Dose Dependent Reduction of Glucose Utilization by Pentobarbital in Rat Brain

Paul D. Crane, Ph.D., Leon D. Braun, B.A., Eain M. Cornford, Ph.D., Jill E. Cremer, Ph.D., James M. Glass, B.S., and William H. Oldendorf, M.D.

SUMMARY: A new method of determining the rate of glucose utilization in brain regions of individual rats has been used to measure the dose dependency of the reduction of the metabolic activity of the cerebral cortex by pentobarbital. Cerebral cortical glucose utilization is depressed to a basal level of 46% of the control rate when cerebral pentobarbital levels exceed 50 μg per g of tissue. The major portion of this effect occurs between the cerebral pentobarbital range of 10-20 μg per g, which can be achieved by 1/5 to 1/10 the normal anesthetic intraperitoneal dosage. If a depression of brain metabolism is responsible for the previously reported protection of the brain from ischemic damage, these data suggest a substantial reduction of brain metabolic rate is achieved in the rat at a barbiturate dosage which may be therapeutically relevant in the human after acute brain ischemia.

CEREBRAL ISCHEMIA leads to deprivation of substrates (glucose and oxygen) and excessive accumulation of metabolic intermediates (e.g. lactate) in the brain. Most protective mechanism seems unlikely in that the intracranial pressure in all these experimental animals would be expected to have been in the normal range since they had had wide temporal craniotomies and therefore subtemporal decompression over the area of swollen brain. It seems more likely that the reduction of intracranial pressure, though beneficial to the survival of ischemic brain, is not a primary phenomenon but is a secondary manifestation of it. It is possibly related to interaction with the metabolic mechanism for the control of cerebral blood flow and therefore of cerebral blood volume. It might be commented that the procedure itself induces the infarction, if this were so then results suggest barbiturate protection from this insult at least. However, tandem occlusion of major vessels is a well established technique for the production of experimental ischemia induced stroke.7,8 While useful intraoperative reduction of ICP by barbiturates has been shown in man,11,12 only isolated attempts have been made to apply this potential protection to the acute stroke victim. Certainly the elderly, generally debilitated patient would withstand substantial barbiturate treatment poorly at best, but more fit persons with isolated cerebral lesions many well benefit from barbiturate intervention. Our study suggests that post-insult therapy is both efficacious and significantly dose dependent allowing for lower dosages to affect a maximal response.

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References

permits the calculation of the rate of brain regional glucose utilization from individual animals, independent of measurements of blood flow and blood brain barrier transport. The additional measurement of brain pentobarbital levels by gas chromatography (GC) has enabled us to report the correlation between brain level of pentobarbital and the rate of glucose utilization.

Methods

Injection of Barbiturate and Radiolabeled 2-DOG

Wistar rats (200–250 g), of either sex, were injected intraperitoneally with 0.5 ml of Ringer's solution containing variable doses of sodium pentobarbital. After 10 min, the rats were placed in a restraining device (Gerling-Moore, Palo Alto) and their tails warmed for 30 sec in warm water. A 23 gauge needle was inserted into the dilated tail vein and ten μCi of (3H(G)) 2-DOG (sp. act., 10 Ci/m mole) in 0.25 ml of Ringers solution injected, followed by 0.1 ml wash of Ringers solution. Two minutes later, 2 μCi of 2-(1-14C)-DOG (sp. act., 54 mCi/m mole) in 0.25 ml of Ringers solution solution injected through the same needle, followed by 0.1 ml of Ringers solution. Two minutes after the 14 C injection, the rats were killed by intense microwave irradiation. Cardiac blood was collected into a heparinized syringe for liquid scintillation counting. The time course of 2-DOG uptake and phosphorylation was determined by single (3H) 2-DOG injections, followed by microwaving at varying times after injection. The brains were excised and stored at −20°C for subsequent analysis.

