ered as an explanation of the geochemical anomalies described at the K/T boundary. I wish to point out that their choice of analytical methods precludes the attainment of their stated objectives and that their data do not support the discarding of the asteroid-impact hypothesis.

First, their study encompassed only the < 2-μm fraction of the boundary clays. With the elimination of all components larger than 2 μm, surely most of the mineralogically exotic materials expected from an asteroid impact will have been missed. If one wishes to distinguish this material from ordinary volcanic ejecta or land-derived detrital components, then one must look at the size fraction in which they would most probably occur (> 2 μm). Studies of altered volcanic ashes (tonsteins) in coal beds have shown that the nonclay mineral components that characterize these rocks as volcanic in origin are much larger than 2 μm (*2*). To ignore this larger-sized component almost eliminates any possibility for determining whether the samples are volcanic or not.

Can the clay fraction < 2 μm be useful at all in determinations of origin? By these investigators' own admission (*1*), the glassy phase of fine ejecta from an asteroid impact might be altered to a clay mineral such as smectite, which might be difficult to distinguish from the volcanogenic smectite present in the marine boundary sections. Glasses of similar compositions, regardless of origin, would be expected to alter to similar clay minerals on the ocean floor. Clay minerals in the limestone and marl beds enclosing the boundary clays also would be expected to be derived mostly from ubiquitous volcanic glass settling from the water column along with the calcareous component. The Late Cretaceous, after all, was a time of intense volcanism, as pointed out by Rampino and Reynolds (*1*). Thus, would one expect to find large differences in clay mineralogy between the boundary clays and the surrounding rocks?

Second, the method of sedimenting the clay fraction (< 2 μm) onto glass slides for x-ray diffraction analysis was a poor choice for this study. Gibbs (*3*) and many other workers since have shown that sedimentation onto glass slides allows size fractionation to occur, masking coarser components with finer-sized clay minerals. Thus, diffraction from the finest clay-sized material of the < 2-μm size fraction will be preferentially enhanced on the x-ray pattern. If any exotic material from the impact event were present in the < 2-μm fraction, the likelihood of its being revealed on the x-ray diffraction pattern with this type of mount is remote.

In summary, because of the choice of analytical methods, the conclusions drawn from this study are based on incomplete data and do not represent a clear assessment of the available mineralogical information from these samples. We still do not know whether these marine K/T boundary clays represent detrital, volcanic, or extraterrestrial events. Consequently, the asteroid-impact hypothesis remains alive and well and living in Berkeley.

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28 February 1983; accpted 8 July 1983

Bohor seems to have missed the point of our investigation. We did not undertake this study to prove or disprove a volcanic origin for the clay layer but to perform what seemed to be a clear-cut test of one of the predictions of the asteroid-impact model, namely, that the fine-fraction mineralogy of the layer should give some indication of an exotic origin.

We concentrated on the < 2-μm fraction specifically because this is the material with significant stratospheric residence times that would be widely dispersed to form the dust cloud of the impact model (*1*). We avoided the > 2-μm fraction precisely because it could (and in fact does) contain local contaminants (*2*). Investigators who have examined the fraction of the boundary layers coarser than 1 μm report no common carrier phase for the supposedly extraterrestrial elements (*3*). Our main findings were that the boundary layers were different mineralogically at each locality studied and that the clay layers were similar to clays stratigraphically above and below them, in contrast to the predictions of an impact origin (*4*).

The glass slide method does indeed enhance the diffraction contribution from the finest grain sizes and diminishes the intensities of diffraction from the coarser material. However, it is much too strong to say that this method makes detection of the coarser material in the < 2-μm fraction remote. In the usual case, intensities may be reduced by 20 to 30 percent of the amount present for minerals concentrated near the bottom of the slide. We claim approximately a 5 percent detection limit; the differences between 5 and 6 or 7 percent are insignificant.

We agree with Bohor that it is difficult to differentiate between smectite produced by volcanic glass and smectite formed from glassy impact debris. Our position here was based largely on philosophical grounds. In the context of Ockham's razor, a line of reasoning seems unnecessarily contrived if it requires that one of the altered glassy horizons in Upper Cretaceous rocks has an origin that is qualitatively different from the many that are clearly of volcanic derivation.

