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Inhibition of protein secretion from liver by cadmium

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Serum enzymes which have been used to diagnose the liver dysfunction can be separated into two groups. One is an enzyme that leaks from damaged liver cells and the other is an enzyme that is secreted by the normal functioning liver.

During the course of a general survey for sensitive indicators of the hepatic injury by cadmium, the serum activity of cholinesterase (CHE; EC 3.1.1.8) (a secreted enzyme from the liver) has been shown to decrease significantly before other indicators in serum show any significant alterations [1]. Further examinations revealed that the serum CHE activity decreases with time after a single subcutaneous injection of cadmium. This activity attained the lowest level on days 2 and 3 post-injection in both sexes of different age groups of rats. However, the activities of the two leaked enzymes, glutamic pyruvic transaminase (GPT; EC 2.6.1.2) and glutamic oxaloacetic transaminase (GOT; EC 2.6.1.1), in the serum were not increased significantly by the doses examined [2].

As the depression of the serum CHE activity was shown not to be caused by a direct effect of cadmium ion on CHE in serum [2], the depression of the activity was deduced to be caused by a decrease of CHE in serum (not a decrease of the activity). The decrease in amount of serum CHE can be explained by a greater degradation rate of serum CHE, a reduced rate of synthesis within the liver, or a reduced rate of secretion. The present study is intended to examine one of these possibilities. At the same time changes in concentrations of serum total protein and albumin with time after cadmium loading were determined to compare the changes with that of the CHE activity in serum.

Materials and methods

Injection of cadmium. Female rats of the Wistar strain (Jcl, Clea Japan Co., Tokyo) were fed on a standard diet (MF diet, Oriental Yeast Co., Tokyo) and distilled water *ad libitum* from 4 to 12 weeks old. The animals (5 rats/group) were injected subcutaneously with a 0.1 ml solution of cadmium chloride (1.5 mg Cd^{2+}/kg body wt) in saline singly and killed by exsanguination at 12 hr, 1, 2, 3, 5 and 7 days after the injection under ether anaesthesia. The control rats were injected with saline in the same manner and killed on days 0, 3 and 7 post-injection.

Determinations of enzyme activities and protein concentration in serum. Blood was collected carefully to avoid hemolysis from the carotid artery, allowed to clot in an ice-bath and then centrifuged at 2300 *g* for 10 min. As the value of serum CHE activity measured with acetylthiocholine as a substrate was about 2.5 times higher than that with butylthiocholine (Y. Mitane, private communication), the serum CHE activity was determined with the former substrate. The activities of CHE, GOT and GPT in serum were determined on a GEMSAEC Fast Analyzer using Cholinesterase-Color-Test® [3], Monotest® GOT opt. [4], and Monotest® GPT opt. [5] (Boehringer-Mannheim Co., Mannheim) according to the manufacturer's monographs. Control serum (Precinorm EA) was used as the reference sample. The concentration of serum total protein was determined colorimetrically by Biuret method [6] and the concentration of albumin by the bromcresol green method [7] using a u.v.-visible spectrophotometer (UV-260, Shimadzu Co., Kyoto). The concentration of globulin was calculated by subtracting the albumin concentration from the total protein concentration, and the A/G (albumin/globulin) ratio was determined.

Determinations of cholinesterase activity and protein concentration in liver. A 1.5 g portion of livers was homogenized in 4 vol. of 0.1 M Tris–HCl buffer solution (pH 7.4, 0.25 M glucose) using a glass–Teflon homogenizer. The homogenate was centrifuged at 1500 *g* for 20 min and the supernatant fraction was subjected to the determinations of the CHE activity and protein concentration as follows. The CHE activity was determined by the Rana *et al.* method [8] and the manufacturer's monograph [3] (Boehringer-Mannheim Co.). A 0.02 ml aliquot of the supernatant fraction and a 0.025 ml portion of a substrate solution (5 mM acetylthiocholine iodide) were mixed with a 0.75 ml solution of 50 mM phosphate buffer (pH 7.2, 0.25 mM 5,5′-dithiobis (nitrobenzoic acid)). The CHE activity was determined at 30° as a change in absorbance at 410 nm during 30 sec intervals ($5863 \times \Delta E/30$ sec [U/l]) on a multipurpose spectrophotometer (MPS-5000, Shimadzu). The total protein concentration in the supernatant fraction was determined by Lowry method [9] using bovine serum albumin as a standard.

