Zinc and alkaline phosphatase in developing rat oral mucosa

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Alkaline phosphatases (AlkPase) of many different tissues and species have been shown to be zinc metalloenzymes. Specific regions of rat oral mucosa have a high activity of AlkPase. Combined autoradiography and enzyme histochemistry showed that they also retained injected radioactive zinc (^{65}Zn). The AlkPase activity was inactivated by EDTA and reactivated with zinc. However, it could not be verified by polyacrylamide gel electrophoresis combined with radioactivity measurements and enzyme analysis that the ^{65}Zn uptake of oral mucosa was incorporated in the AlkPase molecule. □ *Autoradiography; enzyme histochemistry; epithelium; zinc-65*

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Zinc is an essential trace element in biological systems. In mammals it is absorbed in the intestinal mucosa, bound to a protein, and distributed to various target tissues, where it appears, for example, as an integral part of some enzyme systems (for reviews, see Ref. 1–5). Zinc deficiency causes pathological changes in various tissues (2, 5, 6). Deficient animals develop, among other defects, parakeratotic lesions in the oral mucosa and the skin with thickening of epidermis, intra- and inter-cellular edema, and increased mitotic and metabolic activity (2, 7–14). This points to a zinc dependence of keratinizing epithelial cells (15), but the function of zinc in epithelium is unknown.

In an autoradiographic study of the uptake and distribution of radioactive zinc (^{65}Zn) in young rats a distinct uptake in the anterior parts of the oral mucosa was described by Bawden & Hammarström (16). This region of the mucosa contains a fold-like structure in which different types of mucosa merge. The fold is situated in the anterior part of the buccal mucosa and extends from the incisor region of the upper jaw to the incisor region of the lower jaw via the commisural region. It differs from other parts of the oral mucosa by its rich and, for squamous epithelium, unusual contents of alkaline phosphatase (AlkPase EC 3.1.3.1.) (17, 18).

The occurrence of zinc accumulation in the same region that contains AlkPase may be coincidental. However, AlkPases isolated from various tissues and species, including those of rats, have all been identified as zinc metalloenzymes (4, 19–23), making it probable that the accumulation of ^{65}Zn observed by Bawden & Hammarström (16) was incorporated with mucosal AlkPase.

The purpose of the present study was to analyze the distribution of ^{65}Zn in the oral mucosa of the developing rat with autoradiographic techniques and compare it with the distribution of AlkPase; to substantiate the assumption that mucosal AlkPase is also a zinc metalloenzyme by means of an enzyme histochemical inactivation–reactivation test; and to analyze homogenates of the mucosa with the polyacrylamide gel electrophoresis (PAGE) technique, to establish whether accumulated ^{65}Zn is bound to AlkPase in the oral mucosa.

Materials and methods

Autoradiography

Eight 4-day-old Sprague-Dawley rats were

each given intraperitoneal injections of 10 μCi ^{65}Zn every day, on 4 consecutive days, and then left for an additional 2 days. Three of the rats were then anesthetized with ether and rapidly frozen in hexane saturated with solid CO_2 (−75°C). Before the freezing, the rats were embedded on a microtome stage in a mixture of carboxymethyl cellulose. Whole-body horizontal, saggittal, and frontal sections (20 μm) were cut and freeze-dried. The sections were pressed against Structurix X-ray film (Agfa-Gevaert) in a dark room and left for exposure (24, 25). After 1 week the film plates were separated from the sections, developed, and analyzed.

Enzyme histochemistry

The sections used for autoradiography were incubated for AlkPase with 0.09 mM naphthyl-As-Mx phosphate as substrate and Fast Blue RR as coupling agent. They were dissolved in 0.2 M Tris–HCl, pH 8.3 (26). The sections were incubated for 30 min at +37°C, and the reaction was stopped by washing in distilled water. Additional freeze-dried sections were incubated for AlkPase activity with 10 mM β-glycero-phosphate as substrate in 0.08 M Tris–HCl, pH 10.0, containing 3.9 mM $MgCl_2$ and 2 mM lead citrate (27). These sections were incubated at 37°C for 15 min, and the reaction was stopped by washing in 0.9% NaCl. The staining was then developed in 1% ammonium sulfide.

