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INHIBITION OF HEPATIC ALDEHYDE DEHYDROGENASE BY CARBON TETRACHLORIDE: AN *IN VITRO* STUDY

M. F. KENEL*† and A. P. KULKARNI

Toxicology Program, Department of Environmental and Industrial Health, School of Public Health, University of Michigan, Ann Arbor, MI 48109, U.S.A.

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Abstract—1. *In vitro* inhibition of rat liver mitochondrial and microsomal aldehyde dehydrogenase (ALDH) under conditions of active CCl_4 metabolism was investigated.

2. Incubation of microsomes or mitochondria in the presence of NADPH alone caused significant, time-dependent inhibition of mitochondrial and microsomal ALDH. EDTA partially protected ALDH from inhibition.

3. Incubation of microsomes or microsomes plus mitochondria in the presence of NADPH and CCl_4 resulted in marked inhibition of microsomal and mitochondrial ALDH activity. The inhibition was both dose- and time-dependent and was relatively less in the presence of EDTA.

4. It is proposed that the inhibition of membrane-bound ALDH may be one of the early events responsible for the genesis of CCl_4-hepatotoxicity.

INTRODUCTION

Aldehydes occur ubiquitously in nature. In the animal body they are produced enzymatically during the metabolism of endogenous or exogenous chemicals. Tissue aldehyde dehydrogenase (ALDH) represents one of the primary determinants of their biological fate and toxicity (Weiner, 1980).

Recent work has documented a possible role of acetaldehyde in the acute and chronic toxicity of ethanol (Burke and Rubin, 1979; Williams *et al.*, 1980; Lieber, 1980; Lieber *et al.*, 1981). Also, disulfiram-induced aversion of voluntary ethanol consumption has been attributed to its known potential to cause the accumulation of acetaldehyde subsequent to ALDH inhibition (Marchner and Tottmar, 1978; Hoover and Brien. 1981; Cederbaum, 1981). Acetaldehyde forms adducts with protein (Benedetti *et al.*, 1982), phospholipids (Kennedy, 1982), and induces lipid peroxidation in the isolated rat hepatocytes (Stege, 1982) as well as in hepatic microsomes (Kornbrust and Mavis, 1980). Lipid peroxidation whether occurring from normal tissue aging or that triggered by xenobiotics, generates a plethora of toxic aldehydes, some of which, including malondialdehyde (MDA), have been shown to be substrates for ALDH (Horton and Packer, 1970; Hakki and Nodes, 1979; Hjelle *et al.*, 1982c).

The role of lipid peroxidation and covalent binding of reactive metabolites in CCl_4-caused liver injury has been a subject of continued debate for the past several years (Recknagel, 1983; Smith *et al.*, 1983). With the dismissal of the most recent hypothesis that CCl_4 disturbs cell calcium homeostasis (Recknagel, 1983), the question regarding the mechanism of CCl_4 hepatotoxicity remains unanswered. A number of studies have documented potentiation of CCl_4 hepatotoxicity by alcohols (Cornish and Adefuin, 1967; Traiger and Plaa, 1971; Cantillena *et al.*, 1979). An accumulation of acetaldehyde was also shown in the blood of animals receiving CCl_4 and a subsequent dose of ethanol (Hjelle and Petersen, 1981; Hjelle *et al.*, 1982a). The inhibition of liver mitochondrial ALDH resulting from CCl_4-stimulated lipid peroxidation was implicated to explain these observations (Hjelle and Petersen, 1981; Hjelle *et al.*, 1981, 1983). However, appropriate *in vitro* experiments were not performed to elucidate the underlying biochemical mechanisms responsible for the ALDH inhibition in intact mitochondria (Hjelle *et al.*, 1981). Besides mitochondria a significant ALDH activity is associated with the liver microsomal fraction (Tottmar *et al.*, 1973; Lindhal, 1981). Since endoplasmic reticulum is the primary locus of CCl_4 metabolism and concomitant lipid peroxidation, a high degree of inhibition of microsomal ALDH is expected. In view of the fact that rat liver microsomal and mitochondrial ALDH activity accounts for a major portion (>80%) of the total hepatic ALDH activity (Tottmar *et al.*, 1973; Horton and Barrett, 1975; Sjoblom *et al.*, 1978) we have re-examined the issue of susceptibility of these membrane-bound ALDHs to CCl_4 inhibition. The results of *in vitro* experiments reported in this paper demonstrate that under the conditions of CCl_4 metabolism, a significant inhibition of mitochondrial and microsomal ALDH occurs.

