week. These storms represent extremely diffuse clouds that have previously gone undetected.

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Tunneling in Ligand Binding to Heme Proteins

Abstract. *Rebinding of carbon monoxide to the beta chain of hemoglobin after photodissociation by a laser flash is intramolecular below about 200 K. Above 25 K, rebinding occurs via classical over-the-barrier motion; below, quantum-mechanical tunneling dominates. Both are described by an energy spectrum peaked at* $E^{peak} = 4.0$ *kilojoules per mole. The barrier width* d(E), *determined from the energy dependence of the tunneling rate, depends on barrier height,* $d(E) \approx 0.05$ *nanometer* $\times (E/E^{peak})^{1.5}$.

Quantum-mechanical tunneling, in which a particle passes through a classically impenetrable barrier, plays a role even in polymers and biomolecules. Electron tunneling occurs in photosynthesis (*1*); molecular tunneling has been seen in the radiation-induced polymerization of formaldehyde (*2*). We report here the observation of molecular tunneling in the binding of carbon monoxide to the beta chain of hemoglobin (β Hb). Before describing our experiments, we make a few remarks concerning tunneling. Consider a molecule of mass M in thermal equilibrium with its surroundings at temperature T in a well B. Well B shall be separated by a potential barrier of height E and width $d(E)$ from the deeper well A (Fig. 1a). The molecule can move from B to A by hopping over the barrier or tunneling through it (*3*). The two processes are distinguished by their temperature dependences. The classical Arrhenius (over-the-barrier) rate parameter k_a, given by

$$k_a(E) = A \exp(-E/k_B T) \qquad (1)$$

vanishes in the limit $T \rightarrow 0$. Here, $k_B = 8.32$ joule mole^{-1} K^{-1} is the Boltzmann constant and 1 kjoule mole^{-1} = 0.239 kcal mole^{-1} = 0.010 ev. Quantum-mechanical tunneling can also be temperature dependent, but in the limit $T \rightarrow 0$ remains finite and becomes temperature independent (*4*). Tunneling thus is established if the transition rate between two well-defined states becomes temperature independent as $T \rightarrow 0$. For a parabolic barrier with height $E \gg k_B T$, the low temperature limit can be written as

$$k_t(E) = A_t \exp[-\pi d(E)\,(2ME)^{1/2}/2\hbar] \qquad (2)$$

where the exponential is called the Gamow factor (*5*) and $2\pi\hbar$ is Planck's constant.

Experimentally we study the binding of ligands to heme proteins and heme model compounds by flash photolysis. The heme protein H with bound ligand L, HL, is placed in a cryostat and photodissociated by a laser flash. The subsequent rebinding, H + L $\rightarrow$ HL, is followed optically with a transient analyzer with logarithmic time base that records from 2 μsec to 1 ksec in a single sweep (*6*). With this technique we previously investigated the binding of CO and O_2 to myoglobin (Mb) from 40 to 320 K (*7*). Extension of these experiments to 2 K gives evidence for tunneling in Mb and, in fact, in all systems where we looked (MbCO, MbNO, α HbCO, β HbCO, cytochrome P450 CO, carboxymethylcytochrome c CO, hydroxyheme CO, 2-methylimidazoleheme CO, and heme c octapeptide CO). For our work we selected β HbCO and prepared samples (in a mixture of glycerol and water, 3 : 1, by volume, *p*H 7.0) by the method of Geraci *et al.* (*8*).

Figure 1b gives rebinding curves for β HbCO. The quantity $N(t)$ is the fraction of Hb molecules that have not rebound CO at the time t after the laser flash (*9*). Since the curves extend over many orders of magnitude in time, log $N(t)$ is plotted as a function of log t. Figure 1c gives $N(t)$ for $T < 50$ K on an expanded scale. The curves display two conspicuous features: (i) Below about 20 K, they approach temperature independence; and (ii) $N(t)$ is not exponential, but close to a power law. To express feature (i), we characterize each curve in Fig. 1c by $t_{0.75}$, the time at which $N(t)$ drops from 1 to 0.75. The rate $k_{0.75} = 1/t_{0.75}$ is plotted in Fig. 2a versus log T. Above 25 K, $k_{0.75}$ depends exponentially on T; below 10 K, it is independent of T. Quantum-mechanical tunneling thus is established: Initial and final states in the transition are distinct as is proved by their optical spectra; the states are separated by a barrier as demonstrated by the Arrhenius behavior above 25 K, and the transition rate from B to A becomes temperature independent for $T \rightarrow 0$.

