

from a contracting to an expanding phase. Although inflation does not address the cosmic singularity problem directly, it does rely implicitly on the opposite assumption: that the big bang is the beginning of time and that the universe emerges in a rapidly expanding state. Inflating regions with high potential energy expand more rapidly and dominate the universe. If there is a preexisting contracting phase, then the high-potential energy regions collapse and disappear before the expansion phase begins. String theory or, more generally, quantum gravity can play an important role in settling the nature of the singularity and, thereby, distinguishing between the two assumptions.

The cyclic model is a complete model of cosmic history, whereas inflation is only a theory of cosmic history following an assumed initial creation event. Hence, the cyclic model has more explanatory and predictive power. For example, we have already emphasized how the cyclic model leads naturally to the prediction of quintessence and cosmic acceleration, explaining them as essential elements of an eternally repeating universe. The cyclic model is also inflexible with regard to its prediction of no long-wavelength gravitational waves.

Inflationary cosmology offers no information about the cosmological constant problem. The cyclic model provides a fascinating new twist. Historically, the problem is assumed to mean that one must explain why the vacuum energy of the ground state is zero. In the cyclic model, the vacuum energy of the true ground state is not zero. It is negative and its magnitude is large, as is apparent from Fig. 1. However, we have shown that the universe does not reach the true ground state. Instead, it hovers above the ground state from cycle to cycle, bouncing from one side of the potential well to the other but spending most time on the positive-energy side.

Reviewing the overall scenario and its implications, what is most remarkable is that the cyclic model can differ so much from the standard picture in terms of the origin of space and time and the sequence of cosmic events that lead to our current universe. Yet, the model requires no more assumptions or tunings (and by some measures less) to match the current observations. It appears that we now have two disparate possibilities: a universe with a definite beginning and a universe that is made and remade forever. The ultimate arbiter will be nature. Measurements of gravitational waves and the properties of dark energy (11) can provide decisive ways to discriminate between the two pictures observationally.

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Evidence of HIV-1 Adaptation to HLA-Restricted Immune Responses at a Population Level

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Antigen-specific T cell immunity is HLA-restricted. Human immunodeficiency virus–type 1 (HIV-1) mutations that allow escape from host immune responses may therefore be HLA allele-specific. We analyzed HIV-1 reverse transcriptase sequences from a large HLA-diverse population of HIV-1-infected individuals. Polymorphisms in HIV-1 were most evident at sites of least functional or structural constraint and frequently were associated with particular host HLA class I alleles. Absence of polymorphism was also HLA allele-specific. At a population level, the degree of HLA-associated selection in viral sequence was predictive of viral load. These results support a fundamental role for HLA-restricted immune responses in driving and shaping HIV-1 evolution in vivo.

Selection of viral mutations associated with loss of antiviral cytotoxic T lymphocyte (CTL) responses has been described in humans with acute and chronic HIV-1 infection (1), macaques infected with simian immunodeficiency virus (SIV) (2, 3), and rhesus

monkeys challenged with simian-human immunodeficiency virus (SHIV) after vaccination (4). However, the full extent and importance of CTL escape mutation to HIV-1 evolution remains to be established. CTL escape mutations occur at critical sites within HLA-restricted CTL epitopes where an amino acid substitution may abrogate epitope-HLA binding, reduce T cell receptor recognition, or generate antagonistic CTL responses (1). Mutations that affect proteasome cleavage sites flanking CTL epitopes may also disrupt cellular processing of the epitope (5). The capacity of the virus to mutate at any amino acid residue is constrained, however,

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by the functional or structural value of the residue to virus survival (6).

We hypothesized that, as CTL epitopes are HLA-restricted, CTL escape mutations selected within an individual host would be characteristic for specific HLA class I alleles across an HLA-diverse host population. We speculated that such polymorphisms would be particularly evident in viral genes encoding internal proteins such as HIV-1 reverse transcriptase (RT), which is highly expressed in virions (7) and immunogenic in the early response to HIV-1 (8, 9). Therefore, we examined the relationship between HIV-1 RT sequence polymorphisms, known functional constraint, and HLA genotypes in 473 participants of the Western Australian (WA) HIV Cohort Study (10–13).

