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Acute Effects of Carbon Monoxide and Cyanide on Hepatic Mitochondrial Function

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Summary. The effects of carbon monoxide and cyanide on the hepatic redox state and energy charge were investigated. Rats were used for the experiment under pentobarbital anesthesia. Immediately after laparotomy, a rat was placed in an animal chamber made of a transparent plastic box and exposed to a test gas for 3 min. Every test gas was produced in a gas chamber connected to the animal chamber with a flexible tube. HCN was produced from NaCN and H_2SO_4. In the CO inhalation experiment, various amounts of CO were introduced into the gas chamber. Immediately after an exposure, about 2 g liver was frozen in situ with a precooled clamp. Oozed blood from the wound surface was sampled. Concentrations of ATP, ADP, AMP, acetoacetate, and β-hydroxybutyrate in hepatic mitochondria were determined, and the redox state and the energy charge were calculated. For cyanide as well as CO, significant negative correlations were found between the concentration in the blood and the redox state. The same held true for the energy charge. The redox state showed a slight increase at low concentrations of both gases; however, thereafter it began to decrease sharply with increases in concentrations. When concentrations of the toxicant in the blood reached certain levels, a kind of turning point, beyond which the redox state does not decrease any more, was observed. It was about 40% for HbCO and about 2.0 μg/ml for cyanide, and the points seemed to be related to the concentrations, beyond which cells are irreversibly damaged. On the other hand, the energy charge did not change at low concentrations. With an increase in toxicant concentrations, the energy charge decreased drastically. The rate of decrease in the energy charge became higher when blood concentrations exceeded certain levels. It was about 40% for HbCO and 2.0 μg/ml for cyanide. The presence of low levels of blood cyanide did not affect the relationship between the energy charge and the HbCO concentration.

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Zusammenfassung. Der Einfluß von Kohlenmonoxid und Blausäure auf die Funktion der Lebermitochondrien wurde untersucht. Alle Versuche wurden an Ratten in Pentobarbitalnarkose durchgeführt. Unmittelbar nachdem die Bauchhöhle der Ratten geöffnet wurde, wurden einzelne Tiere in einer Versuchskammer aus transparentem Kunststoff für 3 min einem Prüfungsgas ausgesetzt. Jedes Prüfungsgas wurde in einer durch ein Plastikrohr mit der Versuchskammer verbundenen Gaskammer hergestellt. HCN wurde aus NaCN und H_2SO_4 hergestellt. Bei CO-Versuchen wurden verschiedene Mengen von CO in die Kammer eingeführt. Unmittelbar nach den Versuchen wurden etwa 2 g Leber in situ mit einer abgekühlten Klemme gefroren. Eine Blutprobe wurde von der Wunde entnommen. Die Konzentrationen des Azetoazetats, β-Hydroxybutyrats, Adenosintriphosphats, Adenosindiphosphats und Adenosinmonophosphats der Leber wurden bestimmt, und anhand von ihren Werten wurden "redox state" (ein Verhältnis von Azetoazetats zu β-Hydroxybutyrats) und "energy charge" (ein Verhältnis von Summa von ATP und 0.5 ADP zu Summa von ATP, ADP und AMP) berechnet. In vergifteten Ratten verminderten sich "redox state" mit der zunehmenden Konzentration der Toxika im Blut. Als die HbCO-Konzentration 40% überstieg, trat eine Art Wende, bei der die Geschwindigkeit der Verminderung sich reduziert, ein. Für Zyanid war diese Wende 2.0 μg/ml. Im Vergleich zu "redox state" verminderte sich "energy charge" langsamer. Aber die Geschwindigkeit der Verminderung beschleunigte sich, wenn die Blutkonzentration der Toxika eine Grenze überschritt. Die o.g. Wenden waren 40% für HbCO und 2.0 μg/ml für Zyanid. In kombinierten Versuchen wurden Tiere einem Gasgemisch (CO und niederer Konzentration von HCN) ausgesetzt. Es wurde angenommen, daß es keine Wechselwirkung zwischen CO und HCN gibt.

