Biochemical Pharmacology, Vol. 20, pp. 1443–1451. Pergamon Press, 1971. Printed in Great Britain

FUNCTIONAL AND STRUCTURAL CHANGES IN LIVER MITOCHONDRIA OF RATS DUE TO CCl_4 INTOXICATION—II

RESPIRATORY CHAIN AND ION TRANSPORT

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(*Received* 30 *June* 1970; *accepted* 12 *November* 1970)

Abtract—The localization of electron-transport chain damage of NAD-linked substrates in liver mitochondria of rats intoxicated with CCl_4 has been studied in this work. It is shown that in experimental toxic hepatic lesion slow $NADH_2$ generation occurs, which is the cause of a depressed rate of oxidation of α-ketoglutarate, glutamate and pyruvate. In addition, important structural changes in mitochondrial membranes have been observed and may be considered to be the cause of disturbance of ion transport. The possible mechanisms of the appearance of revealed alterations are also discussed. It is presumed that primary impairment of the mitochondrial phospholipids is manifested in animals intoxicated by CCl_4.

CURRENT investigations indicate that a variety of factors causing structural mitochondrial changes result in a disturbance of their functional activity. As it is known, high-amplitude swelling,[1,2] mitochondria treatment by detergents,[3] by fatty acids,[4,5] extraction of phospholipids[6,7] result in the alteration of energy-conserving reactions,[8,9] ion transport,[10] osmotic behaviour of mitochondria.[11]

While studying liver mitochondria metabolism following CCl_4 intoxication, some authors attribute the injuries to the action of this hepatotoxine on the membrane structures, revealed in electron microscope study.[12]

Analysing the available data concerning CCl_4 influence on mitochondria, it should be noted that evidence for the existence of the alteration of ATP synthesis[13,14] is convincingly presented. However, insufficient results have been published on the investigation of other liver mitochondria functions during acute experimental toxic hepatic lesion induced by CCl_4. For example, the work of Malamed and Recknagel,[15,16] devoted to the study of swelling, shows that liver mitochondria treatment with CCl_4 *in vitro* results in volume changes which cannot be prevented by respiratory inhibitors. However, these data are not fully applicable to the discussion of the CCl_4 mechanism of action *in vivo*. From the works on ion transport, the recent accurate reports by Cohn *et al.*[17] and Carafoli and Tiozzo[18] are quite noteworthy. The authors have demonstrated excessive accumulation of Ca^{2+} by liver mitochondria of rats due to CCl_4 intoxication.

This report presents results showing the presence of two limiting factors in the substrate oxidation chain and a description of some ion transport alterations and liver mitochondria volume changes in rats poisoned by CCl_4

EXPERIMENTAL

Animal intoxication and liver mitochondria isolation were carried out according to the pattern described earlier.[19] Respiration, recorded polarographically, pH-shift, registered by pH-meter ЛПМ-60М with the aid of a glass electrode, and absorption decrease, measured photometrically, were observed simultaneously in a 4·5-ml cuvette. The basic medium was described elsewhere.[19] Protein was determined by the Lowry method.[20] The details of the experiments are given in the legends to the figures.

RESULTS

It was previously supposed by us that the changes in the oxidation rates of succinate and NAD-linked substrates by liver mitochondria of rats intoxicated with CCl_4 were caused either by the action of CCl_4 or by parallel development of swelling during incubation of the mitochondria in salt medium. Therefore, to exclude the influence of structural mitochondrial changes induced by swelling, we injected ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) into the incubation medium. As can be seen from Fig. 1, the addition of EGTA stopped the decrease of optical density, but did not influence substrate oxidation by the liver mitochondria of poisoned rats. Thus, it is evident that under our experimental conditions the uncoupling of oxidative phosphorylation and inhibition of oxidation of NAD-linked substrates were not due to swelling in the incubation process.