Biochemical And Radiochemical Analysis

The brains were thawed and the cerebral cortices were weighed and placed in Ten Broeck homogenizers with a measured amount of distilled water (--1.6 ml water – cortex wet wt × 0.8) and 50 μg of sodium secobarbital in 4 ml of methanol to serve as an internal standard in GC. Using a modified procedure of Bligh and Dyer,26 the cortices were homogenized, 2 ml of chloroform were added, and the tissues were rehomogenized. The homogenates were centrifuged, the supernates saved, and the pellets rehomogenized in 7 ml of chloroform-methanol-water (1:2:0.8) and centrifuged. The supernates were combined, 5 ml of chloroform and 5 ml of water added and the aqueous and organic phases separated after centrifugation. The interfaces were washed three times with methanol-water (1:1), and the washes combined with the aqueous phases.

The aqueous phases were further fractionated into neutral (containing glucose and 2-DOG) and anionic (containing 2-deoxy-D-glucose-6-phosphate (2-DOG-P)) fractions by ion exchange chromatography on DEAE Sephadex A25 columns (1 ml bed volume). The neutral fractions were recovered unbound from the column, while the anionic fractions were eluted with 0.5M pyridine acetate, pH 5.0. Aliquots were removed from both fractions for simultaneous 3H and 14 C liquid scintillation counting. The neutral fractions were dried by rotary evaporation and taken up in a 0.1M Tris-HCl (buffered to pH 7.5) and 0.02% sodium azide solution for glucose determination. Glucose was measured by an NADP-linked spectrophotometric assay with hexokinase and glucose 6-phosphate dehydrogenase (Glucose Statpack, Calbiochem, La Jolla, CA).

In control experiments radiolabeled (U-14C)-glucose, 2-(1-14C)-DOG and D-(1-14C)-glucose 6-phosphate were added to separate homogenizers containing 0.2–0.3 g of cerebral cortex, and recovery of the three compounds from the extraction and separation procedures was determined to be greater than 90%.

Water content was determined by weighing the rat brain in a tared glass vial, heating the brain and vial at 100°C for 24 hr, reweighing the vial containing the dried brain, and calculating the percentage of water lost.

Gas Liquid Chromatographic Analysis Of Barbiturate

The organic phase of the tissue extract was dried, taken up in 2 ml of H2O saturated with K2CO3, and extracted with an equal volume of ethyl acetate. The upper layer was transferred to a reaction vial and dried. Methylation was achieved by the addition of 0.2 ml of 1% tetramethyl ammonium hydroxide and by heating at 80°C for 10 min. The methanol was evaporated under nitrogen at ambient temperature and the methylated extract dissolved in 0.1 ml of ethyl acetate for injection into a Packard 419 gas chromatograph.

The recovery of sodium pentobarbital and sodium secobarbital was about 80%, as determined by methylation and injection of the compounds into the GC system before and after their addition to and extraction from cerebral cortex as described above. The GC column was a 6 ft × 2mm (i.d.) glass column packed with 3% SP-2100 on Supelcoport (100–120 mesh). The injection port and detector temperatures were 290°C and 300°C, respectively. The column was operated at 150°C, and the sample was injected in a volume of 1.5 μl. Quantitation was achieved by the ratio of responses of the digital integrator (Varian, 485) to methyl pentobarbital and methyl secobarbital. The response of the integrator was the same for both methylated barbiturate derivatives and was linear within the experimental range. The retention times were approximately 6.7 and 7.9 min respectively.

Effect Of Brain Pentobarbital Content On Animal Physiology

After measurement of rectal temperature, rats were injected intraperitoneally with 0.5 ml of Ringer's solution or variable volumes of Ringer's solution containing 10 mg/ml of sodium barbiturate. Ten minutes after injection, rectal temperatures were again measured; and righting reflex, tail pinch reflex, eye reflex, respiration, and motility were recorded. The rats were then killed by decapitation, the brains excised, and the cerebral cortical pentobarbital levels determined as described above. The significance of alterations in temperature and glucose content are expressed according to Dunnett's method for experiments making multiple comparisons with a control.14