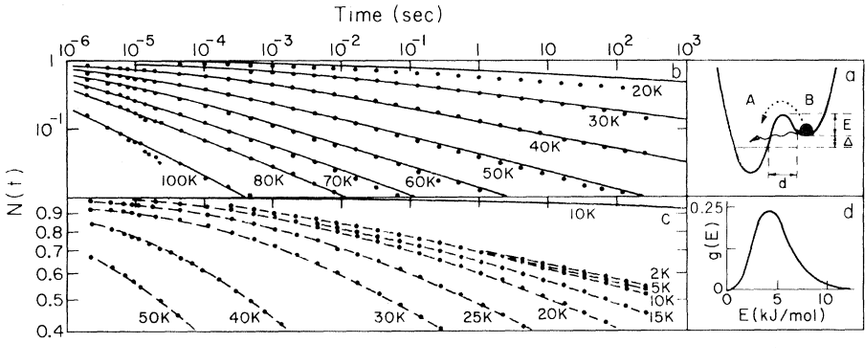

Fig. 1. (a) A molecule in well B can move to A by hopping over the barrier or tunneling through it. (b) Rebinding of CO to β Hb after photodissociation. $N(t)$ is the fraction of β Hb molecules that have not rebound CO at time t after the laser flash. (c) As (b), but with expanded $N(t)$ scale. The solid line labeled 10 K indicates $N(t)$ as expected without tunneling. (d) Activation energy spectrum for β HbCO. The solid lines in (b) are calculated with $g(E)$ as given here.

Before treating tunneling in detail, we elucidate feature (ii), the nonexponential rebinding. We assume that the activation energy governing rebinding shown in Fig. 1b is given by a spectrum (*7, 10*). If $g(E)dE$ denotes the probability of having a β Hb molecule with an activation energy between E and $E + dE$, $N(t)$ becomes

$$N(t) = \int_0^\infty dE\, g(E)\, e^{-k(E)t} \qquad (3)$$

Here $k(E)$ is the sum of classical Arrhenius and quantum-mechanical tunneling rate parameters

$$k(E) = k_a(E) + k_t(E) \qquad (4)$$

k_a dominates above 25 K, k_t below. Since $N(t)$ is nonexponential in the entire temperature range from 2 to 100 K, tunneling and over-the-barrier hopping must be described by the spectral function $g(E)$. We assume that $g(E)$ is unchanged in the entire temperature range. From the experimental points above 25 K, we determine $g(E)$ by inverting Eq. 3 with $k(E)$ given by Eq. 1 (*7, 10*). The resulting spectral function $g(E)$ is shown in Fig. 1d; the corresponding preexponential A is equal to 1.4×10^9 sec^{-1}. The solid lines in Fig. 1b are calculated from $g(E)$ in Fig. 1d and Eqs. 1 and 3; they fit the experimental points above 30 K. Below, rebinding is much faster than predicted classically.

A description of tunneling requires two additional assumptions. First, tunneling is temperature independent only at the lowest temperatures; in general, it can be proportional to a power of T (*3*). We assume that the various tunneling channels all possess the same Gamow factor in Eq. 2, so that the temperature dependence is contained in $A_t(T)$. Second, we assume that the barrier width $d(E)$ in Eq. 2 depends on barrier height through

$$d(E) = d_0(E/E^{\text{peak}})^\delta \qquad (5)$$

where d_0 is the barrier width corresponding to the peak energy $E^{\text{peak}} = 4.0$ kjoule mole^{-1} (Fig. 1d), and δ is a free parameter. We combine the two assumptions by writing

$$k_t(E) = A_t(T) \exp(-\gamma E^{\delta + \frac{1}{2}}) \qquad (6)$$

with

$$\gamma = d_0\pi(2M)^{\frac{1}{2}}/2\hbar(E^{\text{peak}})^\delta \qquad (7)$$

The parameters $A_t(T)$, γ, and δ are determined by inserting Eqs. 1, 4, and 6 into Eq. 3 and fitting by computer the resulting $N(t)$ to the experimental data below 30 K. The term $A_t(T)$ is plotted in Fig. 2b, the parameter $\delta = 1.5 \pm 0.4$. To calculate d_0 from γ, a value for M in Eq. 7 must be assumed. The iron in Hb has

