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EFFECT OF CHLORPROMAZINE ON ISOLATED RAT HEPATOCYTES*

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Abstract—The effect of chlorpromazine hydrochloride (CPZ) (1–500 μM) on plasma membrane permeability and mitochondrial respiratory function of isolated rat hepatocytes was studied. The endogenous oxygen consumption stimulated by 1 mM succinate was increased significantly by 5 μM CPZ, whereas the ability to exclude trypan blue (TB) was decreased significantly by 100 μM CPZ. The release of a cytosomal enzyme, lactate dehydrogenase (LDH), was increased significantly by 50 μM CPZ, whereas the release of glutamic-opalacetic transaminase (GOT) was increased significantly by 100 μM. The endogenous oxygen consumption was decreased significantly by 150 μM CPZ. The respiration control ratio by 2 μM carbonylcyanide-*m*-chlorphenyl hydrazon (CCP) showed significant decreases at all concentrations of CPZ studied; and this might be attributable to the suppression by CPZ of the respiratory stimulation induced by CCP. The results indicated that CPZ at a low concentration (5 μM) first produced a significant change in plasma membrane permeability to low molecular substances such as succinate and then at higher concentrations (50–100 μM) produced significant release of the cytosomal and mitochondrial enzymes, LDH and GOT. They also indicated that the concentrations of CPZ which produced significant effects on respiratory function were higher (above 150 μM) than those which produced significant changes in plasma membrane permeability of hepatocytes.

Chlorpromazine hydrochloride (CPZ), a phenothiazine derivative, has been widely used as a major tranquilizer. The hepatotoxicity of CPZ, however, is an important problem in clinical therapy with chronic use of this drug. The pathogenesis of cholestatic jaundice, which occurred in 1–2% of patients treated with CPZ, has been thought to result from hypersensitivity to CPZ [1]. Other observations suggest that the intrinsic hepatotoxicity of CPZ may contribute to hepatic injury [2]. Almost 50% of patients taking CPZ for prolonged periods of time show abnormal liver function tests, e.g. increased sulfobromophthalein (BSP) retention [3], higher values of GOT (glutamic-oxalacetic transaminase activity) and GPT (glutamic-pyruvic transaminase activity) [4], and morphological abnormalities in liver biopsies [2], suggesting a direct hepatotoxic effect of CPZ. Furthermore, in experiments *in vitro*, CPZ has an adverse effect on liver slices [5], Chang human liver cells [6], isolated rat hepatocytes [7], perfused rat liver [8] and isolated liver plasma membrane [9]. An inhibition of the respiratory chain in isolated rat liver mitochondria has also been reported [10]. Alteration of mitochondrial structure, decreased glycogen, and a rough-surfaced endoplasmic reticulum, in rat liver cells, were observed by electron microscopy after daily injection of CPZ for 3–4 weeks [11].

The purpose of the present study was to determine the effects of CPZ on plasma membrane permeability and mitochondrial respiratory function of hepatocytes in an attempt to elucidate the mechanism of CPZ hepatotoxicity in the initial stage, using isolated rat hepatocytes.

MATERIALS AND METHODS

Isolation of hepatocytes. Male Wistar rats (200–250 g, Nihon Seibutsuzairyo Co., Tokyo, Japan) anesthetized with ether were used as liver donors. Hepatocytes were prepared by the procedure of Berry and Friend [12] as modified by Baur *et al.* [13] and reported previously [14]. A midline abdominal incision was made and the portal vein was cannulated with polyethylene tubing (inner diameter: 1 mm). After the cannula was tied in place, the abdominal vena cava was cut below the renal vein, and perfusion of the liver was started with calcium-free buffer containing 121 mM NaCl, 6 mM KCl, 0.6 mM $MgSO_4$, 0.74 mM KH_2PO_4, 12 mM $NaHCO_3$ and 5 mM glucose oxygenated with 95% O_2–5% CO_2 to a pH of 7.10 at 37° at a flow rate of about 30 ml/min. The thoracic portion of the inferior vena cava was cannulated, and the abdominal vena cava was tied off above the renal vein. The liver was excised and placed in a liver perfusion apparatus (Abe Kagaku Co. Ltd., Funabashi, Japan) with the flow diverted to a beaker. After the liver had been perfused with about 500 ml of the calcium-free buffer, recirculation of the buffer was started and 50 mg of collagenase enzyme (type I, Sigma Chemical Co., St. Louis, MO) and 0.50 ml of 100 mM $CaCl_2$ were added to the 100 ml of perfusate in the system. The liver was perfused for 15 min via the portal vein cannula, followed by 10 min via the vena caval cannula. At the end of the 25-min perfusion, the flow was interrupted and the liver was perfused with about 300 ml of fresh calcium-free buffer. During the entire perfusion procedure, the buffer was kept oxygenated