Calculation Of The Rate Of Glucose Utilization

When the metabolism of glucose is in a steady state, the rate of glucose utilization is equal to the net flux of glucose through the hexokinase catalyzed step.8 The unnatural
glucose analog, 2-DOG, is transported across the blood brain barrier by the same carrier that transports glucose,\textsuperscript{18} but is phosphorylated by hexokinase at a lower rate than glucose.\textsuperscript{18} Phosphorylation of 2-DOG effectively traps it within the cell, since the sugar-phosphate ester cannot be transported, nor can it be converted to fructose 6-phosphate by glucose phosphate isomerase nor oxidized by glucose 6-phosphate dehydrogenase,\textsuperscript{14} nor appreciably dephosphorylated since glucose 6-phosphatase levels are very low in brain.\textsuperscript{14} (The half life of 2-DOGP has been determined to be 9 hours in cerebral gray matter).\textsuperscript{8} Thus, if 2-DOG is presented to the brain, the amount of 2-DOGP formed per unit time divided by the 2-DOG concentration during the time interval is equal to the fraction of 2-DOG utilized. If this fraction (after correction for reduced phosphorylation by hexokinase) is multiplied by the measured brain glucose content, the rate of glucose utilization can be calculated.

This method is dependent on the following conditions: 1) A steady state concentration of brain glucose and glucose utilization must exist. Since the pentobarbital concentration in the cortex has been determined to be constant between 10 and 30 min after intraperitoneal injection,\textsuperscript{16} the measurement of glucose utilization is initiated 10 min after intraperitoneal injection of sodium pentobarbital to permit the content\textsuperscript{17} and utilization\textsuperscript{7} of brain glucose to achieve a new steady state level. 2) Known but tracer concentrations of cerebral 2-DOG and 2-DOGP and a linear rate of 2-DOG phosphorylation must be present during the interval of measurement. In order to conveniently determine the level of 2-DOG during the measured interval, a 2 min to 4 min period after 2-DOG injection has been selected. During this interval the change in cerebral 2-DOG is not significant (.3 \( > p > .2 \)) (fig. 1). An injection of (\(^{14}C\)) 2-DOG is administered 2 min after the initial injection of (\(^{3}H\)) 2-DOG and the rat is killed by microwave irradiation 2 min after the second injection. The \(^{14}C\) dpm/g recovered in 2-DOGP and 2-DOGP fractions are normalized to account for the smaller number of \(^{14}C\) \( \mu \)Ciures injected by multiplying by the ratio, 

\[
R = \frac{^{3}H\text{ dpm injected}}{^{14}C\text{ dpm injected}}.
\]

as determined by liquid scintillation counting of the individual injectate mixes. The normalized \(^{14}C\) values should be equivalent to the brain level of (\(^{14}C\)) 2-DOGP and (\(^{3}H\)) 2-DOG at 2 min after injection. The substrate level of 2-DOG during the interval is approximated by averaging the level at 2 and 4 min. The measurement of substrate and product at two time points permits the calculation of the rate of glucose utilization (expressed as \( \mu \)moles/min/g):

\[
\text{Rate of glucose utilization} = \frac{2-\text{DOGP}}{2-\text{DOG}\times \text{Time}^c} \times \frac{(\text{Glucose})^b}{0.409^c}
\]

where

\[
a = (^{3}H)\ 2-\text{DOGP} - (^{14}C)\ 2-\text{DOGP} \times R
\]

\[
b = (^{3}H)\ 2-\text{DOG} + (^{14}C)\ 2-\text{DOG} \times R
\]

\[
c = 2 \text{ min} - 4 \text{ min measured time interval, i.e.} 2 \text{ min.}
\]

\[
d = \mu \text{moles glucose/g brain}
\]

\[
e = \text{rate of phosphorylation of 2-DOG relative to glucose by brain hexokinase.}
\]

3) The relative rates of phosphorylation of 2-DOG and glucose by rat brain hexokinase must be determined. The \( \text{in vivo} \) \( K_m \) of 2-DOG phosphorylation has yet to be reported. The \( K_m \) of 2-DOG for rat brain hexokinase \( \text{in vitro} \) has been reported to be 1.1 \( \times 10^{-4} \text{M} \), while the \( K_m \) of glucose was 4.5 \( \times 10^{-4} \text{M} \).\textsuperscript{14} Since the \( V_{\text{max}} \) of brain hexokinase is the same for both substrates the relative rates of phosphorylation can be calculated by the ratio of their respective \( K_m \)'s.\textsuperscript{13} The relative rate of 2-DOG phosphorylation used here is a ratio of the kinetic constants given above.