Schlüsselwörter: Kohlenmonoxid, Funktionsstörung der Mitochondrien – Zyanid, Funktionsstörungen der Mitochondrien

Introduction

When burnt cadavers were found in the fire field, the vital reaction to fires, which can be diagnosed by the presence of soot in the respiratory tract and the elevation of carboxyhemoglobin (HbCO) level, should be examined carefully. In general, it is well known that HbCO concentrations in burnt cadavers vary among individuals. It was true of the cases in our institute (Hattori et al. 1985). When HbCO concentrations are higher than 40%, CO is considered to be a major factor contributing to death. On the other hand, in cases in which HbCO cannot be detected, it must be determined whether or not the victim was alive at the start of the fire. When HbCO concentrations are in a sublethal range, the measured value itself has been used as an indicator showing that the victim was

burnt to death. In such a case, factors other than CO poisoning must be examined. Hydrogen cyanide (HCN), which is a thermal degradation product of nitrogen-containing materials, is one of the factors to be taken into account (Yamamoto 1975). It is assumed that some metabolic disturbance would be caused at low levels of HbCO and HCN; however, at present there is no precise information on the relationship between the blood levels of HbCO and cyanide and functions important for life, such as mitochondrial functions. If such a relationship is established, a measured blood value will be one of the useful indices for clarifying the cause of death in fire victims. Ozawa (1983) proposed that the free NAD^+/NADH ratio in the mitochondria has a decisive role in mitochondrial ATP synthesis which relates to the energy charge. In the present experiment, we examined the changes in the hepatic mitochondrial redox state and the hepatic energy charge in acutely CO- or HCN-intoxicated rats.

Materials and Methods

Male Wistar (HLA) rats weighing 250–300 g were used. The animals had been fasted for 15 h prior to the experiment. They were anesthetized with an i.p. injection of sodium pentobarbital (Somnopentyl, 25 mg/kg b.w.). Ten min after the injection, laparotomy was made at the midline. Immediately thereafter, the rat was placed in a supine position in an animal chamber and exposed to a test gas for 3 min. The chamber devised by our laboratory was used (Yamamoto et al. 1982), and consisted of two transparent plastic boxes of the same shape and capacity (measuring $15 \times 15 \times 11$ cm) connected with each other by a flexible tube (3 cm long and 1.2 cm wide). One box was the animal chamber, in which an animal was exposed to the gas, and the other was a gas-producing chamber.

HCN was produced in the gas-producing chamber by addition of 30 mg/ml of NaCN solution into a dish containing 1 ml of concentrated H_2SO_4 through a hole in the upper surface of the chamber. The amount of NaCN solution added was varied from case to case. In the CO inhalation experiment, 5–80 ml pure CO was injected into the chamber. In the combined exposure, the amount of NaCN solution added was fixed at a small dose, and the volume of CO injected was varied. The atmosphere in the chamber was stirred by pushing in and out the plunger of a 50-ml syringe connected to the animal chamber. A gas sample was withdrawn by a glass syringe immediately before the completion of an exposure. The volume withdrawn was about 20 ml for CO determinations and accurately 15 ml for HCN determinations. Animals in the control group were placed similarly in the animal chamber for 3 min.

Immediately after an exposure, about 2 g of the left lateral lobe of the liver was frozen in situ with a Wollenberger clamp precooled in liquid nitrogen. The sampling procedure was completed within 10 s. Oozed blood from the wound surface was sampled. The frozen tissue was pulverized to a fine powder in a metallic tube. The powdered tissue was weighed, and homogenized in 3 vol cold solution of 6% (w/v) perchloric acid in the ice bath. The homogenate was centrifuged at $10{,}000 \times g$ for 5 min at 0°C. The supernatant was used as sample.