MATERIALS AND METHODS

Reagent grade CCl_4 was purchased from the Fisher Scientific Company, Pittsburgh, PA. Amytal, pyrazole, deoxycholic acid, $NADP^+$, NAD^+, and NADPH were

*Present address: Staff Toxicologist, International Research and Development Corporation, Mattawan, MI, U.S.A.

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obtained from the Sigma Chemical Company, St Louis, MO. Disodium EDTA, and monobasic and dibasic sodium phosphate were products of Mallinkrodt.

Male 200–250 g Sprague–Dawley rats were purchased from Charles River, Portage, MI. The rats had free access to water and Purina Laboratory Rodent Chow. Upon sacrifice livers were rapidly removed, weighed, and placed in ice-cold isolation medium (IM) containing 10 mM sodium phosphate buffer, pH 7.4, and 0.25 M sucrose. After mincing and rinsing the tissue sample several times in IM, a 10% (w/v) homogenate was prepared using a hand glass homogenizer. Mitochondria were obtained by the centrifugation of the postnuclear fraction at 10,000 *g* for 20 min. Postmitochondrial supernatant was centrifuged at 104,000 *g* for 1 hr to obtain microsomes and cytosol. Both mitochondria and microsomal fractions were washed by resuspension in an equal volume of IM followed by resedimentation.

Microsomes (2 mg protein/ml) were incubated with or without mitochondria (2 mg protein/ml) in the presence of CCl_4 (0.0–1.0 μl/mg microsomal protein) and/or NADPH (1.0 mM) at 37°C. Reactions were stopped by rapid cooling of reaction flasks in an ice-water bath. Each reaction mixture was then centrifuged at 10,000 *g* for 20 min and/or subsequently at 104,000 *g* for 1 hr to resediment mitochondria and microsomes, respectively. The supernatant containing NADPH was discarded. Microsomes and mitochondria were then resuspended separately in 50 mM sodium phosphate, pH 8.8, and assayed for remaining ALDH activity. The justification for such an experimental design rests with the known continuity of these membranes and direct juxtaposition often found between microsomes and mitochondria in hepatocytes (Pickett *et al.*, 1980).

Both NAD^+- and $NADP^+$-dependent ALDH in rat microsomes and mitochondria were assayed for total ALDH activity (isozymes I + II) using a modification of the procedure described by Tottmar *et al.* (1973). The assay mixture contained 1 mg/ml protein, 0.5 mM NAD^+ or 2.5 mM $NADP^+$, 0.25 mg deoxycholate/mg protein, 2 mM amytal, and 50 mM sodium phosphate buffer, pH 8.8. After 3 min of preincubation the reactions were started with 5 mM acetaldehyde and NAD(P)H production was monitored at 340 nm in an Aminco DW2 spectrophotometer at 37°C. Protein content was determined by the Biuret method (Gornall *et al.*, 1949).

Statistical analysis consisted of analysis for variance followed by Dunnett's *t*-test for comparison of test groups with controls. Significance was assumed at $P \leq 0.05$.

RESULTS

It has been repeatedly documented (see Recknagel, 1983; Smith *et al.*, 1983) that CCl_4 is metabolized when rat hepatic microsomes are incubated in the presence of NADPH. This reaction is also accompanied by covalent binding of reactive metabolites of CCl_4 and extensive peroxidation of membrane lipids.

The effects of preincubation of microsomes or microsomes plus mitochondria in the presence of NADPH alone or NADPH and CCl_4 on ALDH activity are shown in Fig. 1. A significant time-dependent decline in both NAD^+- and $NADP^+$-coupled mitochondrial and microsomal ALDH activity was observed in the presence of NADPH alone. $NAD(P)^+$-dependent microsomal ALDH was found to be more susceptible than mitochondrial activity. As compared to NADPH alone, the preincubation with NADPH and CCl_4 (1 μl/mg protein) resulted in an even greater decrease in the ALDH activity of both the membranes. This decline was time dependent and after 20 min of incubation caused up to 96% inhibition of ALDH. In contrast to the results obtained with NADPH alone, incubation with NADPH plus CCl_4 not only resulted in much higher ALDH inhibition but both mitochondrial as well as microsomal ALDH appeared to be nearly equally susceptible.