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by exchange diffusion of 95% oxygen–5% carbon dioxide. After perfusion, the liver was removed to a beaker containing 100 ml of fresh perfusion buffer and was gently dispersed. The crude cell suspension was then gently filtered through 150 μm mesh nylon and rotated on a rotary evaporator under an oxygen–carbon dioxide atmosphere at 37° for 10 min. The cell suspension was then cooled on ice and centrifuged at 200 rpm for 2 min. The supernatant was aspirated and the loosely packed pellet of cells was gently resuspended in 30 ml of cold wash buffer containing 131 mM NaCl, 5.2 mM KCl, 0.9 mM $MgSO_4$, 0.12 mM $CaCl_2$, and 3.0 mM Na_2HPO_4, (pH 7.40). This washing procedure was repeated three times. Then the final pellet of cells was resuspended in 10 ml of cold Tris–incubation buffer [wash buffer containing 10 mM tris (hydroxymethyl) aminomethane adjusted to pH 7.40 with 1 N HCl], yielding a suspension of 30–40 mg protein/ml of suspension that was stored on ice until use.

Viability tests. Three viability tests were routinely done in every preparation before and after each experiment. Preparations were considered suitable when the following criteria were fulfilled: 0.4% trypan blue (TB) exclusion was greater than 90%, respiratory stimulation by addition of 1 mM succinate was less than 20% in a dissolved-oxygen monitor (Yellow Springs Instruments Co., Yellow Springs, OH), and respiratory stimulation by addition of an uncoupler of oxidative phosphorylation, 2 μM carbonylcyanide-*m*-chlorophenyl hydrazon (CCP, CalBiochem, San Diego, CA), was greater than 50% in the same oxygen monitor described above [14].

Experimental design. To determine the effect of CPZ on the liver cells, isolated rat hepatocytes (1.5 to 2.5 mg protein/ml of air-saturated Tris-incubation buffer in final volume of 5 ml) were incubated with and without CPZ (1–500 μM). CPZ (chlorpromazine hydrochloride; Yoshitomi Pharm. Ind. Co. Ltd., Osaka, Japan) solution was freshly prepared with distilled water just before the experiment to avoid photo-decomposition. After 5 min of preincubation at 37° with gentle shaking, 10 μl of CPZ solution containing various amounts of CPZ was added at time 0, and incubation with gentle shaking was performed for 15 min. A sample of the medium was removed and put in the chamber of the oxygen monitor. After the initial rate was determined, 40 μl of 100 mM sodium succinate or 40 μl of 200 μM CCP was added, and the oxygen utilization rate was measured at 37°. Another 1 ml of the medium was immediately centrifuged at 200 rpm for 2 min to separate the cells from the medium. The loosely packed pellet of cells was gently resuspended in Tris-incubation buffer and used for the TB exclusion test. The supernatant fluid was used for the determination of the released GOT and LDH (lactate dehydrogenase activity). GOT was assayed using a commercial kit (Hepatest A; Daiichi Pure Chemical Co. Ltd., Tokyo, Japan) and LDH was assayed by the method of Zimmerman and Weinstein [15]. The 100% released-enzyme activities were determined as follows: after 0.3 ml of the stored cell suspension was added to 4.7 ml of distilled water (1.5 to 2.5 mg protein/ml) and vigorously agitated for 5 min in a shaking machine, the sample was incubated at 37° with gentle shaking for 1 hr and then centrifuged at 3000 rpm for 30 min. The supernatant fluid was used for the assay of GOT and LDH as described above. Protein concentration was determined by the colorimetric method of Lowry *et al.* [16]. The level of significance between the control and CPZ-treated cells for the two-tailed Student's *t*-test was set at $P < 0.01$ and 0.05.