We first determined the population consensus sequence for HIV-1 RT by assigning the most common amino acid for each position between positions 20 and 227 of all first pretreatment sequences pooled from the cohort. The consensus sequence matched the clade B reference sequence HIV-1 HXB2 at all positions in HIV-1 RT except residues 122 (lysine instead of glutamate) and 214 (phenylalanine instead of leucine) (14). The per-

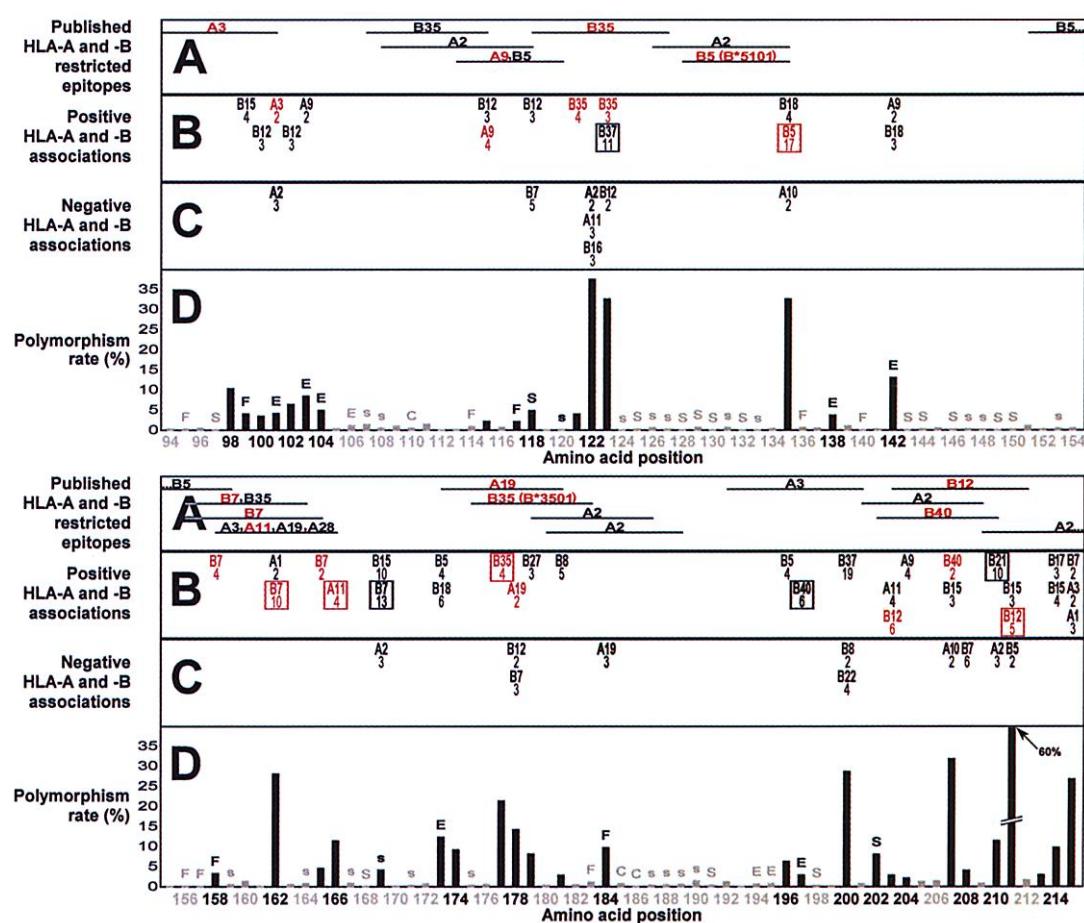
centages of individuals with an amino acid in their own first pretreatment HIV-1 RT sequence different from that of consensus sequence were calculated for each amino acid residue. This rate of polymorphism at single residues varied from 0% to 60% and correlated with the level of known functional constraint at each site (6, 15).