The data given in Fig. 2(A) indicate that this time-dependent inhibition of microsomal ALDH in-

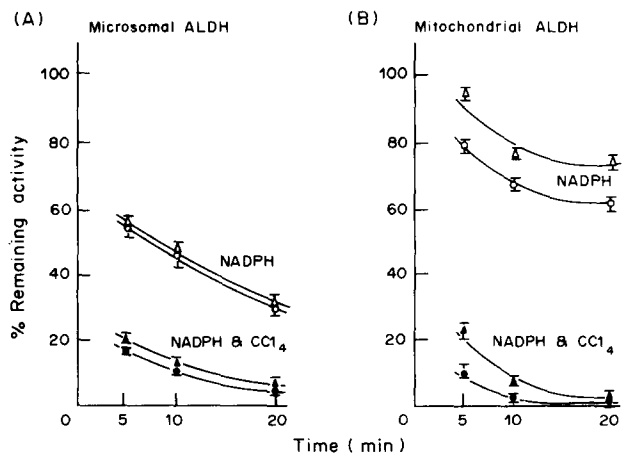

Fig. 1. *In vitro* inhibition of mitochondrial and microsomal ALDH during CCl_4 metabolism: time dependence. Rat hepatic microsomes (2.0 mg protein/ml) (A), or microsomes (2.0 mg protein/ml) plus mitochondria (2.0 mg protein/ml) (B) were preincubated (5 ml final vol) at 37°C for 5, 10, and 20 min in the presence of 1.0 mM NADPH or 1.0 mM NADPH and 1 μl CCl_4/mg protein. ALDH activity of mitochondria and microsomes remaining after incubation was assayed with NAD^+ (0.5 mM) (triangles) or $NADP^+$ (2.5 mM) (circles) as described under Materials and Methods. Control values (at 0 min) for NAD^+- and $NADP^+$-coupled microsomal ALDH were 35.0 ± 1.9 and 14.2 ± 0.5 nmol/min per mg protein, respectively. Control values (at 0 min) for NAD^+- and $NADP^+$-coupled mitochondrial ALDH were 45.4 ± 2.1 and 10.2 ± 0.2 nmol/min per mg protein, respectively. Control values did not significantly change during 20-min incubation period. Each value represents the mean ± S.E. for four separate experiments.

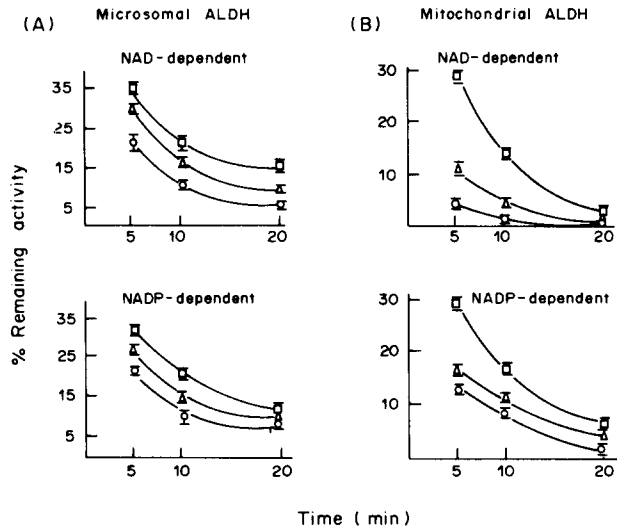

Fig. 2. *In vitro* inhibition of microsomal and mitochondrial ALDH during CCl_4 metabolism: dose dependence. Rat hepatic microsomes (2.0 mg protein/ml) (A) or microsomes (2.0 mg protein/ml) plus mitochondria (2.0 mg protein/ml) (B) were incubated (5 ml final vol) at 37°C for 5, 10, and 20 min in the presence of 1.0 mM NADPH and 0.25 (squares) 0.5 (triangles), or 1.0 (circles) μl of CCl_4/mg protein. ALDH activity of mitochondria and microsomes remaining after incubation was estimated as described under Materials and Methods. Control values (at 0 min) for NAD^+- and $NADP^+$-coupled microsomal ALDH were 44.5 ± 3.0 and 7.5 ± 1.6 nmol/min per mg protein, respectively. Control values (at 0 min) for NAD^+- and $NADP^+$-dependent mitochondrial ALDH were 42.0 ± 1.5 and 12.3 ± 0.9 nmol/min per mg protein, respectively. Control values did not significantly change during 20 min incubation period. Each point represents the mean ± S.E. for four separate experiments.