Acetoacetate and β-hydroxybutyrate were determined according to the method of Williamson and Mellanby (1974). The amounts of adenine nucleotides were measured enzymatically (Lamprecht and Traushold 1974; Jaworek et al. 1974). The mitochondrial redox state was expressed as a ratio of NAD^+ to NADH multiplied by $1/k$. In the present experiment, a NAD^+/NADH ratio was calculated as a concentration ratio of acetoacetate and β-hydroxybutyrate. The k value of the equilibrium constant was 4.93×10^{-2} for β-hydroxybutyrate dehydrogenase. The energy charge was calculated according to a ratio of ATP + 1/2 ADP to total adenine nucleotides (Atkinson 1968, 1970). The concentration of HbCO was determined by the method of van Kampen and Zijlstra (1965). The concentrations of CO in the gas sample were determined by gas-chromatography (Yamamoto et al. 1979). The concentrations of HCN in the gas sample and blood cyanide were determined by the method of Feldstein and Klendshoj (1954).

After HCN in the gas sample was transferred to 2ml of 0.1 *N* NaOH solution in a 30ml flask, the solution was determined for cyanide, and the original HCN concentration in the gas sample was calculated. Student's *t*-test was used for statistical evaluation.

Results

Inhalation of Carbon Monoxide

The concentration of CO in the chamber was maximally about 2%. The HbCO concentrations ranged from 2% to 70%. Respiration was stimulated slightly at an early stage of the exposure when large doses of CO were injected, while stimulation was observed at a later stage when volumes of CO injected were small. All animals survived the 3-min exposure. The change in the redox state is shown in Fig. 1. When the HbCO concentration was below 10%, a slight in-

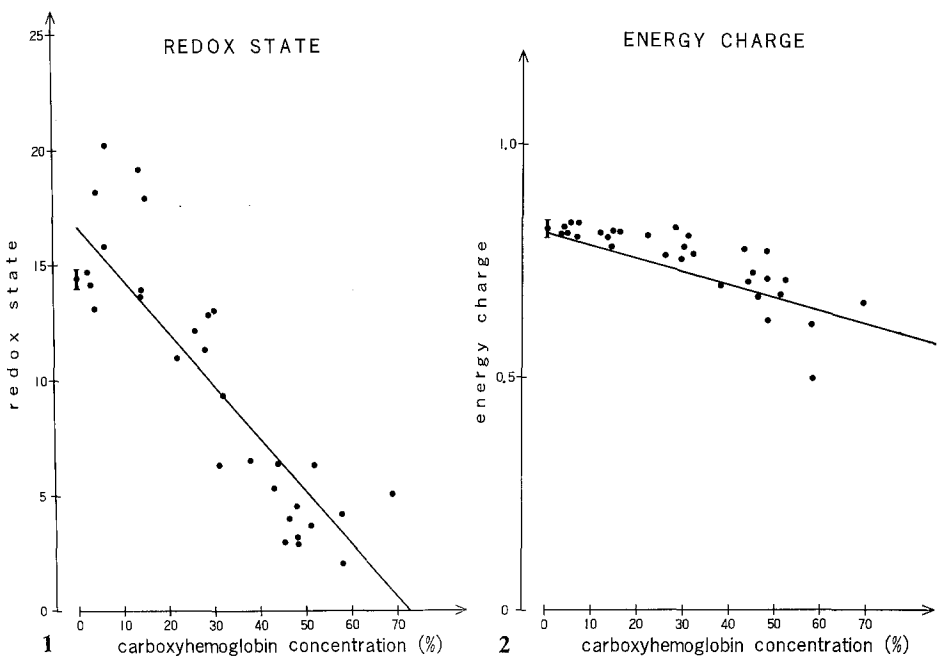

Fig. 1. The mitochondrial redox state and the carboxyhemoglobin concentration. The redox state was calculated as a concentration ratio of acetoacetate to β-hydroxybutyrate. A significant negative correlation was obtained ($r = -0.88$, $P < 0.01$). The regression line of the redox state on the HbCO concentration is shown ($Y = 0.23X + 16.7$). When the HbCO concentration was below 10%, a slight increase from the control level was observed in some cases. The redox state began to decrease when the HbCO concentration exceeded 10%

