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Altered kinetic properties of liver mitochondrial membrane-bound enzyme activities following paracetamol hepatotoxicity in the rat

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Abstract. The effects of treatment with subtoxic (375 mg/kg) and toxic (750 mg/kg) doses of paracetamol on NADH oxidase, succinoxidase and Mg^{2+}-ATPase activities in rat liver submitochondrial particles were examined. In the NADH oxidase system, treatment with subtoxic doses of paracetamol resulted in a 37% increase in activation energy in the high temperature range (E_1) while the phase transition temperature (Tt) for this system decreased by 9°C. Subtoxic doses caused a 43% decrease in E_1. For the succinoxidase system, Tt decreased by 2·4 to 3·4°C after paracetamol administration. E_2 increased by 42% only in the subtoxic-treatment group while E_1 remained unaltered in both paracetamol-treated groups. For the Mg^{2+}-ATPase system, subtoxic doses of paracetamol treatment did not change the values of E_1, E_2 and Tt whereas toxic dose treatment resulted in a 29% decrease in E_2 with a concomitant increase in Tt by 2·4°C without any change in the value of E_1. The results thus suggest that treatment with toxic and subtoxic doses of paracetamol results in possible differential alterations in the membrane lipid milieu.

Keywords. Paracetamol; hepatotoxicity; Arrhenius kinetics; NADH oxidase; succinoxidase; Mg^{2+}-ATPase.

Introduction

Acetaminophen (4-hydroxyacetanilide), commonly known as paracetamol, is a widely used analgesic drug (Mitchell *et al* 1973; Hinson *et al* 1981; Breen *et al* 1982; McClain 1982). It is considered to be safe at therapeutic doses, however, its overdoses are known to produce hepatic centrilobular necrosis associated with structural damage to the mitochondria (Mitchell *et al* 1973; Dixon *et al* 1975; Hinson *et al* 1981; Dixon 1984). Disruption of liver mitochondrial cristae structures and a transient increase in the succinate dehydrogenase activity followed by loss in this enzyme activity have been reported (Cobden *et al* 1982; Newton *et al* 1983; Dixon 1984). High doses of paracetamol are also known to increase the levels of transaminases in the serum (Dixon 1984).

Recently, we have shown impairment in the liver mitochondrial energy metabolism following paracetamol treatment of rats (Katyare and Satav 1989). Thus toxic doses (750 mg/kg) of paracetamol caused impairment in the coupled respiration rates with several substrates with concomitant decrease in the ADP-phosphorylation rates. In addition, this treatment also led to compositional defects

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Abbreviation used: SMP, Submitochondrial particles.

in respiratory chain components e.g., cytochrome contents and dehydrogenase activities. Mg^{2+}-ATPase activities increased indicating structural damage to these organelles. Subtoxic doses (375 mg/kg), on the other hand, caused impairment in the dehydrogenase activities with an increase in the rate of succinate oxidation (Katyare and Satav 1989). Meyers *et al* (1988) have also shown the acetaminophen-induced inhibition of hepatic mitochondrial respiration in mice.

These observations on impairment in the hepatic mitochondrial oxidative energy metabolism (Katyare and Satav 1989), loss of hepatic mitochondrial integrity by paracetamol treatment (Dixon, 1984; Meyers *et al* 1988) and binding of paracetamol and its metabolites to hepatic mitochondria (Jallow *et al* 1973; Ginsberg and Cohen 1985) prompted us to examine the temperature-dependent changes in the mitochondrial respiratory enzymes such as NADH oxidase, succinoxidase and Mg^{2+}-ATPase. The results of such studies when examined in terms of Arrhenius plots would give information on possible alterations in phase transition temperature and activation energy (Raison 1972). These parameters are known to be affected by the integrity of cellular membranes and their lipid composition (Raison 1972; Hulbert *et al* 1976; Dave *et al* 1989); the respiratory enzymes presently examined are membrane-bound and require lipids for their activities (Raison 1972).