We then analyzed the most recent HIV-1 RT sequences obtained from all individuals. We considered single amino acid residues one at a time and the polymorphism of each HIV-1 residue across the host population. We conducted a multivariate analysis with logistic regression for each residue to assess the statistical significance of association(s) between the presence of a polymorphism (defined as any substitution of the consensus amino acid) and the HLA-A and HLA-B alleles of the population. A *P* value and estimated odds ratio (OR) for each HLA allele-polymorphism association were generated. We incorporated initial power calculations to limit analyses to only those HLA alleles and viral polymorphisms that were sufficiently prevalent to have associations between them detected. We applied a further selection procedure based on forward selection and back-

ward elimination. We used randomization tests to determine the “exact” significance levels for associations and designed a customized software program, Epipop, to carry out these analyses (15, 16). This process was repeated for every residue from position 20 to 227 of HIV-1 RT, giving a residue-specific view of the independent selection effects of HLA on HIV-1 RT in vivo and at a population level.

We plotted all the statistically significant HLA-polymorphism associations on a map of HIV-1 RT in relation to polymorphism rate, previously reported functional characteristics of residues (6), and published CTL epitopes (17) (Fig. 1). There were 64 significant positive associations between polymorphisms and HLA alleles (OR > 1, *P* < 0.05 in all cases) (Fig. 1B, fig S1B). Polymorphisms associated with the same HLA allele appeared to cluster along the sequence. For example, HLA-B7 was associated with polymorphism at positions 158, 162, 165, and 169, which are all within or flanking the known HLA-B7-restricted CTL epitope RT(156–165). There was also apparent clustering of associations for HLA-B12, HLA-B35, HLA-B18, and HLA-B15. Fifteen

Fig. 1. Map of polymorphism rate and HLA associations at amino acid positions 94 to 215 of HIV-1 RT. Residues with little power to detect HLA associations are shaded. (A) Published CTL epitopes are shown as black lines with their known HLA restriction to the level of broad serological genotype, except the HLA-B*5101- and HLA-B*3501-restricted epitopes examined in further detail in the study. HLA restrictions of epitopes are marked in red if there is a corresponding HLA-specific polymorphism within the epitope. (B) HLA alleles significantly associated with specific polymorphisms and ORs for the association. HLA-specific polymorphisms within published CTL epitopes restricted to the same HLA allele are red. All other associations are black and may indicate the location of putative CTL epitopes. Boxed associations are those that remain statistically significant after correction for total number of residues examined across the entire gene region. (C) Negative HLA associations with ORs of residue not varying from consensus. (D) Percentages of individuals with a viral amino acid different from that of consensus sequence at each position. Known functional characteristics of residues (6) are marked as stability (S), functional (F), catalytic (C), and external (E).



HLA-specific polymorphisms were at positions within known CTL epitopes, and the HLA allele association matched the known HLA restriction of the epitope. For example, the amino acid at position 162 resides within a known HLA-B7-restricted CTL epitope RT(156–165) and the odds of polymorphism at this site were significantly increased in those individuals with HLA-B7 (OR = 10, $P < 0.001$). Four polymorphisms (at positions 101, 135, 165, and 166) were at primary anchor positions within corresponding CTL epitopes (HLA-A3-, HLA-B51/HLA-B*5101-, HLA-B7-, and HLA-A11-restricted, respectively) where mutation could abrogate binding to the HLA molecule. The remaining 11 associations were at nonprimary anchor positions of CTL epitopes. The number of HLA-specific polymorphisms observed within known CTL epitopes with corresponding HLA restriction was significantly greater than that expected if significant positive associations occurred randomly across residues (15 versus 4.27, $P < 0.001$). Furthermore, an excess of associations over that expected was observed for 10 of the 11 HLA specificities with CTL epitopes in this segment of HIV-1 RT (15). Six HLA allele-specific polymorphisms were not within but flanked CTL epitopes, including the predicted proteasome cleavage sites at positions 26 and 28 flanking HLA-A2- and HLA-A3-restricted CTL epitopes (18) [see fig. S1 (15)].