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27 October 1983

Receptor Binding Studies

In their recent discussions of receptor binding and Scatchard plots, neither Klotz (*1*) nor Munson and Rodbard (*2*) addressed problems in the estimation of free ligand concentrations. In general, the tissue concentrations of cellular receptors are very low and their binding activities are unstable. Consequently, receptor interactions with hormones and other effector molecules have been studied almost exclusively with rapid, nonequilibrium methods that separate and quantify only receptor-bound radioligands. Although the free ligand concentrations that existed at equilibrium prior to the separation step are required for

accurate analysis and interpretation of such binding data, they are rarely mentioned in the literature. Free (*F*) ligand concentrations are usually calculated as the difference between the total ligand concentrations and the corresponding values for receptor-bound (*B*) ligand in order to obtain *B*/*F* ratios for Scatchard analysis (*3*). However, this procedure is invalid if the ligand binds to other components of the system.

A radioligand added to a complex biological sample may bind with low affinity to contaminating cellular or serum proteins, subcellular organelles, or membrane lipids, for example. This binding goes undetected, however, since the ligand rapidly dissociates from such low-affinity sites during separation of receptor-bound ligand. According to the general expression governing multiple binding equilibria (*4*), the distribution of a bound ligand among competing classes of independent binding sites is proportional to nK_A or the product of the concentration of unoccupied binding sites (n) and their affinity constant (K_A) for the ligand for each class of binding sites. Therefore, low-affinity binding sites effectively compete for the ligand when their concentrations are many orders of magnitude greater than that of a receptor in impure tissue preparations. Calculation of free ligand concentrations by difference therefore overestimates the true values to the extent that nonreceptor binding occurs at equilibrium. As a consequence, falsely low *B*/*F* values are obtained and the slope of the Scatchard plot and the K_A derived therefrom will be correspondingly underestimated.

The terms "specific" and "nonspecific," denoting, respectively, receptor and nonreceptor binding of a ligand, are firmly entrenched in the literature. Nonspecific binding is commonly estimated by adding a large amount of nonradioactive ligand, in addition to the radioligand, to samples and processing them in parallel through a receptor assay. Such estimates reflect only the amount of nonreceptor-bound ligand that remains after adsorption, filtration, or washing of the receptor-bound ligand fraction and do not take into account the far greater amount of ligand that may have been bound to other component at equilibrium. Accordingly, they should be designated "post-assay nonspecific binding" values and cannot be used as equilbrium values.

Investigators at my laboratory have estimated the dissociation constant (K_D) for estradiol binding to rat uterine estrogen receptor using a slight modification of the isodialysis technique (*5*) for measurement of the percentages of free estradiol in uterine cytosol. As shown in Fig. 1, the value was about 20-fold lower than that obtained when bound estradiol was measured by the dextran-coated charcoal method and the free concentrations were calculated by difference. The much higher *B*/*F* values obtained when free ligand concentrations were measured demonstrate the potential for error in estimates of binding affinity when nonspecific binding at equilibrium is ignored. Where this approach is not feasible (high molecular weight ligands, extremely high nonreceptor binding) prior enrichment of the receptor by purification of membranes or precipitation of a soluble receptor often allows direct assay of the free ligand concentrations.

In addition to underestimating the affinity of a hormone for a receptor, other kinds of anomalous results and erroneous conclusions are possible if only receptor-bound ligand is measured in impure systems. For example, the discrepancies in receptor number obtained by Scatchard and semilogarithmic saturation plots noted by Klotz (*1*) may be explained easily if the true free ligand concentrations were not high enough to saturate the receptors because of extensive low-affinity binding of ligand at equilibrium. Moreover, apparent changes in receptor affinity associated with physiologic events or in vitro perturbations (changes in *p*H or ionic strength, addition of metal ions, agonists or antagonists, for example) may be due to alterations in nonreceptor binding components which shift the distribution of bound and free ligand at equilibrium rather than changes in receptor binding characteristics. Also, when a series of nonradioactive ligands are allowed to compete with a radioligand for receptor binding in pharmacologic studies of relative drug potencies, the results obtained may reflect differences in their affinity, both for the receptor and nonspecific binding sites, which determines their effective (free) concentration. Finally, displacement of the receptor saturation curve to the right of a biological dose-response curve for a hormone may be caused by erroneously high estimates of the free ligand levels in the receptor assay rather than the presence of "spare" receptors if the assay conditions are not identical.