The glucose utilization and % phosphorylation vs. barbiturate concentration data were fitted to curves described by the equation \( y = ae^{bx} + c \) by means of least squares on an IBM 360 computer.

**Results**

The uptake and phosphorylation of (\(^{3}H\)) 2-DOG in the cerebral cortices of anesthetized rats from 0.25 to 16 min after intravenous injection is shown in figure 1. Approximately 2 min were necessary for equilibrium between the blood and brain. The 2-DOG brain content peaked at 6 min and slowly decreased in conjunction with the blood level. A nearly linear rate of phosphorylation, as shown in figure 2, was observed during the first 6 min which leveled off coincident with the washout of 2-DOG. The 2 min to 4 min interval was chosen as a convenient period to measure glucose utilization as a near constant substrate level of 2-DOG and a linear rate of phosphorylation occurs during this period (see fig. 1).
PENTOBARBITAL AND GLUCOSE UTILIZATION/Crane et al.

The cerebral cortical content of pentobarbital 14 min after intraperitoneal administration of varying dosages of sodium pentobarbital is shown in figure 3. Although the cerebral levels as measured by gas chromatography increased with increasing dosages, a wide variation in cerebral content was observed throughout the range of administered dosages. It is evident from the scatter of the data from the calculated slope (slope, 0.665; standard error of slope, 0.129) of the line of best fit that administered dosage is not an accurate index of brain pentobarbital level. Extraction and analysis of brain pentobarbital by gas chromatography, however, allows the estimation of tissue levels within a 5-10% error in our laboratory.

The fraction of 2-DOG in the brain that is phosphorylated during a steady state time interval is a measure of the percent of glucose entering the brain that is utilized. The effect of the cerebral content of pentobarbital on 2-DOG phosphorylation is given in figure 4. Phosphorylation per min in the conscious rat (after correction for reduced phosphorylation of 2-DOG by hexokinase) was observed to be 19.8 ± 4.0 (± SD, n = 16). Pentobarbital inhibited phosphorylation in a dose dependent, nonlinear fashion, as the change in slope declined at elevated doses. At 90 μg per g, a 75% decrease had occurred. A high scatter of the data from the predicted curve is evident between 0 to 20 μg per g. This correlates with the variable activity observed in control rats and the hyperactivity often seen in those injected with low levels of pentobarbital.

Cerebral glucose content rose with increasing pentobarbital levels from the control level of 2.27 ± 0.47 (± SD, n = 16) μmoles per g in the conscious rat to 3.80 μmoles per g ± 0.67 (± SD, n = 5) at 80 to 100 μg per g pentobarbital. Since these measurements are based on wet weight of tissue, it was necessary to test for water loss incurred by microwave irradiation. No significant difference in water content was observed between microwaved brains or brains analyzed after decapitation, as shown in table 1. The control glucose level is higher than the 1.4 μmole per g obtained for whole brain by freeze blowing, but is in agreement with results observed from microwave-fixed mouse brain in which cerebral cortical levels were significantly higher than whole brain.

The effect of pentobarbital on glucose utilization in the cerebral cortex is depicted in figure 5. The phosphorylation data in figure 4 were corrected for reduced phosphorylation of 2-DOG by hexokinase, and fitted to the rate of glucose utilization equation with the corresponding cerebral glucose levels, as described in the methods section. The rate of utilization in the conscious rat was 0.45 ± .04 (± SD, n = 16) μmol/min/g. The utilization rate was depressed by low levels of pentobarbital, and as the cerebral content of the drug exceeded 50 μg per g, the rate depression leveled off, approaching a basal level of 0.20 μmol/min/g, a 56% decrease from the control rate.

An important feature of the data shown in figure 5 is the marked inhibition of utilization at low pentobarbital levels. At 10 μg per g of pentobarbital, 50% of the total decrease
Table 1: Water Content of Rat Brain After 3.5KW Microwave Irradiation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Water content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irradiation or Decapitation</td>
<td></td>
</tr>
<tr>
<td>Microwave irradiation</td>
<td>77.24 ± 0.25</td>
</tr>
<tr>
<td>Decapitation</td>
<td>77.75 ± 0.71</td>
</tr>
</tbody>
</table>

Each value represents ± S.D. for 3 animals. There is no significant difference between the two groups, 0.3 > p > 0.02.