To take account of the multiple comparisons used in the statistical process over the entire HIV-1 RT protein, we applied Bonferroni-type corrections based on randomization tests. Following this highly stringent correction, 12 associations remained statistically significant ($P < 0.05$). Overall, the finding of

HLA associations, taking all positions and multiple comparisons into account, was statistically significant ($P < 0.001$). We propose that those HLA-associated polymorphisms that were not within published corresponding CTL epitopes may indicate where previously unknown, untested epitopes are located. This is particularly likely for those HLA associations that are strong (with high OR), are clustered, or remain statistically significant after correction for multiple comparisons. It is important to note that our statistical correction for the number of residues examined will be overly conservative in some cases, because the degree of correction depends on the size of the gene region arbitrarily chosen for study. Such correction results in decreasing false associations (higher specificity) at the cost of losing true associations (lower sensitivity). Our gradation of P values uncorrected for multiple comparisons reflects a gradation in strength of statistical evidence for associations. We conclude that independent biological validation rather than statistical means will best determine what P value cutoffs are optimal for sensitivity or specificity.

We further characterized two strong examples of HLA-specific polymorphism, I135x (substitution of consensus isoleucine at position 135) and D177x (substitution of aspartate). These were associated with HLA-B5 and HLA-B35, respectively, in the multivariate models (Fig. 1B). However, these broad serologically defined HLA alleles both have subtypes with distinct epitope binding motifs. We performed high-resolution DNA sequence-based typing on all individuals with HLA-B5 or HLA-B35 and reexamined the associations between their HLA subtypes and viral polymorphisms (Tables 1 and 2). Position 135 is the anchor position of the HLA-

B*5101-restricted epitope RT(128–135 IIIB) (17). Six of the other seven amino acids in the epitope are critical stability residues (6) and were relatively invariant (Fig. 1D). All but 1 of the 40 (98%) individuals in the cohort with HLA-B*5101 had I135x, compared with 127 of the 431 (29%) without HLA-B*5101 ($P < 0.0001$; Fisher's exact test) (Table 1). For the predominant substitution observed, I135T [see fig. S2 (15)], the predicted half-time of dissociation score for the mutant epitope (TAFTIPST) is 11 compared with 440 for the consensus sequence (TAFTIPSI), which indicates that binding to HLA-B*5101 in vivo would be abrogated (19). I135T has also been shown to necessitate a 100-fold increase in the peptide concentration required to sensitize target cells for 50% lysis by CTLs in vitro (20). Notably, the one subject with HLA-B*5101 and consensus sequence at position 135 had taken highly active antiretroviral therapy only days after exposure to HIV. He was asymptomatic, highly viremic, and seronegative at the time of starting treatment, which may have decreased viral replication before selection of I135x by the acute CTL response. This suggests that I135x is characteristically selected during the acute HLA-B*5101-restricted CTL response instead of at transmission or in chronic infection and that protection from viral escape could contribute to the effect of therapy in acute HIV infection, leading to stronger chronic inhibitory CTL responses. Similarly, D177x is within the epitope RT(175–183) known to bind HLA-B*3501 and contain a binding motif distinct from that of other HLA-B35 subtypes (21). After high-resolution HLA typing, D177x was associated with HLA-B*3501 specifically and not with other HLA-B35 subtypes (Table 2). Associations with other HLA-B35-associated polymorphisms in HIV-1 RT, I69x, D121x, and D123x were all strengthened by considering molecular subtypes of HLA-B35.