The inactivation and reactivation of AlkPase was studied on sections that had been kept in 30 mM EDTA for 48 h at +4°C (28, 29) before the histochemical incubation. Some of the ethylenediaminetetraacetic acid (EDTA)-deionized sections were kept in aqueous solutions of $ZnCl_2$ (0.5 mM, for 10 min) and $MgCl_2$ (1 mM, for 10 min) before the histochemical incubation, to study the possibility of reactivating AlkPase activity. In all the inactivation–reactivation studies the sections were incubated with β-glycerophosphate as substrate by the method described above (17). The naphthyl-As-Mx phosphate method was not used in these latter studies because of the contents of zinc in the coupler diazonium salts.

All sections used in the enzyme histochemical studies were mounted in glycerine jelly and examined in the light microscope.

Electrophoresis

The other five rats were decapitated, and the buccal folds of the oral mucosa were rapidly dissected free and homogenized in a glass homogenizer with five volumes of 0.1 M Tris–HCl buffer, pH 7.4, with addition of 1% Triton X-100. The crude extract was centrifuged (40,000 *g* for 30 min) to free the protein solution of whole cells and cell fragments. The supernatant was carefully pipetted off and mixed with an equal volume of 40% sucrose. Thirty microliters of the extract–sucrose mixture was carefully layered on top of a separation gel in a 7.5% polyacrylamide disc electrophoretic system, pH 8.3, as described by Davies (30), with modifications (31, 32). The electrode reservoirs were filled with 0.1 M Tris–glycine, pH 8.3, with bromphenol blue added to the upper chamber to stain the migration front. The electrophoresis was carried out at a constant voltage with 2.0 mA/gel and ran for 5 h.

Radioactivity measurements

The distribution of ^{65}Zn in the gel rods was studied by dividing the gel into 2-mm-thick slices. Each slice was placed in a special plastic tube for radioactivity measurements with an NaI(TI) well crystal in a Selectronic sample changer. ^{65}Zn decays through electron capture (98.3%) and positron emission (1.7%) with a physical half-life of 245 days. The photon radiation emitted is Cu-X-rays, annihilation photons 0.511 MV (3.4%) and gamma radiation 1.115 MV (49%), and is measured in the high-energy channel. A background activity of 0.17 counts/sec was recorded throughout the gels with a coefficient of variation in the net counts of 15% and a total of 3.3 counts/sec for the whole gel.

Zymogram staining

The AlkPase distribution in the gels was studied by incubating the gels in 1 mM α-

naphthyl acid phosphate, 1.5 mM 4-amino-diphenylamide, and 25 mM $MgSO_4 \cdot 7H_2O$ dissolved in 0.25 M Tris-maleate buffer, pH 9.2 (33, 34). One gel was used as control and was incubated without the substrate.

Controls

Control incubations of sections were included regularly when the substrate or the capturing ion was substituted for distilled water.

Materials

Zinc chloride with an activity concentration of 185 MBq/ml (5 mCi/ml) was purchased from New England Nuclear, Boston, Mass., USA. All other chemicals were of analytical grade and obtained from Sigma Chemical Co., St. Louis, Mo., USA.

Results

Autoradiography and histochemistry

Two days after the last of 4 consecutive daily injections of ^{65}Zn, the blood level uptake, as seen in the heart lumen, was low, whereas a high uptake of ^{65}Zn was seen in the teeth and bones. Uptake nearly as high as in mineralized tissues was found in the liver and intestines, whereas a low uptake was seen in the kidney cortex (Fig. 1a). A moderate uptake was present in epithelial cells of the oral mucosa. The ^{65}Zn uptake was localized at the anterior parts of the oral