Further investigations were carried out in order to localize the region limiting the oxidation of NAD-linked substrates. The results of the first communication[19] indicated a defect either in the first complex of the respiratory chain or in the primary dehydrogenases. We were therefore compelled to use substances shunting the first complex

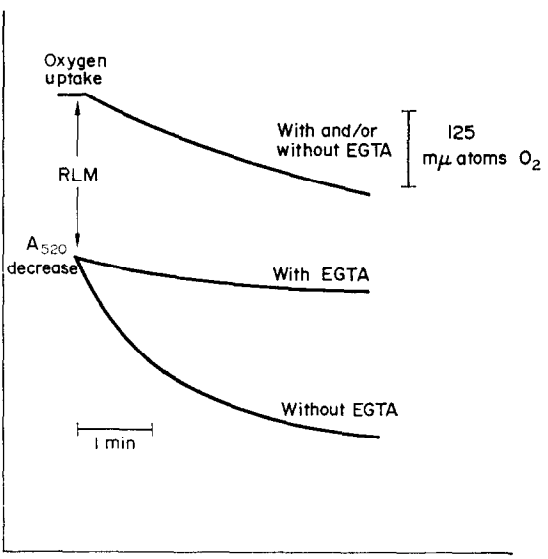

FIG. 1. The effect of EGTA on oxygen consumption by rat-liver mitochondria and mitochondrial swelling 24 hr after administration of CCl_4 (0·25 ml 100 g body wt.). The basic medium contained: 125 mM KCl, 20 mM tris buffer (pH 7·4), 5 mM α-ketoglutarate, 3 mM KH_2PO_4, 300 μM EGTA. The amount of mitochondrial protein was 3 mg. Final volume 3 ml. Temperature, 22°.

region. Figure 2 presents the experiment with the use of the oxidated form *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), so-called Wurster's blue which is known to accept the electrons from $NADH_2$ and pass them to the cytochrome $c_1 + c$.[21,22] Momentary respiratory acceleration, followed by slow O_2 consumption resulted from adding "Wurster's blue" to the mitochondria, which oxidize NAD-linked substrates.

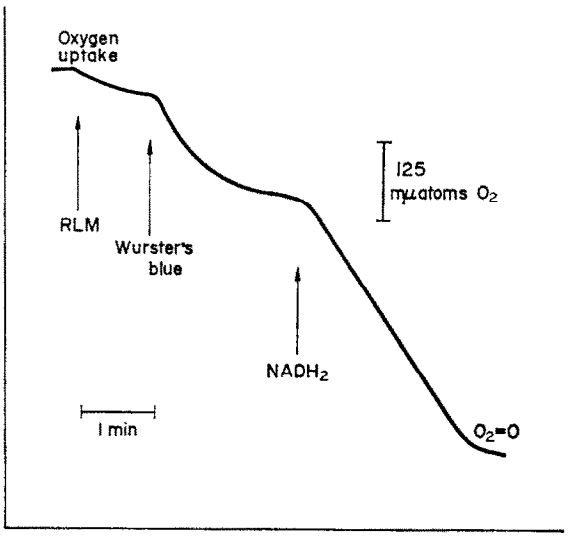

FIG. 2. The oxygen uptake by mitochondria of CCl_4-treated rats. The effect of addition of "Wurster's blue" and $NADH_2$ upon oxygen consumption. Basic medium and experimental conditions as described under Fig. 1, except EGTA. 50 μM "Wurster's blue", 3 mM $NADH_2$.

