Plant Physiol. (1982) 69, 1356–1360
0032-0889/82/60/1356/05/$00.50/0

Thermal Behavior and Lipid Composition of Cauliflower Plasma Membranes in Relation to ATPase Activity and Chilling Sensitivity

Received for publication October 13, 1981 and in revised form January 11, 1982

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ABSTRACT

A plasma-membrane fraction rich in ion-stimulated ATPase activity was isolated from cauliflower (*Brassica oleracea* L.) buds. The activity of the ATPase was dependent on Mg^{2+} and stimulated 4-fold by K^+. The lipids of the membrane fraction contained 57% by weight of phospholipid, 16% glycolipid including sterol glycosides, and 27% neutral lipids. Sterols and sterol esters comprised 9% by weight of the total lipid fraction, and the molar ratio of total sterol to phospholipid was 0.5. Fatty acid unsaturation of the membrane lipids was 75%. Arrhenius plots of the Mg^{2+} and Mg^{2+} + K^+ stimulated ATPase activity were biphasic with an increase in activation energy occurring below about 12°C, a response typical of some membrane-associated enzymes of chilling-sensitive plants. No thermal transitions were detected in the membranes or membrane lipids between 0 and 30°C using differential scanning calorimetry and electron spin resonance spectroscopy. This type of thermal behavior is typical of membranes of chilling-resistant plants. It was concluded that the low temperature increase in activation energy of the ion-stimulated, membrane-associated ATPase is an intrinsic property of the enzyme system and not the result of a transition in the bulk membrane lipid.

Many membrane-bound enzymes associated with mitochondria and chloroplasts of chilling-sensitive plants show nonlinear Arrhenius plots with an increase in Ea[3] below about 10°C (12, 23). The same enzymes from chilling-resistant plants show linear Arrhenius kinetics over the temperature range of 0 to 30°C (12, 23). These thermally-induced changes in function are considered a consequence of a change in membrane lipid ordering as evident from electron spin resonance and fluorescent probe studies (21, 23) and together they provide a molecular explanation of chilling injury (12).

Some soluble enzymes also exhibit nonlinear Arrhenius plots at low temperatures. This can be explained on the basis of a direct, temperature-induced, reversible alteration in the tertiary structure of the enzyme or the enzyme-substrate complex (4). Examples from plants include some key enzymes of carbon metabolism, such as P-enolpyruvate carboxylase (7), but the role of these enzymes in the development of chilling injury has yet to be elucidated. Furthermore, some membrane-associated enzymes from chilling-resistant plants also exhibit nonlinear Arrhenius plots, *e.g.* the photoreduction of DCIP by chloroplasts from barley (17) and pea (18), assayed in the presence of uncouplers. However, no phase separation (21) or change in lipid ordering (24) occurs in the membranes of these plants at temperatures coincident with the altered activation energies and such changes are more likely a direct effect of temperature on the uncoupler-enzyme-substrate complex. Inasmuch as growth of barley and pea continue well into the chilling temperature range, the altered enzyme kinetics observed *in vitro* at chilling temperatures have little influence on growth and development of these plants. These observations stress the importance of establishing whether tissue metabolism reflects the altered kinetics of the isolated enzyme systems before such changes are causally linked to chilling injury.

The question of the relative contribution of changes in enzyme kinetics and/or changes in membrane lipid order to chilling injury is complicated with such enzymes as the K^+-stimulated ATPase. This enzyme is associated with the plasma membrane and reported to be involved in transmembrane K^+ and H^+ transport (8). An early indication of chilling injury is an increase in ion leakage from cells (12).

A purified plasma membrane fraction, based on enhanced sensitivity of the associated ATPase to stimulation by K^+, has recently been obtained from cauliflower (15), a chilling-resistant plant, and this has provided an opportunity to study the thermal behavior of both the lipid structure of the plasma membrane and the associated ion-stimulated ATPase activity.

This paper describes the lipid composition of the cauliflower plasma membrane fraction, the effects of low temperature on the kinetics of K^+-stimulated ATPase activity, and the thermal response of the membranes and membrane lipids. The results show that, while low temperature induces an increase in the Ea of the enzyme, no change in the ordering of the bulk membrane lipids was detected by calorimetry or spin labeling.