RESULTS

Effect of CPZ on plasma membrane permeability. To determine the effect of CPZ (1–500 μM) on the plasma membrane permeability of the hepatocytes, we studied succinate stimulation of oxygen consumption, TB exclusion, and release both of LDH, a cytosomal enzyme, and of GOT which is concentrated in mitochondria. The effect of CPZ on the oxygen consumption that was stimulated by 1 mM succinate is shown in Fig. 1. The ratio of the oxygen uptake rate after addition of succinate to that before addition was increased significantly at 5 μM CPZ ($P < 0.05$), and the maximum ratio was observed at 200 μM; above 300 μM the ratio decreased. The results of the TB exclusion study are shown in Fig. 2. A typical sigmoidal dose–response curve was observed, with a significant increase ($P < 0.01$) being observed at 100 μM CPZ. At 150 μM CPZ 50% of the cells were stained, and almost 100% of the cells were stained at 500 μM. The percentage of released LDH is shown in Fig. 3. The release of LDH increased significantly at 50 μM CPZ ($P < 0.05$), and 50% release of LDH occurred at 350 μM. The percentage of released GOT is shown in Fig. 4. A significant increase of GOT ($P < 0.05$) was observed at 100 μM CPZ; 50% release occurred with 500 μM CPZ which was the highest concentration of CPZ used in this study. The release of GOT was more moderate than that of LDH.

Effect of CPZ on mitochondrial respiratory function. To determine the effect of CPZ (1–500 μM) on the mitochondrial respiratory function, we determined the endogenous oxygen consumption and the response of cells to the oxidative phosphorylation uncoupler, CCP. The effect of CPZ on endogenous oxygen consumption is shown in Fig. 5. A significant decrease ($P < 0.05$) was observed at 150 μM CPZ. A 50% decrease of oxygen consumption was observed with 300 μM CPZ. The results of the respiratory control by 2 μM CCP are shown in Fig. 6. The decrease of the ratio of the oxygen uptake rate after addition of CCP to that before addition was highly significant ($P < 0.01$) with all CPZ doses studied. Similar results were also observed in the study with a lower CCP concentration (0.5 μM).

DISCUSSION

In this study, we examined the effect of CPZ on isolated rat hepatocytes over a 1–500 μM concentration range by measuring changes in both plasma membrane permeability and mitochondrial respiratory function, which are thought to be useful criteria for *in vitro* cytotoxicity of many drugs.

The effects of CPZ on the biological membrane of erythrocytes, mitochondria, microsomes and liver