Fig. 2. The hepatic energy charge and the carboxyhemoglobin concentration. The energy charge was calculated as a ratio of ATP + 1/2 ADP to ATP + ADP + AMP. As HbCO concentrations increased, the energy charge decreased. The rate of decrease became higher when HbCO concentration exceeded 40%. The regression line of the energy charge on the HbCO concentration is shown ($r = -0.83$, $P < 0.01$, $Y = -3.16\text{E-}03X + 0.848$)

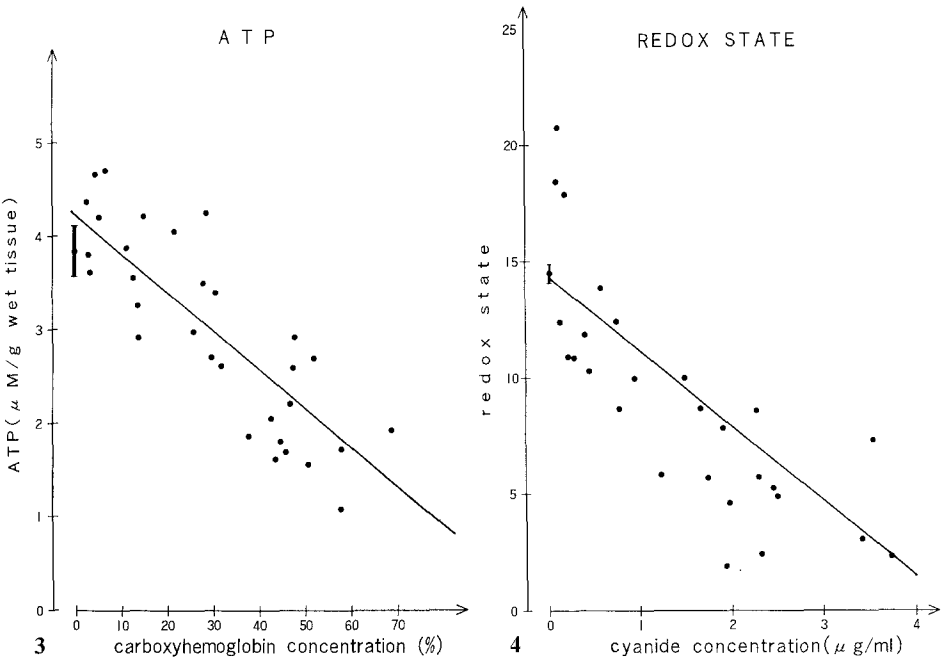

Fig. 3. Changes in the hepatic ATP contents following CO exposure. A significant negative correlation was obtained ($r = -0.81$, $P < 0.01$). The regression line of the ATP content on the HbCO concentration is shown ($Y = -0.42X + 4.27$)

Fig. 4. The mitochondrial redox state and the blood cyanide concentration. A significant negative correlation was obtained. ($r = -0.73$, $P < 0.01$). The regression line of the redox state on the blood cyanide concentration is shown ($Y = -3.46X + 14.97$)

crease from the control level was observed in some cases. When the HbCO concentration exceeded 10%, the redox state, i.e., the $NAD^+/NADH$ ratio, began to decrease linearly. With HbCO concentrations higher than 40%, the rate of decrease became much slower. When HbCO concentrations increased, the concentrations of β-hydroxybutyrate increased, while those of acetoacetate had no correlation with HbCO concentrations. There was no correlation between concentrations of HbCO and total ketone bodies. Figure 2 shows the change in the energy charge. As HbCO concentrations increased, the energy charge decreased. The rate of the decrease became higher when HbCO concentrations exceeded 40%. As compared to the rate of decrease in the redox state, it was much slower in the energy charge. When HbCO concentrations increased from 10% to 40%, the redox state decreased to about 30% of the original value, while the rate of decrease in the energy charge was only about 15%. Figure 3 shows the change in ATP content in the liver. The content of ATP decreased linearly as the HbCO concentrations increased. On the other hand, ADP contents had no correlation with HbCO concentrations and AMP contents increased as HbCO concentrations became higher. The total adenine nucleotide levels had no correlation with HbCO concentrations.