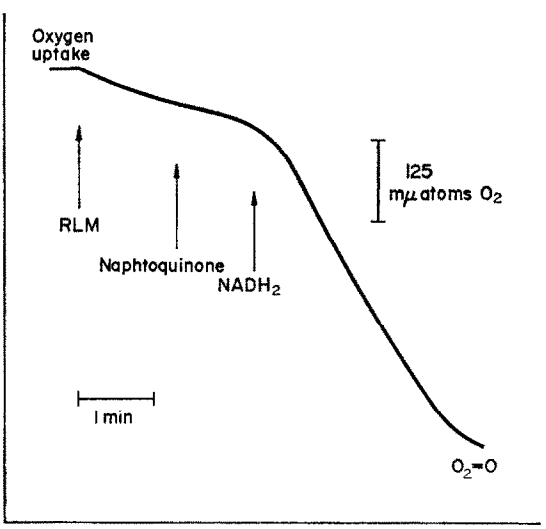

FIG. 3. The absence of by-pass effect of naphthoquinone on the liver mitochondria of poisoned rats which oxidate glutamate + malate. The basic medium contained: 125 mM KCl, 20 mM tris buffer (pH 7·4), 5 mM glutamate, 5 mM malate, 1 mM NAD, 3 mM KH_2PO_4. When added, 1 mM naphthoquinone, 3 mM $NADH_2$. Experimental conditions as in Fig. 1.

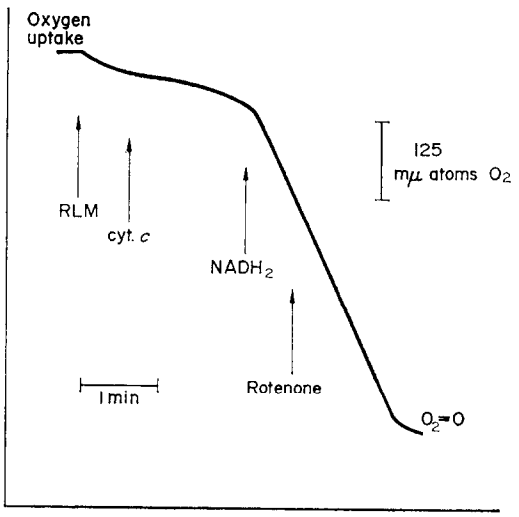

FIG. 4. The oxygen consumption during functioning of the rotenone-insensitive route. Conditions are the same as in Fig. 3. When added, 10 μM cytochrome *c*, 3 mM $NADH_2$, 2 μM rotenone.

Injection of $NADH_2$ into the medium caused rapid O_2 consumption and even its disappearance in the cuvette. After adding 2-methyl,1,4-naphthoquinone for shunting the transfer of the electrons between $NADH_2$ and coenzyme Q,[23] into the incubation medium, respiratory acceleration of the NAD-linked substrates due to oxidation was not observed (Fig. 3). The introduction of $NADH_2$ under these conditions induced acceleration of respiration that was not sensitive to rotenone.

Besides, these mitochondria were able to oxidate exogenetically added $NADH_2$ on the external route through cytochrome b_5 (Fig. 4).

In poisoned rat liver mitochondria preparations, which oxidize succinate or NAD-linked substrates, respiratory acceleration was not usually observed after adding ADP, which is indicative of total absence of respiratory control. However, addition of Ca^{2+} induced respiratory acceleration, but only when succinate was the substrate. These data are in accord with the results of Carafoli and Tiozzo.[18] Figure 5 demonstrates that added Ca^{2+} is rapidly accumulated by CCl_4-treated mitochondria, this process being accompanied by H^+ ejection into the medium and respiratory and swelling acceleration. The ratio $H^+ : Ca^{2+}$ was close to 1·0 and did not differ for intact mitochondria. Ejection of protons in response to Ca^{2+} addition was quickly replaced by their reverse movement whereas subsequent addition of EGTA brought the reappearance of the protons in the medium. It should be noted that the reverse movement process of H^+ in mitochondria under experiment was faster than in intact liver mitochondria. EGTA addition to liver mitochondria of rats poisoned by CCl_4, which oxidate NAD-linked substrates, resulted in the appearance of protons in the medium, but this had not been observed in similar experimentation with intact mitochondria (Fig. 6).