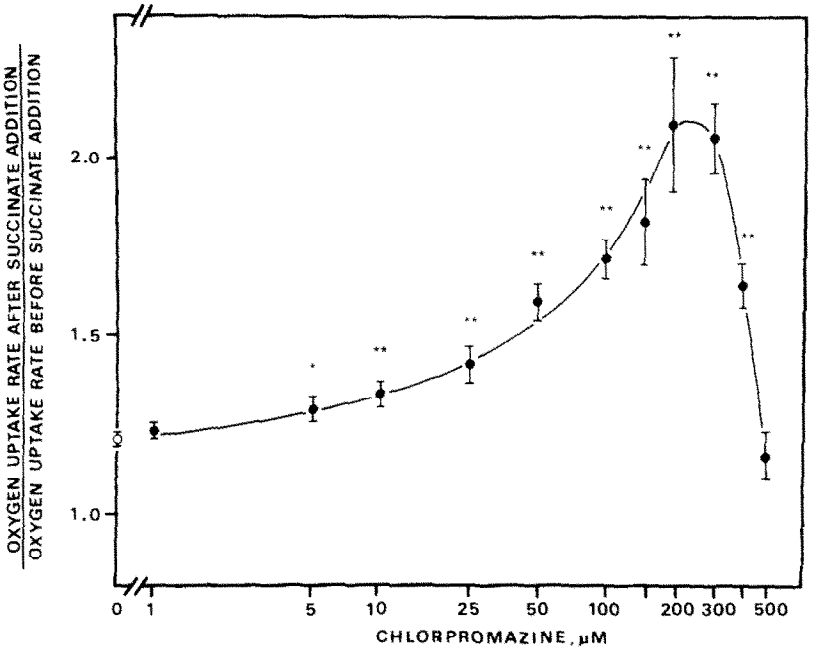

Fig. 1. Effect of CPZ on the succinate permeability of isolated rat hepatocytes after incubation with various concentrations of CPZ at 37° for 15 min. A change in permeability was measured as a change in the endogenous oxygen consumption that was determined using a dissolved-oxygen monitor. Each point and vertical bar represents the mean ± S.E. of five separate experiments. Statistically significant at $P < 0.01$ (**) and $P < 0.05$ (*) when compared with the control. Key: (○) control, and (●) CPZ-treated.

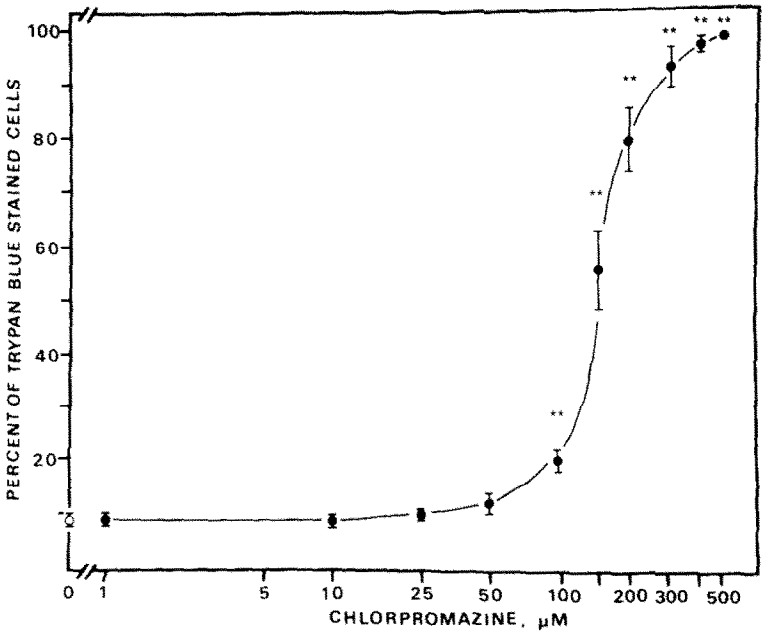

Fig. 2. Effect of CPZ on the ability of isolated rat hepatocytes to exclude trypan blue (TB) after incubation with various concentrations of CPZ for 15 min at 37°. Each point and vertical bar represents the mean ± S.E. of five separate experiments. Statistically significant at $P < 0.01$ (**) when compared with the control. Key: (○) control, and (●) CPZ-treated.