2. Materials and methods

2.1 *Animals*

Male albino rats of Wistar strain, weighing between 250 and 260 g, were used. Animals were fasted overnight and injected with paracetamol the next morning. Paracetamol solutions (35 mg/ml) were prepared in warm (40–50°C) saline. Rats were injected intraperitoneally with 375 or 750 mg/kg of paracetamol (Katyare and Satav 1989); these doses, hereafter, are referred to as 'subtoxic' and 'toxic' respectively. Control animals received an equivalent volume of warm saline. After injections, the animals had free access to food and water. They were killed after 24 h of paracetamol or saline administration for isolation of mitochondria and preparation of submitochondrial particles (SMP).

2.2 *Isolation of mitochondria and SMP*

Liver mitochondria were isolated as described previously (Satav and Katyare 1982; Katyare and Satav 1989), washed once and suspended in 0·25 M sucrose containing 10 mM Tris-HCI buffer, pH 7·4 (10 mg protein/ml). SMPs were isolated after sonication for 4 min (10s sonication followed by 10s rest interval) at 20 kHz in a 'Vibro-cell' ultrasonic disintegrator (Sonics and Materials Inc., USA) by following Standard procedures, suspended in 0·25 M sucrose (6–8 mg protein/ml) and stored at – 25°C. Temperature-dependent changes in enzyme activities were studied within 2–3 days of the preparation of SMP. In separate experiments, it was ascertained that under these storage conditions, the enzyme activities did not change for up to one week.

2.3 *Arrhenius kinetics*

2.3a *NADH oxidase and succinoxidase activities:* NADH oxidase and succinoxidase activities were measured employing a Clark-type oxygen electrode (Chance and Williams 1955; Katyare and Rajan 1988; Katyare and Satav 1989) in a respiration medium consisting of 225 mM sucrose, 10 mM potassium phosphate buffer, pH 7·4, 10 mM Tris-HCI buffer, pH 7·4, 5 mM $MgCl_2$ and approximately 1 mg of SMP proteins in a final volume of 1·3 ml (Satav and Katyare 1982; Katyare and Satav 1989). NADH (2 mM) and sodium succinate (10 mM) were used as substrates to start the reaction. Enzyme activities were measured over a temperature range of 10° to 46°C with a 4°C temperature increase at every step.

2.3b Mg^{2+}-*ATPase activity*: ATPase activities in SMP were examined in a medium (final volume: 1 ml) consisting of 50 mM Tris-HCI buffer, pH 7·4, 75 mM KCl, 0·4 mM EDTA, 6 mM $MgCl_2$ and 150–200 μg of SMP protein (Satav and Katyare 1982). After pre-incubation for 2 min, the reaction, started by adding ATP (neutralized to pH 7·4 with Tris base) in a final concentration of 6 mM, was carried out for 15 min for measurements of the enzyme activity. At the end of the incubation period, the reaction was stopped by adding 0·1 ml of 10% (w/v) sodium dodecyl sulphate (SDS) (Shallom and Katyare 1985) and the liberated inorganic phosphate was estimated following the method of Fiske and Subba Row (1925). The temperature employed ranged from 10° to 46°C with a 4°C temperature increase at every step.

2.3c *Kinetic analysis*: For kinetic analyses, the log-specific activities of the given enzyme system were plotted against the reciprocal of the absolute temperature to obtain Arrhenius plots. The activation energies in high and low temperature ranges (E_1 and E_2 respectively) were determined as reported earlier (Raison *et al* 1971; Raison 1972; Katyare and Rajan 1988). The phase transition temperature was determined from the Arrhenius plots.

Protein was estimated according to Lowry *et al* (1951) with crystalline bovine serum albumin as the standard. Serum levels of glutamate-oxaloacetate transaminase (GOT) were determined by colorimetric assay (Bergmeyer and Bernt 1963).

Results are given as mean ± SE of the number of experiments indicated. Statistical evaluation of the data was by Fisher's Z test.