Investigation of the alteration of mitochondrial volume revealed considerable differences between liver mitochondria behaviour of poisoned and intact rats. CCl_4-treated mitochondria swell very quickly in a medium containing 125 mM KCl when

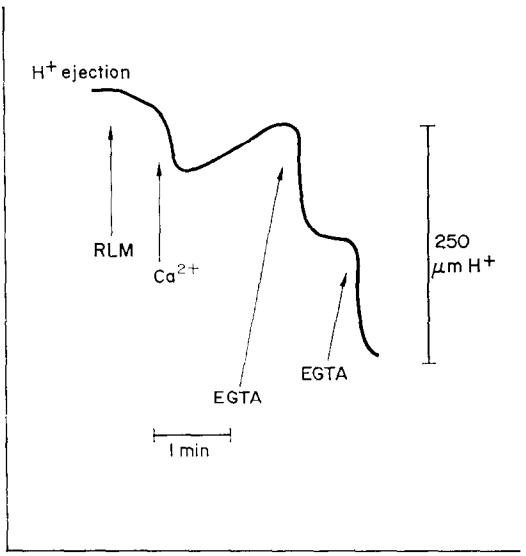

FIG. 5. The proton movement after addition of Ca^{2+} to mitochondria obtained from CCl_4-poisoned rats. The basic medium: 125 mM KCl, 20 mM tris buffer (pH 7·4), 5 mM succinate, 5 mM KH_2PO_4. Additions: 100 μM Ca^{2+}, 200 μM EGTA. The amount of mitochondrial protein was 6 mg. Final volume 4 ml. Temperature, 22°.

inorganic phosphate alone is added (Fig. 7a), whereas inorganic phosphate induces small suspension absorbtion decrease of intact rat liver mitochondria isolated with EDTA. As Fig. 7b demonstrates, intact mitochondria swelling is observed only when Ca^{2+} is added. It should be emphasized that "phosphate-induced" swelling of CCl_4-treated mitochondria had less amplitude than "Ca^{2+}-induced" swelling of intact

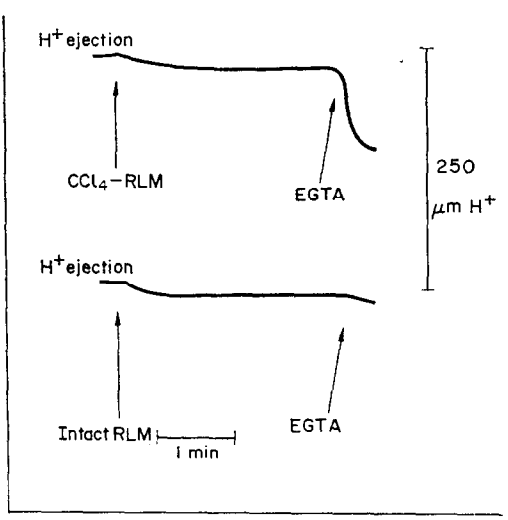

FIG. 6. The ejection of the endogenous Ca^{2+} from liver mitochondria of the CCl_4-poisoned rats during incubation. Conditions are the same as in Fig. 5, except 5 mM α-ketoglutarate + 1 mM NAD was used as a substrate. Addition, 200 μM EGTA.

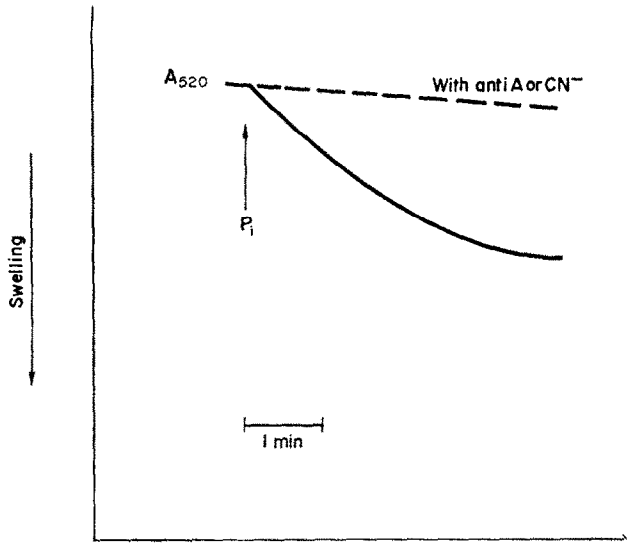

FIG. 7a. The large amplitude swelling of CCl_4-treated mitochondria, induced by P_i. Basic medium: 125 mM KCl, 20 mM tris buffer (pH 7·4), 5 mM succinate, 2 μM rotenone. Addition, 5 mM KH_2PO_4. When inhibitors were used, 4 μg antimycine A, 2 mM NaCN. Amount of mitochondrial protein was 3 mg. Final volume 3 ml. Temperature, 22°.