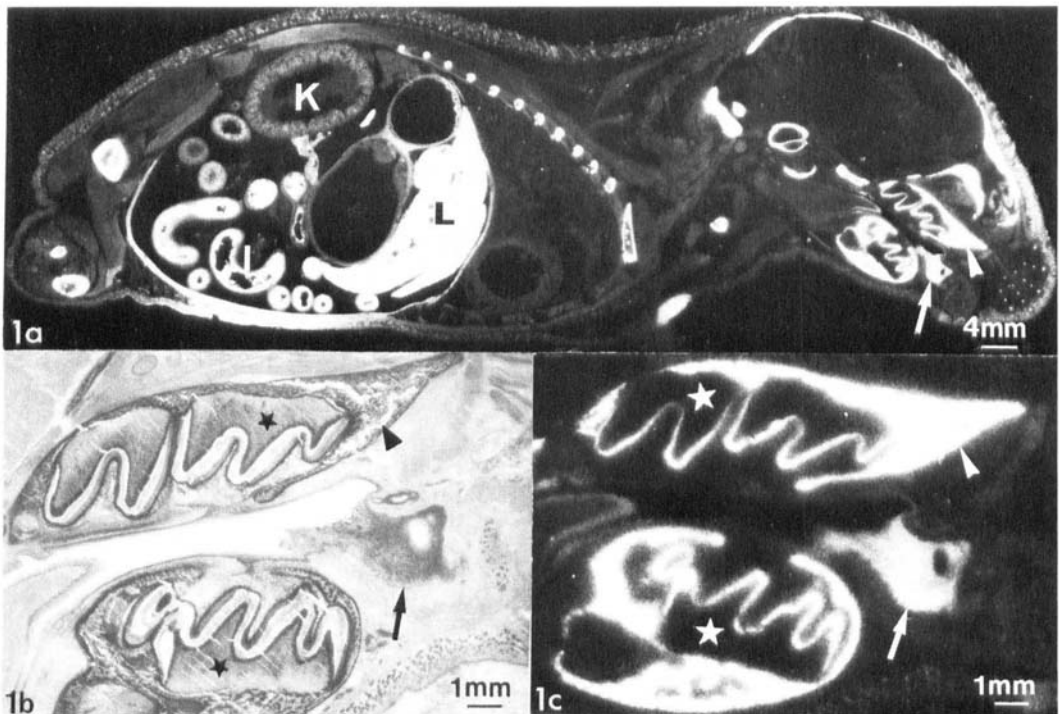

Fig. 1. Sagittal section of 10-day-old rat injected intraperitoneally with ^{65}Zn. 1a. Autoradiogram of whole-body section. Uptake is seen in mineralized tissues like bone (arrowhead) and teeth and also in liver (L), kidney (K), and intestines (I). Also note intense uptake in the anterior part of the oral cavity (arrow). 1b. Detail from section used in the autoradiography in 1a, stained for AlkPase, pH 8.3. Note intense staining of epithelium in the oral mucosa (arrow), which is part of the buccal fold. Also note staining of pulp (asterisk) and mineralized tissues (arrowhead). 1c. Detail of oral cavity from autoradiogram in 1a. Note intense uptake of ^{65}Zn in the anterior part of the oral mucosa (arrow) and compare with the same region in 1b stained for AlkPase. Also note the lack of uptake of ^{65}Zn in pulpal cells of the molars (asterisk) and compare with intense staining for AlkPase in pulp tissue.