2.4 *Chemicals*

Paracetamol (A.R.) was purchased from Aldrich Chemical Co., Milwaukee, WI, USA. Sodium salt of succinic acid, vanadium-free ATP and NADH were obtained from Sigma Chemical Co., St. Louis, Mo, USA. All other chemicals used were of analytical-reagent grade.

3. Results

The extent of hepatic damage in paracetamol-treated animals was ascertained by measuring the levels of GOT. The mean serum levels of GOT was 186 ± 15

units/ml in control group and increased to 937 ± 162 and 3982 ± 406 units/ml respectively in animals which received the 'subtoxic' and 'toxic' doses of paracetamol indicating a moderate and severe hepatic damage in this experimental animal model of paracetamol hepatotoxicity.

The NADH oxidase activity in SMP obtained from control animals increased with temperature and reached a plateau around 46°C. A similar trend was also seen in the two paracetamol groups—subtoxic and toxic. However, in the latter group, the activity was low (15–30%) at all the temperature points examined. The succinoxidase activity showed a steady increase with temperature up to 46°C in all the groups, with the toxic-treatment group showing a significant higher activity at 46°C (data not shown). A more or less similar trend was noticed for Mg^{2+}-ATPase activity (data not shown).

The corresponding Arrhenius plots are given in figures 1 to 3 and the results on energies of activation (E_1 and E_2) and phase transition temperature (Tt) are summarized in table 1. It can be noted that the Arrhenius plots for the NADH oxidase, succinoxidase and Mg^{2+}-ATPase activities from control as well as from paracetamol-treated animals were biphasic in nature showing breaks (figures 1–3). Similar biphasic Arrhenius plots for many other mitochondrial enzyme systems have been reported by other workers (Raison 1972; Hulbert *et al* 1976; Katyare and Rajan 1988; Dave *et al* 1989).

The data in figure 1 and table 1 indicate that treatment with 'subtoxic' doses of paracetamol resulted in a 37% increase in the value of activation energy in the high temperature range (E_1) with a simultaneous decrease in the phase transition temperature by 9°C for the NADH-oxidase system. However, the activation energy

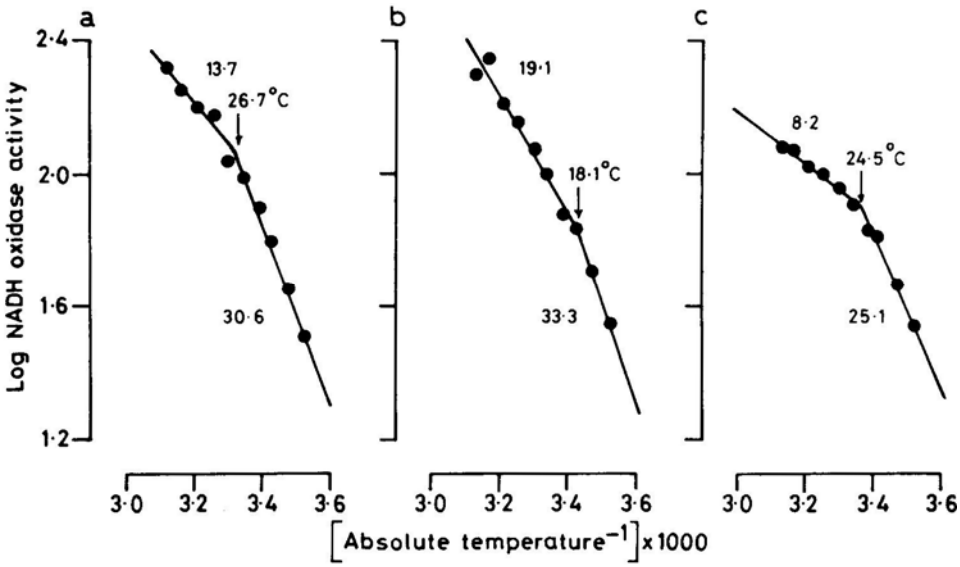

Figure 1. Arrhenius plots of temperature-dependent changes in NADH oxidase activity in rat liver SMP. (**a**) Control; (**b**) paracetamol: subtoxic dose; (**c**) paracetamol: toxic dose. Details of calculations for energies of activation and phase transition temperatures are as given in the text. Each point represents mean of 10 independent experiments. Activation energies are expressed as KJ/mol.