Statistical analysis. As the control values were determined only at the three data points and there were no significant differences among the three data points for all biological data, all control values were expressed as means ± S.D. of 15 samples. Statistical evaluations of alterations from the controls were made by Welch's *t*-test. A value of $P < 0.05$ was accepted as significant and marked with * as follows: *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$.

Results and discussion

The CHE activity in serum began to decrease shortly after the injection of cadmium and attained the lowest level on day 3 after the injection. The time course shown in Fig. 1A is the same as our previous report [2]. On the other hand, the CHE activity in the liver remained at the control level throughout the experiment (Fig. 1B), suggesting that the decrease of serum CHE activity was not caused by a reduced rate of secretion from the liver.

The concentration of total protein in serum also began to decrease after the injection of cadmium. The lowest level was attained on day 2 after the injection. This is one day earlier than that of the lowest level of the CHE activity. The time course shown in Fig. 2A indicates that the concentration of total protein in serum was depressed in a similar mechanism to the CHE activity. This observation coincides with the former observation [2], indicating that the serum CHE activity depressed by cadmium was not due to a direct inhibition of the activity by cadmium but due to a decrease in quantity of the enzyme.

Albumin is the most abundant protein in serum and the decrease of the total protein in serum (Fig. 2A) was largely explained by the decrease of serum albumin as shown in Fig. 2B. The time course of albumin in Fig. 2B is almost identical to that of the total protein in Fig. 2A, suggesting that the change of the total protein in serum was caused mainly by that of albumin.

The serum globulin showed a similar change in quantity and in time course (Fig. 2C). The change was not significant or evident compared to those of the total protein and albumin. The similar change of the globulin concentration to the albumin concentration resulted in an invariant value of the albumin to globulin ratio throughout the experiment (Fig. 2D). This result indicates that some of globulin constituents may also be decreased in concentration.

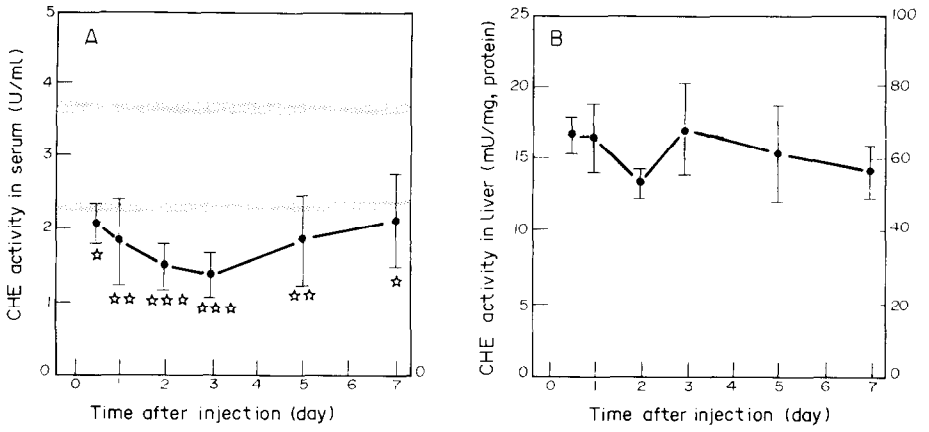

Fig. 1. Changes of cholinesterase activities in serum (A) and liver (B) with time after a single sc injection of cadmium (1.5 mg/kg wt). The control rats were killed on days 0, 3 and 7 (5 rats/group) and the whole data (15 rats in total) were expressed as means ± S.D. with the shaded bars. The Cd-injected rats were killed on days 0.5, 1, 2, 3, 5 and 7, and the CHE activities in the serum and liver were expressed as means ± S.D. of 5 samples.

