Lipid peroxidation caused by chinoform-ferric chelate in cultured neural retinal cells[1]

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Summary. Incorporation of chinoform-ferric chelate was demonstrable in cultured neural retinal cells of chick embryos after 1 h of incubation, and the lipid peroxide level in the cells was increased strikingly 1 h thereafter. On the other hand, free ferric ions were scarcely incorporated into the cells, and a significant increase in the lipid peroxide level in the cells was not observed. These data indicate that chinoform is carrier of iron for its passage through cell membranes and that the incorporated iron induces lipid peroxidation which in turn leads to neural cell degeneration.

Key words. Lipid peroxidation; chinoform-ferric chelate; chick neural retinal cells.

Massive doses of chinoform (5-chloro-7-iodo-8-quinolinol) were proved by epidemiological research to be the cause of subacute myelo-optico-neuropathy (SMON)[2–4]. There are many reports concerning the toxicity of chinoform and its derivatives in vivo[5–14] and various effects of chinoform on cellular functions in vitro[15–18]. These authors, however, focused their attention on the toxicity of chinoform itself, but not on that of chinoform-ferric chelate. For the diagnosis of SMON, chinoform-ferric chelate, which appears on the tongue and in the urine and feces of the patient as a green-colored substance, is regarded as a characteristic substance for this disease[18–19]. In 1976, Yagi et al.[20] proposed that lipid peroxidation induced by chinoform-ferric chelate is a direct cause of the degeneration of nerve tissues which leads to provocation of the neuropathy. According to this supposition, Ohtsuka et al.[21] demonstrated that cultured retinal neuroblasts degenerated only when treated with chinoform-ferric chelate, but not with free chinoform or ferric ions, and this degeneration was prevented by α-tocopherol. From these results, we predicted that chinoform itself should have no toxic effect at low concentrations and that the lipid peroxidation due to the incorporation of iron chelated with chinoform into the nerve tissue would be the most important step in the induction of the neuropathy. To verify this prediction, we examined the incorporation of chinoform-ferric chelate into neural retinal cells and compared its level with the degree of lipid peroxidation in them.

Materials and methods. Preparation and culture of neural retinal cells. Neural retinal cells were prepared by the method of Okada et al.[22]. The cells were resuspended in fresh culture medium (Eagle's minimum essential medium (NISSUI, Tokyo) supplemented with 8% fetal bovine serum (GIBCO, New York), 0.3% glutamine, and 0.14% sodium bicarbonate), and inoculated into Falcon plastic culture dishes (35 mm in diameter) using 1.5 ml of fresh culture medium as described above ($1.5–1.6 \times 10^7$ cells/dish). These cultures were incubated under an atmosphere of 5% CO_2–95% air at 36.5 °C for 5–6 days prior to the experiment. The culture medium was changed every 2 days.

Incorporation of [^{14}C]-chinoform and [^{59}Fe]. [2,3,4-^{14}C]Chinoform (specific radioactivity, 1.63 mCi/mmol, Daiichi Pure Chemicals, Tokyo) was put into the culture medium and sonicated to form an emulsion (concentration, 10 mM). A mixture of unlabeled $FeCl_3$ and [^{59}Fe]Cl_3 (specific radioactivity, 18 mCi/mg Fe, New England Nuclear, Boston) was dissolved in the medium (concentration, 3 mM). The [^{14}C]chinoform-[^{59}Fe] chelate was prepared by mixing and sonicating equal volumes of the above [^{14}C]chinoform and [^{59}Fe] solutions. Unlabeled chinoform-ferric chelate was prepared in the same manner. α-Tocopherol was also emulsified in the medium by sonication. These reagents were diluted with the medium to the desired concentrations. After these reagents were added to the culture medium (2 ml) and incubated at 37 °C for various time intervals, the medium was discarded and the cells were washed exhaustively with Ca^{2+}-Mg^{2+}-free Hank's solution (8–10 times). Then, 2 ml of 0.5 M NaOH was added to the dishes in order to lyse the cells. Aliquots (0.2 ml) of the cell lysate were transferred to scintillation vials containing 2 ml of scintillation cocktail (Scintisol-500, Wako Pure Chemical Industries Ltd., Osaka). The radioactivity was