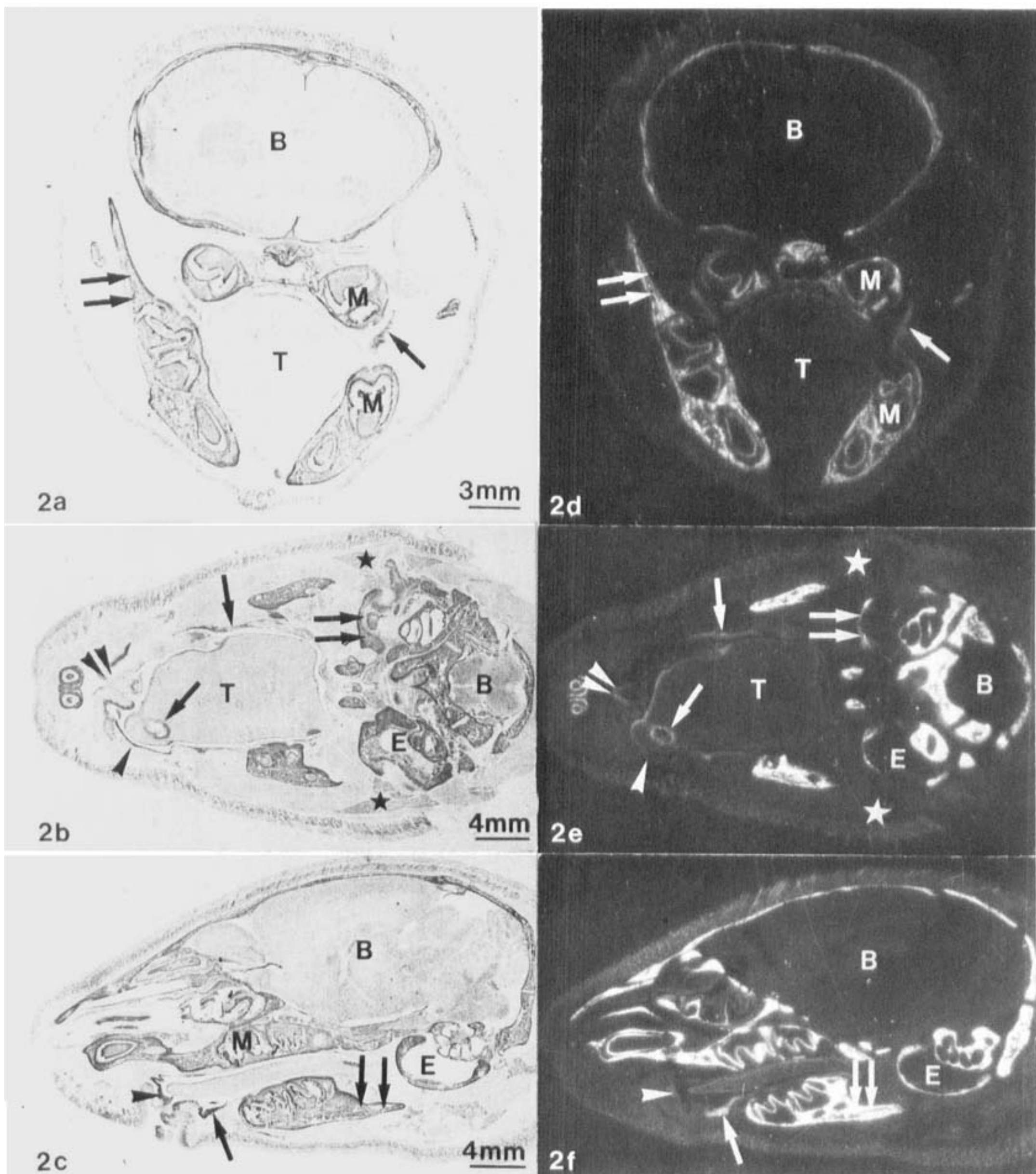

Fig. 2. Frontal, horizontal, and sagittal views of 10-day-old rats. Neighboring sections were used for AlkPase histochemistry and ^{65}Zn autoradiography. 2a–c. Sections incubated for AlkPase, pH 8.3, with activity (dark staining) found in anterior regions of the oral epithelium (arrows and arrowhead). Also note AlkPase activity in mineralized tissues (double arrows) and in the parotid gland (asterisk). 2d–f. Autoradiograms of neighboring sections to the one seen in a–c. The uptake of ^{65}Zn (which indicates radioactivity) in the oral mucosa corresponds well with the enzyme activity demonstrated in the oral epithelium in 2a–c (arrows). However, in some areas of 2e (double arrowheads) ^{65}Zn uptake is evident, whereas the corresponding region in 2b shows no AlkPase activity (double arrowheads). Furthermore, in other regions of 2e and 2f (arrowhead) no uptake of ^{65}Zn is seen, and yet corresponding areas in the sections in 2b and 2c stain for AlkPase (arrowhead). The same is seen in the parotid gland (Fig. 2b and Fig. 2e, asterisk). B = brain; E = inner ear; M = molars; T = tongue.

cavity mucosa and it was mainly associated with the fold-like structure of the buccal mucosa (Figs. 1c and 2d,e,f).