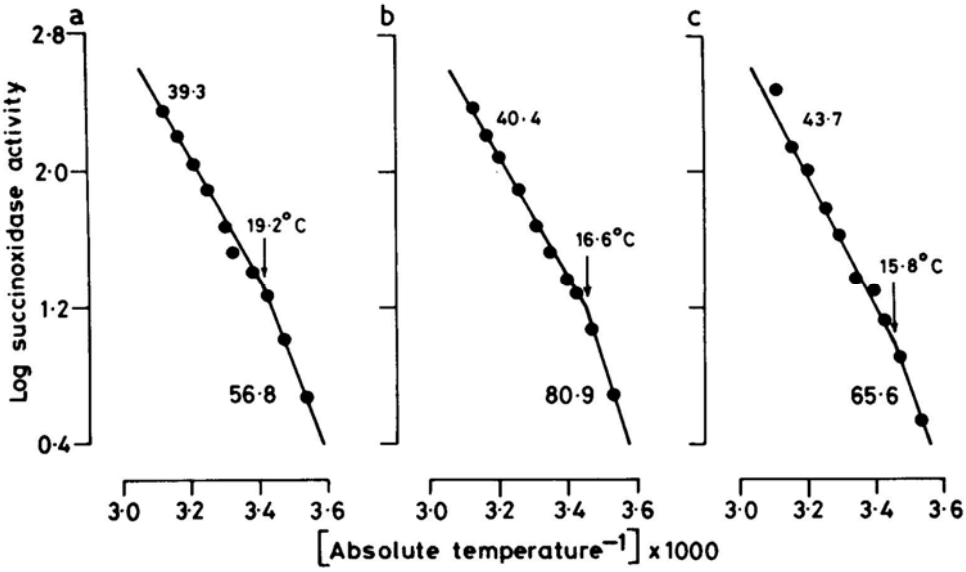

Figure 2. Arrhenius plots of temperature-dependent changes in succinoxidase activity in rat liver SMP. (**a**) Control; (**b**) paracetamol: subtoxic dose; and (**c**) paracetamol: toxic dose. The experimental details are as given in the text and figure 1. Each point represents mean of 9 independent experiments.

in the low temperature range (E_2) was not altered (table 1). In contrast, treatment with toxic doses of paracetamol caused a 43 % decrease in E_1 without any effect on E_2 or Tt although the latter exhibited a tendency to decrease (2°C).

The results in figure 2 show the Arrhenius plots for succinoxidase activity and the values of E_1 and E_2 and Tt are summarized in table 1. It is clear that the subtoxic doses of paracetamol resulted in a 44% increase in the value of E_2 without any change in the value of E_1· However, Tt decreased by 2·4°C which was statistically significant (figure 2, table 1). Toxic doses of paracetamol resulted in a further decrease in Tt (3·4° decrease) without any change in the values of E_1 or E_2.

The Arrhenius plots for Mg^{2+}-ATPase activity are depicted in figure 3. It is apparent that the pattern was practically identical for the control and the subtoxic-dose-group. However, in the toxic-dose-group, the E_2 decreased by 29%, while Tt increased by 2·7°C (table 1).

4. Discussion

In the present studies we have shown that NADH oxidase activity in SMP decreased from 15 to 30% over the entire temperature range examined in the animals receiving toxic doses of paracetamol. The observed decrease in NADH oxidase activity correlates well with our earlier observations on generalized impairment in coupled respiration rates with NAD^+-linked substrates i.e. glutamate, β-hydroxybutyrate and pyruvate + malate (Katyare and Satav 1989). On the other hand, succinoxidase and Mg^{2+}-ATPase activities were generally in the