MATERIALS AND METHODS

Plasma Membrane Preparation. A membrane fraction enriched in K^+-stimulated ATPase activity was prepared from cauliflower

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[3] Abbreviations: Ea, Arrhenius activation energy; ASG, acylated sterol glycosides; CL, cardiolipins; DCIP, dichlorophenol-indophenol; DGDG, digalactosyl diacylglycerols; LPC, lyso-phosphatidyl cholines; LPE, lyso-phosphatidyl ethanolamines; MGDG, monogalactosyl diacylglycerols; PA, phosphatidic acids; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PG, phosphatidylglycerols; PI, phosphatidylinositols; PS, phosphatidylserines; SG, sterol glycosides; SL, sulphoquinovosyl diacylglycerols.

(*Brassica oleracea* L.) buds by differential and discontinuous density gradient centrifugation as described previously (15).

ATPase Assays. K^+-stimulated, Mg^{2+}-stimulated ATPase activity (TMK·ATPase) and Mg^{2+}-dependent ATPase activity (TM·ATPase) were assayed in 25 mM Tris-Mes buffer, (pH 8.0, the optimum for this enzyme [15]), containing 3 mM $MgSO_4$, with and without 50 mM KCl. The temperature sensitivity of the TM·ATPase and TMK·ATPase was determined by using a thermogradient in an aluminum block capable of simultaneously maintaining reaction tubes at 14 different assay temperatures with an accuracy of ±0.2°C. Enzyme assay and protein estimations were carried out as described previously (15).

The activity was determined at ATP concentrations of 1.5, 3.0, 5.0, and 6 mM at 5 and 25°C. Inasmuch as the K_m was not affected by changing temperature and there was no apparent substrate inhibition, a concentration of 3.0 mM ATP was used routinely at all temperatures.

Extraction of Membrane Lipids. The membrane preparation was boiled for 10 min in 20 volumes of isopropanol containing 10 μg/ml of butylated hydroxytoluene as antioxidant. Further extraction was carried out in chloroform:methanol (2:1, v/v) by method of Fishwick and Wright (6). Non-lipid contaminants were removed by the Sephadex treatment of Williams and Merrilees (30).

Fractionation of Membrane Lipids. Purified total lipids were fractionated by column chromatography using 1 g silica gel (Kieselgel 60; Merck)/10 mg lipid. Neutral lipids including sterols and sterol esters were eluted with chloroform (8 mg/g silica gel) followed by 10% (v/v) acetone in chloroform (3 ml/g). MGDG and SG were eluted with 50% (v/v) acetone in chloroform (8 ml/g) and ASG + DGDG with acetone (8 ml/g). SL and phospholipids were eluted with methanol (8 ml/g) followed by 10% (v/v) water in methanol (15 ml/g). Components of lipid fractions obtained from the silica gel column were separated by TLC and were identified by their reaction with specific spray reagents and, with the exception of SG and ASG, by comparison of the R_F values in at least four different solvents with those of standards of known composition.

Quantitative Analysis of Lipids.

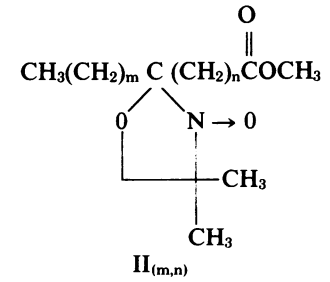
Phospholipids. After separation by TLC, phospholipids were estimated by the method of Kuiper *et al.* (10).

Glycolipids. Fractions containing glycolipids were separated by TLC. Spots containing glycolipid were hydrolyzed in 2 N H_2SO_4. For ASG and SG, the acid hydrolysate was extracted twice with petroleum ether (BP 60–80°C) to remove free sterols. The sugar content was measured by the method of Roughan and Batt (25).

Sterols. Free sterols and sterol esters were separated from the neutral lipid fraction by silica gel column chromatography as described by Carrol (2). Sterol esters were analyzed gravimetrically. Free sterols were derivatized by refluxing with acetic anhydride for 30 min and estimated using a GCQ column (Supelco) 100–103 mesh at 230°C. Cholestane was used as an internal quantitative standard. The absence of brassicasterol in the membrane lipids was confirmed by comparison with the retention times of brassicasterol obtained from an extract of cabbage seed by the method of Ingram *et al.* (9).