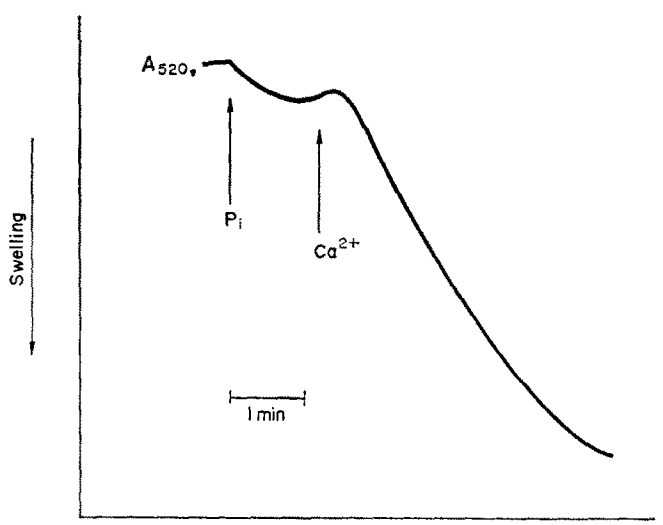

FIG. 7b. The large amplitude swelling of intact rat-liver mitochondria, induced by Ca^{2+} in presence of P_i. Conditions are the same as in Fig. 7a, except amount of mitochondrial protein was 2 mg. Additions, KH_2PO_4 5 mM, Ca^{2+} 100 μM.

mitochondria with inorganic phosphate as an admixture. Transport of ions and water, causing volume increase of CCl_4-treated mitochondria depended on the prevailing energy and was blocked by antimycine A or cyanide.

DISCUSSION

The results of the studies on the electron-transport chain of liver mitochondria of rats intoxicated by CCl_4 presented in this paper and in the previous report[19] are indicative of the presence of two limiting factors in substrate oxidation. One of them is partial loss of cytochrome *c*, inducing respiratory rate decrease in both succinate and NAD-linked substrate utilization.

It is to be pointed out that CCl_4 in a dose of 0·25 ml CCl_4 per 100 g body wt. administered to rats usually causes the changes described in the presented communications, though in some cases certain variations in mitochondrial behaviour are observed. This primarily concerns the state of the succinate electron-transport chain, usually characterized both by the absence of respiratory acceleration after adding ADP or DNP and of some decrease in the rate of O_2 consumption due to partial loss of cytochrome *c*. In some experiments the inhibition of succinate utilization was more pronounced and was not entirely restored by exogenic cytochrome *c* introduction, this testifying to the impairment of other links of the respiratory chain. Sometimes mitochondria preparations reacted to ADP or DNP addition by small respiratory acceleration. Such deviations in mitochondria behaviour were rather rarely encountered, and only in the succinate chain characteristic.

NAD-linked substrate oxidation by liver mitochondria of poisoned rats, proceed at a low rate, provoked not only by cytochrome c loss, but chiefly by slow $NADH_2$ generation. Experiments where use was made of "Wurster's blue", naphthoquinone and oxidation of exogenic $NADH_2$ led us to this conclusion (Figs. 2–4).

Under our conditions mitochondria respiratory chain impairment might have been caused by various factors. Firstly, the swelling effect must be excluded in the process of experiment, as the former had been prevented by EGTA addition (Fig. 1) and therefore it should be supposed that the detected electron-transport changes exist *in situ*.