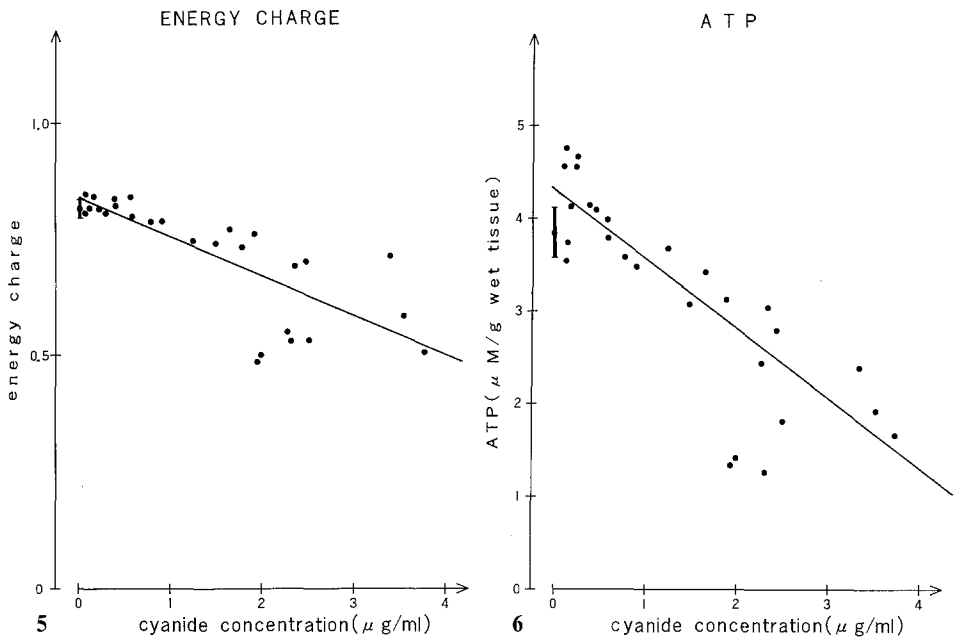

Fig. 5. The hepatic energy charge and the blood cyanide concentration. A significant correlation was obtained ($r = -0.847$, $P < 0.01$). The regression line of the energy charge on the cyanide concentration is shown ($Y = -0.085X + 0.842$)

Fig. 6. Changes in hepatic ATP content following cyanide exposure. A significant negative correlation is present ($r = -0.866$, $P < 0.01$). The regression line of the ATP content on the cyanide concentration is shown ($Y = -0.76X + 4.32$)

Inhalation of Hydrogen Cyanide

The concentration of HCN in the animal chamber was maximally about 1,000 ppm. The maximal blood cyanide concentration was 5 μg/ml. All animals survived the 3-min exposure. A slight degree of respiratory stimulation was observed at an early stage of the exposure when a large amount of NaCN was added. The change in the redox state is shown in Fig. 4. When the cyanide concentration in the blood was below 0.5 μg/ml, the redox state showed a slight increase in some cases, but this increase was statistically insignificant. The redox state began to decrease linearly when the cyanide concentrations exceeded 0.5 μg/ml. However, the redox state did not decrease any more, when the cyanide concentrations exceeded 2.0 μg/ml. Concentrations of acetoacetate decreased as cyanide concentrations increased, while β-hydroxybutyrate concentrations showed no correlations with cyanide concentrations. At low cyanide levels, the concentration of the total ketone body showed a sharp rise; however, it began to decrease when cyanide concentrations reached the level of 0.5 μg/ml. The changes in the energy charge is shown in Fig. 5. When the cyanide concentration was below 0.5 μg/ml, the energy charge level remained virtually unchanged. It began to decrease when cyanide concentrations exceeded 0.5 μg/ml. As cyanide concentrations increased, ATP content decreased (Fig. 6), while