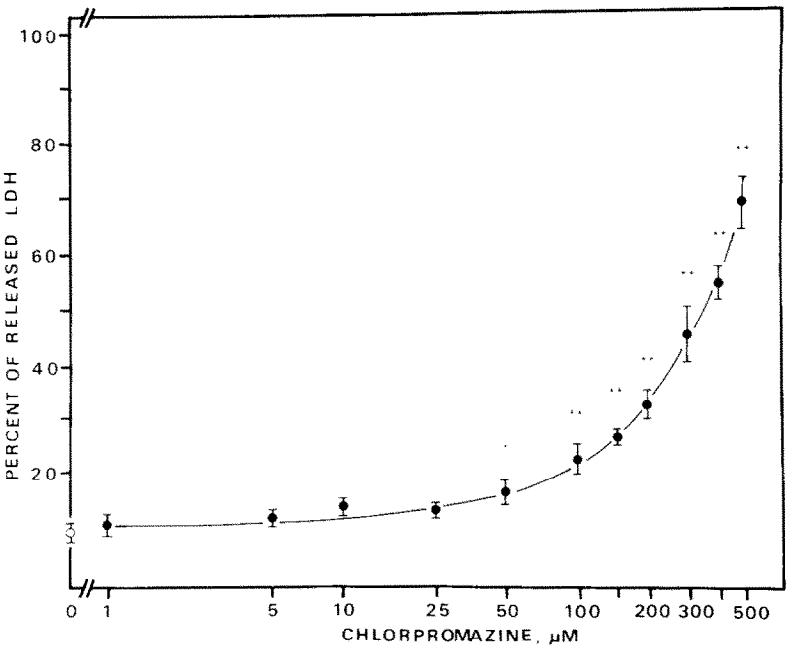

Fig. 3. Effect of CPZ on the release of LDH from isolated rat hepatocytes into the medium after incubation with various concentrations of CPZ at 37° for 15 min. Each point and vertical bar represents the mean ± S.E. of five separate experiments. Statistically significant at $P < 0.01$ (**) and $P < 0.05$ (*) when compared with the control. Key: (○) control, and (●) CPZ-treated.

cells have been studied extensively, revealing: reduction of surface tension and membrane permeability [17]; membrane expansion of erythrocytes, i.e. stabilization at lower, but lysis at higher, concentrations [18]; inhibition of swelling of mitochondrial membranes [19]; and reduction of both membrane fluidity and ATPase activities of rat liver plasma membranes [9, 20]. Also using liposomes as a membrane model, Maoi *et al.* [21] reported that CPZ increased the permeability of the liposomes to low molecular substances such as D-glucose or D-phenylalanine. All these changes of the membrane were thought to result from either the adsorption of CPZ on the membrane surface or the accumulation in the membrane.

Since intact plasma membranes are impermeable to Krebs' cycle intermediates such as succinate and glutamate [22], addition of succinate should have

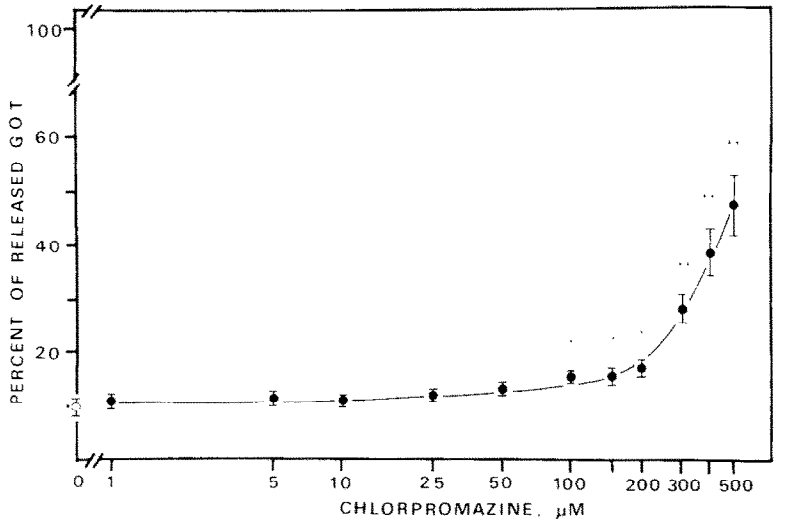

Fig. 4. Effect of CPZ on the release of GOT from isolated rat hepatocytes into the medium after incubation with various concentrations of CPZ at 37° for 15 min. Each point and vertical bar represents the mean ± S.E. of five separate experiments. Statistically significant at $P < 0.01$ (**) and $P < 0.05$ (*) when compared with the control. Key: (○) control, and (●) CPZ-treated.

