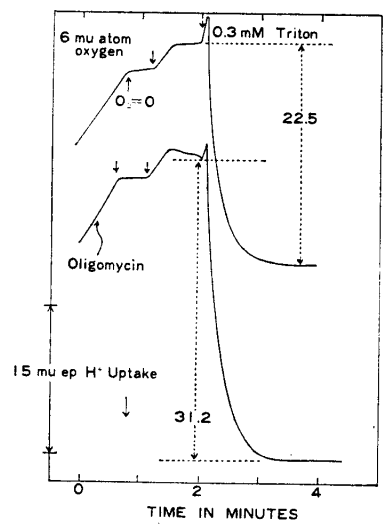


Figure 6. Change of mitochondrial H^+ gradient; effect of oligomycin. Basic conditions were as in Fig. 1. ATP formation was carried out in the presence of 0.3 mM ADP, 0.3 mM phosphate, 1 mM $MgCl_2$, 20 mM glucose and 40 units of hexokinase. Oligomycin concentration was 1 μg/mg protein. A large change of H^+ gradient was observed in the presence of oligomycin which inhibited ATP formation.

and inhibition of ATP formation by oligomycin. It was observed that H^+ gradient still existed during ATP formation and that the gradient was larger if the system contained oligomycin. The observations are further substantiated in Table I. It should be pointed out that hexokinase used in these systems was dissolved in ammonium sulfate the presence of which might influence H^+ gradient observed under conditions of ATP formation. The increase of H^+ gradient upon addition of oligomycin was significantly higher for a system that contained an equivalent amount of ammonium sulfate compared to one that did not contain this chemical. For example, H^+ gradient was 11.25 mμeq/mg for an ATP forming system, but it was 15.6 mμeq/mg protein for a similar system receiving oligomycin-supplemented system. Although we observed a considerable H^+ gradient retained during ATP formation, our experimental results did not rule out the basis of chemiosmotic hypothesis, since mitochondrial H^+ gradient always increased upon addition of oligomycin.

TABLE I EFFECT OF ATP FORMATION ON MITOCHONDRIAL PROTON GRADIENT

MITOCHONDRIA (2 mg PROTEIN/ml) WERE INCUBATED IN A MEDIUM OF 150 mM CHOLINE CHLORIDE AND 5mM SUCCINATE, pH 7.4. ATP SYNTHESIS WAS CARRIED OUT IN THE PRESENCE OF 0.3 mM ADP, 0.3 mM PHOSPHATE, 1 mM $MgCl_2$ 20 mM GLUCOSE AND 40 UNITS OF HEXOKINASE (HK). OTHER ADDITIONS AS SHOWN WERE 0.3 mM TRITON X—100 AND 1μg OLIGOMYCIN /mg PROTEIN

conditions			H^+ Uptake
ATP formation	Glucose and HK	Oligomycin	(mμeq/2 mg protein)
Not occurring	−	−	16.7
Not occurring	−	+	18.7
Occurring	−	−	−16.6*
Occurring	−	+	8.8
Occurring	+	−	24.0
Occurring	+	+	32.9

* Instead of H^+ uptake 16.6 mμeq H^+ was released.

On the basis of the above findings one may expect that uncouplers of oxidative phosphorylation would interfere with mitochondrial H^+ gradient. Fig. 7 shows the effect of 2, 4-dinitrophenol (DNP) on H^+ gradient of mitochondrial membranes upon treatment with Triton X-100. The gradient decreased in the presence of 0.1 mM DNP, a concentration at which oxidative phosphorylation was completely uncoupled. It may be reiterated that such a concentration of DNP did not abolish the mitochondrial H^+ gradient completely. Similar experiments were carried out in the presence of a number of uncouplers and results are summarized in

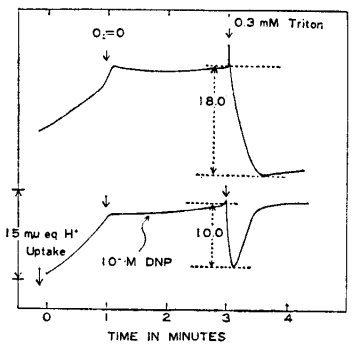


Figure 7. Change of mitochondrial H^+ gradient; effect of dinitrophenol. Basic conditions were as in Fig. 1 except for mitochondrial protein (2.5 mg/ml).