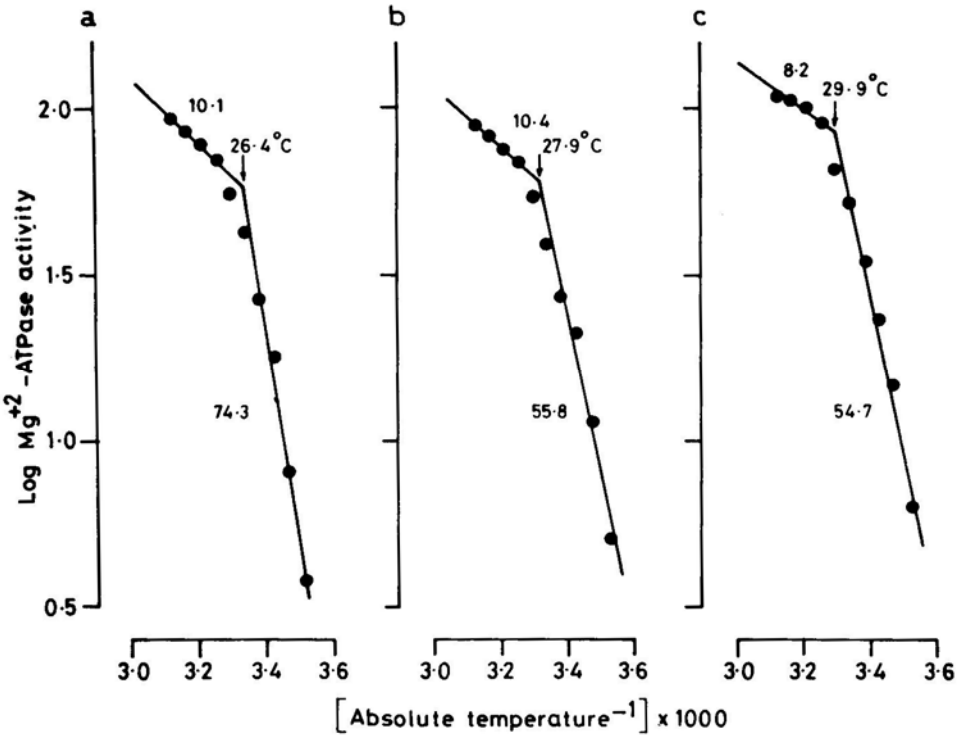

Figure 3. Arrhenius plots of temperature-dependent changes in Mg^{2+}-ATPase activity in SMP from rat liver, (**a**) Control; (**b**) paracetamol: subtoxic dose; (**c**) paracetamol: toxic dose. Other experimental details are as given in the text and figure 1. Each point represents mean of 10 independent experiments.

same range as in the control and the two paracetamol-treated groups (data not given), although the coupled (state 3) respiration rate with succinate had increased in the group receiving the subtoxic dose and decreased in those animals receiving toxic doses of paracetamol (Katyare and Satav 1989).

Arrhenius kinetic studies revealed that the subtoxic doses of paracetamol resulted in an increase in the energy of activation at high temperature range (E_1) for NADH oxidase with concomitant substantial lowering in the phase transition temperature (9°C). Subtoxic doses resulted in an increased value of E_2 in succinoxidase system with a decrease in Tt, while for the Mg^{2+}-ATPase activity all the above parameters were unaffected (table 1).

Toxic dose treatment, on the other hand, brought about a decrease in the values of E_1 only for the NADH oxidase activity and lowered the value of E_2 for Mg^{2+}-ATPase system. The phase transition temperature was lowered for succinoxidase, while its value increased for Mg^{2+}-ATPase. Thus, the effects of subtoxic and toxic doses of paracetamol were differential and sometimes of opposite nature depending upon the enzyme system under study and the dose of paracetamol employed.

The three respiratory enzymes which we have studied are membrane bound and require lipids for their activity (Raison 1972; Ashraf *et al* 1980; Tzagoloff 1982). It is now well recognized that the changes in lipid composition alter membrane fluidity and energies of activation of membrane-associated enzymes (Raison *et al* 1971;

Table 1. Effects of *in vivo* paracetamol treatment on kinetic parameters of NADH oxidase, succinoxidase and Mg^{2+}-ATPase activities in SMP from rat liver.