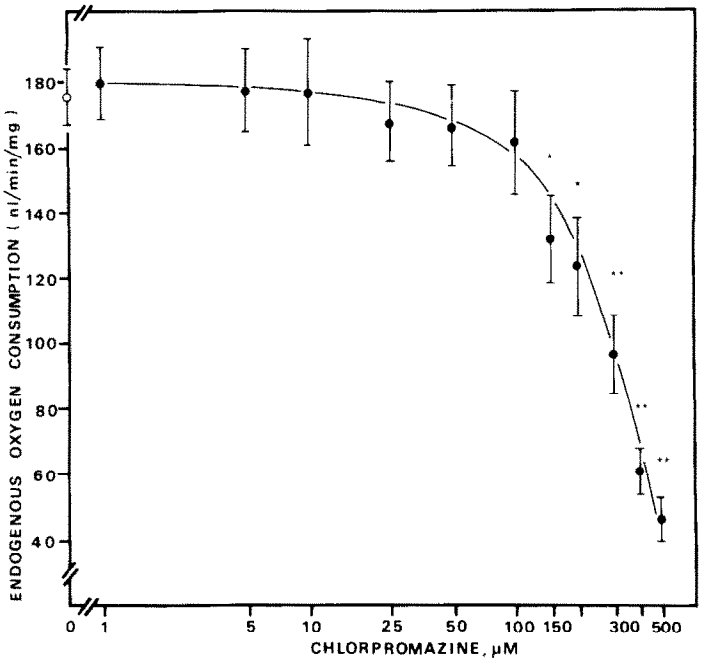

Fig. 5. Effect of CPZ on the endogenous oxygen consumption of isolated rat hepatocytes after incubation with various concentrations of CPZ at 37° for 15 min. Each point and vertical bar represent the mean ± S.E. of five separate experiments. Statistically significant at $P < 0.01$ (**) and $P < 0.05$ (*) when compared with the control. Key: (○) control, and (●) CPZ-treated.

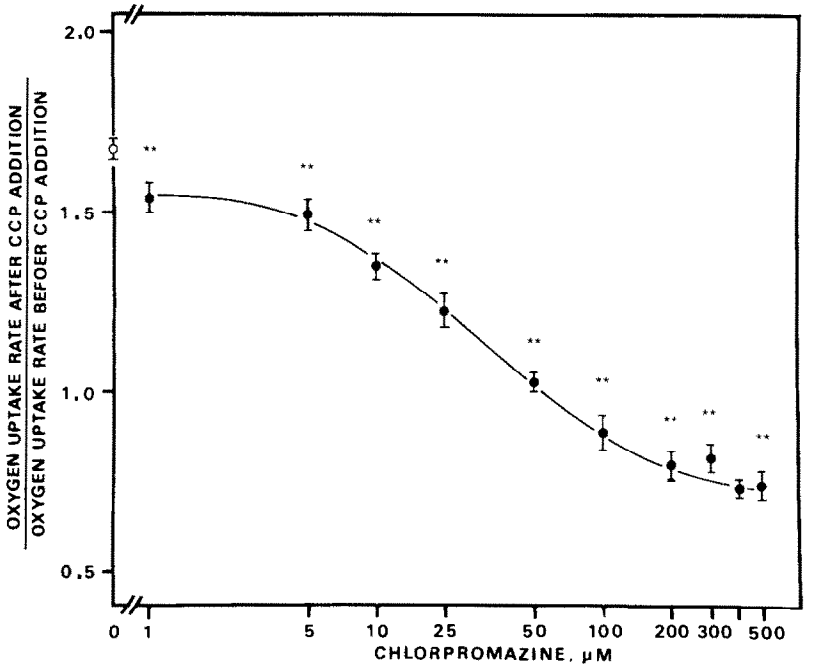

Fig. 6. Effect of CPZ on mitochondrial respiratory control of isolated rat hepatocytes stimulated by 2 μM CCP after incubation with various concentrations of CPZ at 37° for 15 min. Each point and vertical bar represent the mean ± S.E. of five separate experiments. Statistically significant at $P < 0.01$ (**) when compared with the control. Key: (○) control, and (●) CPZ-treated.