In addition to positive HLA associations, we detected 25 negative HLA associations [Fig. 1C, fig. S1C (15)]. For example, polymorphism at positions 32, 101, 122, 169, and 210 was negatively associated with HLA-A2 (OR < 1 , $P < 0.05$ in all cases). This means that HLA-A2 individuals were significantly less likely to vary from the consensus at these sites compared with all non-HLA-A2 individuals. HLA-A2 is the most common HLA-A allele in our cohort and it had 5 of the 25 negative associations (compared with 3 of the 64 positive associations). There appeared to be a predominance of common HLA alleles with negative associations. Unlike positive selection pressure, which causes demonstrable escape over time in individuals, negative selection pressure favors preservation of wild-type virus in vivo and therefore could be made evident only at a population

Table 1. Distribution of the presence of I135x in HIV-1 RT within individuals with HLA-B5, HLA-B*5101 subtype, and non-HLA-B5. High-resolution HLA genotyping strengthens the I135x-HLA allele association. One HLA-B5 individual did not have sufficient DNA sample for high-resolution HLA typing.

HLA type	Present (n)	Absent (n)	Odds ratio	P value
HLA-B5	44	8		
Non-HLA-B5	123	297	13	<0.0001
HLA-B*5101	39	1		
Non-HLA-B*5101	127	304	93	<0.0001

Table 2. Distribution of the presence of D177x within individuals with HLA-B35, HLA-B*3501 subtype, and non-HLA-B35. High-resolution HLA genotyping strengthens the D177x-HLA allele association. Seven individuals with HLA-B35 by serology did not have DNA available for high-resolution HLA typing; in another four, HLA-B35 was not confirmed by sequencing.

HLA type	Present (n)	Absent (n)	Odds ratio	P value
HLA-B35	26	31		
Non-HLA-B35	84	330	3.3	<0.0001
HLA-B*3501	19	14		
Non-HLA-B*3501	86	345	5.4	<0.0001

level. This raises the intriguing possibility that consensus or wild-type virus is adapted to the CTL responses that it has most often encountered during primordial evolution (that is, those CTL responses restricted to the most common or evolutionarily conserved HLA alleles in the host population). It is possible that the consensus HIV-1 sequence in our study population has been selected by the host population's predominant HLA types, as has been shown for weakly immunogenic variants of Epstein-Barr virus in other host populations (22, 23). We speculate that this could explain the apparent lack of HIV-1 escape from HLA-A*0201-restricted responses in studies that have argued against an important role for CTL escape (24, 25), and might even explain why surprisingly few HLA-A2- and HLA-A1-restricted epitopes have been mapped in HIV-1 (26). Primordial viral adaptation to predominant HLA types may account, at least in part, for HIV-1 clade differences. Furthermore, studies of HIV-1-exposed seronegative individuals suggest that CTL responses can alter viral infectivity and susceptibility to established primary HIV-1 infection (27–31). The HLA class I alleles associated with natural HIV-1 resistance or susceptibility appear to differ between racially distinct populations (27–29, 32). To some extent, this may reflect differences in the HLA alleles that are common in different populations and the degree to which a population-adapted wild-type virus can adapt to the individual.

We sought to determine whether HLA-specific polymorphisms were associated with increased plasma HIV RNA levels (viral load) (33). We examined single polymorphisms with sufficient subject numbers for comparison. HLA-A11-associated K166x is at the anchor position of HLA-A11 epitope RT(158–166 LAD). In HLA-A11 individuals ($n = 19$), the median pretreatment viral load was 5.54 ± 0.46 log copies per ml (cps/ml) of plasma (median $\pm$ SD) in those with K166x ($n = 4$) compared with 4.31 ± 0.82 log cps/ml in those without K166x ($n = 15$; $P = 0.045$, Wilcoxon test). A second putative CTL escape mutation within a CTL epitope (but not at a primary anchor position) showed a similar effect. The median pretreatment viral load in HLA-B7 individuals with S162x ($n = 18$) was significantly higher (5.41 ± 1.04 log cps/ml) than in those without S162x ($n = 15$, 4.57 ± 0.83 log cps/ml; $P = 0.046$, Wilcoxon test). A global analysis of factors influencing viral load at a population level showed that the presence of viral polymorphisms in combination with their positively associated HLA alleles or consensus amino acids with their negatively associated HLA alleles was a significantly better predictor of pretreatment viral load than HLA alleles or viral polymorphisms alone ($P < 0.004$) (15). This suggested that the amount of HLA-associated

selection in an individual, as defined by our analyses, explained the viral load variability in the population better than HLA alleles.