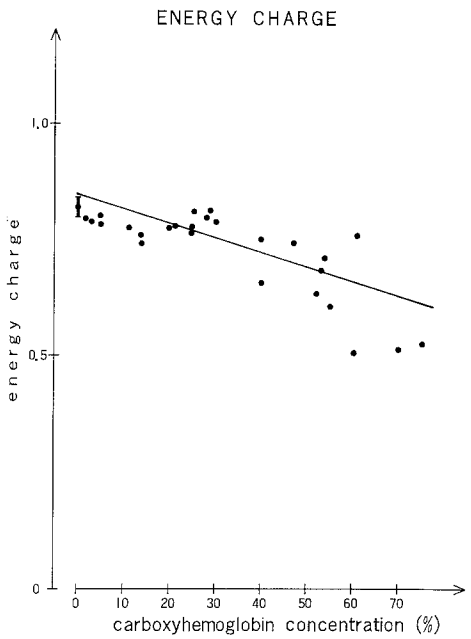

Fig. 7. The hepatic energy charge and the carboxyhemoglobin concentration under the presence of low cyanide concentration (less than 0.5 μg/ml). No significant difference is observed in the regression lines between the single CO exposure (Fig. 2) and the combined exposure ($r = -0.77$, $P < 0.01$, $Y = -0.027X + 0.812$)

both ADP and AMP contents increased. As was the case with CO, the rate of decrease in the redox state was much higher than that of the energy charge. When blood cyanide concentrations increased from 0.5 μg/ml to 2.5 μg/ml, the energy charge showed a slight decrease (from 0.8 to 0.7), while in the redox state the rate of decrease was high.

Inhalations of CO and HCN

In the combined exposure, the HCN concentration in the chamber was maintained at low levels so that blood cyanide concentrations might not exceed 0.5 μg/ml. Under these conditions, over a wide range of HbCO concentration there was no significant difference in the energy charge between the animals exposed to CO alone and those exposed to CO and HCN (Fig. 7). Low levels of cyanide did not change the energy charge level of acutely CO-exposed rats.

Discussion

Carbon monoxide (CO), a gas produced by incomplete combustion of carbon compounds, has an affinity for hemoglobin which is about 210 times that of O_2 for hemoglobin, and forms carboxyhemoglobin (HbCO), which is unavailable for the transport of oxygen. In addition, when HbCO is present, the dissociation curve of the remaining HbO_2 shifts to the left, and the amount of O_2 deliverd to the tissue decreases. Carbon monoxide also combines with hemoproteins other than hemoglobin. Somogyi et al. (1981) histochemically showed a

substantial decrease of the cytochrome c oxidase activity in the rat heart perfused with a CO-saturated solution for 1–2 min. However, because the affinity of CO to hemoproteins other than hemoglobin is very low, the toxicity of CO has been explained by the hypoxia caused by the formation of HbCO. Hydrogen cyanide (HCN) is one of the combustion products from nitrogen-containing polymers. Cyanide causes a histotoxic hypoxia by combining with cytochrome c oxidase. Previously, parameters, such as concentrations of ATP, lactate, and hydrogen ion, have been used as indices for hypoxia. However, they are only indirect measures of hypoxia. In the present study, we used the redox state and the energy charge as indices. These parameters directly relate to mitochondrial functions and are considered to reflect the changes in the state of the electron transport system.

For calculations of the redox state expressed as a ratio of NAD^+ to NADH, the β-hydroxybutyrate dehydrogenase system was used in the present study. The system was considered to be indicative of the redox state of the mitochondria, because this system is located exclusively in the hepatic mitochondria and its activity is high in the liver of the rat (Williamson and Lund 1967). In hypoxia, the mitochondrial oxido-reduction state shifts to the reduced state from the normal state. In the present study, when HbCO concentrations were low (below 10%), a slight, but insignificant increase from the control level was observed in some cases. Similar changes were also observed at low levels of cyanide. According to Shimahara's results, obtained in a hemorrhagic shock model, the redox state sharply increased above the control level after about 5 min, when the shed blood was reinfused into the exsanguinated animal (Shimahara et al. 1981). He explained this over-shoot phenomenon as the complete conversion of the NADH accumulated during the ischemic state to NAD^+. However, this explanation is not applicable to the present case. On the other hand, Takano (1978) observed no change in the redox state of the rat renal tissue at low concentrations of HbCO (below 10%) by use of a redoximeter. The redoximeter indicates the total change of the reduced pyridine nucleotide in the cell, but there is a lag time until a change in fluorescence appears as a result of the induction of hypoxia. Therefore, at low concentrations of HbCO, it is considered that the redoximeter does not indicate the specific oxido-reduction state of mitochondria. The ultimate causes of the slight increase in the present study remain unresolved.