little or no effect on the rate of oxygen consumption. If the membrane has been slightly injured, however, succinate will diffuse into the cells and increase the rate of oxygen consumption by converting the primary electron carrier source from $NADH + H^+$, where three ATPs are produced per pair of electrons, to $FADH_2$, where only two ATPs are produced per pair of electrons transported. In this study, as shown in Fig. 1, CPZ increased significantly the oxygen consumption of the hepatocytes at 5 μM. This may have been the result of membrane expansion [18] or alteration of membrane fluidity [9], which would enable a low molecular substance such as succinate (mol. wt 118) to pass through the plasma membrane of the hepatocytes. Keeffe *et al.* [9] demonstrated a significant alteration of the lipid fluidity of rat liver plasma membrane at 10 μM CPZ by fluorescence polarization. The decrease of the oxygen uptake ratio by concentrations of CPZ above 200 μM may have been due to damage to the mitochondrial membrane by CPZ at higher concentrations, as discussed later. The change of influx permeability was also examined by the TB exclusion test, which is widely used as an estimate of cell viability. As shown in Fig. 2, TB exclusion was not decreased significantly by CPZ concentrations of less than 100 μM, a concentration that is twenty times higher than that needed to produce a significant succinate stimulation of O_2 consumption (Fig. 1); this suggests that TB exclusion could not detect the initial microscopic change of membrane permeability that was detected by succinate stimulation. Increasing release of LDH was linearly related to increasing CPZ concentration ($y = 0.113 + 11.32x$, $r = 0.998$), although Fig. 3 is plotted on a logarithmic scale of the concentration. Since LDH is a cytoplasmic enzyme, its release may indicate an increase in the permeability of the plasma membrane to high molecular substances. The release of GOT from hepatocytes has been widely used as a liver function test both clinically and experimentally. The intracellular distribution of GOT has been reported to be three or four times higher in the mitochondria than in the supernatant fraction [23]. Previously, Abernathy *et al.* [7] reported a significant increase of the release of GOT into the incubation medium from rat hepatocytes at 90 μM. In this study, a significant release of GOT was also shown at 100 μM CPZ, but even at 500 μM CPZ, which was the highest concentration used in these experiments, only 50% of the control was released (Fig. 4). This finding suggests that changes in GOT release reflect cell injury less sensitively than LDH did (Fig. 3). This could be a different reflection of membrane injury, i.e. LDH for the plasma membrane of the hepatocytes and GOT mainly for the mitochondrial membrane.

The endogenous oxygen consumption which decreased significantly at 150 μM CPZ, as shown in Fig. 5, may have been a reflection of the mitochondrial membrane injury caused by CPZ. Francesco and Bickel [24] reported that the major intracellular binders for CPZ were the nonpolar moieties of both mitochondrial and microsomal membrane phospholipids. Bickel and Steele [25] also reported that both mitochondrial and microsomal fractions had higher affinities for CPZ than did other liver subcellular fractions in rats. The effect of CPZ on mitochondrial respiration has been much studied. Dawkins *et al.* demonstrated that CPZ at a concentration of 100 μM uncoupled the phosphorylation coupled to the oxidation of reduced cytochrome *c* [26] and that CPZ also inhibited cytochrome oxidase dose-dependently [10]. Furthermore, CPZ changed the permeability of the mitochondrial membrane to water and sucrose [27], and the swelling of mitochondria was inhibited by CPZ at optimum concentrations [19]. Recently, Dhalla *et al.* [28] studied the effect of CPZ on rat heart mitochondrial and microsomal fractions at concentrations ranging from 25 to 120 μM and found that CPZ produced a significant inhibition of the calcium binding and uptake abilities of both fractions. They also found that the mitochondrial respiratory and oxidative phosphorylation activities were depressed at high concentrations of CPZ. From these findings, the significant decrease of endogenous oxygen consumption at high concentrations (150–500 μM) of CPZ might have been caused by inhibition of mitochondrial respiratory and oxidative phosphorylation activities caused by adsorption and/or accumulation of CPZ on the mitochondrial membrane. This might also explain the decrease of oxygen consumption stimulated by 1 mM succinate at higher concentrations of CPZ (300–500 μM) described before (Fig. 1).

The significant decrease of the respiration control ratio by 2 μM CCP was demonstrated for the entire concentration range of CPZ studied (Fig. 6), and even by a lower concentration of CCP (0.5 μM) (see Results). Since both CCP and CPZ alter mitochondrial respiratory and oxidative phosphorylation activites [29–32], the inhibition of the oxygen uptake stimulated by CCP might be attributable to the suppression by CPZ of the respiratory stimulation induced by CCP, but more elaborate studies might be necessary to elucidate the mechanisms of CPZ–CCP interaction in mitochondria.

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