TABLE 2 EFFECT OF UNCOUPLERS ON TRITON X—100 INDUCED PROTON UPTAKE OF MITOCHONDRIA

BASIC CONDITIONS WERE THE SAME AS IN TABLE 1 EXCEPT FOR MITOCHONDRIAL PROTEIN (4 mg PROTEIN/ml)

Uncoupler	H^+ uptake (mμeq/4 mg protein)
None	49.5
DNP (10 μM)	40.7
DNP (100 μM)	22.0
Dicumarol (5 μM)	39.0
PCP (5 μM)	19.3
Ca^{++} (200 μM)	13.2
Gramicidin (0.03 μg/mg protein)	None*

* In the presence of gramicidin H^+ was released.

Table 2. The data obtained with most of the uncouplers, *e. g.*, dicumerol, pentachlorophenol (PCP) and Ca^{++}, were qualitatively similar to those obtained in the presence of DNP, and decrease of H^+ gradient was dependent upon concentrations of the uncouplers used.

According to CARFOLI *et al.* (7), both Triton X-100 and DNP decrease H^+ gradient, but the action of these two reagents do not follow the same mechanism. This has been exemplified in Fig. 8. Addition of Triton X-100 caused a decrease of H^+ gradient and the subsequent addition of DNP led to a further decrease, but if this second addition were of Triton X-100 instead of DNP, no further decrease of H^+ gradient would be observed. The situation was analogous, *i. e.*, DNP-mediated decrease of H^+ gradient received an additive effect if Triton X-100 instead of DNP was added to mitochondria for a second time.

With Ca^{++}, a typical translocatable ion, a remarkably greater de-

crease of H^+ gradient was observed. As shown in Table 2, under anaerobic conditions Ca^{++}-treated mitochondria had a small H^+ gradient and gramicidin-treated mitochondria had none. These mitochondria in the absence of Ca^{++}, however, had the ability to generate H^+ gradient when an oxygen pulse was introduced (Fig. 9). These data clearly show

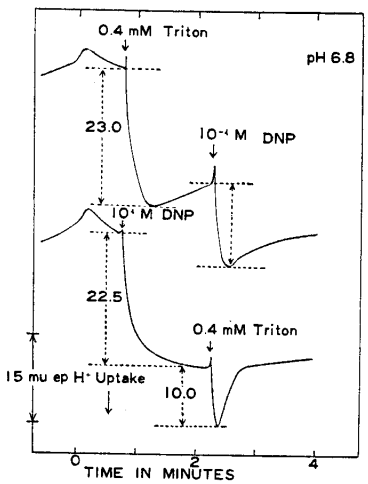


Figure 8. Combined effect of Triton X—100 and dinitrophenol on mitochondrial H^+ gradient. Basic conditions were as in Fig. 1 except for pH (6.8) and mitochondrial protein (2.5 mg/ml).

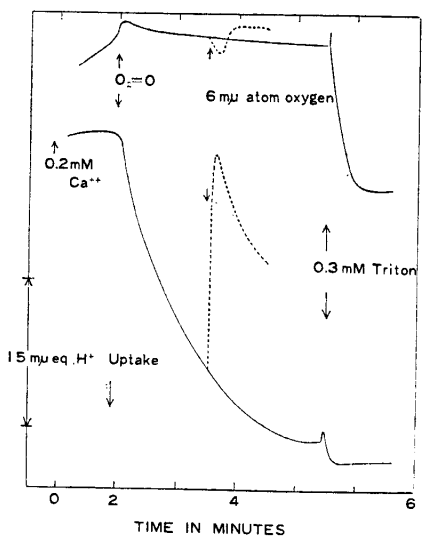


Figure 9. Effect of calcium ion on mitochondrial H^+ gradient. Basic conditions were as in Fig. 1. Under anaerobic conditions no H^+ gradient was observed.

that H^+ gradient under anaerobic conditions still existed to a considerable extent during ATP formation, but it collapsed almost completely during ion translocation. Thus, it is likely that H^+ gradient is more closely related to ion translocation than to ATP formation or any other form of energy transfer reaction.