Discussion

The mechanisms underlying the apparent protective effect of barbiturates in brain from ischemic damage have yet to be elaborated. The work of Gatfield et al., indicates that high energy phosphate intermediates are maintained at a high level after barbiturate therapy which may serve to protect brain function. However, Siesjo and Nilsson have seen no change in the energy state in barbiturate anesthesia and their data suggest that the mild hypothermia induced by barbiturates is responsible for the cerebral protection. Subsequently, Smith et al. observed that barbiturates gave protection from ischemic damage in rats which were kept from becoming hypothermic by supportive surface heating. They theorized that the protective mechanism was primarily a lowering of cerebral blood flow and intracranial pressure and perhaps secondarily a reduction in cerebral oxygen consumption.

Although the present study does not provide a mechanistic explanation of the barbiturate protective effect, since an ischemic model was not used, the results do suggest a new approach to the problem. The data in figure 5 indicate that levels of pentobarbital greater than 50 μg per g produce a basal level of glucose utilization of 0.20 μmol/min/g — a 56% decrease from the control rate, which is in close agreement with results of Sokoloff and Hawkins for thiopental and pentobarbital anesthetized rats respectively. The remaining 44% of glucose utilized may be crucial to brain function as 40% of the energy produced by brain has been estimated to be used for ion transport and maintenance of cellular structure integrity. The data in table 2 negate an involvement of body temperature in barbiturate action on glucose utilization. Des Rosiers and Reivich have demonstrated a high correlation of local cerebral blood flow with local cerebral glucose utilization in both conscious and barbiturate anesthetized rats. Anesthesia per se, however, does not appear to be protective against ischemic damage as halothane alone did not prevent injury in acute focal cerebral ischemia.

It is of interest that subanesthetic doses (4 mg/kg) of pentobarbital have already been observed to depress both mono-and poly-synaptic discharges of the ascending medial longitudinal fasciculus in the adult cat.

In addition, the discharges were reduced in amplitude to 30–40% of control with 25–35 mg/kg pentobarbital, the anesthetic dose for cats. Since the major decrease in glucose utilization occurs at injected doses of 1/5 to 1/10 the level commonly used for anesthesia, substantially lower amounts of barbiturate may be sufficient to effect protection from ischemia than has previously been used (e.g. 40 mg/kg). Subsequent experiments in which prior cerebral ischemia is treated with subanesthetic barbiturate dosages
may indicate whether depressed glucose utilization can provide measurable protection.

The elucidation of quantitative drug-induced effects on brain metabolism requires a method of rate detection that is both responsive to subtle metabolic alterations in the brain and unhampered by concurrent changes in systemic physiology and brain metabolic pools. Methods of brain glucose utilization determination include measurement of brain blood flow and arteriovenous differences, by disappearance of energy reserves after decapitation, and more recently by incorporation of (2-14C) glucose into rat brain. The (2-14C) glucose method allows a rate calculation from in vitro. In vivo metabolism requires a method of rate detection that is independent of a large number of kinetic parameters when in vivo. The elucidation of quantitative drug-induced effects on brain metabolism requires a method of rate detection that is both responsive to subtle metabolic alterations in the brain and unhampered by concurrent changes in systemic physiology and brain metabolic pools. Methods of brain glucose utilization determination include measurement of brain blood flow and arteriovenous differences, by disappearance of energy reserves after decapitation, and more recently by incorporation of (2-14C) glucose into rat brain. The (2-14C) glucose method allows a rate calculation from in vitro. In vivo. Hawkins et al. who reported rates of measurement of the Michaelis Menten constants of rat brain hexokinase for glucose and 2-DOG would serve to pinpoint the actual rate of brain glucose utilization. If future studies define different kinetic values for brain hexokinase, the relative depression of glucose utilization induced by pentobarbital will not be altered. That is, the ratio of the $K_m$'s of hexokinase for glucose and 2-DOG will be the same for both conscious and pentobarbital-treated animals, so that for both conscious and pentobarbital-treated animals, the dose dependent reduction of glucose utilization by pentobarbital will remain constant.