hibition is also a dose related phenomenon. Although a significant decrease in microsomal ALDH activity can be seen in 5 min, the data given in Fig. 2(B) suggest that mitochondrial ALDH is even more susceptible. These results also indicate that, in general, the degree of inhibition is independent of the kind of oxidized pyridine nucleotide used for assaying either microsomal or mitochondrial ALDH.

The effects of EDTA on the inhibition of the mitochondrial and microsomal ALDH activity are given in Fig. 3. The amount of activity remaining After 20-min incubations with NADPH alone was about 30 and 60% for NAD^+-dependent microsomal and mitochondrial ALDH, respectively. The addition of EDTA (0.2 mM) resulted in relatively less inhibition. After incubation in the presence of NADPH

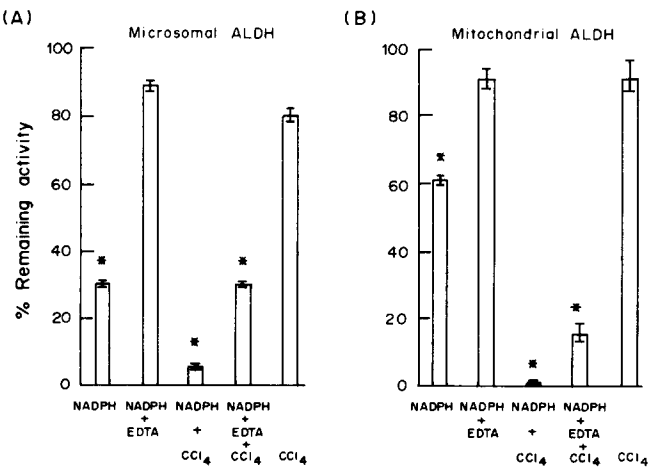

Fig. 3. Effects of EDTA on *in vitro* inhibition of microsomal and mitochondrial ALDH during CCl_4 metabolism. Rat hepatic microsomes (2.0 mg protein/ml) or microsomes (2.0 mg protein/ml) plus mitochondria (2.0 mg protein/ml) were incubated (5 ml final vol) at 37°C for 20 min with 1.0 mM NADPH, and/or 1.0 μl CCl_4/mg and/or 0.2 mM EDTA as indicated. ALDH activity of mitochondria and microsomes remaining after 20-min incubation was estimated as described under Materials and Methods. Control values (at 0 min) for NAD^+-dependent microsomal and mitochondrial ALDH were 35.0 ± 1.9 and 41.9 ± 0.7 nmol/min per mg protein, respectively, in the presence of EDTA. Each value represents the mean ± S.E. for four separate experiments. *Statistically significant ($p \leq 0.05$) from control value.