Fatty Acids. The total lipids and the phospholipid fractions were transesterified in 14% (v/v) boron trifluoride in methanol at 60°C for 30 min. The methyl esters of the fatty acids were extracted with petroleum ether (b.p., 60–80°C) and separated by GLC using a 222 PS column (Supelco) at 175°C.

Electron Spin Resonance Spectroscopy (ESR). Plasma membranes as well as aqueous dispersions of both the total and polar lipids were labeled with nitroxide derivatives of methyl stearate of the general formula:



$II_{(m,n)}$

Spectra were recorded and the motion parameter calculated for the various temperatures as described by Chapman *et al.* (3).

Differential Scanning Calorimetry. The membrane polar lipids were dispersed by sonication in 20 mM Tris-acetate buffer pH 7.2 containing 2 mM EDTA, and the vesicles concentrated by centrifugation at 150,000*g* for 45 min. Samples of the lipid dispersion or the denatured membrane preparation (3–5 mg of lipid) were sealed in 20 μl aluminum pans and thermograms recorded using a differential scanning calorimeter (model DSC-2; Perkin-Elmer, Norwalk, CT) at a scan rate of 10°C/min and a sensitivity of 0.5 mcal/s.

RESULTS AND DISCUSSION

The procedure used for the isolation of the cauliflower plasma membranes gave a 10-fold purification based on a comparison of the enhanced K^+-stimulated ATPase with that of the original homogenate (15).

Membrane preparations rich in ion-stimulated ATPase activity have been isolated from a variety of tissues by different methods. Information on the lipid composition is scant but is essential if meaningful comparisons are to be made with preparations from the same tissue isolated by different methods. The plasma membrane preparation from cauliflower contained 27% neutral lipids, 57% phospholipids and SL and 16% of a mixture of ASG, DGDG, SG, and MGDG determined by fraction weight (Table I). The major phospholipids were PC and PE (Table I). Little CL was present, and since phospholipids of cauliflower mitochondria contain about 11% CL (16), the 1% found in the plasma membrane preparations indicates there was only slight contamination by fragments of mitochondria membranes, which is consistent with the low level of succinate oxidase activity in the preparation (15). Similarly, the low (6%) galactolipid levels in the preparation (Table I) indicate little contamination by plastids, where galactolipid represents about 63% of the total lipids (excluding pigments) (5). In addition to free sterols (4% of total lipid weight) and sterol esters (5%), other sterol components of the plasma membrane preparation are acyl sterol glycosides and sterol glycosides (Table I), the latter in relatively the same proportion as found in plasma membranes isolated from sugar cane leaves (28). The M ratio of free sterol (predominantly β-sitosterol; Table I) to phospholipid in the cauliflower plasma membrane preparation is 0.5. This ratio is similar to that in plasma membranes isolated from soybean root (29) but is lower than the 1:2 found for oat root plasma membranes (8). Considering the wide variation in the sterol to phospholipid ratio in the various membrane preparations, use of this ratio as a marker for purification of plasma membranes is inappropriate and could be misleading. In addition, increases in the free sterol to phospholipid ratio from 0.1 to 0.4 in plasma membranes of *Phaseolus vulgaris* colyledons during senescence have been causally linked to the accompanying changes in the transition temperature of the membrane lipids (11) and this is discussed below in relation to the thermal behavior of the cauliflower plasma membranes.

The lipids of the plasma membrane fraction were particularly rich in linolenic acid (18:3), linoleic acid (18:2), and palmitic acid

Table I. *Lipid Content of Cauliflower Plasma Membranes*

Estimated errors for P and sugar determinations are 10% and 20%, respectively. Free sterol determinations have an accuracy of 5%, whereas the sterol ester content was estimated from the weight of a column fraction only. The calculation of percent of total weight for glycolipids was based on assumed mol wt as follows: MGDG, 778; DGDG, 940; SL, 600; ASG, 900; and SG, 600. The mol wt used for calculation of percent (w/w) of phospholipid were: PC, 750; PE, 744; PS, 788; PG, 745; CL, 1,473; PI, 856; LPC, 513; and LPE, 513. The mol wt of sterol ester was assumed to be 620, and free sterol was 420 from M ratio determinations.