Sections first used for autoradiography and then incubated for AlkPase showed a distinct staining pattern of the epithelium in oral mucosa, which corresponded well with the uptake patterns of ^{65}Zn. The staining was found in the cells of the spinosum and granulosum layers. In the area bordering unstained parts of the mucosa, the staining of the cells became gradually randomized and mixed with non-stained cells. No staining was seen in basal epithelial cells. In the connective tissue cells, subjacent to the epithelium, staining of capillaries could also be seen, whereas the connective tissue fibers and cells were for the most part unstained (Figs. 1b and 2a,b,c). However, some areas of the mucosa without any visible sign of AlkPase activity showed uptake of ^{65}Zn, and areas with intense AlkPase activity lacked uptake of ^{65}Zn (Figs. 2b,c,e,f). ^{65}Zn had also accumulated in teeth at the mineralized parts of the dentin/enamel tissue, but little or no radioactivity was seen in the pulp and enamel organ, where intense activity of AlkPase was found (Figs. 1b,c). The parotid gland also showed AlkPase activity without any sign of zinc accumulation (Figs. 2b,e).

Treatment in 30 mM EDTA for 48 h extinguished all AlkPase activity in the epithelium. Reactivation of EDTA-treated sections with zinc restored the activity of AlkPase, whereas addition of magnesium restored only a minimum of the activity (Table 1).

Electrophoresis

Extracts of oral epithelium from ^{65}Zn-injected rats subjected to disc electrophoretic separation on acrylamide gels contained two significant peaks of different magnitudes, as shown by ^{65}Zn assay, with 0.8 counts/sec in the larger and 0.3 in the smaller (Fig. 3). The coefficient of variation in the net counts for the two peaks was only 2%. Bromphenol blue staining of the migration front proteins showed that the smaller ^{65}Zn peak corresponded to what was estimated to be the albumin/prealbumin region, and the larger ^{65}Zn peak corresponded to the electrophoretic migration front.

The AlkPase staining of the gel rods demonstrated clearly that they contained three fractions. Two of them were narrow and distinct and had migrated only a short distance into the gel. One was moderately stained and the other was intensely stained when the gel was incubated for AlkPase. The third was diffuse, and it was weakly stained. It had moved a considerably longer distance into the gel. Control incubations without the substrate present were done regularly and were always negative.

Discussion

The autoradiographic findings in this study with uptake of ^{65}Zn in mineralized tissues, liver, intestines, kidney and oral mucosa and a low blood level are in good agreement with previous results obtained on the developing and adult rat after a single injection of ^{65}Zn (16, 35). Furthermore, the blood level of

Table 1. Semiquantitative evaluation of freeze-dried rat oral mucosa sections incubated for AlkPase, pH 8.3 (26), and for AlkPase, pH 10.0 (27), to show the effect of EDTA on the AlkPase activity in epithelial cells and the restorative effect of zinc and magnesium on the enzyme activity. Note the lack of response to EDTA in the AlkPase activity at pH 8.3 and compare with the AlkPase activity at pH 10.0. The coupling salt used for the AlkPase activity detection at pH 8.3 contains zinc, which reactivates the enzyme (18, 46, 47)

	No treatment	EDTA treatment	EDTA followed by Zn reactivation	EDTA followed by Mg reactivation
AlkPase, pH 8.3	+++	+++	+++	+++
AlkPase, pH 10.0	+++	0	++(+)	(+)

+++ = Intense staining; ++ = moderate staining; + = low staining; and 0 = no staining detectable in the sections.