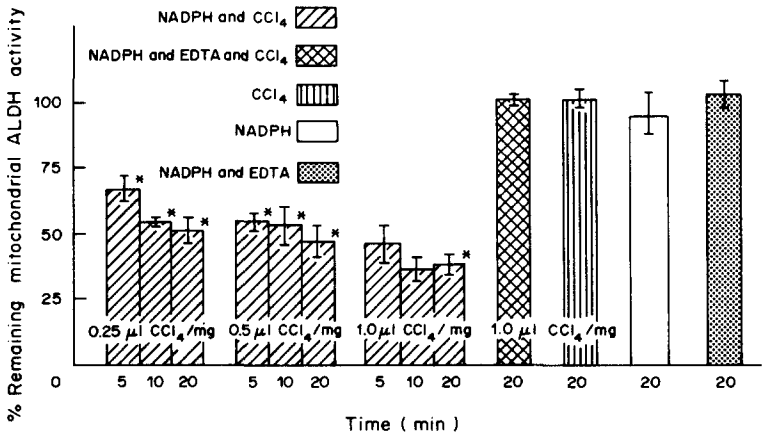

Fig. 4. Effect of CCl_4 on the mitochondrial ALDH activity. Washed rat liver mitochondrial (2 mg protein/ml) were incubated (5 ml final vol) at 37°C for 5, 10, or 20 min in the presence of 1.0 mM NADPH, 0.25, 0.5, or 1.0 μl CCl_4/mg, and 0.2 mM EDTA as indicated. ALDH activity of mitochondria remaining after 20-min incubation was determined as indicated. See text for further details. Control value (after 20 min) for NAD^+-dependent ALDH was 39.7 ± 2.7 nmol/min per mg protein. Each value represents the mean ± S.E. for four separate experiments. *Statistically significant ($p \leq 0.05$) from control value.

and CCl_4 for 20 min only 5 and 0.5% of NAD^+-dependent microsomal and mitochondrial ALDH activity remained, respectively. Under these conditions the inclusion of EDTA resulted in only a partial restoration of microsomal (to 30% of control values) and mitochondrial (to 15% of control values) ALDH activity. Furthermore, the inability of unmetabolized CCl_4 to significantly inhibit ALDH activity clearly attests that the observed inhibition was not due to solvent effects of CCl_4.

Besides microsomes, CCl_4 was shown to be metabolized by rat liver mitochondria (Castro *et al.*, 1984). To assess the role of mitochondrial activation in ALDH inhibition, CCl_4 was preincubated in the presence of NADPH and washed mitochondria. Apparently the isolation procedure employed renders mitochondria permeable to NADPH and retains their capacity to metabolize CCl_4. However, some unavoidable microsomal contamination is likely to be present in the mitochondrial preparations used. The data given in Fig. 4 indicate a substantial time- and dose-dependent inhibition of NAD^+-coupled ALDH when mitochondria were incubated with NADPH plus CCl_4. The decline in specific activity of mitochondrial ALDH was significantly greater with NADPH plus CCl_4 than with NADPH alone and this inhibition was not observed in the presence of EDTA.

DISCUSSION

Bioactivation catalyzed by the microsomal cytochrome P-450 system is a prerequisite well accepted in CCl_4 hepatotoxicity. Despite numerous investigations, the specific biochemical event(s) responsible for hepatocyte dysfunction leading to fatty infiltration and cell death after CCl_4 poisoning is largely unknown. The role of covalent binding of reactive metabolites, lipoperoxidation as well as depression of calcium pump activity have been a subject of continuing debate (Recknagel, 1983; Smith *et al.*, 1983).

Our results confirm the reports (Tottmar *et al.*, 1973; Lindahl, 1981) that significant rat hepatic ALDH activity exists in the microsomal and mitochondrial fractions. When microsomes or microsomes plus mitochondria were incubated in the presence of NADPH alone, a significant time-dependent decline in hepatic NAD^+- and $NADP^+$-coupled mitochondrial and microsomal ALDH was observed (Fig. 1). These results suggest a possible involvement of lipid peroxidation in ALDH inhibitions since similar treatment has been shown to trigger lipoperoxidation in liver microsomal preparations (Levin *et al.*, 1973; Kulkarni and Hodgson, 1981) and it is believed to be due to the presence of metal contaminants in the reagents used. Furthermore, a recent study (Hjelle *et al.*, 1982b) has demonstrated MDA, a major product of lipid peroxidation, to inhibit mitochondrial ALDH.

In vitro metabolism of CCl_4 leading to concomitant lipid peroxidation and covalent binding of reactive metabolites is well documented (Recknagel, 1983). Preincubation with NADPH + CCl_4 significantly increased the loss of ALDH activity in both microsomes and mitochondria, when compared to NADPH alone. This was a dose-related phenomenon. The decline was also time dependent and after 5 min of incubation, microsomal and mitochondrial ALDH was inhibited *ca.* 80%. It has been shown that within 5 min after administration, CCl_4 is detected in liver microsomes and that within 15 min CCl_4-stimulated lipid peroxidation as well as covalent binding of ^{14}C and $^{14}CCl_4$ to macromolecules is maximal (Rao and Recknagel, 1968, 1969). Therefore, our data suggest that CCl_4-dependent inhibition of membrane bound ALDH may occur prior to an overt hepatotoxic response.