The accuracy of the parameters obtained from the analysis of complex receptor binding data is dependent on the correctness of the values for both free and receptor-bound ligand concentrations. Measurement of free ligand con-

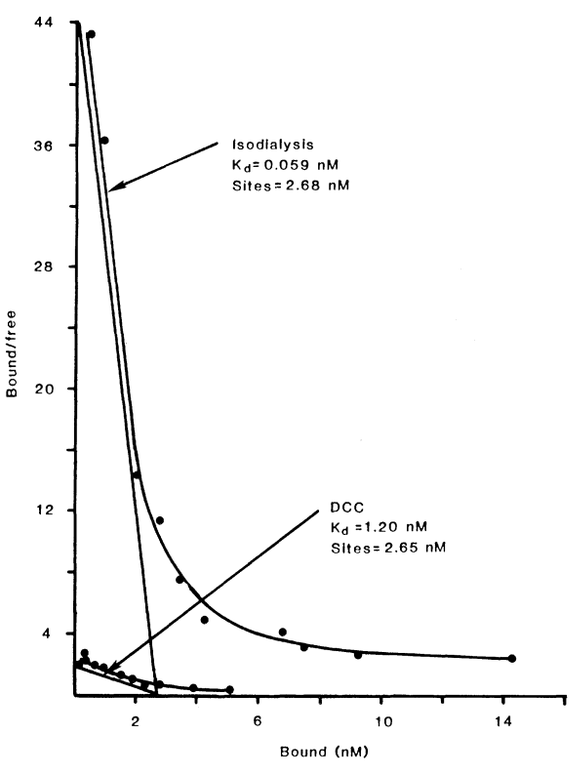

Fig. 1. Scatchard plot analysis of data for [^{3}H]estradiol binding to rat uterine estrogen receptor obtained by measuring bound or free ligand concentrations at 4°C. Uteri obtained from castrated adult female Sprague-Dawley rats were homogenized in 0.05*M* tris buffer, *p*H 7.4. After centrifugation at 800*g*, the supernatant was centrifuged at 100,000*g* for 1 hour to prepare cytosol (protein concentration 1.8 mg/ml). Portions (0.3 ml) were prepared in duplicate for two separate assays in tubes containing sufficient [^{3}H]estradiol to yield 20 final concentrations ranging from 0.05 to 100 n*M*. After incubation for 2 hours at 4°C, one set of samples was assayed for receptor-bound estradiol by the dextran-coated charcoal method (*DCC*) and the other for free estradiol by isodialysis (*5*). Both the free and bound ligand concentrations were calculated as the difference between the total concentrations measured prior to separation. In both cases, the straight lines were obtained by computer-assisted nonlinear least-squares regression analysis. Note that the binding capacity (2.7 pmole per uterus) was identical by both assays.

centrations at equilibrium is essential for assessment of the uncertainty in binding data now available.

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29 June 1983; accepted 27 October 1983