At high concentrations of HbCO and blood cyanide, a turning point, beyond which the rate of decrease in the redox state became much slower was observed. The turning point was about 40% for HbCO and about 2.0 μg/ml for cyanide. Yamamoto showed that in rabbits exposed to combustion products mainly consisting of CO the hydrogen ion concentration in the blood began to increase steeply when the HbCO concentration exceeded 40% (Yamamoto 1975). The value of the redox state corresponding to the turning point was about 5 for each gas. This value is approximately equal to the value reported by Shimahara et al. (1981), beyond which cells are irreversibly damaged.

In living cells, the energy metabolism is a dynamic process and well controlled. Many reactions in metabolism are regulated in part by the energy status of the cell. The amount of adenine nucleotides does not necessarily indicate the

dynamic changes of the cellular energy metabolism (Shimahara et al. 1981). As one index of the energy status, the adenylate energy charge proposed by Atkinson was introduced in the present study. It reflects the energy balance between the energy-generating and the energy-consuming sequences and is a parameter closely related to the regulatory system of metabolism. Therefore, the change in the energy charge can be a suitable index showing the extent to which the energy metabolism is disturbed. Under normal aerobic conditions, the energy charge of a cell is maintained at a constant level. If the energy charge decreases, ATP-generating sequences are accelerated, and ATP-consuming sequences are slowed down (Ozawa 1983). On the other hand, in hypoxia, the electron transfer along the respiratory chain is inhibited and the oxidative phosphorylation is depressed. According to Ukikusa's experiment, in which the rats were placed for 15 min under hypoxic conditions, the hepatic energy charge level began to decrease when arterial PaO_2 values became lower than 50 mm Hg (Ukikusa et al. 1979). In the present study, the energy charge began to decrease linearly, when the concentrations of the toxicants in the blood exceeded certain levels (40% for HbCO and 0.5 μg/ml for cyanide).

There are marked differences in the response to various stressors among mitochondria from different organs. According to MacMillan (1975), in acutely CO-exposed rats the redox state of the brain mitochondria decreased with increases in HbCO concentrations, while there was no change in the energy charge as long as HbCO concentrations were below 60%. In the present data obtained in the liver, both the redox state and the energy charge decreased with increases in HbCO concentrations. Compensatory increase in cerebral blood flow and a large amount of creatine phosphate in the brain may explain this discrepancy. Ozawa et al. (1967) studied the effects of ischemia on the oxidative phosphorylation in mitochondria from various tissues of rats. After 3-min ischemia, the maximal phosphorylation rate in the brain mitochondria fell to one third of the initial rate, while in mitochondria from the liver, heart, and kidney, the initial rate of phosphorylation remained almost constant for 10 min. From these reports the liver as compared with the brain, can be considered to be more vulnerable to carbon monoxide, but more resistant to ischemia.

When CO and HCN coexist, they seem to act additively (Anderson 1981). The presence of such low levels of blood cyanide, as changes neither the redox state nor the energy charge, did not affect the relationship between the energy charge and the HbCO concentration. Under the present experimental conditions, any interaction of CO and low levels of cyanide could be excluded.

The presence of a linear relationship between HbCO concentrations and the impairment of functions important for life, which was delineated in the present study, will make HbCO values a significant index for determining to what extent CO intoxications contributed to the death. The same is considered to hold true of cyanide.

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