Total Lipids			
	μmol/100 mg	*% w/w*	*% phospho-lipid*
Glycolipids			
ASG	12	11	
SG	7	4	
DGDG	5	4	
MGDG	3	2	
SL + (?)	6	4	
Sterols			
Free sterol	10	4	
15% campesterol			
1% stigmasterol			
84% β-sitosterol			
Sterol ester	8	5	
Phospholipids			
PC	26	20	38
PE	10	7	15
PS	5	4	7
PG	3	2	4
CL	0.3	0.4	1
PI	6	5	8
LPC	3	2	4
LPE	2	1	3
Unknowns (2)	10		14
Origin	4		6

Table II. *Fatty Acid Content of Total Lipids and Phospholipids from Caulifower Plasma Membranes*

Acid	Total Lipids	Phospholipids
	%	
14:0	1	T[b]
16:0	20	27
16:1	2[a]	T
18:0	4	1
18:1	12	7
18:2	22	17
18:3	40	48
Σ saturation	25	28
Σ unsaturation	75	72

[a] Two peaks.
[b] Trace only.

(16:0) (Table II). The high proportion of these components in the phospholipids is similar to the proportions of these acids in the phospholipids of ER from the same tissue (16).

The relative proportions of the minor fatty acids were also similar to other plant membranes indicating that fatty acid composition could not be used as a marker for plasma membranes.

Arrhenius transformations of the basal (TM·ATPase) and the K^+-stimulated (TMK·ATPase) activities of the plasma membrane fraction are shown in Figure 1. There is some controversy as to whether such data should be considered in terms of a biphasic system of two Eas, increasing abruptly below a defined temperature or as a system in which the Ea increases continuously as the temperature decreases as shown for the Na^+·ATPase of *Acholeplasma laidlawii* B cells (27). We have used both approaches. Treated as two linear-related functions (Fig. IA), the Ea for the K^+-stimulated ATPase increases from 7.1 to 21 kcal/mol below 11°C. Treated as a continuously variable function, we have fitted the data to equation 1 of Silvius and McElhaney (27) as shown in the caption to Figure 1B. This treatment also shows that Ea (proportional to slope of line) increases at low temperatures and the best fit is obtained using a value of 34 kcal/mol for the enthalpy of transition (ΔH_2) of the enzyme between the high and low activity states and a value of 283°K (10°C) for T_o, the temperature at which the ATPase is assumed to be equally distributed between the two states (27). In the results reported by Silvius and McElhaney (27), T_o was usually about 3 to 4 C degrees below the temperature of the transition of the membrane lipids determined by calorimetry.

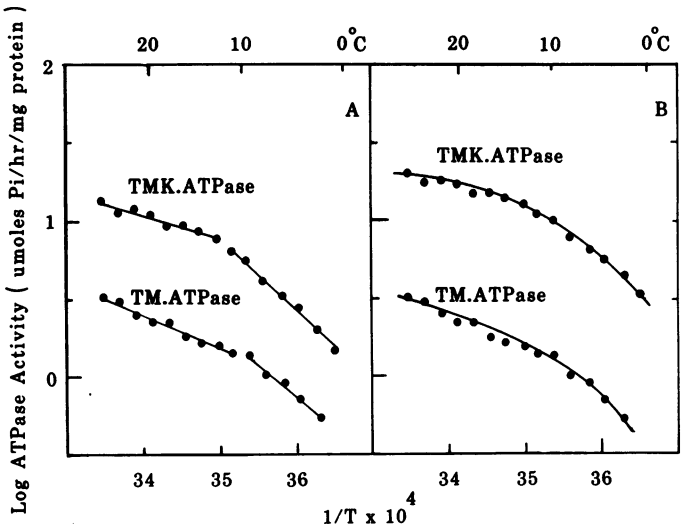


FIG. 1. Variation in the rate of ATPase activity as a function of temperature. A, data points were fitted to two straight lines described by $y = a_o + a_1x$: For TMK·ATPase, $y = 17.39 + (-4712.8)x$ below and $y = 6.32 + (-1553.1)x$ above 11°C. For TM·ATPase, $y = 14.7 + (-4127.3)x$ below and $y = 7.9 + (-2212.8)x$ above 10°C. B, data were fitted to the equation

$$\log \text{rate} = \frac{1}{2.303}\left[K + F(T) - \ln\left\{1 + e^{(\Delta H_{21}/R)(1/T - 1/T_{\omega})}\right\}\right]$$

(27). For TMK·ATPase, the constants were: $K = 0.25$, $F = 0.008$, $\Delta H_{21} = 34$ kcal/mol, and $T_o = 283$°K (11°C); and for TM·ATPase, $K = 0.18$, $F = 0.012$, $\Delta H_{21} = 81$ kcal/mol, and $T_o = 290$°K (17°C). The residual sum of squares for the fit of TMK·ATPase data in A was 0.006, and for B was 0.029.