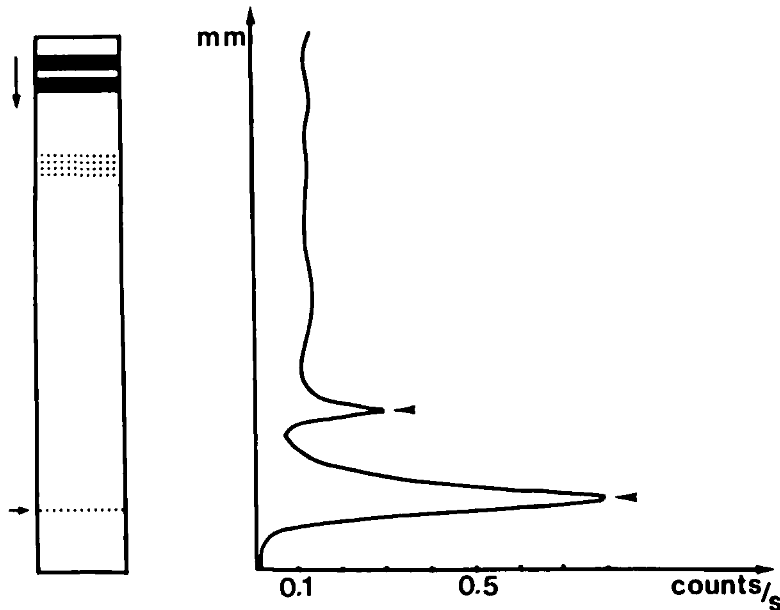

Fig. 3. AlkPase zymogram of buccal mucosa supernatant from 10-day-old rats given repeated intraperitoneal injections of ^{65}Zn. The zymogram consists of two distinctly stained and one diffusely stained AlkPase isozymes. The vertical arrow indicates the migration direction of the electrophoresis and the small arrow the migration front. Gels from the same electrophoretic run were sliced in 2-mm discs and assayed for ^{65}Zn radioactivity. The result is seen in the diagram to the right. The horizontal line indicates the radioactivity assayed in the individual discs. Two peaks of radioactivity were found (arrowheads).

^{65}Zn, as indicated by the autoradiographic image of the blood in the heart, was low, and hence interference from ^{65}Zn in the blood was negligible when the concentration of ^{65}Zn in various tissues was evaluated. The areas with a moderate concentration of ^{65}Zn in the anterior parts of the oral mucosa corresponded to the areas with AlkPase staining found in the same regions of the oral epithelium. However, this similarity in distribution was not altogether complete in every region, and it is therefore obvious that the accumulated zinc did not reflect ^{65}Zn incorporated only with AlkPase molecules and that other components of the mucosa may be associated with the accumulated ^{65}Zn.

From the inactivation tests with EDTA it was further found that AlkPase in situ was sensitive to EDTA, which removes cations like zinc, calcium, and magnesium from different types of tissue. The inactivation of AlkPase which followed the EDTA treatment is in accordance with previous results obtained with different tissues and species (18, 28, 36, 37) and was overcome by addition of zinc but not magnesium. This may indicate that endogenous zinc was extracted from the AlkPase molecule and later replaced by zinc from the medium, leading to a restored activity. Alternatively, another substance was removed from the enzyme and replaced with added zinc, which restored the activity. The fact that the catalytic activity was poorly restored by magnesium addition seems to exclude the possibility that the reactivation of the enzyme was a non-specific cation effect and indicates that it was rather an effect of the zinc ion as described previously (28, 37–39). It corroborates the supposition that oral mucosa AlkPase is a zinc metalloenzyme like all other AlkPases so far investigated (21, 40, 41).

Analysis of the relation between proteins and the retained ^{65}Zn in mucosal extract

showed that most of the retained ^{65}Zn had migrated in or very near the migration front. The beginning and the end of the gel contained negligible amounts of ^{65}Zn, indicating that no diffusion of ^{65}Zn from the peaks had taken place after the electrophoresis was finished. If ^{65}Zn had been incorporated only with AlkPase in the epithelium, it should not have dissociated from the enzyme during the electrophoresis under the experimental conditions of our study (42–44), and all ^{65}Zn should then have been recovered in the AlkPase region of the gel.

Thus the larger of the two ^{65}Zn-containing peaks found in and near the migration front seems to represent unbound ^{65}Zn. The smaller peak contained ^{65}Zn, which must have been firmly bound to unknown proteins migrating short of the front and near the albumin/prealbumin region. The smaller peak was of a low magnitude, and its existence may be questioned. If it reflects a genuine zinc protein complex, it qualifies as a zinc metalloprotein (22). One such protein that may be considered is metal theoneine, a metal-binding protein that is recovered in the body as zinc-theoneine after exogenous administration of zinc (38, 41) and which provides the cells in different parts of the body with zinc. In this context it may be pertinent to note that sufficient time (6 days) seems to have passed for incorporation of ^{65}Zn with mucosal AlkPase molecules (4). Moreover, the turnover time for rat buccal epithelium cells, which is approximately 7–10 days (45), should give ample time for synthesis of AlkPase and incorporation of ^{65}Zn.

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