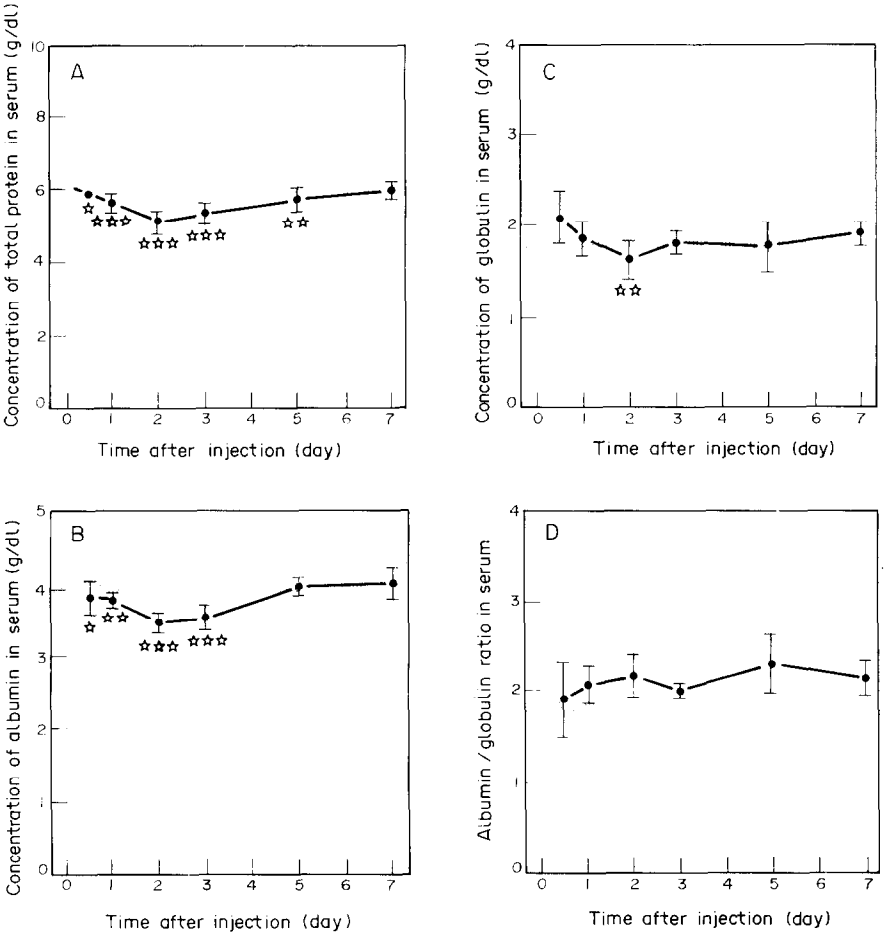

Fig. 2. Changes in the concentrations of total protein (A), albumin (B) and globulin (C) and albumin/globulin ratio (D) in serum after a single sc injection of cadmium (1.5 mg/kg body wt). The control rats injected with saline were killed on days 0, 3 and 7 (5 rats/group). The data of the three groups were combined and expressed as means ± S.D. of 15 samples with the shaded bars. The Cd-injected rats were killed on days 0.5, 1, 2, 3, 5 and 7, and the data were expressed as means ± S.D. of 5 samples in each group.

In contrast to the serum CHE activity and albumin concentration (and possibly globulin concentration), the GPT and GOT activities in serum remained within the control levels throughout the experiment (data not shown).

The present study revealed that cadmium depresses the serum levels of not only CHE but also other secretory proteins from the liver, and this inhibitory action is observed before the onset of serum increase of leaked enzymes from the liver.

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Non-selective inhibition of GABA and 5-HT uptake systems in rat brain by *N*-*n*-alkyl hydroxybenzylamine and *N*-*n*-alkyl phenylethylamine derivatives

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GABA (4-aminobutyric acid) is believed to be the major inhibitory neurotransmitter used by the mammalian central nervous system (CNS) [1]. Accordingly, drugs which impair the inhibitory effects of GABA, by blocking its synthesis, release or post-synaptic actions, possess proconvulsant activity, whereas agents which enhance GABA mediated inhibition often exhibit anticonvulsant effects [2]. One approach to enhancing the inhibitory effects of synaptically released GABA is to block the high affinity, sodium dependent uptake process which transports GABA into GABAergic neurones and glial cells [3, 4]. When these GABA uptake mechanisms are inhibited, both the inhibitory effects of iontophoretically applied GABA, and physiologically evoked GABA mediated postsynaptic inhibition have been shown to be enhanced [5, 6]. Likewise, the central administration of GABA uptake inhibitors has been shown to protect genetically susceptible mice against sound-induced seizures [7]. Thus, it has been suggested that a potent and selective inhibitor of central GABA uptake mechanisms could be beneficial in the treatment of various neurological disorders, including epilepsy [2].