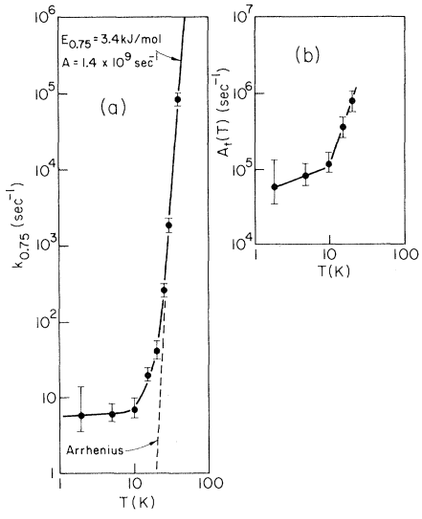

Fig. 2. (a) The rate $k_{0.75} = 1/t_{0.75}$ plotted as a function of log T. The time $t_{0.75}$ at which $N(t)$ drops from 1 to 0.75 is taken from Fig. 1c. The steeply dropping part corresponds to the classical Arrhenius hopping over the barrier. The observed rate is much faster than the classical one for $T < 20$ K and provides evidence for quantum-mechanical tunneling. (b) The tunneling preexponential $A_t(T)$, plotted as a function of log T, indicates the presence of two regions.

spin $S = 2$, and is displaced from the heme plane by 0.07 nm; in HbCO it lies in the heme plane with $S = 0$ (*11*). We postulate that the binding process corresponds to the simultaneous motion of Fe into the heme plane and CO from the pocket closer to the Fe, and thus take M in Eq. 7 to be the reduced mass of Fe and CO, $M = M_{Fe}M_{CO}/(M_{Fe} + M_{CO})$; $d(E)$ and d_0 then become

$$d(E) = d_0(E/E^{\text{peak}})^{1.5 \pm 0.4},$$
$$d_0 = (0.05 \pm 0.01)\text{ nm} \qquad (8)$$

The width $d(E)$ at the bottom of the barrier (Fig. 1a) increases with increasing barrier height E; the value for d_0 agrees approximately with the displacement of the iron atom in the transition $S = 2 \rightarrow S = 0$. The fact that we can extract $A_t(T)$ and $d(E)$ separately can be understood by first looking at the Arrhenius equation (Eq. 1), where preexponential A and energy E are separated by varying T. Similarly, in tunneling (Eq. 2) A_t and d can be determined individually if either M or E can be varied. The first approach is the isotope effect; the second is possible here because the energy spectrum allows us to educe k_t as a function of E.

Tunneling, as described by $A_t(T)$ in Fig. 2b, shows two regions: Below $T_0 = 10$ K, $A_t(T)$ is essentially temperature independent; above T_0, it can be fit by a power law, $A_t(T) \propto T^{4 \pm 2}$, or by an exponential, $A_t(T) \propto \exp(-\epsilon/k_BT)$ with $\epsilon = (0.6 \pm 0.2)$ kjoule mole^{-1} (*12*). For a tentative interpretation we assume for simplicity that tunneling occurs from the lowest level in well B to a single excited level in well A that lies lower than B by an energy difference Δ (Fig. 1a). Energy conservation then demands that the energy Δ be taken up by one or more phonons. The only temperature-independent phonon process is spontaneous emission and we consequently assume that tunneling below T_0 is accompanied by the spontaneous emission of a phonon of energy Δ. Above T_0, induced phonon emission and scattering can take place. In some solids, T_0 is less than 1.5 K (*13*). The high value of T_0 found here can be explained in two ways: (i) Δ can be large compared to k_BT_0 or (ii) the finite size of the biomolecule can produce a low-energy cutoff in the phonon spectrum (*14*).

Our work shows that below 25 K binding of CO to β Hb occurs by quantum-mechanical tunneling. Tunneling also occurs in other heme proteins and compounds. While molecular tunneling has no biological importance, it yields information of interest to the understanding of biological processes. The width of the barrier governing binding at the active center can be deduced; the temperature dependence of the tunneling rate is connected to the phonon spectrum at the heme and the spectrum of excited states of the ligand.

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160 K, is unchanged when the concentration is changed by a factor of 30,000; rebinding consequently must be intramolecular.

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Wastewater Renovation and Reuse: Virus Removal by Soil Filtration

Abstract. *Secondary sewage effluent and renovated water from four wells at the Flushing Meadows Wastewater Renovation Project near Phoenix, Arizona, in operation since 1967, were assayed approximately every 2 months in 1974 for viruses during flooding periods. Viruses, regularly found in the secondary effluent, were not detected in any renovated water samples. Our results indicated that human viral pathogens do not move through soil into the groundwater, but are apparently absorbed and degraded by the soil and reduced in numbers by a factor of at least 10^4 (99.99 percent removal).*

The fate of viruses in wastewater as they contact the soil is an important consideration in land disposal systems (*1*). We need more information on virus movement and survival in soil, especially under field conditions, to properly assess the pollution hazard to our resources. Once a sound data base is obtained for evaluating virus removal mechanisms, then management can implement practices for renovation and reuse of wastewater.