DISCUSSION

It is well known from the work of MITCHELL (1-3) that freshly prepared mitochondria have a low permeability to H^+. Therefore, if mitochondria build an H^+ gradient through respiration, the gradient is maintained even under anaerobic conditions provided mitochondria do not undergo any energy transfer reactions, *e. g.* ion translocation. In the present study, we have isolated mitochondria using an EDTA-containing medium to ensure removal of Ca^{++}, if any, the uptake of which involves energy transfer reactions. These mitochondria, when freshly prepared, maintain an H^+ gradient even under anaerobic conditions. However, H^+ gradient is gradually discharged during a prolonged anaerobic incubation. This effect may be due to an impairment of mitochondrial membrane structure. Again, introduction of an oxygen pulse to mitochondria, which have lost H^+ gradient through aging, will readily generate the gradient, but it will be discharged immediately after exhaustion of oxygen. This suggests that mitochondrial permeability to H^+ increases upon aging or prolonged incubation under anaerobic conditions. It may be reiterated that introduction of an oxygen pulse to anaerobic mitochondria, which are structurally intact and maintain a large H^+ gradient, may not elevate the existing gradient any further. Oxygen pulse, of course, will restore H^+ gradient in anaerobic mitochondria which have lost the gradient but potentially are capable of sustaining it.

Production of H^+ and building of an H^+ gradient following administration of an oxygen pulse are observed not only in aged or loosely coupled mitochondria but also in Ca^{++}-loaded mitochondria. We have observed that in the presence of Ca^{++} mitochondria did not have any H^+ gradient even though they were freshly prepared. In contrast to this situation, H^+ gradient was not discharged at the aerobic-anaerobic transition if Ca^{++} were absent. Since Ca^{++}-translocation requires energy and upon anaerobiosis mitochondria release accumulated Ca^{++}, the separated protons are also discharged simultaneously. It is possible that the discharge of H^+ gradient in the Ca^{++}-supplemented system is a result of energy utilization for Ca^{++}-loading under anaerobic conditions. In order to

establish this possibility it would be necessary to follow the kinetics of H^+ disappearance from medium and Ca^{++} leakage from mitochondria. It should be mentioned that according to BALTOZAR *et al.* (8) the acid base gradient between mitochondria and suspending medium induced by Ca^{++}, *i. e.* the energy thereof, was not available for ATP formation. This gradient was utilized as energy source to maintain the level of translocated Ca^{++} in mitochondria. In fact, the energy utilization for Ca^{++} translocation was preferable to ATP formation (9). On the other hand, the accumulated Ca^{++} was available for release (as an energy source) in exchange of K^+ uptake in mitochondria in the presence of valinomycin (10, 11).

In addition to Ca^{++} effect on H^+ gradient, we have also observed a partial discharge of H^+ gradient in mitochondria either in the presence of uncouplers during aerobic respiration or during ATP formation under anaerobic conditions. Our findings, therefore, are in agreement with MITCHELL's hypothesis on mitochondrial electron and energy transfer (1-3).

SUMMARY

Changes of H^+ gradient at various energy states of mitochondria were studied. There was a close relation between the extent of H^+ gradient and the level of ATP formation ; the former decreased as a result of ATP synthesis but was not completely abolished. A partial depression of H^+ gradient was also observed in the presence of uncouplers of oxidative phosphorylation. The H^+ gradient seemed to be more closely related to the ion translocation than ATP formation. In the presence of Ca^{++} the energy of H^+ gradient was utilized in translocating Ca^{++} rather than synthesizing ATP. These findings further substantiate the chemiosmotic theory of MITCHELL on mitochondrial electron and energy transfer.

ACKNOWLEDGEMENT

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