This study encompasses demographic, clinical, and laboratory data collected over 2210 person-years of observation. Our findings support a model of HIV-1 evolution in vivo in which CTL escape mutations are selected within functional limits within individuals, and this selection during viral passage through a population determines the wild-type or consensus viral sequence. Thus, the HLA alleles present in a population may explain in large part both the polymorphism (viral adaptation to individuals) and the consensus (primordial adaptation) of HIV-1 sequences in that population. Our data suggest that CTL escape mutation is common and widespread and selected by responses restricted to a much wider array of HLA alleles than have been studied to date.

These results are especially notable, considering the factors that reduce the likelihood of observing significant associations in such analyses. First, the power to detect associations is not constant for all combinations of HLA allele and viral residue. Large numbers of individuals would be needed to observe any polymorphism at residues under CTL pressure but with strong functional constraint or any associations with HLA alleles that are rare. The use of formal power calculations identifies those HLA associations that cannot be excluded until larger data sets are examined. Second, as suggested by the enhancement of associations between HLA-B5 and I135x and between HLA-B35 and D177x by high-resolution HLA typing, the molecular subtype of an HLA allele better predicts its binding properties in vivo. Other alleles with multiple splits of similar frequency (HLA-A10 or HLA-A19) may have had associations that we did not detect because only broad alleles were considered. Furthermore, molecular splits that have opposing effects at the same viral residue would negate any association with the broad allele. Lastly, published epitopes are more likely to be in conserved regions, because studies tend to use laboratory reference strains as target antigens and conserved regions are more likely to generate measurable CTL responses in vivo (34). This approach, in contrast, preferentially detects putative CTL epitopes in polymorphic regions and, thus, may be complementary to standard epitope mapping.

Application of this method to other HIV-1 genes in larger populations with more complete high-resolution HLA genotyping is needed. To that end, an international collaboration to pool data and provide the Epipop program to participating centers has been initiated. This approach could be used to screen or prioritize standard testing of candidate epitopes in all HIV-1 proteins. In the HIV

envelope, effects associated with antibody responses to HIV, CCR5 and CXCR4 genotype, and any other polymorphisms of genes encoding products that target envelope proteins could also be considered. Future analyses of HIV-1 RT and other antiretroviral drug targets should adjust for drug selection effects to examine the interactions between drug resistance mutations and putative CTL escape mutations. If CTL responses and antiretroviral drugs compete at sites within viral sequence (35), a greater or lesser tendency to drug resistance and response may be observed depending on HLA genotype. Individualization of antiretroviral therapy conceivably could be improved if synergistic or antagonistic interactions between immune pressure and drug pressure were better understood. Ultimately, it may be possible to generalize these approaches to examine host immune effects on hepatitis B, hepatitis C, and other chronic human pathogens.

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12. HIV-1 DNA was extracted from buffy coats (QIAMP DNA blood mini kit; Qiagen, Hilden, Germany) and codons 20 to 227 of RT were amplified by polymerase chain reaction (PCR). A nested second round PCR was done, and the PCR product was purified with Bresatec purification columns and sequenced in forward and reverse directions with a model 373 ABI DNA sequencer. Raw sequence was manually edited with software packages Fatura and MT Navigator (PE Biosystems).
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REPORTS

Magneto-Opto-Electronic Bistability in a Phenalenyl-Based Neutral Radical

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A new organic molecular conductor, based on a spiro-biphenalenyl neutral radical, simultaneously exhibits bistability in three physical channels: electrical, optical, and magnetic. In the paramagnetic state, the unpaired electrons are located in the exterior phenalenyl units of the dimer, whereas in the diamagnetic state the electrons migrate to the interior phenalenyl units and spin pair as a π -dimer. Against all expectations, the conductivity increases by two orders of magnitude in the diamagnetic state, and the band gap decreases. This type of multifunctional material has the potential to be used as the basis for new types of electronic devices, where multiple physical channels are used for writing, reading, and transferring information.