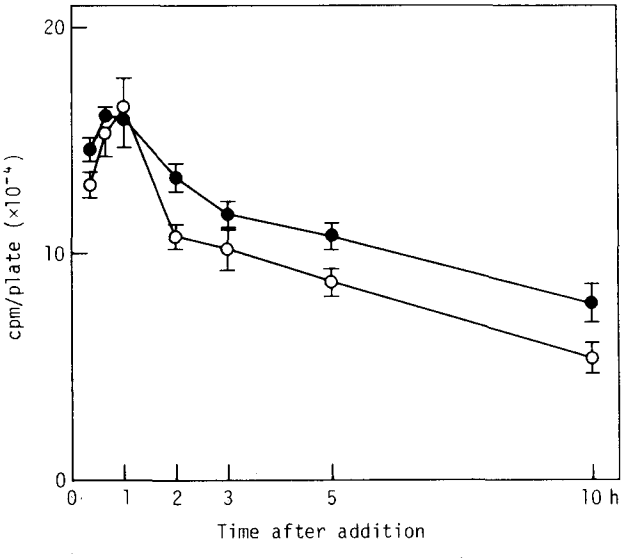

Figure 1. Incorporation of chinoform into neural retinal cells. ○, [^{14}C]chinoform (50 μM); ●, [^{14}C]chinoform-[^{59}Fe] chelate (50 μM as chinoform). See materials and methods for details. Each point represents the mean of four plates. Vertical bars indicate standard errors.

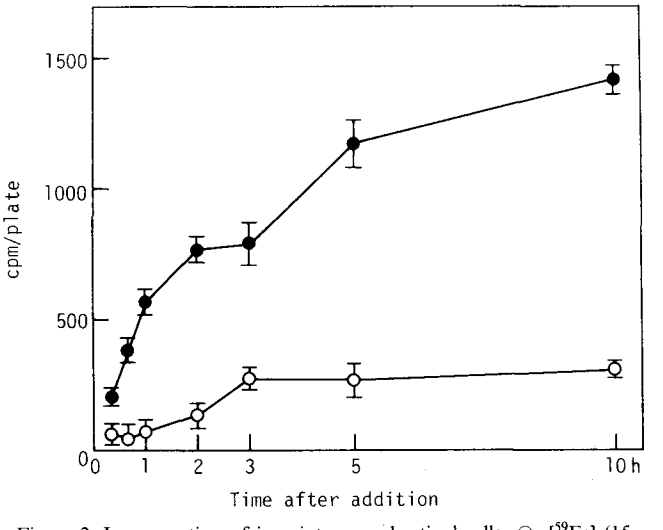

Figure 2. Incorporation of iron into neural retinal cells. ○, [^{59}Fe] (15 μM); ●, [^{14}C]chinoform-[^{59}Fe] chelate (15 μM as ferric ions). See materials and methods for details. Each point represents the mean of four plates. Vertical bars indicate standard errors.

counted with a liquid scintillation counter (Beckman). Aliquots (0.2 ml) of the cell lysate were also transferred to test tubes for counting [^{59}Fe] with an autowell γ-ray counter (Aloka). [^{14}C]Chinoform and [^{59}Fe] in the culture medium were also counted.

Measurement of lipid peroxides. After the culture medium was discarded, cells were collected using a rubber policeman and washed by centrifuging 3 times with 0.9% NaCl solution. Then, the cells from each dish were resuspended in 0.8 ml of distilled water and assayed for lipid peroxides by the thiobarbituric acid method[23]. The lipid peroxide level was expressed as nanomoles per plate in terms of malondialdehyde using tetramethoxypropane as an external standard.

Results and discussion. When neural retinal cells were cultured for 5–6 days, a sheet of flattened epithelial cells was formed, upon which small neuroblasts with axonal processes were superimposed. These features are the same as reported previously[21]. To such cultures the agents to be examined were added, and their incorporation was measured.

As shown in figure 1, radioactive [^{14}C]chinoform was rapidly incorporated into the cells, and the maximal incorporation was observed at 1 h of incubation. Thereafter, incorporated radioactivity gradually decreased until 10 h of incubation. On the other hand, the radioactivity of [^{14}C]chinoform in the culture medium decreased within 1 h and then increased, indicating that the incorporated radioactivity was released into the medium (data not shown). The incorporation pattern of chinoform into the cells was similar to the above when chinoform-ferric chelate was used, and the maximum incorporation was found at 40–60 min of incubation. Then the radioactivity was gradually released into the medium as in the case of free chinoform. In the presence of 100 μM of α-tocopherol, a similar incorporation pattern was observed. Although the reason why the incorporated chinoform was released into the medium is not known at present, it may be possible that chinoform, which was taken up by the cells, was gradually metabolized in the cells during incubation at 37 °C and then released.