Alcohol-Induced Tolerance in Mitochondrial Membranes

Lieber and his colleagues (*1*) state that they have found no evidence for a correlation between the structure of membrane phospholipids and function of mitochondrial membranes from ethanol-fed rats, or for resistance to disordering by ethanol, which we reported earlier (*2*). However, extensive studies in our laboratory show that the decreased rate of respiration, first described by Cederbaum *et al.* (*3*) in intact mitochondria, can be explained as a direct consequence of decreased content and activity of individual protein components of the respiratory chain in mitochondrial inner membranes (*4*). What causes this decrease is not clear. It might be due to direct inhibition of mitochondrial protein synthesis (*5*) or to interference with membrane assembly. The latter may be influenced by the phospholipid composition of the membranes. Moreover, the respiratory activity indeed may be influenced by the phospholipid composition. We have shown that mitochondrial membranes from ethanol-fed rats display an increased saturation in the acyl chains of cardiolipin (*6*), an essential phospholipid component of the electron transport chain, which may contribute to the regulation of the respiration rate. Therefore, we suggested that "The phospholipid composition . . . probably plays a role in other modulations of membrane structure and function . . . " (*6*). Since Lieber and his colleagues (*1*) analyzed only total fatty acid composition of the mitochondrial membranes, and cardiolipin is a minor component, they could not detect this increased saturation of cardiolipin acyl chains. Nevertheless, their data regarding total fatty acid composition actually confirm our findings of a significant increase in stearic acid and a decrease in palmitic acid in both phosphatidylcholine and phosphatidylethanolamine.

Our evidence for increased resistance to disordering by ethanol is based on studies with electron paramagnetic resonance (EPR) spin probes (*2*). The order parameter, measured by 5-doxyl stearic acid or 12-doxyl stearic acid, is significantly decreased by low concentrations of ethanol in liver mitochondria from normal rats but not in ethanol-fed rats. Similarly, the partition of doxyl-decane is greatly enhanced by ethanol in control rats but not ethanol-fed rats. Similar results were obtained earlier by several other groups in synaptosomal membranes and red blood cells (*7*) and more recently by us in liver microsomes (*8*). We, therefore, believe that this is a general phenomenon relevant to all membranes in all tissues (*9*). It is necessary to explain why Lieber and his colleagues could not confirm this observation in their studies. We have found that the resistance to disordering by ethanol is observed at high temperature (35°C) but not at low temperature (15°C) (*2*). Lieber and his colleagues measured 12-(9-anthroyloxy) stearic acid (12 AS) fluorescence anisotropy at 28°C, where the difference, if it exists, is expected to be small. The sensitivity of 12 AS anisotropy to small structural changes is at least one order of magnitude lower than that of the EPR technique, particularly in highly scattering membranes such as mitochondria. In fact, we suspect that scattering artifacts were not properly corrected for in their studies. Vanderkooi and Chance (*10*) studied fluorescence anisotropy of 12 AS in mitochondria at 20° to 45°C (Fig. 3 in *10*); their measured polarization values ranged from 0.125 to 0.1. This value corresponds to an anisotropy range of 0.087 to 0.069. We measured the fluorescence anisotropy of 12 AS in mitochondria and obtained a value of 0.08 at 28°C. This value is in excellent agreement with those obtained by Vanderkooi and Chance (*10*), but is one-third those reported by Lieber and his colleagues (*1*). Nevertheless, because of the low values of fluorescence anisotropy and the large corrections for light scattering, the effect of low concentrations of ethanol on membrane fluidity cannot be easily detected. We suspect that Lieber and his colleagues, in fact, measured the considerable effect of alcohol on light scattering, which is caused by mitochondrial swelling.

In summary, there is sufficient evidence from studies of rat liver mitochondria and other membrane systems to indicate that chronic alcoholism is associated with changes of membrane structure, composition, and function and that these changes lead to tolerance to the acute effects of ethanol.

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2 August 1982; accepted 12 November 1982

Gordon *et al.* (*1*) examined the basis of a challenging theory proposed by Rottenberg and his colleagues (*2*) that the "chronic consumption of ethanol induces an adaptation of membrane composition causing increased membrane rigidity (decreased fluidity). . . . The increased rigidity impairs normal membrane function . . . but in the presence of moderate concentrations of ethanol the membrane becomes sufficiently fluid to resemble normal membranes (dependence)" (*2, 3*).

To examine this theory, we designed a controlled nutritional experiment, which included a group of Chow-fed rats (for which respiratory functions have been well defined), ethanol-fed rats, and the pair-fed controls of the latter. Mitochondrial membranes from the Chow-fed animals contained a larger amount of saturated fatty acids than the mitochondrial preparations from the pair-fed controls and were more resistant to the fluidizing effects of ethanol, although respiratory functions in the membranes of the two groups were similar. In contrast, the