Irrespective of how the data is presented or treated, it is apparent that the Ea increases at low temperature and the greatest increase occurred at about 12°C. This temperature response is in marked contrast to the constant Ea shown by the oxidative enzymes associated with the mitochondrial membranes of cauliflower buds (13), and of a number of other membrane-associated enzymes of chilling-resistant plants (23).

To resolve whether the change in Ea of the cauliflower ATPase is induced by a change in ordering of the associated membrane lipids, or is an intrinsic property of this enzyme, an examination was made of the thermal response of the membrane and membrane lipids by spin labeling and calorimetry. The temperature coefficient for motion of spin labels with the plasma membrane preparation and with total and polar membrane lipids is constant over the temperature range of 0 to 40°C indicating that there is no abrupt change in the molecular ordering of the membrane lipids

coincident with the change in Ea of the ATPase (Fig. 2). In addition, also shown in Figure 3, exothermic phase transitions above 0°C were absent in thermograms of the plasma membranes (trace A) and in dispersions of the polar lipids (trace B) from the membranes. It is of interest to note that the presence of sterols and sterol esters in the total lipids compared with their absence in the polar lipid fraction (Figs. 2 and 3) did not alter the thermal behavior of the lipids. Thus, sterols do not appear to modulate the thermal response of the plasma membrane fraction from cauliflower, as they do in the plasma membrane lipids of *Phaseolus vulgaris* (11).

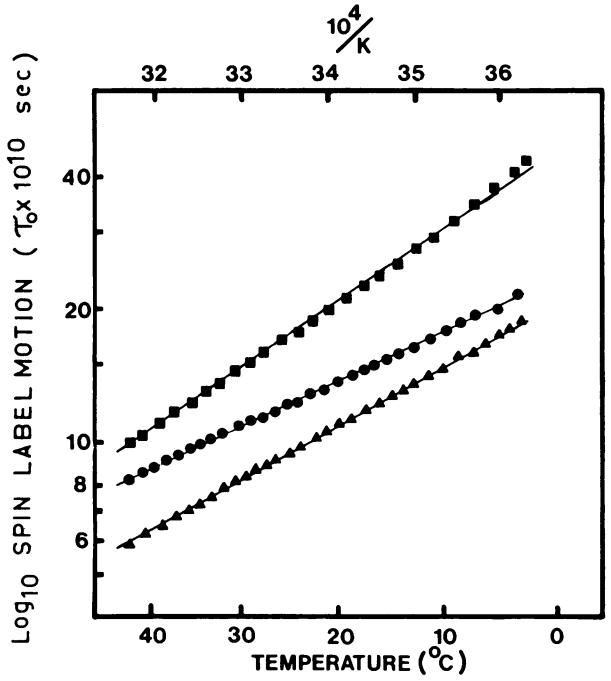


FIG. 2. Variation in the motion (τ_o) of spin label infused into plasma membranes and plasma membrane lipids isolated from cauliflower buds as a function of temperature. Intact plasma membranes (●) and an aqueous dispersion of the total lipids extracted from the plasma membranes (▲) were labeled with II (1, 14). The aqueous dispersion of polar lipids (■) was labeled with II (5, 10).