The processing of information is based on the ability to control and retrieve changes in a particular physical property of a material, such as the electrical, magnetic, or optical response. Usually, at the level of the basic unit, one of these physical channels is used. When two different physical channels of the material are simultaneously involved, a new breadth of applications and even new fields of research often appear, such as optoelectronics, magnetooptics, and spintronics (1), which, as Das Sarma (2) noted, "is a seamless integration of electronic, optoelectronic and magnetoelectronic multifunctionality on a single device. . . ." We now report that a phenalenyl-based neutral radical organic conductor exhibits bistability just above room temperature so that one state is paramagnetic, insulating, and infrared (IR) transparent. These properties reverse in the other state to produce a material that is diamagnetic, conducting, and IR opaque.

By definition, neutral organic radical mole-

cules contain an unpaired electron just as in a classical (mono)atomic metal, and they are promising basic units for the construction of an intrinsic molecular metal or superconductor (3). The basic molecular building block on which we report consists of two phenalenyl ring systems, spiro-conjugated through a boron atom (Fig. 1), so that the two halves of the molecule are orthogonal to one another. This nonplanar

unit prevents the formation of a one-dimensional structural chain (4, 5), thus obviating the occurrence of a Peierls transition to an insulating ground state (6).

A variety of alkyl (C_nH_{2n+1}) groups can be attached to the nitrogen atoms to modify the crystal packing; the first phenalenyl-based neutral radical to be crystallized [1, with a hexyl group ($n = 6$) attached to nitrogen] is a resonance-stabilized carbon-based free radical (7). The existence of the monomeric, neutral carbon-based free radical 1 in the crystalline state is unprecedented; all prior structures have required steric hindrance to inhibit dimerization (4, 5, 8, 9). Furthermore, the hexyl radical 1 shows the highest conductivity of any neutral organic molecular solid, despite the fact that all intermolecular contacts are outside of the van der Waals spacing (7).

Changing the alkyl group from hexyl 1 to butyl 2 or ethyl 3 leads to a crystal structure containing π -dimers as the basic building block (Fig. 1) (10). In the high-temperature limit [3, temperature (T) > 350 K], the interplanar distance within the π -dimer is about 3.3 Å and the interaction is sufficiently weak that the electron spins remain unpaired. The dimerization to a diamagnetic state occurs at a structural phase transition when T decreases below 320 K and

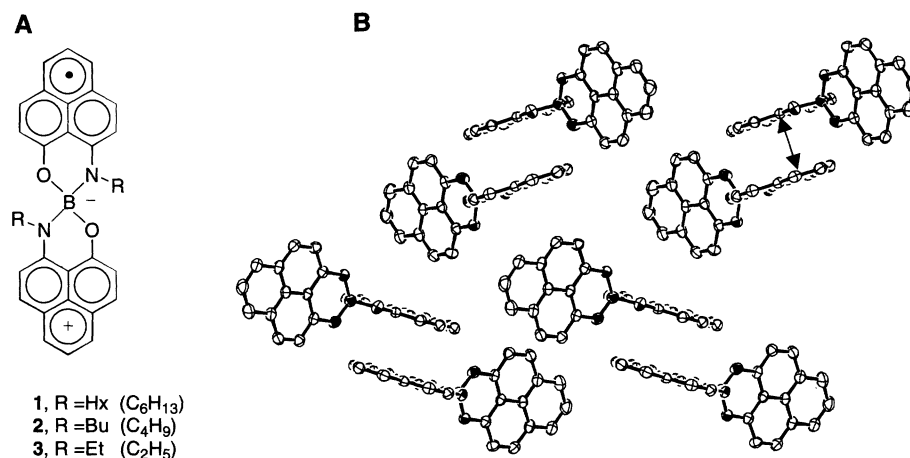


Fig. 1. (A) Molecular structure of neutral radicals. (B) X-ray crystal structure of ethyl radical 3, showing the intra-dimer distance, d , indicated by the double-headed arrow. d increases from ~ 3.2 to ~ 3.3 Å as the spins unpair and the compound becomes paramagnetic.

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