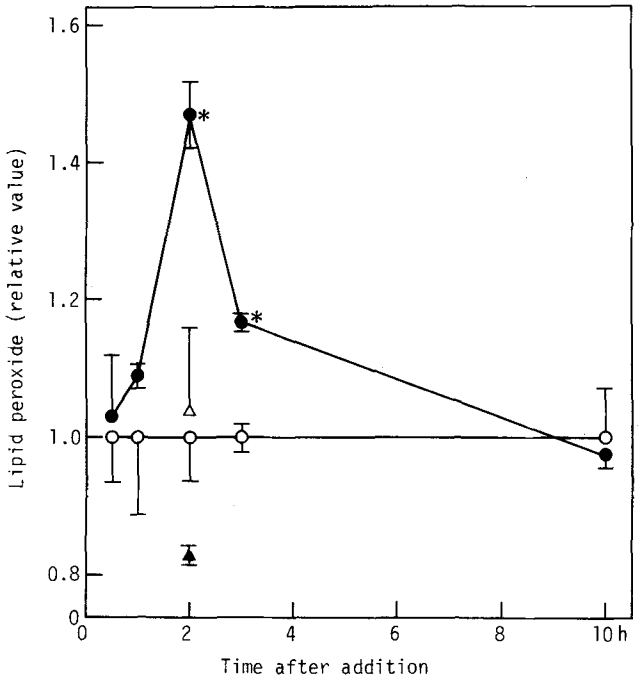

Figure 3. Lipid peroxide levels in neural retinal cells. ○, no addition (control); ●, chinoform-ferric chelate (50 μM as chinoform); △, chinoform (50 μM); ▲, chinoform-ferric chelate (50 μM as chinoform) + α-tocopherol (100 μM). After the addition of these reagents to the cells and incubation at 37 °C for various time intervals, the cells were washed with 0.9% NaCl and analyzed for their lipid peroxide levels by the thiobarbituric acid method. Lipid peroxide levels are expressed as relative values. Values of control cultures (no addition) were 0.88, 0.91, 0.99, 1.1, and 0.99 (nmol/plate) at 0.5, 1, 2, 3, and 10 h, respectively. Points marked by asterisks indicate that the difference from the control is statistically significant ($p < 0.01$). Each point represents the mean of five plates. Vertical bars indicate standard errors.

The incorporation of iron into the cells is shown in figure 2. Iron was efficiently incorporated into the cells by 1 h of incubation when chelated with chinoform, but scarcely incorporated when in its free form. From this result, it appears that chinoform, being lipotropic in nature, plays a role in carrying iron across the plasma membranes. α-Tocopherol had no significant effect on the incorporation of iron chelated with chinoform.

We observed a remarkable difference between the incorporation patterns of chinoform and iron, when the chinoform-ferric chelate was added to the medium. After 1 h of incubation, chinoform taken by the cells was gradually released into the medium, whereas the incorporation of radioactive iron increased gradually. Probably, iron chelated with chinoform would be transferred to proteins such as transferrin present in the cells[24-25], and chinoform would be metabolized and released from the cells.

Under the same conditions, the lipid peroxide levels of neural retinal cells were examined. As shown in figure 3, the lipid peroxide level in the presence of the chinoform-ferric chelate increased significantly at 2–3 h after its addition as compared to the control (no addition). Chinoform itself had no significant effect at 2 h after addition. The increase in lipid peroxide level in the presence of free ferric ions is not significant, and the data involved large deviations (data not shown). In accordance with these data, the incorporation of free ferric ions into the cells was very little, as shown in figure 2, and the addition of ferric ion had no deleterious effect on the morphology of the retinal neuroblasts[21]. Therefore, the incorporation of iron in the form of chinoform-ferric chelate should be considered as deleterious. As shown in figure 3, the addition of α-tocopherol inhibited the lipid peroxidation induced by chinoform-ferric chelate. Although it was found that α-tocopherol suppressed the thiobarbituric acid reaction (unpublished result), its inhibitory effect was minute at a concentration of 100 μM. Therefore, the data obtained in the present study probably reflect mainly the inhibition of lipid peroxidation due to chinoform-ferric chelate.