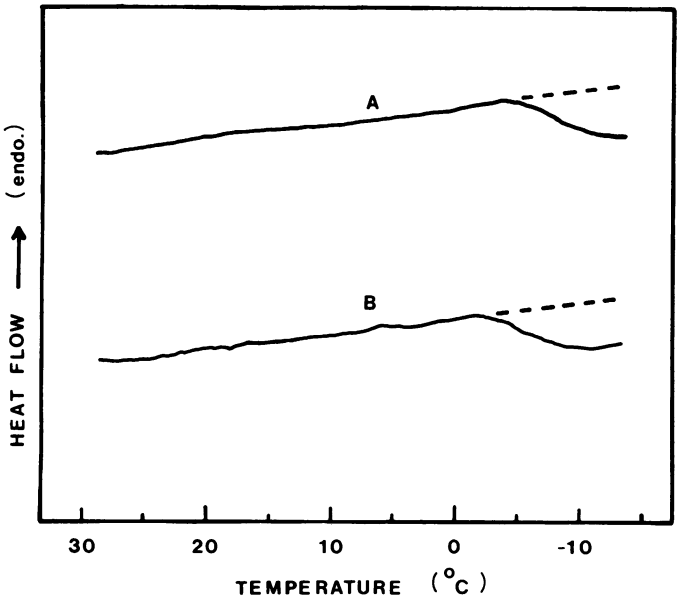


FIG. 3. Calorimetric traces showing the thermal behavior, in the cooling mode, of (A) the denatured plasma membrane fraction and (B) the polar lipids extracted from the plasma membrane fraction. The cooling rate was 10°C/min, and the sensitivity range was 0.5 mcal/s.

The thermal response of simple, artifical lipid systems is modulated in part by the degree of unsaturation of the fatty acids of the component lipids (20). On this basis, the high proportion of unsaturated fatty acid in the cauliflower plasma membrane (Table II) would be consistent with the absence of a thermal transition above 0°C. However, in complex mixtures of lipids the situation is not straightforward, and in plant membrane lipids the degree of fatty acid unsaturation is not always directly related to the thermal behavior of the lipids (19).

Based on these results, the increase in Ea for the K^+-stimulated ATPase cannot be attributed to a change in structure of the bulk lipid phase and is thus more likely an intrinsic property of the enzyme. Alternatively, it is possible that the change in function at low temperature might be a consequence of the thermal property of some particular boundary lipids specifically associated with the ATPase. Since no transitions were observed in denatured membranes or in the total membrane lipids (Fig. 3), it is unlikely that a transition in boundary-type lipids caused the change in Ea of the ATPase unless a transition in the boundary lipids was obscured by their interaction with the bulk lipids. In addition, the increase in Ea shown by the isolated enzyme system might reflect an artefact induced by inappropriate selection of assay conditions, particularly substrate concentration as suggested by Silvius *et al.* (26). This is also unlikely because it was experimentally determined that the enzyme was saturated with respect to ATP and not subject to substrate inhibition in both the high and low temperature regions.

The data presented are relevant to the interpretation of results of the effect of temperature on the rate of ion uptake by roots of plants of varying sensitivity to low temperatures. For both chilling-sensitive (maize) and chilling-resistant (barley) plants, the Ea for ion accumulation (measured as Rb^+ uptake), increases below 10°C (1). The net accumulation of Rb^+ by roots is the result of an active uptake, closely linked to ATP hydrolysis (22), less the efflux. The marked decrease in ion accumulation by maize roots at chilling temperatures might thus be attributed to a decrease in the rate of active uptake and an increase in efflux induced by a phase change which occurs in the membrane lipids of this plant at 12°C (23). This same explanation, however, cannot be extended to include barley. Barley is a chilling-resistant plant and no thermal transitions have been detected in the chloroplast membranes (24) or leaf phospholipids (21) of this plant at temperatures near 10°C. It is therefore more likely that the plasma membranes of barley respond to changes in temperature in a manner similar to those of cauliflower (Figs. 2 and 3) with no transition above 0°C. Since the plasma membrane ATPase shows a marked increase in Ea below about 11°C in both chilling-resistant (Fig. 1) as well as chilling-sensitive plants (14), the decrease in ion accumulation by both types of plants (1) is most likely a direct effect of temperature on the kinetics of the transport enzyme involved.

The independence of the thermal behavior of the cauliflower plasma membrane K^+-stimulated ATPase, relative to the bulk membranes lipids, is in direct contrast to the lipid-induced changes displayed by a number of enzymes of chilling-sensitive plants and demonstrates that caution is warranted when interpreting increases in the Ea of enzymes induced by low temperature in terms of possible mediators of the metabolic dysfunction associated with chilling.

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