Enzyme	Treatment	group	Phase transition temperature (Tt) (0°C)	Energy of activation (KJ/mol)	
				High temperature range (E_1)	Low temperature range (E_2)
NADH oxidase	Control	(10)	27·3 ± 0·98	13·9 ± 1·11	30·9 ± 1·54
	Subtoxic	(10)	18·3 ± 0·85****	19·1 ± 1·02***	33·1 ± 1·57^{NS}
	Toxic	(10)	25·5 ± 1·69^{NS}	8·0 ± 0·74****	27·6 ± 2·12^{NS}
Succinoxidase	Control	(9)	19·1 ± 0·74	39·7 ± 1·53	55·1 ± 3·51
	Subtoxic	(9)	16·7 ± 0·63*	39·6 ± 0·74^{NS}	79·1 ± 4·49****
	Toxic	(9)	15·7 ± 0·35****	43·9 ± 2·10^{NS}	64·4 ± 3·19^{NS}
Mg^{2+}-ATPase	Control	(10)	27·0 ± 0·99	11·9 ± 0·90	74·5 ± 7·25
	Subtoxic	(10)	25·8 ± 1·60^{NS}	11·5 ± 0·66^{NS}	58·7 ± 3·98^{NS}
	Toxic	(10)	29·7 ± 0·61*	9·3 ± 1·14^{NS}	52·8 ± 3·42**

The experimental details are as described in the 'materials and methods' section. Results are given as mean ± SE of number of independent experiments indicated in the parentheses.

*$P < 0·05$; **$P < 0·02$; ***$P < 0·002$; ****$P < 0·001$; ^{NS}not significant.

Raison 1972; Hulbert et al 1976). Therefore the observed differential changes in activation energies (E_1 and E_2) and phase transition temperatures (Tt) in the three enzyme complexes studied could be due to alterations in lipid microdomains of these enzyme systems caused by paracetamol treatment. Drug-induced changes in lipid metabolism have been previously reported with Imipramine and desipramine (Albouz *et al* 1982; Fauster *et al* 1983). It would therefore be of interest to examine if paracetamol intoxication also leads to alterations in membrane lipid composition.

The observed impairment in the mitochondrial energy-linked functions (Katyare and Satav 1989) and alterations in phase transition temperatures and activation energies could also be due to membrane lipid peroxidation, since lipid peroxidation has been suggested to be responsible for paracetamol-induced tissue damage (Wendel *et al* 1979). Paracetamol-induced lipid peroxidation would change the degree of unsaturation of fatty acids in the lipid domains of the membrane resulting in alterations in the membrane fluidity and activities of the membrane bound enzymes (Raison 1972). Alternatively, it is also possible that the two doses of paracetamol employed may have led to differential adduct formation with mitochondrial proteins and enzyme proteins (Hinson *et al* 1981; Dixon 1984). Interestingly, Potter *et al* (1974) have reported that a reactive metabolite of paracetamol, N-acetyl *p*-benzoquinoneimine is generated by the hepatic mixed-function oxidase system in amounts sufficient to exceed its detoxification. This metabolite binds to critical macromolecules causing disturbed cellular homeostasis and eventual cell death (Potter *et al* 1974). In addition, paracetamol is known to alter the Ca^{2+} permeability, Ca^{2+} release and cause swelling of mitochondria (Beatrice *et al* 1984; Meyers *et al* 1988). Therefore, it is also likely that altered intracellular Ca^{2+} homeostatis may be responsible for impairment in the mitochondrial functions (Schanne *et al* 1979), however, we have not examined this possibility in the present studies.

The data presented here do not pinpoint which of the factors discussed above play a predominant role. More direct experiments will be necessary to ascertain these possibilities. Nevertheless, the present studies clearly show that the toxic and even the subtoxic doses of paracetamol bring about alterations in the kinetic properties of mitochondrial enzyme systems which are intimately associated with energy transduction processes.

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