It is well known that lipid peroxidation causes cell degeneration[26-28]. We further observed that neural retinal cells degenerated within a day, when they were cultured in the presence of ascorbic acid (0.2–0.5 mM) or in an atmosphere of 5% CO_2–95% O_2. These conditions are considered to induce lipid peroxidation[29-33]. This degeneration was prevented by catalase and α-tocopherol, respectively (data not shown).

From the results described in this paper, we conclude that a) chinoform itself has no toxic effect even though it is incorporated into cells, but has only a carrier function for delivery of iron into the cells, and b) iron incorporated into the cells in the form of chinoform-ferric chelate is an inducer of lipid peroxidation, which in turn leads to neural cell degeneration. Details of the mechanism of lipid peroxidation induced by chinoform-ferric chelate have been worked out in our laboratory using isolated rat liver microsomes and will be presented elsewhere.

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Effect of pyrophosphate and orotidine monophosphate on cytosine deaminase regulatory properties

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Summary. The maximal velocity of the reaction (V_{max}) and the half-saturation constant ($K_{0.5}$) values of the *S. typhimurium* cytosine deaminase were altered in the presence of its effectors, pyrophosphate and orotidine monophosphate. From the kinetics of orotidine monophosphate inhibition of cytosine deaminase, it was characterized as a mixed-type noncompetitive inhibitor.
Key words. Cytosine deaminase; kinetics; pyrophosphate; orotidine monophosphate.

The deamination of cytosine to uracil with the concomitant release of ammonia is catalyzed by the enzyme cytosine deaminase (cytosine aminohydrolase, EC 3.5.4.1)[2]. Cytosine deaminase in *Salmonella typhimurium* is an anabolic salvage enzyme in pyrimidine metabolism whose synthesis is repressed by pyrimidines[3]. In *S. typhimurium*, cytosine deaminase has been purified and is tetrameric in structure with its subunits being identical[4]. It is active at high temperatures with a neutral pH optimum and a substrate specificity for cytosine or 5-fluorocytosine. The cytosine deaminase reaction, which exhibits Michaelis-Menten kinetics, can be activated by pyrophosphate as well as inhibited by orotidine monophosphate. The present report examines the influence of pyrophosphate and orotidine monophosphate on *S. typhimurium* cytosine deaminase regulatory properties.

Materials and methods. Cytosine deaminase from *S. typhimurium* was purified from strain HD11-AE2 as previously described[4]. The preparation showed a single protein band after 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis[5]. The specific activity of the enzyme specimen was 21.4 μmoles uracil formed/min/mg protein. The cytosine deaminase assay used in this study measured enzyme activity by utilizing the difference in molar extinction coefficients between cytosine and uracil in acid at 295 nm ($2.38 \times 10^3\ M^{-1} \cdot cm^{-1}$). The 0.5 ml assay mixture, which contained 50 mM Tris-HCl buffer (pH 7.3), 1.34 μg/ml purified enzyme and cytosine, was incubated at 37 °C for 10 min. The reaction was terminated by the addition of 1 N perchloric acid (1 ml) at 4 °C. Enzyme activity is expressed as nmoles uracil formed/min/ml.

Results and discussion. Pyrophosphate is best characterized as a nonessential activator of cytosine deaminase since the deamination proceeds in its absence. In the table, it can be observed that the half-saturation constant ($K_{0.5}$) for cytosine decreased from 0.77 mM with no pyrophosphate present to 0.43 mM in the presence of 10 mM pyrophosphate. The concentration of activator giving 50% of maximal stimulation is 4.27 mM pyrophosphate. The maximal velocity of the reaction (V_{max}) values were also noted to increase for the deamination of cytosine as the pyrophosphate concentration was elevated (table). Therefore, the affinity for cytosine and the rate of product formation by cytosine deaminase was enhanced as pyrophosphate was added. The cytosine deaminase from *Serratia marcescens*, another enteric microorganism, has been purified[6] and pyrophosphate was noted to stimulate its enzyme activity[7]. Interestingly, the stimulation by pyrophosphate of the *S. marcescens* deaminase increased its V_{max} while its affinity for cytosine remained constant.

Influence of effectors on kinetic properties of cytosine deaminase

Effector	Concentration (mM)	$K_{0.5}$ (mM)	V_{max} (μmoles/min)
None	–	0.77	48.93
Pyrophosphate	2	0.63	52.94
	4	0.55	56.87
	10	0.43	62.12
Orotidine monophosphate	0.2	1.20	36.15
	1.0	2.00	25.57

Cytosine deaminase was assayed as stated in text. Values were derived from plots of velocity versus substrate concentration and Hill plots[15].