In 1967 an experimental project, the Flushing Meadows Project, in the Salt River bed west of Phoenix, Arizona, was installed to study renovation of secondary sewage effluent by land disposal. Effluent from an activated sludge-type secondary sewage treatment plant was allowed to infiltrate into six parallel horizontal basins (6 m by 210 m, 6 m apart) consisting of 60 to 90 cm of fine loamy sand underlain by several coarse sand and gravel layers to a depth of 75 m, where a clay layer begins. Observation wells for sampling renovated sewage were installed in line midway across the basin area, except that one well was located 22.5 m from the end of the basin area midway between basins 3 and 4. The wells were cased to the bottom with nonperforated steel pipe (15 cm in diameter), except for one well 76.2 m deep, which was perforated from 3 to 9 m. The other wells ranged in depth from 6.1 to 9.1 m. The infiltration rate was about 100 m of wastewater per year. Each year the wastewater was usually applied intermittently with 14-day flood and dry periods. Bouwer *et al.* (*2*) described the infiltration and hydraulic aspects of the project as well as its water quality improvement and economic aspects. Our research showed that wastewater viruses did not move through the soil infiltration system used for renovated wastewater.

Table 1. Numbers and types of viral isolates from the secondary sewage effluent and the renovated wastewater wells. (Numbers are averages for duplicate samples from the sewage effluent and the four well sites combined.)

Sampling dates (1974)	Viruses per 100 liters		Types of viruses in sewage effluent
	Sewage effluent	Renovated water	
7 to 11 January	786	0	Poliovirus 2, echovirus 15
12 to 18 March	2745	0	Poliovirus 2, echovirus 7
5 to 9 May	2378	0	Poliovirus 2 and 3
25 June to 9 July	158	0	Poliovirus 2, coxsackievirus B4
27 August to 12 September	7475	0	Reovirus 1 and 2*
19 November to 11 December†	1142	0	Reovirus (undetermined)*
Range	158–7475	0	

*No plaques were noted until after 14 days under agar overlay. †Data for sewage effluent were obtained in the November flood period and data for renovated water (East Well and Well 7, only) in the December flood period.

A more detailed paper covers other microbial aspects of the Flushing Meadows Project (*3*).

Secondary sewage effluent and renovated water samples for virus analysis were collected every 2 months throughout 1974 during the second week of the 14-day flood periods so that the four wells (*4*) sampled would yield "fresh" renovated water (the underground detention time was 5 to 10 days). Each sampling period was about 1 week. During this time, duplicate samples of 174 to 454 liters from each well were processed through a portable virus concentrator by using methods previously described (*5*), but modified (*6*).

The concentration of naturally occurring enteric viruses in the secondary effluent flowing into the infiltration basins was determined from samples of 4 to 20 liters. Viruses were concentrated from the effluent samples by a method similar to that described by Homma *et al.* (*7*).

Viruses were isolated by using primary baboon kidney cells that were obtained from immature baboons, trypsinized, and grown as described by Melnick and Wenner (*8*). Usually only 30 to 50 percent of the total sample was assayed at a time. The sample was divided into two equal volumes, and one subsample was assayed by bottle culture and the other by the overlay method. In the overlay method, 0.1 to 0.2 ml of inoculum was placed on a monolayer of cells in 30-ml flat glass bottles (with a cell surface area of 12 cm^2) and incubated at 37°C for 1 hour. The bottles were then washed with 5 ml of Eagle's minimal essential medium (MEM) to reduce toxicity, drained of excess fluid, and overlaid with 5 ml of agar. The agar overlay medium consisted of single-strength Eagle's MEM without phenol red; 1.5 percent agar (Difco); 23 m*M* $MgCl_2$; neutral red in a final concentration of 1 part in 54,000; 100 units of penicillin; streptomycin, 100 μg/ml; and 0.4 percent $NaHCO_3$. When plaques appeared, virus was "plucked" from the plaques and passed to fresh cultures maintained under fluid media. Progeny virus harvested from these bottles was identified by antiserum pools (*9*) and by specific antiserums.

The second subsample was placed in bottles (0.2 to 0.5 ml per bottle) with drained cell cultures and incubated for 6 minutes at 37°C. Then 4 to 5 ml of Eagle's MEM was added to each of the bottles, which were examined daily for cytopathic effects. When cytopathic effects were observed, the culture fluid was inoculated in fresh bottle cultures to