Acknowledgments

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References


### Table 2: Comparison of Cerebral Glucose† and Pentobarbital Content With Body Temperature‡, Response to Stimuli, and Clinical Signs

<table>
<thead>
<tr>
<th>n</th>
<th>Pentobarbital (mg/g)</th>
<th>Glucose§ (μmol/g)</th>
<th>∆T (°C)</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>0</td>
<td>2.27 ± 0.47</td>
<td>0.02 ± 0.8</td>
<td>normal or overactive</td>
</tr>
<tr>
<td>9</td>
<td>2-10</td>
<td>1.78 ± 0.49*</td>
<td>0.49 ± 1.01</td>
<td>sluggish; staggering</td>
</tr>
<tr>
<td>3</td>
<td>10-20</td>
<td>2.12 ± 0.40</td>
<td>-0.04 ± 0.8</td>
<td>lying prone; somewhat immobile; unable to right</td>
</tr>
<tr>
<td>3</td>
<td>20-45</td>
<td>2.41 ± 0.43</td>
<td>-0.14 ± 0.77</td>
<td>unconscious; only slight response to tail pinch; slow respiration; cyanotic</td>
</tr>
<tr>
<td>3</td>
<td>45-90</td>
<td>3.26 ± 0.20*</td>
<td>-0.77 ± 0.71</td>
<td>loss of eye reflex; very hallow, jerking respiration; cyanotic</td>
</tr>
<tr>
<td>3</td>
<td>90-120</td>
<td>4.14 ± 0.60**</td>
<td>-1.70 ± 0.58*</td>
<td>loss of eye reflex; very hallow, jerking respiration; cyanotic</td>
</tr>
</tbody>
</table>

*n = number observed.
‡ ∆T = change in rectal temperature 10 min after IP saline or sodium pentobarbital injection. The mean temperature before treatment was 38.25 ± 0.58 ± SD.
†The cerebral glucose levels of those rats could not be determined, as the rats were killed by decapitation rather than microwave fixation. The glucose data given here are means ± S.D. of the levels measured in microwave-fixed brains in the appropriate cerebral regions; but it is subject to correction for CO2 loss and systemic biotransformation of the 14C glucose, both of which will remain constant.
§ Significant differences were determined according to Dunnett's method for making multiple comparisons with the untreated control where, *p <.05 and **p <.01.

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Intracranial Hemorrhage and Infarction in Anticoagulated Patients with Prosthetic Heart Valves

ABRAHAM LIEBERMAN, M.D., WILLIAM K. HASS, M.D., RICHARD PINTO, M.D., WAYNE O. ISOM, M.D., MARK KUPERSMITH, M.D., GEORGE BEAR, M.D., AND RANDOLPH CHASE, M.D.

SUMMARY In 1 year 6 patients with prosthetic heart valves (PHVs) treated with anticoagulants suffered intracranial hemorrhage. In 4, hemorrhage occurred into the site of a recent non-hemorrhagic infarction. In the others, both of whom had endocarditis, hemorrhages probably occurred as the result of rupture of a mycotic aneurysm. Five patients were treated with warfarin; 1 with heparin. In all patients the level of anticoagulant activity was greater than 1.5 times control. Five patients were in atrial fibrillation; 1 was hypertensive. The diagnosis of intracranial hemorrhage was made and its location and extent accurately determined by computed tomography.

A LARGE NUMBER OF patients are now living with artificial non-organic prosthetic heart valves (PHVs). Although operative mortality and morbidity have been dramatically reduced, emboli and infection continue to be problems. In the absence of anticoagulants the frequency of systemic and cerebral emboli is variable and has been related to the type of prosthesis, the covering of the prostheses, and the cardiac rhythm. After embolization serious disability and death frequently occur since the majority of symptomatic emboli lodge in the cerebral circulation. Three patients underwent surgery and 2 are alive with only minor neurological deficits. Among the 3 patients who did not undergo surgery 2 died and 1 is alive with a moderate neurological deficit. The management of PHV patients with use of anticoagulants is discussed in terms of the mechanisms involved in intracranial bleeding. Emphasis is placed on prevention of emboli, discontinuation of anticoagulants once non-hemorrhagic infarction has occurred and the primacy of CT scan in diagnosis when hemorrhage is suspected. The special problems of anticoagulation in the presence of endocarditis are also discussed.

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