The larger quantities of mitochondrial protein required for attaining initial optical density of suspension similar to the density of suspension of normal mitochondria and the considerable drop in liver mitochondria swelling amplitude of poisoned rats justified the supposition that these mitochondrial preparations had been partially swelled before their isolation. Hence, it may be concluded that in liver cells of rats intoxicated by CCl_4 the mitochondria are already in a markedly changed structural state. The supposition that these structural changes may disturb the cytochrome *c* bond with membrane phospholipids and result in the partial loss of this respiratory carrier seems quite probable. In contrast to other respiratory carriers, cytochrome *c* is known[24,25] to be connected with membrane phospholipids (cardiolipin and phosphatidyl inositol) by electrostatic bonds and is easily lost during mitochondria volume increase and salt treatment.[26,27]

Slow generation of $NADH_2$ by mitochondria which oxidize NAD-linked substrates is probably due to structural changes. It should be pointed out that this assumption does not reject the possible effect of CCl_4 (or its metabolites) on the respiratory chain

enzymes, though it is difficult to conceive such surprising likeness in NAD-linked dehydrogenases injury and what is more, localized in different mitochondrial compartments.[28] Another likely explanation of our results is that the apparent changes in the electron-transport liver mitochondria chain of intoxicated rats are caused by impairment of phospholipids in the mitochondrial membranes. It is well known how these phospholipids affect the functioning of various enzymes[29–33] in maintaining the osmotic properties[11,34] in ion binding processes.[11,35] The works by Fleischer *et al.*,[6] Scarpa and Azzone,[11] Rendi,[34] Brierly *et al.*,[29] Lester and Fleischer[30] are clearly indicative of the fact that phospholipid depletion results in the loss of the above mentioned properties of the mitochondria while addition of phospholipid restores them.

The facts confirming decrease of the ratio $H^+:Ca^{2+}$ in liver mitochondria of rats intoxicated by CCl_4 testify to a possible quantitative decrease of anionic phospholipid sites for binding of Ca^{2+}. Osmotic liver mitochondria behaviour changes of poisoned rats (the work is in preparation) also evidence the injury of the phospholipid component of the mitochondria membrane.

In the light of the data set forth we consider the assumption that the initial mechanism causing a disarrangement of the mitochondria structure is the interaction between CCl_4 (or its metabolic products) and the phospholipids of the membrane. This may result in a decrease of the respiratory chain oxidation capacity due either to the injury of the phospholipid component of separate electron-transport chain complexes or to the impairment of the phospholipids which provide the general stability of the mitochondrial structure.

As to the oxidation of exogenic $NADH_2$ along the external route through cytochrome b_5 (inasmuch as its physiological role is insufficiently studied), the only alternative on the basis of the data offered by Sottocasa *et al.*[36] is to take into consideration the existence of a mitochondrial outer membrane 24 hr after intoxication by CCl_4.

Some facts given in Figs. 5 and 6 show important differences between the transport processes and Ca^{2+} retention by liver mitochondria of intact and poisoned animals. When using NAD-linked substrates, Ca^{2+} uptake was not revealed either by respiratory acceleration or by H^+ ejection and what is more, EGTA addition to these mitochondria (without adding Ca^{2+}) caused H^+ appearance in the medium, that is probably indicative of the loss of intramitochondrial Ca^{2+} in the course of experiment. Uptake of Ca^{2+} during succinate oxidation was accompanied by respiratory acceleration, swelling and H^+ ejection. However in this case a more rapid release of accumulated Ca^{2+} as compared to intact mitochondria, was observed, this being evidenced by the reverse uptake of H^+ in liver mitochondria of intoxicated rats and the appearance of H^+ in the medium due to further EGTA addition.

Mention should be made of the important distinctions of CCl_4-treated mitochondria behaviour when analysing the changes of the optical density of suspension. Inorganic phosphate addition to intact mitochondria, isolated with EDTA, brings about a small decrease in optical density; the development of large amplitude swelling may be provoked only by subsequent addition of Ca^{2+} ions. Liver mitochondria of the experimental rats employed swells in the presence of phosphate alone.

The described results are indicative of the injury of the retention mechanisms of

exogenic and (or) accumulated Ca^{2+} by liver mitochondria of rats intoxicated by CCl_4. This may be a reflection of the observed structural and functional changes in mitochondria resulting in a decrease of ATP generation and the rate of electron transport.

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