However, in addition to blocking GABA uptake, such a drug must also be able to cross the blood brain barrier. We have previously synthesized a series of *N*-*n*-alkyl hydroxybenzylamine derivatives (5–7) which inhibit the uptake of [^{3}H]GABA into rat brain synaptosomes [8]. Furthermore, the presence of long, hydrophobic, alkyl side chains on these molecules might be expected to facilitate their entry into the CNS. Sulphation of (6) on oxygen afforded a very potent GABA uptake inhibitor (IC_{50} 3.5 μm) which we regarded as a major lead because of its lipophilicity. This compound was, however, somewhat unstable to hydrolysis. With the apparent ability of the *n*-octyl chain to mask a zwitterion, it was clearly important to establish the generality of this structural device for the design of neurotransmitter analogues. In the present study, a number of *N*-octyl phenylethylamines (9–11) were also tested as inhibitors of [^{3}H]GABA uptake into crude P2 fractions prepared from rat cerebral cortex. Furthermore, to investigate their pharmacological specificity, the above compounds were examined for their ability to inhibit [^{3}H]5-HT uptake into crude P2 fractions prepared from rat hypothalamus.

Materials and methods

Chemical syntheses. Hydroxybenzylamines (5–7) were samples prepared and used in our previous studies [8, 9]. *N-octyl-2-methylbenzylamine* (8) was prepared by the method of Dewar *et al.* [9]; the hydrochloride had m.p. 169°. *Found:* C,71.1; H, 10.3; N, 5.0; Cl, 13.4; $C_{16}H_{28}ClN$ requires: C, 71.2; H, 10.5; N, 5.2; Cl, 13.1%. δ_H ($CDCl_3$) 7.8–7.1, 4H, m; 4.02, 2H, s; 2.43, ^{3}H, s; 2.8, 2H, m; 2.0–0.7, 15H.

The *N-octylphenylethylamines* (9–11) were prepared by borane reduction of the corresponding amides which were obtained from the acid chlorides. *N-octyl-3,4-dimethoxyphenylacetamide* (93%) b.p. 112–114°, 0.1 Torr ν_{max} (Nujol): 3320, 1640, 1605, 780 and 720 cm^{-1}. δ_H ($CDCl_3$): 0.7–1.7, 15H, m; 3.2, 2H, *m*; 3.51, 2H, s; 3.88, 6H, s; 6.83, ^{3}H, s; 5.65, D_2O, b. *Found:* C, 70.1; H, 9.4; N, 4.2%. $C_{18}H_{29}NO_3$ requires C, 70.3; H, 9.5; N, 4.6%.

N-octyl-3,4-dimethoxyphenylethylamine hydrochloride (11) (61%) m.p. 116–118° ν_{max} (Nujol): 3200–2300, 1590, 800 and 720 cm^{-1}. δ_H ($CDCl_3$): 0.7–2.1, 15H, m; 2.96, 2H, m; 3.20, 2H, s; 3.86, 6H, s; 6.77, ^{3}H, s. *Found:* C, 65.1; H, 9.8; N, 4.4; Cl, 10.5%. $C_{18}H_{32}ClNO_3$ requires C, 65.6; H, 9.7; N, 4.3, Cl, 10.8%.

N-octyl-3,4-dihydroxyphenylethylamine hydrobromide (10) was prepared by demethylation of (11) using boron tribromide (63%) m.p. 108°. ν_{max} (Nujol) 3500–2500, 1600, 800 and 720 cm^{-1}. δ^H ($DMSOd_6$): 0.7–1.8, 15H, m; 2.88, 2H, m; 3.37, 2H, s; 6.4–6.8, ^{3}H, m; 8.4–8.9, b, D_2O. *Found:* C, 54.3; H, 8.1; N, 3.9; Br, 24.0%. $C_{16}H_{28}BrNO_2$ requires C, 55.5; H, 8.2; N, 4.1; Br, 23.1%.

N-octyl-4-hydroxyphenylacetamide (73%) m.p. 142–143° δ_H ($CDCl_3$): 0.7–1.7, 15H, m; 3.17, 2H, m; 3.44, 2H, s;