The facts that postnuclear supernatant of rat liver when similarly incubated in the presence of NADPH and CCl_4 does not result in a significant inhibition of mitochondrial ALDH (data not shown) and EDTA protects mitochondria from inhibition (Fig. 4) tend to suggest a predominant role of lipoperoxidation in the observed ALDH inactivation. This view is consistent with reports on inhibition of microsomal lipid per-

oxidation by the cytosolic constituents (Kamataki *et al.*, 1974; Kulkarni and Hodgson, 1981).

To further test this hypothesis, incubations were performed in the presence of 0.2 mM EDTA (Fig. 3), a concentration shown to block metal-, but not CCl_4-, stimulated lipid peroxidation (Kornbrust and Mavis, 1980; Waller and Recknagel, 1982). The protective effect noted suggests that the observed inhibition of ALDH, due to NADPH alone, could be attributed to metal-dependent, NADPH-stimulated lipid peroxidation. The EDTA-dependent partial restoration of microsomal and mitochondrial ALDH suggests that the binding of free radicals or products of CCl_4-stimulated lipid peroxidation may be responsible for the observed inhibition of ALDH. Similar protective effects were also observed by Hjelle *et al.* (181) in the presence of glutathione. The inability of CCl_4 alone to significantly inhibit microsomal or mitochondrial ALDH (Fig. 3) eliminates the role of solvent effects and suggests that bioactivation of CCl_4 is a prerequisite for ALDH inactivation.

A significant decrease in NAD^+-dependent ALDH activity observed in the presence of NADPH + CCl_4 as compared to NADPH alone (Fig. 4) suggests that mitochondria may be capable of CCl_4 bioactivation. However, it should be kept in mind that contaminating microsomes may be partially contributing to the activation process. In any case, the magnitude of inhibition of mitochondrial ALDH observed in these experiments (Fig. 4) is much smaller than that observed in incubations in the presence of both mitochondria and microsomes (Fig. 3b). This is rather expected from the relatively lower efficiency of mitochondria to metabolize CCl_4 than microsomes.

Hjelle *et al.* (1981) incubated isolated mitochondrial ALDH preparation containing 1 mM dithiothreitol in the presence of microsomes, NADPH and CCl_4 and the incubates were sampled periodically and directly assayed for ALDH activity. The authors reported essentially complete inhibition of mitochondrial ALDH within 5 min. The reliability of their data is somewhat questionable since the mitochondrial ALDH was apparently measured in the presence of microsomes and other components of the incubation mixture. The inhibition of microsomal ALDH (Figs 1–3), the stimulation of lipid peroxidation by dithiothreitol (Kulkarni and Hodgson, 1981), the measurement of ALDH in the presence of NADPH, a regulatory inhibitor of ALDH (Kenel and Kulkarni, unpublished observations) will each introduce an error in the estimation of mitochondrial ALDH activity. It is known that lipophilic aldehydic products of lipid peroxidation are preferentially contained in the membranes and only a small fraction escapes into the surrounding (Esterbauer *et al.*, 1982). In view of this, the reported inhibition of mitochondrial ALDH (Hjelle *et al.*, 1981) may have been partly due to diffusable products of lipid peroxidation. Our data based on re-sedimented mitochondria and microsomes suggest that membrane-bound products of lipid peroxidation also possess high inhibitory potency.

Ability of CCl_4 to decrease the specific activities of these membrane-bound ALDH may severely compromise the cells' capacity to metabolize acetaldehyde or aldehydic products of lipid peroxidation. Because of their high toxicity, reactivity, and in some cases high stability (Benedetti *et al.*, 1982, 1984; Esterbauer *et al.*, 1982) these aldehydes are capable of binding to tissue macromolecules causing enzyme inactivation or capable of being transported to distant loci to produce toxicity at sites other than their origin. Although the precise events leading to cellular pathology remain unknown, it has been suggested (Lowrey *et al.*, 1981) that the underlying biochemical mechanism may involve inhibition of several vital enzyme systems. Our data suggest that ALDH inhibition may represent one of the important enzyme systems that is significantly affected during early stages of CCl_4 poisoning. The results of the *in vivo* experiments (reported elsewhere) confirm this view.

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